ANALYTICAL STUDIES ON POLYSACCHARIDES

WITH PARTICULAR REFERENCE TO GUM EXUDATES

FROM THE GENUS *ACACIA*

By

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A

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PREFACE

The Papers on which this Thesis is based have not been submitted for any other degree.

The Papers record and discuss the results of an original research programme which was planned and initiated by the candidate.

The experimental work involved was carried out within the period 1957 - 1967 by the candidate, or by post-graduate research workers studying for the degree of Doctor of Philosophy under the candidate's direct supervision. The only exception to this involves Papers 42 and 44, for which the light-scattering measurements were made in the University of Leeds under the supervision of Dr. G. Stainsby.

D.M.W. Anderson

REFERENCES TO THE PUBLICATIONS OF D.M.W. ANDERSON

Section A. "Analytical Applications of Infra-red Spectroscopy".

		Reference
1.	Part I: "The identification and determination of g.l.c. fractions".	Analyst, 1959, 84, 50-55.
2.	Part II: "Observations on some aspects of the Zeisel alkoxyl determinations". (With J.L. Duncan)	Talanta, 1960, 7, 70-79.
3.	Part III: "The simultaneous determination of methoxyl and ethoxyl groups". (With J.L. Duncan)	Talanta, 1961, 8, 1-7.
4.	Part IV: "The causes of anomalous alkoxyl determinations given by sugars and polyhydric alcohols". (With J.L. Duncan)	Talanta, 1961, 8, 241-8.
5.	Part V: "The retention of water and organic solvents by carbohydrate materials". (With N.J. King)	Talanta, 1961, 8, 497-504.
6.	Part VI: "A review of recent advances in infra-red spectroscopy".	Talanta, 1961, 8, 832-5.
7.	Part VII: "The behaviour of thioalkyl compounds under Zeisel reaction conditions". (With S.S.H. Zaidi)	Talanta, 1962, 9, 611-5.
8.	Part VIII: "An anomalous Zeisel alkoxyl reaction". (With M.A. Herbich and S.S.H. Zaidi)	Talanta, 1962, 9, 620-1.
9.	Part IX: "The differentiation of esters and acetals from ethers in the Zeisel reaction". (With J.L. Duncan)	Talanta, 1962, 9, 661-6.

10. "Analytical applications of infra-red spectroscopy". (Review). In "Analytical Chemistry, 1962". pp. 339-344.

11.	Part X: "The Zeisel reaction of tertiary-butoxyl groups, and the anomalous reaction of tertiary butyl phenols". (With J.L. Duncan, M.A. Herbich, and S.S.H. Zaidi)	Analyst, 1963, 88, 353-8.
12.	Part XI: "The determination of 1,2-diols by modified Zeisel reactions". (With S.S.H. Zaidi)	Talanta, 1963, 10, 691-2.
13.	Part XII: "The behaviour of propoxyl and butoxyl groups in the Zeisel reaction". (With S.S.H. Zaidi)	Talanta, 1963, 10, 1235-1242.
14.	Part XIII: "The determination of small amounts of alcohols in aqueous solution". (With S.S.H. Zaidi)	Analyt. Chim. Acta, 1964, 30, 303-7.
15.	Part XIV: "The determination of small amounts of acetaldehyde in aqueous solution". (With J.F. Stoddart). In "Proceedings of the S.A.C. Conference, Nottingham 1965".	pp. 232-8.

Section B. "Analytical Applications of Chromatographic Methods".

16.	"The use of 'Biogel P' in the gel filtration of polysaccharides". (With I.C.M. Dea, S. Rahman, and J.F. Stoddart).	Chem. Commun., 1965, 145.
17.	"Separations of sugars on 'Chromagrams'". (With J.F. Stoddart).	Carbohyd. Res., 1966, 1, 417-8.
18.	"Some observations on molecular weight estimations by molecular-sieve chromatography". (With J.F. Stoddart).	Analyt. Chim. Acta, 1966, 34, 401-6.
19.	"The use of molecular-sieve chromatography in studies on Acacia senegal gum". (With J.F. Stoddart).	Carbohyd. Res., 1966, 2, 104-114.
20.	"Theories of molecular-sieve chromatography". (With J.F. Stoddart)	Lab. Practice, 1967, 16, 841-6.

Section C. "Polysaccharides of the Characeae".

No.	Title	Reference
21.	Part I: "Examination of a starch-type polysaccharide from *Nitella translucens*". (With N.J. King).	*J. Chem. Soc.*, 1961, 2914-9.
22.	Part II: "The carbohydrate content of *Nitella translucens*". (With N.J. King)	*Biochim. Biophys. Acta*, 1961, *52*, 441-9.
23.	Part III: "The carbohydrate content of *Chara australis*". (With N.J. King).	*Biochim. Biophys. Acta*, 1961, *52*, 449-454.
24.	Part IV: "A non-esterified pectic acid from *Nitella translucens*". (With N.J. King).	*J. Chem. Soc.*, 1961, 5333-8.

Section D. "Studies on Uronic Acid Materials".

No.	Title	Reference
25.	"The elimination of errors in uronic acid determinations".	*Talanta*, 1958, *1*, 283-4.
26.	Part I: "An apparatus for semi-micro estimations of uronic acid contents".	*Talanta*, 1959, *2*, 73-8.
27.	Part II: "The variation in composition of gum nodules from *Combretum leonense*". (With E.L. Hirst and N.J. King).	*Talanta*, 1959, *3*, 118-126.
28.	Part III: "An investigation, using ^{14}C compounds, of acid decarboxylation reaction times". (With S. Garbutt).	*Talanta*, 1961, *8*, 605-611.
29.	Part IV: "Aqueous decarboxylation of uronic acids, and the decarboxylation of pectic materials during extraction". (With A.J. Bews, S. Garbutt and N.J. King).	*J. Chem. Soc.*, 1961, 5230-34.
30.	Part V: "The thermal decarboxylation method of analysis". (With S. Garbutt and J.F. Smith).	*Talanta*, 1962, *9*, 689-697.

31. Part VI: "The variation in composition and properties of gum nodules from Acacia seyal Del. (With M.A. Herbich). — J. Chem. Soc., 1963, 1-6.

32. Part VII: "The kinetics and mechanism of the decarboxylation of uronic acids". (With S. Garbutt). — J. Chem. Soc., 1963, 3204-3210.

33. Part VIII: "Some colorimetric methods of estimating the uronic acid content of polysaccharides". (With S. Garbutt). — Analyt. Chim. Acta, 1963, 29, 31-8.

34. Part IX: "The simultaneous determination of uronic acid and alkoxyl groups in polysaccharides". (With S. Garbutt and S.S.H. Zaidi). — Analyt. Chim. Acta, 1963, 29, 39-45.

35. Part X: "The analytical importance of the methoxyl content of Acacia gum exudates". (With G.M. Cree, M.A. Herbich, K.A. Karamalla, and J.F. Stoddart). — Talanta, 1964, 11, 1559-1560.

36. Part XI: "The carbohydrate content of the oleo-resin from Boswellia papyrifera". (With G.M. Cree, J.J. Marshall and S. Rahman). — Carbohyd. Res., 1965, 1, 320-3.

37. Part XII: "The composition of Acacia gum exudates". (With K.A. Karamalla). — J. Chem. Soc., (C), 1966, 762-4.

38. Part XIII: "The composition of gum exudates from Albizia species". (With G.M. Cree, J.J. Marshall and S. Rahman). — Carbohyd. Res., 1966, 2, 63-9.

39. Part XIV: "Methylation with the sodium hydride-methyl iodide-dimethylsulphoxide system". (With G.M. Cree). — Carbohyd. Res., 1966, 2, 162-6.

40. Part XVI: "Inter-nodule variation and the acidic components in Acacia nilotica gum". (With K.A. Karamalla). — Carbohyd. Res., 1966, 2, 403-410.

41. Part XVII: "Some structural features of *Acacia senegal* gum". (With Sir Edmund Hirst and J.F. Stoddart).	*J. Chem. Soc.*, (C), 1966, 1959-1966.
42. Part XVIII: "Light-scattering studies on some molecular-weight fractions from *Acacia senegal* gum". (With Sir Edmund Hirst, S. Rahman, and G. Stainsby).	*Carbohyd. Res.*, 1967, *3*, 308-317.
43. Part XIX: "The composition of the gum from *Acacia laeta* var. *hashab*". (With R.N. Smith).	*Carbohyd. Res.*, 1967, *4*, 55-62.
44. Part XX: "The viscosity-molecular weight relationship for *Acacia* gums". (With S. Rahman).	*Carbohyd. Res.*, 1967, *4*, 298-304.
45. Part XXI: "Some structural features of *Acacia arabica* gum". (With Sir Edmund Hirst and J.F. Stoddart).	*J. Chem. Soc.*, (C), 1967, 1476-1486.
46. Part XXII: "The composition of the gum from *Acacia drepanolobium*". (With I.C.M. Dea).	*Carbohyd. Res.*, 1967, *5*, 461-9.
47. Part XXIII: "Possible degradations with the methyliodide-sodium hydride-dimethylsulphoxide system". (With I.C.M. Dea, P.A. Maggs and A.C. Munro).	*Carbohyd. Res.*, 1967, *5*, 489-491.
48. Part XXIV: "Analytical studies of some unusual forms of gum from *Acacia senegal*". (With I.C.M. Dea, K.A. Karamalla and J.F. Smith).	*Carbohyd. Res.*, 1968, *6*, 97-103.
49. Part XXV: "Structural studies of some unusual forms of gum from *Acacia senegal*". (With I.C.M. Dea).	*Carbohyd. Res.*, 1968, *6*, 104-110.
50. Part XXVI: "The aldobiouronic acids in gums from the *Acacia* genus". (With G.M. Cree).	*Carbohyd. Res.*, 1968, *6*, 214-9.
51. Part XXVII: "Structural features of the gum from *Acacia nubica*". (With G.M. Cree).	*Carbohyd. Res.*, 1968, *6*, 385-403.

52. Part XXVIII: "Some structural features of _Acacia drepanolobium_ gum". (With I.C.M. Dea).	_Carbohyd. Res._, 1968, _7_, 109–120.
53. Part XXIX: "Some structural features of _Acacia laeta_ gum". (With I.C.M. Dea and R.N. Smith).	_Carbohyd. Res._, 1968, _7_, 320-333.
54. Part XXX: "Examination of three fractions obtained from _Acacia drepanolobium_ gum". (With I.C.M. Dea).	_Carbohyd. Res._, accepted for publication.
55. Part XXXI: "Some structural features of the water-soluble fraction of _Acacia drepanolobium_ gum". (With I.C.M. Dea).	_Carbohyd. Res._, accepted for publication.
56. Part XXXII: "Structural features of the gum from _Acacia seyal_ Del". (With I.C.M. Dea and Sir Edmund Hirst).	_Carbohyd. Res._, accepted for publication.

Analytical Studies on Polysaccharides, with particular reference to gum exudates from the genus *Acacia*.

N.B. In this section of the Thesis, arabic numbers in the text refer to the publications already listed on pp. 2-7. References to other publications are given thus (Hirst and Jones, 1955), and these are listed at the end of this section (p. 33).

This Thesis is based on four inter-related Series of Studies:-

A. Analytical Applications of Infrared Spectroscopy. (15 Papers).

B. Analytical Applications of Chromatographic Methods. (5 Papers).

C. Polysaccharides of the Characeae. (4 Papers).

D. Studies on Uronic Acid Materials. (32 Papers).

These Series of Studies represent (a) attempts to develop new methods of analysis, or to improve some of the analytical methods available generally in 1957 for polysaccharide materials (Anderson and Sands, 1945; Hirst and Jones, 1955); and (b) subsequently to use these improved techniques as part of an

attempt to introduce a more strongly based analytical approach than had been adopted by earlier investigators in studying polysaccharide materials.

One of the shortest - and best - of the definitions of "Analytical Chemistry" is that it is "The science of chemical characterisation and measurement" (Laitinen, 1966). Analytical chemistry, which is concerned with the determination of the composition and structure of all matter, is therefore basic and fundamental to all chemistry and is required (Taylor, 1963) to answer questions such as:-

Question	Type	
What is present?	Qualitative	Analysis
How much?	Quantitative	
In what fragments?	Functional group	
How arranged?	Structural	

In 1957, the writer tried to assess which of the many specialised topics within the broad field of polysaccharide chemistry offered the most attractive prospects for a more fundamental analytical approach. It was particularly desirable, furthermore, that this should supplement, and neither compete with nor duplicate, the approaches of the established workers. A broad survey indicated that the period 1945 - 1957 had seen significant developments and progress in studies dealing with starches, glycogens, dextrans, cellulose, hemicelluloses, fructosans, pectic materials, marine polysaccharides, and algal,

bacterial, and muco-polysaccharides. Many of the advances in these fields had resulted from the comparatively recent introduction of methods such as periodate oxidation, paper and column chromatography, enzymic degradation and syntheses, electrophoresis, and methods for investigating the molecular size and shape, and the homogeneity or heterogeneity, of polymers. To the writer, it appeared clearly that the aspect of polysaccharide chemistry that had not advanced at a commensurate pace involved the plant gum exudates. Although several authoritative reviews (Jones and Smith, 1949; Hirst, 1951; Hirst and Jones, 1951; Hirst and Jones, 1955) had been published within the period under consideration, with further reviews appearing soon after (Hirst, 1958; Hirst and Jones, 1958), the number of Papers on gum chemistry was comparatively small in the period 1950 - 55, and even appeared to decrease in 1956 and 1957. It had been recognised (Hirst, 1951) that "The problems involved were among the most difficult and complicated in the whole field of carbohydrate chemistry" and, later, that the study of plant gums "poses the most formidable problems in present-day carbohydrate chemistry" (Hirst, 1956); by 1959 it was obvious that newer methods of investigation would be required for the elucidation of detailed structural features (Hirst, 1959).

What further challenge was there to seek? To a new investigator, seeking to find the particular polysaccharide

problem calling out most for a fresh analytical approach, the gum exudates stood out clearly as offering the strongest practical challenge. How frequently does it occur that the most perplexing problem at one particular time turns out to be the simplest in the end? The plant gums, paradoxically, through their uronic carboxyl groups, offered the most attractive possibilities of applying the various spectroscopic and chromatographic methods of analysis which, in 1957, obviously still had great latent analytical potential within the polysaccharide field.

One further point deserves comment. To the writer, in 1957, it appeared that too great emphasis was possibly being put on work which investigators at that time chose to describe as "structural studies". With hindsight, the writer is now convinced that this was so. Much of the difficulty - and many of the doubts - had arisen because of a strange bias against basic analytical chemistry, with the resultant lack of appreciation that the correct elucidation of complex structures can only come as the final stage of a very carefully conducted, detailed, series of chemical analyses. The doubts and confusion had arisen - in at least several instances - because the final, most complex analyses were being attempted on materials for which the more preliminary essential analytical stages had been neglected or omitted. Frequently the overall task had not been seen as one of

the most complex problems in analytical chemistry that require to be undertaken, with the result that the basic approaches inherent in good analytical practice had not been employed. There are, for instance, no standard methods for the purification of polysaccharides (Bouveng and Lindberg, 1960) - each biological material presents its own specific problems. The disastrous repercussions of neglecting the analytical chemists' proper attention to control of sampling, particularly with natural products, has received comment[27]; the importance of much early work is unfortunately considerably diminished (Hirst and Jones, 1958). When it is considered that all the analytical stages involved in a full structural investigation of a new polysaccharide material may take a few years, doubts regarding its authenticity, homogeneity and purity, or the correct botanical identification of its source, cannot be entertained.

The structural studies presented in this Thesis were accordingly approached from the view-point that their immediate importance and interest would arise from the new analytical difficulties presented. Whenever an analytical problem was encountered, its solution was given priority; after it was overcome, the sequence of analyses was either resumed as originally planned or amended if necessary.

The writer's earliest experiences in polysaccharide chemistry had indicated that the most unsatisfactory of the essential

analytical procedures used at that time (1950 - 55) were the methods for the quantitative determination of methoxyl groups and uronic acid residues. Fundamental studies of these analyses were therefore planned. The use of both vapour-phase infrared spectroscopy and gas-liquid chromatography was invoked, and it was soon realised[1] that the conjoint use of these techniques provided an extremely powerful analytical weapon. Other workers and commercial instrument makers quickly realised the importance of this; during the past ten years the development of ancillary instrumentation[6,10] has led to great increases in the sensitivity of the technique, and it seems likely that the ultimate has not yet been reached.

A number of the variable reaction conditions in the Zeisel alkoxyl determination were examined, and it was concluded[2] that several faulty recommendations had been proposed by earlier investigators of this classical method of analysis. It was also found that the degradation products of sugars and polyhydric alcohols include furan derivatives[4]; these lead to analytical errors if the non-specific iodometric finish to the analysis is used. A further development was also possible; the determination of methoxyl groups in the presence of ethoxyl[3] and other alkoxyl groups[13] is difficult - if possible at all - when titrimetric or gravimetric methods are used for materials containing mixed alkoxyl groups. After gum purification processes - or extraction

stages with the carbohydrate-resin type exudates[36] - it is extremely difficult to remove the final traces of ethanol; it was recognised (Hirst and Jones, 1955) that, as a precaution, both the crude and purified gums should be analysed. Calculations, from gravimetric or titrimetric measurements, of the apparent methoxyl content of a polysaccharide containing even traces of higher alkoxyl groups[13] leads to an erroneous result, but this danger had not been recognised[38].

The extent to which a number of polysaccharides retained both moisture and organic solvents was also investigated[5]: the possibility of inaccurate results and misleading artifacts arising from such retentions was stressed. In due course, the availability of a sensitive, specific analytical method[35] for methoxyl groups enabled a significant structural feature involving the aldobiouronic acids of gums from the Acacia genus to be recognised.

Thioalkyl[7] and tertiary butoxyl[11] functional groups are involved in some aspects of natural products chemistry; the applications of Zeisel-type reactions to these groups were investigated, and some anomalies and potential sources of analytical error were found. Further investigations of modified Zeisel reactions, involving HCl and HBr in place of HI, led to the description of methods for the differentiation of esters, acetals, and alcohols from ethers[9], for the determination of

small amounts of alcohols in aqueous solution[14], and for the determination[12] of 1,2-diols. The latter method was subsequently useful for examining some of the cleavage products from Smith degradations, and the products from reduction experiments with hydroxyethyl and hydroxypropyl esters of acidic polysaccharides.

The presence of rhamnose[37] is a useful structural feature of *Acacia* gums. The earliest species studied fortuitously contained similar molar proportions of rhamnose and uronic acid, although it is now quite common[37] for the rhamnose content to be low (≑ 1%) in some species, with no unit molar correspondence to the uronic acid content. When this was first encountered, the determination of methylpentose in the 0 - 2% range was not possible with the degree of reliability required, and a simple, accurate, vapour-phase infrared method[15] was therefore developed.

Following the rapid development of gas chromatographic methods for investigating carbohydrate materials, thin-layer chromatography[17] and molecular-sieve chromatography[16] (originally called "gel filtration") began to offer analytical advantages. The development of molecular-sieve chromatography was so rapid that, by 1966, several sources of confusion and apparent contradictions clouded the literature. The situation was therefore reviewed[18] critically. The theories proposed for the mechanism of separation, and the bases for effecting the

calibration of columns in terms of molecular weight, were also discussed[20]. Great caution must be exercised at present when interpreting the behaviour of polymer molecules on molecular-sieve columns, but once a wider range of fully characterised fractions suitable for effecting valid calibrations becomes available, this new technique will undoubtedly facilitate physico-chemical studies of all types of polysaccharide molecules and their degradation products. To date, the molecular-sieve technique has provided interesting results in structural studies of the gums from <u>Acacia senegal</u>[19,41], <u>A.arabica</u>[45], <u>A.laeta</u>[53], <u>A.drepanolobium</u>[54] and <u>A.seyal</u>[56]. The recent introduction of molecular-sieves based on porous glass and on agarose preparations has extended the upper molecular-weight range ($\bar{M}_w$) of the technique from approx. 0.5×10^6 to 15×10^6; at the lower molecular-weight range monosaccharides can be separated from disaccharides, and de-salting and other processes previously effected by dialysis can now be effected alternatively. Molecular-sieve chromatography also gives an additional technique to those available previously for assessing molecular-weight distributions and for determining whether a polymer is hetero- or homogeneous. Regretably, molecular-sieve chromatography is still not being used as extensively in polysaccharide chemistry as it is with other natural products.

The other major analytical series of investigations

undertaken involved all aspects of the quantitative determination of uronic acid groups. The sources of error inherent[25] in the classical method of Lefèvre and Tollens were explored fully, and an apparatus and procedure facilitating routine estimations on the semi-micro scale was developed[26]. Earlier investigators had disagreed considerably regarding the reaction-time necessary for complete decarboxylation; since amounts of carbon dioxide — comparatively small but significant — are given linearly with time by non-acidic sugar residues, it follows that a high result is obtained if the reaction is allowed to proceed for the full time required for complete decarboxylation of the uronic acid units present. There are two solutions to this difficulty: <u>either</u> a reaction-time slightly less than theoretical is allowed, so that the correct result is given by a compensation of errors, <u>or</u> the amount of carbon dioxide evolved in the reaction-time from the particular combination of other non-acidic sugar residues present is calculated and deducted - as a "blank" value - from the total. In an attempt to resolve these difficulties, the correct reaction-time for quantitative decarboxylation of uronic acids was found[28] by experiments with carbon-14 labelled carboxyl groups, and the mechanism and kinetics of the acidic decarboxylation of uronic acids was investigated[32]. An analytical method based on thermal decarboxylation in the solid state was assessed[30] and found to have no advantages over the acidic reaction in solution.

For many purposes, particularly for comparative analyses - or "screening" - of large numbers of samples and for routine investigations of well-characterised materials, colorimetric methods of analysis are useful. Many such methods[33] had been proposed for colorimetric estimations of uronic acid groups, and there were conflicting reports[33] regarding their specificity and validity. A comparative study[33] of the results given for a range of polysaccharides by some of the more commonly used colorimetric methods was therefore undertaken. For some classes of polysaccharide, surprisingly good agreement with the acidic decarboxylation value was obtained; for other polysaccharides the discrepancies were alarmingly large, with an unpredictable positive or negative bias.

Previous work[2] involving the Zeisel reaction of hydriodic acid with methoxyl groups had shown that carbon dioxide was a reaction product from acidic, methylated sugars. The conditions for obtaining quantitative recoveries of carbon dioxide from uronic acids were therefore investigated, and it was found[34] that simultaneous determinations of the methoxyl and carboxyl contents were possible on one sample, if the ratio of methoxyl:carboxyl groups fell within a certain favourable range. Decarboxylation with hydriodic acid is still the method of choice; an exhaustive list[34] of the amounts of carbon dioxide evolved from neutral sugars in side-reactions was also prepared.

Methylation is still a vital procedure in the structural analysis of polysaccharides, and recently there have been attempts to improve yields and also to reduce the number of repetitive stages required with the classical methods of Purdie and Haworth. Dimethyl sulphoxide and N,N-dimethylformamide have become fashionable as solvents, and recent developments involving these have been reviewed[39]. A useful methylation system involving sodium hydride-methyl iodide-dimethylsulphoxide, which gives good yields after very few additions of reagents, has been proposed[39]. The method may not be applicable to the complete range of polysaccharides; for example it gives excellent results for Acacia gums containing small amounts of rhamnose, but degradation occurs[47] for these species containing amounts of rhamnose that correspond, on a molar ratio basis, with the uronic acid content.

The ways in which these analytical developments were applied to polysaccharide problems can now be considered.

Fresh-water green algae of the Characeae family have long, filamentous, internodal cells which facilitate biochemical and physiological experiments. Little was known of their chemical constitution. In a series of experiments the carbohydrate constitutions of Nitella translucens[22] and Chara australis[23] were examined; Nitella translucens was found to contain a starch-type polysaccharide[21] and an interesting non-esterified

pectic acid.[24]

In polysaccharide chemistry there are frequently difficulties concerned with extracting or purifying the natural product without simultaneously causing some modification or degradation[29]; this is equally true for both land and marine sources (Hirst, 1958a). It has been all too common practice merely to extract a polysaccharide under what is considered to be the mildest convenient conditions without subsequently testing - possibly by putting part of the material isolated through the same extraction process for a second time - that modification or degradation had not indeed been caused. The labile nature of some of the uronic acid groups in pectic materials, and in polyuronides, was not fully appreciated until it was shown[29] that decarboxylation occurs during extraction with hot water; drastic chemical modification, and/or physical degradation, was found under some of the extraction procedures employed by previous investigators.

An interesting class of acidic polysaccharide can be obtained by solvent extraction procedures from the oleoresin-type exudates given by species of the Boswellia and Commiphora genera. Such polysaccharides have not been studied extensively in the past, and several conflicting statements occur[36] in the few references that deal with exudates from Boswellia serrata and Boswellia carteri. For the latter exudate, Jones and Nunn (1955) found that 4-O-methylglucuronic acid was present; in contrast,

El Khadem and Megahed (1956) reported that galacturonic acid was present, and that the polysaccharide contained acidic and neutral components. The sample studied by El Khadem and Megahed was of commercial origin. Investigation[36] revealed that the commercial samples of gum Frankincense (Boswellia spp.) and gum Myrrh (Commiphora spp.) are liable to be heavily adulterated with other gum species. A sample of Boswellia papyrifera, collected personally by the Sudanese Gum Research Officer, was found[36] to contain large amounts of 4-O-methyl-glucuronic acid, but no galacturonic acid, and the attempts to fractionate the gum polysaccharide were unsuccessful[36]. In further studies of these types of gum exudate it is obvious that commercial samples must be avoided; if samples cannot be verified authoritatively during collection, their identity ought to be substantiated subsequently by expert examination of botanical specimens of the leaves and inflorescence[36] collected for this purpose at the same time as the gum resin.

A further gum-bearing genus regarding which contradictory statements have appeared in the literature is the genus Albizia. Much of the difficulty was found to have resulted from the confusion that exists regarding the botanical nomenclature of species in this genus, and the situation required clarification. This has been done[38]. It is particularly important to realise that, in the past, gum exudates from the Acacia genus were

frequently ascribed to the Albizia genus. Samples of the gums from Albizia sericocephala and Albizia glaberrima were secured for study, and the results indicated[38] that aspects of the earlier work on the exudates from Albizia procera and Albizia glaberrima require re-investigation.

By 1957 it was apparent in certain fields of carbohydrate chemistry e.g. those dealing with starch and glycogen, that sampling procedures and botanical verification of the specimens require strict attention if results of any permanent significance are to be obtained. Even if strict control of botanical species or variety proves possible, variations caused by seasonal effects occur in complex natural products; wide variations in chemical composition had been found in seaweeds, cereal crops, grasses, and clovers. Although it had become widely recognised (Hirst and Jones, 1958) that confusion had arisen in early studies of gum arabic because commercial samples, suspected to be mixtures of Acacia species, had been used, it was not known "whether one tree produces gum with identical analysis from year to year" (Hirst and Jones, 1955). Although it had been found (Jones and Smith, 1949) that there was a "striking uniformity in the structure of gums isolated from different trees of the same type e.g. damson trees and gum arabic from Acacia trees", it was later (Hirst and Jones, 1955) recorded that "there are several varieties of damson, and it is possible that the gums exuded may

vary in composition between varieties and perhaps from tree to tree". Very few earlier investigators had taken the trouble to examine individual nodules of gum: for Fagara xanthoxyloides and Brachychiton diversifolium (cf. ref. 27) it was concluded that the nodules showed no significant variations. To the writer it appeared that these examinations had been too superficial, and that, for a natural product as complex as a gum polysaccharide, some differences, either in chemical or physical properties, could well exist. If this were indeed so, structural studies made on a bulk sample of many nodules from many trees - as was the custom - would be expected to lead to analytical difficulties; in addition to this important factor, it was clear that the nature of any variation from tree to tree - and eventually, from nodule to nodule on a single tree - might provide useful evidence regarding the precursors, biosynthetic processes, and reasons for gum exudation by certain botanical genera.

Inter-nodule experiments are difficult with several gum-forming genera because the gum either forms in small nodules or does not form discrete nodules at all. It was observed, however, that the gum from Combretum leonense existed in discrete, large, nodules weighing 8 - 12 grams. An analytical study of six such nodules was undertaken[27]; by every analytical test made, on both the crude and purified forms of the gum, analytical differences much greater than could arise from analytical error were found.

It became essential to study inter-nodule differences of gums from other genera, and to secure for examination several nodules exuded simultaneously from different parts of one tree. After considerable difficulty, particularly over the latter requirement, the specimens necessary were secured, and the results of these studies are summarised below.

The remainder of this part of the thesis summarises results from studies of gum exudates from the commercially important genus Acacia, which contains many botanically distinguishable species, estimated by one botanical authority (J.P.M. Brenan, private communication) to number 750 - 850.

By 1957, only seven species of Acacia gums had been studied to any great extent[37]. Analytical studies of a further seven species revealed that the atypical features of one of the species studied earlier (Acacia karroo; positive optical rotation and low rhamnose content) also occurred[37] in other species quite frequently. For one of these new species, Acacia seyal Del., eight separate nodules were analysed[31] together with a representative bulk sample from many trees. As found for Combretum leonense, the chemical composition and physical behaviour of A.seyal gum differed from nodule to nodule by amounts greater than could be ascribed to analytical error[31]. A similar analytical study was made[40] of ten separate nodules of gum from Acacia nilotica; nine nodules differed from each other by amounts similar

to those found for C.leonense and A.seyal gums, but one nodule differed to such an extent that it may belong to some other species - not so far studied - even although each nodule analysed had been collected by the most authoritative fieldsman in Sudanese Acacias. Only future studies of new species can clear up this doubt. The fact gives, however, an illustration of how apparent heterogeneity can arise when studies are based on bulk samples obtained from many nodules.

Further studies on A.nilotica revealed[40] that it contained four aldobiouronic acids, only two of which had been detected previously in Acacia exudates. The relative amounts of these acids differed from nodule to nodule, and this gave an indication of the occurrence and extent of some fine structural differences.

Studies of the aldobiouronic acids present in the gums from a further seventeen Acacia species showed[50] that species having positive optical rotations contained all four acids; species with negative rotations do not appear, so far, to contain more than two acids. It is unlikely, however, that this structural difference alone can account for the wide range of optical rotation ($-49^{\circ} \rightarrow +108^{\circ}$) that occurs[37] with the species studied to date.

Further inter-nodule studies were made[43] on the gum from Acacia Laeta var. Hashab. The specimens available facilitated a study of the seasonal variation and of the variation between six

nodules obtained simultaneously from a single tree. Eighteen samples in all were analysed; calculations gave average values for the analytical parameters expressing the composition and properties of A.laeta gum. For the eighteen samples, the extent of the variations from the calculated average values for A.laeta were similar to the variations found for A.seyal[31], but were more marked than for A.nilotica[40]. The variation between the six nodules from one tree was considerably less than that between samples from different trees.

The final experiments on inter-nodule variation were made[48] on Acacia senegal gum. In an extensive study, twelve samples of tapped gum from three different districts of the Sudan (chosen for their different soil profiles) were compared with thirteen different nodules of natural exudate gum. In addition, three nodules from one tree (A) were compared with two nodules from tree (B) and two from tree (C). Average values for the analytical parameters of A.senegal gum were calculated; the variations from the average value were similar in extent to those found previously for other gums[27,31,40,43]. Furthermore, in support of the results for A.laeta gum[43], the three nodules from A.senegal tree A were very closely alike; the same effect was shown by the two nodules from tree B, and by the two nodules from tree C. There is no doubt that whilst gum exudates are characteristic of the species, they are in turn much more

characteristic of particular trees of that species.

It is clear from these inter-nodule studies, which involved much analytical effort, that structural studies should preferably be made on one large nodule of a gum; for species exuding small nodules, the total gum collected from one tree should be used. In either case, several similar samples from other trees of the same species should be analysed to an extent sufficient to ascertain that the structural studies are being made on a specimen that is acceptably typical of the species; this simultaneously establishes the extent of the variation liable to be found in other specimens.

Interesting atypical variants of A.senegal were also studied[48]. These included gum exuded at holes made by wood-boring beetles; this gum was closely similar, after purification, to typical samples of natural exudate or tapped gum. In contrast, a dark-colored, sweet-tasting gum that exudes from the lower, main stem of A.senegal trees differed in two respects from gums obtained by normal tapping of the upper branches. The ash content of a clean sample of gum (pH of solution = 6) was much higher than usual, and few, if any, of its uronic carboxyl groups could have been in the free acid form. Secondly, the rhamnose content was only 50% of that present in typical forms of the gum.

Thus authentic nodules of A.senegal gum can contain different

amounts of rhamnose; furthermore the gum from some *Acacia* species contains 12 - 14% of rhamnose, others 6 - 7%, and some less than 1% of rhamnose. Much of the early evidence for chemical heterogeneity of gum arabic was based on the depleted rhamnose content of certain fractions obtained from commercial samples: the classical work of Heidelberger should be repeated (Whistler, 1959) on specimens from one variety of *Acacia* or, preferably, from a single tree.

The gum from *Acacia drepanolobium* is not completely water-soluble, giving about 20% of an insoluble gel. A graded extraction procedure gave three gum fractions, and an analytical study did not reveal any significant differences in their chemical composition. It was found[54] that dissolution of the water-insoluble gel could be achieved in cold 1% sodium borohydride solution, and Smith-degradation and methylation analyses showed[54] the water-soluble fraction to be very similar structurally to the borohydride-solubilised gel. Molecular-sieve chromatography subsequently indicated that the borohydride-solubilised gel had a much higher molecular weight than the water-soluble gum, and this was confirmed[54] by light-scattering measurements.

It was found[19] that precipitation with nearly saturated solutions of sodium sulphate gave molecular-weight fractions from *Acacia senegal* gum; molecular-sieve chromatography was used to

estimate their values of $\bar{M}_n$. Light-scattering measurements[42] subsequently showed that the whole gum had $\bar{M}_w$ = 580,000, with a very broad molecular weight distribution; the fractions had $\bar{M}_w = 11.85 \times 10^5$, 3.56×10^5, 3.20×10^5, and 1.00×10^5 respectively. The viscosity-molecular weight relationship was then investigated[44], and the constants in the Mark-Houwink modification of the Staudinger equation were found to be $K' = 1.3 \times 10^{-2}$ and $\alpha = 0.54$ for A.senegal gum. A.nubica and A.arabica gums had $\bar{M}_w = 8.71 \times 10^5$ and 18.9×10^5 respectively, and it was shown that the values of K' and α for A.senegal gum are not applicable to all species of Acacia gum. A number of A.senegal gum specimens were subsequently shown[48], however, to have values of $\bar{M}_w$ ranging from $0.26 - 1.16 \times 10^6$; this explained the lack of agreement in previous investigations which had reported $\bar{M}_w$ for A.senegal gum to be 0.58×10^6 and 1.00×10^6 respectively: both results were quite possibly correct for the particular samples studied.

There only remains to summarise the full structural studies made on the gums from Acacia senegal[19,41,49], A.arabica[45], A.nubica[51], A.drepanolobium[52,55], A.laeta[53], and A.seyal[56]. The conventional structural investigations involving partial hydrolyses, linkage analyses, and methylation analyses of the whole and degraded gums were carried out; in addition, a feature of these studies has been the introduction of a procedure involving several

consecutive Smith-degradations. A portion of the degradation product at each stage is subjected to structural analysis, and the decrease in molecular weight is also estimated. The sequence of degradations is continued until all the rhamnose, uronic acid, and arabinose residues, together with some of the galactose, are removed, leaving a residual, periodate-resistant, branched galactan core. This pattern of events occurred with A.senegal[19,41] and A.laeta[53]. For A.arabica[45], A.nubica[51], A.drepanolobium[52,55], and A.seyal[56], a periodate-resistant galactan core is never attained; for these species, the degree of branching of the galactan framework is greater than for A.senegal and A.laeta. The number of Smith degradations necessary to remove all of the arabinose residues was four for A.seyal[56], four for A.senegal[41], and five for A.laeta[53]: an arabinose-free degradation product is not given by A.arabica[45] after four treatments, nor for A.nubica[45] and A.drepanolobium[55] after five treatments. The minimum length of the longest arabinose chains is therefore 5 units for A.arabica, at least 6 units for A.nubica, and - by calculation[55] - at least 8 units for A.drepanolobium. The structural and physico-chemical evidence suggests that the most compact structures are given by A.laeta and A.senegal. There is no evidence for a galactan "back-bone"; the cores are branched galactan frameworks, the degree of branching varying from species to species.

An earlier statement (Jones and Smith, 1949) that "it is not unlikely that while plant gums differ in detail, they may be assembled on one or perhaps two common structural frameworks which are relatively simple" therefore appears to be an oversimplification. Structural variations between gums of different species within the Acacia genus are certainly more pronounced than was at one time supposed. It is particularly important that concepts that such gums are based on "a stable backbone to which is attached side-chains" (Hirst, 1956) or on a "main-chain" (Hirst, 1962) should be superceded. Acacia gums are of relatively low viscosity (Stoloff, 1958) and the concept of a main-chain or "backbone", which suggests linearity, is rather misleading. An early review (Jones and Smith, 1949) suggested that gum arabic had "a highly branched chain structure", but the writer considers the most appropriate description to be "branched galactan frame-work", or "branched core".

The essential features and differences between the gum structures studied are most conveniently conveyed by the diagrams for A.senegal and A.seyal shown on pages 34 - 37. It is essential to realise the limitations of these models: they merely represent one possible assemblage of 100 sugar units that would satisfy the structural evidence available. For clarity in the three-dimensional models all of the short chains attached to the core rise upwards, and the actual galactose residue in the

branched core to which any particular short chain is attached is, of course, arbitrary. In the diagrams that depict the sequence of events in successive Smith degradations, the models are shown as flat, two-dimensional arrays.

It is believed that these structural studies have led to the most detailed elucidations of structure achieved to date for any plant gums. Despite the rapid progress that these models represent, a long programme of work remains before our knowledge of the structure, function, and biosynthesis of these complex natural products could be regarded as adequate.

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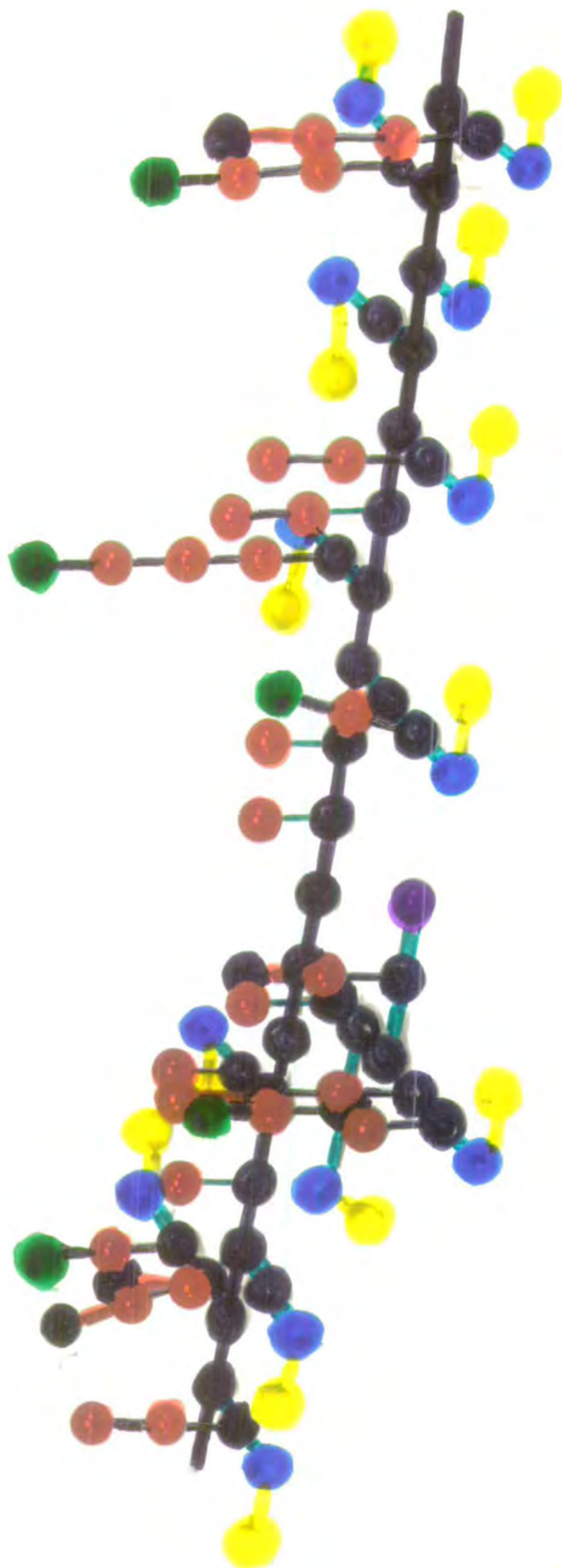


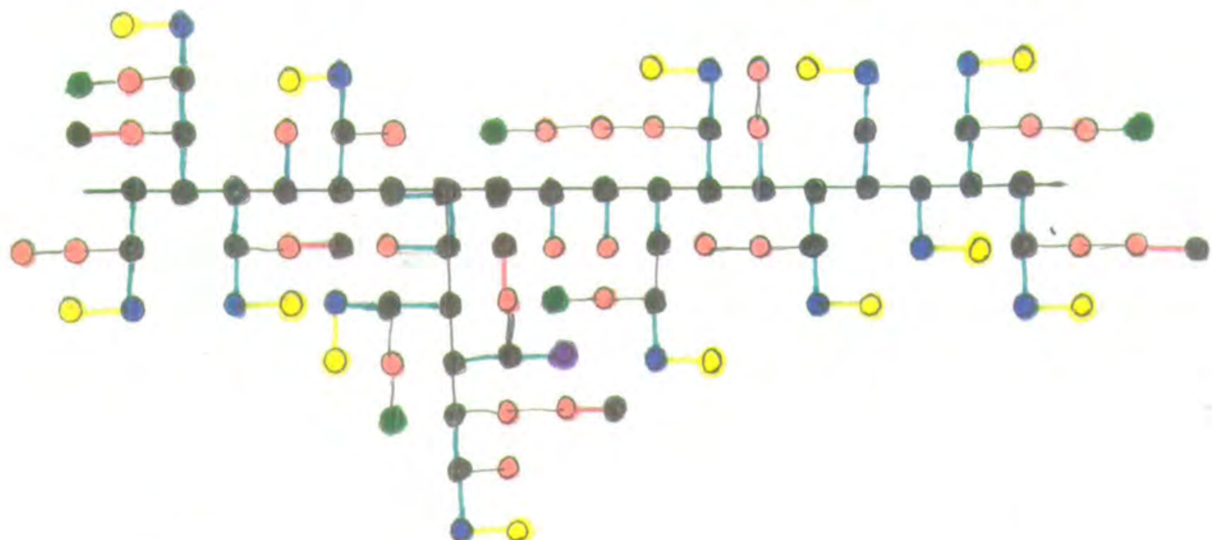
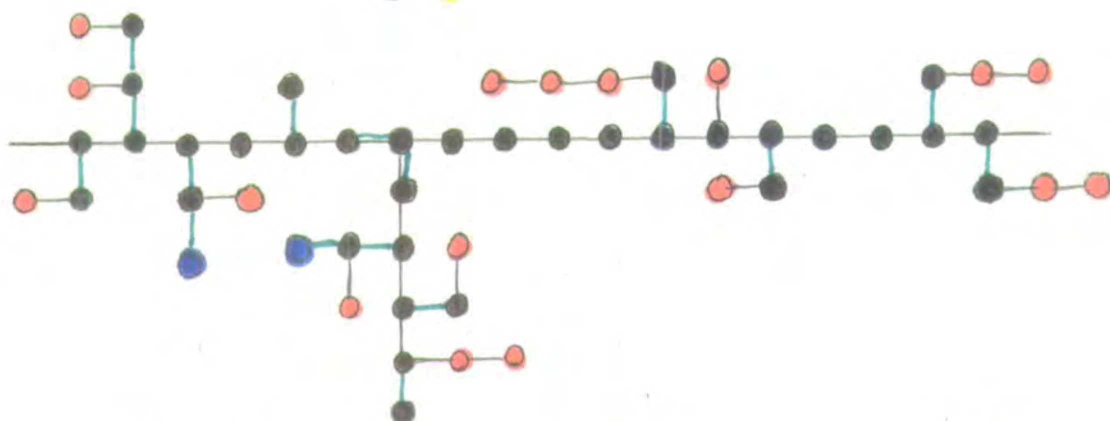
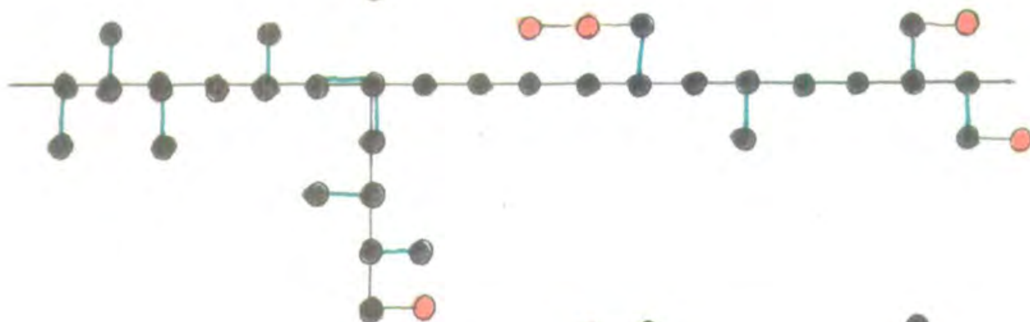
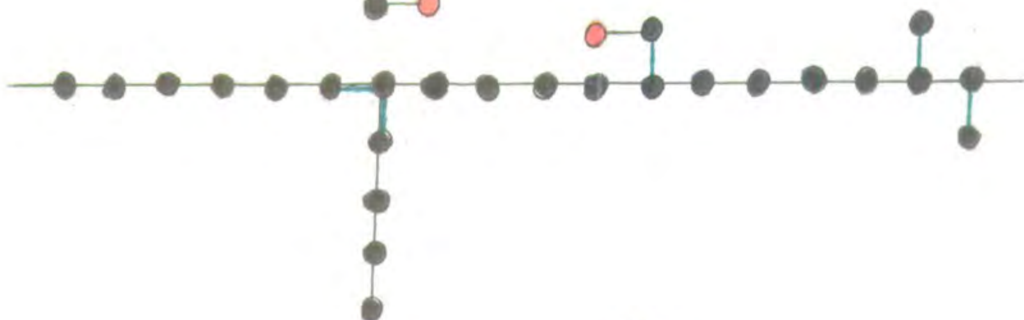
Acacia Senegal gum

Colour Code

Colour	Sugars	molar proportions
Black	D-galactose	42
Red	L-arabofuranose	27
Green	L-arabopyranose	5
Yellow	L-rhamnose	13
Blue	D-glucuronic acid	13
Purple	4-O-methyl-D-glucuronic acid	1

Colour	Bond
Black	$\beta 1,3$-
Red	$\alpha 1,3$-
Green	$\beta 1,6$-
Yellow	$\alpha 1,4$-

Acacia Senegal gum

Acacia senegal gum

Smith Degradation Sequence

← Whole gum.

Molar ratios:

Sugar	Ratio
Galactose	42
Arabofuranose	27
Arabopyranose	5
Rhamnose	13
Glucuronic acid	13
4-O-methylglucuronic acid	1

← First degradation product

Sugar	Ratio
Galactose	34
Arabofuranose	17
Glucuronic acid	2

← Second degradation product

Galactose/arabinose = 32/5

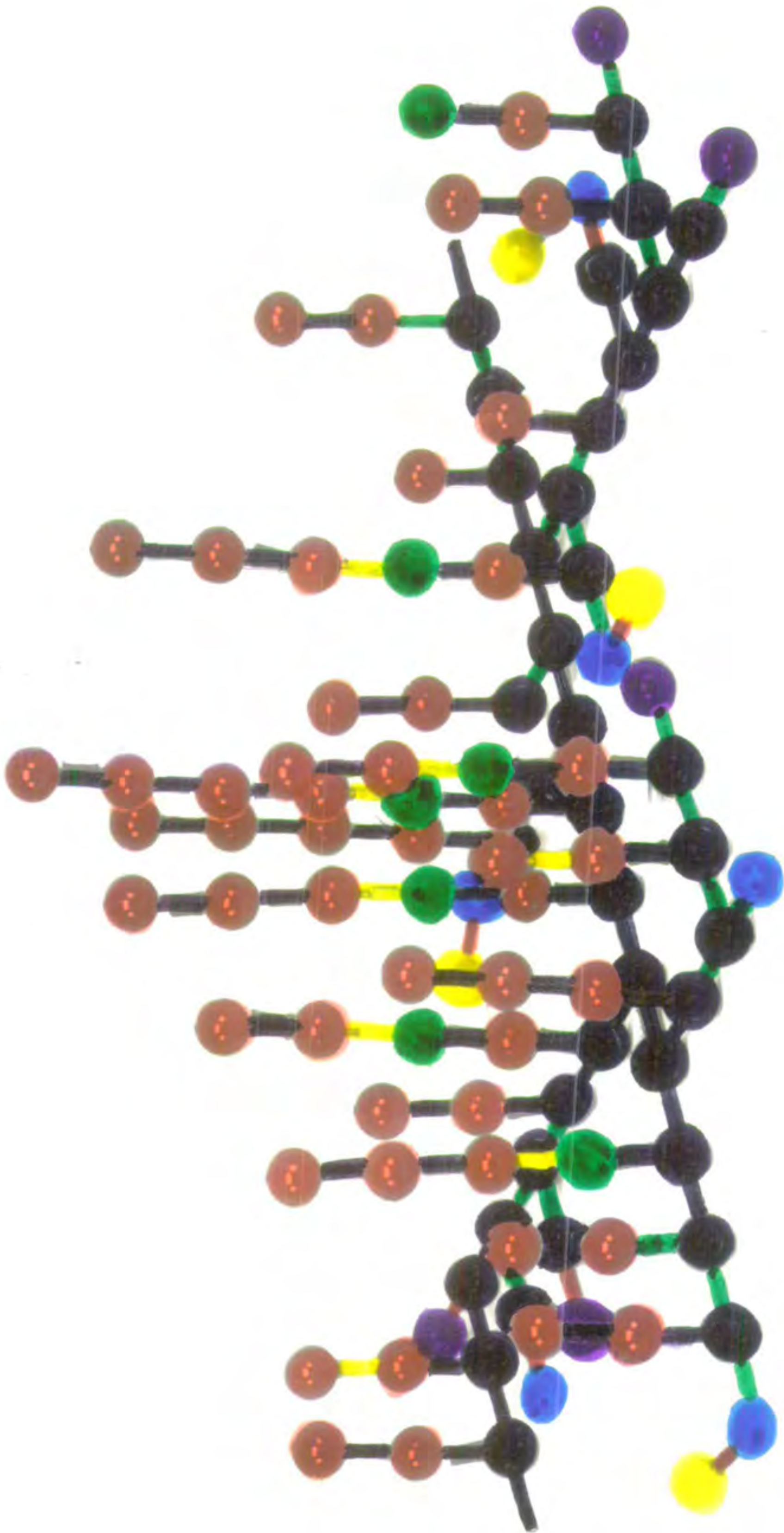
← Third degradation product

Galactose/arabinose = 25/1

← Fourth degradation product

A branched galactan

Colour Code	
Colour	Sugars
Black	D-galactose
Red	L-arabofuranose
Green	L-arabopyranose
Yellow	L-rhamnose
Blue	D-glucuronic acid
Purple	4-O-methyl-D-glucuronic acid
Colour	Linkages
Black	β1,3-
Red	α1,3-
Green	β1,6-
Yellow	α1,4-



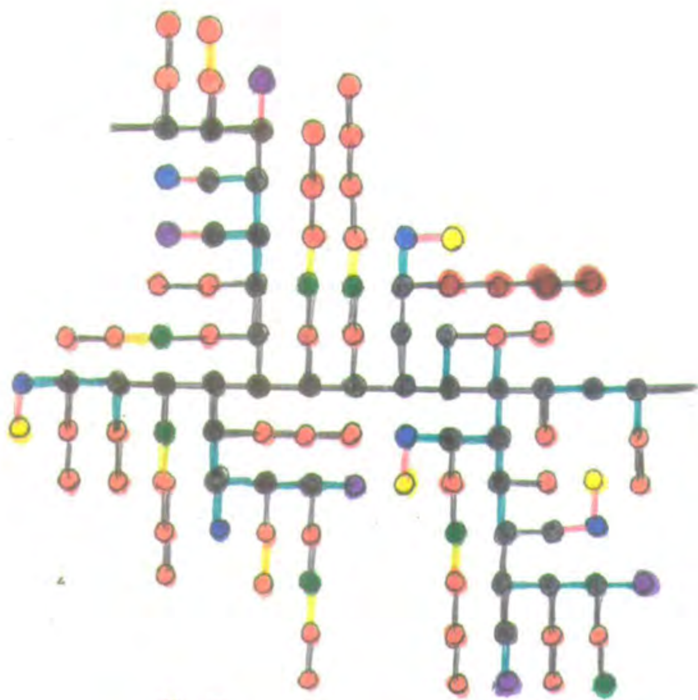
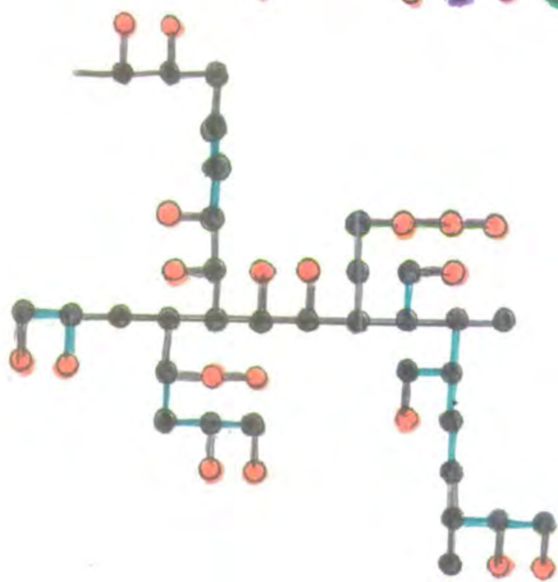
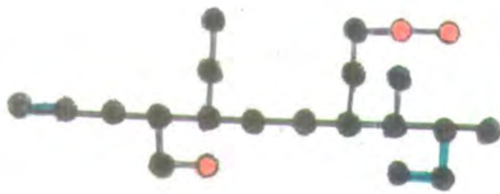
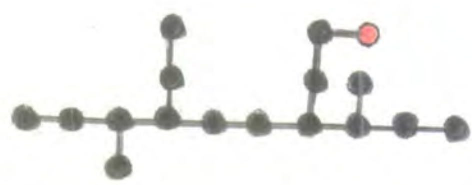
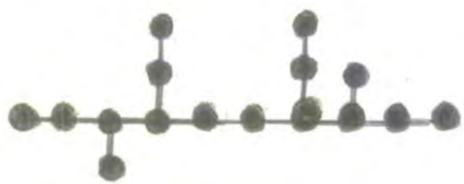
Acacia seyal gum

Colour Code

Colour	Sugars	Molar proportions
Black	D-galactose	38
Red	L-arabofuranose	50
Green	L-arabopyranose	7
Yellow	L-rhamnose	4
Blue	D-glucuronic acid	6
Brown	4-O-methylglucuronic acid	5

Colour	Bond
Black	β1,3-
Red	α1,4-
Green	β1,6-
Yellow	1,2-

Acacia seyal gum

Acacia seyal gum

Smith Degradation Sequence

Colour Code	
Colour	Sugars
Black	D-galactose
Red	L-arabofuranose
Green	L-arabopyranose
Yellow	L-rhamnose
Blue	D-glucuronic acid
Purple	4-O-methyl-glucuronic acid
Colour	Linkages
Black	β1,3-
Red	α1,4-
Green	β1,6-
Yellow	1,2-

Whole Gum

Molar ratios:-

Galactose	38
Arabofuranose	50
Arabopyranose	7
Rhamnose	4
Glucuronic acid	6
4-O-methylglucuronic acid	5

First degradation product

Galactose/arabinose = 33/19

Second degradation product

Galactose/arabinose = 19/3

Third degradation product

Galactose/arabinose = 16/1

Fourth degradation product

A branched galactan

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Of my own teachers, I owe most to Dr. Christina C. Miller and (the late) Dr. Mowbray Ritchie for inculcating that good experimental techniques and good analysis are essential in chemistry. I am also indebted to Dr. (now Professor) Neil Campbell, O.B.E., and Professor F. Bell for their preaching, in 1958, that it was still possible to carry out original academic research efficiently without the necessity of great financial expense: the studies described in this Thesis were completed in eleven years, in association with only eleven research students, without the assistance of major financial grants.

It is a pleasure to acknowledge the industry and experimental skill attained by my research students, who received maintenance awards from the Science Research Council, (to Drs. N.J. King, S. Garbutt, G.M. Cree, J.F. Stoddart, and I.C.M. Dea); the Carnegie Trust (to Dr. J.L. Duncan); the Sudanese Department of Education (to Dr. K.A. Karamalla); the Pakistani High Commission (to Drs. S.S.H. Zaidi and S. Rahman); and Messrs. Rowntree & Co. Ltd., Samuel Jones & Co. Ltd., and Laing-National Ltd. (to Drs. M.A. Herbich and R.N. Smith).

In conclusion I must record the tolerance and understanding shown by my wife, who has had to endure, for ten years, a person obsessed with the complexity of the challenge to chemical analysis presented by plant gums. There are still sufficient problems left to form the basis for a life-time of chemical investigation.

1.

Applications of Infra-red Spectroscopy: The Identification and Determination of Gas-chromatographic Fractions

BY

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Applications of Infra-red Spectroscopy: The Identification and Determination of Gas-chromatographic Fractions

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The desirability of identifying products by a method other than comparison of retention times is stressed. Details of a simple method of collecting products separated from mixtures by gas chromatography are given, and the technique subsequently used for their identification and quantitative determination by vapour-phase infra-red spectroscopy is described. The many advantages of infra-red examination of vapours rather than liquids, when only fractional milligram amounts are available, are outlined. Amounts of about 5 μmole of all vapours or liquids having a boiling-point of up to about 175° C can be identified and quantitatively determined to within ±2 per cent. without difficulty. Experiments are in progress to increase the sensitivity and applicability of the method.

IN routine analyses of products from well characterised reaction systems, and possibly for research investigations in certain fields,[1] it is often possible to obtain quantitative and qualitative results of acceptable accuracy from relative retention times on a carefully controlled column. For many purposes, however, identification of separated components on gas-chromatographic evidence alone is inadequate.

Column-retention times are purely relative and not always exactly reproducible; even when the chemical class (*e.g.*, ketone, hydrocarbon etc.) of the expected reaction products is known, identification by comparison of the retention time of the unknown with those of standards requires exact reproduction of column operating conditions.[2]

Difficulty can also arise when non-symmetrical peaks are produced[3]; the peak-maximum retention time of a component then depends on concentration. Peaks can also overlap,[4,5] and certain combinations of substances are often difficult to separate.[5] Chromatographically, a single peak is no criterion of purity, since more than one substance may be present[6]; the components present could often be resolved if their presence was suspected and alternative column operating conditions were selected.

Confirmation of homogeneity of fractions is therefore required, together with unequivocal identification and accurate quantitative determination of the product. The analytical method used should involve some property of the molecule other than boiling-point. Often,

only fractional milligram amounts of material can be recovered from a column; few methods therefore remain applicable. Mass-spectroscopic examination of fractions on this scale has been described,[4,7,8,9] but instances have been reported[10] in which infra-red spectroscopy was also required.

The tremendously powerful technique of coupling gas-chromatographic separation with subsequent infra-red identification has long been recognised and advocated.[11,12,13,14,15] Greater use of the combined techniques has not so far been made because samples for gas chromatography are normally smaller by a factor of about 10^2 than are those required for spectroscopy. However, attention is now being given commercially to production of infra-red cells and accessories for micro-analysis; for instance, a liquid cell of capacity 80 μl has been described.[16] Progress is also being made with scale-expansion techniques and development of infra-red microscopes, by which single fibres and crystals may be examined.

This paper describes a technique that has been used in this department during the past 2 years for, *e.g.*, the identification of gas-chromatographically fractionated products from the following investigations—

(*i*) Reactions of methylene radicles with propylene and *cyclo*propane,[17] *iso*butene[18] and acetylenes and allenes.[19]
(*ii*) High-temperature oxidation of propane.[20]
(*iii*) Photolysis of *cyclo*pentanone.[21]
(*iv*) Irradiation of organic chloro compounds with cobalt-60.[22]
(*v*) Retention of solvents and moisture by carbohydrates.[23]
(*vi*) Acidic decomposition products of carbohydrates.[24]

Gas - liquid chromatography has been used throughout for separation, but the technique is applicable to products separated by gas - solid chromatography. In addition, it has been used for the collection and infra-red examination (without prior separation by gas - liquid chromatography) of mixtures of reaction products, such as methyl and ethyl iodides from semi-micro alkoxyl determinations[25] and carbon dioxide and furan from uronic acid determinations.[26]

The method has the advantages of (*a*) ease of handling, particularly of volatile liquids, (*b*) greatly increased sensitivity, (*c*) increased accuracy of quantitative determination, and (*d*) ease of quantitative recovery of all samples. No originality is claimed for the technique; it is described in the belief that its simplicity, scope and advantages may not be fully recognised. No expensive ancillary infra-red equipment is required.

Experimental

Apparatus and procedure—

Fig. 1 (*b*) shows the dimensions of the trap used. The exit of the gas-chromatographic column is connected (via an anhydrone tube if necessary) by a B10 ground-glass joint to A (traps made more recently have hemispherical joints). With an anhydrone guard-tube fitted to B, trap XY is immersed in a flask of liquid nitrogen. Any carrier gas can be used, although carbon dioxide, if used, must be removed before passage through the trap. At flow rates of 15 to 20 ml per minute, milligram amounts of vapours and liquids having boiling-points greater than about $-120°$ C are quantitatively trapped with no retention of carrier gas. Under these conditions, the trap appears to have an adequate safety factor, and discrete frozen droplets, which could be swept as such through the trap, do not form.[27] Should, however, this simple trap prove to be inadequate under certain conditions, a trap for recovery of extremely small fractions has been described.[28] A simple 3-way stopcock junction system similar to that already described[5] has been used to facilitate collection, in separate traps, of fractions emerging from a column in close succession.

The trapped samples, stoppered at A and with tap T_1 closed, are kept in liquid nitrogen until required. The B10 cone of an evacuated gas-cell is inserted into A, shown in Fig 1 (*b*), tap T_2 being closed. The cone B is then attached to a vacuum-line, with trap XY still immersed in liquid nitrogen. Taps T_1 and T_2 are then opened and the whole system is evacuated to a pressure of between 0·5 and 1 mm of mercury. Tap T_1 is then closed, the assembly is removed from the vacuum-line and an anhydrone guard-tube is fitted to B. Trap XY is then withdrawn from the liquid nitrogen and immersed in boiling water or warmed gently in a bunsen flame. The former method of heating is preferred, but the latter was found to be necessary for higher-boiling liquids. When the trapped substance has completely

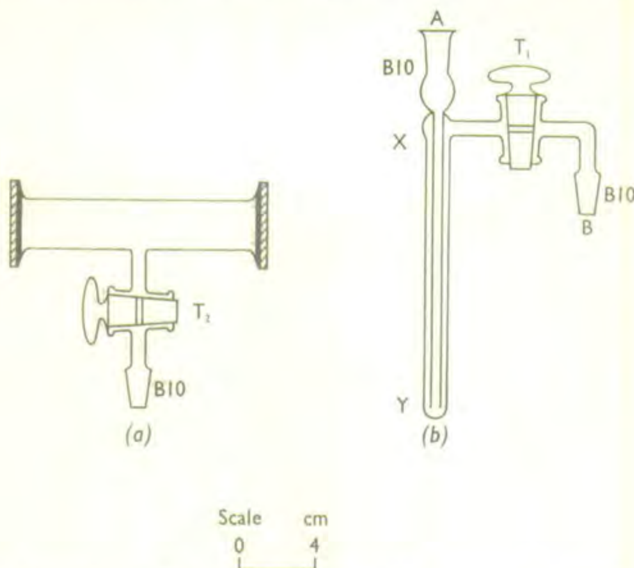

Fig. 1. Apparatus used for collecting separated products: (*a*) gas-cell, (*b*) trap

vaporised, tap T_1 is quickly opened so that the residual vapour in trap XY is swept into the gas-cell; the contents are then at atmospheric pressure. This serves as a standard condition for the examination; daily variations in atmospheric pressure are insignificant and the conditions for automatic double-beam compensation for atmospheric carbon dioxide and water vapour are not upset. The procedure also gives increased sensitivity; when the total pressure is made atmospheric by adding infra-red inactive gases to a gas-cell containing a compound at a partial pressure of 1 mm, the peak height is greater than that produced when the total pressure is allowed to remain at 1 mm. "Pressure broadening" effects are considered later.

RECOVERY OF SAMPLES—

Replace the cone of the gas-cell in socket A, and attach B to the vacuum-line. With tap T_2 closed, evacuate trap XY and immerse it in liquid nitrogen. Close tap T_1 and open tap T_2. Close tap T_2, and, after 10 to 20 seconds, open tap T_1 and re-evacuate trap XY. Repeat this cycle several times, then finally evacuate completely with taps T_1 and T_2 both open. Quantitative recovery has been repeatedly proved by returning the recovered product to the gas-cell in the usual way and re-running the spectrogram; the peak heights previously obtained are duplicated. When it is found that insufficient material has been available for analysis, recovery of the sample into the trap and collection therein of the identical peak from a second chromatographic separation generally permits sufficient material to be collected. No undesirable retention of products in the traps or gas-cells has been observed if continuous evacuation for 10 to 15 minutes is carried out between analyses. The amount of Apiezon or silicone grease on taps and ground-glass joints should, however, be kept to a minimum.

DISCUSSION OF THE METHOD

APPLICABILITY—

When conventional infra-red unheated gas-cells fitted with sodium chloride end-plates secured by Bedacryl 122X resin (kindly supplied by Imperial Chemical Industries Ltd., Dyestuffs Division) are used, the method is applicable to all vapours and liquids of low or medium boiling-point. Generally, partial pressures of 1 to 2 mm admitted to a cell of volume 64 ml (the pressure subsequently being made atmospheric in the way already described) give satisfactory spectra. No condensation of the substance occurs during the period of examination (usually 10 to 15 minutes) if the boiling-point is not too high. Spectra for furfuryl alcohol (boiling-point 170° C) and for benzaldehyde (boiling-point 179° C) have been obtained in this way, but boiling-points of 175° to 180° C probably represent the upper limit for unheated cells. The use of heated cells is well known,[29,30] however, and experiments are in progress to discover how far their use will extend the applicability of this technique.

SENSITIVITY—

The infra-red absorption of different compounds varies widely, and the method has maximum sensitivity for compounds (*a*) of a polar character, and (*b*) of low molecular weight. The data in Table I, however, are representative; for predominantly non-polar compounds, the sensitivity will only decrease by a factor of about 2. When a gas-cell of length approximately 12·5 cm, internal diameter approximately 2·5 cm and measured internal volume 64 ml is used, the sensitivity shown in Table I is attainable.

TABLE I

SENSITIVITY OF THE METHOD

Compound	Minimum amount of compound detectable, μg	Minimum amount of compound detectable, μmole	Partial pressure required: Minimum, mm of mercury	Partial pressure required: Maximum, mm of mercury
Methanol	130	4 to 5	1·0	2·0
Ethanol	190			
Acetone	240			
*iso*Propyl alcohol	250			
Diethyl ether	300			
Chlorinated solvents	—			
Benzene	470	6 to 7	1·5	2·5
Furan	410			
Dioxan	530			

Use of the partial pressures shown in Table I gives satisfactory spectra and permits positive identification of the compound; possible information regarding freedom from contaminants is also provided. In certain limited instances, when the reaction product is almost certainly known, confirmation of identity and an estimate of the amount present can be made on much smaller amounts (about 10 per cent. of those in Table I) by referring only to the most intense absorptions in the spectrum.

QUANTITATIVE DETERMINATIONS—

Spectra for the liquid and vapour forms of a compound usually differ. Identification must therefore be made by comparison with spectra for standard vapours. A series of standard spectra can be obtained over as wide a concentration range as is desired for each compound under investigation by using a simple manometer system, the essential features of which are shown in Fig. 2. Initially, a partial pressure of the vapour under test equal to 16 mm of mercury is admitted to the 64-ml gas-cell, this pressure being read from the scale (graduated in millimetres) attached to the manometer limb. With care, satisfactory

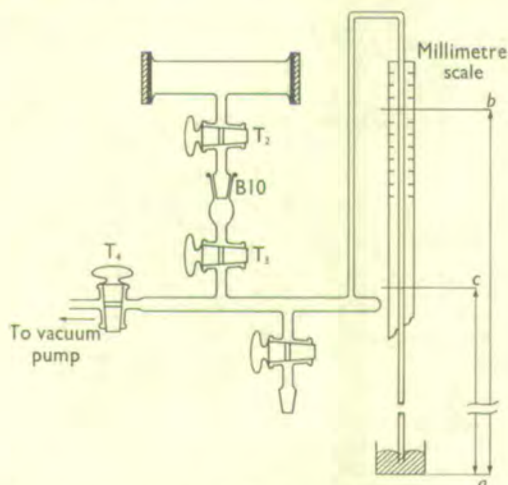

Fig. 2. Manometer system used for determinations

reproducibility in this operation was possible. Accurate dilutions in the gas-cell, which, in turn, give partial pressures of 8, 4, 2, 1 and 0·5 mm of mercury, are subsequently conveniently made by using the manometer, which can readily be constructed by successive glass-working refinements so that its internal volume, when a pressure equal to 50 per cent. of atmospheric is being indicated, is equal to that of the gas-cell. This is easily checked experimentally. The gas-cell, with contents at atmospheric pressure and tap T_2 closed, is fitted to the manifold, as shown in Fig. 2. The manifold system is completely evacuated with tap T_3 open; mercury rises to level *b*. The correct internal volume of the manifold is attained when opening tap T_2 causes the mercury level to drop from *b* to *c*, where $ac = ab/2$. Construction of a manifold satisfying this condition to within 3 mm of mercury was easily achieved; this was considered to be adequate, as daily variations in atmospheric pressure were not taken into account.

When the partial pressure of the gas under examination has been halved in this way, air is admitted to restore atmospheric pressure in the gas-cell; the infra-red spectrum for this concentration is then recorded.

The method described is considered to permit determination of concentrations to within ± 2 per cent. Standardisation need not be tedious, since, for any vapour under test, calibration on any one carefully selected characteristic absorption in the spectrum is usually adequate. Peak areas, not peak heights, are proportional to concentration.[31,32] The relationship between peak height and concentration is not linear, and care should be taken to compare peak areas when comparisons are made with spectra representing standard concentrations.

Obvious refinements to the apparatus can be introduced should increased accuracy of determination be desired. Consideration would then have to be given to the effect of "pressure broadening"; in a gaseous mixture, each gas (even homopolar diatomic gases that have no infra-red absorption) affects the optical density of the others, thereby causing deviations from Beer's law. Calibration curves for correcting these deviations can be experimentally plotted and applied, so permitting concentrations to be measured to within $\pm 0 \cdot 1$ per cent.[33]

DETAILS OF INFRA-RED SPECTROMETER—

A Hilger H800 double-beam instrument was used; it has a large area available for the introduction of sample cells and can accommodate cells up to 60 cm in length. Experiments are in progress to evaulate the ultimate sensitivity of this method without recourse to the use of optical cells of multiple path length.[34,35] For instance, recent work has shown that a gas-cell (length 32 cm, internal diameter 1·5 cm and volume 51 ml) constructed by fusing together the non-flange ends of two ground-glass flanges (Quickfit and Quartz type FG15) increases the sensitivity shown in Table I by a factor of 40 to 50. The use of a gas-cell of this length introduces appreciable energy losses in the sample beam (an effect not caused by a 12·5-cm cell) and it is necessary to insert a compensating cell of similar dimensions in the reference beam. Further work is in progress, however, and will be reported elsewhere at an early date.

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APPLICATIONS OF INFRARED SPECTROSCOPY—II*

OBSERVATIONS ON SOME ASPECTS OF THE ZEISEL ALKOXYL DETERMINATION

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Summary—A sensitive infrared method for quantitative determination of vapours has been used to study some of the reaction variables in the Zeisel alkoxyl determination. Several conflicting reports in the literature have been clarified. Reaction conditions giving rapid and accurate determinations on solids and volatile liquids, even in presence of large sulphur concentrations, are described: for determinations on vanillin the standard deviation is 0·16%. Interaction between alkyl iodides and sulphuretted hydrogen has been found to occur only in aqueous solutions; the use of soda-asbestos as a solid scrubber therefore has fundamental advantages over aqueous solutions, and gives excellent results.

ALTHOUGH the determination of alkoxyl groups is still based on Zeisel's classical method,[1] an iodometric procedure[2] has largely superseded the original gravimetric technique. In addition, an almost continuous catalogue of modifications to procedure, reagents, scrubber composition and apparatus design has been published. (See, for instance, references 3, 4, 5, 6, 7, 8, 9 and 10.) Since conflicting recommendations and statements still exist in the literature, an attempt to clarify the present position seemed desirable.

METHOD OF INVESTIGATION

A sensitive infrared technique,[11,12] developed recently for the quantitative determination of substances in the vapour phase, has been used to study certain stages of the alkoxyl determination. Milligram-quantities of the lower alkyl iodides can be trapped quantitatively in liquid nitrogen[11] and can subsequently be determined by referring the heights of selected characteristic peaks in their infrared spectrum to a carefully constructed calibration curve. This is previously obtained under carefully standardised spectroscopic conditions by quantitatively volatilising weighed amounts of purified alkyl iodides into the infrared gas-cell. (Experimental details of the procedures involved will be given in a subsequent paper[35] describing an infrared method for the simultaneous determination of methoxyl and ethoxyl groups.) Other vapours, *e.g.* hydrogen sulphide, hydrogen iodide and iodine, do not interfere with the determination since there is (*a*) no reaction between these and alkyl iodides in the vapour phase, and (*b*) no overlapping of peaks in their infrared spectra.

Since it is necessary in infrared spectroscopy to exclude water-vapour, the volatile reaction products are trapped after passage through Anhydrone; an Anhydrone guard-tube must be fitted to the trap, as shown in Fig. 1. Careful preliminary tests showed that, of the normal volatile reaction products, Anhydrone retained only water-vapour.

* Part I: D. M. W. Anderson, *Analyst*, 1959, **84**, 50.

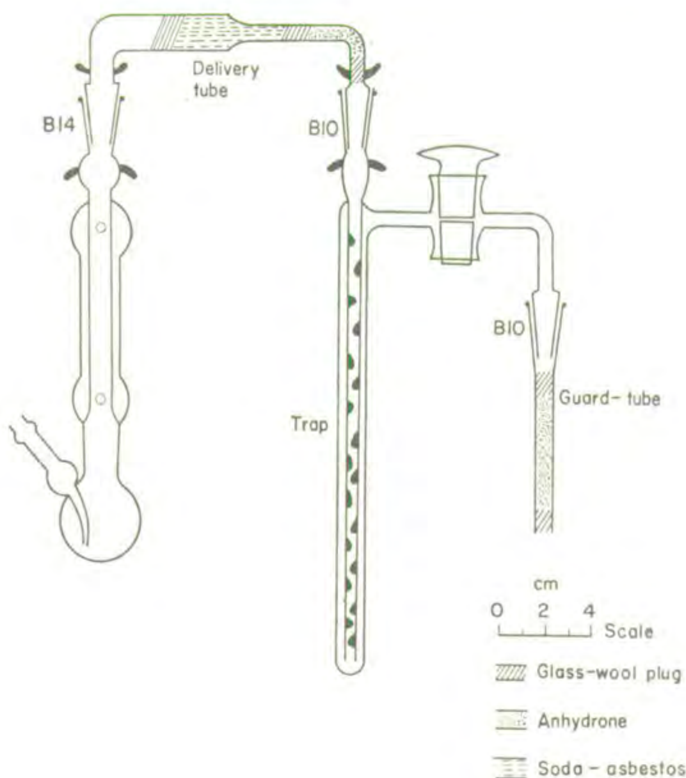

FIG. 1. Assembled apparatus.

The reaction products could therefore be trapped either before or after passage through scrubbers or absorbers of the type normally used in Zeisel determinations. The subsequent spectroscopic examination not only revealed which alkyl iodide had been produced and permitted simultaneous measurement of its concentration, but also revealed if any other volatile product affecting the validity of the result had been produced. The high sensitivity and adequate accuracy of this infrared technique conveniently gave information on (*a*) efficiency of removal of hydrogen sulphide by scrubbers (*b*) sources of loss of alkyl iodide, and (*c*) anomalous reaction of certain compounds.

EXPERIMENTAL

Reagents

Hydriodic acid: M.A.R., sp. gr. 1·7 (6-ml ampoules).
Phenol: AnalaR.
Anhydrone: M.A.R., 14–22 mesh.
Soda-asbestos: M.A.R.
Alkyl iodides: for calibration purposes, the reagent grade was re-distilled three times; the still-head was packed with Anhydrone and the middle fraction collected each time.

Standard compounds

α-Methyl-D-glucoside
Vanillin
Phenacetin } organic analytical standards.

In addition, *vanillin* (microanalytical standard grade) purified by zone-melting was used.

Apparatus

This was assembled as shown in Fig. 1, and consisted of:

(*a*) the combined reaction-flask and condenser described in B.S. 1428: part C 1:9154 (type-2 apparatus).

(*b*) a delivery-tube, which could be packed with Anhydrone and soda-asbestos. Ground-glass joints were lightly coated with silicone grease.

(*c*) a trap, similar to that previously described.[11] Recent traps have been made with Vigreux-type indentations in the inner absorption tube, as shown in Fig. 1.

(*d*) the design of scrubber described in B.S. 1428: part C 1:1954 was used to investigate the efficiency of aqueous scrubbing solutions.

Calibration curves

Fig. 2 shows a typical calibration curve, constructed by making about 5 different measurements per 5-mg range of alkyl iodide using a Hilger double-beam infrared spectrometer. The gas-cells described

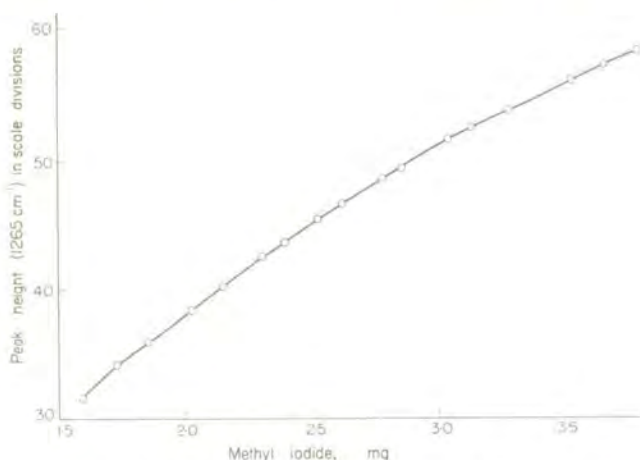

FIG. 2. Calibration curve for determination of methyl iodide.

in references 11 and 12 were used: gas-cells "A" (length 12·5 cm) and "B" (length 31·5 cm) respectively permitted determination of 4–10 mg and 1–4 mg quantities of methyl iodide.

Weighing of samples

For solids, a long-handled weighing-spoon[10] was used. Volatile liquids were conveniently weighed in a long-handled micro weighing-bottle[10] fitted with a leak-proof ground-glass stopper.

Flow-gas

The infrared technique required nitrogen to be used. Nitrogen ("N.O.F." grade) was passed through soda-asbestos, silica gel and Anhydrone. The flow-rate, stabilised by needle-valves and passage through a large reservoir fitted with capillary outlet, was adjusted by a Rotameter, Type 704, calibrated over the range 4–25 ml per min.

Heating of reaction-flask

An electrothermal flexible heating mantle, Type MBJ 1822, gave very steady ebullition. Recent papers[13] still report difficulty with "bumping", although electrical heating was earlier reported to be advantageous.[14]

Preliminary determinations using standards

(*a*) *Conditioning of acid and apparatus:* The contents of one 6-ml ampoule of hydriodic acid was added to the reaction flask and refluxed for 30 min under a steady flow of nitrogen (6–8 ml per min).

This procedure is advisable: it conditions the apparatus, ensures that the acid is constant-boiling, and decomposes any excess hypophosphorous acid which may have been added. (In any infrared investigations, all traces of phosphine must be removed, since its absorption peaks overlap the particular alkyl iodide peaks used for determination).

(*b*) *Determination of reaction-times and optimum flow-rate:* Recommended flow-rates have varied between 4[7,15] and 25[8] ml per min. For 2–5 mg samples and a flow-rate of 6–8 ml per min, we obtained quantitative recovery of methyl iodide from vanillin in 10–15 min and of ethyl iodide from phenacetin in 15–20 min from start of reflux. These times vary little from those found by Kirsten[8] (25 ml per min) and by Inglis[9] (12–15 ml per min). Since some compounds could possibly react more slowly than these standards, a reflux period of 30 min was adopted for methoxyl and ethoxyl compounds, and has since proved adequate for many organic compounds containing a wide range of other functional groups and analysed as "unknowns" by one of us (J. L. D.)

Although Inglis used carbon dioxide flow-gas at 12–15 ml per min with one scrubber (to minimise the tendency for methyl iodide to dissolve in aqueous solution), it had earlier been established[10] that a flow-rate of 6 ml per min caused high results if a second scrubber was not used. Pending investigation of these conflicting reports of scrubber efficiency, we considered that, since the reaction-times quoted above were sufficiently short for routine analyses, and were not in any case decreased appreciably by using faster flow-rates, the use of a slow flow-rate was fundamentally correct experimentally in minimising tendencies towards (*a*) inadequate gas scrubbing, and (*b*) incomplete alkyl iodide retention in the final stage. Any scrubber solution in which alkyl iodides react or are appreciably soluble at low flow-rates is unsatisfactory in any event. A flow-rate of 6–8 ml per min was therefore adopted as standard.

(*c*) *Results:* Table I shows the results obtained for standard compounds. This gives a true representation of the poorest results that may be expected, since none of the results obtained was rejected; the standard deviation is also shown.

TABLE I. RESULTS OF ALKOXYL DETERMINATIONS ON STANDARD COMPOUNDS

Compound	Range of sample weights taken, *mg*	No. of detns.	Alkoxyl content		Stand. Devn., %	Max. divergence from theory	
			Theory, %	Av. found, %		+	−
Vanillin	1·84–3·39	15	20·40	20·39	0·16	0·28	0·28
α-Methyl-D-glucoside	2·38–4·36	5	15·99	15·99	0·08*	0·11	0·07
Phenacetin	2·82–3·67	6	25·15	25·18	0·17*	0·20	0·23

* Calculated by the range method.

Having established that the reaction conditions described and the spectroscopic method of determining alkyl iodides gave satisfactory analyses of pure reference compounds, the following investigations were made.

INVESTIGATIONS

1. *Concentration and colour of hydriodic acid*

Most analysts have found constant-boiling hydriodic acid adequate, but the use of more concentrated acid (sp. gr. 1·96) has been proposed,[16] and the addition of a few drops of this concentration to the constant-boiling acid suggested.[5] Reflux for 30 min over red phosphorus before use has been recommended.[6,17] We have found the M.A.R. constant-boiling acid, conditioned as already described, to be satisfactory; tests showed that, at 6–8 ml per min, only trace amounts, if any, of hydriodic acid vapour were volatilised. The use of more concentrated acid would give increased amounts of distilling acid vapour, so introducing a possible source of error.

Furter[4] stated that the use of fresh colourless acid was essential. Steyermark,[18] however, found that the colour was not critical if the acid was refluxed before use: more recently it was observed[19] that the presence of free iodine in the hydriodic acid was in fact advantageous. Belcher[7] obtained acceptable results titrimetrically in the presence of 20 mg added iodine, although an increased blank was observed. Our experiments confirm that the acid colour has no effect on the yield of alkyl iodide. Appreciable volatilisation of iodine does, however, occur from very dark mixtures, particularly if the compound under analysis contains sulphur or strong reducing groups, or, as can occur with carbohydrate materials, is readily decomposed to give carbon. As Kirsten pointed out,[8] this may impose an additional test on the scrubber efficiency, although much of the iodine condenses out before reaching the scrubber.

2. *Repeated use of acid in routine analyses*

In routine analyses when compounds are known to decompose quantitatively in the normal reaction-time, one charge of acid can safely be used for 8 determinations (av. sample weight = 5 mg) if the mixture is refluxed between each determination: to allow a safety margin, we have always used a reflux period of 30 min, but a shorter period may be found adequate. In one series of experiments, 14 successive determinations were satisfactorily made without change of acid. Care must always be exercised, however: we have found certain compounds[20] which liberate alkyl iodides continuously for several hours.

3. *Determinations on volatile liquids*

Earlier investigations found double distillation[9,18,21] or the use of modified apparatus[8] necessary for quantitative results. Double distillation is not essential when the following procedure is used:—

Weigh the sample as already described. Transfer to a reaction flask charged with a mixture of hydriodic acid (6 ml) + phenol (approximately 0·25 g) which has been conditioned as described for 30 min, *then allowed to cool completely*. The small stoppered weighing-bottle must submerge completely in the phenol-hydriodic acid mixture. Shake the reaction flask to loosen the weighing-bottle stopper. Using a nitrogen flow-rate of 2–4 ml per min, heat gently so that reflux begins after 8 to 10 min, then increase the flow-rate to 6 to 8 ml per minute and reflux for 30 minutes.

Compounds such as methanol, anisole, 1:1- and 1:2-dimethoxyethanes, and ethyl orthoformate have been analysed without difficulty in this way. Infrared experiments with anisole revealed that no detectable traces of unreacted anisole were volatilised together with the methyl iodide unless the flow-rate was increased to >15 ml per min.

4. *Effect of presence of sulphur*

The presence of sulphur has long been known to complicate alkoxyl analyses, causing low results. Sulphur present in organic functional groups is usually reduced to hydrogen sulphide by reflux with hydriodic acid; inorganic sulphate reacts similarly. Commonly only 1 sulphur atom is present, but the ratio of sulphur atoms to alkoxyl groups in compounds can exceed 1:1. It is of importance that the analysis of compounds mixed with relatively large amounts of inorganic sulphate has caused particular difficulty.[22,23]

An interaction between hydrogen sulphide and alkyl iodides, resulting in mercaptan formation,[1,24] is considered to cause the low results; Bethge and Carlson[25] suggested

that this reaction is favoured in alkaline solution. The infrared technique has shown that *no* reaction between hydrogen sulphide and alkyl iodides occurs in the vapour phase: alkoxyl compounds containing sulphur, or to which had been added sodium sulphate, gave theoretical yields of alkyl iodide in presence of the evolved hydrogen sulphide. The undesired interaction must therefore occur only *in aqueous solution*.

Mere absorption of hydrogen sulphide by scrubbing solutions is therefore insufficient; quantitative removal of sulphide ions from solution in a rapid reaction is essential. Scrubbers must also effectively remove iodine and hydrogen iodide vapours: although the use of (*a*) low flow-rates, and (*b*) pre-conditioning of the hydriodic acid should ensure that the amounts of these involved are normally very small, there will be a tendency for increased volatilisation of iodine when much hydrogen sulphide is released. Thus any scrubber able to remove satisfactorily the large amounts of hydrogen sulphide liberated when excess inorganic sulphate is present will have a safety-factor when sulphur-containing organic compounds are analysed. The following investigation of scrubber efficiency was made from this viewpoint.

5. *The efficiency of aqueous scrubbing solutions*

The functional efficiency of the design of scrubber used must also be considered here; the volume of scrubbing solution and the flow-rate used will be contributing factors. The efficiencies of some scrubbing solutions were therefore initially compared at a flow-rate of 6–8 ml per min, 4 ml of the solution being used in the B.S. (1954) design spiral scrubber.

Our results confirmed[10] that aqueous sodium thiosulphate is unsatisfactory because of reaction with methyl iodide.[26,27] Table II summarises the other results obtained. For each solution tested, several determinations were made under the conditions shown; the range of recoveries obtained is quoted, since averaged results at the 100% level can be misleading.

(*a*) *In absence of sulphur*, all the solutions listed were satisfactory. Water itself, however, gave slightly low results; this is in agreement with Heron *et al.*,[10] who reported 94–98% recovery. These negative errors are undoubtedly due to the solubility of methyl iodide in water; the effect can be minimised by keeping the scrubber temperature at $42° \pm 1°$, but this adds a complicating factor to the scrubber design. Moreover, whilst heated scrubbing solutions have been recommended,[1,6,28] Colson[21] has pointed out that the increased possibility of hydrolysis of methyl iodide may lead to low results. As shown in Table II, the negative error was dependent on the volume of water used, and large errors were introduced when sulphur was present (*cf.* the results obtained with water saturated with hydrogen sulphide).

(*b*) *In presence of* 3–4 *mg of added sodium sulphate* (equivalent to 1 sulphur atom per methoxyl group for 3 mg samples of vanillin) only sodium acetate and sodium bicarbonate of the solutions tested were clearly unsatisfactory (*cf.* ref. 25).

(*c*) *In presence of larger amounts of sulphur*, both sodium antimonyl tartrate and the sodium thiosulphate + cadmium sulphate mixture began to give low results. This finding explains the differing opinions expressed regarding their efficiency (*cf.* 7 and 25, 8, 10 and 27). In agreement with earlier investigators,[23,29] cadmium sulphate gave good results; it was the only satisfactory solution when a large excess of sulphur was present. The formation of heavy cadmium sulphide precipitates did not influence its efficiency, as has been suggested.[30]

Table II. Efficiency of some scrubbing solutions* in methoxyl determinations in the presence and absence of sulphur

Range of weight of vanillin, 2·92–3·39 mg, ∴ methoxyl present approx 0·2 mEquivs.

Composition of scrubbing liquid†		Wt. of sodium sulphate added, *mg*	∴ Sulphur present, *mEquivs*	Range of methoxyl recovery, %
5% aq. sodium bicarbonate		0	0	99·4–100·4
		3	0·2	95·1–95·3
		10	0·7	92·5–92·7
		27	1·9	90·0–90·4
25% aq. sodium acetate		0	0	100·0–100·5
		4	0·3	98·1–98·5
		30	2·1	91·1–91·2
10% aq. sodium antimonyl tartrate		0	0	99·5–100·2
		3	0·2	99·6–100·0
		10	0·7	95·1–95·3
		15	1·0	94·1–94·2
5% aq. sodium thiosulphate + 5% aq. cadmium sulphate (1 + 1 V/V)		0	0	100·0–100·5
		3	0·2	100·0–100·1
		10	0·7	99·1–99·5
		18	1·3	97·7–98·0
5% aq. cadmium sulphate		0	0	99·8–100·6 (for 0, 0·2, 0·7, 1·0)
		3	0·2	
		10	0·7	
		15	1·0	
		32	2·2	99·0–99·2
Water	2 ml	0	0	99·5–99·7
	4 ml	0	0	98·8–99·2
	2 ml	4	0·3	97·5–97·9
	4 ml	4	0·3	96·8–97·1
Water satd. with hydrogen sulphide	2 ml	0	0	95·2
	3 ml	0	0	94·0
	4 ml	0	0	91·3
	5 ml	0	0	90·7

* Used in the spiral scrubber described in BS1428 : C1 : 1954. Flow-rate = 6–8 ml per min. Temp. of solutions = 20° ± 1°.

† Volume used = 4 ml, unless otherwise stated.

With high concentrations of hydrogen sulphide, the decreased efficiency of sodium antimonyl tartrate may be caused by complex thio-anion formation from the original Sb_2S_3 precipitate, and subsequent interaction between the thio-anion and alkyl iodide.* For cadmium sulphate the concentration of hydrogen sulphide is apparently not so critical since cadmium sulphide does not form complex sulphides.

In earlier experiments, Gran[30] found a preliminary water scrubber essential (to remove hydrogen iodide vapour), since otherwise the scrubber acidity increased

* We are grateful to one of the referees for this suggestion.

sufficiently to cause some dissolution of the cadmium sulphide precipitate. This effect, however, resulted from his use of acid more concentrated than the azeotrope. Although the introduction of a water scrubber removed one source of error, another was simultaneously introduced, as already described; Gran found that this in turn could be minimised by increasing the flow-rate to 40 ml per min. Experiments at this very high flow-rate revealed that the absorption of hydrogen sulphide by water is indeed much less than at 6–8 ml per min; the high flow-rate either decreases the efficiency of scrubbing, or creates a superior competitive "degassing" effect.

In solutions containing sodium thiosulphate + cadmium sulphate, complexing of thiosulphate ions by cadmium will undoubtedly occur. The decreased efficiency of this scrubbing mixture at high sulphur concentrations (at which cadmium sulphate itself remains efficient) is probably due to removal from solution of cadmium as cadmium sulphide. Some of the previously complexed thiosulphate is therefore liberated, so increasing its possibility of reaction with alkyl iodide.[26,27]

Many other aqueous scrubbers have been proposed, *e.g.* red phosphorus suspensions,[1,2,4,28] solutions of organic compounds,[27] acetic acid[8] and hydrochloric acid.[25] The efficiency of these was not investigated.

The B.S. design spiral scrubber gives adequate scrubbing efficiency under the experimental conditions described, but not at greatly increased flow-rates. When aqueous cadmium sulphate was used in two spiral scrubbers joined in series, no detectable leakage of hydrogen sulphide to the second scrubber occurred at a flow-rate of 10 ml per min; slight leakage occurred at 15–20 ml per min. This supports the earlier warning by Heron *et al.*[10] regarding scrubbing efficiency.

6. *The use of a solid scrubber*

Having established that reaction between hydrogen sulphide and alkyl iodides occurred only in aqueous solution, it appeared that the use of a suitable solid scrubber would eliminate several possible sources of error. The use of solid scrubbers was apparently first suggested by Fierz-David *et al.*,[31] but their use in alkoxyl determinations attracted little attention until recently.[13,32,33,34]

We have found soda-asbestos (M.A.R.) to be a very efficient solid scrubber, although Večeřa and Spěvák[34] apparently rejected it after trial experiments. It quantitatively absorbs and firmly retains hydrogen iodide, iodine, hydrogen sulphide

TABLE III. EFFICIENCY OF A SODA-ASBESTOS SOLID SCRUBBER
Flow-rate = 6–8 ml/min

Compound	Range of weights taken, *mg*	∴ Alkoxyl present, *mEquivs*	Wt. of sodium sulphate added, *mg*	∴ Sulphur present, *mEquivs*	Range of alkoxyl recovery, %
Vanillin	2·736–3·01	approx. 0·2	0	0	99·6–100·5
			10	0·7	99·7–100·5
			30	2·1	99·7–100·5
			60	4·2	99·7–100·1
Phenacetin	2·98–3·61	approx. 0·2	0	0	99·5–100·4
			30	2·1	99·8–100·6
			60	4·2	100·0–100·6

and carbon dioxide; even when quantities of these vapours have been absorbed there is no retention of alkyl iodides. Table III shows its high efficiency under testing conditions. In addition, when 40 mg of sodium sulphate was refluxed for 3 hr, using a flow-rate of 40 ml per min, no hydrogen sulphide passed the scrubber.

Experimentally, the use of a solid scrubber simplifies the apparatus and procedure. The use of nitrogen as flow-gas is, of course, essential. The spiral liquid scrubber is replaced by a straight connecting tube, as shown in Fig. 1; a packing of soda-asbestos 2 inches long is adequate, and is renewed for each analysis.

The reaction conditions described in this paper and the results presented on scrubbers are totally applicable to alkoxyl determinations in which the standard gravimetric or iodometric finish is employed.

Details of the investigation of reaction conditions for determination of propoxyl and butoxyl groups, of the differentiation of ester from ether groups, and of the infra-red method for analysis of compounds containing methoxyl and ethoxyl groups will be given shortly in further parts of this series.

Acknowledgements—We thank Professor E. L. Hirst, C.B.E., F.R.S., for his interest; the Carnegie Trust for the award of a Scholarship (to J. L. D.); Messrs. I.C.I. Ltd., and the Rockefeller Foundation for research grants; and Messrs. I.C.I. (Dyestuffs Division) Ltd., for supplying zone-refined vanillin.

Zusammenfassung—Eine empfindliche Infrarotmethode zur quantitativen Bestimmung von Dämpfen wurde verwendet um die Reakationsvariablen der Alkoxylbestimmung nach Zeisel zu studieren. Verschiedene Widersprüche in der Literatur wurden klargestellt. Die Raktionsbedingungen zur raschen Erzielung genauer Resultate bei der Analyse von festen Körpern und verdampfbaren Flüssigkeiten, selbst in Gegenwart grosser Mengen an Schwefel, werden beschrieben. Es wurde gefunden, dass die Reaktion zwischen Alkyljodid und Schwefelwasserstoff nur in wässriger Lösung stattfindet; die Anwendung von Soda-Asbest als festes Absorptionsmittel hat daher fundamentalen Vorteil gegenüber einer wassrigen Lösung und gibt exellente Resultate. Mit Vanillin ausgeführte Testbestimmungen zeigen eine Standardabweichung von 0·16%.

Résumé—Une méthode infra-rouge sensible pour la détermination quantitative des vapeurs a été utilisée pour l'étude des variables de la réaction dans le dosage des alkoxyl de Zeisel. Différents rapports contradictoires dans la littérature ont été clarifiés. Les conditions de réaction, donnant des dosages rapides et précis pour des solides et des liquides volatils, même en présence d'une grande concentration de soufre, sont décrites; pour des dosages de vanilline, l'écart standard est 0,16%. Les auteurs ont trouvé que l'interaction entre les iodures d'alkyl et l'hydrogène sulfuré se produisait seulement en solutions aqueuses; l'utilisation d'amiante sodée comme laveur solide a cependant des avantages fondamentaux sur les solutions aqueuses, et donne d'excellents résultats.

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3.

APPLICATIONS OF INFRARED SPECTROSCOPY—III*

THE SIMULTANEOUS DETERMINATION OF METHOXYL AND ETHOXYL GROUPS

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Summary—Quantitative vapour-phase infrared spectroscopy permits the simultaneous determination of methoxyl and ethoxyl groups as alkyl iodides or bromides: when the methoxyl/ethoxyl ratio exceeds 4 : 1, determinations are only possible as bromides. The reaction-time involved is 30 min. Results, correct to within ±1% for each alkoxyl group, can be obtained on the micro-scale. The presence of sulphur does not interfere.

PART II[1] of this series described a spectroscopic investigation of some of the reaction variables of the Zeisel determination, and results were reported for micro alkoxyl determinations on reference compounds. These showed that the spectroscopic method of determining the liberated alkyl iodides not only gave results comparable in accuracy and reproducibility with those obtainable by the more conventional methods, but also distinguished in the same analysis between different alkoxyl groups. On unknown compounds, this selective information cannot be obtained directly if the conventional iodometric or gravimetric finishes are used.[2,3]

Gran[2] reviewed the techniques available for the simultaneous determination of methoxyl and ethoxyl groups; the majority require time-consuming modification or extension to the standard Zeisel method. Techniques based on Martin and Vertalier's[4] use of gas chromatography were later introduced[5,6] which, although not comparable in accuracy with the more conventional methods, offered possibilities for future development. More recent publications, however, have merely reverted to modifying[7] the Willstätter and Utzinger[8] technique or to describing modifications[9,10] of the combustion method.[11]

This paper describes how the quantitative infrared technique[1,12] can be extended, without modification or loss in accuracy, to the simultaneous determination of both methoxyl and ethoxyl groups. Results, correct to within ±1% for both groups, can be obtained on the microscale. The reaction-time is 30 min, and only one weighing of the sample is involved. The presence of excess sulphur (which continues to cause difficulty[13]) in any form or quantity does not interfere.

EXPERIMENTAL

Gas-cells

Descriptions of the gas-cells used, and data giving their useful concentration range and sensitivity have been given.[1,12,14]

* Part II—see ref. 1.

Spectrometer

The spectrometer used was a Hilger H800 double-beam instrument, which has the following advantages: (*a*) accommodation of gas-cells up to 56 cm long, (*b*) linear recording of percentage absorption, with an accuracy of $\pm 0 \cdot 5\%$. This fixes the limiting accuracy of the method, and obviates the necessity of weighing 2–5 mg samples to the nearest microgram. (By adjustment of tares, all weighings were made within the range of the 0–30 mg graticule scale of an aperiodic direct-reading balance having an accuracy of $\pm 10\ \mu g$.)

Spectrometer conditions

For the calibration curve to be of maximum accuracy, the standard operating conditions (slit-width, scan speed, electronic gain etc.) must be carefully selected initially and subsequently rigorously controlled during determinations. The gas-cell carrier must be rigidly fixed, or fitted with some simple locating device ensuring replacement each time in identical position with respect to the infrared beams.

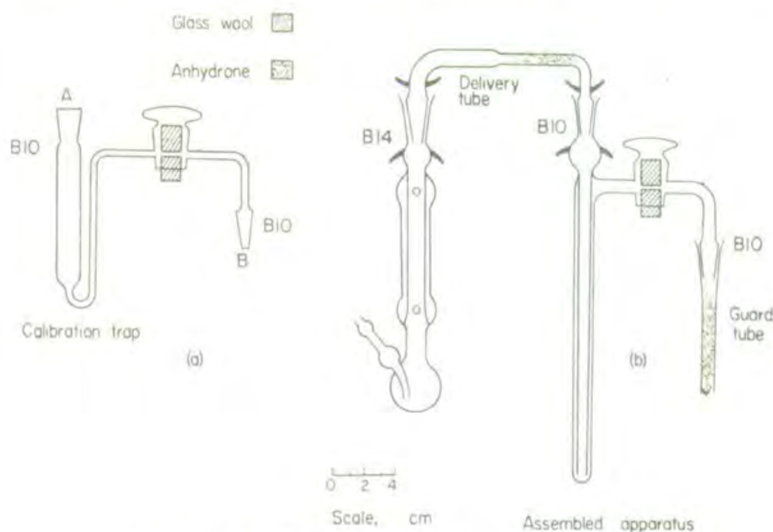

FIG. 1.—(*a*) Calibration trap. (*b*) Assembled apparatus.

Reproducibility of percentage absorption for a given concentration depends on the recorder zero-stability; any peak height must always be measured with respect to some arbitrarily fixed base-line or zero associated with the wave-length involved. All other conditions being constant, the base-line may be affected by small energy losses caused by fogged or scratched cell-windows, which must therefore be kept in good condition. Small local variations in base-line caused by such factors may, however, be satisfactorily compensated by adjustment of some auxiliary compensating device interposed in the reference beam.

Construction of calibration curves

Weigh the required amount of alkyl iodide in a micro weighing-bottle (12×4 mm) fitted with a leak-proof ground-glass stopper. Via *A*, place the bottle in the trap shown in Fig. 1(*a*), then immerse the limb of the trap in liquid nitrogen. Insert the requisite evacuated gas-cells at *A*, connect *B* to a suitable vacuum-line,[12] and evacuate the system with the trap still in coolant.

Vaporise the trapped alkyl iodide into the gas-cell, making the total pressure equal to atmospheric, by the technique already described.[12] Quantitative transfer, within the experimental limits of $\pm 1\%$, is easily achieved. Record the particular absorption peak selected for calibration (see below) six times, then calculate the mean peak height: this minimises any error in the peak heights drawn by the pen recorder, the reproducibility of which is $\pm 0 \cdot 25\%$.

Fundamentally, peak areas are proportional to concentration, but at the partial pressures used

peak heights are sensitive to concentration changes and are, under standard conditions, much easier to measure. "Pressure-broadening" effects do not cause complications.[12] Consequently, calibration curves can be constructed with reference to peak heights. Fig. 2 shows typical curves obtained.

Efficiency of trapping

Various weights of alkyl iodides were added to the reaction-flask and volatilised using flow-rates between 4 and 20 ml per min. At each flow-rate, recoveries ranged from 99·0–101·0% with an equal distribution of high and low results. In several experiments, two absorption traps were connected in series: in no case was alkyl iodide detectable in the second trap. These experiments led to ±1%

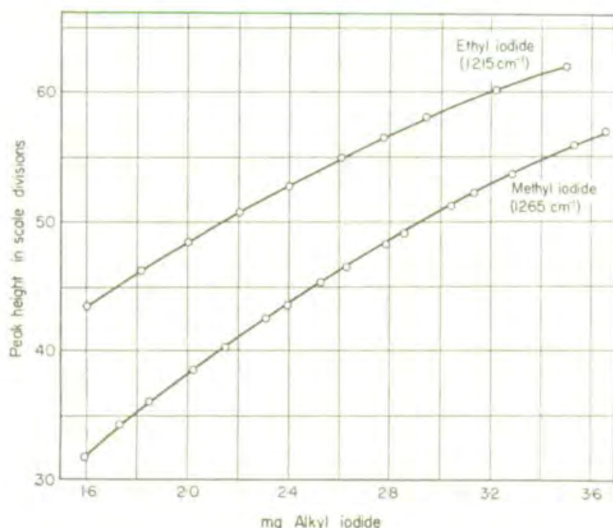

FIG. 2.—Calibration curves for methyl iodide and ethyl iodide.

being claimed as the accuracy of the method. In practice, results better than these limits can be expected (see Table I).

Alkoxyl determinations

Details of the apparatus, reagents, flow-gas, heating, reaction-time, and weighing of samples have been described.[1] For volatile samples, the described technique obviating use of double distillation[1] is used; it may also be applied to non-volatile and solid samples. The following technique, however, has been successfully used for solids: weigh the sample in a long-handled weighing spoon.[1] Add 20–40 mg phenol to the spoon, and warm *gently* over a hot-plate to form a homogeneous melt at as low a temperature as possible. Place the spoon in a reaction-flask to which 6 ml of hydriodic acid had been added, pre-conditioned as described,[1] and cooled.

Procedure

Attach the delivery tube (Fig. 1(*b*)), packed with fresh Anhydrone, to a clean trap fitted with an Anhydrone guard-tube: immerse the trap in liquid nitrogen. (This order of assembly minimises collection of atmospheric water-vapour in the trap.) Attach the B14 cone of the delivery tube to the reaction condenser, so assembling the apparatus as shown in Fig. 1(*b*). Adjust the nitrogen flow-rate to 6–8 ml per min, and begin heating the reaction-flask.

After 30 min reflux, remove and stopper the trap, keeping it immersed in coolant. When convenient, volatilise the trapped reaction products into the appropriate gas-cell. Draw the spectrum of the products—this reveals immediately which iodides are present. Then re-draw 6 times (ensuring that the correct base-line is given) the characteristic peak on which calibration of each iodide is based. Refer the average peak height for each iodide to the appropriate calibration curve, and hence determine the weight of alkyl iodides liberated.

RESULTS

Interferences

Since iodine, hydrogen sulphide and hydrogen iodide vapours do not interfere with the spectroscopic determination, conventional Zeisel scrubbers are not necessary. Phosphine, however, does interfere; preconditioning of the hydriodic acid as described[1] is required, and subsequent addition of hypophosphites to decolourise dark reaction mixtures should be avoided. The use of colourless hydriodic acid is not necessary for quantitative results. Since water vapour must be excluded in spectroscopic determinations, the reaction products are trapped after passage through Anhydrone[1].

Choice of calibration peaks

Fig. 3 diagramatically represents the spectra given in the 800–1500 cm^{-1} region,

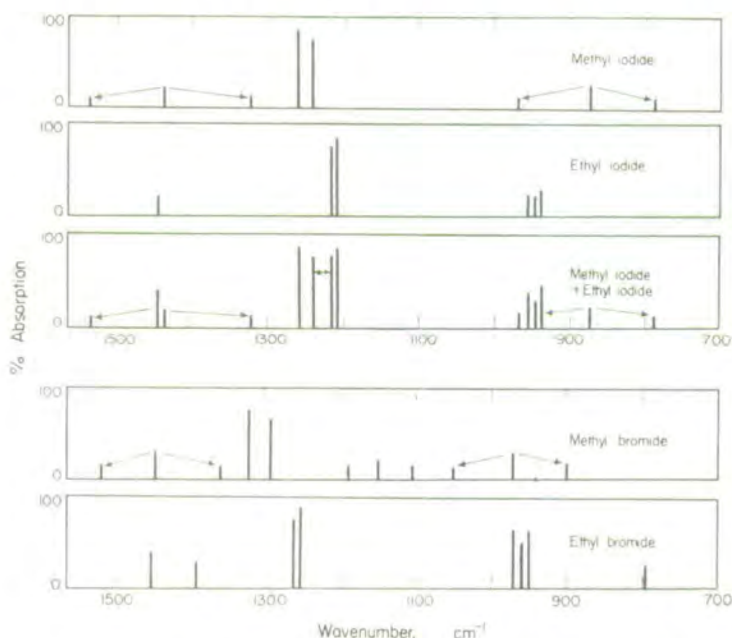

FIG. 3.—Representation of spectra given by methyl iodide, ethyl iodide, methyl bromide and ethyl bromide.

at a scan speed of 127 cm^{-1} per min, by methyl iodide, ethyl iodide, and by a mixture of the two. If a vapour contains only one component, calibration can be based on any convenient absorption peak in the spectrum, although maximum sensitivity and accuracy will clearly be obtained if calibration is based on the most intense absorption. In mixtures, however, over-lapping or coincidence of peaks can occur. Calibration for a particular component must then be based on the most intense absorption which is not masked or reinforced by peaks given by the other components present.

In the composite spectrum (Fig. 3) given by a mixture of methyl + ethyl iodides, very slight overlapping of peaks occurs at the normal scanning speed, so that the 1244 cm^{-1} peak of methyl iodide fractionally increases the height of the 1215 cm^{-1} ethyl iodide peak. The 1265 cm^{-1} methyl iodide peak is not masked, and so calibrations based on it give correct methoxyl values as shown by the typical results given in Table I.

Calibrations based on the 1215 cm^{-1} ethyl iodide peak, however, lead in this circumstance to high ethoxyl values: the more minor peaks given by ethyl iodide at 1450 cm^{-1} and 950 cm^{-1} are also subject to overlapping with minor methyl iodide peaks and so cannot be used.

Fortunately, this positive ethoxyl error was found to be a linear function of the

TABLE I. SIMULTANEOUS METHOXYL/ETHOXYL DETERMINATIONS

Compound or mixture	Weight taken, *mg*	Wt. alkyl iodide found		Corr. wt. $\overline{\text{Et}}$I found,* *mg*	Methoxyl		Ethoxyl	
		$\overline{\text{Me}}$I, *mg*	$\overline{\text{Et}}$I, *mg*		found, %	theory, %	found, %	theory, %
Vanillin	7·41	6·94			20·4	20·4		
Phenacetin	3·29		3·00	2·86			25·2	25·1
Vanillin	10·24	9·52			20·3	20·4		
Phenacetin	3·02		2·82	2·63			25·3	25·1
Vanillin	2·03	1·90			20·5	20·4		
Phenacetin	4·28		3·75	3·71			25·1	25·1
Anisic acid	9·43	8·72			20·2	20·4		
Cmpd X†	7·20		3·15	2·98			12·0	12·0
Cmpd Y†	2·60	6·26			52·7	52·3		
			2·40	2·28			25·8	25·3
Cmpd Z†	1·42	0·98			15·1	15·0		
			3·22	3·20			65·1	65·5

* Corrected weight $\overline{\text{Et}}$I = (wt. $\overline{\text{Et}}$I found) − (0·019)(wt. $\overline{\text{Me}}$I found).

† Origin of samples:

Cmpd X: 4-chloro-2-ethoxy-p-tosyl-1-naphthylamine (Prof. F. Bell)

Cmpd Y: 1:1:3-trimethoxy-3-ethoxypropane } L. Light and Co. Ltd. (redistilled).

Cmpd Z: 1:1:3-triethoxy-3-methoxypropane }

weight of methyl iodide present, and a correction factor (0·019), applicable to a particular set of standard spectrometer conditions, was calculable. When

$$(0{\cdot}019) \times (\text{weight of methyl iodide found})$$

was subtracted from the actual weight of ethyl iodide found, the results shown in Table I were obtained for methoxyl-ethoxyl compounds or for synthetic mixtures ranging in composition from methoxyl/ethoxyl = 3:1 to 1:3.

At low ethoxyl contents, the 1215 cm^{-1} ethyl iodide absorption occurs as a shoulder on the 1244 cm^{-1} methyl iodide peak and determinations become inaccurate: at methoxyl/ethoxyl ratios $> 4:1$, there is no measurable ethyl iodide peak height and the method fails. Although such combinations must be rare in actual chemical compounds, they could readily be met in determinations on mixtures of methoxyl and ethoxyl compounds. Such mixtures can be analysed by using hydrobromic acid in place of hydriodic acid during reflux.

Alkoxyl determinations using hydrobromic acid

Constant-boiling hydrobromic acid (sp. gr. 1·47) is effective in cleaving alkoxyl groups: it produces alkyl bromides quantitatively from esters and ethers almost as quickly (15–20 min for methyl and ethyl) as hydriodic acid forms the corresponding iodides. Alkyl bromides are as conveniently estimated as alkyl iodides by the infrared technique if the required calibration curves are constructed (the manometric technique[12] must be used for methyl bromide, b.p. 3·6°). Moreover, Fig. 3 shows that the 1320 cm^{-1} absorption of methyl bromide is so widely separated from the 1250 cm^{-1} peak of ethyl bromide that calibrations based on each of these are valid for the analysis of all possible methoxyl/ethoxyl percentage compositions. The only slight disadvantage in the use of the bromides is a small decrease in sensitivity. Gas-cell "B"[14] permits determination of approximately 2–6 mg of alkyl bromide as opposed to 1–4 mg of alkyl iodide; this requires that sample weights of alkoxyl compounds must be approximately doubled when determination as the bromide is used. Results equal in accuracy to those shown in Table I are, however, obtainable without the necessity of using a correction factor.

CONCLUSIONS

The method described permits satisfactory simultaneous determinations of methoxyl and ethoxyl groups on the micro scale. Although Table I shows only a few typical results, many determinations have been made during the past 18 months on a wide range of polyfunctional compounds, each of which contained, in addition to alkoxyl, one or more of the following groups: —F, —Cl, —Br, —I, —NO, $—NO_2$, $—NH_2$, =NH, —N=N—, =S, $—SO_3H$, $≡PO_4$, —PS, =Se. Satisfactory results were obtained on pure samples; in our experience, some organic compounds are surprisingly difficult to purify sufficiently for satisfactory results to be obtained in the alkoxyl determination, which is a rigorous functional group analysis.

The accuracy obtainable, small samples required, short reaction time, elimination of conventional scrubbers, and absence of interference from sulphur combine to make this spectroscopic method undoubtedly superior to any method previously described for simultaneous determination of alkoxyl groups. At the present time, when ever-increasing numbers of chemists have access to an infrared spectrometer, this method should be of general interest.

Acknowledgements—We thank Professor E. L. Hirst, C.B.E., F.R.S., for his interest in this work, and the Carnegie Trust for the award of a Scholarship (to J. L. D.).

Zusammenfassung—Quantitative Infrarotspectroskopie in der Gasphase erlaubt die gleichzeitige Bestimmung von Methoxyl- und Äthoxylgruppen als Alkalijodide oder Bromide. Wenn das Verhältnis Methoxyl:Äthoxyl den Betrag von 4:1 überschreitet kann die Bestimmung nur über die Bromide durchgeführt werden. Die benötigte Reaktionszeit ist 30 Minuten. Die Resultate, innhalb von $\pm 1\%$ genau, können für Proben im Mikrobereich erhalten werden. Gegenwart von Schwefel stört nicht.

Résumé—La spectroscopie infra-rouge quantitative en phase vapeur permet le dosage simultané des groupements méthoxy et éthoxy à l'état d'iodures ou de bromures d'alkyl: quand le rapport méthoxy/éthoxy dépasse 4/1, les dosages ne sont possibles que pour les bromures. Le temps de réaction nécessaire est de 30 minutes. Les résultats, corrects dans l'intervalle de ± 1 pour cent pour chaque groupement alkoxyl, peuvent être obtenus à l'échelle microscopique. La présence de soufre ne gêne pas.

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4.

APPLICATIONS OF INFRARED SPECTROSCOPY—IV*

THE CAUSES OF ANOMALOUS ALKOXYL DETERMINATIONS GIVEN BY SUGARS AND POLYHYDRIC ALCOHOLS

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Summary—The volatile reaction products from Zeisel determinations on sugars and related compounds have been identified. One of the products from certain common sugars is 2,5 dimethylfuran, which interferes with the Vieböck iodometric determination of alkyl iodide, and has been shown to be a contributing cause of apparent alkoxyl values. Suggestions that hexyl iodide is evolved from sugars and that vinyl iodide is a product from polyhydric alcohols have been disproved.

ZEISEL determinations on unmethylated sugars and polysaccharides have been reported[1,2] to give small apparent alkoxyl values. This implied that all carbohydrate materials would give slightly high analytical results, and made particularly difficult the interpretation of analyses of polysaccharide functions having a small but real methoxyl content.

In contrast, Goldstein and Smith[3] found that certain periodate oxidation products gave very high results. They proposed that the liberation of "extra" alkyl iodide from polyhydric alcohols such as glycerol was responsible. Von Rudloff[4] had earlier reported that polyhydric alcohols gave anomalous alkoxyl values. Strangely, he also obtained very unsatisfactory results for α-methyl-D-glucoside, a recommended reference standard[5] which gives excellent results.[6–9] Although it had been clearly established that the volatile products from ethylene glycol were ethylene + ethyl iodide, von Rudloff claimed that vinyl iodide + ethyl iodide were formed.

It is significant that the apparent methoxyl values quoted for standard compounds *e.g.* glucose and sucrose, ranged from zero to 0·4% (*cf.* refs. 1 and 2). Modification of Willstätter and Utzinger's trimethylamine technique[11] was found[1–3] to give results more satisfactory (although still high) than were obtained by the more convenient Vieböck iodometric titration. Recently, in a paper[13] which apparently repeats the published description[14] of a combustion method for alkoxyl determinations, Fukuda states that the method is not applicable to carbohydrates or related substances; the combustion method fails, in fact, for compounds liberating any non-acidic organic vapour in addition to alkyl iodides.

The identity of the volatile products from carbohydrate materials, and the sources of error contributing to the anomalous results, have hitherto not been clearly established. For simple sugars, Gran[2] showed that the apparent methoxyl content was not caused by traces of contaminating alcohols *etc.* He suggested that the formation of volatile hexyl iodides was responsible, although he could not identify or isolate such products.

* Part III—D. M. W. Anderson and J. L. Duncan, *Talanta*, 1961, **8**, 1.

The proposals of von Rudloff and Gran were clearly based on inadequate experimental evidence: further investigation was desirable. Following our studies[9,15] of certain aspects of the Zeisel determination, it appeared that vapour-phase infrared spectroscopy could be applied to identify the volatile products from sugars and related compounds.

EXPERIMENTAL

Apparatus

The reagents, reaction conditions, and apparatus used for alkoxyl determinations were as described.[9] The technique for trapping volatile products, and details of the infrared method for their subsequent identification, have been given.[16,17]

Reagents

Glucose, sucrose, mannitol—all M.A.R.

5-Hydroxymethylfurfural was prepared by Haworth's method.[18]

5-Methylfurfural, furan, 2-methylfuran, 2,5-dimethylfuran and compounds not described in footnotes to Table I were commercial samples which conformed, after purification where necessary, to literature description.

Procedure

Samples (30–60 mg) of the compounds listed in Tables I and II were refluxed for 1 hr with hydriodic acid (6 ml, constant-boiling, pre-conditioned[9]) using nitrogen as flow-gas (6–8 ml per min.). The mixture of volatile products was collected, after passage through Anhydrone, in a trap immersed in liquid nitrogen. The components of each mixture were subsequently identified and quantitatively determined by vapour-phase spectroscopy[16,17]: since 2-iodohexane (b.p. 165°) was insufficiently volatile, it was identified as a pure liquid. All components were identified without difficulty; none of the mixtures was sufficiently complex for prior separation of the components by vapour-phase chromatography to be necessary.

RESULTS

Table I shows the products liberated in 1 hr from 30-mg to 60-mg samples of sugars and polysaccharide materials. The products from polyhydric alcohols are shown in Table II.

Effect of varying reaction-time and sample weights

The results obtained from experiments with glucose are shown in Table III.

Volatilisation of iodine

With 60 mg of glucose, only small amounts of iodine were released, insufficient to form a sublimate on the condenser walls: iodine did not pass the condenser. With 250-mg and 500-mg samples, however, extensive volatilisation occurred.[9] Although most of this condensed, some iodine reached the cold trap in which the volatile products were collected. The efficiency of removal of such quantities of iodine by 10% aq. sodium antimony tartrate in the B.S. design of scrubber[9] was therefore tested. When 100 mg of iodine were refluxed with pre-conditioned hydriodic acid, the amount of iodine passing the scrubber increased the reagent blank from 0·03 ml of 0·01N sodium thiosulphate to 0·06 ml (*cf.* ref. 8). When 500 mg of glucose was refluxed, the apparent methoxyl content found iodometrically was exactly equivalent to the amount of methyl iodide found by the infrared method in a duplicate run.

TABLE I

Compound*	Yield of volatile products†‡					
	n-Heptane	n-Hexane	n-Pentane	n-Butane	2,5 Dimethyl-furan	Acetone
Glucoheptose	tr	—	—	—	—	—
2-Deoxyglucose	—	tr	—	—	—	—
5-Hydroxymethylfurfural	—	tr	—	—	—	—
Glucose, hexoses	—	tr	—	—	2%	—
Fructose, sorbose	—	1%	—	—	2%	—
Rhamnose, fucose	—	1%	—	—	2%	—
Glucosamine HCl	—	tr	—	—	tr	tr
Sucrose	—	tr	—	—	2%	1%
Glucurone[a]	—	—	tr	—	—	—
Xylose, pentoses	—	—	tr	—	—	—
Furfural	—	—	tr	—	—	—
5-Ketogluconolactone[b]	—	—	tr	—	—	tr
Galactono-γ-lactone[c]	—	—	tr	—	—	tr
Erythrose	—	—	—	tr	—	1%
Glyceraldehyde	—	—	—	—	—	1%
Oat starch[d]	—	tr	—	—	1%	—
Waxy maize starch	—	tr	—	—	3%	tr
Glycogen[e]	—	tr	—	—	3·5%	tr
Inulin[f]	—	tr	—	—	4·5%	tr
Agar[f]	—	tr	—	—	2%	tr
Cellulose[g]	—	—	—	—	2%	tr
Araboxylan[h]	—	—	tr	—	—	—

* 30–60 mg in 6 ml of HI under reflux for 1 hr.

† expressed as % of wt. of sample taken; *tr* denotes ≯ 0·02%.

‡ In addition to the products shown, all compounds in this table gave a trace of CO_2, except for a, b and c, which gave approx. 0·75 mole of CO_2 per mole of compound.

d, Anderson and Greenwood, *J. Sci. Food Agric.*, 1955, **6**, 587.

e, ex Mussel; L. Light and Co. Ltd.

f, B.D.H. Ltd.

g, ex *Nitella translucens;* Anderson and King, in preparation.

h, ex Rye flour; Aspinall and Sturgeon, *J. Chem. Soc.*, 1957, 4469.

TABLE II

Compound*	Volatile products†
Sorbitol, mannitol	CO_2 (tr) + n-hexane (tr) + 2-iodohexane
Ribitol	CO_2 (tr) + n-pentane (tr) + 2-iodopentane
Erythritol	CO_2 (tr) + 2-iodobutane
Glycerol	isopropyl iodide
Ethylene glycol	ethylene + ethyl iodide
Allyl alcohol	isopropyl iodide
Vinyl ethyl ether	ethyl iodide

* Reaction details as in Table 1, footnote †.

† For yields of alkyl iodides, see Discussion: tr denotes ≯ 0·02%.

DISCUSSION

When a carbohydrate material is suspected to have a low methoxyl content, large sample weights must be employed if the methyl iodide released is to be determined accurately. There is also a tendency for reflux periods longer than normal to be used (*cf.* ref. 2). Table III shows that *up to a certain ratio* (100 mg of sample per 6 ml of hydriodic acid) the volatile products from sugars are independent of the sample weight taken: for larger samples, different decomposition mechanisms are clearly involved. Hexyl or other iodides are only liberated, however, when hexahydric alcohols are present. This, therefore, refutes the suggestion made by Gran.[2] Table III shows that methyl iodide is only formed when exceptionally large weights of sugars are reacted; the very small amount liberated (equivalent to 0·03% and 0·1% of methoxyl after 1 and 3 hours respectively) does not explain in full the anomalous results previously reported.

TABLE III

Wt. of glucose, *mg**	Reflux period, *hr*	Volatile products†
20–100	1	CO_2 (tr) + n-hexane (tr) + 2,5-dimethylfuran (2%)
20–100	4	CO_2 (tr) + n-hexane (tr) + 2,5-dimethylfuran (4%)
250	1	CO_2 (tr)
500	1	CO_2 (tr) + methyl iodide (0·12%)
500	3	CO_2 (tr) + methyl iodide (0·4%)

* treated with 6 ml of HI under reflux. † yields expressed as in Table I.

Alternative possible sources of error were therefore investigated. Those included (*a*) volatilisation of iodine, (*b*) formation of 2,5-dimethylfuran and of hydrocarbons and acetone, (*c*) retention by polysaccharides of organic solvents used in their preparation.

Volatilisation of iodine

Previous workers[7,8] have commented on the volatilisation of small but significant quantities of iodine. In particular, Belcher[8] showed that volatilisation could occur, although correct results were obtainable if the appropriate increased "blank" value was taken into account. This, however, indicates that iodine vapour, when liberated in quantity, is not *totally* retained by aqueous scrubbers. Our results are in agreement, but the amounts of iodine passing the scrubber are very small, even under testing conditions.

Volatilisation of iodine is therefore not the only possible source of error, although it may well have made a major contribution to some of the high results reported by previous workers, particularly when very large sample weights, fast flow rates and long reflux periods were used. Any tendency for errors to arise through volatilisation of iodine can be minimised by use of the solid scrubber, soda-asbestos.[19]

Result of formation of 2,5-*dimethylfuran*

The observation that 2,5-dimethylfuran is one of the volatile reaction products from certain sugars permits a new explanation of anomalous high results to be given. Soda-asbestos and the scrubbing solutions normally used in the Zeisel determination do not absorb 2,5-dimethylfuran.

When 100 mg of this compound were added to 10 ml of the conventional oxidising solution (glacial acetic acid/bromine/potassium acetate) used in the iodometric determination of alkyl iodides,[12] a reaction, clearly involving bromine, took place. A precipitate, found to be potassium bromide, was produced. When the determination was completed in the usual way, the thiosulphate titre indicated, when carried out immediately, an apparent addition of 0·9 mg of methyl iodide. If 10 min were allowed to elapse before titrating,[6] the titre was equivalent to 1·8 mg of methyl iodide, indicating 0·4 % of methoxyl. Slow liberation of iodine continued for 5 hours, when the apparent methoxyl content was 1·2 %. Furan and 2-methylfuran reacted similarly, but liberated smaller quantities of iodine than 2,5-dimethylfuran. The effect of 2,5-dimethylfuran

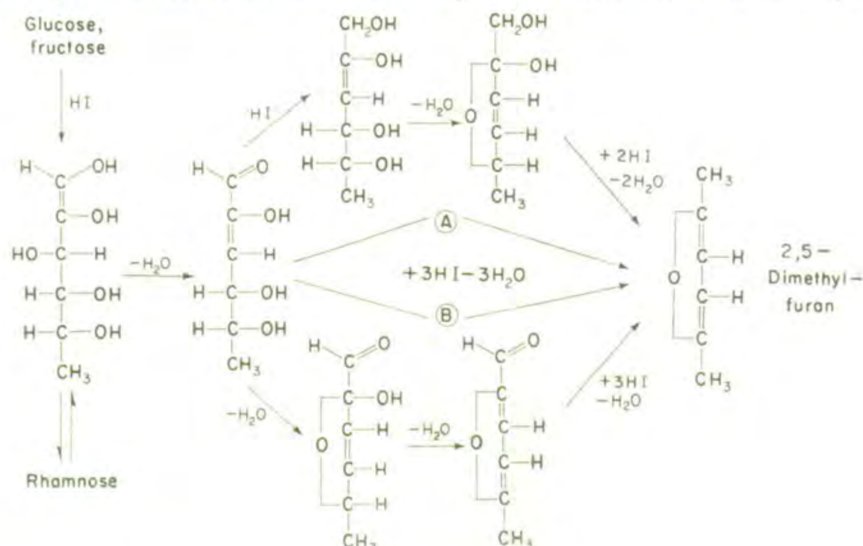

FIG. 1.

was not associated with addition of excess formic acid[20] or excess sodium acetate and sulphuric acid,[21] and clearly contributes to the anomalous Zeisel reactions of sugars.

When furan is added to a cold solution of bromine in acetic acid containing potassium acetate, potassium bromide is precipitated and 2,5-diacetoxy-2,5-dihydrofuran, which is hydrolysed to malealdehyde, is produced in good yield.[22] Our experiments show that both 2-methyl- and 2,5-dimethylfuran react analogously to furan, although 2,5-diacetoxy-2-methyl-5-hydrofuran was too unstable[23,24] to be isolated from the 2-methylfuran reaction mixture. The following facts suggest that the hydrolysis products are involved in the liberation of titratable iodine: (*a*) iodine is released continuously for 4–5 hour, (*b*) the relative instabilities of the diacetoxy addition products increase in the order, furan, 2-methylfuran, 2,5-dimethylfuran, which is the order of increasing yields of iodine by these substances.

Pathway *A* in Fig. 1 shows a possible reaction mechanism for the unexpected formation of 2,5-dimethylfuran. When 5-methylfurfural was refluxed with hydriodic acid, extensive resinification and polymer formation occurred; negligible amounts of 2,5-dimethylfuran were produced. Pathway *B* is therefore not operative. From Tables I and II, it is also apparent that reaction of glucose with hydriodic acid does not involve the intermediate formation of sorbitol.

Quantitative infrared experiments showed that 100 mg of glucose refluxed with hydriodic acid (6 ml) for 1 hour liberated 2 mg of 2,5-dimethylfuran. This, it can be calculated from the results already given, would liberate iodine equivalent to an apparent methoxyl content of 0·01 %. The yields of 2,5-dimethylfuran that are found explain positive errors of the magnitude reported by Adams and Castagne,[1] whose results have been duplicated in experiments using the Vieböck titration.

Result of liberation of hydrocarbons and acetone

The n-hydrocarbons liberated (see Table I) are, like 2,5-dimethylfuran, not retained by the scrubbers normally employed. Pentane, hexane and heptane did not interfere with the Vieböck determination, although a sluggish starch/iodine end-point, caused presumably by slight solvent-extraction of the iodine, was observed.

Table I shows that certain substances liberate small amounts of acetone. Morgan[10] considered that acetone might interfere in Vieböck determinations. The blank values found in experiments using 100 mg of acetone were, however, negligible.

Products from polyhydric alcohols

Table II shows the volatile products given by polyhydric alcohols. Propylene was not produced from glycerol (*cf.* ref. 10). We confirm, however, that the relative amounts of ethylene and ethyl iodide produced from ethylene glycol are affected by several factors;[10] we found that the concentration of phenol used as solubiliser was also critical, and this effect is under investigation. For the present purpose, one mole of ethylene glycol gives 0·4–0·6 mole of ethyl iodide.

In 1 hour, 1 mole of glycerol liberates 0·9–1·0 mole of iso-propyl iodide. The relative involatility ascribed[7,10] to iso-propyl iodide is difficult to understand; even 2-*iodobutane* is sufficiently volatile for approximately 0·8 mole to be given by 1 mole of erythritol in 1 hour. 2-*Iodopentane* and 2-*iodohexane* are less volatile, but the amounts volatilised in 1 hour indicated apparent methoxyl contents of 40 % for ribitol and 15 % for mannitol. (*cf.* ref. 4).

Formation of vinyl iodide

Von Rudloff's proposal that vinyl iodide was formed from ethylene glycol in the Zeisel reaction was not substantiated. Allyl alcohol gives 1 mole of iso-propyl iodide, whilst vinyl ethyl ether gives 1 mole of ethyl iodide as the only volatile product (see Table II). Vinyl propionate gives no volatile products. The reaction

$$R{\cdot}O{\cdot}CH{:}CH_2 \xrightarrow{H^+} R{\cdot}OH + CH_3CHO$$

(which represents the essential reaction involved in the standard method[25] for analysis of vinyl ethers) explains our findings. Vinyl groups are converted to acetaldehyde, which immediately polymerises so that no volatile products result. Vinyl iodide is therefore not a possible volatile reaction product in Zeisel determinations.

Von Rudloff's proposal[4] was based on the detection of formaldehyde after passage of the volatile products from the reaction of ethylene glycol with HI (*i.e.*, ethylene and ethyl iodide) through a permanganate-periodate reagent. Tests show that this oxidising reagent produces some formaldehyde from ethylene; there is therefore no need to postulate formation of vinyl iodide in order to explain the production of formaldehyde.

CONCLUSIONS

It is evident that several factors can contribute to cause anomalous results. Samples should not be larger than required to give the minimal amount of methyl iodide determinable. The reflux period should not be longer than normal; true methoxyl compounds react within 8–12 minutes,[7,9,13,26] and it is suggested that reflux of carbohydrate materials for longer than 1 hour leads only to increased error. High results from volatilisation of iodine can be eliminated by using the soda-asbestos scrubber.[9,19] Small positive errors of the magnitude reported by Adams and Castagne[1] are given in the Vieböck procedure; these are caused by the production of 2,5-dimethylfuran.

The larger errors reported by certain authors (*cf.* refs. 1,2) cannot be explained by the 2,5-dimethylfuran contribution alone; two suggestions can be offered. Firstly, that abnormally large samples were allowed to react for excessive periods, so creating errors through liberation of 2,5-dimethylfuran and volatilisation of iodine; and possibly also, in extreme cases, through formation of methyl iodide. Secondly, that the polysaccharide samples concerned retained small amounts of organic solvents, particularly alcohols, ethers or esters used in their preparation or isolation. Jansen[27] warned that pectic materials were particularly liable to give this effect; the warning has been repeated more recently[28] with regard to wood products. Experiments[29] have shown that organic solvents used to de-fat or dehydrate polysaccharide materials are retained, up to temperatures at which the materials begin to decompose, despite oven and high-vacuum drying at temperatures much higher than the boiling-point of the solvents involved (*cf.* ref. 27). Although pectic materials and plant gums gave the greatest retention, starches retained smaller but easily measurable quantities (*cf.* ref. 30).

Polysaccharide materials isolated with the aid of organic solvents should therefore be re-dissolved, dialysed and freeze-dried wherever possible, otherwise misleading results will be given in alkoxyl and other analyses: care should still be taken with freeze-dried samples, which can retain solvents.[31] When freeze-drying is not possible, the use of methanol and methoxy compounds should be avoided; analysis by one of the valid methods[15,32] for methoxyl in presence of ethoxyl can then be used. For materials suspected to contain both methoxyl and ethoxyl groups, the use of isopropanol or acetone during preparation will allow the infrared method[15] to give the true content of both alkoxyl groups: methoxyl determinations may also be made in the presence of polyhydric alcohols, since none of these (see Table II) liberate methyl iodide. Indeed, examination of the spectrum of the mixture of volatile reaction products would also reveal which polyhydric alcohols, or mixtures thereof, were present, if this were not otherwise known. Valuable evidence in structural studies, in the detection of anomalous linkages, and in the examination of products obtained by periodate oxidation[3] or hydrogenolysis[33] may thus be given by the infrared method, which also offers a rapid method for "difficult" analyses such as the determination of glycerol in presence of residual sugar substrates and 2,3-butanediol.[34]

Acknowledgements—We thank Professor E. L. Hirst, C.B.E., F.R.S., for his interest in this work, and the Carnegie Trust for the award of a Scholarship (to J. L. D.)

Zusammenfassung—Die flüchtigen Reaktionsprodukte, die während der Zeiselbestimmung von Zuckern und ähnlichen Substanzen entstehen, wurden identifiziert. Ein Produkt aus gewöhnlichen Zuckern ist 2,5-Dimethylfuran, welches die Bestimmung von Alkyljodid nach Vieböck stört. Es

wurde gezeigt, dass diese Substanz dazu beiträgt, dass scheinbare Alkoxylwerte erhalten werden. Die Annahmen, dass Hexyljodid gebildet wird und dass Vinyljodid als Produkte aus Polyalkoholen enstehen, wurde als unzutreffend bewiesen.

Résumé—Les produits de réaction volatiles obtenus dans les dosages de sucres et de composés apparentés par la méthode de Zeisel ont été identifiés. Un de ces produits obtenu à partir de certains sucres courants est le 2,5-dimethylfuranne, qui gêne dans le dosage de l'iodure d'alkyle par la méthode de Viebock; les auteurs ont montré que ce produit était la cause des valeurs apparentes d'alkoxyle. Les suggestions suivantes ont été réfutées: de l'iodure d'hexyle se dégage à partir des sucres, l'iodure de vinyle est un produit obtenu à partir des alcools polyhydriques.

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5.

APPLICATIONS OF INFRARED SPECTROSCOPY—V*

THE RETENTION OF WATER AND ORGANIC SOLVENTS BY CARBOHYDRATE MATERIALS

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Summary—Studies have been made of the extent to which some carbohydrate materials retain water and organic solvents. The water evolved was determined gravimetrically, the solvents by an infrared method. "Drying to constant weight" in an oven at 103°, or *in vacuo* at 80°, does not give true "moisture" contents. Both water and organic solvents are retained up to temperatures at which the carbohydrate materials begin to decompose: the polarity and boiling point of the solvent do not determine the extent to which it is retained. Freeze-dried samples can retain organic solvents, and have significant moisture contents. The possibility of inaccurate results and misleading artefacts arising from such retentions or from interaction with solvents is stressed.

ORGANIC solvents are generally employed at some stage in the extraction or purification of carbohydrate materials. Thus, water-soluble polysaccharides are isolated, and plant gums purified, by precipitation from aqueous solution; Soxhlet extraction or refluxing with solvents removes plant pigments and soluble sugars, and inactivates enzymes; starches are "defatted" with 80% methanol or methanol-benzene, and are subsequently fractionated by the use of butan-1-ol or other polar organic solvents. The purified material is often "dried" by successive immersion in acetone, ethanol and ether, with final oven drying at temperatures slightly above the boiling points of the solvents used. Since such treatment may irreversibly decrease the solubility of some materials, *e.g.* starches, these are often stored under methanol or toluene until required.

The retention of moisture and organic solvents by pectins,[1] cellulose,[2] starches,[3,4] gum arabic[5] and biological polymers[6] has been reported: a mathematical treatment of moisture desorption isotherms has been given.[7] For cereals, "free water" (*i.e.* water lost at a stated drying temperature) has been distinguished[8] from "bound water", which is difficult to remove and is considered to be associated with the protein content. The effect of bound water has been considered in ultrasonic studies of hydration effects in sugars.[9]

In this paper we report quantitative observations on some polysaccharide materials in the belief that the extent to which retention of moisture and solvents can occur is not widely appreciated. The experiments were based on the use of a micro-scale, vapour-phase, quantitative infrared technique, developed recently[10] for the examination of fractions separated by GLC and used[11] for a study of the Zeisel alkoxyl reaction.

EXPERIMENTAL

Drying methods

Method I: Oven drying; temperatures and periods as stated.

Method II: Drying over phosphorus pentoxide in a conventional pistol-dryer, heated by refluxing organic solvent and evacuated by a water-pump.

* Part IV: D. M. W. Anderson and J. L. Duncan, *Talanta*, 1961, **8**, 241.

Method III: Vacuum drying (usually at 80°) in a small electrically heated, thermostatted, glass drying chamber attached to a suitable high vacuum line (< 0·02 mm mercury pressure).

Method IV: Heating in a stream of dry nitrogen. The sample was weighed in a small 3-necked flask, which was then placed in a temperature-controlled oil-bath. The central neck of the flask carried a mercury thermometer pocket which was in contact with the sample. One of the two outer necks served as an inlet for CO_2-free nitrogen, dried by passage through Anhydrone; the third neck served as the outlet, to which was connected a tared Anhydrone absorption tube. This served in turn as the inlet to a cold trap immersed in liquid nitrogen. The outlet of the trap was fitted with an Anhydrone guard-tube (*cf.* ref. 11). The nitrogen flow-rate was 15 ml per min.

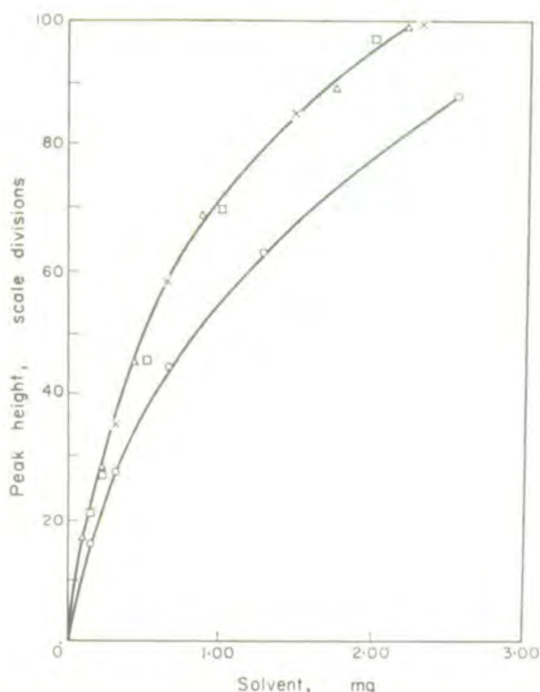

FIG. 1.—Infrared calibration curves for determination of solvents
○ Ethanol (1070 cm^{-1})
× Ether (1140 cm^{-1})
□ Acetone (1740 cm^{-1})
△ Methanol (1040 cm^{-1})

Determination of water released

The Anhydrone absorption tube was weighed at intervals. Anhydrone (B.D.H., M.A.R.) does not retain any of the solvents involved in these experiments.

Determination of organic solvents released

These were quantitatively retained in the cold-trap, and were subsequently determined by a quantitative vapour-phase infrared technique. This has been described,[10] together with details of the design of trap, method of quantitative transfer from trap to gas-cell, and methods of constructing calibration curves for each solvent involved (*cf.* ref. 11). The absorption peaks given by acetone at 1740 cm^{-1}, ethanol at 1070 cm^{-1}, methanol at 1040 cm^{-1}, and ether at 1140 cm^{-1} were used for calibration; the calibration curves obtained are shown in Fig. 1. (It is, of course, fortuitous that the sensitivity of detection of acetone, methanol and ether were all identical, as shown by their sharing a common calibration curve). No overlap or interaction occurred for the absorption peaks chosen, so that all four solvents could be determined simultaneously. The results from some typical experiments are reported below.

RESULTS

1. A sample of the gum from *Combretum leonense*[12] was precipitated with acidified acetone (0·1*N* with respect to HCl) and dried with 5 changes of acetone (each in contact for 2 days, the powder being progressively ground as it dried). The powder was stored in a desiccator (continuously evacuated) for 2 weeks at room temperature, and was then dried for 1 hr at 60° by method II. A weighed sample (554 mg) was then treated by method IV at various temperatures for lengths of time as shown in Table I. The total

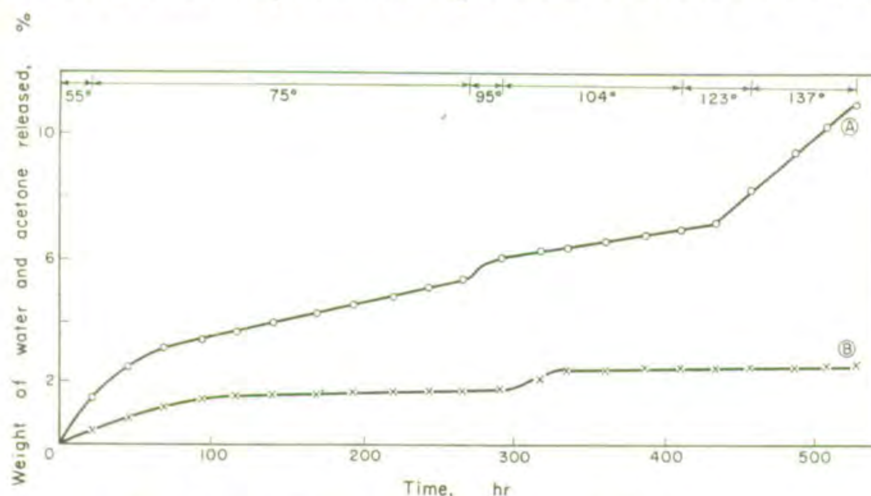

Fig. 2.—Release of water and acetone from a sample of *C. leonense* gum.
Curve OA—water
Curve OB—acetone

amounts of water and acetone evolved were found gravimetrically and spectrometrically respectively. Several measurements were made within each of the periods quoted in Table I; the results are shown graphically in Fig. 2. After heating for 525 hr, weight of gum recovered = 469 mg, *i.e.*, loss in weight = 85 mg. The weights of water

Table I

Period of heating, *hr*	Temp., °*C*	Total weight released from 554-mg sample.	
		water, *mg*	acetone, *mg*
1–20	55	7·9	2·2
20–264	75	29·2	9·8
264–288	95	33·0	10·0
288–406	104	38·5	12·3
406–454	123	46·3	13·1
454–525	137	60·0	14·3

+ acetone found total only 74·3 mg. Very slight decomposition of the gum began at 123° and was significant at 137°; in the period 430–525 hr (Fig. 2) 6 mg of CO_2 were evolved. This evolution of CO_2 would be accompanied by water produced in the decomposition, and this explains the sharp rise in the amount of total water released between 430 hr and 525 hr in Fig. 2.

2. Crude *C. leonense* gum was precipitated with acidified ethanol, dehydrated with 5 changes of acetone (as in 1 above) and finally washed with ether. After preliminary drying by method II for 6 hr at 60°, a weighed sample (474 mg) was dried by method IV at 70° for the period 1–5 hr, at 90° for 5–68 hr, at 105° for 68–116 hr, at 130° for 116–164 hr, at 140° for 164–188 hr, at 150° for 188–236 hr, and at 155° for 236–260 hr. The total weights of water, ether, ethanol and acetone liberated are shown graphically in Fig. 3. Decomposition of the gum was slight at 130° and pronounced at 140°: the graphs show that evolution of organic solvents continued steadily after the onset of

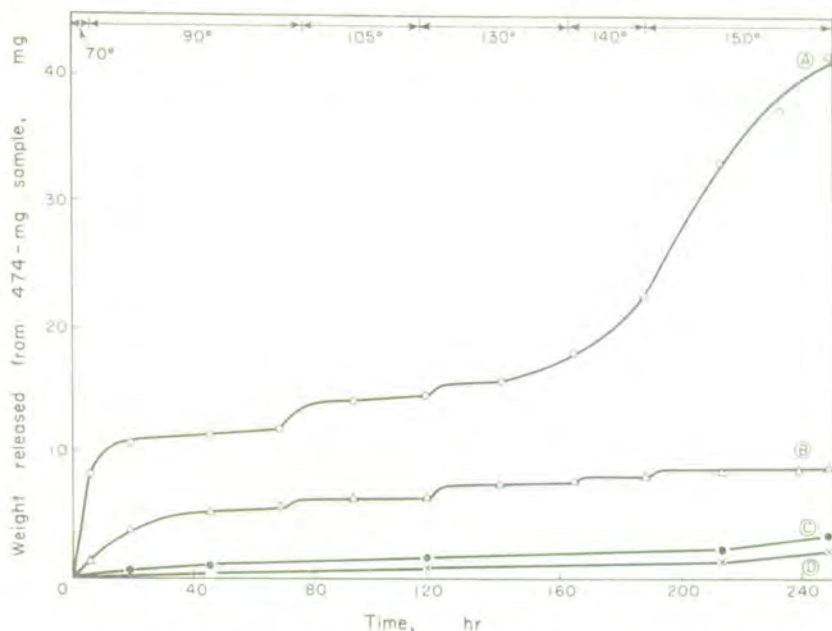

FIG. 3.—Release of water, ether, ethanol, and acetone from a sample of *C. leonense* gum.
Curve OA—water
Curve OB—ether
Curve OC—ethanol
Curve OD—acetone

decomposition. It is noteworthy that, of the three solvents involved, ether was the most strongly retained: this is surprising, since (*a*) its boiling point is the lowest, (*b*) it is the least polar, (*c*) it was used in smallest amount for the shortest contact time and only for superficial washing. Apparently the ethanol (used in the precipitation process) and the acetone (used in the dehydration procedure) are accessible to and extracted by the ether used in the final washing stage. This effect was verified in the next experiment.

3. A further sample of crude *C. leonense* gum was precipitated with acidified ethanol, dehydrated with absolute ethanol (5 changes, each in contact with the gum for 2 days), progressively ground to a powder, and finally washed with ether. After air drying, a weighed sample (845 mg) was dried by method II at 60° for 24 hr, when the total loss in weight (*i.e.*, water + solvents) was 3·5%, as represented by the curve OA given in Fig. 4. The sample was then quickly transferred and dried by method IV at 78° for a further 138 hr, *i.e.*, for the composite drying period of 24–162 hr. This gave part AB of the dehydration curve. Further drying at 108° for the period 162–234 hr gave curve BC; drying at 120° for period 234–258 hr gave curve CD. The dotted

curves AE, AF and AG give the weights evolved of water, ether and ethanol respectively; curve ABCD gives the sum of AE + AF + AG. The curves clearly show that solvents are still being liberated after drying for 280 hr, and confirm that the ether used as a final wash is more strongly retained than the ethanol used for both precipitation and dehydration.

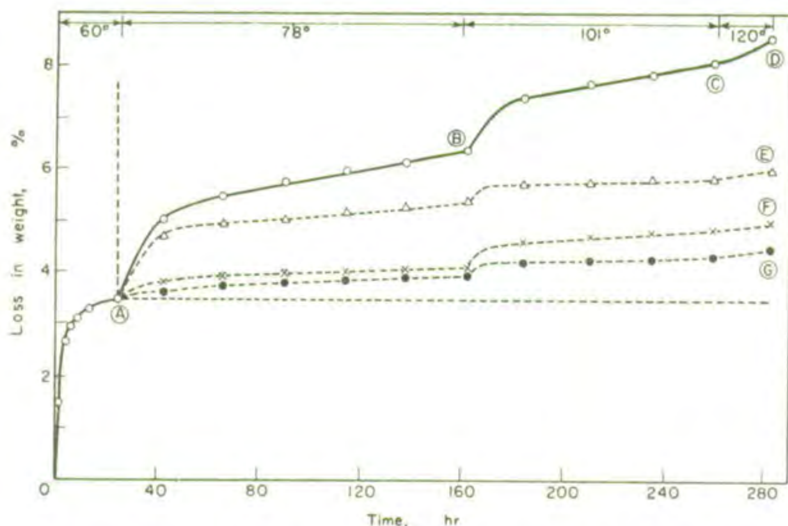

Fig. 4.—Release of water, ether, and ethanol from a sample of *C. leonense* gum.
Curve OABCD—total % loss in weight
Curve AE—water contributing to total AD
Curve AF—ether contributing to total AD
Curve AG—ethanol contributing to total AD

4. To test the effect of particle size on the retention, a sample of crude *C. leonense* gum was precipitated and dehydrated with methanol (6 changes, each in contact for 2 days). After air-drying, the sample was ground finely and sieved. Two fractions (*a*) passing 200 mesh and (*b*) passing 100 mesh were retained, and dried by method II at 60° for 9 hr. Weighed samples were then dried by method IV at 65° for the period 0–365 hr, at 95° for 365–437 hr, at 98° for 437–509 hr, and at 101° for 509–629 hr. For the 200-mesh sample, the weights of water and methanol evolved are given in Fig. 5 by curves *A* and *B* respectively, and for the 100-mesh sample by curves *C* and *D* respectively. These curves again emphasise that the drying temperature is more effective than the period of drying; methanol was still being released at the end of the experiment.

5. Parallel experiments to those described for *C. leonense* gum were made on samples of gum ghatti;[13] the retention of water and solvents by the two gums was very similar.

6. Further experiments were made with gum ghatti to test whether (*a*) solvents less polar than ethanol, methanol, acetone and ether *e.g.*, carbon disulphide and dioxan, or (*b*) solvents containing bulkier functional groups *e.g.*, isopropanol would be less strongly retained. For all of these solvents, however, retentions similar to those for acetone, ethanol, methanol and ether were given.

7. The retention of methanol by some starches was investigated. (*a*) A sample of potato starch (500 mg) was refluxed with methanol for 4 hr. After heating by method

III at 80° for 24 hr, it was then dried by method IV at 98° for 0–18 hr, at 102° for 18–40 hr, at 108° for 40–64 hr, at 112° for 64–84 hr and at 134° for 84–88 hr. As much methanol was evolved in the final 4-hr period of heating at 134°, after steady evolution for 84 hr, as was evolved in the 18-hr initial period at 98°. (*b*) Rye starch, which had been stored under methanol at 0° for 2 years, was treated by method II at 61° for 24 hr.

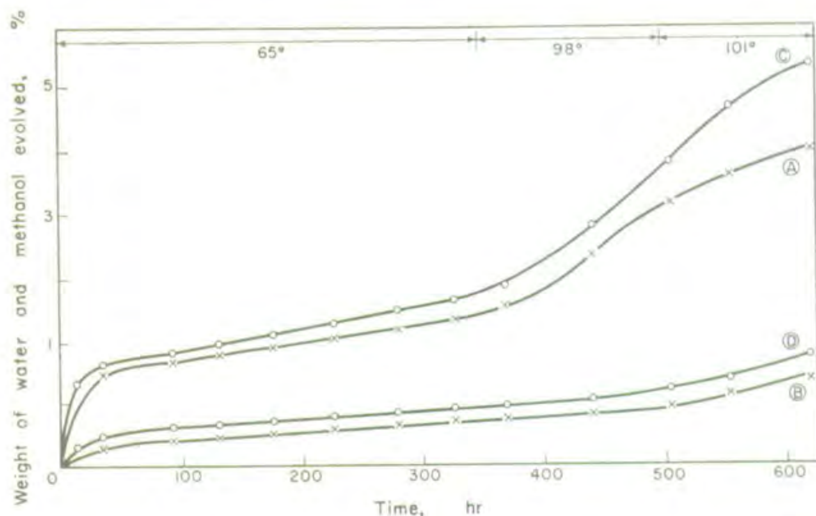

FIG. 5.—Effect of particle size on release of water and methanol from a sample of *C. leonense* gum.
Curve A—water, Curve B—methanol: from 200-mesh
Curve C—water, Curve D—methanol: from 100-mesh

A sample (500 mg) was then dried by method IV, the evolution of methanol being reported in Table II (+ and ++ indicate 0·1 – 0·5 % and 0·5 – 1 % w/w respectively). Similar results were obtained with other starch samples.

8. A sample of *C. leonense* gum which had been purified by ethanol precipitation, dialysed, and freeze-dried for 4 days was found to lose 1 % by weight after heating at

TABLE II

Temperature, °*C*	78			92	98	115	128	140
Period of heating, *hr*	0–18	18–42	42–66	66–90	90–106	106–154	154–178	178–202
Methanol evolved	++	++	+	++	+	++	+	+

103° for 5 hr, and a further 1 % on raising the temperature until decomposition began. Similar experiments with a freeze-dried sample of a pectic acid isolated from *Nitella translucens*[14] gave a loss in weight of 6·4 % (duplicate runs) when the sample was taken direct from the freeze-drier; samples stored in a desiccator overnight after freeze-drying gave 8·5 % loss in weight when treated by method III.

DISCUSSION

The results show that both water and organic solvents are retained tenaciously, even on prolonged drying under vacuum at temperatures much higher than the boiling point of the solvents concerned. Indeed, retention continues up to the temperature at which the polysaccharide material begins to decompose, as revealed by yellowing in colour and the starting of the evolution of CO_2. Furthermore, although apparent "constant weight" is reached after drying at a certain temperature for a determinable number of hours, continued drying at a slightly increased temperature gives further liberation of water and solvents, until eventually "constant weight" is again reached. This cycle can be repeated many times, by raising the temperature in small increments, until eventually the decomposition temperature of the carbohydrate material is reached.

Freeze-dried samples also retain moisture tenaciously, although in smaller amount; this agrees with Robson,[15] who found that a solution containing 16% glucose + 3% gelatin required freeze-drying for 500 hr at room temperature for *all* moisture to be removed. In practice, freeze-drying is not normally continued for longer than about 100 hr, and it is often assumed that samples so treated have a negligible moisture content: many quantitative inaccuracies must arise in this way. Freeze-dried samples may also retain organic solvents; cellulose retains 1·5% (w/w) of benzene on freeze-drying.[16]

Water and vapours can be occluded[17] in crystalline sugars; indeed, it was found recently[18] that when phenylboronate ester derivatives of methyl glycopyranosides were recrystallised from benzene, significant amounts of solvent were retained unless special care was taken to ensure its complete removal. Other authors[19] have recently reported similar solvent occlusion effects.

To obtain accurate quantitative results with carbohydrate materials, it is clear that great attention must be paid to drying procedures. Furthermore, any previous treatment of a sample with organic solvents must be considered if inaccurate and misleading functional group analyses *e.g.*, methoxyl, acetyl are to be avoided (*cf.* ref. 20). Application of the infrared alkoxyl method[11] has shown that mere reflux with ethanol can create artefacts *e.g.*, ethoxylation of the fructose from lucerne[21] and of certain plant gum components:[22] we are grateful to Dr. R. J. Ferrier for the information[18] that a dry, powdery xylan of normal appearance prepared by him contained 70% of ethanol. These findings serve to support the timely warning given by Bell[23] regarding the possibility of formation of non-reducing ethyl glycosides when plant materials are treated with hot 80–95% ethanol. It now appears likely that the occurrence of an ethyl riboside,[24] so far unconfirmed,[25] also arose in this way.

Acknowledgements—We thank Professor E. L. Hirst, C.B.E., F.R.S., for his interest in these studies, and the Department of Scientific and Industrial Research for a maintenance grant (to N. J. K.). We are grateful to Dr. R. S. Fanshawe, Dr. R. J. Ferrier and Dr. E. E. Percival for supplying materials for examination, and for providing experimental details.

Zusammenfassung—Es wurde untersucht, bis zu welchem Ausmasse einige Kohlenwasserstoffe Wasser und organische Lösemittel festhalten. Das Wasser wurde gravimetrisch und die Solventien mittels einer Infrarotmethode bestimmt. "Trocknen zu konstantem Gewicht" bei 103°C im Ofen oder bei 80°C im Vakuum gibt nicht den "wahren Feuchtigkeitsgehalt". Wasser sowohl als auch organische Lösemittel werden bis hinauf zu Temperaturen festgehalten, wo bereits Zersetzung der Kohlenwasserstoffe stattfindet. Polarität und Siedepunkt sind für das Ausmass der Retention nicht bestimmend. Frostgetrocknete Proben können organische Lösemittel zurückhalten und zeigen

signifikante Gehalte und Feuchtigkeit. Die Möglichkeit ungenauer Ergebnisse und irreführenden Verhaltens, die durch diese Art von Retention hervorgerufen werden können, werden betont.

Résumé—Les auteurs ont étudié l'importance avec laquelle les hydrates de carbone retiennent l'eau et les solvants organiques. L'eau fixée était dosée par gravimétrie, les solvants par une méthode infra-rouge. Le "séchage à poids constant" dans un four à 103° ou sous vide à 80° ne donne pas des "teneurs en humidité" réelles. L'eau et les solvants organiques sont retenus tous les deux jusqu'à des températures auxquelles les hydrates de carbone commencent à se décomposer; la polarité et le point d'ébullition du solvant ne déterminent pas l'importance de sa liaison. Des échantillons séchés à froid peuvent retenir des solvants organiques et ont des teneurs en eau importantes. La possibilité de résultats inexacts et de produits aberrants provenant de fixation ou d'intéraction avec les solvants est soulignée.

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Applications of infra-red spectroscopy—VI*

Recent developments in techniques of increased sensitivity for the analysis of GLC fractions and other small scale samples—a brief review

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THE relative cost of acquiring the appropriate apparatus has probably contributed significantly to the unfortunate fact that, of absorption spectroscopic methods, colorimetry and ultraviolet have been more frequently applied to analytical problems than infrared. Recently, however, the availability of less expensive equipment has fostered greatly increased interest in the analytical uses of infrared spectroscopy. The emphasis, however, has been on qualitative applications; the tremendous potential which infrared offers to quantitative analysis does not appear to have been adequately appreciated. Infrared not only identifies unequivocally, but simultaneously gives a basis for accurate quantitative analysis if the appropriate calibration curves on standards are constructed. Moreover, the sample is not destroyed, and the nature of any contaminants is revealed. Generally, contaminants rarely interfere with quantitative determination of the desired compound if the operating conditions are carefully chosen, but if interference does occur, adequate warning is given by the appearance of the spectrum. The literature continues to describe analytical procedures which could be carried out more rapidly and accurately, with decreased interference from other components, by infrared methods.

General interest in the analysis of smaller and smaller amounts of material continues, and has naturally stimulated efforts to increase the sensitivity of all spectroscopic techniques. For infrared this can be approached by the use of auxiliary instrumentation such as scale-expansion,[1] multi-pathlength cells,[2] or beam condensers—all of which are relatively expensive—or by improving the design of single-pass cells. Techniques are now clearly established[3] for the quantitative analysis of 20–100 μg of material in solution or in the liquid, solid, or gas phase; various techniques and accessories permitting the examination of <1 μl of some samples have also been described[4] or commercially advertised.

The greatest stimulus to the development of increased sensitivity has doubtless come from the challenge for spectroscopists to approach the sensitivity attainable in chromatographic separations. This challenge has been invaluable, since the resulting spectroscopic developments are equally applicable to samples which are not of chromatographic origin. Throughout the development of both techniques, samples obtained as GLC fractions have always tested the minimum limits detectable by infrared; it now appears that spectroscopic techniques may never match the chromatographic sensitivity attained recently through the development of capillary columns.

Indeed, some authorities[5] consider that the identification of GLC fractions may have to be based solely on the use of retention-volume characteristics. Although this is doubtless satisfactory for routine applications, its serious deficiencies when applied to entirely unknown mixtures from research investigations, or to systems which have not been completely characterised, have already received comment.[6] A triangulation method using three stationary phases of differing polarities has been proposed[7] for the positive identification of GLC components, but many investigators are agreed[4,6,8–10] that identification based solely on retention characteristics is inadequate. Compounds can have closely similar retention volumes, even on different column materials; components may only be separable[9] as "shoulders"; and mixtures of similar hydrocarbons[10] or of members of different homologous series[8] may be found under a single chromatographic peak. The collection of fractions and subsequent examination by infrared[4,6,11] or mass spectroscopy[9,10] is therefore desirable; continuous infrared detection of functional groups has also been proposed.[12] If spectroscopic equipment is not available, then functional group analysis[8,13] of the eluted fractions is desirable, and can be operated at the 20-μg level.

Considering now only infrared techniques, it is clear that the dominant difficulty lies in matching the sensitivity of GLC. When co-operation between the two techniques is essential, a possible solution is to operate below maximum GLC sensitivity; this, however, could only be taken a certain length before leading to column overloading or to decreased chromatographic resolution. All too frequently, the particular component to be studied is present in trace amount in a mixture: even although an

* Part V: D. M. W. Anderson and N. J. King, *Talanta*, 1961, **8**, 497.

adequate supply of the mixture is available, insufficient amounts of the required fraction for infrared examination may be given by each chromatographic fractionation. It appears, therefore, that there will always be a demand for the technique of trapping the appropriate peak from a number of consecutive GLC runs, so that eventually a quantity sufficient for spectroscopic identification is collected.[6] Techniques involving collection in liquid nitrogen traps are now frequently used.[4,14] Other methods, such as mixing the eluted fraction with a vapour solvent introduced *via* a side-arm to the trap,[15] or depositing the desired component at room temperature on finely ground potassium bromide, which is subsequently pressed to form a disc,[16] have also been described.

Although it has clear limitations[6] (*cf.* ref. 4), the vapour-phase technique remains invaluable for certain types of sample, and any increase in its sensitivity[11] would be advantageous. Although metal cells of uniform rectangular cross-section[4] can increase the sensitivity given by circular glass-cells,[11] they do not, however, give the maximum sensitivity attainable with single-pass cells. Metal cells having tapered bores of rectangular cross-section, such that all essential rays of the energy beam are exactly enclosed, became available commercially for certain spectrometers during 1960: tapered cells made here for use with the Hilger H800 instrument were shown to visitors to this department during the 3rd International Gas Chromatography Symposium. It is of interest that the asymmetrically placed focus of the H800 spectrometer gives some advantage in designing such a tapered cell for insertion in the energy beam between the point of focus and the spectrometer entrance slit, rather than (*a*) between the energy source and the point of focus or (*b*) symmetrically about the focus.

That such rectangular tapered cells are more conveniently constructed of metal rather than of glass introduces no disadvantages; the examination of corrosive or reactive compounds merely requires the surfaces of the cell to be thinly covered with an epoxy or other coating suitably inert to the compound being examined. Indeed there now appears to be a distinct advantage in also constructing the trap and stop-cocks of metal; when trap dimensions are reduced, the increasing fragility of glass is off-set. A trapping device employing hypodermic needle tubing has been described.[17]

Suitably small vacuum-tight metal stop-cocks are now available commercially; these facilitate all-metal construction, although there is no difficulty in incorporating glass stop-cocks[4] in an otherwise metal assembly. It has long been known, however, that the presence of stop-cock grease is undesirable.[18] The importance of using minimal amounts of lubricants was stressed earlier,[6] but in working with ever decreasing amounts of material, there are now distinct indications that *all* traces of lubricants must be eliminated. High-vac greaseless taps are now offered commercially, and greaseless laboratory devices have been described.[18,19]

The future development of minimum volume, all-metal, greaseless trap-cells may also simultaneously assist the incorporation of heating devices so that the applicability of the vapour-phase technique may be extended above its present limit of about 125° (*cf.* refs. 4 and 11). Development of heated cells[6] suitable for accurate quantitative work has proved troublesome; difficulty has also been reported by other investigators.[4] Nevertheless a vapour cell operating at $\pm 1°$ up to 180° has been developed.[20] The results of continued efforts in this laboratory will be published in due course.

Finally, the development of new infrared transmitting glass such as "Irtran-2" (Eastman-Kodak Ltd.) which gives $>70\%$ transmission from 2-10 μ, decreasing to 40% at 14 μ, and which has good thermal shock properties, may facilitate the development of a single unit trap-cell which could be cooled directly to trapping temperature, then heated to volatilise the sample. Such cells may also be suitable for aqueous systems. The technique of trapping directly in evacuated gas-cells has recently been used.[14]

Acknowledgement—I am grateful to Dr. W. Zehden (Messrs. Hilger and Watts) for helpful discussions, in November last year, regarding the design of single-pass gas-cells of maximum sensitivity for use in Hilger H800 spectrophotometers.

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Summary—The advantages of quantitative infrared spectroscopy recommend the technique for wider application to analytical procedures than at present. Micro-scale samples can already be dealt with, and recent developments leading to increased sensitivity are briefly reviewed, with particular reference to the examination of samples in the vapour phase.

Zusammenfassung—Die sehr Vorteilhafte quantitativer IR-Spectroskopie wird zur ausgedehnteren Anwendung empfohlen. Es ist bereits möglich mit Mikroproben zu arbeiten; jüngste Entwicklungen führten zu erhöhter Empfindlichkeit, was im Hinblick auf die Untersuchung von Proben in der Dampfphase behandelt wird.

Résumé—L'auteur recommande les avantages de la spectroscopie infrarouge quantitative pour une application plus importante aux méthodes analytiques. Des échantillons miscroscopiques peuvent déjà être traités; les développements récents conduisant à une sensibilité accrue sont brièvement passés en revue, avec une référence particulière pour l'étude des échantillons en phase vapeur.

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APPLICATIONS OF INFRARED SPECTROSCOPY—VII*

THE BEHAVIOUR OF THIOALKYL COMPOUNDS UNDER ZEISEL REACTION CONDITIONS.

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Summary—Vapour-phase infrared spectroscopy has been used to study the behaviour of a wide range of thioalkyl compounds when refluxed with constant-boiling hydriodic acid. A very wide range of reactivity has been observed. Unusually labile compounds exist which react quantitatively in less than 3 hr; the kinetics of the decomposition of such compounds must be investigated individually, since over-production of methyl iodide can occur. Many thiomethyl compounds, however, do not yield methyl iodide, and others react very slowly, giving variable non-quantitative yields of methyl iodide after reflux for 16-20 hr. It is therefore concluded that the mere extension of Zeisel reaction conditions for prolonged reflux periods does not usefully provide a general method for the functional group analysis of thioalkyl compounds.

THIOALKYL compounds, which are of current interest in sugar chemistry,[1] yeast metabolism,[2] choline-esterase inhibition,[3] and the chemistry of food-stuffs,[4] have received considerable attention in recent years.

A modified Zeisel reaction[5] has long been used in protein studies to determine the thiomethyl group in methionine. Kassel and Brand[6] suggested improvements to Baernstein's method,[5] and the method of Kuhn, Birkofer and Quackenbush[7] was modified[8] so that both alkoxyl and thiomethyl groups could be determined.

Much is known of the chemistry of thioethers.[9,10,11] The analytical methods applicable to mercapto groups, disulphides and sulphides (thioethers) have been reviewed,[12,13,14,15] and special methods continue to be described,[16,17,18] particularly for biological materials. Many thioethers are cleaved at pH 8–10 by silver or mercury salts;[10] fission of the carbon-sulphur bond has been more extensively studied in alkaline solution[19] than in acids.[20] In general, thioethers and thiolesters react much more slowly in aqueous acids than do their oxygen analogues,[20,21] although the ease of cleavage of carbon-sulphur bonds is influenced by the presence of substitution[22] and β-unsaturation[11] in the molecule.

Early investigators[23,24] reported applications of a modified Zeisel reaction to aromatic thiomethyl compounds, in which a longer reaction period (which was not specified) was required (*cf.* ref. 25). It has been observed,[26] however, that some compounds do not give quantitative results; *S*-methylthiamin[27] gives only 33% of the theoretical yield of methyl iodide. It is apparent that early investigators were involved in considerable experimental difficulties,[23,24] particularly when gravimetric methods were used. A wide range of thioethers and thiolesters has therefore been studied by the infrared method[28] in an attempt to clarify some of the reported anomalies.

* Part VI: D. M. W. Anderson, *Talanta*, 1961, **8**, 832

EXPERIMENTAL

Apparatus

The apparatus has been described elsewhere,[28] together with the technique for trapping volatile reaction products and the infrared method for their subsequent identification and estimation.

Compounds

The majority of compounds investigated were reagent-grade commercial samples which conformed, after recrystallisation or distillation where necessary, to literature description. The other compounds investigated were research specimens (kindly provided by Dr. D. Leaver of this department and by Dyestuffs Division, Messrs. I.C.I. Ltd.) for which satisfactory elemental analyses existed or were obtained (Messrs. Weiler and Strauss, Oxford, England).

Procedure

Samples of the compounds listed in Table I were refluxed, for the periods indicated, with hydriodic acid (6 ml of constant-boiling azeotrope, pre-conditioned[28]) using cylinder nitrogen ("N.O.F." grade) as flow-gas (6-8 ml per min). Sample weights giving 1-4 mg of methyl iodide were normally taken: larger samples were used to permit the identification of minor reaction products. The compounds were dissolved with care in molten phenol[28] before addition to the reaction-flask; a recent report[29] has confirmed the effectiveness of this technique. The mixture of volatile products was collected, after passage through Anhydrone, in a trap immersed in liquid nitrogen. The components of each mixture were subsequently identified by vapour-phase infrared spectroscopy by reference to spectra obtained from authentic compounds. None of the mixtures was sufficiently complex for prior separation of the components by G. L. C. to be necessary, and the presence of other components did not interfere with the determination of methyl iodide.

RESULTS

All compounds containing sulphur produce some hydrogen sulphide on reflux with hydriodic acid; only volatile products other than hydrogen sulphide are reported below.

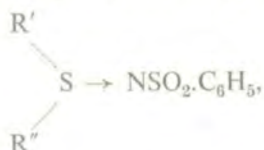
1. *Compounds giving no reaction*

The following thiolesters and thioethers gave no volatile products under Zeisel reaction conditions for reflux periods of 18 hr: methylchlorothiolformate, ethylchlorothiolformate, methyl phenyl sulphide (thioanisole), methyl-α-naphthyl sulphide, 2-thiomethyl-1,4-naphthoquinone, 3-methyl-2-thiomethyl-1,4-naphthoquinone.

The following compounds were partially volatilised, without reaction, from the reaction-flask: methyl and ethyl mercaptan, dimethyl and diethyl sulphide, dimethyl and diethyl disulphide.

2. *Compounds which react but do not yield alkyl iodide*

Dimethyl sulphide was produced from dimethylsulphoxide (fast) and from dimethylsulphone (slow), whilst compounds of the type



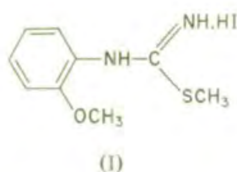
where R′, R″ = *methyl* or *ethyl*, liberated the corresponding dialkyl sulphide. Phenacyl methyl trithiocarbonate gave carbon dioxide, carbon monoxide, and methyl mercaptan.

3. *Compounds which react, yielding alkyl iodide and other volatile products*

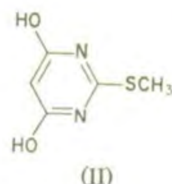
Thiodiglycol and compounds containing the $—S(CH_2.CH_2OH)_2$ grouping gave

ethyl iodide + ethylene in 1-2 hr. Such compounds therefore react similarly to glycols and glycol ethers.[30]

Thiocyanato-methane gave 1 mole of methyl iodide in 2·5 hr, together with some thiocyanogen. The same product was also given by (I) which gives 1 mole of methyl iodide (from the alkoxyl group) in 0·5 hr; the total yield of methyl iodide reached 2 moles after reflux for 20 hr.



(I)



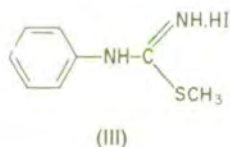
(II)

4. *Compounds which yield only methyl iodide*

Table I lists the kinetic results obtained.

TABLE I.—THE RATE OF PRODUCTION OF METHYL IODIDE FROM SOME THIOMETHYL COMPOUNDS

Compound	Moles of methyl iodide produced per mole of compound
2-(Methylthio)benzo-thiazole	0·44 (0·5 hr); 0·76 (1 hr); 0·87 (2 hr); 0·99 (3 hr); 1·12 (4 hr)
S-Methylthiouronium sulphate	0·35 (2 hr); 0·60 (3 hr); 0·98 (4 hr); 1·05 (4.5 hr)
II	0·30 (1 hr); 0·45 (2 hr); 0·55 (2·5 hr); 0·76 (3-4 hr, const.)
III	0·50 (16 hr); 0·52 (20 hr)
IV	0·49 (20 hr)
V	0·25 (12 hr); 0·30 (18 hr)
Methyl-*p*-tolyl sulphide	0·25 (16 hr)
Methyl-*p*-nitrophenyl sulphide	0·15 (17 hr)
Methionine	0·86 (0·5 hr); 0·96 (1 hr); 0·99 (2 hr); 1·15 (2·5 hr); 1·25 (3 hr)
N-Acetyl-DL-methionine	0·86 (1 hr); 1·00 (1·5 hr); 1·22 (2 hr)
Glycyl-DL-methionine	0·1 (15 hr)
S-Methyl-D(+) cysteine	0·15 (2 hr); 0·36 (4 hr); 0·60 (8 hr); 0·70 (16 hr); 0·76 (22-40 hr, const.)
S-Methylglutathione	0·14 (2 hr); 0·46 (8 hr); 0·57 (12 hr); 0·66 (18 hr); 0·75 (22-40 hr, const.)



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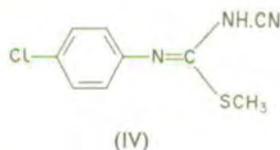

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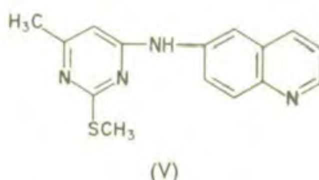

(V)

5. *Investigation of amino-acids*

Table I shows the rate of production of methyl iodide from methionine and S-methyl derivatives of amino acids. In connection with the possible over-production

of methyl iodide, it was observed that Baernstein had found[31] all samples of leucine to give small amounts of methyl iodide. Whilst Baernstein considered that this indicated the presence of methionine, the methyl iodide could have arisen from general decomposition of the amino-acid molecules. Whilst one sample of L-leucine (shown by paper chromatography to contain some methionine) did give approximately 0·1 mole of methyl iodide, no methyl iodide was given by two different commercial samples of DL-leucine which gave only one spot when examined by paper chromatography. Similarly no methyl iodide was evolved from the following: cystine, homocystine, djenkolic acid, glutathione (reduced), taurine, glycine, alanine, DL-α-amino-n-butyric acid, DL-α-amino-iso-butyric acid, DL-β-amino-iso-butyric acid, L-valine, DL-valine, DL-nor-valine, DL-nor-leucine, DL-iso-leucine.

DISCUSSION

The results quoted show that, under the conditions of the Zeisel alkoxyl determination, the behaviour of thioalkyl groups is extremely variable. It would be misleading to suggest that the determination of *S*-methyl groups generally follows the procedure for *O*-methyl groups, even if the time of reaction be extended by several hours (*cf.* ref. 25 and 32). Indeed, as might be expected from the known stability of the sulphur-carbon bond,[10,11,20,21] the few labile compounds which do give quantitative yields of methyl iodide can be regarded as exceptions to the general rule; those compounds which react rapidly must be considered as possible sources of anomalous alkoxyl determinations. No generalisations regarding reaction conditions or reflux period required can be made; obviously, the kinetics of decomposition of each thiomethyl compound must be investigated individually (*cf.* ref. 33).

The observed rate of formation of methyl iodide from methionine agrees with that reported by previous workers,[5,6] who observed[6] that small differences in reaction rate can be attributed to variations in the design of apparatus. Baernstein's earliest procedure[5] (1932) recommended a reaction period of 15 hr, but this was later (1934) amended to 3 hr. Our results show the possibility (which does not appear to have been appreciated previously) of over-production of methyl iodide from those thiomethyl compounds which are sufficiently labile to react in short periods.

Theoretical reasons for the failure of aqueous halogen acids to cleave carbon-sulphur bonds as effectively as carbon-oxygen bonds have been discussed (*cf.* refs. 11, p. 36, and 21, p. 677). The very wide range of reactivities observed in thioethers is not unique: a remarkable range of reactivity is also to be found in their oxygen analogues.[34] It is of interest that compound II is not particularly labile, whereas methoxy compounds of this type were found[34] to be unusually reactive in acid solution.

The behaviour of thiomethyl compounds in Herzig and Meyer's pyrolytic procedure[35] for alkimide groups has not been studied. Experiments were made, however, with acid more concentrated (67% HI, sp. gr. 1·94) than the constant-boiling azeotrope (55% HI, sp. gr. 1·70). In agreement with previous reports,[23] the more concentrated acid did not cause appreciably faster liberation of methyl iodide. The addition of large amounts of phenol, propionic anhydride, or mercuric chloride to the reaction mixture[23,24] (*cf.* ref. 11, p. 39) did not influence the kinetic results.

Acknowledgements—We thank Dr. D. Leaver and Messrs. I.C.I. (Dyestuffs Division) Ltd., for providing samples, and the P.C.S.I.R., Karachi, for granting study leave to S. S. H. Z.

Zusammenfassung—Infrarotspectroskopie in der Gasphase wurde angewandt um das Verhalten von Thioalkylverbindungen zu studieren, wenn sie unter Rückfluss mit konstant siedender Jodwasserstoffsäure behandelt werden. Ungewöhnlich labile Komponenten existieren, welche weniger als 3 Stunden zur quantitativen reaktion benötigen. Die Kinetik der Zersetzung solcher Komponenten muss individuell studiert werden, da Überproduktion von Methyljodid auftreten kann. Viele Verbindungen jedoch geben kein Methyljodid, ander reagieren sehr langsam, sodass die Ausbeute selbst nach 16–20 Stunden nicht quantitativ ist. Es wird daher geschlossen, dass die blosse zeitliche Ausdehnung der Zeiselmethode keine allgemeine Methode zur Analyse funktioneller Gruppen in Thioalkylverbindungen ergeben kann.

Résumé—Les auteurs ont utilisé la spectroscopie infra-rouge en phase vapeur pour l'étude du comportement d'un grand nombre de composés thioalcoylés chauffés au reflux avec de l'acide iodhydrique bouillant constamment. Ils ont observé un domaine de réactivité très large. Il existe des composés inhabituellement labiles qui réagissent quantitativement en moins de trois heures; la cinétique de la décomposition de tels corps doit être étudiée individuellement, car il peut y avoir surproduction d'iodure de méthyle. Cependant, de nombreux composés thiométhylés ne donnent pas d'iodure de méthyle et d'autres réagissent très lentement en donnant des rendements variables non quantitatifs en iodure de méthyle après reflux pendant 16–20 heures. On peut donc conclure que le simple développement des conditions de la réaction de Zeisel pour des périodes de reflux prolongées n'apporte pas de méthode générale d'analyse du groupement fonctionnel des composés thioalcoylés.

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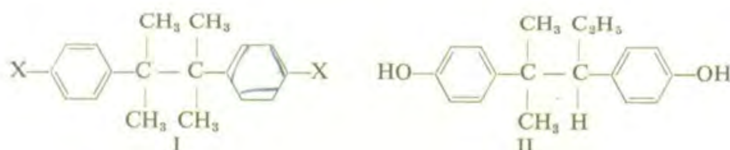
Talanta, 1962, Vol. 9, pp. 620 to 621. Pergamon Press Ltd. Printed in Northern Ireland

Applications of infrared spectroscopy—VIII*: Investigation of a reported anomalous Zeisel alkoxyl reaction

(*Received* 23 *February* 1962. *Accepted* 28 *February* 1962)

SEVERAL examples of molecules which react anomalously in the Zeisel alkoxyl reaction have been reported,[1,2,3] and solvent retention is known[4,5,6] to be a potential source of error in alkoxyl determinations.

Huang and Morsingh[7] reported that 2,3-dimethyl-2,3-diphenylbutane (I, X = H) and certain of



its derivatives (I, X = OH, NO_2) reacted anomalously in the Zeisel reaction, giving an apparent methoxyl content of 3·3%. Such a result appeared surprising; when it was observed that the experimental results quoted[7] were somewhat variable, and that the specimen used[7] had been prepared by a method[8] involving crystallisation from ethanol, it appeared that the reported anomaly might be, in effect, a further example of solvent retention. If so, application of the infrared alkoxyl method[9] would reveal that ethyl iodide, and not methyl iodide, was the reaction product.

2,3-Dimethyl-2,3-diphenylbutane, prepared by Farmer and Moore's method,[8] was re-crystallised from ethanol. After drying in the normal way, the product had m.p. 118° (lit., 118–119°). Analysis (Weiler and Strauss, Oxford,) England gave %C = 90·51 %H =, 9·34; required, %C = 90·75, %H = 9·25. When treated with constant-boilinghydriodic acid, under the conditions described by Anderson and Duncan,[9] this compound gave no volatile reaction products, even after prolonged reflux overnight. Indeed, so stable is this hydrocarbon that it was recovered unchanged (identity of infrared spectrum) from the hydriodic acid reaction medium. This compound therefore neither retains solvent of crystallisation nor reacts anomalously under *normal* Zeisel conditions.

It is perhaps significant that Huang and Morsingh reported[7] that neither the dimethoxy derivative (I, X = OCH_3) nor compound II reacted anomalously, and that drastic conditions, normally reserved for the analysis of *N*-methyl groups, were used[10] in their analyses. Thus the sample was dissolved in phenol and acetic anhydride and refluxed with hydriodic acid; after evaporation to dryness, the residue was heated above 300°. It is quite unreasonable for results obtained by such a procedure to be described[10] as anomalous Zeisel methoxyl determinations.

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Summary—2:3-dimethyl-2:3-diphenylbutane is stable to reflux with constant-boiling hydriodic acid under standard Zeisel alkoxyl reaction conditions. A previous report that this compound reacts anomalously is therefore incorrect.

Zusammenfassung—2,3-Dimethyl-2,3-diphenylbutan ist stabil, wenn es unter Rückfluss mit konstantsiedender Salzsäure gemäss den Bedingungen einer Alkoxylbestimmuhg nach Zeisel gekocht wird. Eine frühere Mitteilung, dass die Verbindung abnormales Verhalten zeigt, ist daher unrichtig.

Résumé—Le 2-3-diméthyl-2-3-diphénylbutane est stable quand il est chauffé au reflux avĕc de l'acide iodhydrique bouillant constamment dans les conditions de la réaction standard de Zeisel pour les alcoyles. Un rapport antérieur prévoyant que ce composé réagit de facon anormale est donc incorrect.

* Part VII: *Talanta*, 1962, **9**, 611.

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Talanta, 1962, Vol. 9, pp. 661 to 666. Pergamon Press Ltd. Printed in Northern Ireland

APPLICATIONS OF INFRARED SPECTROSCOPY—IX*

THE DIFFERENTIATION OF ESTERS AND ACETALS FROM ETHERS IN THE ZEISEL ALKOXYL REACTION

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(*Received* 30 *January* 1962; *Accepted* 5 *February* 1962)

Summary—After determination of the total alkoxyl content (ester + ether groups) by reflux with hydriodic acid, the contribution from methyl and ethyl ester groups is found by refluxing a second sample of the compound in constant-boiling hydrochloric acid for 3 hr. Acetals cannot be distinguished from esters; the yields of alkyl chloride from propyl and butyl esters are diagnostic, although not quantitative. Certain classes of compounds, which have unusually labile ether linkages, restrict the general applicability of the method; examples of such compounds are given.

THE Zeisel alkoxyl determination gives the total alkoxyl content of a compound, and does not differentiate[1] between esters, acetals, alcohols and ethers; such information would be useful in structural studies on complex molecules.

Analytical methods for esters[2] and acetals[3] have been reviewed: colorimetric[4] and oximation[5] methods for determining acetals, ketals and vinyl ethers continue to be described. Differences in reaction-rate can sometimes be used to analyse mixtures of organic compounds containing the same functional group.[6,7] The Zeisel reaction-time for quantitative yields of alkyl iodide from methoxy and ethoxy compounds is so rapid, however (approx. 15 min for methyl iodide) that kinetic differentiation between esters and ethers is not feasible (*cf.* ref. 8).

Methods for the determination of methanol, methyl esters and methyl ethers were considered in an early review,[9] and the determination of glycosidic methoxyl was investigated by Hoffpanir and Reeves.[10] The use of hydrobromic acid as a demethylating agent is well known;[11,12] when it was found[13] that constant-boiling hydrobromic acid produced alkyl bromides quantitatively from esters and ethers almost as quickly as hydriodic acid formed the corresponding iodides (*cf.* refs. 12 and 14), the rates of reaction in constant-boiling hydrochloric acid were investigated. Quantitative yields of the corresponding alkyl chloride were obtained from methyl or ethyl esters and acetals in 3 hours; in that time, only trace amounts of alkyl chloride were produced from true ethers (*e.g.*, 0·05 moles from vanillin, 0·12 moles from 2,3-dimethoxybenzaldehyde, 0·02 moles from phenacetin) (*cf.* ref. 15). This modification to the Zeisel reaction therefore gives the possibility of differentiating between esters and ethers.

EXPERIMENTAL

The reagents, reaction conditions and apparatus used for determination of total alkoxyl, using constant-boiling hydriodic acid, were as described elsewhere.[16] The ester content is found by refluxing a sample (sufficient to yield 1–4 mg of alkyl chloride) for 3 hr with constant-boiling hydrochloric acid (6 ml) to which approx. 0·25 g of phenol has been added.

* Part VIII: D. M. W. Anderson, M. A. Herbich and S. S. H. Zaidi, *Talanta*, 1962, **9**, 620.
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The alkyl chloride evolved is determined by the infrared method, using the manometric method of calibration:[17] laboratories not equipped with an infrared spectrometer can use a modified Fujiwara method.[18]

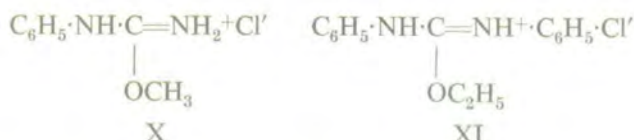
No difficulty was experienced with volatile alcohols, acetals and esters when the reaction conditions proposed by Anderson and Duncan[16] were used (*cf.* refs. 19 and 20).

RESULTS

The method has been applied to a wide range of methoxy and ethoxy compounds having (*a*) ether groups only, (*b*) ester groups only, and (*c*) ether and ester groups in various combinations. Some typical results are shown in Tables I and II, together with the structural deductions possible had only the molecular weight of the compound been known.

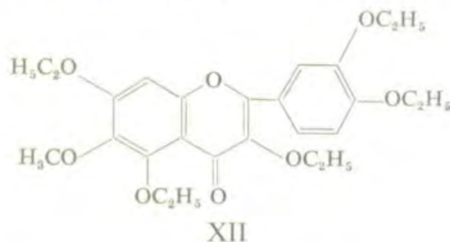
Whilst the method is clearly applicable to many compounds, it is not of general applicability. Several classes of compound contain abnormally reactive ether groups, which are labile in constant-boiling hydrochloric acid. The following examples of these have been encountered in this work, and others doubtless exist (*cf.* ref. 12):

(1) *Substituted urea salts*, such as X and XI.



(2) *Certain methoxy-pyrimidines*[21] *and -pyridines*.[22]

(3) *Flavone derivatives*[23] containing alkoxyl substituents at position 5. Thus 3,5,7,3′,4′-pentaethoxy-6-methoxyflavone (XII) gives 1 mole of ethyl chloride (from position 5) when treated with hydrochloric acid, and 1 mole of methyl iodide + 5 moles of ethyl iodide on reflux with hydriodic acid.



Reaction with constant-boiling hydrochloric acid is therefore a diagnostic test for alkoxyl substituents at position 5 in flavones if it is known (infrared spectroscopy) that ester groups are not also present in the molecule.

(4) *Certain dimethoxy compounds*,[24] such as the *N*-benzene-sulphonyl derivative of XIII.

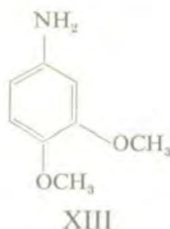
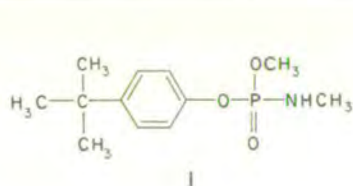


TABLE I.

Compound	Reflux with hydriodic acid: total methoxyl, % Theory	Reflux with hydriodic acid: total methoxyl, % Found	Reflux with hydriodic acid: total ethoxyl, % Theory	Reflux with hydriodic acid: total ethoxyl, % Found	Reflux with hydrochloric acid: ester methoxyl, % Theory	Reflux with hydrochloric acid: ester methoxyl, % Found	Reflux with hydrochloric acid: ester ethoxyl, % Theory	Reflux with hydrochloric acid: ester ethoxyl, % Found	Deductions
Vanillin	20·4	20·4	—	—	0	0·9	—	—	ether groups only.
Phenacetin	—	—	25·2	25·1	—	—	0	0·5	
Yohimbine HCl	7·94	7·92	—	—	7·94	7·67	—	—	ester groups only.
Ethyl-*p*-nitrobenzoate	—	—	23·1	22·9	—	—	23·1	22·7	
"Ruelene" (I)	10·7	10·5	—	—	10·7	10·5	—	—	
"Ronnel" (II)	19·3	19·3	—	—	19·3	19·0	—	—	
"Menazon" (III)	22·1	21·4	—	—	22·1	21·2	—	—	
"Mecarbam" (IV)	—	—	41·0	40·6	—	—	41·0	40·1	
Methyl anisate	37·4	37·4	—	—	18·7	19·2	—	—	methyl ether and ester groups, ratio 1:1
Methyl-3:4-dimethoxy-benzoate	47·4	47·3	—	—	15·8	16·1	—	—	methyl ether and ester groups, ratio 2:1
Reserpine	30·6	30·4	—	—	5·10	5·36	—	—	methyl ether and ester groups, ratio 5:1
Methyl mangifate trimethyl ether (V)	29·7	29·3	—	—	7·43	7·71	—	—	methyl ether and ester groups, ratio 3:1
Ethyl-α-ethoxy-propionate	—	—	61·6	61·2	—	—	30·8	31·7	ethyl ether and ester groups, ratio 1:1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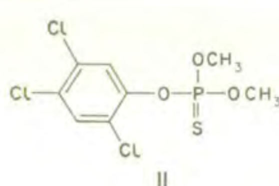
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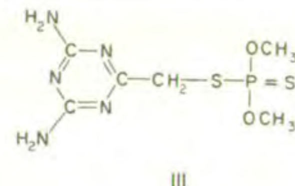
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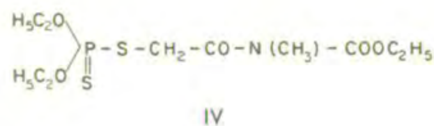
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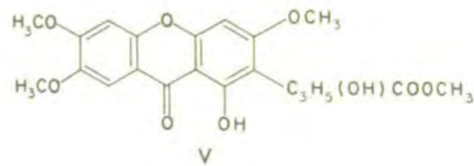
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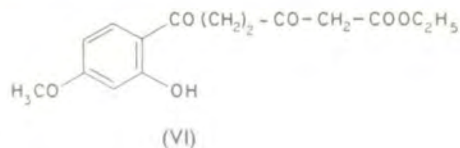
Table II

Compound	Reflux with hydriodic acid				Reflux with hydrochloric acid				Deductions
	total methoxyl, %		total ethoxyl, %		ester methoxyl, %		ester ethoxyl, %		
	Theory	Found	Theory	Found	Theory	Found	Theory	Found	
Methyl-3-ethoxy-4-methoxybenzoate	29·5	29·3	21·4	21·1	14·7	15·0	—	—	methyl ester, methyl and ethyl ether, ratio 1:1:1.
Ethyl-3:4-dimethoxy-benzoate	29·5	29·3	21·4	21·6	—	—	21·4	20·8	all ethoxyl present as ester, all methoxyl as ether. Ratio of methoxy/ethoxy = 2/1.
(VI)	10·0	10·3	14·5	14·1	—	—	14·5	13·9	all ethoxyl present as ester, all methoxyl as ether. Ratio of methoxy/ethoxy = 1/1.
(VII)	26·0	26·0	—	—	13·3	13·0	—	—	methoxy only; ratio of ester/ether = 1/1.
2-hydroxy-5-methoxy-w4:6-triethoxy-acetophenone (VIII)	10·4	10·6	45·3	44·7	—	—	—	—	methoxy and ethoxy, present as ethers only. Ratio = 1/3.
Ethyl angolensin (IX)	10·3	10·0	15·0	14·8	—	—	—	—	methoxy and ethoxy, present as ethers only. Ratio = 1/1.



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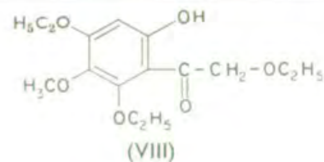

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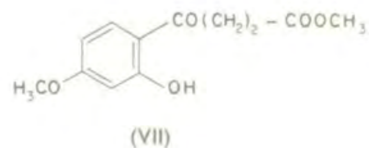

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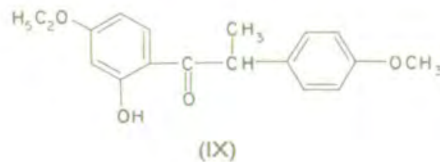

(IX)

(5) *Compounds containing tertiary butyl*[25] *groups*. Two effects must be distinguished: (*a*) non-alkoxy tert-butylated compounds yield alkyl halide, so giving an anomalous reaction; (*b*) in certain alkoxy tert-butylated compounds, such as tert-butylated-*p*-hydroxyanisole, both the tert-butyl and alkoxyl groups are labile so that the compound does not react like a true ether. In addition, high alkoxyl results are given if a method which does not differentiate between methyl and tert-butyl halides is used (*cf*. refs. 8 and 26).

(6) *Methyl glycosides and* O-*methyl carbohydrate derivatives*. A method for differentiating between methyl ester groups at C_6 and methyl linkages at C_2, C_3 and C_4 would be of great value in investigations of plant gums[27] and pectins; investigations of mild de-alkylating reagents[22] are in progress.

Propyl and butyl esters give non-quantitative yields of the corresponding alkyl chloride (approx. 70% for propyl, 30% for butyl chloride) even when zinc chloride is added to the reaction mixture. It is well-known, however, that propyl and butyl ethers react much more slowly in acid solutions than methyl or ethyl ethers. Small yields of propyl and butyl chlorides on reflux with constant-boiling hydrochloric acid therefore indicate the presence of the corresponding ester groups. (The exceptional case of tert-butyl groups has already been mentioned.)

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Zusammenfassung—Nach Bestimmung des geamten Alkoxylgehaltes (Ester- und Äthergruppen) mittels Rückflussdestillation mit Iodwasserstoffsäure, wird der Anteil der Methyl- und Äthylgruppen durch Rückflussdestillation (3 Stunden) mit konstant siedender Salzsäure bestimmt. Acetale können von Äthern nicht differenziert werden. Die Ausbeuten der Alkylchloride von Propyl- und Butylestern sind nicht quantitativ, haben aber diagnostischen Wert. Gewisse Klassen von Komponenten mit besonders labilen Ätherbindungen begrenzen die allgemeine Anwendbarkeit der Methode. Beispiele für solche sind mitgeteilt.

Résumé—Après dosage de la teneur totale en alcoyle (groupes ester + éther-oxyde) par reflux avec l'acide iodhydrique, la contribution des groupes esters méthylique et éthylique est déterminée en chauffant au reflux un deuxième échantillon du composé dans de l'acide chlorhydrique bouillant constamment pendant trois heures. Les acétals ne peuvent pas être distingués des éther-oxydes; les rendements en chlorure d'alcoyle à partir des esters propylique et butyrique sont déterminés, bien que non quantitatifs. Certaines catégories de composés, qui possèdent des liaisons étheroxyde inhabituellement labiles, restreignent l'application générale de la méthode; des exemples de tels composés sont donnés.

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10.

ANALYTICAL APPLICATIONS OF INFRARED SPECTROSCOPY

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It has been said[1] that it is very unusual to find a spectroscopist who is familiar with the complete range of spectroscopic methods. This is not surprising when one considers that nowadays the entire electromagnetic spectrum is used, ranging from the very short wavelengths of X-rays, through the ultraviolet, visible, and infrared regions, up to the long wavelengths of microwave spectroscopy: furthermore, the techniques at any wavelength may involve measurements based on emission, absorption, polarisation, reflection or fluorescence. Since the word "spectroscopy" is involved, we must also mention the techniques of mass, atomic absorption, nuclear magnetic resonance, and electron spin resonance spectroscopy. None of these spectroscopic techniques is all-powerful or of universal applicability: there will always be some need for basic chemical skills in chemical analysis. Nevertheless, within its own sphere of applicability, each of the spectroscopic methods offers such power to the analyst in terms of speed, accuracy, and micro-scale capability, that today any chemist who does not have access to the entire range is working at a distinct disadvantage. The main difficulties are, of course, the capital cost of acquiring the necessary instruments and problems of their subsequent maintenance. Some techniques are still prohibitively expensive to all but the largest laboratories, but most laboratories can now afford apparatus for measurements in the visible and ultraviolet regions. It has been said[2] that with the recent advent of low-cost recording infrared spectrometers, infrared techniques are now practical for even the most modest laboratories, but it does seem that analytical chemists are still insufficiently aware of the advantages of infrared analysis.

Infrared spectroscopy can be applied in some way to the analysis of almost all chemicals, pure or in admixture, be they vapours or solids, liquids or solutions. There may, of course, be some disadvantage if an examination *must* be made in a certain phase, but there can be few chemists who cannot utilise infrared spectroscopy whatever their field of activity, be it fundamental or applied, involving pigments and paints[3], polishes[4], pharmaceuticals[5], polypropylene[6], copolymers[7], polyurethanes[8], rubbers[2], proteins[9], nucleic acids[10], textiles[11], wool[12], or explosives[13].

To date, the emphasis in infrared spectroscopy has been on structural diagnosis and qualitative applications in organic chemistry. It is true that the major use of infrared spectroscopy in the everyday 2.5–16 μ region (4000–650 cm^{-1}) lies in the provision of a unique "finger-print" of a molecule together with certain information concerning the presence or absence of important functional groups. The interpretation of the infrared spectrum of a complex molecule is a skilled job which demands

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considerable experience. Beginners can do no better, after reading some basic introduction such as that of DAVIES[14], than base their approach on the books of CROSS[15] and BELLAMY[16]. An excellent review of the trends in infrared structural diagnosis was given[17] by BELLAMY AND WILLIAMS, who indicated the importance of absolute intensity measurements. Intensity studies of analytical importance continue to be published[18] and a possible intensity standard has been proposed[19].

In a short review, no author could do justice to all possible analytical applications of infrared spectroscopy. Some restricted selection of topics must be made; the present review is intended for the general chemist, not for the specialist, and emphasis is laid on several quantitative topics and techniques which deserve to be better known.

Quantitative infrared analysis can be carried out in any of the physical phases, and we shall consider liquids, solids, and vapours in turn. Accuracies of $\pm 2\%$ can generally be achieved, with $\pm 1\%$ or better often attainable. As usual in spectroscopy, analysis is based on the Lambert-Beer Law, with the particular set of spectroscopic conditions imposed or selected for use being carefully standardised. SHREWSBURY[20] has described a procedure for checking the linearity of the percentage transmission scale. Many individual sources of error can arise, and these were particularly well reviewed by PHILPOTTS AND MADDAMS[21], who also discussed the reasons why spectroscopists prefer to examine solids in solution.

Although CCl_4, CS_2, $CHCl_3$ and CH_2Cl_2 are the solvents of choice, many other solvents can be used, as shown in a paper by BELLAMY *et al.*[22]. Dimethylsulphoxide is a solvent currently fashionable in several fields of study[23]. Solute–solvent interactions are a well-known source of quantitative error[24], but these same interactions can often be turned to good use as a means of identifying unusual or anomalous qualitative group frequencies[25]. Often, of course, a suitable solvent may be difficult to find; many compounds do not dissolve satisfactorily in the preferable solvents. Sulphonic acids give trouble in this way, but treatment of such compounds with a liquid anion exchanger gives a salt which is soluble in carbon disulphide[26]. Sometimes it is necessary to use unusual solvents, such as liquid sulphur dioxide[27] or antimony trichloride, which melts at 73°C and is a good spectroscopic solvent[28] for water and D_2O.

Mention of water leads to a consideration of the examination of aqueous solutions. Water absorbs strongly at certain infrared wavelengths, and it has an undesirable effect on the usual, but not inexpensive, alkali halide window materials. This does not mean that it is impossible to examine aqueous solutions, although it is certainly less convenient to do so. Aqueous solutions were, in fact, first examined in 1905 by COBLENTZ, and the use of optical silver chloride cells was described in 1949[29]. The technique has been used more frequently since 1956, becoming almost commonplace around 1959[30]. The studies by GOULDEN[31] and PARKER[32] are worthy of note. Barium fluoride, calcium fluoride, and thallium bromoiodide (KRS-5) are all suitable window materials for aqueous solutions, and special glass, such as Irtran-2, has recently become available. This glass is also useful when reactive compounds such as sulphur trioxide are being examined[33].

An interesting application is the determination of active hydrogen[34,35] in solids and liquids by exchange with deuterium on treatment with D_2O. Determinations can be completed in 30 min with an accuracy of 2%.

As distinct from aqueous solution work, determinations of trace amounts of water

can be made conveniently by "near" infrared spectrometry[36]. The near infrared region extends from 1 to 2.5 μ (10,000–4000 cm^{-1}); the spectra arise from overtone and combination bands. Although such spectra were first observed photographically in 1881, the analytical advantages of this region have not yet been fully appreciated. Excellent reviews have been published[37,38], and the near infrared has been used for determinations of unsaturation[39], terminal epoxides[40], aldehydes[41], and moisture determinations on solvents[42].

At this point the far infrared region, nominally covering 16 to 100 μ, should also be mentioned. The analytical applications of this region have been adequately treated in a review listing over 500 references[43]. The availability of low-cost spectrometers fitted with KBr prisms should stimulate exploitation of at least part of the far region.

To return to the 2.5–16 μ region, recent papers have described studies made on water-soluble inorganic carbonates[44], and on the simultaneous determination of sulphate, nitrate and nitrite in water samples[45]. So many papers dealing with the study of organic molecules by infrared methods appear nowadays that the applicapility to inorganic compounds is often overlooked. Any attempt to utilise infrared spectroscopy for the study or analysis of inorganic molecules is therefore welcome[46]. Recent inorganic applications have included the determination of traces of sulphate[47] and the identification of niobium and tantalum phosphates[48]. Structural information on oxinates and other divalent metal chelates has been obtained, and the number of molecules of water of hydration can be determined[49]. GATEHOUSE observed that reaction occurred between rock-salt windows and reactive inorganic materials such as nitrato coordination compounds; the difficulty was overcome[50] by coating the windows with thin films of polystyrene. It has now been suggested[51] that thin plastic foil can, with certain advantages, replace rock-salt windows for more general purposes.

For fundamental reasons it is preferable to examine solids in solution, and good quantitative results are most readily obtained in this way. Sometimes, however, there may be good reasons why an examination cannot be made in solution. The alternative methods of preparing samples have been reviewed[52]; the same paper also describes the use of synthetic diamond and sapphire as window materials.

The method of mulling (*i.e.* suspending the solid as a finely ground dispersion in an inert liquid) can be applied on the micro scale[53], and the addition of an internal standard gives the basis for a quantitative method, which is probably accurate to about $\pm 5\%$. Calcium carbonate, potassium and lead thiocyanates, and sodium azide[54] are suitable internal standards.

Agar films have been proposed[55] as specimen carriers for biological materials. Porous Vycor glass[56] is useful for studies of adsorption phenomena and catalytic processes, and the behaviour of carbon monoxide and carbon dioxide on zinc[57], nickel[58] and titanium dioxide[59] surfaces has been investigated. Supplementary optics for such studies have been described[60].

The KBr disk method is widely used for the examination of solids; from the point of view of reproducibility and precision in quantitative analysis it is preferable to the use of mulls. Unfortunately, many serious difficulties and sources of error can arise, and the analyst must constantly be on the alert if misleading results are to be avoided. The advantages and limitations of the method have been reviewed[61], and DUYCKAERTS[62] has discussed the sources of the anomalies which can occur. So

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many reports of anomalous results have been published that there is little excuse for underestimating the possible dangers of the disk method. Papers continue to draw attention[63,64] to the influence of moisture.

KBr disks are being increasingly used in the study of polymeric materials and fibres, both natural and synthetic[65,66,67]. Single fibres can be studied with the aid of beam condensing optics[1], and a recent paper describes the examination of samples as small as 0.05 μg[68]. The fact that infrared microspectrophotometry has not yet been used to full advantage in the analysis of trace constituents and biological materials[68] is largely due to the expensive nature of the auxiliary optical systems which are required. The application of polarised radiation is an established technique[9,69] which has been little used in recent years.

The use of anion-[45] or cation-exchange[70] before pressing the disk can lead to increased selectivity[45] and sensitivity[70]. Aqueous samples can be examined by a combined freeze-drying/disk technique[45], and substantially the same technique can be applied to non-aqueous systems[71]. Care is required[71] to prevent the contamination of freeze-dried specimens by high-vacuum lubricants. Aromatic solvents have been collected[72] on finely ground KBr which is then pressed to form a disk for examination.

The applications of vapour-phase spectroscopy should also be considered. These, naturally, are limited in scope[73], but a surprisingly large range of compounds can be examined, and, by proper choice of instrumental and operating variables, vapour-phase spectroscopy is adaptable to a wide variety of analytical problems[74]. Where it is practicable, examination in the vapour phase is the quickest, cleanest and most generally satisfactory technique of all, from which samples are most easily recovered if required. Calibration curves can be constructed by both weighing and manometric methods[75], and quantitative results are usually accurate and reproducible to within $\pm 1\%$.

For everyday use, mg quantities can be analysed in inexpensive cells of simple construction[75,76]. For particular purposes, minimum volume cells of long path-length[77] and cells of special construction[78] continue to be described. It is often of interest to study reactions at low temperatures[79] or to study decompositions, *e.g.* of plastics, at high temperatures[80,81], and there are many examples of cells specially designed for these purposes, and for the collection[82] of eluates from gas–liquid chromatographic columns. Recent advances in the application of vapour phase spectroscopy to the identification of components in gas chromatography fractions have been reviewed[73]. The danger of relying solely on retention data for the identification of compounds in eluates has been pointed out[73,75] and authorities on gas chromatography continue to stress the dangers and difficulties[83]. Errors arising from non-resolution of components within single peaks continue to come to notice[84], and the importance of combining gas chromatography with infrared spectroscopy has been discussed[85]. It is unfortunate that the spectroscopic sensitivity available is several powers of ten less than that now given by capillary chromatography columns[86]: this discrepancy is likely to remain, and it appears that the repetitive trapping of fractions[73] will remain a useful technique.

The tremendous analytical advantage of infrared spectroscopy is due to the fact that, in a determination, the spectrum reveals exactly what is being measured. The presence of impurities, or the occurrence of an unwanted decomposition in an analyt-

ical procedure, is immediately revealed. The vapour-phase technique can be applied to investigate undesirable side-reactions in analytical procedures such as the ZEISEL reaction[87,88], and it is also a powerful method of investigating anomalous analytical reactions[89-91]. Furthermore, in simple mixtures of known components there is generally no difficulty in determining all the components simultaneously. Thus the infrared method is undoubtedly the most convenient of the available methods for the simultaneous determination of mixed alkoxyl groups[92], or for investigations of the physical retention of organic solvents[93], a process which can cause inaccurate analytical results in natural product chemistry.

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11.

Applications of Infrared Spectroscopy

Part X.* The Zeisel Determination of t-Butoxyl Groups, and the Anomalous Reactions of t-Butylphenols†

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Zeisel determinations on t-butoxyl compounds give non-quantitative and variable results. t-Butyl iodide decomposes thermally to isobutene, the equilibrium involved being affected by the reaction variables and by the addition of phenolic compounds. The over-all effect is therefore particularly complex for aromatic t-butoxyl compounds, since phenolic compounds are formed within the reaction medium as de-alkylation occurs.

Results are presented showing that more satisfactory analyses can be obtained when hydrobromic acid is used in place of hydriodic acid. t-Butyl bromide suffers ≯2 per cent. decomposition to isobutene when boiled under reflux with constant-boiling hydrobromic acid; moreover, this decomposition is reproducible under given reaction conditions, and correction factors can therefore be applied. Since t-butoxyl compounds are de-alkylated almost as quickly in hydrobromic acid as in hydriodic acid, the reaction periods required are not significantly longer; the period required varies from 2 to 3 hours, and is dependent on the nature of the sample.

Boiling under reflux with constant-boiling hydrochloric acid offers a method of differentiating between true t-butoxyl compounds and those t-butyl compounds that react anomalously in hydriodic and hydrobromic acids.

THE utilisation of t-butyl and t-butoxyl compounds has increased greatly in recent years, *e.g.*, in antioxidants,[1,2] perfumery chemicals,[3] free-radical reactions,[4,5] graded oxidants[6,7] and in chromatographic separations.[8] The relatively easy removal[9,10] of t-butyl and t-butoxyl groups makes them useful in reaction intermediates[11] and as protective groups in syntheses, *e.g.*, of peptides.[12] Steric effects,[9] rearrangements[13] and instability[14,15] are factors that combine with the property of ease of removal to complicate the functional analysis of t-butoxyl groups. t-Butylphenols react anomalously in Zeisel determinations,[16,17] and some of the attendant analytical difficulties have been indicated.[18]

Only a few papers have discussed the application of the Zeisel reaction to butoxyl compounds in general. Of these, only two—so far as we are aware—have quoted results for the tertiary isomer. Houghton and Wilson[19] reported, without comment, a recovery of only 18·8 per cent. of the theoretical yield of t-butyl iodide from t-butyl alcohol; under different reaction conditions, Kirsten and Nilsson[20] obtained 60 to 70 per cent. recoveries, and stated that "tertiary butanol appears to give a fairly stable volatile iodide, although the reproducibility of recovery is not good."

It has long been known that t-butyl iodide is unstable at its boiling-point (103° C), the equilibrium—

$$C_4H_9{}^tI \rightleftharpoons C_4H_8 + HI$$

being established.[21] Some decomposition must therefore occur in Zeisel determinations (compare Campbell and Chettleburgh[16]), in which the reaction temperature is 127° C. In view of the discrepancies between the recoveries reported,[19,20] a spectroscopic[22] study of the recovery of t-butyl iodide from reflux in hydriodic acid was undertaken in an attempt to improve the accuracy of determining t-butoxyl groups.

It became clear that the use of hydriodic acid was analytically unsatisfactory when it was found that: (*i*) variation of the reaction conditions gave recoveries of t-butyl iodide ranging from 19 to 80 per cent.; (*ii*) under standardised reaction conditions, the yields of t-butyl iodide were affected by the presence of phenolic compounds in the reaction medium.

* Part IX appeared in *Talanta*, 1962, **9**, 661.

† Presented at the Joint Meeting of the Scottish and North of England Sections in Belfast, June 28th and 29th, 1962.

t-Butoxyl compounds react rapidly[23,24] with aqueous hydrobromic and hydrochloric acids, and t-butyl bromide and chloride are more stable thermally than is the iodide; the possibility of basing analytical reactions on boiling under reflux with those acids was therefore investigated.

Experimental

Compounds—

(*a*) Samples of t-butyl alcohol[25] and t-butyl halides conforming to literature description were obtained by redistillation under reduced pressure of reagent-grade commercial samples. Since t-butyl iodide quickly develops a dark colour, small amounts were redistilled daily. Isobutene was prepared by dehydration (with concentrated sulphuric acid) of purified t-butyl alcohol.

(*b*) *t-Butyl ester*—t-Butyl 3,5-dinitrobenzoate was prepared; the specimen conformed to literature description.

(*c*) *t-Butyl ethers*—t-Butyl phenyl ether, t-butyl-*p*-tolyl ether and t-butyl-1-naphthyl ether were prepared by Grignard reactions with t-butyl perbenzoate[26,27]; the specimens gave satisfactory elemental analyses (Weiler and Strauss, Oxford). Dark colours developed on storage, and these specimens were redistilled under reduced pressure as required.

(*d*) *t-Butylphenols*—Samples were supplied by Dr. R. L. Williams, Messrs. Kodak Ltd. and Messrs. I.C.I. (Dyestuffs Division) Ltd. Liquids were purified by redistillation. Most of the samples, however, were low-melting solids not readily purified by recrystallisation; these were purified by zone-melting.

Apparatus, reagents and procedure—

These have been described,[28,29] together with details of (*i*) the technique for trapping volatile reaction products and (*ii*) the infrared vapour-phase method for their subsequent identification and determination. Particular care is necessary when transferring the contents of the trap to the gas-cell; t-butyl iodide decomposes so readily that direct warming of the trap over a flame is inadvisable. Satisfactory results were obtained by immersing the trap in water at 80° to 90° C, the sodium chloride cell windows being suitably protected (with plastic covers) during this operation.

A slight reaction occurred between t-butyl halides (particularly the iodide) and the sodium chloride cell windows, so that the windows "fogged" much more quickly than usual. The validity of calibration curves had therefore to be checked more frequently than in previous investigations.

Use of solid scrubbers—

Aqueous solutions hydrolyse t-butyl halides to t-butyl alcohol; hydrolysis of the iodide occurs extremely rapidly.[30] It is therefore essential (compare Campbell and Chettleburgh[16]) to use a solid scrubber in determinations of t-butyl halides. Soda asbestos[31] has given satisfactory results throughout our studies.

Results

Experiments with constant-boiling hydriodic acid—

(*a*) *Rate of reaction of t-butoxyl compounds*—Zeisel determinations were conducted on t-butyl alcohol, t-butyl 3,5-dinitrobenzoate and t-butyl-1-naphthyl ether under standard conditions. The conditions were: volume of hydriodic acid, 6 ml (sp.gr. 1·70, pre-conditioned[28]); nitrogen flow rate, 6 to 8 ml per minute; weight of phenol added, 30 mg. Sample weights yielding 2 to 4 mg of t-butyl iodide were taken. The yields of t-butyl iodide at the reaction times stated were as shown in Table I. Burwell, Elkin and Maury[32] have already commented on the fact that ethers are not always less reactive than alcohols.

(*b*) *Recovery of t-butyl iodide*—Samples of t-butyl iodide (in small weighing bottles fitted with ground-glass stoppers—see Anderson and Duncan[28]) were placed in the Zeisel reaction flask; the recovery from boiling under reflux in hydriodic acid was investigated, the reaction conditions being the same as those outlined in (*a*) above. The maximum recovery varied from 58 to 80 per cent.; about 80 per cent. of the total recovery in each determination distilled within 20 minutes. For fixed weights of samples of t-butyl iodide, small variations in recovery

TABLE I: YIELD OF t-BUTYL IODIDE FROM DIFFERENT COMPOUNDS

Compound	Yield of t-butyl iodide (as percentage of theoretical)		
	After reflux for 1 hour	After reflux for 2 hours	After reflux for 3 hours
t-Butyl 3,5-dinitrobenzoate	76·0	80·8 (max.)	—
t-Butyl-1-naphthyl ether	40·5	66·6 (max.)	—
t-Butyl alcohol { 1st determination	42·5	48·7	56·6 (max.)
t-Butyl alcohol { 2nd determination	39·5	45·0	60·9 (max.)

resulted when: (*i*) the volume of hydriodic acid was decreased from 6 to 1 ml, (*ii*) the flow rate was varied from 4 to 12 ml per minute and (*iii*) the weight of phenol was varied from 0 to 100 mg. Little variation in the rate of recovery was found when the temperature of the condenser water was increased (compare Belcher, Fildes and Nutten[33] and Inglis[34]).

(*c*) *Production of isobutene*—In all these determinations some isobutene was produced, the sum of the molar recoveries of t-butyl iodide and isobutene accounting for the t-butyl iodide taken.

A time - recovery experiment with 2,6-di-t-butyl-4-methoxyphenol in which Campbell and Chettleburgh's[16] experimental conditions were used gave results agreeing well with those reported[16]; boiling under reflux for 1 hour gave the theoretical yield of methyl iodide, together with isobutene and a yield of t-butyl iodide that, calculated as methyl iodide, gave an apparent methoxyl content of 21 to 22 per cent. The ratio of the molar yields of isobutene and t-butyl iodide was, however, constant over the whole reaction period, *e.g.*, boiling under reflux for 10 minutes gave approximately 70 per cent. of the total yield of isobutene and also approximately 70 per cent. of the total yield of t-butyl iodide. This does not support Campbell and Chettleburgh's implication[16] that the isobutene results from decomposition of some t-butyl iodide that does not distil in the earlier stages of the reflux period.

The molar ratio of isobutene to t-butyl iodide (*i.e.*, the extent of decomposition of the t-butyl iodide) was also much greater than in any of our previous experiments. It was suspected that this resulted from the changes made in the reaction conditions in order to duplicate Campbell and Chettleburgh's experiments.[16] These workers, in testing scrubber effects, determined the total apparent methoxyl content of 2-t-butyl-4-methoxyphenol under four different reaction conditions, and found four different values ranging from 18·06 to 22·56 per cent. This range was confirmed when these experiments were repeated with a solid scrubber. Changes in reaction conditions, and not scrubber hydrolysis effects, therefore cause the variable results. This effect was further investigated as described below.

(*d*) *Variation in yield of t-butyl iodide with reaction conditions*—In a series of experiments, a constant weight of t-butyl-4-hydroxyanisole (mixed 2 and 3 isomers) was allowed to react under different conditions; these are shown, together with the yields of t-butyl iodide obtained, in Table II. Further experiments showed that cresols and other phenolic compounds caused similar variations in the results. The conjoint addition of other solubilisers, such as propionic anhydride and hypophosphorous acid, further complicated the effect.

Other experiments indicated that phenolic compounds, formed in the reaction medium during the de-alkylation reaction, contributed to the decomposition of t-butyl iodide. (1) t-Butyl-4-hydroxyanisole (5 mg) was allowed to react in hydriodic acid (6 ml), with no added phenol. The recovery of t-butyl iodide was 80 per cent., in agreement with the result

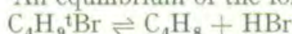
TABLE II: YIELD OF t-BUTYL IODIDE FROM 5-mg SAMPLES OF BUTYLATED HYDROXYANISOLE UNDER DIFFERENT REACTION CONDITIONS

Nitrogen flow rate, ml per minute	Hydriodic acid (sp.gr. 1·70) used, ml	Phenol added, mg	Yield (as percentage of theoretical)
4	1	30	19
4	6	30	51
6 to 8	1	10	53
6 to 8	6	0	80
6 to 8	6	10	72
6 to 8	6	30	62
6 to 8	6	60	50
12 to 15	1	30	45
12 to 15	6	30	73

in Table II. (2) The reaction medium was boiled for a further 4 hours to eliminate all traces of alkyl iodides, and the mixture was then cooled. A further 5 mg of sample and 30 mg of phenol were added; the recovery of t-butylphenol was 52 per cent., compared with 62 per cent. (see Table II) for the corresponding "straight" reaction with 30 mg of phenol. (3) The reaction medium was again boiled for 4 hours, and then cooled; a further 5 mg of sample and 30 mg of phenol were added. The recovery of t-butyl iodide was then only 42 per cent., compared with 50 per cent. for 60 mg of phenol in Table II. Thus the effect of the total phenol added (60 mg) was apparently augmented by the sum (approximately 6 mg) of the weights of phenolic compounds formed in determinations (1) and (2).

EXPERIMENTS WITH CONSTANT-BOILING HYDROBROMIC ACID—

(*a*) *Recovery of t-butyl bromide*—When 6 ml of hydrobromic acid, 30 mg of phenol and a nitrogen flow rate of 6 to 8 ml per minute were used, the recovery of samples (2 to 5 mg) of t-butyl bromide after boiling under reflux for 1 hour was 90·7 per cent. and, after 2 hours, 98·4 per cent. (maximum yield). An equilibrium of the form—



$$C_4H_9{}^tBr \rightleftharpoons C_4H_8 + HBr$$

must exist,[35] but under the stated reaction conditions (reflux temperature 115° C) the extent of decomposition to isobutene does not exceed 2 per cent. Indeed, only traces of isobutene were detectable in the infrared spectrum of the reaction products; some polymerisation[35] of isobutene may occur. The recoveries of t-butyl bromide were reproducible and were not strongly influenced by small changes in reaction conditions or in the amounts of phenol added.

(*b*) *Rate of reaction of t-butoxyl compounds*—With use of the same reaction conditions as in (*a*) above, the rate of evolution of t-butyl bromide from some t-butoxyl compounds was determined. The results are shown in Table III. The reaction time required varies from 2 to 3 hours, depending on the compound being analysed. When these results are corrected by +1·6 per cent. (the percentage loss of t-butyl bromide during recovery), only the results for two of the ethers are slightly low; this may well reflect the state of purity of these specimens.

(*c*) *The anomalous reaction of t-butylphenols*—Some t-butylphenols were boiled under reflux in constant-boiling hydrobromic acid for 2 to 3 hours. Nearly quantitative yields of t-butyl bromide were produced. Such compounds cannot therefore be distinguished from t-butoxyl compounds by this reaction.

TABLE III: YIELD OF t-BUTYL BROMIDE FROM t-BUTOXYL COMPOUNDS

Compound	Yield of t-butyl bromide (as percentage of theoretical)		
	After reflux for 1 hour	After reflux for 2 hours	After reflux for 3 hours
t-Butyl alcohol	95·4	98·3 (max.)	—
t-Butyl 3,5-dinitrobenzoate	97·0	98·6 (max.)	—
t-Butyl phenyl ether	86·5	91·1	96·2 (max.)
t-Butyl-p-tolyl ether	86·8	93·3	95·6 (max.)
t-Butyl-1-naphthyl ether	90·8	93·6	97·5 (max.)

EXPERIMENTS WITH CONSTANT-BOILING HYDROCHLORIC ACID—

Under the reaction conditions specified in "(*a*) Recovery of t-butyl bromide" above boiling under reflux with constant-boiling hydrochloric acid for 2 hours gave the results listed below.

(*a*) Recovery of added t-butyl chloride was nearly quantitative (>98 per cent.) (compare Kistiakowsky and Stauffer[36]).

(*b*) t-Butyl alcohol and t-butyl 3,5-dinitrobenzoate gave 98 per cent. of the theoretical yields of t-butyl chloride.

(*c*) t-Butyl ethers gave 60 to 65 per cent. of the theoretical yield of t-butyl chloride.

(*d*) The reactions of the t-butylphenols were—

(*i*) No t-butyl chloride formed—

2,4-di-t-butylphenol;
5-methyl-2-t-butyl-4,6-dinitroanisole;
4-t-butylphenol.

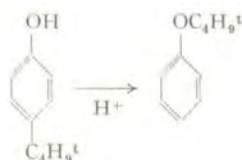
(*ii*) <10 per cent. of t-butyl chloride formed—
5-methyl-2-t-butylphenol;
t-butylated-4-hydroxyanisole (mixed 2 and 3 isomers);
2,6-di-t-butylphenol.

(*iii*) <20 per cent. of t-butyl chloride formed—
2,4-dimethyl-5-t-butylphenol;
3-methyl-4,6-di-t-butylphenol.

Conclusions

Boiling under reflux with constant-boiling hydrobromic acid is a satisfactory method of analysis for t-butoxyl groups. Under the reaction conditions described, decomposition of t-butyl bromide does not exceed 2 per cent., and the appropriate correction factor can be applied to the analytical results. The reaction period varies from 2 to 3 hours, depending on the compound being analysed. When the infrared method of determination[22] is being used, prolongation of the reaction period is not critical, although this might be inadvisable for volumetric or gravimetric determinations of the t-butyl bromide. Boiling under reflux with constant-boiling hydrobromic acid does not distinguish between true t-butoxyl compounds and t-butylated phenols.

Boiling under reflux with constant-boiling hydrochloric acid, however, offers a method of making this distinction. The yields of t-butyl chloride vary from 60 to 98 per cent. for t-butoxyl compounds, and from 0 to 20 per cent. for the range of t-butylphenols studied. It is possible that a rearrangement[27] of the form—



occurs in acid solution, the extent of the rearrangement depending on the concentration of acid and the nature of the substituent groups and substitution pattern in the phenolic compound.

The results presented show clearly that boiling under reflux with hydriodic acid does not give a satisfactory analytical reaction for t-butoxyl groups. The equilibrium—

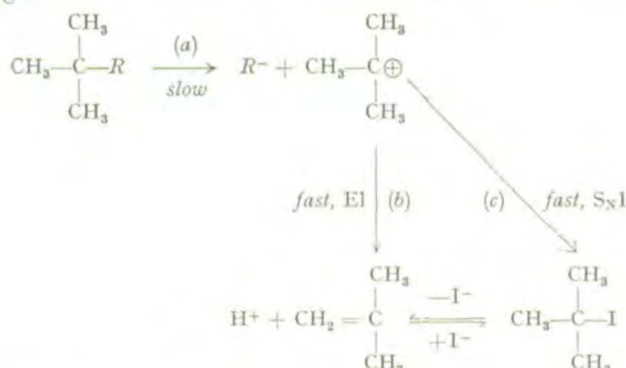
$$C_4H_9{}^tI \rightleftharpoons C_4H_8 + HI$$

is clearly dependent on the ratio of sample weight to volume of acid used, on the flow rate of scavenging gas and on the amounts of phenolic compounds added as solubilisers or formed within the reaction medium during the de-alkylation reaction.

Our results indicate that production of isobutene does not increase markedly in the latter stages of a determination; there is no extensive decomposition of undistilled portions of t-butyl iodide. Two effects must be distinguished. In the recovery of t-butyl iodide, the yield varied from 58 to 80 per cent. and was not strongly dependent on changes in flow rate or on the addition of phenolic compounds. The rate of distillation was rapid (approximately 80 per cent. in 15 minutes), and the recovery of added t-butyl iodide appears to be mainly dependent on the thermal decomposition equilibrium. In the formation of t-butyl iodide from t-butoxyl compounds, however, several striking differences are apparent. (*a*) The relative yields of t-butyl iodide vary much more widely (19 to 81 per cent.); (*b*) the rate of distillation (now dependent on the rate of formation) is much slower (approximately 50 per cent. in 1 hour); (*c*) the relative amounts of t-butyl iodide and isobutene formed are strongly dependent on the reaction conditions, and, particularly, on the presence of added compounds.

Mechanisms of the reactions of t-butyl compounds have been extensively studied,[37,38] and the relative stability of the t-butyl carbonium ion is well known. Olefine-forming

elimination reactions involving butyl compounds proceed via competitive S_N1 and E1 unimolecular reactions, in which the rate of formation of the carbonium ion (step (*a*) below) is rate-determining.



It appears that the relative extent to which reactions (*b*) and (*c*) occur in Zeisel determinations is dependent on the reaction conditions and additives employed.

We are grateful to Dr. R. L. Williams, Ministry of Aviation, Waltham Abbey, Essex, Messrs. Kodak Ltd., Kirkby, Lancs., and Messrs. I.C.I. (Dyestuffs Division) Ltd. for providing specimens of t-butylphenols. We thank the P.C.S.I.R., Karachi, for granting study leave and financial assistance to one of us (S.S.H.Z.).

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12.

PRELIMINARY COMMUNICATION

Applications of infra-red spectroscopy—XI*
The determination of 1,2-diols by modified Zeisel reactions

(*Received* 11 *February* 1963. *Accepted* 12 *February* 1963)

DERIVATIVES of 1,2-diols, and compounds made by condensations with ethylene or propylene oxides, are becoming increasingly important as solvents, plasticisers, detergents and emulsifiers.

Specialised methods have been developed for the analysis of ethylene oxide condensates,[1] and for the separation, by azeotropic distillation[2] and by chromatographic methods,[3–5] of aqueous solutions of simple glycols and their derivatives. For quantitative determinations, the Malaprade[6] periodate oxidation involves the determination of formaldehyde[7,8] or silver iodate.[9] Other investigators have used an acetylation method,[10] cerate oxidation[11] (which releases formic acid), and dichromate oxidation[12] (which produces carbon dioxide).

A more general method, however, involves the well-known Zeisel reaction, in which 1,2-diols yield an olefine and an alkyl iodide. The method has been used on the micro scale.[13] Although early attempts to use this reaction quantitatively were unsuccessful, it has been claimed[14–16] that reasonable results are obtained if the sum of the molar yields of both the volatile products is taken: the relative yields of the two reaction products appear to be dependent on the compound under study and on the precise reaction conditions. Although Etienne[16] found that iodine chloride was more satisfactory than bromine as an absorbant for ethylene, all attempts[14] to simplify the analysis, by introducing a single chemical absorption system for both volatile products, have failed. A method[17] based on determination of the iodine liberated in the reaction medium through decomposition of the primarily formed di-iodo alkanes has been adapted to the micro scale.[18]

Vapour-phase infra-red spectroscopy gives a simple, sensitive and specific method for the simultaneous determination of ethylene, or propylene, together with alkyl iodides. For ethane-diol, calibration is based on the absorption by ethylene at 955 cm^{-1} and by ethyl iodide at 1215 cm^{-1}. Routine analyses can be made without difficulty on 5-mg samples. The analytical apparatus required has been described (see Fig. I, ref. 19): details of the simple gas-cells,[20] construction of calibration curves,[21] and a procedure for the measurement of infra-red absorptions[21] have been given.

Part X: D. M. W. Anderson, S. Garbutt, M. A. Herbich and S. S. H. Zaidi, *Analyst*, 1963, **88**, in press.

Under our standard conditions[22] for Zeisel determinations, [*i.e.*, 5-mg samples refluxed with 6 ml of hydriodic acid (55%) using nitrogen as flow-gas at 6–8 ml per min] ethanediol gives 85 moles per cent of ethylene and 15 moles per cent of ethyl iodide. This agrees well with the observations of Kaintz[13] but not with those of Morgan.[14] The evolution of ethylene is extremely rapid. From start of reflux, the evolution of ethylene is complete within 10 min; evolution of ethyl iodide is slower, being complete in 30 min, a time slightly less than is usual in determinations on ethoxy compounds (*cf.* ref. 13).

A pure sample of 1,2-di-iodoethane decomposed rapidly (*a*) in boiling water, giving a quantitative molar yield of ethylene, (*b*) in hydriodic acid, giving 90 moles per cent of ethylene and 10 moles per cent of ethyl iodide. In agreement with Etienne,[16] the formation of 1,2-di-iodoethane may therefore be the essential reaction intermediate (*cf.* ref. 14). The suggestion[16] that low results may be caused by the loss of traces of 1,2-di-iodoethane from the reaction flask must be questioned, however; the decomposition of 1,2-di-iodoethane in boiling aqueous solutions is so rapid that its existence as a reaction intermediate must be very short under the analytical reaction conditions. Further experiments are in progress to investigate all aspects of the determination of 1,2-diols and their derivatives in an attempt to improve the sensitivity, reproducibility and accuracy of this modified Zeisel method: the results will be published in due course.

Recently, constant-boiling hydrochloric[23] and hydrobromic[24] acids have been used to advantage in modified Zeisel reactions. Because both 1,2-dibromo- and 1,2-dichloroethane are relatively more stable than 1,2-di-iodoethane, it was suggested[25] that reaction of 1,2-diols with hydrochloric or hydrobromic acids might lead to the formation of a single volatile reaction product. Unfortunately, this does

not occur. Ethanediol does not react to any significant extent with constant-boiling hydrochloric acid. Reaction with constant-boiling hydrobromic acid gives 1,2-dibromoethane, ethyl bromide and ethylene: furthermore, the total molar yield of these products is only about 50% after reflux for 2 hr. Hydrobromic acid is therefore less satisfactory than hydriodic acid as an analytical reagent. When 1,2-dibromoethane is treated with constant-boiling hydrobromic acid, some dibromoethane distils unchanged, and some is decomposed to ethylene, a small proportion of which (as shown in separate experiments) is converted to ethyl bromide.

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Summary—Vapour-phase infrared spectroscopy offers a simple, sensitive and specific method for simultaneous determinations of the olefines and alkyl iodides liberated in Zeisel determinations on 1,2-diols.

Zusammenfassung—Die Infrarotspektroskopie in der Dampfphase bietet eine einfache, empfindliche und spezifische Methode für die gleichzeitige Bestimmung der bei Zeisel-Bestimmungen an 1,2-Diolen gebildeten Olefine und Alkyljodide.

Résumé—La spectroscopie infra-rouge en phase vapeur constitue une méthode simple, sensible et spécifique du dosage simultané des oléfines et des iodures d'alcoyle formés dans le dosage des diols-1,2 par la méthode de Zeisel.

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13.

APPLICATIONS OF INFRARED SPECTROSCOPY—XII*

THE BEHAVIOUR OF PROPOXYL AND BUTOXYL GROUPS IN THE ZEISEL REACTION†

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Summary—Vapour-phase infrared spectroscopy has been used to study the behaviour of n-propoxy, iso-propoxy, n-butoxy, iso-butoxy and sec-butoxy groups in Zeisel's reaction. Within each group, the reaction rate varies with the compound under study. The equilibrium iso-$C_3H_7I \rightleftharpoons HI + C_3H_6$ is involved in the determination of iso-propoxy compounds with hydriodic acid; low recoveries of iso-propyl iodide therefore result. Reflux with hydrobromic acid gives a more nearly quantitative analytical reaction, since iso-propyl bromide is more stable to reflux with hydrobromic acid than iso-propyl iodide is to reflux with hydriodic acid. n-Propoxy, n-butoxy and sec-butoxy groups can be determined successfully with hydriodic acid; in the determination of iso-butoxy groups a rearrangement occurs, and the reaction product is a mixture of iso-butyl and sec-butyl iodides.

ALTHOUGH there have been many investigations of Zeisel's reaction (see, for example, literature cited in ref. 1) very few of these have considered its application to the determination of propoxyl and butoxyl groups. In continuation of our previous investigations of aspects of the Zeisel reaction (see earlier parts of this series) we have therefore studied the behaviour of propoxy and butoxy compounds, which are now very frequently used, *e.g.*, in plastics, resins, paints and agricultural chemicals, and also as anticholinesterases and solvent extractants.[2]

Problems encountered in the analysis of the *tertiary* butoxyl isomer were found to merit individual attention: a method of analysis, involving the use of hydrobromic acid, has been published.[3]

In an extensive study, Kirsten and Nilsson[4] confirmed (*cf.* ref. 5) that iso-propoxyl groups gave low results, but these authors did not establish the cause clearly and did not suggest a remedy. They did observe, however, that reaction rates for propoxyl and for butoxyl compounds varied[4] from compound to compound; previously Inglis[6] had reported a similar effect for methoxyl compounds, which, comparatively, react much more quickly.

Several previous papers on propoxyl and butoxyl compounds can therefore be criticised on the grounds that only a few (<3) compounds were studied, or that no analytical results were quoted (*cf.* refs. 5–9). Furthermore, although these investigations used the Vieböck titrimetric finish, the other reaction conditions employed varied so widely that it is difficult to draw any critical conclusions; for example, the reaction-time recommended for n-butoxy groups varied from $7\frac{1}{2}$ min[6] to 3 hr.[8] This

* Part XI: D. M. W. Anderson and S. S. H. Zaidi, *Talanta*, 1963, **10**, 691.

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lack of agreement has been maintained in more recent papers in which non-aqueous titration[10] and gas chromatography[11] have been used to determine the alkyl iodides formed in Zeisel reactions; reaction periods of 1 hr [11] (at N_2 flow-rate of 1–2 bubbles per sec) and 2 hr[10] (at 2–3 bubbles per sec) were proposed for iso-propoxy groups.

Rearrangements and decompositions of alkyl groups are known to be caused by the action of hydriodic acid,[12–16] but the extent to which these effects occur under the conditions operative in a Zeisel determination had not been investigated. Since standard analytical reference compounds for propoxyl and butoxyl groups have not yet been proposed, the present study was based on an investigation of as wide a range of compounds as could conveniently be obtained.

EXPERIMENTAL

Apparatus and general reaction conditions

These have been described,[1] together with details of the techniques for trapping volatile products and for their determination by vapour-phase infrared spectroscopy.[17,18]

Reagents

Hydriodic acid, phenol, Anhydrone and *soda asbestos* were all as previously described.[18]
Hydrobromic acid: AnalaR, about 48%, sp. gr. 1·46–1·49 (B. D. H. Ltd.)

Reference compounds

Specimens of *propane* and *propylene* (purity > 99% by vapour phase chromatography) were kindly provided by Dr J. H. Knox.

Propyl and *butyl alcohols, iodides:* Reagent grade (B.D.H. Ltd.) samples were re-distilled, then re-distilled immediately before use. Sec-butyl iodide was particularly difficult to obtain sufficiently dry (infrared spectroscopy) for calibration purposes; distillation of the vapour through a packing of Anhydrone was required.

Compounds investigated

The range of compounds investigated included alcohols, esters and ethers; their origin is shown in footnotes to the Tables. The research specimens kindly given by Messrs. I.C.I. (Dyestuffs Division) Ltd., by Dr. W. J. Kirsten, and by Dr. E. S. Lane were used as received. Commercial samples were recrystallised or redistilled carefully before use.

Procedure

Samples (2–6 mg.) were refluxed with 6 ml of hydriodic acid (constant-boiling azeotrope, pre-conditioned as described,[1] or with hydrobromic acid (48% w/w). The flow-gas was nitrogen (N. O. F. grade) at 6–8 ml per min. Crystalline samples were added to the reaction-flask after *careful* dissolution in molten phenol;[1] for liquid samples, phenol (200 mg) was added to the reaction acid before the addition of the sample. For some samples (*e.g.*, di-n-propyl ether, tri-iso-propyl phosphate, di-n-butyl ether, tri-sec-butyl phosphate) the addition of a few drops of propionic anhydride was also required to prevent their distillation, unreacted, from the reaction-flask.

In all the experiments, kinetic runs were timed from the start of ebullition of the reaction mixture and a steady flow of cold water was passed through the reaction flask condenser. The volatile reaction products were collected in a cold-trap after passage through soda asbestos and Anhydrone.[1]

RESULTS

1. *Iso-propoxy compounds*

(*a*) *Recovery of iso-propyl iodide from reflux with hydriodic acid:* When iso-propyl iodide (freshly redistilled, 2–5 mg) was refluxed with constant boiling hydriodic acid, the recovery of iso-propyl iodide was 76% after 30 min and 92% after 1 hr (averages of several runs). The use of (1) reflux for longer periods, (ii) increasing the nitrogen flow-rate to 15 ml per min (iii) passing warm water through the condenser jacket (*cf.* refs. 8, 19), and (iv) adding xylene[10] to the reaction-flask as a "carrier" did not in separate experiments, increase the percentage recovery.

(*b*) *Reaction of iso-propoxy compounds with hydriodic acid:* In experiments with iso-propanol, the molar recovery of iso-propyl iodide did not exceed 92%, and the infrared spectrum of the vapour products showed that iso-propanol had not distilled unchanged from the reaction-flask. Similar results were obtained from experiments with the esters and ethers listed in Table I.

This low but constant recovery of iso-propyl iodide indicated that some decomposition or rearrangement was involved, rather than incomplete reaction of the compounds with hydriodic acid. To facilitate the detection and identification of any decomposition product, experiments involving larger samples (50–60 mg) of iso-propyl iodide were carried out; small amounts of propylene were indicated in the infrared spectrum of the reaction products, and this was confirmed independently by vapour phase chromatography (experiments by courtesy of Dr. J. H. Knox). (In order to eliminate the possibility of thermal decomposition of iso-propyl iodide to propylene in the chromatography column, the propylene was separated from the iso-propyl iodide in the Zeisel reaction products before the chromatographic examination).

(*c*) *Recovery of iso-propyl bromide from reflux with hydrobromic acid*: Kinetic experiments showed that the recovery of iso-propyl bromide when refluxed with hydrobromic acid (48%, w/w) was quantitative in 1 hr. The recovery was 99·2% (average of several runs).

(*d*) *The reaction of iso-propoxy compounds with hydrobromic acid*: A number of iso-propoxy compounds were refluxed with constant-boiling hydrobromic acid. As shown in Table I, quantitative recovery of iso-propyl bromide was given in 1 hr for many of the compounds studied, although iso-propanol required a reaction period of 3 hr and di-iso-propyl ether required 4 hr. Analysis of the last compound was achieved by the procedure already described[1] for analysis of volatile compounds; the addition of a few drops of propionic anhydride to the reaction mixture eliminated the tendency for traces of di-iso-propyl ether to distil unreacted.

TABLE I.—YIELDS OF ISO-PROPYL BROMIDE (AS PERCENTAGE OF THEORETICAL) FROM REFLUX OF ISO-PROPOXY COMPOUNDS WITH 48% AQ. HYDROBROMIC ACID

Compounds	Reaction period, *hr*			
	1	2	3	4
1. Propan-2-ol	90·7	94·5	99·6 (max.)	
2. iso-Propyl-β-glucoside tetra-acetate	100·2			
3. Tri-iso-propyl phosphite	99·5			
4. Tri-iso-propyl phosphate	99·4			
5. iso-Propyl-*N*-(α-naphthyl)-carbamate	99·6			
6. iso-Propyl-(2,4,5-trichlorophenyl) acetate	100·2			
7. iso-Propyl-*N*,phenyl-carbamate	97·4			
8. iso-Propyl-(2,4-dichlorophenyl) acetate	100·2			
9. 2-iso-Propoxyethanol	98·3			
10. p-iso-Propoxydiphenyl	100·2			
11. 2-iso-Propoxybenzthiazole	100·2			
12. Di-iso-propyl ether	77·8	89·4	95·6	98·0 (max.)

Origin of samples: Commercial samples—1, 3, 4, 6, 8, 12.
Research specimens—5, 7, 10.
Given by Messrs. I.C.I. Ltd.—9, 11.
Given by Dr. W. Kirsten—2.

2. *n-Propoxy compounds*

(*a*) *The recovery of n-propyl iodide from reflux with hydriodic acid*: Kinetic experiments showed the recovery of n-propyl iodide to be 83·5% (0·5 hr); 89·5% (1 hr); 95% (2 hr); 98·0% (3 hr, max.)

(*b*) *Reaction of n-propoxy compounds with hydriodic acid*: The recoveries of n-propyl iodide from several compounds are shown in Table II.

TABLE II.—YIELDS OF n-PROPYL IODIDE (AS PERCENTAGE OF THEORETICAL) FROM REFLUX OF n-PROPOXY COMPOUNDS WITH 55% aq. HYDRIODIC ACID

Compound	Reaction period, *hr*				
	1	2	3	4	5
1. Propan-1-ol	92·8	95·5	99·4(max.)		
2. n-Propyl-2-chloro-3,5-dinitrobenzoate			99·6		
3. *p*-n-Propoxydiphenyl			99·4		

Origin of samples: Commercial samples—1.
Research specimens—2, 3.

(*c*) *Recovery of* n-*propyl bromide from reflux with hydrobromic acid*: The recoveries of n-propyl bromide from reflux with hydrobromic acid were:— 83·5% (1 hr); 92·6% (2 hr); 98·0% (3 hr, max.)

(*d*) *Reaction of n-propoxy compounds with hydrobromic acid*: The recoveries of n-propyl bromide from the compounds studied are shown in Table III.

TABLE III.—YIELDS OF n-PROPYL BROMIDE (AS PERCENTAGE OF THEORETICAL) FROM REFLUX OF n-PROPOXY COMPOUNDS WITH 48% aq. HYDROBROMIC ACID

Compound	Reaction period, *hr*				
	1	2	3	4	5
1. Propan-1-ol	69·8	76·5	88·2	94·5	99·8
2. Di-n-propyl ether	57·6	68·9	77·5	86·8	93·7
3. *p*-n-Propoxydiphenyl	82·2	89·3	99·6(max.)		
4. n-Propoxyacetic acid	90·0	94·0	100·2(max.)		
5. n-Propyl-α-naphthylurethane	84·0	90·3	99·3(max.)		
6. n-Propyl-3,5-dinitrobenzoate			97·6(max.)		
7. n-Propyl-2-chloro-3,5-dinitrobenzoate			99·7(max.)		
8. 2-n-Propoxybenzthiazole			99·5(max.)		
9. Di-n-propoxypentaerythritol			100·0(max.)		

Origin of samples: Commercial samples—1, 2.
Given by Messrs. I.C.I. Ltd.—4, 8, 9.
Research samples—3, 5, 6, 7.

3. *Butoxy compounds*

(*a*) *Recovery of the isomeric butyl bromides when refluxed with hydrobromic acid:* The recoveries obtained were:—

n-butyl bromide: 86·5% (0·5 hr); 90·2% (1 hr); 95·5% (2 hr); 98·2% (3 hr, max.)
iso-butyl bromide: 96·3% (1 hr, max.)
sec-butyl bromide: 96·6% (1 hr, max.)

(*b*) *Reaction of butyl alcohols with hydrobromic acid*: The recoveries of butyl bromides (as percentage of theoretical yield) were:—

n-butyl bromide from n-butanol: 68·8% (1 hr); 70·5% (2 hr); 75·6% (3 hr, max.)
sec-butyl bromide from sec-butanol: 77·5% (1 hr); 83·3% (2 hr, max.)
iso-butyl bromide from iso-butanol: 66·5% (1 hr); 67·7% (2 hr, max.)

These low recoveries indicated that reflux with hydrobromic acid does not give quantitative analytical reactions for n-, iso- and sec-butyl compounds. There was no increase in yield in experiments in which small amounts of the catalysts[14] zinc chloride and sulphuric acid were added to the reaction mixture. Further experiments using the "carrier" technique[10] were also made; toluene, mesitylene, α-methylnaphthalene, nitrobenzene and carbon tetrachloride were all tested for carrier activity, but with no significant success.

(*c*) *Reaction of butyl alcohols with phosphoric acid + potassium iodide*: A mixture of *ortho*-phosphoric acid + potassium halide is well-known[20] as a reagent for converting ethers into the corresponding halide: the use of this reagent was proposed recently[21] for the determination of methoxyl and ethoxyl groups. This reaction mixture gives a much higher reflux temperature than HI or HBr, and it appeared that its use might lead to quantitative recoveries of the butyl bromides. This was found not to be the case. It is noteworthy that Stone and Shechter[20] also reported yields of only 80–90% for the conversion of dibutyl ether and di-iso-propyl ether to the corresponding iodides.

(*d*) *Recovery of butyl iodides from reflux with hydriodic acid*: The recoveries obtained were:—

n-butyl iodide: 74·1% (0·5 hr); 80·3% (1 hr); 91·2% (2 hr); 96·4% (3 hr, max.)
iso-butyl iodide: 90·2% (0·5 hr); 94·5% (1 hr, max.)
sec-butyl iodide: 81·3% (0·5 hr); 85·5% (1 hr); 94·1% (2 hr, max.)

(*e*) *Reaction of n-butoxy compounds with hydriodic acid*: The recoveries of n-butyl iodide from a number of compounds are recorded in Table IV, which shows that the reaction-time required for maximum recovery of iodine depends on the compound under study and varies from 1–4 hr.

(*f*) *Reaction of iso-butoxy compounds with hydriodic acid*: When iso-butoxy compounds were refluxed with hydriodic acid it was observed that reaction was complete in 1 hr and that the volatile reaction product was a mixture of iso-butyl and sec-butyl iodides. The relative yields varied with the compound studied, as shown in Table V. Since iso-butyl iodide can be recovered unchanged from reflux with hydriodic acid, partial rearrangement involving a carbonium ion probably occurs during a step-wise reaction mechanism with the formation of a mixture of iso- and sec-butyl iodides, which then distil unchanged.

(*g*) *Reaction of sec-butyl compounds with hydriodic acid*: Only two compounds of sufficient purity were available for study. The molar yields of sec-butyl iodide were as follows:—

tri-sec-butyl phosphate: 85·7% (1 hr); 89·8% (2 hr); 96·7% (3 hr):
sec-butanol: 89·7% (1 hr); 95·8% (2 hr); 97·9% (3 hr).

A rearrangement of sec-butyl groups to tert-butyl has been reported to occur,[16] but it was not observed to take place to any significant extent in the present study. The sec-butanol used was purified according to Failes and Stimson.[22]

Table IV.—Yields of n-butyl iodide (as percentage of theoretical) from reflux of n-butoxy compounds with 55% aq. hydriodic acid

Compound	Reaction period, hr			
	1	2	3	4
1. Piperonyl butoxide	98·6 (max.)			
2. 2-Butoxyethanol†	97·4 (max.)			
3. Tri-butyl phosphite	93·9 (max.)			
4. Allyl dibutyl phosphate*	95·5 (max.)			
5. Diethyl dibutyl ether	93·5	95·6 (max.)		
6. Dibutyl hydrogen phosphonate	96·8	97·7 (max.)		
7. Dibutyl butyl phosphonate	95·7	98·8 (max.)		
8. Butyl lactate	72·8	92·9 (max.)		
9. Butyl salicylate	72·4	93·2 (max.)		
10. Butyl vinyl ether†	85·2	92·8 (max.)		
11. 1-Butoxy-3-*N,N*-diethylcarbamoylbenzene	84·3	96·2 (max.)		
12. Butyl phenyl ether	86·6	99·0 (max.)		
13. Butyl chloromethyl ether	82·7	93·6	97·5 (max.)	
14. Butan-1-ol	78·6	91·3	97·6 (max.)	
15. 2-Amino-2′-butoxydiethyl ether†	71·5	91·3	97·2 (max.)	
16. Titanium tetra-butoxide	65·6	82·0	93·2 (max.)	
17. *N,N′*-Bisbutoxymethylurea	66·1	88·6	97·8 (max.)	
18. 1,1′-Dibutoxy-n-butane	56·5	63·5	78·2	92·6 (max.)

* Also gives iso-propyliodide.
† Also gives ethylene + ethyl iodide.

Origin of samples: Commercial samples—1, 2, 3, 8, 9, 10, 14.
Research samples—12.
Given by Messrs. I.C.I. Ltd.—11, 13, 15, 16, 17.
Given by U.K.A.E.A., Harwell—4, 5, 6, 7.

Table V.—Yields of butyl iodides (as percentage of theoretical) from reflux of iso-butoxy compounds for 1 hr with 55% aq. hydriodic acid

Compound	Recovery, %	
	As iso-butyl iodide	As sec-butyl iodide
1. Vinyl iso-butyl ether	63	35
2. iso-Butyl-chloromethyl ether	54	45
3. Boron-tri-iso-butoxide	58	42
4. iso-Butanol	86	12
5. Tri-iso-butyl phosphate	60	38

Origin of samples: Commercial samples—1, 2, 3, 4.
Given by U.K.A.E.A., Harwell—5.

DISCUSSION

These studies have confirmed earlier evidence[4,6] that reaction rates in the Zeisel reaction vary considerably from compound to compound; for a particular alkoxyl group, no single reference compound can be taken as a standard. This is particularly important for propoxyl and butoxyl groups; for methoxyl and ethoxyl groups the reaction rates involved are very much faster, and the effect is therefore not of such great practical significance. The lack of agreement in earlier papers[6–9] regarding the

reaction-periods required can now be ascribed, at least in part, to the fact that the range of samples studied in each case was too restricted.

The thermal decomposition of iso-propyl iodide to propylene has been extensively studied[23,24] in the gas phase, but the reaction conditions involved are not directly applicable to the comparatively low temperature of the Zeisel reaction. Evidence has been obtained that under our Zeisel reaction conditions the equilibrium

$$\text{iso-propyl iodide} \rightleftharpoons \text{HI} + \text{propylene}$$

exists to a small extent which is, however, sufficient to cause low analytical recoveries of iso-propyl iodide. The molar amount of propylene present is equivalent to 6–8 moles per cent of iso-propyl iodide, and this small amount of propylene could only be detected and estimated satisfactorily when the weight of iso-propyl iodide taken was increased from 5 to 50 mg. [This is unlikely to cause any significant change in the reaction mechanism since this range of sample weights is small compared with the large excess of hydriodic acid used (6 ml of 55% w/w).]

The increased thermal stability of iso-propyl bromide permits nearly quantitative recoveries from refluxing hydrobromic acid azeotrope. This constitutes the third important application of hydrobromic acid in modified Zeisel reactions—earlier papers have outlined its use in simultaneous determinations of mixed ethoxyl-methoxyl groups[1] and in the determination of tert-butoxyl groups.[3] Unfortunately, the comparative thermal stability of iso-butyl bromide cannot be invoked—because of the inadequate reactivity of hydrobromic acid—to give an alternative method of analysis for iso-butoxyl groups. The rearrangement to sec-butoxyl which occurs in hydriodic acid would not, of course, cause any difficulty in a determination using the Vieböck iodometric finish; the rearrangement would, however, invalidate an attempted determination, by the vapour phase infrared method, of a mixture of iso-butoxy and sec-butoxy compounds.

The difficulty of securing pure commercial samples of propyl and butyl halides, and of avoiding thermal and acidic rearrangements to other isomeric forms during purification, has recently received attention.[25,26] The standard samples used for calibration purposes in this study were probably of 98–99% purity: a paper[27] on the purification of propyl and butyl alcohols was published as this report was being prepared for publication. Many of the compounds studied were probably of 94–99% purity, as indicated by the maximum recoveries of alkyl halides recorded in the Tables; this is not considered to effect the validity of the conclusions which have been reached.

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Résumé—La spectroscopie infra-rouge en phase vapeur a été utilisée pour étudier le comportement des groupes n-propoxy, iso-propoxy, n-butoxy, iso-butoxy et sec-butoxy, dans la réaction de Zeisel. Pour chaque groupe, le taux de réaction varie avec le composé étudié. L'équilibre iso $C_3H_7I \rightleftharpoons HI + C_3H_6$ est compris dans la détermination des composés iso-propoxy avec l'acide iodhydrique; par conséquent,

il en résulte un taux de récupération assez bas d'iodure d'isopropyle. Le reflux avec de l'acide bromhydrique donne une réaction analytique plus quantitative, car le bromure d'isopropyle est plus stable au reflux avec l'acide bromhydrique, que ne l'est l'iodure d'isopropyle avec l'acide iodhydrique. Les groupes n-propoxy, n-butoxy et sec-butoxy peuvent être dosés avec succès au moyen d'acide iodhydrique; dans le dosage du groupe iso-butoxy, il apparait un réarrangement et le produit de réaction est un mélange d'iodure d'isobutyle et d'iodure de butyle secondaire.

Zusammenfassung—Das Verhalten von n-Propoxy-, iso-Propoxy-, n-Butoxy-, iso-Butoxy- und sek-Butoxygruppen bei der Zeisel-Reaktion wurde mittels Dampfphasen-Infrarotspektroskopie untersucht. Innerhalb jeder Gruppe hängt die Reaktionsgeschwindigkeit von der untersuchten Verbindung ab. Bei der Bestimmung von Isopropoxyverbindungen mit HJ spielt das Gleichgewicht $C_3H_7J \rightleftharpoons C_3H_6 + HJ$ eine Rolle, daher ist die Ausbeute an Isopropyliodid zu klein. Der Rückfluß mit HBr gibt bessere quantitative Ergebnisse, da Isopropylbromid unter Rückfluß mit HBr stabiler ist als das Iodid mit HJ. n-Propoxy-, n-Butoxy- und sek-Butoxygruppen können mit HJ bestimmt werden. Bei der Bestimmung von iso-Butoxygruppen findet Umlagerung statt; es resultiert ein Gemisch von Isobutyliodid und sek-Butyliodid.

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14.

APPLICATIONS OF INFRARED SPECTROSCOPY

PART XIII[1]. THE DETERMINATION OF SMALL AMOUNTS OF ALCOHOLS IN AQUEOUS SOLUTION

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A simple method was required for the identification and determination of small concentrations (*ca.* 0.05%) of lower alcohols in aqueous solution. Since simple mixtures of alcohols were liable to be present, many of the standard methods of analysis were inapplicable.

A literature survey indicated that methanol and ethanol are still most frequently determined by distillation or diffusion[2], followed by oxidation with permanganate[3] or dichromate[4]. Although such methods can be extended to butan-1-ol[5] and to secondary alcohols[6,7], errors are liable to occur through incomplete reaction or over-oxidation[8]. Some investigators prefer to determine the aldehydic or acidic oxidation products[4,6,9]; others determine the excess dichromate electrometrically[10] or spectroscopically[8].

Considerable attention has been devoted in recent years to the development of new methods of analysis, including those based on the use of acetylation[11], colorimetry[12,13], chemiluminescence[14], reaction with vinyl cyanide[15], conversion to nitrite[16], formation of vanadium–oxinate complexes[17,18], and gas–liquid chromatography[19–21].

For mixtures of alcohols, differential rates of reaction can be used[22], but gas–liquid chromatography[23] undoubtedly provides an extremely sensitive method (a detection limit[24] of 0.1% in water for a 5-μl sample). For positive identification of the chromatographic peaks, a non-aqueous solvent extraction method[25] and a combined infrared–GLC technique[26] have been described.

When only a small number of determinations are required, however, it is not always economic in time and cost to prepare a special gas chromatography column and to carry out the modifications necessary for the analysis of aqueous solutions. This paper describes a simple modification of the Zeisel reaction which has been found to give satisfactory results for the analysis of aqueous solutions containing low concentrations of alcohols.

EXPERIMENTAL AND RESULTS

Compounds

For development of the method, dilute solutions (1 ml of alcohol per l of distilled water, *i.e. ca.* 0.079%, w/v) were prepared from methanol and ethanol (B.D.H. Ltd.; "special for spectroscopy"). In later work, similar dilutions of the propanols and butanols were prepared from reagent-grade samples after redistillation. Finally,

independent test solutions were prepared both on a weight/volume and volume/volume basis, the concentration being calculated from the density of the alcohol used.

Zeisel apparatus and reagents

These have been described[27], together with details of the technique for trapping volatile reaction products and of the infrared vapour-phase method for their subsequent identification and determination.

Procedure for determinations as iodides

An aliquot (1.00 ml) of the sample solution is pipetted into the reaction flask of the micro Zeisel apparatus. A few crystals of phenol (about 25 mg) are added, followed by 3 ml of hydriodic acid (AnalaR, about 66%, sp. gr. 1.94). The reaction flask consequently contains the unknown amount of alcohol(s) dissolved in 4 ml of hydriodic acid of specific gravity 1.70, *i.e.* closely approximating to the constant-boiling azeotrope. Small differences from the azeotropic composition are not critical when the liberated alkyl iodides are determined spectroscopically; in determinations where a volumetric or gravimetric finish is used, the specific gravity must not exceed 1.70, so that water, and not hydriodic acid, distils with the volatile reaction products. (In this connection it has been found advisable to determine the density of the commercial acid, since this is frequently lower than 1.94.)

The reflux periods required are: methanol and ethanol, 1 h; *n*-propanol and *n*-butanol, 3 h. If the alcohol(s) present are not known, a preliminary qualitative analysis should be based on the products from a 3-h reaction.

The sensitivity depends on the gas-cell used[28]. The procedure described here can be carried out adequately with the aid of the less expensive, bench-type spectrometers now available commercially. Such instruments can usually accommodate a gas-cell of about 10 cm length. With a simple, conventional cell of this length, 1–5 mg of methyl iodide can be determined to within $\pm 1\%$. Because of the very favourable factor incurred through conversion to the iodide, methanol concentrations within the range 0.2–1.1 g/l can be determined. The ranges for ethanol, propanols and butanols are slightly less sensitive, since the conversion factors are progressively less favourable (4.4 for methanol, 2.5 for butanols).

Limitations on determinations as iodides

When determined as iodides, *tert.*-butanol[29] and isopropanol[1] give low results as a result of olefine formation. Determinations as bromides overcome this difficulty[1,29]. Any of the lower alcohols can, of course, be determined as bromides, although some loss of sensitivity results. This is because (*a*) the conversion factor for bromides is less favourable than for iodides, and (*b*) the intensity of absorption is less for alkyl bromides than for iodides. Thus 2–6 mg of methyl bromide, for example, can be determined in a 10-cm gas-cell; taking 1-ml aliquots, methanol concentrations in the range 0.5–2 g/l can therefore be determined.

Procedure using hydrobromic acid

The procedure using hydrobromic acid is similar to that described for hydriodic acid, except that 4 ml of hydrobromic acid (66%, sp. gr. 1.70) is added to a 1-ml

aliquot of the unknown aqueous solution. This gives a reaction mixture (5 ml) which is virtually azeotropic in composition (sp. gr. 1.48).

The possible binary mixtures of the C_1–C_4 alcohols are shown in Table I: in only a few mixtures is a component not determinable.

TABLE I

DETERMINACY OF BINARY COMBINATIONS OF THE LOWER ALCOHOLS AS BROMIDES[a]

Mixture of alcohols	*Bromides liberated*							
	Methyl	*Ethyl*	n-*Propyl*	*Isopropyl*	n-*Butyl*	*Isobutyl*	sec.-*Butyl*	tert.-*Butyl*
Me/Et	D	D						
Me/*n*-Pr	D		D					
Me/iPr	D			D				
Me/*n*-Bu	D				D			
Me/iBu	D					D		
Me/*s*-Bu	D						D	
Me/*t*-Bu	D							D
Et/*n*-Pr		ND	D(MP)					
Et/iPr		D(CF)		D(MP)				
Et/*n*-Bu		ND			ND			
Et/iBu		D(CF)				D(MP)		
Et/*s*-Bu		ND					D	
Et/*t*-Bu		D						D
n-Pr/iPr			D(MP)	D(MP)				
n-Pr/*n*-Bu			D(MP)		D(MP)			
n-Pr/iBu			D(MP)			D(MP)		
n-Pr/*s*-Bu			D(MP)				D(MP)	
n-Pr/*t*-Bu			D					D
iPr/*n*-Bu				D	D(MP)			
iPr/iBu				D(MP)		D(MP)		
iPr/*s*-Bu				D(MP)			D(MP)	
iPr/*t*-Bu				D				ND
n-Bu/iBu					D	D(MP)		
n-Bu/*s*-Bu					D(MP)		D	
n-Bu/*t*-Bu					D			D
iBu/*s*-Bu						D	D	
iBu/*t*-Bu						D(MP)		ND
s-Bu/*t*-Bu							D	ND

[a] D: Determinable.
ND: Not determinable.
D(CF): Determinable using correction factor } see ref. 27.
D(MP): Determinable using minor peak }

DISCUSSION

The simple modifications described allow the Zeisel reaction to be applied to the analysis of dilute aqueous solutions of the lower alcohols. Results accurate to within $\pm 1\%$ were obtained for test solutions. Analyses of solutions containing only one component can, of course, be made using any of the conventional finishes to the Zeisel reaction; for mixtures of alcohols, the spectroscopic method undoubtedly offers great advantages. Determination as iodides is not satisfactory for isopropanol and

tert.-butanol, which require the use of hydrobromic acid.

Concentrations greater than about 0.1% can readily be determined by making an appropriate dilution; concentrations greater than about 10% can be found by normal Zeisel determinations. Concentrations less than about 0.02% can be analysed (*1*) by using longer conventional gas-cells, minimum volume gas-cells, or scale-expansion devices, etc., (*2*) by taking larger aliquots and scaling up the volumes of reactants proportionately, using a larger reaction-vessel, (*3*) by adding a known weight of the alcohol under test to the unknown solution, subsequently calculating the weight present in the unknown solution from the total weight recovered[30]. Concentrations down to about 0.002% can be determined by careful choice of the weight of alcohol added so that the most sensitive portion of the calibration curve for the gas-cell is involved.

The method is limited to systems known to contain alcohols only. At the low concentrations involved, ethers, esters and acetals may be sufficiently water-soluble to interfere. Ethylene glycol and its derivatives also interfere, but their presence is revealed by the formation of ethylene on reaction[31] with hydriodic acid.

We thank Professor E. L. HIRST for his interest in these studies, and the P.C.S.I.R., Karachi, for granting study leave and financial support (to S.S.H.Z.).

SUMMARY

Recent developments in methods of analysing dilute solutions of alcohols are briefly reviewed, and a simple modification to the Zeisel reaction is described. This permits the analysis, on a 1-ml aliquot, of dilute aqueous solutions of alcohols alone or in admixture.

RÉSUMÉ

Les auteurs passent en revue les récents développements dans les méthodes d'analyse de solutions diluées d'alcools. Ils décrivent une modification simple de la réaction de Zeisel; le dosage peut se faire sur une prise de 1 ml.

ZUSAMMENFASSUNG

Über die neueren Entwicklungen zur Analyse von verdünnten Alkohollösungen wird kurz berichtet und eine einfache Modifikation der Zeisel-Reaktion beschrieben. Sie ermöglicht die Analyse von 1-ml verdünnten wässrigen Alkohollösungen.

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15.

An Infrared Method for the Determination of Small Amounts of Acetaldehyde in Aqueous Solution*

By D. M. W. ANDERSON and J. F. STODDART
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A specific and accurate method is described for determining acetaldehyde in aqueous solutions on the microgram scale. After quantitative aspiration from solution with a rapid flow of nitrogen, the acetaldehyde is determined by vapour-phase infrared spectroscopy. Other lower aldehydes, acids and alcohols in the aqueous solution do not interfere. The method is therefore particularly useful when used in conjunction with analytical procedures involving periodate oxidation, *e.g.*, the determination of methyl pentoses in carbohydrates, and of threonine in proteins.

As knowledge of the chemistry of natural products increases, there is always a need for more accurate and specific methods of analysis, so that consolidation, correction and refinement of earlier studies, often exploratory, can be made.[1]

For polysaccharides of both land and aquatic origin, investigations centred upon the methyl pentose sugars have given useful information about the presence of heterogeneity[2] and the nature of structural fragments.[3] Although several methods, *e.g.*, distillation as methyl furfural,[4] colorimetric[5] and titrimetric,[6] for determining methyl pentoses have been proposed, each has been deficient in either sensitivity, specificity or accuracy, with the result that some doubt has existed[7] about the significance of some observations.[8,9] Our immediate requirement, for studies on *Acacia* gum exudates, was for a method giving results to within $\pm 0{\cdot}1$ per cent. absolute at the 0 to 1 per cent. level of rhamnose, and $\pm 0{\cdot}2$ per cent. at the 1 to 15 per cent. level.

A review of the procedures available indicated that a method based on the periodate-oxidation release of acetaldehyde[10] offered the greatest chance of success. The task therefore involved finding a method for improving the existing methods for determining small amounts of acetaldehyde in complex aqueous reaction mixtures. The necessity for a more selective and sensitive method to be found has already received comment,[11] and several investigations with this aim have been reported recently.[12,13,14] Gas chromatography has also been used in a more original approach,[15] although the more specific (if slightly less sensitive) technique of infrared spectroscopy was, whenever possible, preferred for the present study. When it was realised that the accurate determination of rhamnose was also required in studies of bacterial metabolism,[16,17] and that the determination of threonine, as acetaldehyde, is of current importance in studies of proteins[18] and lipids,[19] it was decided to investigate the determination of acetaldehyde from a more general analytical viewpoint.

The method proposed involves the production of acetaldehyde from the protein or carbohydrate hydrolysate by periodate oxidation at pH 7·0, the removal of

* Part XIV of the series, "Applications of Infrared Spectroscopy."

the acetaldehyde from the aqueous solution by an aspiration technique (apparently first used by Clausen,[20] and subsequently used frequently[10,21,22,23]) and the determination of the acetaldehyde by the specific method of vapour-phase infrared spectroscopy. The flow-gas emerging from the reaction vessel is passed through a preliminary cold-trap (acetone - solid carbon dioxide) to remove water vapour; the acetaldehyde is then collected quantitatively in a trap immersed in liquid nitrogen and is subsequently transferred[24] as a vapour to a gas-cell for quantitative determination.

Experimental

Apparatus—

The apparatus required for aspirating acetaldehyde from aqueous reaction mixtures at room temperature is shown in Fig. 1. The outlet from the three-necked flask (100-ml capacity) is connected, *via* a wide-bore U-tube, to a liquid-nitrogen trap, details of which have already been described.[25] The U-tube is immersed in a

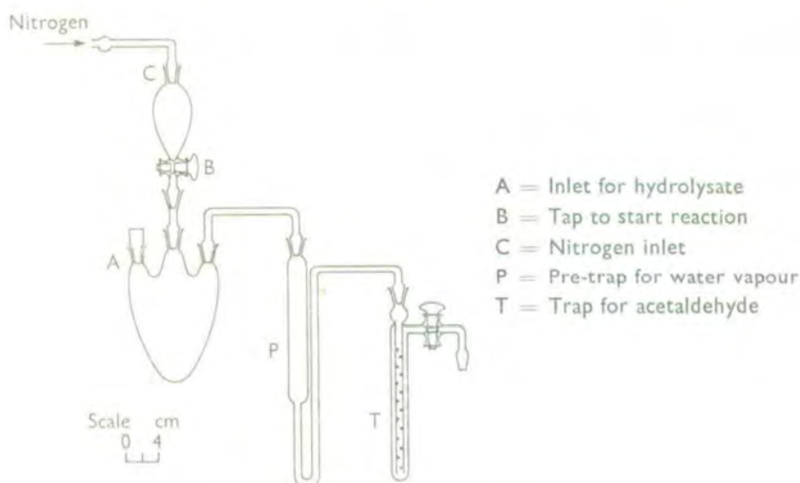

Fig. 1. The assembled apparatus

Dewar flask (1-pint capacity) containing solid carbon dioxide in acetone; this serves to trap the water vapour without retaining any of the acetaldehyde. Cylinder nitrogen, at a flow-rate of 300 to 350 ml per minute, is used for aspirating the acetaldehyde. The inlet tube reaches to the bottom of the flask, and carries a dropping funnel (25-ml capacity); at the start of a reaction, the periodate solution is introduced from this dropping funnel under pressure from the nitrogen.

Reagents—

Sodium m-*periodate solution*, 0·5 M.

Potassium arsenite solution, N.

Phosphate buffer solution, pH 7·0—A solution 0·0906 M with respect to disodium hydrogen phosphate and 0·0426 M with respect to potassium dihydrogen phosphate was used.

Acetaldehyde, twice redistilled.

Rhamnose monohydrate—Obtained from T. Kerfoot and Co. Ltd., Vale of Bardsley, Lancs.

DL-α-*Alanine*—M.A.R. grade.

Spectroscopic determination of acetaldehyde—

Acetaldehyde was transferred quantitatively from the liquid-nitrogen trap to a gas-cell of suitable sensitivity,[26] by the technique previously described.[24] Immersion of the cold-trap in hot water was sufficient to effect conversion of the acetaldehyde to the vapour-phase. The calibration is based on the strong carbonyl absorption given by acetaldehyde vapour at 1750 cm^{-1}. A typical calibration curve is shown in Fig. 2, and was constructed, under standardised spectrometer operating

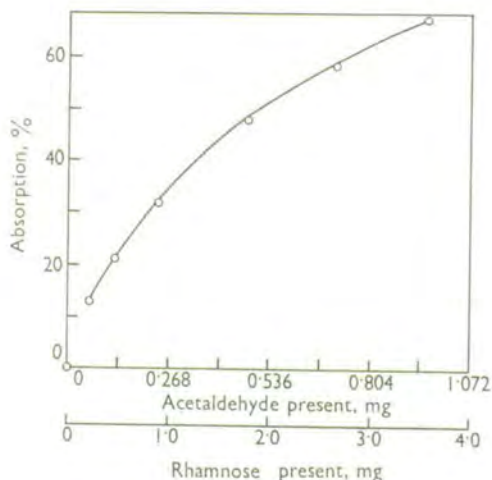

Fig. 2. Calibration curve for rhamnose in terms of acetaldehyde absorption at 1750 cm^{-1}

conditions, by using the stoppered weighing-bottle technique already described[25] for volatile liquids. During this investigation, it was necessary to use two gas-cells of different sensitivities, each having their respective calibration curves; a relatively insensitive cell was used when experiments (rate of aspiration, etc.) directly involving weighed amounts of acetaldehyde were in progress, and a cell of standard sensitivity (length, 12·5 cm; internal volume, 31 ml) was used for experiments with the much smaller amounts of acetaldehyde evolved from suitable weights of rhamnose and polysaccharides.

Rate of aspiration of acetaldehyde—

Preliminary experiments showed that amounts of acetaldehyde of up to 1 mg were quantitatively aspirated within $1\frac{1}{2}$ hours at nitrogen flow-rates of 300 to 350 ml per minute. In routine determinations, an aspiration period of 2 hours is allowed, and this time is in good agreement with that found by other investigators.[6]

Recovery of acetaldehyde after periodate oxidation of rhamnose—

A standard solution, made by dissolving 400 mg of rhamnose monohydrate in 500 ml of water, was used in preliminary experiments to establish the reaction conditions for quantitative periodate oxidation, volumes of this solution ranging from 0·50 to 5·00 ml being used. Considerable amounts of formic acid are usually released in periodate oxidations of carbohydrate materials, and the reaction medium must be adequately buffered to prevent an increase in acidity as the oxidation proceeds. The most satisfactory results were obtained when a pH of 7·0 was maintained with the phosphate buffer system described above. For individual weights

of rhamnose monohydrate in the range 1·5 to 6·0 mg, weights of acetaldehyde corresponding to recoveries of rhamnose monohydrate of between 96 and 104 per cent. were obtained by the procedure given below. Similar recoveries were obtained for fucose and for rhamnose, to which was added equal weights of arabinose, galactose and glucuronic acid.

Method

The apparatus and reagents are as described under "Experimental."

Procedure—

Transfer the standard rhamnose solution, or the weighed sample of rhamnose, to the three-necked flask *via* neck A (see Fig. 1). Add 1 ml of potassium arsenite solution, 5 ml of phosphate buffer solution and sufficient water to make the final volume up to 17 ml. Place in the dropping funnel, by pipette, 3 ml of sodium *m*-periodate solution. Assemble the apparatus as shown in Fig. 1, immersing the pre-trap, P, in an acetone - solid carbon dioxide mixture, and trap T in a flask of liquid nitrogen. Start the reaction by opening tap B, simultaneously inserting the nitrogen-delivery tube into socket C. Maintain a nitrogen flow-rate of 300 to 350 ml per minute for 2 hours. During this time a considerable amount of ice is deposited in pre-trap P, but there is no retention of acetaldehyde, which is collected in trap T. The wide-bore entry shown for pre-trap P is necessary to prevent the deposition of ice from blocking the flow system.

When the reaction is completed, transfer the acetaldehyde quantitatively[24] from trap T to a gas-cell of suitable sensitivity, previously calibrated (under standardised instrumental conditions) with known weights of acetaldehyde.

Spectroscopic conditions for calibration

Under the conditions described above, infrared spectroscopy showed that formaldehyde and formic acid were not present in admixture with the acetaldehyde. The strong carbonyl absorption by acetaldehyde vapour at 1750 cm^{-1} can therefore be used for calibration purposes. In unusual reactions, however, other carbonyl compounds could conceivably be trapped together with the acetaldehyde; under such circumstances (which should always be tested for) the choice for calibration purposes of suitable individually separated peaks in the infrared absorption spectrum of each of the components present in the mixture will permit their individual concentrations to be determined simultaneously.[27]

Traces of carbon dioxide and water vapour will not interfere with normal determinations of acetaldehyde; the function of the pre-trap, P, is to reduce the water vapour collected in trap T to a minimum, so that the sodium chloride windows usually fitted to gas-cells will not become fogged more quickly than can be avoided, with resulting invalidation of the calibration curve being used. Recently, we have used windows of "Irtran-4" glass for our infrared cells, and traces of water vapour are then entirely without effect, and calibration curves remain valid much longer. The sole disadvantage is the high cost of this type of optical glass.

The ffect of adding alanine to the reaction mixture

In the method described by Nicolet and Shinn,[10] the addition of 200 mg of alanine to all reaction mixtures was recommended, presumably to react with any formaldehyde produced and thus eliminate the possibility of its being aspirated together with the acetaldehyde. Under the reaction conditions given above, the volatilisation of formaldehyde does not occur, and the addition of alanine is not necessary. The effect of the addition of alanine was, however, investigated, and it was found, unexpectedly, that the addition introduces a source of error that leads to high results. Nicolet and Shinn[10] reported that alanine undergoes reaction with

periodate at a rate which is one-thousandth of that of the hydroxy amino-acids, serine and threonine. Other authors have reported[28] that the oxidation of alanine with periodate at room temperature is negligible at pH 7·6, the optimum pH being 8·0.

In experiments with threonine and alanine (micro-analytical reagent grade) under the reaction conditions given above, it was found that threonine is quantitatively oxidised to acetaldehyde. In contrast, alanine undergoes slow oxidation, resulting in the production of 0·30 mg of acetaldehyde (average of five determinations) from 200-mg samples of alanine in 2 hours. A similar result was obtained with two other specimens of alanine from other sources. (Although an aspiration period of 2 hours was allowed, the effective duration of the periodate-oxidation reaction, which is arsenite controlled, is much less.)

The implication of these results is that, if 200 mg of alanine are added to 1·64 mg of rhamnose, the recovery of acetaldehyde after aspiration for 2 hours will be 0·44 mg (from the rhamnose) *plus* 0·30 mg (from the alanine). The addition of alanine should therefore not be made, since high recoveries will be obtained.

The hydrolysis stage for polysaccharides before periodate oxidation

Polysaccharides containing rhamnose must be completely hydrolysed, so that all the rhamnose is available in the free form for the periodate-reaction stage. The hydrolysis conditions required by two gum exudates, differing widely in their rhamnose content, were investigated; the samples used had been carefully purified by electrodialysis, since it is known[29] that traces of heavy-metal ions can cause decomposition of acetaldehyde. It was found that hydrolysis under reflux in N sulphuric acid and in 2 N sulphuric acid followed first-order kinetics; the times required for complete hydrolysis were 6 hours and 2½ hours, respectively. The decomposition of rhamnose was studied under these conditions, and although no detectable decomposition occurred with either system in the times stated, the hydrolysis with 2 N acid for 2½ hours was preferred for the *Acacia* specimens. For different polysaccharide materials, however, the hydrolysis conditions required would have to be established before the remainder of the analytical procedure can be carried out.

After the hydrolysis stage, the strongly acidic hydrolysate must be neutralised before the start of the periodate oxidation, and the procedure described below is proposed.

Hydrolysis with 2 N sulphuric acid is carried out in a 50-ml flask fitted with a short water-condenser, the flask being heated in a water-bath at 100° C for 2½ hours. A suitable weight of the polysaccharide (approximately 200 mg for a rhamnose content of 1 per cent., and approximately 50 mg for a rhamnose content of 10 per cent.) is placed in the flask, and dissolved in 12 ml of 2 N sulphuric acid before the heating is started. After it has been heated for 2½ hours at 100° C, the hydrolysate is transferred with careful rinsing to a 25-ml calibrated flask. The hydrolysate is then made neutral to methyl orange indicator by the addition of the required volume of 5 N sodium hydroxide, the volume being finally adjusted to 25 ml with water. Portions (3, 4 or 5 ml) are then placed in the three-necked reaction flask (Fig. 1) for periodate oxidation. (If desired, smaller samples of polysaccharide, *e.g.*, 5 to 10 mg for a rhamnose content of 10 per cent., can be hydrolysed, neutralised and transferred directly to the three-necked flask for single determinations.)

When the amount of rhamnose present is extremely small, however, a useful technique is to transfer a known weight of rhamnose to the periodate-oxidation flask with the portion of polysaccharide hydrolysate. The rhamnose content of the polysaccharide can then be calculated from the apparent excess in the recovery of the added rhamnose. This technique allows the spectroscopic measurement to be made with respect to the most sensitive portion of a calibration curve, and its use has already been described in greater detail.[30]

RESULTS

Determinations on samples of *A. senegal* (gum arabic) gave a rhamnose content of 13·0 per cent., a value in excellent agreement with that commonly accepted for this polysaccharide. Determinations on the exudate from *A. karroo* gave a rhamnose content of 2·05 per cent., a result that substantiates the provisional value reported by Charlson, Nunn and Stephen,[9] who found this amount "too small to estimate with accuracy." Results obtained by the proposed method for some other *Acacia* specimens, hitherto unstudied, are given in Table I; the replicates indicate the confidence that can be shown in determinations of extremely low rhamnose contents.

TABLE I

THE RHAMNOSE CONTENT OF SOME *Acacia* SPECIES

Acacia species	Weight of gum sample in portion taken, mg	Weight of rhamnose added (*a*), mg	Weight of acet-aldehyde found (*b*), mg	Total recovery of rhamnose ($c = b \times 164/44$), mg	Weight of rhamnose in gum sample ($c - a$), mg	Rhamnose present, %
A. nilotica	40·4	0·38	0·148	0·55	0·17	0·4
	40·4	0·38	0·145	0·54	0·16	0·4
A. arabica	41·6	0·38	0·148	0·55	0·17	0·4
	41·6	0·38	0·150	0·56	0·18	0·4
A. nubica	40·0	0·38	0·166	0·62	0·24	0·6
	40·0	0·38	0·169	0·63	0·25	0·6
A. drepanolobium ..	48·3	Nil	0·129	0·48	0·48	1·0
	48·3	Nil	0·137	0·51	0·51	1·1
A. campylacantha ..	12·5	Nil	0·236	0·88	0·88	7·0
	12·5	Nil	0·231	0·86	0·86	6·9

CONCLUSIONS

The method described has proved to be straightforward and has given satisfactory results in routine use in this laboratory during the past 6 months. Although it has been used specifically to investigate samples of gum exudates, it is clear that the method is of general application for methyl pentose determinations in the polysaccharide group, provided that the hydrolysis conditions required are established for each material under examination. The method gives an over-all accuracy greater than has been available to date; further, the method gives reliable results at lower levels of rhamnose than could previously be determined.

The procedure described gives quantitative recovery of threonine, and the method outlined should facilitate determinations of this amino-acid in proteins. Other analytical reactions that can be based on a conversion to acetaldehyde include the determination of lactic acid,[15,20,31] and that of alanine after reaction with ninhydrin.[32]

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16.

The Use of "Biogel-P" in the Gel Filtration of Polysaccharides

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LATHE and RUTHVEN[1] suggested that columns of swollen starch might provide a simple chromatographic method for determining the molecular size of proteins and polysaccharides. Gel filtration on cross-linked dextrans ("Sephadex") has, however, been much more successful, particularly for proteins,[2-5] and molecular weights of up to 225,000 can now be determined.[6] Cross-linked polyacrylamide gels have been used for gel-filtration studies on proteins.[7] These are now available commercially ("Bio-Gel P") and they offer the opportunity of studying the gel-filtration behaviour of polysaccharides on noncarbohydrate material.

Accordingly, we have examined different grades of "Biogel-P" for their possible application to the estimation of the molecular size of polysaccharides. For "Bio-Gel P 300" the empirical relationship between log $\bar{M}_n$ and elution volume[2,8] is linear for values of $\bar{M}_n$ between 5000 and 125,000; although the useful working range extends slightly beyond these values (cf. ref. 9) the exclusion limit of "Biogel-P 300" for polysaccharides appears to fall considerably below the value of 300,000 quoted commercially and found, presumably, for proteins. We have used columns measuring $2 \cdot 5 \times 50$ cm. and $5 \cdot 0 \times 50$ cm. at a loading of 2—10 mg. of polysaccharide. Calibration can be effected with dextran fractions having known values of $\bar{M}_n$ and, when molar sodium chloride is used as eluant, we have found such a calibration to be valid for acidic polysaccharides. Thus two gum fractions (obtained[10] from *Acacia senegal* gum), for which osmotic-pressure measurement had indicated $\bar{M}_n$ 105,000 and 37,000, respectively, gave $\bar{M}_n = 99{,}000 \pm 10{,}000$ and $\bar{M}_n = 35{,}000 \pm 3{,}000$ by gel filtration. A sample of the degraded (autohydrolysis) gum, having $\bar{M}_n = 4400$ (periodate end-group analysis, as formaldehyde) gave $\bar{M}_n = 4800 \pm 500$ by gel filtration. The elution pattern of a de-ionised sample of the whole gum indicated that the molecular-weight distribution extended over a very wide range.

More experiments with "Bio-Gel P" materials are necessary to assess the importance of their application in fractionation and degradative studies and to establish their validity, applicability, and useful working range for molecular-weight estimations of polysaccharides.

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17.

Separations of Sugars on "Chromagrams"

Recently, "Chromagram" sheets for thin-layer chromatography have become available commercially (Messrs. Kodak Ltd., Kirkby, Liverpool). We have examined the polycarbonate and silica-gel forms of these, and have obtained very rapid, good separations of many sugars, without the tedium of plate preparation. The uniformity of coating allows reproducible R_F values to be obtained without difficulty; moreover, the solvent front runs evenly, and the absence of "edge-effect" facilitates comparison of R_F values of unknown sugars with those of standards on the same chromagram.

EXPERIMENTAL

Chromagrams were developed by the ascending technique in a small tank (Shandon, for thin-layer chromatography) lined with filter sheet to assist vapour-phase equilibration.

The polycarbonate coating was pre-treated by immersion in phosphate buffer (0.2M, pH 6.8). After the chromagram had been air-dried, 0.5 μl of a 5% aqueous solution of sample was applied. The solvent system, propan-1-ol–ethyl acetate–water (10:3:1), recommended by Messrs. Kodak, Harrow Division, was used; development for 5 h was required. The chromagrams were sprayed with (*a*) saturated ethanolic aniline oxalate (followed by heating in an oven at 140° for 2–3 min), to detect reducing sugars, or (*b*) an aqueous solution of periodate and alkaline permanganate[1] to detect sugar alcohols. Table I gives the R_F values obtained.

TABLE I

R_F VALUES OF SOME SUGARS ON POLYCARBONATE (0.2M PHOSPHATE BUFFER, pH 6.8) CHROMAGRAMS USING PROPAN-1-OL–ETHYL ACETATE–WATER (10:3:1)

D-Galactose	0.13	D-Xylose	0.44
D-Mannose	0.31	D-Ribose	0.49
D-Glucose	0.18	L-Arabinose	0.29
L-Rhamnose	0.58	Maltose	0.10
L-Fucose	0.49	Lactose	0.05
D-Glucuronic acid	0.03	D-Arabinitol	0.37
D-Galacturonic acid	0.03	Erythritol	0.48
D-Fructose	0.28		

The behaviour of some sugars on the silica-gel chromagrams, pre-treated with 0.1 N boric acid, was examined in two different solvent-systems[2]: solvent 1, butan-1-ol–acetone–water (4:5:1); solvent 2, butanone–acetic acid–water (3:1:1). For these solvents, development of 10 cm required only 1 h. The chromagrams were sprayed with the same reagents as before. The R_F values observed are given in Table II.

TABLE II

R_F VALUES OF SOME SUGARS ON SILICA-GEL (0.1 N BORIC ACID) CHROMAGRAMS

	Solvent 1[a]	*Solvent 2*[a]
D-Galactose	0.32	0.36
D-Mannose	0.43	0.46
D-Glucose	0.41	0.41
L-Rhamnose	0.61	0.58
L-Fucose	0.50	0.48
D-Glucuronic acid	0.05	0.34
D-Galacturonic acid	0.06	0.42
D-Fructose	0.31	0.43
D-Xylose	0.47	0.54
D-Ribose	0.45	0.57
L-Arabinose	0.43	0.48
Maltose	0.29	0.30
Lactose	0.20	0.25
Galactitol	0.14	0.43
D-Arabinitol	0.28	0.47
Erythritol	0.44	0.52
Glycerol	0.49	0.56

[a]Solvent 1: Butan-1-ol–acetone–water (4:5:1). Solvent 2: Butanone–acetic acid–water (3:1:1).

The speed and ease with which good separations of sugars can be achieved, especially on the silica-gel chromagrams, make these analytical aids attractive to the carbohydrate investigator.

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18.

SOME OBSERVATIONS ON MOLECULAR WEIGHT ESTIMATIONS BY MOLECULAR-SIEVE CHROMATOGRAPHY

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Use of the so-called "gel filtration" technique for separating substances on a molecular size basis is increasing rapidly. "Gel filtration" columns can also be calibrated for direct molecular weight estimations of polystyrenes[1], proteins[2-6], peptides[7], and polysaccharides[8] if suitable molecular-sieves and valid standards of known molecular weight are available.

Unfortunately, several sources of confusion exist in the literature. Many of the papers concerned have been published in either remote or applied biological journals; we choose to draw attention to these in an international analytical journal in the hope that increased fundamental interest in some of the present sources of difficulty will result.

The validity of PORATH AND FLODIN's term[9] "gel filtration" as a suitable description of the essential process(es) involved has been questioned legitimately[10-14]. The discussion regarding the relative merits of the alternatives "exclusion chromatography", "restricted diffusion chromatography", and "gel permeation chromatography" has been reviewed by DETERMANN[15], who proposed a differentiation between "gel filtration" and "gel chromatography". The term "molecular-sieve filtration" has also been used[16].

ACKERS[17] suggested that "molecular exclusion" and "restricted diffusion" are distinguishable primary molecular-sieving effects, the relative importance of which varies with the type of molecular-sieve used. Almost simultaneously, and in direct contrast, LAURENT AND KILLANDER[10] concluded that the "decreased diffusion rate" was not a contributory process on the basis of the insensitivity of elution position to rate of flow. Until this disagreement is resolved—and, moreover, since gel-formers are not the only type of bed materials that can give the essential molecular-sieving effect[11,18,19]—it appears that none of the terms referred to above is generally acceptable. Although TISELIUS, PORATH AND ALBERTSSON[20] stated (*cf.* ref. 15) that the technique "differs basically from common chromatography", some of their supplementary remarks support the view that the essential process is described more appropriately as "chromatography" than as "filtration". Assuming, however, that the term "molecular-sieve" is accepted to have the physico-chemical significance required to describe those porous column-packing materials which allow large molecules to be eluted faster than small molecules, the name "molecular-sieve chromatography" (M-SC)[21] appears to be the most appropriate of those suggested to date. Perhaps it is not yet too late for the adoption of this term in preference to "gel filtration".

Confusion also arises at present from the lack of agreement regarding the best method of relating elution volumes to molecular weights. IWATSUBO AND CURDEL[22] plotted elution volumes directly against molecular weight, and obtained a smooth curve. In order to obtain linear relationships, several authors[2,3,5,23,24] have each elected to plot some different function of the elution volume against the logarithm of the molecular weight, whilst others have related the cube root of the "distribution coefficient", K_d, linearly to the square root of the molecular weight[26]. In contrast, SANFELIPPO AND SURAK[27] observed a linear relationship between K_d and the *reciprocal* of the logarithm of the molecular weight for hormonally-active proteins and peptides on cross-linked dextran gels.

Defining K_d as the fraction of the internal volume, V_i, that is accessible to the solute, then

$$K_d = \frac{V_e - V_0}{V_i} \tag{1}$$

where V_0 is the void volume of the column and V_e is the elution volume[28]. ACKERS[17] has interpreted "molecular-sieve" processes in terms of a restricted diffusion mechanism in which he relates K_d to the Stokes' radius, a, of a macromolecule that is diffusing within a restrictive barrier of effective pore radius, r, by the RENKIN[29] equation

$$K_d = (1 - a/r)^2 \left[1 - 2.104\, a/r + 2.09\, (a/r)^3 - 0.95\, (a/r)^5\right] \tag{2}$$

From this, ACKERS computed theoretical values for K_d for known values of a/r; plotting these values of K_d *directly* against a/r gave good agreement with the experimental values. By plotting all ACKERS' computed values of K_d against the logarithm of a/r, we obtain curve A in Fig. 1.

An alternative approach follows from PORATH's theory[26]; on the assumption

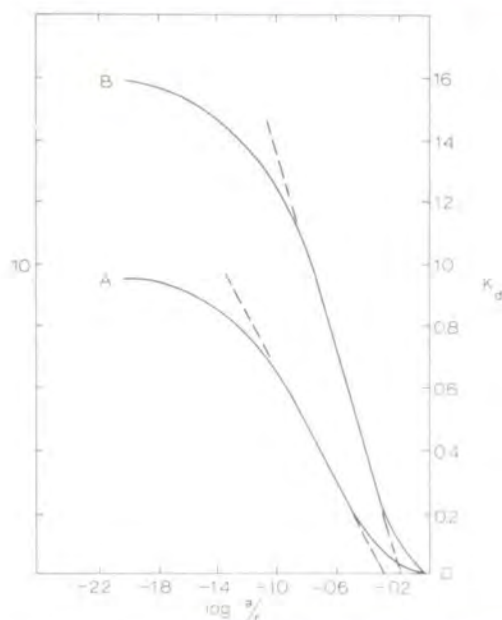

Fig. 1. Plot of K_d *versus* log a/r. Curve A: from ACKERS' theory; curve B: from PORATH's theory.

that gel pores are conical in shape, PORATH derived, for the exclusion process, a theoretical formula which, in terms of the symbols already introduced here, is equivalent to

$$K_d = k(1 - a/r)^3 \quad (3)$$

where k is a constant, equal to 1.64 for dextran fractions on "Sephadex" gels. For this value of k, we have calculated values of K_d for known values of a/r; a plot of K_d against log a/r gives curve B in Fig. 1.

These theoretical treatments can be used in a qualitative way to account for the empirical correlations of other investigators. For either curve A or B in Fig. 1, any restricted central portion that can be considered essentially linear is of the general form

$$K_d = -k_1 \log a/r - k_2 \quad (4)$$

where k_1 and k_2 are constants. Since a is proportional to some fractional power of the molecular weight M, eqn. (4) can be expressed as

$$K_d = -\mathrm{b} \log M + \mathrm{c} \quad (5)$$

where b and c are constants. A plot of K_d against log M is therefore linear[24] over a restricted range which is dependent on the nature of the solute molecules and on the type of molecular-sieve. Departures from linearity[2,5] for solutes which can penetrate the molecular-sieve pores to only a very small extent are predicted by either curve A or B in Fig. 1.

Since V_0 and V_i are constants for a particular column, eqn. (1) can be re-written as

$$V_e = -\mathrm{b}' \log M + \mathrm{c}' \quad (6)$$

where b′ and c′ are constants. This form of plot, used by ANDREWS[2], is the simplest possible correlation between the elution volume and log M; we have found it the most convenient and reliable plot for routine use. The introduction of other constants, such as V_0[5], may facilitate comparison of solute behaviour on dimensionally different columns of the same gel-former, but this is not necessary for calibration purposes.

Almost all the other logarithmic forms of plot to which reference has been made[2,3,5,23,30] may be regarded as variants (merely involving changes in the associated constants) of eqn. (5). The equation

$$\log M = -\mathrm{a}(V_e/V_0 - 1) + \mathrm{b} \quad (7)$$

published (with an incorrect sign) by WHITAKER[5], and subsequently used correctly by LEACH AND O'SHEA[3], appears to be needlessly complex. The relationship of SAN-FELIPPO AND SURAK[27] is exceptional in that it does not follow directly from eqn. (5).

Values of K_d, as defined by eqn. (1), are dependent on the values found for V_i, and there is disagreement regarding how this can best be determined experimentally. Values for V_i have been determined (*a*) from expressions using the water regain of the gel[1,6,23-25], and (*b*) from the difference between the elution volumes of tritiated water and some substance that is completely excluded from the molecular-sieve[17]. Such determinations of V_i include, with the internal volume, the water of hydration which is associated with the gel and is generally inaccessible to polymer solute molecules[28].

In addition to the poor theoretical justification for determining V_i from an expression using the water regain, there are experimental difficulties; the internal volume of a gel packed in a column may differ from that determined experimentally under centrifugal force[24].

It is therefore suggested that K_d be defined according to the practice introduced by ANDREWS[2]; V_i is defined as the difference between the elution volume at which molecular-sieving ceases to be operative for small molecules (of the polymer system under test) and the void volume, V_0, of the column. This point becomes more important in the light of a recent theoretical treatment of "Sephadex gel filtration" by SQUIRE[31], who claims that his equations might apply throughout the entire range of molecular weights, even for very small molecules. The molecular-sieve process breaks down for relatively small molecules; on a "Bio-Gel P300" column, capable of molecular weight estimations of polysaccharides[8] in the range 5,000–125,000, the elution volumes of glucose and sucrose are identical. On ANDREWS' basis[2] this elution volume equals $V_0 + V_i$, and K_d is therefore unity for both glucose and sucrose. The introduction of constant k into eqn. (3) is necessary to account for the experimental observation[1,2,26] that, in a linear plot of $K_d^{1/3}$ against $M^{1/2}$, the *intercept* on the $K_d^{1/3}$ axis is greater than unity. This fact was not realised, apparently, in earlier papers[6,15] in which the straight line drawn through a number of observations would have represented the experimental data better had it not been made to pass through unit value for $K_d^{1/3}$ at $M^{1/2} = 0$.

LAURENT AND KILLANDER[10] have pointed out that PORATH's assumption[26] that a is directly proportional to $M^{1/2}$ is true for flexible macromolecules only[32]. In contrast, SQUIRE[31] assumed a to be directly proportional to $M^{1/3}$, and he deduced a linear relationship between $(V_e/V_0)^{1/3}$ and $M^{1/3}$ for proteins and dextran fractions. Both PORATH and SQUIRE claim good agreement with the experimental data, although CARNEGIE has found that SQUIRE's relationship is not as satisfactory as PORATH's for peptides on columns of "Sephadex G25" with phenol–acetic acid–water (1:1:1, w/v/v) as solvent. At present, however, practical tests of such theories are not very sensitive (*cf.* ref. 10). In contrast, the relationship between K_d and log M has the merit of holding regardless of the value of the fractional power to which M has to be raised so as to be proportional to a, provided that the fractional power is constant for the polymer series under investigation.

ALBERTSSON[33] has shown that the BRÖNSTED relationship[34] between the partition coefficient and the molecular weight of globular proteins applies to their distribution in certain two-phase aqueous systems. If such a relationship holds in molecular-sieve chromatography, log K_d would be expected to be proportional to $M^{2/3}$ (*cf.* ref. 21) if the temperature is constant.

Thin-layer chromatography of proteins on "Sephadex" has also been used for molecular weight estimations[2,35], and this technique has some features in common with molecular-sieve chromatography. Thus eqn. (1) can be written

$$K_d = \frac{V_e - V_0}{V_i' - V_0} \tag{8}$$

where

$$V_i' = V_0 + V_i \tag{9}$$

Now the elution volume for a solute molecule is inversely proportional to the distance

moved by that molecule down a column in a given time. Comparing the migration distances on a thin-layer chromatogram with those on a column, eqn. (8) may be re-written in the form

$$K_d = \frac{(d_0 - d_e)d_i'}{(d_0 - d_i')d_e} \tag{10}$$

where d_0, d_e and d_i' are the migration distances corresponding to the elution volumes V_0, V_e and V_i' respectively. This relationship is similar to that used by CARNEGIE[7] in his peptide mapping technique. Since $d_i'/(d_0 - d_i')$ is a constant, it follows from eqn. (10) that

$$K_d = c_1(d_0/d_e - 1) \tag{11}$$

where c_1 is a constant. If the R_0 value be defined by d_e/d_0, then

$$K_d = c_1(1/R_0 - 1) \tag{12}$$

so that a plot of K_d against the reciprocal of R_0 should be linear. In contrast, MORRIS[35] has found that a plot of K_d against R_{Hb} (which corresponds directly to R_0) is linear for proteins on "Sephadex" G100 and G200, and CARNEGIE[7] found an approximately linear relationship between d_e and log M for peptides on "Sephadex" G25.

These comparatively new analytical techniques for estimating molecular weights are clearly in their infancy. The potential applications are so wide that it is important for the fundamental difficulties referred to here to be resolved, if progress is to be made on other than an empirical basis.

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SUMMARY

A critical review is given of theoretical aspects of the quantitative application of molecular-sieve chromatography to the estimation of molecular weights of macro-molecules. Particular reference is made to some of the inconsistencies, controversies, and sources of confusion which exist in the literature at present.

RÉSUMÉ

Une revue critique est présentée sur les aspects théoriques de l'application quantitative de la chromatographie sur colonne avec remplissage de tamis moléculaire, en vue d'une estimation de poids moléculaires. Les auteurs se sont référés spécialement à quelques controverses et sources de confusion existant actuellement dans la littérature.

ZUSAMMENFASSUNG

Es wird ein kritischer Überblick gegeben über theoretische Aspekte der quantitativen Anwendung der Molekularsieb-Chromatographie auf die Abschätzung von Molekulargewichten von Makromolekülen. Besonders werden einige kontroversen und Unstimmigkeiten in der gegenwärtigen Literatur berücksichtigt.

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19.

STUDIES ON URONIC ACID MATERIALS

PART XV*. THE USE OF MOLECULAR-SIEVE CHROMATOGRAPHY IN STUDIES ON *Acacia senegal* GUM (GUM ARABIC)

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INTRODUCTION

Lathe and Ruthven[2], and Andrews and Roberts[3], have suggested the possibility of applying molecular-sieve chromatography (gel "filtration") to molecular-weight estimations on polysaccharides. Some of the experiments described in this paper on the application of this technique to *Acacia senegal* gum have been reported in a preliminary communication[4].

MATERIALS AND METHODS

The nodules of *A. senegal* (syn. *verek*) were collected by (the late) Mr. M. P. Vidal-Hall, Gum Research Officer, Republic of the Sudan, at Qala en Nahal, Kassala Province, as the first collection of the 1960 gum season.

Nitrogen, ash, and moisture determinations

Nitrogen was determined by a semi-micro Kjeldahl method, moisture by heating to constant weight at 105°, and ash by heating (muffle furnace) to constant weight at 550°.

Viscosity measurements

Determinations were made in M sodium chloride, in a suspended-level, dilution viscometer at 25° (flow time for M sodium chloride, 189.9 sec).

Uronic acid determinations

Uronic acid content was determined by a vapour-phase, i.r. method after decarboxylation with hydriodic acid[5].

Methoxyl determinations

A vapour-phase, i.r. method was used[5].

*For Part XIV see ref. 1.

Polysaccharide hydrolyses

Polysaccharides were hydrolysed with N sulphuric acid for 7 h at 100°. These conditions do not cause any extensive hydrolysis of the uronic acid linkages in *A. senegal* gum; this was taken into account when determining proportions of galactose. Hydrolysates were neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H^+ form) and concentrated at *ca.* 35° on a rotary evaporator.

Sugar ratios

These were determined by chromatographic separation on Whatman 3MM paper, followed by elution and colorimetric estimation by the phenol–sulphuric acid method[6]. After periodate oxidation, rhamnose was also determined, as acetaldehyde, by a vapour-phase, i.r. method[7]. Sugar compositions were calculated as anhydro-sugar residues.

Periodate oxidations

Unless otherwise stated, these were carried out at room temperature in darkness using excess of sodium metaperiodate. Formic acid was estimated potentiometrically[8]. Formaldehyde was estimated colorimetrically with chromotropic acid[9].

Paper chromatography of sugars

Whatman No. 1 and 3MM papers were used with the following solvent systems (v/v): (*a*) benzene–butan-1-ol–pyridine–water (1:5:3:3, upper layer); (*b*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); (*c*) butan-1-ol–ethanol–water (4:1:5, upper layer); (*d*) butan-1-ol–acetic acid–water (4:1:5, upper layer); (*e*) ethyl acetate–pyridine–water (10:4:3); (*f*) butanone–acetic acid–water (9:1:1, saturated with boric acid). Chromatograms were developed with aniline oxalate, *p*-anisidine hydrochloride, alkaline silver nitrate, or the periodate–permanganate reagent.

Thin-layer chromatography of sugars

This was carried out on "Chromagram" sheets (Kodak Ltd., Kirkby, Liverpool) of polycarbonate or silica gel[10], using the following solvent systems (v/v): (*g*) propan-1-ol–ethyl acetate–water (10:3:1) with polycarbonate sheets; and (*h*) butan-1-ol–acetone–water (4:5:1), or (*i*) butanone–acetic acid–water (3:1:1) with silica-gel sheets.

Molecular-sieve chromatography

This was carried out on columns (6.0 × 50 cm) of "Bio-Gel P300" (Bio-Rad Laboratories, Richmond, California) using M sodium chloride as eluant[4]. To prevent deformation by "wall effects", columns were pre-treated with 1% dichlorodimethylsilane in benzene at 60°. After oven-drying, columns were packed with gel that had been allowed to swell in M sodium chloride for 2 days. The gel slurry was added continuously to the column; a thin layer of glass beads supported the gel and kept the "dead space" to a minimum. To stabilise the soft top-surface of the P300 gel, 1-cm

layers of "Bio-Gel P200" and "Bio-Gel P10" were applied successively to the column. Eluant was allowed to flow for 2 days before the columns were calibrated with dextran fractions (Pharmacia Ltd., Uppsala) of known, number-average, molecular weight ($\overline{M}_n$). Polysaccharide (*ca.* 10 mg), dissolved in 1.5M sodium chloride (1 ml), was applied to the column by careful layering beneath the M sodium chloride. Fractions, collected from a 2-ml siphon by an automatic collector, were screened by the phenol–sulphuric acid method[6]. Elution volumes (V_e) were estimated to the nearest ml from peak maxima.

RESULTS

Fractional precipitation of A. senegal *gum with sodium sulphate*

The gum (40 g) was dissolved in water (800 ml), filtered, and electrodialysed. Analyses on the freeze-dried product are shown in Table I.

A solution of the purified gum (25.4 g) in water (500 ml) was maintained at 28°. Anhydrous sodium sulphate was added in small portions with constant stirring. Precipitation commenced at concentrations approaching 40% (w/v); at 40%, a pale-brown material rose to the surface and was removed (Fraction I). Two further fractions, II and III, much lighter in colour, were obtained on continued, slow, stepwise addition of very small portions of sodium sulphate; eventually, the supernatant solution contained polysaccharide material which was not precipitated from a saturated solution of sodium sulphate, and this yielded Fraction IV. The fractions were dialysed against tap water until free of sulphate and were then electrodialysed to ensure complete removal of inorganic ions. Analytical data for the freeze-dried fractions are given in Table I.

Autohydrolysis of A. senegal *gum*

A sample (4 g) of electrodialysed gum was dissolved in water to give a 2% solution (pH, 2.8). Autohydrolysis on a boiling water-bath was followed polarimetrically[11]. After 50 h, the solution was cooled, filtered (to remove denatured protein), and dialysed against water (3 × 2 l). Dialysis was completed against running tap-water, and freeze-drying gave the degraded gum (2 g), $[\alpha]_D$ −11° (*c* 1.0, water) (Found: moisture, 9.7; uronic acid, 19.2; galactose, 68; arabinose, 2%). Hydrolysis of the degraded gum indicated the presence of two aldobiouronic acids, which had R_{Gal} values of 0.22 (major component) and 0.59 (minor component) in solvent (*b*), and were chromatographically identical with 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose and 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose, respectively. Chromatographic examination of the diffusate from the degraded gum showed it to contain galactose, arabinose, rhamnose, three (major) neutral disaccharides, traces of the two aldobiouronic acids, and oligosaccharide material. Further hydrolysis of a portion of the diffusate yielded more of the same aldobiouronic acids found for the degraded gum.

TABLE I

ANALYTICAL DATA FOR ELECTRODIALYSED *A. senegal* GUM AND FRACTIONS OBTAINED BY PRECIPITATION WITH SODIUM SULPHATE

	A. senegal gum	*Fractions*			
		I	*II*	*III*	*IV*
Yield, %	—	23.6	29.5	33.7	1.2
Moisture, %	11.0	14.7	7.3	8.6	—
Ash, %	0.01	0.01	0.02	0.01	—
N, %	0.33	1.01	0.12	0.02	—
Protein, % [N % × 6.25]	2.1	6.3	0.75	0.13	—
[η], cm^3g^{-1}	20.0	33.5	14.8	10.8	—
Rhamnose, %[a]	12(14)	10(13)	12(13)	12(13)	—
Arabinose, %[a]	25(28)	24(30)	26(28)	23(25)	—
Galactose, %[a]	34(39)	29(37)	37(40)	40(44)	—
Uronic acid, %[a,b]	16.7(19)	16.0(20)	17.5(19)	16.7(18)	15.5
Methoxyl, %	0.23	0.23	0.23	0.23	0.22
$[\alpha]_D$ (*c* 1.0, water)	−31.5°	−32.7°	−32.7°	−31.5°	—
Equiv. wt.[c]	1290	—	—	—	—
Formic acid released on periodate oxidation (mole/g) × 10^3	1.58	1.56	1.59	1.60	—
Ratio of galactose/arabinose[d]	1.40	1.23	1.43	1.76	—

[a]Values in parentheses are corrected for all non-carbohydrate material.
[b]Calculated as the anhydride of glucuronic acid.
[c]By direct titration (potentiometric) with 0.02N sodium hydroxide.
[d]Calculated from the values corrected for non-carbohydrate material.

Borohydride reduction of degraded gum

Degraded gum (500 mg) was dissolved in water (100 ml), and sodium borohydride (400 mg) was added. The solution was kept for 24 h at room temperature before further sodium borohydride (100 mg) was added. After the solution had been stirred for 6 h, it was dialysed against running tap-water for 2 days. The freeze-dried product was hydrolysed to yield the same aldobiouronic acids and neutral disaccharides found in the degraded gum. In addition, paper chromatography in solvent (*f*), and t.l.c. on silica gel with solvent (*h*) indicated the presence of galactitol. No arabinitol was detected.

Periodate oxidation of degraded and reduced, degraded gum

Degraded gum did not give detectable amounts of formaldehyde on periodate oxidation. The production of formaldehyde with time from reduced, degraded gum (34.32 mg, dry wt.) was as follows: 0.25 h, 160 μg; 0.5 h, 200 μg; 1 h, 215 μg; 2 h, 220 μg; 6 h, 220 μg; 24 h, 235 μg. Assuming production of one formaldehyde molecule per average polymer unit, a value for $\overline{M}_n$ of 4,400 was calculated for the degraded gum. Taking into account its composition, this corresponds to a number-average degree of polymerisation ($\overline{P}_n$) of 27.

Controlled Smith-degradation[12] *of degraded gum*

Periodate oxidation was carried out at 2°. Degraded gum (1 g) was dissolved in water (25 ml), 50% (w/w) periodic acid (1.75 ml) was added, and the solution made up to 50 ml. After 2 days, the reaction was stopped by addition of excess of ethylene glycol. Following dialysis against running tap-water for 2 days, the solution was treated with sodium borohydride (250 mg) for 36 h. Further dialysis for 2 days was followed by hydrolysis of the acetal linkages with N sulphuric acid for 2 days at 18°. The acidic solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H^+ form), and concentrated on a rotary evaporator. Chromatographic examination revealed the presence of glycerol and glycolic aldehyde. Molecular-sieve chromatography on a column (2.5 × 75 cm) of "Bio-Gel P10" was used to separate such low molecular-weight materials from the Smith-degraded product (180 mg). Hydrolysis of a small portion of the latter product, with examination by paper chromatography, gave galactose, arabinose (a trace) and arabinitol [solvent (*f*)], but no galactitol or erythritol.

Molecular-sieve chromatography

Figure 1 shows the calibration plot of elution volume (V_e) against log $\overline{M}_n$ obtained with dextran fractions of known $\overline{M}_n$. For "Bio-Gel P300", this relationship[13-15] is approximately linear for values of $\overline{M}_n$ from 5,000 to 125,000; although the useful working range may extend slightly beyond these values, the exclusion limit of "Bio-Gel P300" *for the polysaccharides investigated* is apparently less than 300,000

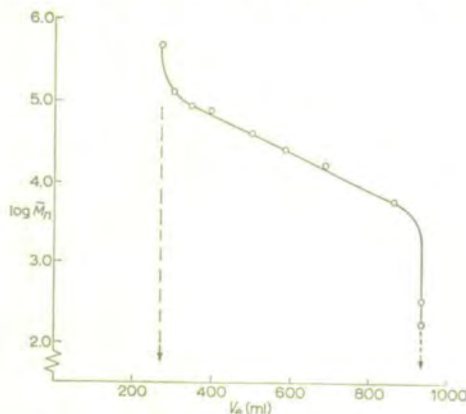

Fig. 1. Plot of elution volume (V_e) against log $\overline{M}_n$ for dextran fractions of known $\overline{M}_n$ values. ["Bio-Gel P300" column (5.0 × 50 cm), elution with M sodium chloride]. The arrows shown correspond with those on Fig. 2.

Sucrose and glucose have the same elution volume, which is defined as being equal to $V_o + V_i$, where V_o is the void volume and V_i the internal volume[14,16]. The elution volume of "blue dextran" (Pharmacia Ltd., Uppsala) was taken as the void volume, and values for the distribution coefficient (K_d) were calculated from the relationship[17], $K_d = (V_e - V_o)/V_i$. Figure 2 shows the elution patterns for *A. senegal*

gum, for the fractions (I–IV) precipitated by sodium sulphate, and for the degraded gum obtained by autohydrolysis of *A. senegal* gum. Table II gives the values found for K_d and $\overline{M}_n$; estimation of $\overline{M}_n$ for the whole gum was rendered difficult by the asymmetric nature of its elution curve.

TABLE II

ESTIMATION OF $\overline{M}_n$ BY MOLECULAR-SIEVE CHROMATOGRAPHY

	V_e	K_d	$\overline{M}_n$
A. senegal gum	(276)	—	—
Fraction I	270	0.00	—
Fraction II	294	0.04	140,000 ± 20,000
Fraction III	351	0.12	99,000 ± 10,000 (105,000)[a]
Fraction IV	532	0.40	35,000 ± 3,000 (37,000)[a]
Degraded gum[b]	884	0.92	4,800 ± 500 (4,400)[c]

[a]By osmometry; the authors thank Mr. S. Rahman for these determinations.
[b]Obtained by autohydrolysis.
[c]Periodate end-group analysis, as formaldehyde.

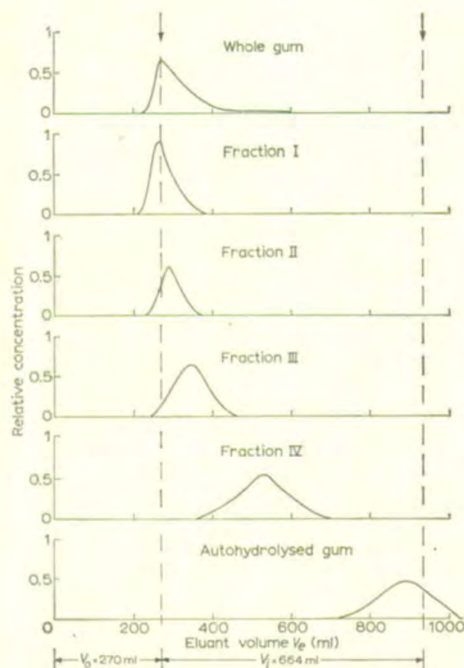

Fig. 2. Elution patterns for *A. senegal* gum, sodium sulphate fractions, and autohydrolysed gum. ["Bio-Gel P300" column (6.0 × 50 cm), elution with M sodium chloride].

DISCUSSION

Fractional precipitation of gum arabic with propan-2-ol[18] and acetone[19] has been reported. Van Beek[19] has suggested that there is a correlation between the limiting-viscosity numbers of the fractions precipitated by acetone and their content of divalent cation. In spite of the fact that metal ions were reported[20] to cause aggregation of complex acidic polysaccharides in solution, all attempts in this laboratory to repeat the experiments of Van Beek have failed.

Prolonged contact of *A. senegal* gum with organic solvents leads to insolubility difficulties, and the possibility of using salt as a fractional precipitant was therefore examined. Since the feasibility of fractional precipitation depends upon the polysaccharide in question having a broad molecular-weight distribution, *A. senegal* gum, and fractions obtained by fractional precipitation with sodium sulphate, were studied by molecular-sieve chromatography. With such fractional precipitations, the number of fractions isolated is arbitrary and is usually governed by the amount of material required for the analyses necessary to characterise the fractions. The elution patterns obtained on molecular-sieve chromatography (Fig. 2), and the limiting-viscosity numbers (Table I) of the fractions, clearly demonstrate that fractionation by "molecular" size was effected. Careful electrodialysis eliminated the possibility of traces of di- and poly-valent cations causing aggregation. Molecular-sieve chromatography and viscosity measurements were carried out in solutions having a constant concentration of univalent cations (*i.e.*, M sodium chloride). Aggregation by metal ions cannot, therefore, explain our results. Aggregation of the polysaccharide by protein is also unlikely to occur in M sodium chloride, since coacervates are broken down on addition of simple electrolytes[21]. Our results can, however, be explained by fractionation according to the molecular size of the polysaccharide.

The chemical composition of the fractions was investigated, and the results are summarised in Table I. The similar yields of formic acid released on periodate oxidation indicate that there is little variation in the degree of branching of the polysaccharides in the fractions, but the varying proportions of galactose to arabinose indicate that the gum is chemically heterogeneous. Previous evidence of chemical heterogeneity in commercial gum arabic was obtained by Heidelberger and Adams[22]; the small fraction of gum precipitated by Type II antipneumococcal horse-serum was depleted in rhamnose.

Confusion has arisen over the use of the terms *homogeneous* and *heterogeneous* in relation to plant gums and other polysaccharides. Smith and Lewis[23] claimed that the *heterogeneity* of *A. senegal* gum is revealed by electrophoresis on glass-fibre paper, whilst Jermyn[24] observed no sharp discontinuity in the properties of the molecular species after chromatography on DEAE-cellulose. *Combretum leonense* gum has been described by Aspinall and Bhavanandan[25] as *micro-heterogeneous*, *i.e.*, "a mixture of polysaccharides composed of the same structural units, which are linked in a similar manner, but are in slightly differing proportions". Norman[26] has stated that *A. senegal* gum is "not a substance of constant composition, but is con-

structed in a particular pattern from varying amounts of constituent units", and Hirst[27] has referred to it as "a mixture of closely related, molecular species". Other terms, such as *grossly heterogeneous*[25], *polydisperse*[3,28], and *polymolecular*[29], have also been employed. Unfortunately, their usage has not always been in accordance with their accepted definitions; *polydisperse* describes polymer systems containing more than one component; *polymolecular* denotes a *homogeneous* polymer having a variation in molecular weight (*cf.* ref. 20).

There is no evidence from our present investigations, nor from those of Jermyn[24], that *A. senegal* gum is *polydisperse*. On the other hand, if the gum is claimed to be *polymolecular*, the above definition of this term implies that it is a *homogeneous* polymer. The term *homogeneous* has been used[30,31] to indicate that polysaccharides are not *polydisperse*, even although, chemically, they are undoubtedly *heterogeneous*. To avoid this ambiguity, it is suggested that the term *polymolecular* be reserved for the description of those polymer systems having only a distribution in molecular weight, and the term *heteropolymolecular* be used to describe polymer systems having either a variation in monomer composition and/or a variation in the mode of linking and branching of the monomer units, in addition to a distribution in molecular weight. Defined in this way, the term *heteropolymolecular* conveys a more comprehensive description of the spectrum of related polysaccharides that comprise *A. senegal* gum.

Molecular-sieve chromatography of the degraded gum obtained on autohydrolysis (Fig. 2) indicates that $\overline{M}_n = 4,800$ (Table II). Degradation of the whole gum to produce units of this small size is much greater than would be expected to result from removal of labile sugar-residues (such as L-arabinofuranose and L-rhamnopyranose) from the periphery of the molecule. This observation was made by Smith and Montgomery[32], and it led them to suggest that some labile sugarresidues were present in the interior of the gum molecule. They postulated that blocks of degraded units might have been interconnected by labile residues of arabinofuranose. If this were so, it should be possible to show that some, if not all, of this arabinose is sited at the reducing end of the degraded molecules resulting from autohydrolysis. Arabinose was not, however, reported by Smith[11] to be present in the degraded gum.

This investigation shows that autohydrolysis of *A. senegal* gum results in the release of galactose residues, in addition to arabinose and rhamnose residues, with the formation of a degraded gum containing 2% of arabinose. Although autohydrolysis is sufficient to break galactopyranosidic bonds to give galactose, arabinose was not completely removed from the degraded portion that remained behind after dialysis (*cf.* ref. 33). In order to discover whether arabinose was present as the reducing end-group, the autohydrolysed, degraded gum was reduced with borohydride. The presence of galactitol, and the absence of arabinitol, in the hydrolysate of this reduced material shows that galactose occupies the reducing end-group. The 2% of arabinose in the degraded gum appears, therefore, to be sited other than at the reducing end.

Autohydrolysis is not very selective as a means of degradation. In our autohydrolysis experiments, traces of aldobiouronic acids are released, together with oligosaccharides which are small enough to pass through cellophane dialysis-tubing

(Kalle Aktiengesellschaft, Wiesbaden). Acidic material of low molecular-weight has also been obtained from autohydrolysis of gums from *A. karroo*[34] and *A. cyanophylla*[35], and this led Hirst[36] to suggest that acidic residues may occur in labile side-chains.

The methoxyl content of *A. senegal* gum has already received comment in a preliminary communication[37]. The methoxyl group is not present as the ester of D-glucuronic acid, since the methoxyl content does not decrease on treatment with N sodium hydroxide. Methoxyl groups are now known to occur commonly in plant gums in residues of 4-*O*-methyl-D-glucopyranosyluronic acid. A careful, chromatographic re-examination of the degraded gum from *A. senegal* resulted in the detection of the aldobiouronic acid, 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose, the presence of which had not been recognised by earlier investigators. The sample of *A. senegal* gum used in the present study has a methoxyl content of 0.23%; this corresponds to a content of 1.4% of 4-*O*-methyl-D-glucopyranosyluronic acid. The presence of this residue in *A. senegal* gum accounts for some of the 2,3,4-tri-*O*-methyl-D-glucuronic acid obtained after hydrolysis of the methylated whole-gum[38].

Periodate oxidation of the degraded gum produces no formaldehyde, so it may be concluded, in conjunction with methylation evidence[39], that the reducing galactose residue is substituted at C-6. As a result, borohydride-reduced, degraded gum was assumed to produce one formaldehyde molecule per average unit on periodate oxidation. On this basis, a value of 4,400 for $\overline{M}_n$ was calculated for the degraded gum.

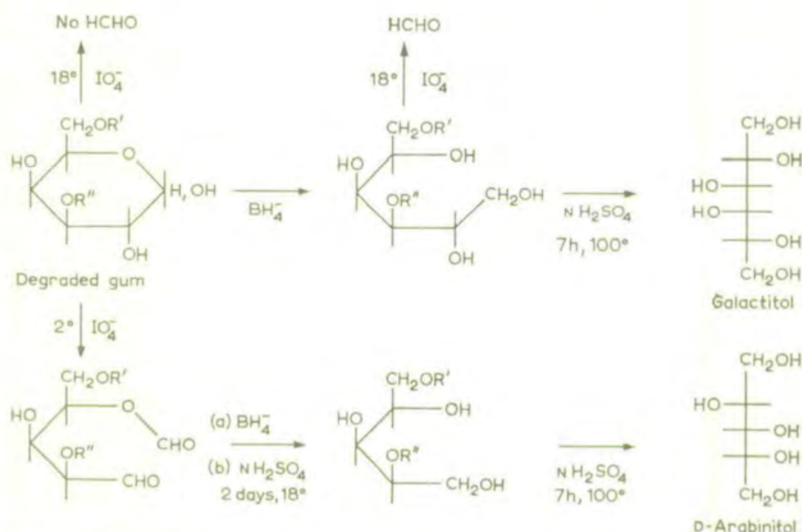

Fig. 3. Scheme of reactions carried out on autohydrolysed gum from *A. senegal*. R′ and R″ represent the remainder of the degraded gum.

The reactions carried out on the degraded gum are summarised in Fig. 3. A controlled Smith-degradation (*cf.* ref. 40) carried out at 2° provides additional

information that galactose constitutes the reducing end-group. The series of reactions proposed[32] to account for the appearance of arabinitol is evidence that the reducing galactose residues are also substituted at C-3.

If the extensive degradation of the macromolecule observed on autohydrolysis is not due to the presence of internal, labile, arabinofuranosidic bonds, certain galactopyranosidic bonds must be unusually reactive towards very mild conditions of hydrolysis, which would not normally be expected to cleave such bonds. As a result of studies on *Virgilia oroboides* gum, Stephen[41,42] has suggested that the carboxyl groups of the uronic acid residues may be responsible for "deep-seated decomposition ". This fact, and the overall geometry of the *A. senegal* gum molecule, may provide the explanation for the unexpected lability of some pyranosidic bonds. More knowledge is required on the degree of branching within the molecular framework of *A. senegal* gum before a theory of more heuristic value may be advanced.

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SUMMARY

Investigations involving fractional precipitation of *A. senegal* gum by sodium sulphate lead to a discussion on the type of heterogeneity exhibited by the gum. Molecular-sieve chromatography is used to estimate number-average molecular weights. Results obtained using this chromatographic technique on the degraded gum produced on autohydrolysis indicate that such mild conditions of hydrolysis are not always very selective as a means of degradation. The degraded gum is shown to have galactose residues as reducing end-groups. There is no evidence for labile, internal, arabinofuranosyl linkages in the whole gum. In addition, chromatographic evidence is obtained for the presence of 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose residues in *A. senegal* gum.

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Theories of Molecular Sieve Chromatography

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Abstract

Two aspects of the theory of molecular sieving are distinguished: (a) a thermodynamic aspect, which relates to the differential partition of solute molecules between porous materials and interstitial solvent, and (b) a hydrodynamic aspect, which concerns the restricted diffusion of solute molecules within porous particles. These aspects are discussed, together with related facts concerning the molecular sieving method of molecular weight estimation, and the effects of temperature, flow-rate and column parameters on elution patterns from molecular sieve columns.

Théories de la chromatographie sur les tamis moléculaires

On distingue deux aspects de la théorie du tamisage moléculaire: (a) un aspect thermodynamique, qui a rapport à la répartition différentielle des molécules dissoutes entre des matériaux poreux et le solvant interstitiel, et (b) un aspect hydrodynamique qui concerne la diffusion restreinte des molécules dissoutes dans les particules poreuses. Ces aspects sont discutés avec les faits qui s'y rapportent, et qui concernment la méthode de tamisage moléculaire de l'estimation du poids moléculaire, et les effets de la température, du taux d'écoulement, des paramètres de colonnes sur la formation des dessins à partir des collonnes de tamis moléculaires.

Theorien über die Molekularsieb-Chromatographie

Es wird zwischen zwei Gesichtspunkten der Theorie der Molekularsiebung unterschieden und zwar (a) einem thermodynamischen Gesichtspunkt, der sich auf die differentielle Teilung gelöster Moleküle zwischen porösen Materialien und nichtstöchiometrischen Lösungsmitteln betrifft, und (b) einem hydrodynamischen Gesichtspunkt, der die beschränkte Diffusion gelöster Moleküle innerhalb poröser Teilchen betrifft. Diese Gesichtspunkte werden zusammen mit zugehörigen Gegebenheiten über die molekulare Siebmethode der molekularen Gewichtsermittlung besprochen, und zwar unter Berücksichtigung der Auswirkung von Temperaturen, Durchflußmengen und Säulenkenngrößen im Zusammenhang mit Eluierung aus Molekularsiebsäulen.

Introduction

Molecular sieve chromatography is a form of partition chromatography, which utilizes the distribution behaviour of solutes between solvent phases in two physically distinguishable environments. If solvent is allowed to flow through a column packed with porous particles, the proportion of unbound solvent imbibed within the pores constitutes the stationary phase and the proportion of unbound solvent occupying the interstices between the porous particles constitutes the mobile phase. The remainder of the solvent is strongly bound to the porous particles and is inaccessible to the solute. Therefore the total volume V_t of the column is given by

$$V_t = V_g + V_o + V_i \qquad \ldots (1)$$

where V_g is the volume of the solvated porous particles, V_o is the volume of the unbound solvent in the interstices between the solvated porous particles (i.e. the volume of the mobile phase), and V_i is the volume of the unbound solvent inside the solvated porous particles (i.e. the volume of the stationary phase).

The distribution coefficient

The behaviour of a solute passing through a molecular sieve column is described by a distribution coefficient K_d, which is defined (Wheaton & Bauman, 1953; Gelotte, 1960) as the fraction of the internal volume V_i that is accessible to the solute. Thus it follows that

$$K_d = \frac{V_e - V_o}{V_i} \qquad \ldots (2)$$

where V_e is the elution volume of the solute and V_o is the so-called void volume or exclusion volume of the column. For large solute molecules which cannot enter the stationary phase $V_e = V_o$, and so $K_d = O$. For small solute molecules, which can distribute themselves equally between the two phases, $K_d = 1$ because $V_e = V_o + V_i$. Molecular sieving occurs for solute molecules which obey the condition, $0 < K_d < 1$. Clearly the partitioning of solute molecules between the mobile and stationary phases will be influenced to some extent by adsorption of solute molecules to the solvated porous particles (i.e. the support phase). Preferential adsorption of certain solute molecules may result in $K_d > 1$.

The distribution coefficient is a useful quantity for comparing solute behaviour on molecular sieve columns of different dimensions packed with the same porous material. The difficulties involved in obtaining experimental values for K_d have been discussed by Anderson & Stoddart (1966*a*), who recommended that values for K_d

be obtained from Equation (2), according to the practice introduced by Andrews (1964). Thus, the void volume V_o of a molecular sieve column is taken as the elution volume at which molecular sieving ceases to be operative for large solute molecules of the homologous series of macromolecules under test. Likewise, the internal volume V_i is taken as the difference between the elution volume at which molecular sieving ceases to be operative for small molecules and the elution volume corresponding to the void volume of the column.

The distance moved by a solute down a column in a given time is inversely proportional to the elution volume of the solute. Writing Equation (2) in the form

$$K_d = \frac{V_e - V_o}{V'_i - V_o} \quad \ldots (3)$$

where $V'_i = V_o + V_i$, we have

$$K_d = \frac{(d_o - d_e)d'_i}{(d_o - d'_i)d_e} \quad \ldots (4)$$

where d_o, d_e, and d_i are the migration distances corresponding to the elution volumes V_o, V_e, and V_i respectively. This relationship may be used to obtain values for K_d from thin layer chromatograms.

The formulation of a theory of molecular sieve chromatography is dependent on the interpretation of K_d, or some related quantity, in terms of the mechanisms of interactions of solute molecules with porous materials (Ackers, 1964). Two aspects of the interaction of solute molecules with porous materials need to be distinguished (Ogston, 1966): (*a*) there is a thermodynamic aspect, which relates to the differential partition of solute molecules between porous materials and interstitial solvent, and (b) there is a hydrodynamic aspect, which leads to restricted diffusion of solute molecules within porous particles.

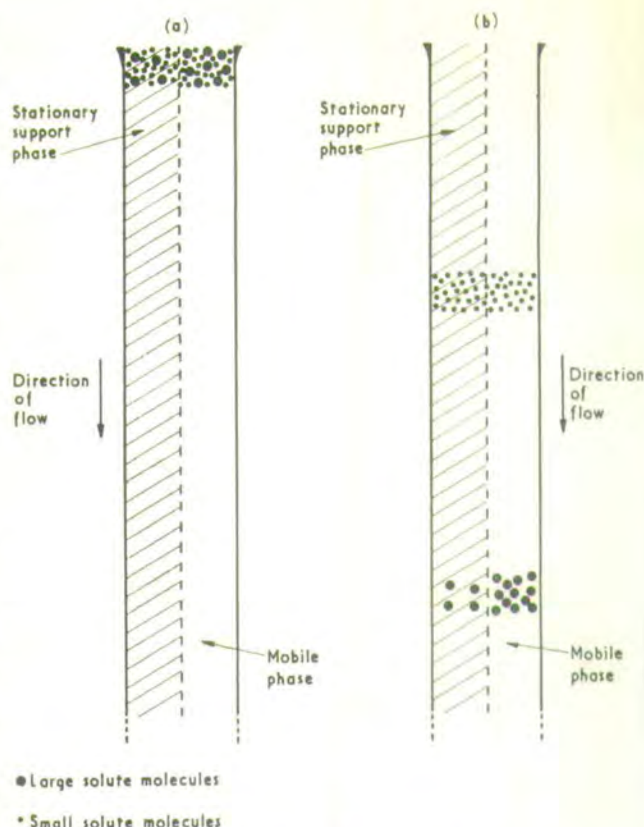

Fig. 1. The separation of large from small solute molecules on molecular sieve columns.
a. **Before chromatography commences.**
b. **During chromatography.**
Note that the volume of the mobile phase is given by V_o, and the volume of the stationary support phase by $V + V_i$.

The thermodynamic aspect

A general phenomenon exhibited by aqueous polymer mixtures in phase separation (Albertsson, 1958 and 1960). Aqueous two-phase systems may be obtained by mixing small amounts of two water-soluble polymers in water. The partition behaviour of isochemical substances in two-phase systems has been studied by Brønsted (1931), who found that for large solute molecules the partition behaviour could be described approximately by the relationship

$$K = e^{\lambda M/RT} \quad \ldots (5)$$

where K is the partition coefficient, M is the molecular weight of the solute, R is the gas constant, T is the absolute temperature, and λ is a constant, the sign and magnitude of which is dependent on the nature of the solute and of the solvent phases. Albertsson (1958) has shown that the distribution behaviour of different globular proteins in certain two-phase aqueous systems obeys approximately the above relationship if M is replaced by $M^{2/3}$. The exclusion properties of solutions of hyaluronic acid have also been examined (Ogston & Phelps, 1961) in a two-phase system, and a simple steric theory has been put forward to explain the exclusion of large solute molecules from hyaluronic acid solutions. Several authors (Hjertén & Mosbach, 1962; Tiselius, Porath & Albertsson, 1963; Ogston, 1966) have suggested that the mobile and stationary support phases in molecular sieve chromatography constitute the two phases of a system in which large solute molecules are excluded from one phase, namely the stationary support phase, to a greater extent than small solute molecules. Columns of cross-linked dextran gels (Laurent & Killander, 1964) and of cross-linked hyaluronic acid gels (Laurent, 1964) have been compared to two-phase systems exhibiting these exclusion properties. If the solute molecules in the mobile phase are in equilibrium with those in the stationary support phase, the exclusion mechanism forms the basis for a chromatographic separation of solutes according to molecular size, with large solute molecules being eluted from columns in advance of small solute molecules. This concept is illustrated diagrammatically in Figure 1. The distribution behaviour of solutes in this kind of system may be described (Laurent & Killander, 1964) by the relationship

$$K_{av} = \frac{V_e - V_o}{V_g + V_i} \quad \ldots (6)$$

This equation relates the partition coefficient of a solute to the volume fraction of the cross-linked dextran gel available to the solute.

Several attempts have been made to explain molecular exclusion processes in terms of steric models which describe the behaviour of solute molecules interacting with porous materials. Laurent & Killander (1964) have

assumed that cross-linked dextran gels are composed of straight, rigid polysaccharide chains of infinite length and of random distribution within the gel particles. According to Ogston (1958) the volume fraction available to a spherical solute molecule in a network composed of randomly oriented straight rods of infinite length is given by the expression

$$K = e^{-[\pi L(a+r')^2]} \quad \ldots (7)$$

where L is the concentration of rods expressed in cm rod per cm^3, a is the Stokes radius of the solute molecule, and r′ is the radius of the rods. Laurent & Killander (1964) have shown that the calculated values obtained for K from Equation (7) for a large number of proteins and for dextran fractions are in good agreement with those determined using Equation (6) from a knowledge of the elution volume of the solute, and the void volume and the total volume of the gel column. These authors have also shown that the volumes available to proteins in cross-linked dextran gels are approximately the same as those available in dextran solutions of the same concentrations as the gels.

Porath (1963) has derived a theoretical formula relating K_d to the Stokes radius a of a solute molecule on the assumption that the pores in cross-linked dextran gel particles are conical in shape. The cones are assumed to have an average radius r and an average height h′. A solute molecule with an effective radius a is assumed to penetrate to within a volume represented by a cone of average radius, $r-a$, and of average height h″. This steric model is represented diagrammatically in Figure 2. The total volume, v′, of the cone is given by

$$v' = \frac{\pi}{3} r^2 h' \quad \ldots (8)$$

and the volume, v″, of the cone available to the solute molecule by

$$v'' = \frac{\pi}{3} (r-a)^2 h'' \quad \ldots (9)$$

Thus

$$\frac{v''}{v'} = \frac{h''}{h'}\left(\frac{r-a}{r}\right)^2 \quad \ldots (10)$$

and as

$$\frac{h''}{h'} = \frac{r-a}{r} \quad \ldots (11)$$

it follows that

$$\frac{v''}{v'} = \left(\frac{r-a}{r}\right)^3 \quad \ldots (12)$$

If K_d is assumed (Porath, 1963) to be proportional to the ratio between v″ and v′, then

$$K_d = k(1-a/r)^3 \quad \ldots (13)$$

where k is a constant. For flexible macromolecules Zimm & Stockmeyer (1949) have shown that a is proportional to $M^{1/2}$, and so it follows from Equation (13) that $K_d^{1/3}$ should be proportional to $M^{1/2}$ for such molecules. Porath (1963) has shown that this relationship holds for dextran fractions eluted from columns of cross-linked dextran gels (Granath & Flodin, 1961). Several authors (Wieland, Duesberg & Determann, 1963; Andrews, 1964; Determann, 1964; Auricchio & Bruni, 1964) have also found the $K_d^{1/3}$ is proportional to $M^{1/2}$ for globular proteins. Theoretically this relationship should hold for flexible macromolecules only, and not for globular proteins (Laurent & Killander, 1964).

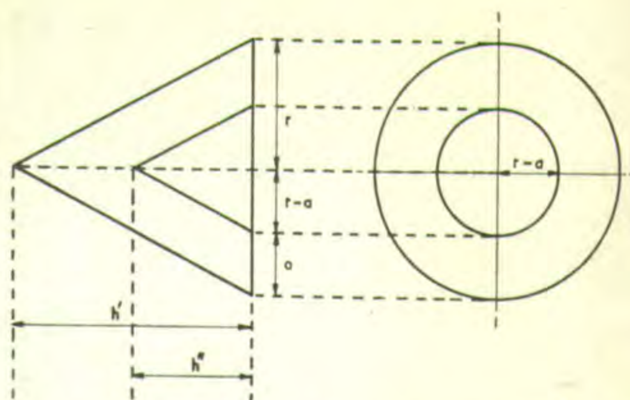

Fig. 2. The conical pores in gel particles. This steric model was proposed by Porath (1963).

The steric model used by Porath (1963) has been extended by Squire (1964) to include cylinders and "crevices" as well as cones. The mathematical treatment of this more sophisticated steric model yields the equation

$$\frac{V_e}{V_o} = \left[1 + g(1-a/r)\right]^3 \quad \ldots (14)$$

which relates V_e to a, where g is a constant. Following the assumption that a is proportional to $M^{1/3}$, Squire (1964) has proposed a linear relationship between $(V_e/V_o)^{1/3}$ and $M^{1/3}$, and claims good support for his theory from empirical data for globular proteins and for dextran fractions on columns of cross-linked dextran gels.

The hydrodynamic aspect

Pedersen (1962) has drawn attention to the enhanced migration of large with respect to small solute molecules in capillaries of diameters about 10 times those of the solute molecules. Provided solution flow is optimal, the capillary volume for solute molecules is reduced by a layer on the surface of the capillary, the thickness of which is approximately proportional to the diameters of the solute molecules. Thus large solute molecules are confined to smaller volumes within a capillary than are small solute molecules. This results in enhanced movement of large with respect to small solute molecules. This effect, which is illustrated diagrammatically in Figure 3, means that separation of solute molecules in very long capillaries with diameters of macromolecular dimensions is theoretically feasible. Indeed Pedersen (1962) has noted that blood corpuscles move faster than the surrounding plasma in blood capillaries (cf. Determann, 1964), and has shown that large protein molecules experience enhanced movement with respect to small protein molecules in columns packed with glass spheres of diameters between 20 and 35μ. These experimental observations have led Pedersen (1962) to suggest that separations of solute molecules according to size by molecular sieve chromatography may be related to the enhanced movement of large with respect to small solute molecules in capillaries.

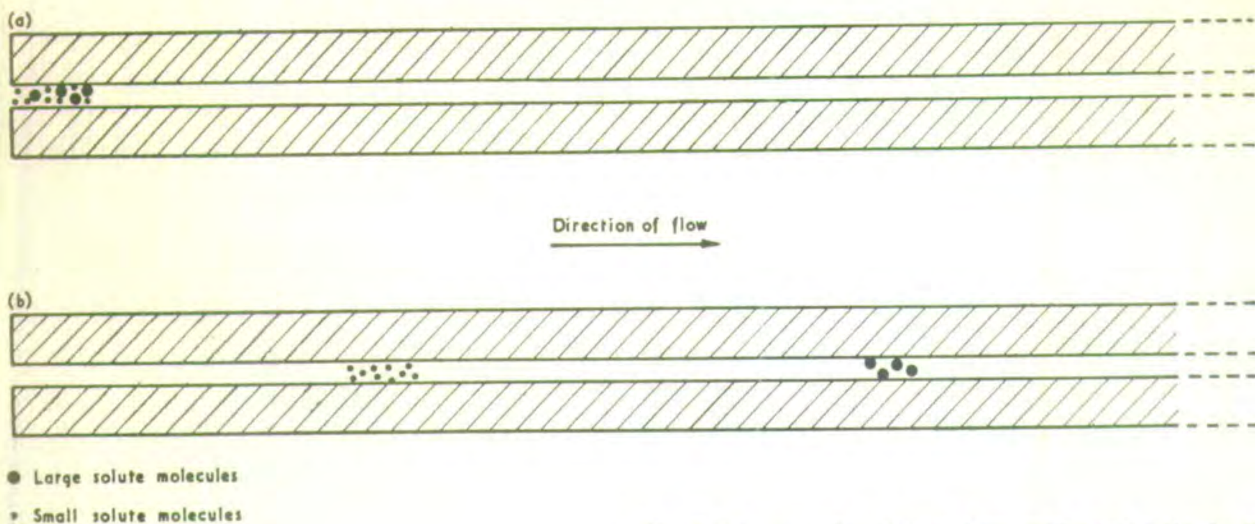

Fig. 3. The separation of large from small solute molecules in a capillary.

a. A mixture of large and small solute molecules in a capillary.

b. Separation of large from small solute molecules during solution flow in the capillary.

Direction of flow

Direction of flow

Large solute molecules

Small solute molecules

Fig. 4. The interaction of large and small solute molecules with a gel particle. Note that the restricted diffusion mechanism referred to by Ackers (1964) is the same as the exclusion process described by Pedersen (1962).

Ackers (1964) has shown that a process of simple molecular exclusion by itself does not explain the properties of columns packed with loosely cross-linked dextran gels, and has interpreted K_d for these columns in terms of steric hindrance (molecular exclusion) and hydrodynamic resistance to molecular diffusion within the gel matrix. The pores within the gel particles are represented by uniform capillaries, which act as diffusion barriers to solute molecules in the mobile phase. Two kinds of interactions of solute molecules with capillary-like pores of gel particles must be distinquished. The first is a simple molecular exclusion effect. The probability that a solute molecule in the mobile phase will enter a capillary (stationary phase) is less for a large than for a small solute molecule. The second kind of interaction is experienced by those solute molecules which enter the capillaries. On account of increased hydrodynamic resistance to diffusion, the movement of large solute molecules is enhanced with respect to the movement of small solute molecules. (Pedersen, 1962). This means that large solute molecules diffuse through gel particles faster than small solute molecules. The two kinds of interactions of solute molecules with gel particles are illustrated diagrammatically in Figure 4. Ackers (1964) has interpreted these interactions by equating K_d to the ratio of restricted to free areas for diffusion in the Renkin (1955) equation to yield the expression

$$K_d=(1-a/r)^2[1-2.104(a/r)+2.09(a/r)^3-0.95(a/r)^5] \quad \ldots (15)$$

where a is the Stokes' radius of a solute molecule diffusing within a capillary of pore radius r. The first term on the right hand side of Equation (15) is a molecular exclusion term. The second term accounts for the increased hydrodynamic interactions experienced by solute molecules within the capillaries of the gel particles. Ackers (1964) has obtained good support for his theory from empirical data for globular proteins on cross-linked dextran gels.

Successful correlation of empirical data with different theoretical equations probably reflects the insensitivity of the practical measurements (Laurent & Killander, 1964; Porath, 1964; Anderson & Stoddart, 1966*a*) as well as the inadequacies of the theories. This criticism is not intended to detract from the attempts that have been made to formulate theories of molecular sieve chromatography in terms of steric models. Progress towards an understanding of the mechanisms of interactions of solute molecules with porous materials at a molecular level is fraught with formidable problems (Ogston, 1966).

The effect of temperature, flow-rate and column parameters on the elution pattern

Those who have based theories of molecular sieve chromatography on steric models have usually recognized the idealized nature of the models in relation to the actual structures of the porous materials. In this connection Ogston (1966) has pointed out that fibres of molecular dimensions in gel particles must be subject to thermal disturbances. It follows that some allowance should be made in theoretical treatments of molecular sieve chromatography for the temperature dependence of elution volumes of solutes from gel columns (Leach & O'Shea, 1965).

The elution volumes of solutes from molecular sieve columns do not vary appreciably with the rate of flow of the eluent (Porath & Flodin, 1959; Flodin, 1962; Tiselius *et al.*, 1963; Laurent & Killander, 1964; Moore & Hendrickson, 1965). This independence of elution volumes on the rate of flow is strong evidence that molecular sieve chromatography is a steady-state process, and that the diffusion of solute molecules between the mobile and stationary phases is a near-equilibrium controlled mechanism. Whilst changes in the rate of flow may affect the diffusion of solute molecules in the mobile phase, differences in the flow rate are not expected to influence the diffusion of solute molecules within the stationary phase (Ackers, 1964; Cazes, 1966). Moreover, the restricted diffusion mechanism (Ackers, 1964), operative on solute molecules within the stationary phase, needs to be distinguished from the diffusion process governing the transfer of solute molecules across the hypothetical membrane between the mobile and stationary phases.

From the preparative point of view, zone spreading during molecular sieve chromatography is as important as elution volume in obtaining good resolution between different components. Giddings & Mallick (1966) have characterized zone spreading in terms of a general plate-height equation and have suggested how separations on molecular sieve columns may be improved. In particular they recommend a reduction in column diameter (Stouffer, Oakes & Schlatter, 1966; Nyström & Sjövall, 1966) as an aid to more efficient separations but recognize that this may involve a compromise with a reduction in sample capacity.

Molecular weight estimation

The possibility of estimating the dimensions or the molecular weights of proteins, polysaccharides, and a variety of other polymers from their elution behaviour on molecular sieve columns has been realized by a number of authors (see P. Andrews, p.851 of this issue). Approximately linear relationships between the logarithms of the molecular weights M and the elution volumes V_e have reported. Using either Equations (13) or (15), it has been shown (Anderson & Stoddart, 1966*a*) that theoretical plots of K_d against log a/r give S-shaped curves, restricted central portions of which are essentially linear. These linear portions may be described by equations of the general form

$$K_d=-k_1 \log a/r-k_2 \quad \ldots (16)$$

where k_1 and k_2 are constants. If a is assumed to be proportional to M^x, where x is some constant fractional power, then Equation (16) may be expressed as

$$K_d=-b \log M+c \quad \ldots (17)$$

where b and c are constants. Thus a linear correlation will exist between K_d (and hence V_e) and log M for a homologous series of macromolecules over a restricted range of M, provided the proportionality between a and M^x is obeyed. This latter condition may not hold strictly for a series of globular proteins which differ in shape as well as in size. This may be the reason why Ackers (1964) obtained better agreement between theory and practice by plotting K_d against a/r, rather than against log M. Nevertheless, Squire (1964) has shown that differences in the shape of globular proteins may

have little effect on the elution volume, whilst Andrews (1965) has suggested that the close linear conformity of V_e with log M for a large number of globular proteins is in accord with the view that the majority are almost alike in both shape and density in solution. Squire (1964) has also drawn attention to the effect of the density of the macromolecule on V_e. This factor no doubt accounts for the elution behaviour of polysaccharides in relation to that of globular proteins. Polysaccharides have more expanded structures (Andrews, 1964; Anderson & Stoddart, 1966*b*), probably on account of their greater hydration, than globular proteins of approximately the same molecular weight. For this reason polysaccharides are eluted from molecular sieve columns in advance of proteins of comparable molecular weights. This means that a simple linear correlation between V_e and log M would not be expected to hold for glycoproteins, where the carbohydrate to protein content is variable (Ward & Arnott, 1965). Indeed Andrews (1965) has found this to be the case, and has shown that elution volumes correlate better with the diffusion constants of glycoproteins (Ackers, 1964). Clearly, linear relationships between V_e and log M or between $K_d^{1/3}$ and $M^{1/2}$ are only expected to hold for a homologous series of macromolecules (Meyerhoff, 1965, *a* and *b*). Provided this criterion is fulfilled, the above relationships are useful and easy to apply in practice. Nevertheless it should always be borne in mind that the technique only provides a means of *estimating* molecular weights and should not be looked upon as a method for *determining* molecular weights, as it may be impossible to ascertain whether a macromolecule is behaving typically or atypically during molecular sieve chromatography.

Conclusions

It is to be hoped that advances in the application of molecular sieve chromatography to studies on proteins, polysaccharides, nucleic acids, and synthetic polymers will proceed on a co-operative basis. Unfortunately some synthetic polymer chemists have appeared to convey the impression that they have been developing a new technique under the synonym of gel permeation chromatography. Mention of this term raises the vexed problem of nomenclature. Clearly there will continue to be dispute on this question for some time to come, because it is so strongly associated with attempts to achieve an understanding of the fundamental processes involved.

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21.

559. *Polysaccharides of the Characeae. Part I. Preliminary Examination of a Starch-type Polysaccharide from* Nitella translucens.

By D. M. W. Anderson and N. J. King.

The fresh-water green alga, *Nitella translucens*, gives an iodophilic glucan in 4% yield (dry-weight basis). This material, which is very difficult to extract and purify satisfactorily, has been examined by chemical, enzymic, and physical methods.

Extraction by chloral hydrate yields a severely degraded product which has an abnormal potentiometric iodine-titration curve. Extraction by perchloric acid gives a more satisfactory product having a limiting viscosity number of 40; the amylose content is 12%, and the amylopectin component, which has an average chain length of 19 glucose units, appears to be similar in structural properties to plant amylopectins.

The fresh-water green alga, *Nitella translucens* (class, Chlorophyceae; order, Charales; family, Characeae) is of current interest since its long, filamentous, internodal cells (up to 40 cm. long) facilitate certain biophysical and physiological experiments.[1,2] Little is known of the chemical constitution of the Characeae; at the request of the Biophysics Department of this University, the polysaccharide components of both *Nitella translucens* and *Chara australis* have been studied. This paper reports a preliminary study of the iodophilic glucan isolated from *Nitella translucens.*

Both salt-water and fresh-water green algae are included in the Chlorophyceae, and authorities [3] consider species of protozoa, such as *Polytoma, Polytomella,* and *Prototheca,* to be colourless members of the Chlorophyceae. Although much is known of the metabolism of algae,[3] few studies of the carbohydrate systems in such materials have been reported.[4,5] Investigations of algal cell components [5,6] must be distinguished from studies of the soluble extracellular materials exuded by some algae.[7]

According to Fogg,[3] the Chlorophyceae tend to be ancestral to land flora, having starch and fat as reserve materials; the starch is considered to be essentially similar to that from land plants (Fogg, p. 93). Although it is clear that the " paramylon granules " [8] reported in early studies may be starch-like (*e.g.*, for *Polytoma uvella* [9] and *Polytoma obtusum* [10]), recent work has shown that the " paramylon " from *Ochromonas malhamensis* [11] and from *Euglena* species [12] (which do not belong to the Chlorophyceae) resemble laminarin. Recent chemical investigations on *Polytomella coeca* [13] and *Dunaliella bioculata* [5] however support Fogg's statements, but it must not be presumed that the polysaccharide systems of all members of the Chlorophyceae will be similar to those of land plants. Indeed, *Cladophora rupestris* [6,14] is reported to contain laminarin rather than starch. This is of interest since Cronshaw *et al.*[15] found, by *X*-ray and electron-microscopical studies, that *Cladophora rupestris* and *Chaetomorpha melagonium*, from four green marine algae examined, had cell-wall structures resembling those of land plants: it was also observed that chemically differing carbohydrates can give the same *X*-ray diffractogram.[15] Recent *X*-ray evidence [16,17] has suggested the presence of differing " degrees of order " in tuber and algal starches, including an unidentified species of *Nitella*.

An early study [18] of an un-named species of *Nitella* showed a cellulose-type polyglucan to be present; tests for starch were negative. However, iodophilic starch-like granules, enclosed within a membrane, were later observed [1] in *Nitella* species.

Experimental and Results

Analytical Methods.—Conventional carbohydrate techniques were used throughout.

Collection of Material.—*Nitella translucens* (authenticated by Dr. A. J. Brook) was collected on May 19th, 1959, from Loch Cardney, Dunkeld, Perthshire. The filamentous algal cells were easily snapped, with resultant loss of the cell inclusions, and were therefore handled as carefully as possible during collection. The cells were individually freed by hand from traces

of debris and other pond-weeds. The raw material contained: N 2·3, 2·1%; ash 14·1, 13·7%; uronic anhydride 24·1, 24·4%. A methoxyl content of $<0{\cdot}2\%$ may arise from the chlorophyll present.

Preparation of Crude Starch.—The cells were homogenised in water chilled to 0° by brief treatments in an "Atomix" homogeniser. The turbid fluid was poured through a double layer of muslin. The combined residues were re-homogenised with chilled water; this extraction process was carried out 4 times in all.

The dark-green sediment was exhaustively defatted (Soxhlet), giving material which was stained blue-black with iodine solution (yield, 21%, dry-weight basis) (Found: N, 4·6; uronic anhydride, 13·0; ash, 24·4; SO_4'', 0·8%). Paper chromatography of a hydrolysate showed that galactose, glucose, arabinose + mannose, and xylose were present in the ratio 1·5 : 92 : 5·5 : 1; cuprimetric determinations [19] of the total reducing power after hydrolysis indicated 21% of free sugar (as glucose). Calculation therefore shows that the total amount of starch present was $\ngtr 4\%$ (dry weight basis).

Attempted Deproteinisation.—The action of (*a*) proteolytic enzymes, (*b*) trichloroacetic acid, and (*c*) a modified Sevag denaturation process, previously found effective in freeing cereal starches from protein,[20] failed to reduce the nitrogen content significantly. Since other protein precipitants had been ineffective on material of somewhat similar origin,[6] methods of extracting the glucan preferentially from the inorganic and proteinaceous material were applied.

(1) *Alkali-swelling and leaching with hot water.* These gave a cream-coloured powder (Found: N, 1·8%). An aqueous solution was stained blue with iodine ($\lambda_{max.}$ 585 mμ). Hydrolysis gave glucose with very small amounts of xylose and arabinose; cuprimetric titration showed that the glucan was 76% pure. On α-amylolysis,[21] conversion into maltose (P_M) = 60% in 6 hr., 76% in 24 hr., and addition of iodine–potassium iodide solution then gave no reaction. On β-amylolysis, P_M = 54 (24 hr.), 59 (48 hr.). 1·15 mol. of periodate were reduced [22] per anhydroglucose unit after 190 hr.

(2) *Extraction by chloral hydrate.* Exhaustive extraction [23] with 33% aqueous chloral hydrate at 70° under nitrogen gave a pale cream-coloured product (26%). A solution in hot water did not reduce Fehling's solution, had $[\alpha]_D^{16}$ +163° (*c* 1·0% in water), and gave a blue colour with iodine solution (Found: N, 0·8%). Hydrolysis and paper chromatography gave only glucose and xylose (98 : 2). Cuprimetric determinations,[19] with correction for the trace of xylose present, gave the purity of the glucan as 91, 90, 92, 92% (control determinations on purified oat starch [20] gave 96, 99, 97, 96%). On α-amylolysis, P_M = 70% (4 hr.), 82% (24 hr.), 83% (48 hr.). On β-amylolysis at pH 4·6, P_M = 56% (24 hr.), 62% (48 hr.). Viscosity measurements were made [24] in 0·1M-potassium chloride solution in a modified Ubbelohde viscometer. Extrapolation to zero concentration of the usual viscosity graph gives $[\eta] = 24{\cdot}4$.

The glucan (51·2 mg.) was oxidised at 0° with sodium metaperiodate [25] in a total volume of 50 ml. with the following results:

Time of oxidation (days)	1	3	6	9	11
Periodate (mol.) reduced per anhydroglucose unit (ref. 26)	0·91	0·95	0·99	1·01	1·03
0·00714N-Formic acid (ml.) released per 5 ml. (ref. 27)	0·25	0·29	0·30	0·30	0·31

Hence the average chain length = 15 anhydro-glucose units, if no amylose was present.

The "blue-value" [28] (B.V.) of the glucan and of ten starches (whose amylose content had earlier been found by the potentiometric method [29]) was determined. The values found are shown in Table 1.

TABLE 1.

Source of starch *	Amylose (%) *	Blue value	Source of starch *	Amylose (%) *	Blue value
Oat	26·0	0·365	Pearl manioc	15·7	0·241
Barley	22·0	0·370	Parsnip	11·1	0·159
Potato I	20·4	0·372	Oat amylopectin	3·2	0·071
Sweet potato	17·8	0·287	Waxy maize	1·4	0·044
Banana	16·8	0·294	*Nitella translucens*	?	0·161
Tapioca	16·7	0·270			

* Origin of samples and amylose contents as quoted in ref. 29.

The graph of B.V. against amylose content (see Fig. 1) indicated that the *Nitella* starch contained 10—11% of amylose. Although there is no simple general relation between blue value

and amylose content, an approximately linear relation apparently holds for amylose contents of <18%.

Potentiometric iodine titrations [29] of the glucan gave curve A in Fig. 2, from which it was not possible to obtain the amylose content by extrapolation. The glucan gave no reaction with " concanavalin A " (result by courtesy of Dr. D. J. Manners and Dr. A. Wright).

(3) *Exhaustive extraction with* 30% *aqueous perchloric acid*. Such extraction at room temperature [30] gave a product which was precipitated and purified as iodine complex. The final product was freeze-dried (yield, 5%); it had $\lambda_{max.}$ 585 mμ, $[\alpha]_D^{16}$ +170° (*c* 1% in water) (Found: N = 0·8%). After hydrolysis, glucose + xylose (98 : 2) were detected by paper chromatography; cuprimetric determination [19] showed that the glucan was 90% pure. On α-amylolysis, P_M = 53% (3 hr.), 77% (24 hr.); β-amylolysis at pH 4·6 gave P_M = 50% (24 hr.), 61% (48 hr.). From viscosity measurements, extrapolation of the graph gave $[\eta]$ = 40. Viscosity determinations on parsnip starch [31] and on a commercial sample of waxy maize starch gave $[\eta]$ = 44 and 40 respectively.

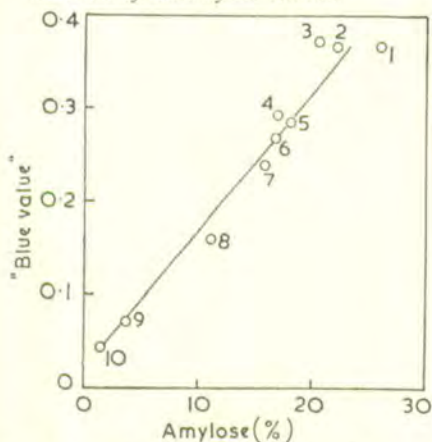

Fig. 1. *Plot of amylose content (potentiometric titration) against " blue value " for materials of low amylose content.*

1, oat starch; 2, barley starch; 3, potato starch; 4, banana starch; 5, sweet potato starch; 6, tapioca; 7, pearl manioc starch; 8, parsnip starch; 9, oat amylopectin; 10, waxy maize starch: origins as quoted in ref. 29.

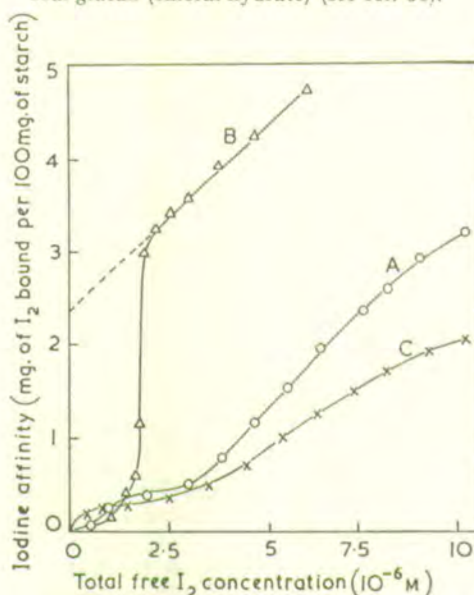

Fig. 2. *Potentiometric iodine-titration curves (standard conditions as in ref.* 29) *for*: A, *N. translucens* glucan (chloral hydrate), B, *N. translucens* glucan (perchloric acid), C, Protozoal glucan (chloral hydrate) (see ref. 36).

The glucan (101·2 mg.) was oxidised [27] at room temperature with potassium metaperiodate (final vol. 50 ml.) with the following results:

Time of oxidation (days)	3	6	9	11
Periodate (mol.) reduced per anhydroglucose unit (ref. 26)	0·60	0·76	0·95	0·97
0·00714N-Formic acid (ml.) released per 5 ml. (ref. 27)	0·28	0·38	0·42	0·43

Hence the average chain length is 21 units, and that of the amylopectin component (if the glucan contains 12% of amylose) is 19 glucose units. The product resulting from periodate oxidation for 11 days was isolated, dialysed, and hydrolysed. Paper chromatography revealed a small amount of xylose but no glucose.

Potentiometric iodine titration [29] of the glucan (20·02 mg.) gave curve B in Fig. 2. This curve is of similar shape to that given by plant starches and indicates the presence of 12% of amylose.

Discussion

The crude material extracted as a cold-water sediment was very difficult to purify. Initial attempts to deproteinise the starch consumed considerable amounts of material without success (cf. ref. 6). Extraction with cold alkali and hot water gave, in poor yield, a glucan of 76% purity, a result similar to that (69%) reported [5] for *D. bioculata*.

Chloral hydrate gave a relatively pure glucan (90%; cf. ref. 32) which was severely degraded (periodate oxidation and viscosity results) and gave an abnormal potentiometric iodine titration curve (see below).

Although the yield was poor, the glucan extracted by perchloric acid was, comparatively, much less degraded. Indeed, its limiting viscosity number and amylose content were similar to that of parsnip starch which is therefore included in Table 2, together with the present results and the values reported for commercial waxy maize starch, a salt-water alga,[5] and a green seaweed.[33] The properties of algal starches can now be compared [32,33,34] with those of glycogens and floridean, plant, and protozoal starches, though only a preliminary investigation of our glucan could be made with the material available.

TABLE 2.

Source of glucan	*N. translucens* * (fresh-water alga)		*D. bioculata* [5] (salt-water alga)	*C. filiformis* [33] (green seaweed)	Parsnip starch [31]	Waxy maize starch ¶
Method of extraction	perchloric acid	chloral hydrate	perchloric acid	see ref. 33	cold water	commercial sample
$[\alpha]_D$ (c 1% in H_2O)	+170°	+163°	+169°	+154°	+166°	+170°
$\lambda_{max.}$ of I_2 complex (mμ)	585	590	600	540	590	555
Blue value	0·16	0·16	—	—	0·16	0·04
Amylose (%) (I_2 titrn.)	12	?	12—14	0	11 [29]	1 [29]
β-Amylolysis limit	61	62	62	57	72 ‡	54 [34]
α-Amylolysis limit	77	82	85	90	85	88 †
IO_4^- reduction (mole per anhydroglucose unit)	0·97	1·03	—	0·95	1·03 ‖	1·05 [27]
Av. chain length ($\overline{C.L.}$)	21	15	18	21	23 [27]	20 [27]
Hence amylopectin $\overline{C.L.}$	19	13	15—16	21	20·4 [27]	20 [27]
Limiting viscosity no. [η]	40	24	—	15	44	40
Av. internal C.L.§	5—6	3—4	3—4	6—7	6—7	6—7 [34]
Av. external C.L.§	13—14	9—10	12	14—15	13—14	14—15 [34]

* Analytical values are based on the determined glucose content (cf. ref. 32). † Data from *J.*, 1956, 2831. ‡ Data from *Stärke*, 1960, **12**, 169. § As calculated in ref. 21. ‖ D. M. W. Anderson, Ph.D. Thesis, Edinburgh, 1956. ¶ Same sample as used in refs. 27 and 29. A different sample, having $[\alpha]_D$ 212°, [η] 150, is presumably referred to in refs. 33 and 34.

The differences between the products obtained by use of chloral hydrate and perchloric acid suggest that the amylose-type component in algal starches may be highly labile and very easily degraded. Although chloral hydrate was stated [13] to be the best extractant for similar materials, it was later found [5] to cause more extensive degradation (cf. ref. 32) than perchloric acid.

Abnormal iodine-titration curves, similar in shape to that given by the chloral hydrate product, have been noted previously [35] for proteinaceous, floridean, and protozoal starches, for degraded samples, and for certain amylopectins obtained by fractionation; degradation, contamination with protein or waxy materials (cf. ref. 29) and possibly also the presence of abnormal linkages can all distort the normal shape of the titration curve. A value for the amylose content, in good agreement with that found by iodine titration for the perchloric acid product, was, however, deduced from the "blue-value" of the chloral hydrate material. This suggests that the degradation, although severe, had not proceeded below the achroic limit. Since both glucans had very similar nitrogen contents, it is possible that in this instance the abnormal curve resulted primarily from the degradation caused during extraction. The iodine-titration curve (Fig. 2, curve C) given by a protozoal starch [36] is strikingly similar in shape; since the amylose content was stated—on the basis of a low B.V.—to be negligible,[36] re-examination of protozoal starches extracted [37]

with chloral hydrate may now be desirable. It is unusual for no amylose to be detectable in amylopectin-type glucans, *e.g.*, in the " waxy " starches or in fractionation products.

On this basis, the results quoted for an amylopectin-type glucan [33] may also bear re-investigation since the mode of extraction involved addition of a quaternary ammonium salt. Amylose is precipitated by such reagents,[38] and some fractionation may inadvertently have been effected.

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22.

POLYSACCHARIDES OF THE CHARACEAE*

II. THE CARBOHYDRATE CONTENT OF *NITELLA TRANSLUCENS*

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SUMMARY

The chemical constitution of cells of *Nitella translucens* has been studied. A graded extraction procedure gave 22 fractions, which have been analysed. The free sugars include galacturonic acid, galactose, glucose, arabinose, sucrose and fructose; a small amount (4%) of an iodophilic, starch-type, glucan is present. The pectic fractions have a very small methoxyl content. The uronic acid content of the cells accounts for the ion-exchange capacity previously reported for the cell walls. The outer cell wall appears to contain an almost pure cellulose as well as hemicellulosic materials.

INTRODUCTION

The fresh-water alga, *Nitella translucens* (Class, Chlorophyceae; order, Charales; family, Characeae) is of current interest since its long, filamentous internodal cells facilitate biophysical[1–4], physiological[5–7] and metabolic[8] experiments.

Little is known of the chemical constitution of the genus *Nitella*, other than an investigation[9] of the lipid and sterol content of *N. opaca* and a report[10] that the polysaccharide content of an unidentified species of *Nitella* was composed largely of a cellulose-type glucan. Indeed, only rarely have complete investigations of plant cells from any source been made[11,12]. The aim of the present work, undertaken at the request of the Biophysics Department of this University, was to investigate the carbohydrate components of *N. translucens* and to determine the nature of any acidic polysaccharide systems which could contribute to the ion-exchange properties[3] of the cell wall.

Because of the diversity of plant materials, no single extraction scheme is of general application for fractionating the polysaccharides in plant cells. It is therefore customary to devise a scheme applicable to the particular material under study[13–15]; the scheme may require refinements or revision once some indication of the nature of the materials actually present becomes known. The extraction scheme used here was based on the methods of SINCLAIR AND CRANDALL[16] and of WILLIAMS AND BEVENUE[17]; separation of (a) cell inclusions, (b) waxy, fatty and pigment materials, (c) cold water soluble materials, (d) hot water soluble materials, (e) pectic substances, (f) oxidised lignin, (g) hemicellulosic substances and (h) cellulose was achieved. From

* Part I: D. M. W. ANDERSON AND N. J. KING, *J. Chem. Soc.*, in the press.

the "cell inclusions" fraction a starch-rich sediment was obtained, and the studies made on this material have been reported[18].

MATERIALS AND METHODS

Details of the source, collection and authentication of the alga, and the results of analyses made on the dried starting material, have been given[18]. The standard analytical methods used for determining free sugars, nitrogen, uronic acid anhydride and methoxyl contents have been indicated[18]; conventional carbohydrate techniques were used for hydrolyses, paper chromatography and the subsequent cuprimetric estimation of free sugars.

Extraction and fractionation

The composite scheme used is shown diagramatically in Fig. 1. The algal cells (estimated dry wt., 23.6 g) were homogenised in portions by treatment for 2-3 min

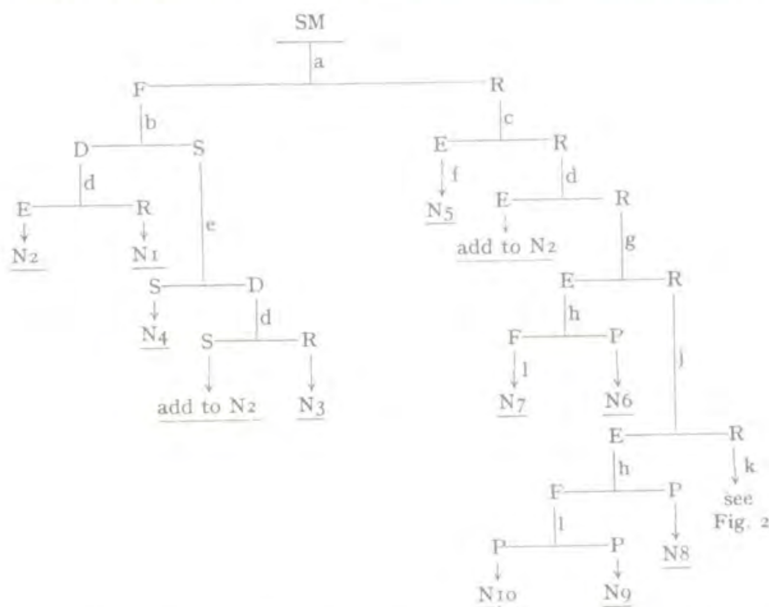

Fig. 1. Scheme for extraction of *N. translucens* fractions. SM, starting material; F, filtrate; R, residue; E, extract; P, precipitate; S, supernatant; D, deposit given on centrifugation. a, Homogenised then filtered; b, centrifuged; c, extracted with hot water; d, extracted with benzene-methanol; e, reduced in volume, then centrifuged; f, freeze-dried; g, extracted with cold oxalate; h, stood overnight; j, extracted with hot oxalate; k, divided into 2 parts, I and II; l, precipitated by addition of ethanol.

in an "Atomix" blender, each portion (approx. 3 g) being treated with 500 ml chilled water (0°). The turbid fluid passing through a double layer of muslin after homogenisation was added to a fresh portion of weed which was then homogenised. The combined residues were re-homogenised with a fresh 500-ml portion of chilled water. This homogenisation and extraction process was carried out 4 times in all. Finally, the combined residues were mechanically shaken with 2 portions (each 1 l) of cold water, each extraction lasting 2 h. The total extract was filtered through a double layer of

muslin, then centrifuged. As far as possible (cold room and refrigerated centrifuge) these extractions were made at 0°.

The dark-green sediment obtained from the centrifugation was extracted exhaustively (Soxhlet) with methanol, then methanol–benzene (1:1, v/v). After extraction it was ground to a coarse powder as it dried (Fraction N1). The combined methanol and methanol–benzene extracts were taken to near dryness under reduced pressure (Fraction N2). When the greenish-yellow centrifugate from the cold-water extractions was reduced in volume at 30° to 500 ml, a dark green precipitate appeared; this was removed by centrifuge, then treated as for N1, giving N3. The supernatant, now amber in colour, was freeze-dried (N4).

The residue from the cold-water extractions consisted of ruptured cell fragments which were pale green-brown in colour; most of the chloroplasts appeared to have been removed by the cold-water treatment. This residue was extracted (Soxhlet) for 6 h with 600 ml water. This hot-water extract (approx. 85°) was filtered free of small debris (which was returned to the main residue) and freeze-dried, giving fraction N5. The main residue was then extracted for 6 h with 90 % aqueous methanol then with methanol–benzene (1:1, v/v); these extracts were taken to near dryness and added to fraction N2.

A solution (1.5 l) of 0.25 % ammonium oxalate + 0.25 % oxalic acid was used to extract the pectic fractions[17] from the main residue by shaking overnight at room temperature. The extraction was repeated with 2 further portions (1 l each) of the oxalate solution, and the extracts pooled. After standing for 24 h at 0°, this extract gave a greenish-grey precipitate and a pale-yellow opalescent solution. The precipitate was collected at the centrifuge, washed with water, 70 % ethanol, absolute ethanol, and ether and then dried *in vacuo*, giving fraction N6. After the clear solution was reduced in volume, fractionation was attempted by stepwise additions of alcohol (0.05 N with respect to HCl). Only one fraction (N7) was obtained (with 0.5 volume ethanol) although up to 4 volumes ethanol were added. Additions of cetavlon and borate[19] to the alcoholic solution gave a very slight precipitate ($<$ 20 mg) which was not considered significant and was discarded. Fraction N7 was re-dissolved and re-precipitated twice with acidified ethanol, dialysed for 3 days against tap water and freeze-dried.

The residue from the cold-oxalate extraction was heated to 90° with 1.5 l of the oxalate–oxalic acid solution and was maintained at this temperature for 30 min with frequent stirring. The mixture was kept at room temperature for 2 h, and was then poured through muslin. This extraction was repeated with two further portions (1 l each) of oxalate solution. The residue was washed with hot water, and the extracts and washings pooled. On being kept overnight at 0°, a greenish-grey precipitate appeared; this was removed, washed, and dried (as for fraction N6) giving fraction N8. The clarified extract was reduced in volume to about 1 l: acidified ethanol was then added stepwise as in the isolation of N7. A bulky gelatinous precipitate, obtained with 0.5 volume ethanol, was removed by centrifuge and isolated by the procedure used for N7, giving fraction N9. A further bulky fraction, obtained on addition of 1–2 volumes ethanol was isolated as for N7, giving fraction N10. The mother liquor from these precipitations was reduced in volume, dialysed against tap water and treated with borate and cetavlon[19]. Only a slight precipitate resulted ($<$ 20 mg), which was not considered significant and was discarded.

The residue from the hot oxalate extractions was divided into 2 approximately equal parts, referred to below as part I and part II; Fig. 2 shows their subsequent treatments.

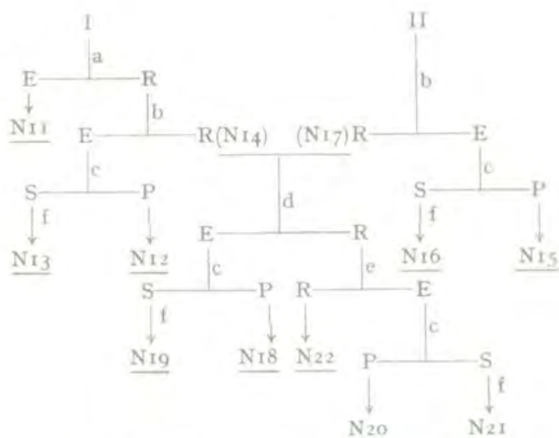

Fig. 2. Scheme for extraction of lignin, hemicellulose and cellulose fractions from *N. translucens*. I and II, see Fig. 1; E, extract; R, residue; S, supernatant; P, precipitate. a, Chlorite treatment; b, extracted with cold 5.5 % aqueous potassium hydroxide; c, acidified; d, extracted with hot 5.5 % aqueous potassium hydroxide; e, extracted with cold 24 % aqueous potassium hydroxide; f, freeze-dried.

Part I was stirred into cold distilled water (250 ml) containing glacial acetic acid (1 ml). The mixture was heated to 70° and four 1-g portions of sodium chlorite added[20] at hourly intervals with stirring. The mixture was then cooled and centrifuged. After washing the residue thoroughly with water, the mother liquor and washings were dialysed for 3 days against tap water, reduced in volume and freeze-dried, giving fraction N11.

The chlorite treated material was extracted with 250 ml 5.5 % aqueous potassium hydroxide by shaking for 18 h at room temperature. The extract was centrifuged off, and the extraction repeated with a fresh portion of alkali. The combined extracts were acidified with glacial acetic acid, and the gelatinous precipitate which formed was removed by centrifuge. This fraction was re-dissolved in alkali and re-precipitated twice with acid, washed with 80 % ethanol, absolute ethanol, and ether, powdered, and dried *in vacuo*, giving fraction N12. The mother liquor from this precipitation was dialysed for 3 days against tap water, reduced in volume, and freeze-dried, giving fraction N13. The residue from the alkaline extraction was washed with water, 80 % ethanol, absolute ethanol, and ether, and dried *in vacuo*, giving fraction N14.

Part II was treated similarly to part I, except that the chlorite oxidation was omitted. Fractions N15, N16 and N17 therefore correspond to N12, N13 and N14.

Fractions N14 and N17 still contained a fairly high percentage of uronic acid (see Table I), and they were therefore combined and extracted with 200 ml of 5.5 % aqueous potassium hydroxide at 100° for 2 h. After cooling, the extract was removed by centrifuge. The extraction was repeated with fresh alkali, the residue washed with hot water, and the extracts and washings combined. This extract was acidified with glacial acetic acid; fraction N18 was centrifuged off, purified and dried as for N12. The mother liquor was reduced in volume, dialysed for 4 days, then freeze-dried, giving fraction N19.

The residue was extracted twice at room temperature by shaking for 6 h with 24% aqueous potassium hydroxide. The residue was washed with warm water, and the extracts and washings combined. Fractions N20 and N21 were isolated in the same way as were N18 and N19 respectively. The final residue was washed with alcohol and ether, then dried *in vacuo*, giving fraction N22.

RESULTS

Table I shows the yields obtained for fractions N1 to N17, together with their ash, protein, and uronic acid anhydride contents. Table II shows the yields and uronic acid contents for fractions N18 to N22. Table III shows the carbohydrate content of the fractions and gives the molar ratios of the sugars present, as found by standard paper chromatography procedures. Rhamnose was also present in fractions N12, N13, N16, N17 and N22 in small amounts which were not estimated.

TABLE I

YIELDS AND ANALYSES OF FRACTIONS FROM *Nitella translucens*

Fraction	Yield (g)	Yield corrected for losses (%)	Ash (%)	Weight of ash in fraction (g)	Uronic acid anhydride (%)	Weight of UAA in fraction (g)	Protein (%)	Weight of protein in fraction* (g)
N1	4.30	21.2	24.4	5.17	13.0	3.76	28.5	6.04
N2	1.19	5.9	41.2	2.43	7.3	0.43	13.1	0.77
N3	1.22	6.0	20.1	1.21	7.6	0.46	51.3	3.08
N4	1.70	8.4	48.9	4.11	4.7	0.39	15.5	1.30
N5	0.52	2.5	26.4	0.66	10.6	0.25	18.4	0.46
N6	0.42	2.1			12.8	0.27	18.2	0.38
N7	0.53	2.6			34.0	0.88	4.6	0.12
N8	0.25	1.2			39.3	0.47	18.0	0.22
N9	1.75	8.6			62.7	5.39	1.0	0.09
N10	0.09	0.4			65.8	0.26	4.7	0.02
N11	0.27	1.3			13.2	0.17	35.0	0.45
N12	0.03	0.1			32.6	0.03	0.2	0.00
N13	0.36	1.8			19.0	0.34	7.9	0.14
N14	3.39	16.7			20.2	3.38	1.7	0.28
N15	0.05	0.2			18.5	0.04	19.1	0.04
N16	0.20	1.0			30.0	0.30	6.3	0.06
N17	4.06	20.0			22.3	4.46	1.6	0.32
Totals	20.35	100.0		13.55		21.28		13.77
Starting** material	23.6 g			13.6%		24.0%		14.6%
% recovery	85			100		89		94

* Based on 6.25 × %N (Kjeldahl).

** Analysis of starting material (see ref. 18) gave 13.6% ash, 24.0% uronic acid anhydride, 14.6% protein, < 0.5% methoxyl.

Fractions N9 and N10 contained galacturonic acid; fractions N12, N13 and N16 contained galacturonic and glucuronic acids (solvent system: pyridine–ethyl acetate-acetic acid–water, 5:5:1:3, v/v).

Fractions N17 and N22 gave an insoluble cellulosic residue even after prolonged

TABLE II

FURTHER EXTRACTION OF FRACTIONS N14 + N17 WITH HOT DILUTE AND COLD CONC. ALKALI

Fraction	Extraction procedure	Yield (g)	Corrected yield (%)	Uronic acid anhydride (%)	Weight of total UAA present (g)
N18	Hot dilute alkali extract; precipitate given on acidification	0.33	6.1	18.6	1.12
N19	As above, acid soluble fraction	0.67	12.4	39.3	4.86
N20	Cold conc. alkali extract; precipitate given on acidification	0.30	5.6	27.1	1.52
N21	As for N20; acid soluble fractions	0.64	11.8	34.4	4.07
N22	Final residue	3.47	64.1	7.0	4.49
	Totals	5.41	100.0		16.06
	Weight of N14 + N17 taken	6.50 g			21.3%
	Recovery	83%			75%

hydrolysis with sulphuric acid (2 N) at 102–105°. These fractions were therefore hydrolysed on a larger scale[21] (approx. 200 mg). The finely divided material was added in small amounts to 4 ml of chilled (0°) 72% sulphuric acid. After 8 days at room temperature, the mixture was diluted to 100 ml (*i.e.* 1 N) and heated at 100° for 8 h. After cooling, the hydrolysate was neutralised with barium carbonate, then filtered. The filtrate was concentrated to small volume at 30°, and investigated by paper chromatography for neutral and acidic sugars.

Fraction N17 contained glucose, xylose, rhamnose and arabinose in the ratio 10:3:1:1; glucuronic acid (possibly also galacturonic acid) was also present. Fraction N22 contained glucose and trace amounts of xylose, rhamnose, and mannose. On addition of alcohol to the hydrolysate, a precipitate formed which, after re-crystallisation, had a melting point and mixed melting point with authentic D-glucose = 143°; $[\alpha]_D = +52°$ (equilibrium; concn. 0.5% in water). The infrared spectrum was also identical to that of D-glucose. From fraction N22 was obtained[22] cellobiose octaacetate, having a melting point and mixed melting point, 224–226°, and having the same infrared spectrum as an authentic sample.

TABLE III

CARBOHYDRATE CONTENT AND MOLAR RATIOS OF SUGARS OBTAINED ON HYDROLYSIS OF *Nitella* FRACTIONS

Fraction	Carbohydrate present (%)	Ratios of sugars contributing to carbohydrate content				
		Uronic acid	Galactose	Glucose	Arabinose + mannose	Xylose
N1	49	20	1	74	4	1
N3	14	34	14	26	21	5
N4	38	11	19	34	31	5
N5	56	16	19	37	23	5
N6	56	19	3	53	22	4
N7	21	60	8	15	9	9
N9	23	74	6	7	6	7
N10	31	68	7	12	10	3
N12	62	31	9	22	7	31
N13	80	19	10	35	27	10
N16	75	29	8	37	21	5

Examination of fraction N4

This fraction could not be examined directly for free sugars because of the large amount of inorganic salts present, and a charcoal column[23] packed with a mixture of charcoal (ultrasorb-ZF grade)–Hyflo-supercel (1:1) was used to fractionate the sugars present. Elution with water, gradient elution with ethanol (0.2–15 %) and gradient elution with pyridine were used in turn: 100-ml fractions were collected, taken to near dryness, re-dissolved in water and examined by paper chromatography for acidic sugars, aldoses and ketoses. The presence of galacturonic acid, sucrose, fructose, glucose, arabinose, galactose, hexose oligosaccharides (having $R_G = 0.00$, 0.13–0.18 and 0.23–0.26) hexose and pentose disaccharides (having $R_G = 0.68$–0.86) was indicated.

DISCUSSION

The extraction scheme separated the material into the following broad fractions: (a) Starchy sediment from cold-water extraction (N1 + N3). (b) Fatty, waxy and pigment materials (N2). (c) Cold water soluble materials (N4). (d) Hot water soluble materials (N5). (e) Pectic substances (N6, N7, N8, N9 and N10). (f) Oxidised lignin (N11). (g) Hemicellulosic materials (N12, N13, N15, N16, N18, N19 and N20). (h) Cellulose (N22).

Based on the weight of starting material (23.6 g, estimated by drying two aliquots (see ref. 18)), the weight of the 22 fractions isolated accounted for 85 % of the original material. Yields of 85–90 % are usual in this type of study[24]; manipulative losses occur in the early stages of the extraction process when large volumes of material are submitted to mechanical processes, and loss of some volatile components may also occur. At later stages, degradation of polymeric material to small molecular weight species, which are either water-soluble or able to pass through dialysis membranes, can occur.

Fractions N1 and N3 contained large amounts of protein and inorganic salts, and had a significant uronic acid content. These fractions also contained about 20 % of a starch-type glucan, and studies on this have been reported[18]. The cold-water extracts (N4) contained 49 % of inorganic material, 15 % protein and 36 % of water-soluble sugars and oligosaccharides.

The hot-water extracts (N5) contained large amounts of ash and protein, about 50 % carbohydrate material being present. On hydrolysis the major sugar component was glucose; this was shown to come mainly from an α,1-4′-linked glucan by detecting maltose as the major sugar present in a 24-h digest with salivary α-amylase, and also by the bluish colour given by solutions of this fraction on addition of iodine solution. It is well-known[25] that complete extraction of starch from plant tissues by cold water is difficult to achieve. Although galacturonic acid was present, the amount involved was relatively small and the methoxyl content (shown by the infrared method to be real[26]) was also very small (< 0.5 %). Since water-soluble pectic materials usually have relatively high methoxyl contents[27], it is clear that only insignificant amounts of water-soluble pectic materials are present in the *Nitella* cells.

The results indicate that fractions N6, N7, N9 and N10 are pectic materials. None of these fractions had any true methoxyl content[26], but all had $[\alpha]_D < +200°$. A detailed study of a pectic acid isolated from these fractions has been made and

will be reported as a subsequent part of this series. Since it is unlikely that the extraction conditions used would completely de-esterify pectins, it must be concluded that these pectic fractions were not esterified; this appears to be the first unmethylated pectic acid to be reported. The small methoxyl content (approx. 0.5 %) of the cells as collected probably arose from the chlorophyll present, which has a methyl ester group. The pectic fractions therefore probably exist in the plant as calcium and magnesium salts of pectic acid; on the basis that all the uronic acid groups present can participate in salt formation, calculation[3] has shown that the uronic acid content is sufficient to account for the ion-exchange capacity of the *Nitella* cell walls. It is significant that the *Nitellae* are not influenced by plant hormones of the auxin type[28], which are believed to act upon the pectic substances of plants by affecting the mechanism causing esterification and de-esterification of the galacturonic acid groups present[29].

The hemicellulose fractions (N12-N20) were not investigated further than as shown in the Tables of results: from the relative amounts of the sugars present on hydrolysis, they appear to be typical plant hemicelluloses. Since glucuronic acid occurs commonly in such materials, it is probable that the galacturonic acid detected arose from residual traces of pectic materials which were not extracted by the oxalate treatments.

The cell wall appeared to be a cellulosic material as shown by its conversion to cellobiose octaacetate etc. The presence in hydrolysates of small amounts of other sugars in addition to glucose is commonly found with cellulosic materials.

The present work shows that *Nitella* resembles land plants in containing fatty material, starch and cellulose; the starch and lignin contents are very low, however, and the pectic content (which is non-methylated) is high, so that some differences from land plants are apparent.

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23.

POLYSACCHARIDES OF THE CHARACEAE*

III. THE CARBOHYDRATE CONTENT OF *CHARA AUSTRALIS*

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SUMMARY

The chemical constitution of cells of the fresh-water green alga *Chara australis* has been studied; analyses of 12 fractions, obtained by a graded extraction procedure, are reported. The uronic acid content, largely present as a non-methylated pectic acid, accounts for the ion-exchange capacity of the cell wall. The results show that *Ch. australis* and *Nitella translucens* are very similar in chemical constitution; the chemical composition of their cell walls is compared with that of some typical land plants.

INTRODUCTION

In the preceding paper we reported[1] the results of an investigation of the polysaccharide systems in *Nitella translucens*. We now give the results of a similar investigation made on cells of the fresh-water alga *Chara australis* R.Br.var. *nobilis* (Class, Chlorophyceae; order, Charales; family, Characeae), which has been used recently in studies[2-4] of the ionic relations in plant cell walls.

In very early work, ZACHARIAS[5] concluded that *Ch. foetida* contained cellulose; a proximate analysis of dried cells of *Ch. lispida* has been published[6]. More recently, the cellulose and hemicellulose fractions from an unidentified species of *Chara* were found[7] to resemble the corresponding polysaccharide fractions obtained from land plants. The object of the present work was to investigate the carbohydrate components of *Ch. australis*, to determine the nature of any acidic polysaccharide systems which

* Part II: see ref. 1.

could contribute to the ion-exchange properties of the cell wall, and to compare the results with those already obtained[1] for the related alga, *Nitella translucens*.

The comparatively small quantity of dried cells of *Ch. australis* available necessitated some simplification of the detailed extraction scheme used[1] for the study of *N. translucens*. Fortunately, the experience and knowledge gained in investigating the *Nitella* allowed the number of fractions to be reduced from 22 to 12 for *Ch. australis* without invalidating any of the possible bases for comparison with the results obtained for *N. translucens*.

MATERIALS AND METHODS

Cells of *Ch. australis*, authenticated and collected in Australia, were available by courtesy of Dr. J. DAINTY. After collection, the cells had been freed from foreign matter by hand, rinsed, washed with ethanol, and dried in an oven at 60° before being sent to this laboratory in a sealed cellophane package.

The analytical methods and all procedures used were as described[1] previously. Analysis of the starting material as received gave the following results: 10.1 % moisture, 14.9 % ash, 2.78 % N, hence 17.4 % protein, 16.4 % uronic acid anhydride, $<$ 0.5 % methoxyl. There was no sulphate present in the ash.

Extraction and fractionation

The algal cells (dry weight estimated at 29.3 g) were submitted to the extraction scheme shown in Fig. 1. Homogenisation, exhaustive cold-water extraction, filtration

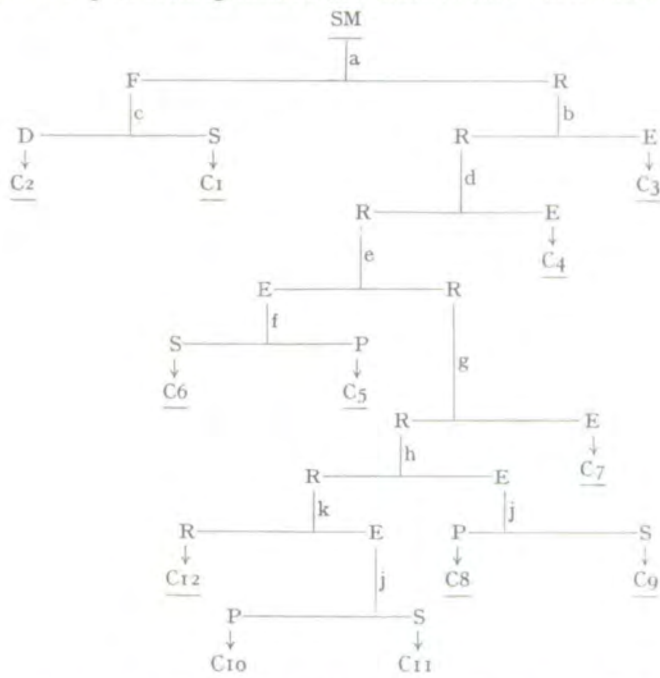

Fig. 1. Scheme for extraction of *C. australis* fractions. SM, starting material; F, filtrate; R, residue; D, deposit on centrifugation; S, supernatant; E, extract; P, precipitate. a, Homogenised and filtered; b, extracted with hot water; c, centrifuged; d, extracted with benzene–methanol; e, extracted with hot oxalate; f, addition of 0.5 volume ethanol; g, chlorite treated; h, extracted with cold 6% aqueous KOH; j, acidified; k, extracted with cold 24% aqueous KOH.

through layers of muslin, centrifugation, and reduction in volume at 30° under reduced pressure followed by further centrifugation gave two fractions: C1 (clear supernatant), C2 (combined residues from the centrifugation). Both fractions were freeze-dried. Exhaustive hot-water extraction gave C3; the residual cellular material was extracted with benzene–methanol giving C4 on removal of solvent. Hot-oxalate extractions gave C5 when acidified ethanol (0.5 volume) was added: addition of smaller or larger volumes of ethanol gave no fractionation effect. Fraction C6 was obtained when the supernatant from the oxalate extractions was dialysed and freeze-dried. The supernatant from chlorite treatment gave C7 after dialysis and freeze-drying. Acidification of the cold 6 % aqueous potassium hydroxide extracts gave C8; the supernatant from this precipitation gave C9 after dialysis and freeze-drying. Acidification of the cold 24 % aqueous potassium hydroxide extracts gave C10 and the supernatant from this fraction gave C11 after dialysis and freeze-drying. The final residue gave fraction C12.

RESULTS

Table I shows the yields obtained for fractions C1 to C12, together with their ash, protein and uronic acid anhydride contents. Table II shows the sugars detected by paper chromatography when the fractions were hydrolysed [2 *N* HCl, sealed tube, 105° (oven) for 6 h].

DISCUSSION

The extraction scheme gave fractionation into (a) cell inclusions, including cold water soluble material but excluding fatty materials (fractions C1, C2); (b) hot water soluble material (C3); fatty substances (C4); pectic substances (C5, C6); oxidised lignin (C7); hemicellulosic materials (C8–C11); cellulose (C12). As shown in Table I, the recovery of material as isolated fractions was 75 %, a satisfactory result considering the small amount of material available.

Examination of fraction C2 under the microscope showed that granules staining blue with iodine were present. Comparison with the corresponding fraction isolated from *N. translucens*[8] indicated that the amounts, and granular size, of the starch present were very similar. The amount of material available was insufficient to allow the isolation of any starch. As found previously[1], the hot-water extract (C3) also contained some starch.

Of the pectic fractions, C5 had a very high uronic acid content: neither C5 nor C6 had any methoxyl content. It appears, therefore, that *Chara* resembles *Nitella* in containing a non-esterified pectic acid which accounts for about 40 % of the total uronic acid content. Since the methoxyl content of the whole cells was almost negligible, the results shown in Table I indicate that non-methylated uronic acid groups occur in all the carbohydrate fractions of the cells, including the water-soluble polysaccharides and those systems which are closely associated with, and difficultly separable from, the cellulose of the cell wall. Assuming that these un-methylated uronic acid groups act as the indiffusible anions of the *Chara* cell wall, calculation has shown[3] that the uronic acid content of the cells is sufficient to account for their high ion-exchange capacity.

TABLE I

YIELDS AND ANALYSES OF FRACTIONS FROM *Chara australis*

Fraction	Yield (g)	Yield corrected for losses (%)	Ash (%)	Weight of ash in fraction (g)	Uronic acid anhydride (%)	Weight of UAA in fraction (g)	Protein* (%)	Weight of protein in fraction (g)
C1	4.17	18.8	44.4	8.34	10.4	1.95	15.7	2.94
C2	2.34	10.5	24.0	2.52	14.1	1.48	22.8	2.38
C3	0.98	4.4	27.2	1.19	12.5	0.55	14.0	0.63
C4	1.02	4.6	39.8	1.83	11.8	0.54	8.9	0.44
C5	2.44	11.0			52.0	5.72	11.6	1.25
C6	1.84	8.3			16.2	1.35	10.7	0.94
C7	1.44	6.5			10.4	0.68	45.4	2.94
C8	0.32	1.4			10.5	0.15	6.8	0.13
C9	0.85	3.8			21.2	0.81	6.6	0.25
C10	0.41	1.8			11.8	0.21	5.6	0.13
C11	0.41	1.8			42.8	0.77	9.5	0.19
C12	6.00	26.9			11.5	3.08	9.4	2.50
Totals	22.22 g	100%		13.9%		17.2%		14.7%
Starting material	29.3 g			14.9%		16.4%		17.4%
Recoveries	75%			93%		105%		85%

* Based on 6.25 × %N (Kjeldahl).

TABLE II

SUGARS DETECTED AFTER HYDROLYSIS OF *Chara* FRACTIONS

In addition to the sugars listed, all fractions contained a trace of rhamnose. Key: tr, < 5%; +, 5–10%; ++, 10–20%; +++, 20–30%; ++++, 30–40%; +++++, 40–50%; ++++++, 50–60%.

Fraction	Uronic acid	Galactose	Glucose	Arabinose	Mannose	Xylose
C2	++	tr	++++++	+	?	tr
C3	++	+	++	+++	+	tr
C5	++++++	+	+	+	?	+
C6	++	+++	+++	+	tr	+
C7	+	++++	+++	+	?	tr
C8	+	+++	++++	tr	?	+++
C9	++	+	+++	+	tr	+++
C10	+	++	++++	++	?	+
C11	++++	tr	++	+	+	++

TABLE III

THE CHEMICAL CONSTITUTION OF *Ch. australis* AND *N. translucens*

	Ch. australis				*N. translucens*			
	Fractions	%	Total uronic acid anhydride content (%)	Total ash content (%)	Fractions	%	Total uronic acid anhydride content (%)	Total ash content (%)
Cell contents	C1 + C2	29.3	20	73	N1 + N3 + N4	35.6	22	77
Fatty materials	C4	4.6	3	13	N2	5.9	2	18
Hot-water solubles	C3	4.4	3	9	N5	2.5	1	5
Pectic fractions	C5 + C6	19.3	40		N6 — N10	14.9	34	
Oxidised lignin	C7	6.5	4		N11	2.6*	1	
Hemicelluloses	C8 — C11	8.8	12		N12 — N20	15.0**	32	
Cellulose	C12	26.9	18		N22	23.4	8	

* 1.3% added to the value for N11 to allow for the material not treated with chlorite.
** 1.3% subtracted from totals of these fractions.

The ratio of sugars found on hydrolysis and the iodine-staining properties of a hemicellulose fraction from a species of *Chara* studied by AMIN[7] suggest that his preparation was heavily contaminated with starch. If it is assumed that 90 % of the glucose reported arose from this starch, then the uronic acid content of the true hemi cellulose increases from 2 % to 12 %, a value similar to that found in the present work for the materials extracted by dilute alkali.

Fraction C12 contained fibrous fragments, an infrared spectrum of which was very similar to that of the corresponding fraction isolated from *N. translucens* and to that of a thin piece of transparent paper.

Table III compares the chemical constitution of *Ch. australis* and *N. translucens*. Because of the pretreatment given to the *Chara* cells (washed with ethanol then dried at 60°), the fat and ash contents may have been reduced. Some denaturation of protein may also have been caused, since the protein was not almost completely extracted by cold and hot water (as in the case of *Nitella*) but appeared in appreciable amounts in all the fractions isolated. However, not only do the two algae have remarkbaly similar chemical constitutions but also very similar distributions of their total uronic acid and ash contents.

TABLE IV

THE CHEMICAL COMPOSITION OF CELL WALLS (%)

	Fresh-water algae		*Land plants**			
	*Nitella*** *translucens*	*Chara**** *australis*	*Oat coleoptiles*	*Oat straw*	*Wheat straw*	*Corn cob*
Protein	4	11	12	2	1	3
Pectic substances	26	28	8	1	1	0.5
Lignin	4	9	0	19	19	17
Hemicellulose	26	13	38	32	35	42
Cellulose	40	39	42	44	43	37
TOTALS	100	100	100	98	99	99.5

* Results quoted in refs. 9 and 10.
** Data from ref. 1.
*** Data from this paper.

Table IV shows results (calculated from the analytical data obtained for the relevant fractions) for the chemical constitution of the cell walls only (as distinct from the overall composition of the cell walls + cell contents). Once again *Chara* and *Nitella* are distinctly similar. When compared with results for the cell-walls of a number of land plants (as quoted by BONNER[9] and NORTHCOTE[10]) it is clear that the major differences lie in the percentages of lignin and pectic substances present. In land plants, the highest pectic contents are usually found in the roots, but even the values reported for these (5–13 %, depending on the plant[11]) do not approach the values found in *Chara* and *Nitella*.

CONCLUSIONS

It is almost certain that these Charaphytes, like other algae, obtain their nutrients through the cell walls, their basal rhizoids acting merely as organs of attachment.

Thus it seems reasonable to conclude that the pectic materials found in the walls perform ion-exchange functions similar to those found in the roots of higher terrestrial plants.

The cylindrical internodal cell walls of *Chara* and *Nitella* are from this study envisaged as consisting primarily of a semi-permeable cellulosic sheath, embedded in which are layers of acidic polysaccharide systems. These act as ion-exchange membranes by virtue of their uronic acid groups which are non-methylated and therefore free to participate in salt formation. The cell vacuoles contain proteinaceous and fatty materials, free sugars, oligosaccharides and considerable accumulations of inorganic salts. In addition, iodophilic starch-like granules have been found associated with the chloroplasts. Indeed, from a chemical standpoint these Charaphytes show distinct affinities with higher terrestrial plants. However, the relative proportions and fine structure of some of the components (*e.g.* starch, non-methylated pectic acid) show interesting differences.

In view of the debatable systematic position of the Charaphytae in relation to the Chlorophytae on the one hand, and to the higher terrestrial plants on the other, the similarity of their chemical systems with those of the latter is of considerable interest, especially when PRESTON *et al.*[12], in cell-wall studies of marine algae, have shown that of four members of the Chlorophyceae examined, only two, *Cladophora rupestris* and *Chaetomorpha melagonium* showed any resemblance in chemical composition to the higher terrestrial plants. The similarities shown were, moreover, only slight.

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1052. *Polysaccharides of the Characeae. Part IV.*[1] *A Non-esterified Pectic Acid from* Nitella translucens.

By D. M. W. ANDERSON and N. J. KING.

The pectic complex extractable from the fresh-water green alga *Nitella translucens* contains a non-esterified pectic acid. This is best purified by precipitation as the copper complex, followed by incubation with α-amylase to remove contaminating glucans. The pectic acid had $[\alpha]_D$ +245° and contained 74% of uronic anhydride; hydrolysis gave galacturonic acid, with galactose, arabinose, xylose, and rhamnose in the ratio 4 : 6 : 3 : 1. Partial hydrolysis indicated that a high proportion of the arabinose and xylose residues were labile, the galactose being resistant to hydrolysis. Passage through diethylaminoethylcellulose gave one main fraction only. Methylation studies established the presence of chains of 1,4′-linked-α-D-galacturonic acid. This pectic acid is of interest in studies of the ion-exchange capacity of the algal cell-wall.

RECENT studies [1] revealed that the fresh-water green algæ *Nitella translucens* and *Chara australis* have clear similarities to higher terrestrial plants on the basis of their carbohydrate content. This is of interest since the botanical relationship of the *Charaphytae* to (*a*) *Chlorophytae* and (*b*) higher terrestrial plants is debated.[1]

The cell-walls of *Chara* and *Nitella* contain significant quantities of pectic materials; [1] these appear to perform ion-exchange functions similar to those of pectins in the roots of higher terrestrial plants.[1] Pectin has so far not been isolated from marine sources.[2] The precise function of pectic materials in plant cells has long been in question.[2] When it was found that the pectic acid in these Characeae was non-esterified, it became important to establish the extent of its differences from the known plant pectic acids. Algal polysaccharides have previously provided many novel structural features: [3,4] indeed, *N. translucens* contains also an interesting labile starch-type glucan.[5]

The origin and collection of the alga, analyses of the dried cells (MeO $\not>$ 0·1%), and the extraction procedures have been described.[1] Although the material extracted by water at 90° (fraction N5, ref. 1) contained 10·6% of galacturonic acid and had no methoxyl content, it was shown (Table 1, ref. 1) that this fraction accounted for only 1% of the total uronic anhydride content of the cells. Negligible amounts of pectic substances were therefore extracted in the hot-water and other preliminary treatments. In general, the lower the methoxyl content, the more difficult is the hot-water extraction of pectic substances from plant tissues.[6]

Extraction of the algal residues with hot aqueous ammonium oxalate–oxalic acid solution [7] gave ammonium pectate. Paper chromatography of a hydrolysate showed that galacturonic acid, galactose, glucose, arabinose, xylose, and rhamnose were present (Found: $[\alpha]_D$ +205°; uronic anhydride, 51%; Ac, SO_4, and OMe, 0). Despite attempted purification by precipitation with organic solvents, cetylpyridinium bromide, ammonium sulphate, as the calcium salt, and as the copper complex, no fractionation was achieved and none of the neutral sugars originally present was eliminated. Regeneration from the copper complex gave the best product, having $[\alpha]_D$ +240°, uronic anhydride 72% (both as free pectic acid). Subsequent incubation with salivary α-amylase removed the glucose-containing contaminant; the incomplete extraction of starch by cold and hot water [8] and the contamination of pectin by starch are well known.[9,10] The glucose-free pectic acid, which contained 74% of uronic anhydride, together with galactose, arabinose, xylose, and rhamnose in the ratio 4 : 6 : 3 : 1, was used in the experiments reported in this paper. Decarboxylation studies made later indicated [11] that this material had probably under-

gone about 8% of decarboxylation during its extraction (these studies assumed that all the carbon dioxide evolved came from 6-carboxyl groups).

Fractionation on a column of diethylaminoethylcellulose [12] gave one major component (see Table) which accounted for 74% of the material recovered from the column and for

Fractionation of pectic acid on diethylaminoethylcellulose.

Fraction	Total vol. eluted (ml.)	Wt. of fraction (mg.)	Gal. Acid	Sugars identified Gal	Ara	X	Rha	$R_{rhamnose}$ of unidentified sugars (solvent C)
1	40	2	++	++	++		+	
2	220	7	+	++	++		+	
3	420	7	+	++	++		+	
4	670	4	+	++	++		+	
5	690	3	+	++	++			1·21(+), 1·89(+)
6	830	5	+	++	++		+	1·24(+)
7	960	6	+	+	+			
8	1130	7	+	+	+		+	1·74(+)
9	1150	3	+	+++	+			
Elution by sodium hydroxide begun.								
10	1350	10	+	+++	+	+	+	1·22(+)
11	1410	11	+	++++	+	++		oligos. ++
12	1560	3	++	++	+	+		oligos. ++
13	1610	9	++	++	+			oligos. +
14	1840	295	71%	+	+			oligos. +++
15	1930	9	+	+++	+			oligos. +
16	2150	8	+	+++				
17	2250	9	—	+++				
18	2400	3	—	+++	+			
		401						

which the optical rotation, uronic anhydride, and proportions of neutral sugars were almost identical with those of the unfractionated material. Seventeen other fractions, none of which accounted for more than 2·5% of the material recovered, were also investigated. The significance of these must await further investigation with larger quantities of material; however, hydrolysis of some of the fractions eluted before the main peak gave two sugars of high chromatographic mobility (cf. refs. 13 and 14), and one of the fractions eluted after the main peak gave only galactose on hydrolysis, indicating that minor quantities of a galactan were present in the pectic complex (cf. ref. 2).

Hydrolysis at pH 5·0 with fungal pectinase gave the crystalline sodium–calcium double salt [15] of galacturonic acid in good yield. Partial hydrolysis (0·1N-hydrochloric acid, 2 hr., 100°) gave an insoluble, degraded polysaccharide having 83% of uronic anhydride; the sugars in the hydrolysate were galactose, arabinose, xylose, and rhamnose (3 : 10 : 6 : 1), with galacturonic acid also present. The pectic acid therefore contained labile arabinose and xylose. Further hydrolysis (2N-sulphuric acid, 6 hr., 100°) of the degraded polysaccharide gave galacturonic acid, and galactose, arabinose, xylose, and rhamnose (3 : 1 : 1 : 1), but hydrolysis was incomplete. Hydrolysis with 90% formic acid was also incomplete.

Periodate oxidation of the ammonium pectate required 1·05 moles of periodate per anhydrogalacturonic acid unit. The oxidised polysaccharide was reduced with sodium borohydride; chromatography of the hydrolysed product gave arabinose, xylose, and small amounts of galacturonic acid in addition to the expected threonic acid. The presence of arabinose and absence of galactose is in agreement with the structures proposed [2] for the araban and galactan components of pectic complexes. The unoxidised galacturonic acid may equally have arisen from incomplete periodate oxidation or from some galacturonic acid residues linked other than in the α-1,4′-manner commonly found in pectic acids.

Methylation of the pectic acid was difficult and was accompanied by extensive degradation: similar difficulties have been encountered previously.[2,16,17] Reasonable yields and methoxyl contents greater than 37—39% appear to be difficult to achieve with

pectic materials (cf. refs. 13 and 16). After successive repeated treatments with thallium hydroxide–methyl iodide, dimethyl sulphate–sodium hydroxide, silver oxide–methyl iodide, a product having 37·4% of methoxyl (88% of the theoretical) was eventually obtained. After methanolysis, the product was reduced with lithium aluminium hydride, and the reduction product was hydrolysed. Chromatographic separation of the resultant mixture of sugars gave 7 fractions; from the two major fractions, crystals of the aniline derivative of 2,3-di-*O*-methyl-D-galactose were prepared.

Supplies of the authenticated alga are difficult to procure; only a limited amount of information could be obtained with the amount of material available, and it is hoped to obtain a larger supply in due course. It is apparent, however, that the pectic complex in *Nitella translucens* is similar in many respects to that from land plants, containing a pectic acid which has chains of 1,4'-linked α-D-galacturonic acid residues; these are not, however, present as methyl esters. This is clearly not the result of inadvertent demethylation or de-esterification during extraction, since the methoxyl content of the dried alga was ≯0·1%. The significance of this interesting pectic acid has been discussed [1] in relation to the lack of auxin-type hormonal activity in the *Nitellae* and to the possible ion-exchange function of these materials in the algal cell. The material may be of interest in investigations of the validity of current theories which relate the stability of pectins to their ester content.[18,19]

Experimental

Paper partition chromatography was carried out on Whatman No. 1 paper with the following solvent systems (v/v): (A) butan-1-ol–benzene–pyridine–water (5 : 1 : 3 : 3, upper layer); (B) pyridine–ethyl acetate–acetic acid–water (5 : 3 : 1 : 3); (C) butan-1-ol–ethanol–water (4 : 1 : 5, upper layer); (D) butan-2-one, half saturated with water containing 1% of ammonia. Optical rotations were measured at 18° ± 2°. Uronic anhydride was found by Anderson's decarboxylation apparatus; [20] methoxyl by the vapour-phase infrared method; [21] nitrogen by Kjeldahl semimicro-determinations; ash by heating to constant weight at 550—600° (muffle).

Isolation and Examination of the Water-soluble Polysaccharides.—The origin and pre-treatment of the algal cells have been described, and the extraction scheme used has been detailed.[1] The yields and analytical results obtained for the cold- and hot-water extracts [1] show that insignificant amounts, if any, of the pectic content were extracted in these pre-treatments.

Isolation and Examination of the Pectic Substances.—The pectic substances were extracted from the cold- and hot-water residues by aqueous oxalate solution (0·25% with respect to both oxalic acid and ammonium oxalate [7]) at 80—90° for 1 hr. (three treatments) [yield, 31 g. of ammonium pectate from a batch of algal cells (dry wt. estimated at 320 g.)]. This crude product had $[\alpha]_D$ +205° (*c* 0·2 in H_2O), uronic anhydride 51%. After hydrolysis, chromatography showed that galacturonic acid was present, and also galactose, glucose, arabinose, xylose, and rhamnose (3 : 4·5 : 3 : 3 : 1).

Purification and Attempted Fractionation of Ammonium Pectate.—(1) The crude product (5 g.) was dissolved in water (400 ml.); ethanol, acidified with acetic acid (5 ml. per l.), was slowly added with stirring. Two fractions, which were not sharp, were collected: (A) at 40% ethanol, 3·7 g., $[\alpha]_D$ +220° (uronic anhydride, 60%); (B) at 60% ethanol, 0·8 g., $[\alpha]_D$ +220° (uronic anhydride, 65%). Reprecipitation failed to increase the uronic acid content significantly. Hydrolysis gave the same sugars as had been found in the crude product; the two fractions were therefore combined in view of their similarity.

(2) These combined fractions (3·8 g.) were dissolved in water, and 10% calcium chloride solution was added until precipitation of calcium pectate was complete. The precipitate was heated in water at 80° for 30 min. with a slight excess of ammonium oxalate; calcium oxalate was removed by filtration and the solution dialysed against distilled water. Pectic acid was precipitated by addition of acidified ethanol, isolated, then re-dissolved and re-precipitated a further three times. The product (3·4 g.; uronic anhydride, 64%) gave a cloudy solution, even after filtration through Celite on a No. 2 glass sinter. This was probably due to colloidal calcium oxalate; clarification was achieved by making the solution slightly alkaline, then adding sufficient ethylenediaminetetra-acetic acid to make the solution 0·05N in it. The ammonium pectate was precipitated after 30 min. by addition of ethanol, isolated, redissolved,

and reprecipitated. This material had $[\alpha]_D$ +240° (*c* 0·3% in H_2O) (uronic anhydride, 69%); hydrolysis gave galacturonic acid, with galactose, glucose, arabinose, xylose, and rhamnose (4 : 1 : 5 : 4 : 1).

(3) Pectic acid (5 g.), a product of procedures 1 and 2 above, was dissolved in water and Fehling's solution added until precipitation of the copper complex was complete. Attempted decomposition of this complex, by adding it to 50% aqueous ethanol which was 4N with respect to acetic acid, removed only a little of the copper. The complex was therefore re-dispersed in water which was made just alkaline by sodium hydroxide; disodium ethylenediaminetetra-acetate was added and the mixture stirred at room temperature until the copper complex dissolved. The sodium pectate was precipitated with ethanol, redissolved, and reprecipitated twice. The pectic acid (4·6 g.) finally isolated had $[\alpha]_D$ +240° (*c* 0·3% in H_2O) (uronic anhydride, 72; N, 0·16%).

(4) Ammonium sulphate (100 g.) was stirred into an aqueous solution (100 ml.) of a portion (2 g.) of the product from procedure (3) above. The solution was kept at 0° for 3 days. The precipitate was isolated, dissolved in water, and dialysed till free from salts. The pectic acid 1·6 g.), isolated in the usual way, had $[\alpha]_D$ +238° (*c* 0·2% in H_2O) (uronic anhydride, 68%).

(5) Cetylpyridinium bromide (1% aqueous solution) was added to a solution of ammonium pectate (a product of treatments 1 + 2) until precipitation was complete. This complex was very stable. Attempted regeneration of the pectic material by stirring it with 4N-acetic acid (400 ml.) for 1 hr. (three treatments) or 15% aqueous sodium chloride failed to dissolve the complex. Heating it at 60° with 0·5% aqueous ammonium oxalate was finally successful. The pectic acid finally obtained had $[\alpha]_D$ +205° (*c* 0·4% in H_2O) (uronic anhydride, 54%).

Removal of Glucose.—Each of the purified products described above gave a positive reaction with iodine solution and gave, on hydrolysis, galacturonic acid with galactose, glucose, arabinose, xylose, and rhamnose. Only the amounts of glucose present were variable. Digestion with salivary α-amylase, followed by dialysis, removed all the glucose-containing contaminant,[6,10] leaving the amounts of other sugars unchanged. The polysaccharide having the highest uronic anhydride content was obtained by treatment with α-amylase of the product from procedure (3) above. This pectic acid had $[\alpha]_D$ +245° (*c* 0·3% in H_2O) (uronic anhydride, 74%); hydrolysis and paper chromatography gave galactose, arabinose, xylose, and rhamnose (4 : 6 : 3 : 1), and galacturonic acid. The subsequent experiments were made on this material.

Chromatography on Diethylaminoethylcellulose.[12]—Pectic acid (420 mg.) was treated on a column (22 × 3 cm.) of diethylaminoethylcellulose, with gradient elution with phosphate buffer (pH 6, 0·5M, 750 ml. siphoning into 500 ml. of water, flow-rate 40 ml. per min.) followed by gradient elution with aqueous sodium hydroxide. The fractions (20 ml.) were screened for uronic anhydride content by the carbazole method.[22] The Table shows the sugars found in each fraction after dialysis, freeze-drying, hydrolysis, and chromatography; 401 mg. of material were recovered.

Enzymic Hydrolysis.—Crude fungal pectinase (50 mg.) was shaken with acetate buffer (0·1M, pH 5·0, 50 ml.); the filtrate was added to an aqueous solution of ammonium pectate (300 mg. in 100 ml.). After incubation at 37° for 2 days, the solution was concentrated to about 30 ml., then fractionated on a charcoal–Celite column [23] [2 × 30 cm.; Celite + ultrasorb ZF charcoal (1 : 1)]. Gradient elution was by aqueous ethanol (0·5—15%; total vol. 1·25 l. in 150 ml. fractions), then by aqueous pyridine (0·5—3%). The pyridine fractions were found to contain only galacturonic acid and were therefore combined and reduced in volume. After addition of sodium hydrogen carbonate (to pH 4·0) and calcium carbonate (1 g.), the mixture was heated at 80° for 10 min. Reduction in volume after filtration gave a thin syrup which crystallised (92 mg. of sodium calcium galacturonate hexahydrate [15]). From the free acid, the 2,5-dichlorophenylhydrazone, m. p. 179°, and mucic acid, m. p. 205°, were prepared; these derivatives had infrared spectra identical with those of authentic specimens.

Periodate Oxidation.—Ammonium pectate (190 mg.; $[\alpha]_D$ +240°, uronic anhydride, 69%) was oxidised at 2° in darkness with 0·025M-sodium metaperiodate (100 ml.). The reduction of periodate [24] (mol. per uronic anhydride unit) was: 0·32 (17 hr.); 0·47 (42 hr.); 0·77 (95 hr.); 0·94 (138 hr.); 1·05 (173 hr.); 1·06 (320 hr.). The periodate oxidation was repeated on a larger sample of pectate (490 mg.). Ethylene glycol (5 ml.) was added after 320 hr. and the oxidised polysaccharide was then treated with sodium borohydride (0·8 g.) overnight. The product was precipitated with ethanol, reprecipitated twice, then taken to dryness several times with small portions of methanol to remove borate (yield, 520 mg., $[\alpha]_D$ +36° (*c* 1·2% in

$H_2O)$}. Dialysis against distilled water gave a dialysable fraction {160 mg., $[\alpha]_D$ +18° (c 1·6% in H_2O)} and a non-dialysable fraction {140 mg., $[\alpha]_D$ +10° (c 1·0% in H_2O)}. On hydrolysis by 0·5N-hydrochloric acid for 4 hr. at 100° each fraction gave the same products (paper chromatography; solvents A, B, and C)—galacturonic acid, arabinose, and xylose (galactose absent). With solvent C, the presence of threonic acid ($R_{rhamnose}$ 1·27) was indicated.

Methylation of Ammonium Pectate.—Complete methylation was difficult to achieve and several experiments were conducted with little success. Eight treatments with dimethyl sulphate and sodium hydroxide gave, in 26% yield, a product having 14·6% of methoxyl; three treatments with silver oxide and methyl iodide then gave, in 15% yield, a product having 32·3% of methoxyl. The best result (OMe, 37·4%; yield, 8%) was obtained by a procedure involving the use in turn of repeated treatments with thallium hydroxide–methyl iodide, dimethyl sulphate–sodium hydroxide, and silver oxide–methyl iodide.

Fractionation of Methylated Methyl Pectate.—Light petroleum (b. p. 40—60°) was added to a solution of the methylated polysaccharide (380 mg.) dissolved in chloroform (20 ml.), giving the following fractions:

Ratio of chloroform to light petroleum	Fraction no.	Wt. of fraction (mg.)	$[\alpha]_D$
2 : 1	I	33	+180°
1 : 1	II	57	—
1 : 2	III	154	+186
Unprecipitated	IV	110	+123

Methanolysis of Methylated Methyl Pectate; Reduction, Hydrolysis, and Separation of Methylated Sugars.—Fraction III (154 mg.) was heated in a sealed tube with methanolic 6% hydrogen chloride (3 ml.) at 100° for 12 hr. After neutralisation (silver carbonate) and removal of solvent, the residue was extracted several times with chloroform. The resulting syrup was dissolved in dry tetrahydrofuran (15 ml.), and lithium aluminium hydride in tetrahydrofuran (0·2 g. in 10 ml.) was added dropwise to the boiling solution. After refluxing for 30 min., the solution was cooled and the excess of hydride destroyed by addition of ethyl acetate and water. The mixture was taken to dryness under reduced pressure, and the residue extracted with acetone and ethanol. The extracts were diluted with water, de-ionised, and reduced in volume to a syrup (107 mg.). This was hydrolysed with N-hydrochloric acid (5 ml.) at 100° for 4 hr. Neutralisation by silver carbonate, filtration, and evaporation gave a syrupy mixture of sugars (89 mg.). This was chromatographed on thick paper (50 × 15 cm., Whatman seed-test grade), upward development with solvent B being used to give seven fractions. After elution with hot 7 : 3 aqueous methanol, these fractions were reduced in volume and examined by paper chromatography and solvent B: two of the fractions contained three components and the remainder contained two components (cf. ref. 13) as shown below:

Fraction	$R_{rhamnose}$, colour with aniline oxalate, and intensity			
a	0·62 R–B +++	0·80 Y–B +		
b	0·63 R–B +	0·81 R–B ++		
c	0·63 R–B +	0·80 R–B +++		
d		0·83 R–B +++	1·14 O–R +++	
e	0·60 R–B +	0·86 R–B +	1·17 O–R ++++	
f		0·86 R–B +	1·10 O–R +	1·45 O–R +++
g		0·82 R–B +		1·76 O–R +++

R–B, red-brown; Y–B, yellow-brown; O–R, orange-red.

The largest individual fractions were f and g. These were combined (total 35 mg.) and refluxed with aniline (0·5 ml.) in methanol (5 ml.) in darkness. From this, crystals, m. p. 151° (20 mg.), were obtained which were identical (infrared spectroscopy) with the aniline derivative of 2,3-di-*O*-methyl-D-galactose.

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PRELIMINARY COMMUNICATION

Elimination of errors in uronic acid determinations

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DURING the past two years a systematic study of the Lefèvre and Tollens[1] decarboxylation method has been made. The form of apparatus described by McCready, Swenson and Maclay[2] has been extensively modified for use on the semi-micro scale. All unnecessary traps and joints have been eliminated; there is no apparatus or reagent "blank", and reproducible results are obtained using 10–15 mg samples of a pure uronic acid, or 20–30 mg of heterogeneous carbohydrate fractions containing 10–50% uronic acid. The CO_2 liberated in the decarboxylation reaction is determined titrimetrically in an absorption trap of special design. The entire apparatus was designed for ease of use in routine analyses, and has been widely used for analysis of carbohydrate fractions isolated by several workers in this department.[3] The apparatus, together with some of the results obtained, will be described fully in a forthcoming issue of this journal.

Using 19% (w/v) aqueous HCl, kinetic studies have shown that, under the experimental contions used, quantitative decarboxylation is not complete in less than 2½ hours. Deviation from the kinetic values of Huber[4] have been found, and decarboxylation using metal ions[5] was not successful. It has been found, however, that traces of metals[6] do have a catalytic effect on decomposition of the non-acidic carbohydrates generally present in a complex uronic acid containing polysaccharide.

It has been reported[7] that amino-sugars and proteins interfere in uronic acid estimations, and also that, in acidic hydrolysis of protein/carbohydrate materials, by-product 5-hydroxymethylfurfural reacts with the protein present.[8] This may be important in analysis of mucopolysaccharides. To date, no interference in uronic acid estimations caused by presence of protein or amino-sugars has been found.

The nature of (*a*) volatile decomposition products from the reaction and (*b*) the solid polymeric materials which form in the reaction flask have been investigated by infra-red spectroscopy and gas chromatography. Contrary to the findings of Bowman and McKinnis,[9] small quantities of carbon monoxide have been found, and the decarboxylation reaction shown to be oxygen-sensitive; further work on this aspect is in progress.

Under the analytical conditions used, the volatile decomposition products from *non-methylated* acidic and non-acidic carbohydrates, either simple sugars or polymers, have been shown to be furan (*not* furfural) and CO_2; the molar ratio of CO_2 to furan produced is dependent on internal configuration. The quantities of CO_2 reported in earlier investigations[10] to be evolved from simple sugars have been generally confirmed. It has been found possible, however, to decrease in some instances the amount of CO_2 evolved by non-acidic substances, by the addition of complex-forming reagents. It is hoped that further work will lead to the general suppression of evolution of CO_2 from undesirable side-reactions to some extent, so making the analytical reaction more specific.

The volatile decomposition products from methyl sugars and from acidic and non-acidic methylated polysaccharides have been shown to include CO_2, methyl chloride, methyl formate, 2-methylfuran and dimethylformal. In analysis of pectins, etc., precautions are therefore necessary to ensure that such substances capable of acidic reaction are not determined together with CO_2. It is hoped that further investigations into these volatile decomposition products will provide some evidence regarding the decarboxylation mechanism.

Full details of the investigations outlined here will be published elsewhere in due course.

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STUDIES ON MATERIALS CONTAINING URONIC ACID—I

AN APPARATUS FOR ROUTINE SEMI-MICRO ESTIMATIONS OF URONIC ACID CONTENT

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Summary—The design features of some of the many forms of apparatus previously proposed for the estimation of uronic acids using the Lefèvre and Tollens decarboxylation reaction have been critically examined. Several sources of error have been eliminated, and a simple, yet reliable, apparatus facilitating routine estimations on a semi-micro scale has been developed. Details of the experimental procedure and apparatus finally devised are given.

IN recent years, carbohydrate chemists have shown an ever-increasing interest in polyuronides and acidic polysaccharides of plant and animal origin. Having carried out during previous investigations many uronic acid estimations on a wide variety of polysaccharide fractions,[1,2,3] the author found, in common with several other workers in this department, that reasonably reproducible results were not easily obtained, even when 250 to 500 mg samples were taken and a facsimile of the apparatus described[4] by McCready, Swenson and Maclay was used (cf. ref. 5). Non-homogeneity of samples was ruled out since the same difficulty was found with pure standards. As the method also gave an unsatisfactorily large and variable "blank", it was considered that, in addition to the well-known sources of error arising from lack of specificity of the reaction, certain errors were present in the experimental procedure. A critical study of some of the many forms of apparatus previously published was therefore made with the intention of devising a simplified apparatus having the following fundamental features: (*a*) no unnecessary joints and traps, (*b*) no "blank", (*c*) reproducible results from semi-micro samples, (d) ease of use for routine analyses and for kinetic studies of the decarboxylation reaction.

It was found that more than 100 papers on the decarboxylation reaction have been published. No recent critical review appears to be available. Several analytical methods other than that based on the classical decarboxylation reaction exist; these, and the reasons why there is often lack of agreement between the values they give, have been discussed.[5,6,7,29]

A multiplicity of colorimetric methods has been described, based on such reagents as (*a*) naphthoresorcinol[8,9,10] (*b*) anthrone[11,12] (*c*) carbazole[13,14,15] (*d*) thioglycollic acid[16] and (*e*) alkaline hydroxylamine.[17] Very recently, a method based on ultra-violet estimation of the degradation products resulting from heating with concentrated H_2SO_4 has been proposed.[18] Such colorimetric methods are often useful in micro-scale analyses, although they are not based on quantitative reactions,[29,30] and many conflicting reports regarding their specificity and validity exist.[8,9,12]

In addition the accuracy obtainable, detailed procedures involved, and time required per analysis make these methods no more advantageous for routine determinations than the classical decarboxylation reaction, several adaptations of which for micro-scale analyses have been described.[19,20,21,22] No known method for determination of uronic acids is entirely selective;[23,24] *estimations* only are possible at present. Nevertheless, it was considered that elimination of all apparatus errors, subsequent critical study of the reaction kinetics and mechanism, and an investigation into possible methods of minimising the amounts of CO_2 evolved from (*a*) undesirable side reactions (*b*) non-uronic acid materials, could increase the accuracy and specificity of the decarboxylation method. A preliminary note[25] has indicated the extent of the investigations already carried out: various aspects will be reported as subsequent parts of this series elsewhere in due course. This paper deals only with the description of the apparatus and analytical method evolved, a preliminary account of which was given during its development.[26]

EXPERIMENTAL

(1) *Choice of decarboxylation agent*

The use of 19% (w/w) aqueous HCl as decarboxylating agent was introduced[4] with the claim that decarboxylation was complete in 1½–2 hours at an oil-bath temperature of 145°, as opposed to the 4–5 hours required with 12% HCl. Since a method suitable for routine analyses was under development, the use of 19% HCl was retained. (The claims of several authors[27,28] that use of 19% HCl leads to less accurate estimations is now being investigated.)

(2) *Choice of scavenging, with introduction of by-pass system*

The apparatus was designed for scavenging by a slow stream (pressure stabilised at 15 ml/min) of CO_2-freed "spot" nitrogen, rather than by sucking through CO_2-free air. (Recently, indications that the small content of oxygen in "spot" nitrogen may affect the course of the reaction in certain cases have been obtained. A study of this effect is in progress.) A scavenging by-pass system, operated by two 2-way taps, was designed; this greatly facilitates routine operation.

(3) *Design of titration cell for CO_2 absorption*

Choice of method for estimating the CO_2 evolved was difficult. The iodometric method[24] (which is insensitive to traces of HCl retained by the carrier-gas), potentiometric titration,[29] and gravimetric[31,32,33] methods were all considered, but the titrimetric method, which is the most convenient for routine use, was retained. A trap permitting addition of all reagents directly into a nitrogen atmosphere was designed. *Quantitative* absorption of CO_2 *at twice the flow-rate normally used in analyses* was proved (*a*) by fitting a similar cell to the exit as a guard-tube (*b*) by trapping the exit gas at −180° and testing for CO_2 by an infra-red technique which has been described.[36]

(4) *Elimination of all unnecessary traps and joints*

Zinc dust,[4] mossy zinc,[34] aq. $AgNO_3$,[35] Ag_2SO_4 paste,[19] and water[24,28] traps have been used to remove traces of HCl carried over by the scavenging-gas. Maher,[27] however, found such traps unnecessary if a long reflux condenser was used. This was confirmed, provided (*a*) low flow-rates were used (10–15 ml/min), (*b*) the carrier-gas inlet did not reach below reflux level in the reaction flask. To keep the overall size and internal volume of the apparatus small, further experiments were conducted using a fairly short ether-type condenser. Blank determinations and infra-red analyses showed no escape of HCl even at flow-rates twice that normally used. The same infra-red analyses showed that *non-methylated* uronic-acids, hexoses, pentoses and polysaccharides gave CO_2 + furan as the volatile reaction products. Pure furfural was refluxed under the analytical conditions used; only traces of furan (no furfural) were detected in the scavenging-gas. It has been stated[37] that refluxing furfural for 2 hours in 19% HCl destroys 60%, whilst steam distillation gives 99% recovery of the furfural: under the conditions of low flow-rate, non-aeration of reaction mixture, and high reflux efficiency used in this analytical method no furfural is volatilised. Furan gives no reaction in the

CO_2 absorption trap. The aniline or phloroglucinol scrubber included in previous methods was thus eliminated, and the design of a compact, single-unit, apparatus having only one critical joint was possible.

(5) *Heating of reaction-flask*

In numerous previous methods, heating by oil-bath maintained at 135–145°, or even higher, was used. 19% HCl refluxes at about 109° and bath temperatures much in excess of this were found to produce charring on the reaction-flask walls, with resultant evolution of spurious and non-reproducible quantities of CO_2. This effect also results if heat is directly applied to a level higher than that of the refluxing liquid inside the flask. Complete elimination of such charring was achieved by heating the 100-ml flask with an electrothermal mantle designed for 50-ml flasks. The heat available just maintains reflux, and the flask area to which heat is applied is suitably limited.

Construction of apparatus

The apparatus is shown to scale in Fig. 1. It is readily constructed by a competent glass-blower. The ether-type condenser has two concentric coils wound closely round a central vertical conductor. The outer coil is a sliding fit inside the outer wall, and the concentric coils fit closely inside each other.

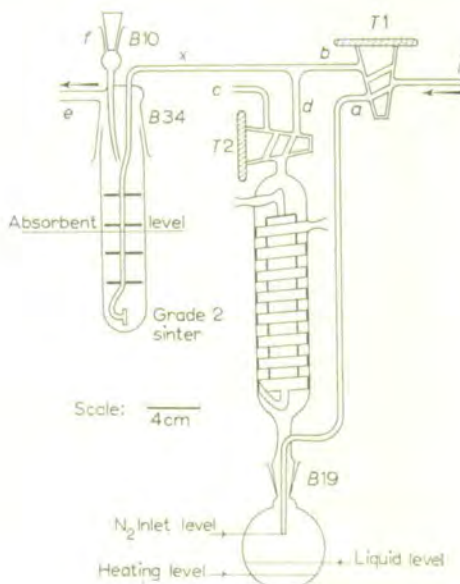

Fig. 1.

The condenser jacket is virtually filled with cooling coils, and the internal volume of condenser and absorption trap is only 150 ml. Since decarboxylation requires about 2½ hr for completion, a flow-rate faster than 15 ml/min is unnecessary. The absorption-trap is constructed from a B34 cone and socket. About 50% of the ground-glass cone is cut off; this permits a closer fit of the baffle plates inside the absorption-tube. Baffles can be made either of glass (the Q. and Q. "Disc Distributor EX10/20" supplied with Soxhlet extractors is easily adaptable), or of thin polythene discs, in which a central hole and radial slot are cut to permit a tight fit on the delivery tube, which ends in a 1-cm diameter sinter-disc. A space left between this disc and the bottom of the tube allows the coagulated $BaCO_3$ ppt. to settle. Grade 2 porosity is the most suitable; a stream of fine bubbles is desired without creation of a back-pressure great enough to test either the B19 joint on the reaction-flask or the taps T_1 and T_2. Several discs may have to be made before one fulfilling these conditions is obtained; the average pore-size usually decreases with heating and annealing. For kinetic measurements, a two-way junction stop-cock system is introduced at x in Fig. 1, so that the gas stream can be switched through different traps. A pair of traps fitted with closely matched sinter-discs is then essential, otherwise differing back-pressures are created, which, it was found, alter the reflux temperature sufficiently to affect reaction rates.

At the start of an analysis, sufficient time is allowed for nitrogen to scavenge the apparatus and trap. 15·00 ml of 0·05*N* $Ba(OH)_2$ is then added at *f* from a CO_2-free storage system, and the delivery-tube *f* washed down until the absorbent level in the trap is just below the third baffle. The baffles create turbulence, so that quantitative absorption of CO_2 is achieved and the absorbent concentration remains uniform. A slight pumping action develops and the third baffle is kept awash. The top baffle acts as an anti-spray device. When absorption is complete, back-titration of excess $Ba(OH)_2$ to phenolphthalein end-point is possible by addition of 0·05*N* HCl from a burette *via f*. Adequate trap volume remains if care is taken with the quantity of wash-water used. Turbulence inside the trap produces homogeneity within about 1 minute. Excellent end-points are obtainable with a little care: should the end-point be exceeded, direct titration of the slight excess of acid can in turn be made.

Routine operation sequence

The apparatus is permanently held by clamps round the B34 socket of the absorption-cell and round the condenser. The pressure-stabilised flow of carefully purified "spot" nitrogen, regulated by needle valves and rotameter at 15 ml/min, enters the apparatus at *i*. The reaction-flask containing the weighed sample and 35 ml of 19% HCl is placed in position. A trace of silicone grease is used; a good joint is essential. T_1 and T_2 are positioned so that nitrogen flows through *a* and out at *c*. After 10 minutes (or longer as considered necessary if carbonate is suspected in the sample) the condenser will have been swept out, and T_2 is turned so that the nitrogen flows through *d*. Heating of the reaction-flask is begun. When reflux begins (after about 10 minutes) the trap will have been swept out (with no stopper in position at *f*). 15 ml of 0·05*N* $Ba(OH)_2$ etc. are now added as already described; the first traces of CO_2 from the decarboxylation will not reach the trap until 10 minutes later. When decarboxylation is complete, T_1 is turned so that nitrogen flows through *b* and through the trap. T_2 is turned to release the slight pressure inside the reaction-flask and condenser through *c*. The heating-mantle and flask are removed, and a second flask containing sample II positioned. The absorption trap contents are now back-titrated, and T_1 is then turned so that nitrogen flows through *a* to *c*. Whilst the reaction-flask and condenser are thus being swept, the absorption trap is lowered, thoroughly rinsed and replaced. T_2 is then turned to position *d*, heating is begun, and the cycle of operations completed as already described.

The by-pass system therefore ensures that the steady flow-rate need never be interrupted (important in kinetic studies) and that the minimum time is lost between analyses. Three analyses can be completed in 8 hours, and the apparatus needs no attention during each 2½ hours decarboxylation period.

RESULTS

CO_2-free distilled water and *AnalaR* grade chemicals are used; reagents are stored in vessels protected with Sofnolite guard-tubes. The standard analytical conditions are:— Flow-rate = 15 ml/min; Vol. of 19% (w/w) HCl used = 35 ml; reflux temp. = 108–109°; decarboxylation time = 2½ hrs from start of reflux; sample weights sufficient to liberate 1–5 mg CO_2 are preferable, but 0·5 mg CO_2 can be determined without loss of accuracy (see Table II).

1. "*Blank*" *determinations*

(*a*) No blank is given under standard analytical conditions.

(*b*) 50 ml 19% HCl refluxed continuously *for 96 hrs* at *25* ml/min flow-rate gave:— (1) acidity absorbed in $Ba(OH)_2$ $\not>$ 0·02 ml 0·05*N*. (2) CO_2free distilled water, initially colourless to phenolphthalein, turned very faint pink. (3) alkalinity absorbed in HCl $\not>$ 0·02 ml 0·05*N*.

2. *Determination on National Bureau of Standards "Dolomite 88"* (% CO_2 = *47·25*)

A reaction flask fitted with a side-arm dropping-funnel for addition of acid was used. Wt. of sample taken = 12·48 mg. CO_2 found = 47·1%. First traces of cloudiness in $Ba(OH)_2$ trap appeared 12 minutes after first addition of acid. Liberation of CO_2 was fast, but infra-red analysis (sensitive[36] to 0·2 mg CO_2) showed that absorption of CO_2 was complete.

3. *Determinations on standards*

Uronic acid standards of 100% purity are difficult to obtain. Glucurone (American Corn Products Refining Co. Ltd.) once recrystallised, and having Neutralisation Equivalent = 171 (mean of 5 detns.), and galacturonic acid monohydrate (Roche Chemicals Ltd.), were used to obtain the results shown in Table I.

TABLE I

Sample	% purity found from neut. equivt.	Apparent % uronic acid found,							
		Decarboxylation time, *mins*							
		20	40	60	90	120	150	240	480
Glucurone	97·2*	57·0	80·2	86·7	94·3	95·6	96·7 97·2 97·4 96·8	98·3	101·5
Galacturonic acid monohydrate	96·0†	73·0	89·0	92·1	93·8	95·0	95·8 95·4 95·6	98·5	103·0

* Calculated as uronic acid anhydride.
† Calculated as monohydrate.

A decarboxylation period of 2½ hours is therefore used in routine analyses. In this time, the CO_2 evolved from non-uronic materials varies from apparent uronic acid anhydride contents of 3·5% for pure L(+)rhamnose and D(+)mannose to 1·8% for D(+)glucose and D(+)xylose. A full account of the kinetic results obtained and their detailed application to the method of analysis wll be given as a later part of this series.

4. *Results obtained for Hakea gum samples**

TABLE II

		Wt. taken *mg*	CO_2 found *mg*	% Uronic acid anhydride
Crystalline Hakea gum.	Detn. 1	116·85	3·35	11·4
	2	110·71	3·06	11·1
	3	103·20	2·88	11·2
	4	70·72	1·98	11·2
	5	48·23	1·35	11·4
	6	21·00	0·59	11·2
Fractionated Hakea gum.	Detn. 1†	35·05	4·73	56·2
	2	23·61	3·31	56·0
	3	11·63	1·64	56·4

* Prepared by Dr. A. M. Stephen, Univ. of Cape Town.
† Neut. Equivt. subsequently found (by Dr. A. M. Stephen) = 315, *i.e.* % Uronic acid anhydride = 55·8.

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Zusammenfassung—Die Eigentümlichkeiten von einigen der vielen zur Bestimmung der Uronsäure mittels der Lèfevre und Tollens-Dekarboxylation-Reaktion schon vorgeschlagenen Apparat-Typen werden kritisch untersucht. Mehrere Fehlerquellen sind eliminiert worden und ein einfacher, zuverlässiger Apparat, der Normal-Bestimmung auf einer Halb-mikroskala erleichert, wurde entwickelt. Die Nebenumstände des experimentellen Verfahrens und des vorgeschlagenen Apparats werden angegeben.

Résumé—Les caractéristiques de quelques uns des nombreux appareils proposés jusqu'ici pour la détermination des acides uroniques en utilisant la réaction de décarboxylation de Lefèvre et Tollens ont été examinés d'un point de vue critique. Plusieurs sources d'erreur ont été éliminées et un appareil simple, cependant fidèle, permettant les déterminations de routine à l'échelle semi-micro a été proposé. On donne le détail des conditions expérimentales et de l'appareil tel qu'il a été conçu finalement.

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STUDIES ON URONIC ACID MATERIALS—II[1]

THE VARIATION IN COMPOSITION OF GUM NODULES FROM *COMBRETUM LEONENSE*

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Summary—Several gum nodules from the Nigerian tree *Combretum leonense* have been individually investigated. The amount of material present in each nodule has permitted a study of the variation in constitution of both crude and purified forms of the gum. The variations found are much greater than can be explained by analytical error. They are considered to indicate that inter-nodule differences in fine structure exist.

IN certain fields of carbohydrate chemistry, *e.g.* those dealing with starch[2,3,4] and with glycogen,[5] sampling procedures require strict attention when material for investigation of fine structure is being selected. Even if strict control of botanical variety and strain proves possible, variations in growth and maturity caused by seasonal effects are difficult to control, and wide variation in chemical composition can occur.[6] Several extensive studies of such effects, *e.g.* in some varieties of seaweed,[7] barley,[8] grasses and clovers[9,10] have now been reported.

Severe sampling difficulties exist in the study of plant gums. Usually the gum is taken from the tree bark in small nodules which can vary greatly in colour and general appearance. Any guarantee of constancy of originating botanical species is difficult to obtain, particularly when collection overseas is arranged. Furthermore, gum is normally exuded to minimise bacterial attack at localised sites where superficial injury from a variety of possible causes has been sustained at some unknown time. Thus nodules from the barks of a number of trees may possibly vary greatly in maturity and chemical composition, even although each of the trees was authentically of the same botanical species.[11]

Consequently, when a batch of nodules collected in such a way is purified by precipitation in acidified alcohol from aqueous solution, a complex mixture of different polymeric carbohydrate systems may result. Although early studies[12] showed that damson gum from several trees grown in different districts was "essentially a homogeneous chemical entity," recent work has suggested that gum ghatti may be heterogeneous[13] and that sisal pectic acid[14] may contain a series of closely related molecular species.

There have been few investigations of inter-nodule variation. Torto[15] analysed one nodule of gum from *Fagara Xanthoxyloides* for acetyl and methoxyl content, and stated that specimens prepared from nodules from different trees did not show significant variations in optical rotations and equivalent weights; this was claimed to indicate the essential homogeneity of the gum. In a study of the gum from *Brachychiton diversifolium*, three separate nodules were powdered, dried, and analysed

for ash, nitrogen, methoxyl and acetyl content: it was deduced that the nodules were "not significantly different in composition".[16]

A sample of the gum from *Combretum leonense** was observed to contain a number of unusually large clean nodules, each approximately the size of a walnut. This appeared to make possible a study of the composition of each nodule.† This paper gives the results of an attempt to investigate the composition of each nodule as fully as the amount of material available permitted.‡

Initially, the six largest, whole nodules, whose weight varied from 8–12 grams, were selected for study. They were alike in appearance, being dark brown in colour with characteristic surface markings, and had a marked odour of acetic acid. The nodules, numbered I–VI, were individually ground to a coarse powder. It was immediately observed that nodules I, III and V gave a pale yellow powder, whilst that from the others was a dark reddish-brown.

EXPERIMENTAL

Analytical methods

(*a*) Moisture contents were found by heating to constant weight at 103°.

(*b*) % ash was found by heating in a muffle at 550°; constant weight was reached in 4 hours.

(*c*) % nitrogen was found by a semi-micro Kjeldahl method.

(*d*) Acetyl contents were determined by the Weisenberger method.[22]

(*e*) Anderson's apparatus[1] was used to determine uronic acid contents.

(*f*) Free titratable acidity was found by direct titration with 0·0247*N* sodium hydroxide to phenolphthalein end-point in a stream of CO_2-free nitrogen.

(*g*) Viscosity measurements were made using a suspended-level dilution viscometer (Polymer Consultants Ltd; water = 184 secs) in a thermostat constant to 0·01°; results are expressed using g/ml as concentration units.[23]

Autohydrolysis experiments

The quantity (calculated on an ash-free, dry weight basis) of each nodule to give 100 ml of a 1% (w/v) solution was dissolved in a standard flask. The solutions were transferred to clean, dry flasks which were placed in a boiling water bath after being fitted with short reflux condensers. Aliquots (2 ml) were withdrawn at intervals by pipette (considerably lengthened above the graduation mark) introduced *via* the reflux condenser, which was fitted with an anhydrone guard-tube except when withdrawals were being made.

Paper partition chromatography

This was carried out on Whatman No. 1 paper at 20° with the upper layers of the following systems (v/v): (*A*) butan-1-ol–benzene–pyridine–water (5 : 1 : 3 : 3); (*B*) pyridine/ethyl acetate/acetic acid/water (5 : 5 : 1 : 3).[24] Sugars were located by heating after spraying with either saturated aqueous aniline oxalate solution or with a solution of *p*-anisidine hydrochloride in butan-1-ol. All sugars were identified by comparison with reference sugars run on the same chromatograms.

Quantitative estimation of sugars

This was by the Somogyi method;[25] D-ribose was used as reference sugar after being shown absent in the gum. In the autohydrolysis experiments, total free reducing sugars were calculated as arabinose, which was the main sugar liberated.

Electrophoresis

The electrophoresis of neutralised solutions of purified nodules VII and VIII was attempted (Shandon Universal Filter Electrophoresis Apparatus) using 300 volts for 2–6 hours in 0·05*M* borate

* Collected in Nigeria in 1952, and kindly provided by Dr. R. J. McIlroy.

† Dr. G. O. Aspinall kindly provided several nodules for this purpose, although the total weight of gum available was not large.

‡ The structure of the gum is now being studied in this Department by Dr. Aspinall.

buffer at pH 8 on (*a*) 20 × 5 cm paper strips (Whatman No. 1), (*b*) glass-fibre paper. No movement was observed on the paper strips: small mobilities (< 0·5 cm after 6 hours) were obtained on the glass-fibre paper, but no separation of components could be detected. Electrophoresis for 6–18 hours on glass-fibre paper in 2*M* alkali[13] resulted in movements of 3–4 cm as a continuous smear, from which no conclusive result could be obtained.

Studies on crude material

Table I shows the results of duplicate determinations of the moisture, ash, nitrogen, methoxyl, acetyl and uronic acid content of the crude powdered material from each nodule. Results of analyses of the ash are also shown.* Chloride, sulphate and phosphate were all absent; carbonate, formed during ashing, was found to account for 55% of the ash from nodule VI.

Table II shows the values for pH, free titratable acidity, total free reducing sugars, and flow-time number obtained from aliquots of aqueous solutions (1% w/v, corrected for moisture and ash content) of each powdered nodule. Each 1% solution was then autohydrolysed at 98°. At intervals, up to a

TABLE I.—DETERMINATIONS ON CRUDE POWDERED MATERIAL

	Nodule					
	I	II	III	IV	V	VI
% Moisture	14·9 14·9	15·3 15·6	14·0 14·0	15·5 15·6	17·1 16·9	14·9 14·9
% nitrogen	0·13 0·14	0·06 0·07	0·16 0·16	0·05 0·05	0·11 0·13	0·12 0·12
% methoxyl	nil	nil	nil	nil	nil	nil
% acetyl	5·1 5·2	6·2 6·2	4·4 4·4	5·9 5·9	6·3 6·2	3·0 3·0 3·1
% Uronic acid anhydride	15·4 15·7	18·7 19·0	15·9 16·0	18·9 19·0	17·7 17·8	15·1 15·4
% Ash	2·56 2·54	4·03 3·96	2·38 2·43	3·79 3·83	3·02 3·12	1·71 1·69
Composition of ash (as parts per million)						
Ca	235,000	235,000	205,000	257,000	257,000	270,000
Na	5,200	5,200	4,700	3,700	3,000	4,400
K	136,000	91,000	180,000	127,000	136,000	37,400
Mg	n.d.	n.d.	n.d.	n.d.	n.d.	84,000
Cu	166	59	538	56	82	272
Mn	25,000	11,400	17,400	17,100	13,400	18,400
Fe	4,800	1,240	2,310	1,200	1,200	1,540
Ba	680	590	620	630	550	540
Sr	1,590	1,290	1,490	1,560	1,170	1,060

* We are greatly indebted to Dr. R. L. Mitchell, Macauley Institute for Soil Research, Aberdeen, who obtained from the small quantities of ash available the values for Cu, Mn, Fe, Ba and Sr.

TABLE II.—DETERMINATIONS ON 1% AQ. SOLUTIONS OF CRUDE MATERIAL

	Nodule					
	I	II	III	IV	V	VI
% cold water insoluble	0·3	0·5	0·6	0·2	0·2	0·3
pH	4·25	4·30	4·10	4·25	4·20	4·10
Free titratable acidity (*ml* 1*N* NaOH per gram) Hence apparent equiv. wt.	0·378 0·374 2645	0·374 0·384 2645	0·324 0·330 3125	0·426 0·404 2380	0·398 2500	0·354 2820
% Free reducing sugars (Somogyi,[25] as arabinose)	0·74	1·02 1·02	0·72 0·84	2·10 2·16	0·74	0·90
Flow-time at 26·4° (water = 184 secs) Hence $(t - t_0)/t_0$	468 466 1·52	491 491 1·73	425 429 1·32	620 618 2·41	449 451 1·45	366 364 1·00

total of 360 hours, aliquots were withdrawn for determination of total reducing power and total free acidity; the values found are shown in Figs. 1 and 2 respectively. Table III shows the ratios of free sugars present after 70 and 168 hours' autohydrolysis; values of the flow-time number were also found at 70 hours. Hydrolysis with H_2SO_4 (2*N*) at 100° for 24 hours was incomplete, but formic acid (90%) in a sealed tube at 100° for 5 hours gave complete hydrolysis. Within the limits of experimental error, the ratios of the sugars found were the same for each of the samples, *viz.* arabinose/galactose/rhamnose = 9/10/1.

Studies on purified material

Preliminary purification experiments were carried out on nodules I and V, which were the largest samples remaining. After dissolution in cold distilled water, the solutions were poured into 3 volumes of acidified ethanol (0·1*N* with respect to HCl). The white curdy gum precipitate was removed by centrifuge. After one such precipitation, the material from nodule I contained 0·04%*N* and 0·11% ash; two precipitations of the material from nodule V gave purified gum having 0·03%*N* and 0·02% ash. Since re-precipitations and ash determinations at this level consume considerable quantities of material, it was decided to accept this as a reasonable standard of purification. Each of the remaining nodules was therefore precipitated twice, and, to conserve material, it was assumed that their values of %*N* and % ash would be similar to those found for V.

After removal by centrifugation, the precipitated material was dried by immersion in acetone for 10 days: the acetone was changed daily and the gum was progressively ground to a fine powder. Samples I and V were then placed in a vacuum pistol at 60° over P_2O_5 for 1 hour, then stored in a vacuum desiccator for 14 days. After this treatment, oven drying to constant weight at 103° showed 6·83% "moisture" in sample I and 4·64% in V. Purified samples II, III, IV and VI were dried, after acetone dehydration as before, under vacuo at 80° for 48 hours in an attempt to decrease the moisture content; oven drying to constant weight at 103°, however, showed 5·25%, 4·86%, 5·73% and 6·20% "moisture" for these samples respectively. Since these values were surprisingly high, further drying experiments were carried out on small quantities of each sample. Using an infra-red quantitative technique,[17] it was found that both water and acetone (or any other organic solvent used for precipitation or preliminary dehydration) were retained up to temperatures at which decomposition of the gum began (about 135°) as indicated by slight browning and the start of evolution of CO_2. To augment the material available, a further two nodules (numbers VII and VIII) were obtained; after two

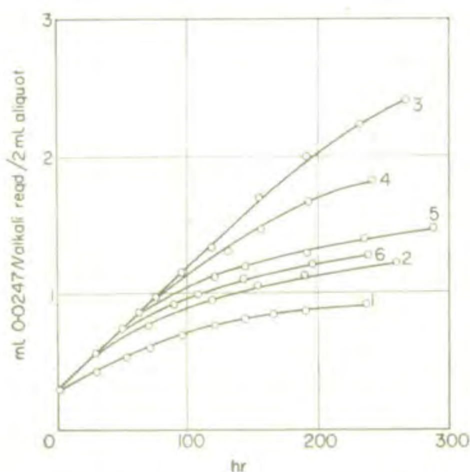

FIG. 1.—Increase in total free acidity on autohydrolysis.

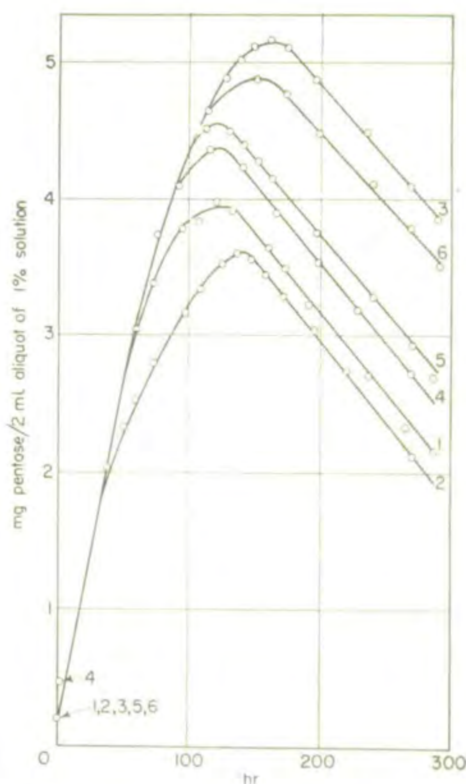

FIG. 2.—Increase in total free reducing power on autohydrolysis.

TABLE III.—DETERMINATIONS ON 1% AQ. SOLUTIONS AFTER AUTOHYDROLYSIS

	Nodule					
	I	II	III	IV	V	VI
After 70 *hours:*						
$(t - t_0)/t_0$	0·04	0·02	0·07	n.d.	0·10	0·06
ratio of sugars: hexose disacch.	1	1	1	n.d.	1	1
pentose disacch.	1	1	1	n.d.	1	1
galactose	1·6	1·2	1·5	n.d.	1·5	1·6
arabinose	12	10	20	n.d.	15	20
rhamnose	1	1	1	n.d.	1	1
After 168 *hours:*						
ratio of sugars: hexose disacch.	1	1	1	1	1	1
pentose disacch.	1	1	1	1	1	1
galactose	2	2	2	2	2	2
arabinose	15	12	14	10	13	13
rhamnose	1	1	1	1	1	1

TABLE IV.—DETERMINATIONS ON PURIFIED MATERIAL

	Nodule							
	I	II	III	IV	V	VI	VII	VIII
% nitrogen	0·04 0·03	n.d.	n.d.	n.d.	0·03 0·03	n.d.	0·04	0·06 0·07
% acetyl	2·9 2·8	3·4 3·3	3·3	2·9 2·9	4·3 4·4	1·7 1·7	3·0 2·9	3·6
% uronic acid anhydride	15·3 15·5	19·2 19·3	15·2 15·4	19·7 19·6	18·1 18·4	14·9 14·9	19·2 19·4	20·1 19·9
Results from 1% aq. solutions:								
% Free reducing sugars	0·21	n.d.	n.d.	n.d.	0·28	n.d.	n.d.	n.d.
Free titratable acidity*	0·882	1·058	0·868	1·106	1·060	0·856	1·072	1·149
Hence equivalent wt.	1133	945	1152	904	943	1168	933	870
Hence % uronic† acid anhydride	15·5	18·6	15·3	19·4	18·6	15·1	18·9	20·2
Limiting flow-time‡ number	0·63	0·76	n.d.	0·98	0·68	n.d.	0·61	0·67

* as mls. 1·0*N* NaOH per gm
† if all acidity due to uronic acid groups
‡ at 25·0° in 1% NaCl solution.

precipitations in acidic ethanol, the purified material was redissolved, dialysed against distilled water for 3 days, then isolated by freeze drying. The resultant material was found (*a*) to lose 1% of water on drying to constant weight at 103°, and (*b*) to retain a further 1% of water up to the temperature at which decomposition began. Robson[18] has reported that freeze drying for 500 hours is required to remove *all* traces of moisture from glucose–gelatine solutions. A full account of the dehydration and solvent retention studies made will be given in a later communication.

Determinations of nitrogen, acetyl, and uronic acid content were made on the purified samples, the results (corrected for the moisture contents reported above) being shown in Table IV, which also gives the results of determinations of free reducing sugars and free titratable acidity made on 1% (w/v) aqueous solutions. Calculation of the equivalent weight of the gum acid, based on the free titratable acidity value, shows that, in each case, the uronic acid content accounts for the total acidity found.

Before studying the viscosity behaviour of each purified sample, the polyelectrolyte effect was investigated. Fig. 3 shows that 1% salt solution gives adequate suppression of the polyelectrolyte effect. The limiting flow-time number of the purified samples was subsequently found at 25·0° in 1% sodium chloride solution; the viscosity curves obtained are shown in Fig. 4. Use of a zero shear viscometer (Polymer Consultants Ltd.) showed that Newtonian behaviour was given by an 0·5% solution of sample VIII in 1% sodium chloride.

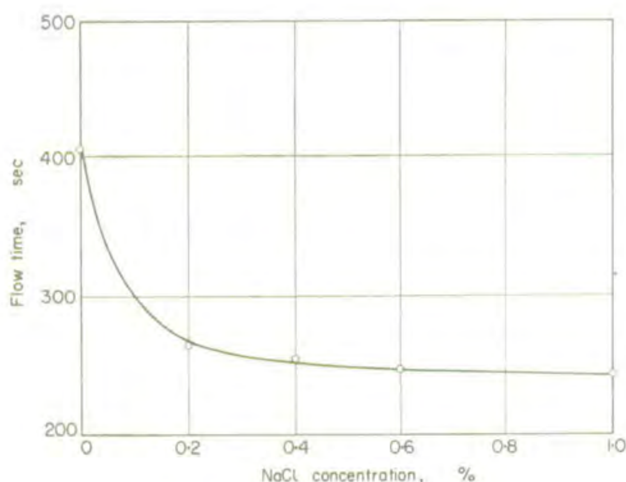

FIG. 3.—Decrease in electroviscous effect.

When a plant gum is purified by acidic ethanol precipitation from aqueous solution (which may, as in this case, have pH = 4) some form of auto-degradation or fractionation could possibly occur. Such an effect would become increasingly serious as the number of re-precipitations—and consequently the percentage of free-acid groups—was increased. To investigate this possibility, the twice precipitated material from nodule VII (curve VII(a), Fig. 4) was given two further purifications. During each of these, the possibility for degradation to occur was increased by storing the aqueous solution at 40° for 1 hour before precipitation in ethanol. (In the original precipitations, no heating was employed, and addition to ethanol was made as soon as solution was achieved). Since the material recovered from these treatments gave viscosity curve VII(b) in Fig. 4, no degradation of this gum apparently occurs whilst it is in the free acid form during purification processes. The effect of dilute alkali was also tested. An aqueous solution (1 % w/v) of the gum was neutralised to phenolphthalein end-point; a calculated excess of alkali was then added to make the solution 0·1N with respect to

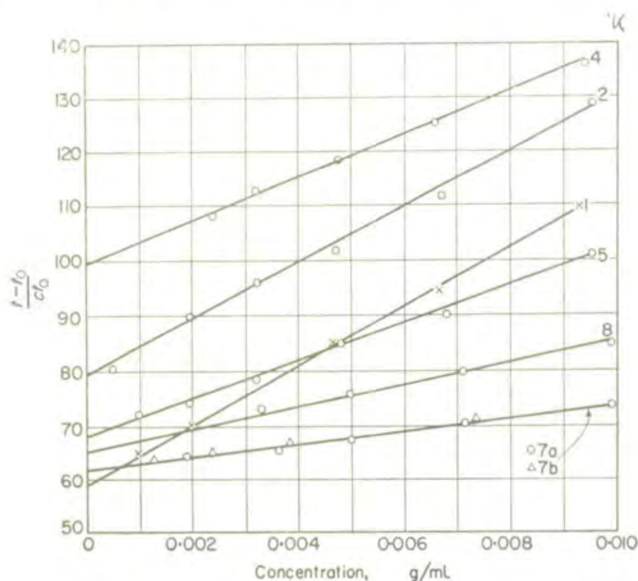

FIG. 4.—Viscosity curves for purified samples.

NaOH. The limiting flow-time number of this solution was determined at 25·0° on portions of the solution (*a*) *immediately*, (*b*) *after storage* at 25·0° for 72 hours. No difference in viscosity behaviour, before and after storage, was detectable.

No tendency for fractionation to occur was observed when the ethanol used in precipitations was varied from 2 to 5 volumes. After centrifugation of the main precipitate, the supernatants were stored at 0° for several days; no further material precipitated. No precipitation of neutral material was given on addition to the supernatants of borate + "cetavlon".[19] Electrophoresis experiments on nodules VII and VIII were inconclusive. (see above).

DISCUSSION

The available material was used to obtain, as far as possible, information supplementary to that which will be obtained in the structural study now in progress on the bulk material. Each of the samples was remarkably homogeneous; good replicates, as shown in the tables, being obtainable without difficulty. Each nodule, however, differed strikingly in composition from each of the others. In view of the reproducibility of results obtained in each of the analytical procedures used, the internodule differences are greater than could be ascribed to any possible experimental error, and are considered to be significant. In only one procedure—determination of the ratio of sugars liberated on complete hydrolysis—was it not possible to distinguish between each nodule; the error involved in such a determination is, however, at best $\pm 5\%$ and is more probably $\pm 10\%$.

Correlations between the properties of the nodules are difficult. The nodules cannot easily be assigned to sub-groups; a gradation between the maximum and minimum values of each analysis appears to be given. That the nodules are broadly similar in essential detail is not surprising, considering the care with which they were selected for study from a batch. Yet, in the crude material, 100% variation occurred in the nitrogen, ash, and acetyl contents. Calculation shows that, in the crude material, about 50% of the uronic acid carboxyl groups are in the free acid form.

In the purified samples, considerable variations occur in the acetyl and uronic acid anhydride contents, and in the viscosity behaviour. The acetyl contents shown in Table IV are stable in solution under the precipitation conditions used; when stored in the solid state, however, the purified material develops a strong odour of acetic acid, a behaviour reminiscent of the original nodules. The nitrogen content of the crude material was reduced, but not completely eliminated, by the purification process used. Re-precipitations were found to reduce ash contents significantly, but little reduction in residual nitrogen content was achieved. Small residual nitrogen contents persist in other carbohydrate systems[20] despite extensive attempts at purification; their significance remains in doubt.[21]

In only one case (nodule III) is the uronic acid content of the purified sample significantly smaller than that of the crude material. This is difficult to explain since, on a dry weight basis, purification should lead to increased uronic acid content unless some sort of fractionation has occurred. Agreement between the uronic acid values found directly and by calculation from the neutralisation equivalent is good, and strengthens the evidence that the variation from 14·9% to 20·1% is real. Such a variation must reflect fine structural differences; the inter-nodule variation in viscosity and acetyl content, and the kinetic differences found in the autohydrolysis experiments support this indication.

The variations found are sufficiently great to emphasise the need for more careful

sampling procedures than have generally been used in the past. It is hoped at an early date to extend the present investigation by securing for study* a number of gum nodules obtained (*a*) from the bark of one tree as a result of several injuries made simultaneously and (*b*) from the barks of a number of trees, all of the same species, as the result of one similar injury inflicted simultaneously on each of them.

Acknowledgments—We thank the Rockefeller Foundation and Messrs Imperial Chemical Industries Ltd. for grants, and the Department of Scientific and Industrial Research for a maintenance allowance (to N. J. K.).

Zusammenfassung—Gummitropfen vom Baume Combretum leonense aus Nigeria wurden individuell untersucht. Die Mengen verschiedener Substanzen in einzelnen Tropfen wurden als stark variierend gefunden und gestatteten Rückschlüsse auf Verschiedenheiten in der Zusammensetzung sowohl von Roh- als auch gereinigtem Latex. Verschiedenheiten der Zusammensetzung waren weitaus grösser als dass sie hätten durch Schwankungen der Analysenwerte erklärt werden können. Der Schluss wurde gezogen, dass sie durch Unterschiede in der feineren Stuktur der Tropfen verursacht werden.

Résumé—Les auteurs ont étudié individuellement différents nodules de gomme provenant de l'arbre nigérien, Combretum leonense. La quantité de matière présente dans chaque nodule a permis une étude de la variation de la constitution des deux formes: brute et purifiée de la gomme. On trouve des variations beaucoup plus grandes que ne l'expliquerait l'erreur analytique. On considère que ces variations indiquent qu'il existe des différences internodules dans la structure fine.

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28.

STUDIES ON URONIC ACID MATERIALS—III*

AN INVESTIGATION, USING ^{14}C COMPOUNDS, OF ACID DECARBOXYLATION REACTION-TIMES

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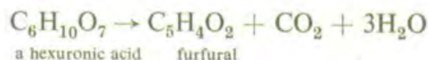
Summary—The reaction-times for complete (*i.e.* 99%) decarboxylation of glucuronic acid labelled at C6 with ^{14}C are respectively 200 min and 360 min, from start of reflux, for 19% and 12% (w/w) aq. hydrochloric acid. The corresponding first-order rate constants are $7\cdot4 \times 10^{-4}$ sec^{-1} and $1\cdot6 \times 10^{-4}$ sec^{-1}. Because of the simultaneous production of carbon dioxide from side reactions, however, analytically correct results are given, by compensation of errors, in shorter times, *i.e.* in 140–160 min for 19% acid and in 270–290 min for 12% acid. The results also indicate that the use of 19% acid minimises the error arising from spurious production of carbon dioxide in side reactions: this is contrary to some published opinions.

THE quantitative analysis of uronic acid groups is of great importance in investigations of plant gums[1] and other carbohydrate materials, in the analysis of foodstuffs and of organic matter in soils, and in animal[2] and plant[3] biochemistry. The analytical problems presented by uronic acid materials offer a considerable challenge to the chemist.

Lefèvre and Tollen's classical decarboxylation method[4] was based on Mann and Tollen's earlier experiments[5] with glucuronic acid and 12% (w/w) aq. hydrochloric acid. Although the reaction was formulated as



$$\underset{\text{a hexuronic acid}}{C_6H_{10}O_7} \rightarrow \underset{\text{furfural}}{C_5H_4O_2} + CO_2 + 3H_2O$$

the yield of furfural is always much less than theoretical[6] at the reaction-time giving 1 mole of carbon dioxide. Side-reactions, which are complex,[7,8,9] therefore take place; they begin as soon as decarboxylation of a uronic acid molecule, resulting in the formation of an acid-labile decomposition product, is first achieved. Since carbon dioxide is liberated from these side-reactions slowly and approximately linearly with time, yields in excess of 1 mole must result[10] if the reflux period necessary for 100% decarboxylation is used.‡ There is no known method of eliminating these side-reactions. The analytically correct time, even for pure reference uronic acids, is therefore, by compensation of errors, less than the full time required for complete decarboxylation.

The complex system of reaction products cannot readily be reconstructed for study as a synthetic mixture; the uronic acid and its successive reaction products form a dynamic, integrated system which at present can only be studied as a whole. Consequently, it is not surprising that widely differing times for complete decarboxylation have been proposed. Thus for 12–13% hydrochloric acid the literature includes such

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‡ Since first-order reactions are never absolutely complete, > 99% decarboxylation is here referred to as "complete" or "total" decarboxylation.

periods as 3 to 4 hr,[4,6,11], 8 hr[12,13] and 15 hr;[14] 70 min[15] and 3–4 hr[16] have been proposed for 12–13% hydrochloric acid saturated with sodium chloride. A reaction period of 4 hr was suggested[17] for *both* 13% and 18% hydrochloric acid. For 19% acid, reflux for 1·5 hr was first proposed;[18] this has been increased in more recent papers to 2 hr[19] and to 2·5 hr.[20]

A study of the analytical chemistry of uronic acids has been made over the past 5 years.[21] Comparison of the results given by the classical decarboxylation reaction with those obtained by (*a*) thermal decarboxylation[22] and (*b*) colorimetric methods (*cf.* ref. 20) indicated that the decarboxylation method was by far the most reliable. It was therefore important to verify the correct reaction-time for a given set of conditions. This was facilitated when potassium-D-glucuronate, labelled at C_6 (the carboxyl group) with ^{14}C, became available.

EXPERIMENTAL

^{14}C-labelled compounds

Barium carbonate-^{14}C and potassium-D-glucuronate-6-^{14}C were purchased from the Radiochemical Centre, Amersham, England. The certificated radiochemical purity of the glucuronate at the time of purchase was quoted as 101%, but this material was kept in storage for 2 yr, and its purity was only 92–93% at the time of use in these experiments. (*cf.* ref. 23).

Counting

The carbon dioxide evolved from labelled compounds was swept by the nitrogen scrubbing gas through a scintillation cell [Nuclear Enterprises (G.B.) Ltd.] mounted in a photomultiplier: the cell was designed for high efficiency counting of ^{14}C labelled vapours. The photomultiplier was attached to a probe unit (Type 1014A) whose pulse output was counted on a two-decade fast scaler (Type 1009B) fitted with a three-decade electronic register. Preliminary experiments showed, however, that absorption of carbon dioxide occurred on the plastic surfaces of the scintillation cell, giving a large residual count which persisted for several hours. The small amounts of furan, *etc.*, evolved[24] as reaction products from uronic acids may have had a slight solubilising effect on the plastic surfaces, leading to some entrainment of carbon dioxide. The scintillation cell was therefore replaced by a flow-cell of special design*; the mica end-window (1·9 mg/cm² window density) of a Geiger tube acted as one wall of the cell. The Geiger tube (fed from a power unit, Type 1007 with a suitable potentiometer) was not so efficient as the photomultiplier, but showed much better response to changes in the ^{14}C concentration. It was therefore used in all the experiments reported.

Preliminary experiments with labelled barium carbonate

The decarboxylation apparatus used was originally designed[20] to facilitate both analytical determinations and kinetic studies. The amount of "dead-space" had been minimised, but a potential source of error lay in the possible absorption of carbon dioxide in droplets of water adhering to the cold condenser surfaces. At the outset it appeared that this effect was unlikely to be significant, since the total volume of carbon dioxide liberated in any determination (*e.g.* from 30 mg of a pure uronic acid) does not exceed 4 ml; with a steady nitrogen flow-rate of 15 ml per min through an apparatus of internal volume 150 ml, the partial pressure of carbon dioxide, even at the time of maximum rate of production, is very small. Although the recovery of all carbon dioxide liberated from a carbonate had been carefully verified[20] during the development of the apparatus, the extent of the retention of carbon dioxide by the apparatus was nevertheless investigated.

Labelled barium carbonate was weighed in a small glass bucket and placed in a special reaction flask fitted with a side-arm dropping-funnel for addition of acid (*cf.* ref. 20). The ground-glass joint of this flask was joined to that of a standard decarboxylation apparatus[20] which, immediately previously, had been used in a routine determination so that the surfaces of its cooling coils were coated with the normal equilibrium amount of water. After scavenging the assembled apparatus

* We are grateful to Dr. R. Scott, Biophysics Dept., University of Edinburgh, for making this cell and for placing at our disposal the counting equipment used in these experiments (see *J. Sci. Instr.*, 1961, **38**, 31).

with nitrogen in the usual way,[20] acid was added from the dropping funnel. The experimental conditions were therefore reproduced in all respects except that more dilute acid was used. The stream of flow-gas + reaction products was passed through the counting cell into the standard titration-cell[20] charged with baryta. Table I shows the results of a typical experiment in which the

TABLE I. RECOVERY OF CO_2 FROM $BaCO_3$-^{14}C

Time,* *min*	Total count	Count per min	Dead-time† correction	∴ Corrected count per min	∴ True count per min	Total corrected count
0		47 (background)				
5	3,400	3,539	85	3,624	3,577	3,871
10	40,000	9,900	701	10,601	10,554	42,373
20	196,500	21,000	4,454	25,454	25,407	223,139
30	331,600	8,300	487	8,787	8,740	327,341
40	380,000	2,700	49	2,749	2,702	422,070
60	398,980	300	1	301	251	440,265
90	—	72	—	72	25	—

* Measured from time of switching on heater; reflux began 10 min later.
† From tables.

weight of labelled barium carbonate taken was 7·34 mg, and the weight of carbon dioxide found, as determined by the usual titration-cell procedure, was equivalent to 7·41 mg of barium carbonate. In another experiment, 3·26 mg of labelled barium carbonate were mixed with 10·50 mg of AnalaR barium carbonate; the cell titration gave carbon dioxide equivalent to 13·94 mg of barium carbonate; the initial background count was 54 and the true count per min, after 1 hr, was 11.

Procedure for decarboxylation experiments

The labelled uronic acid was weighed in a small glass bucket and added to the reaction-flask.

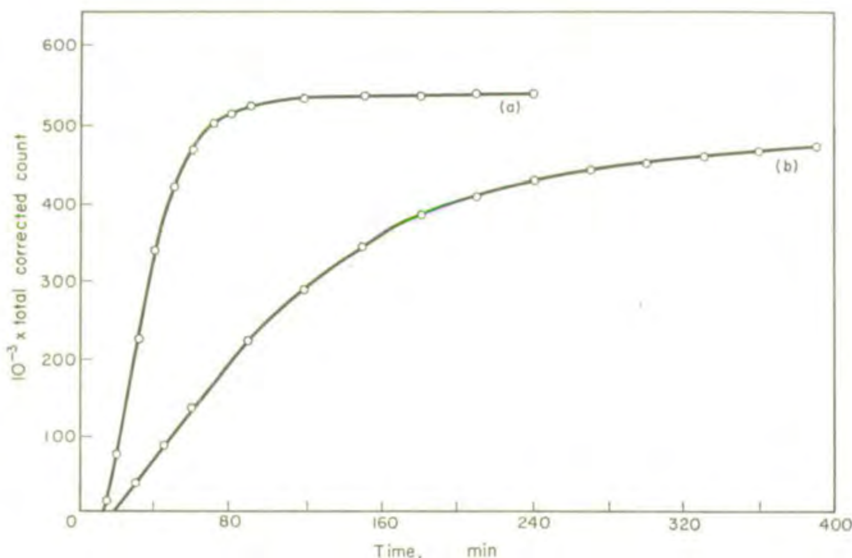

FIG. 1.—Rate of liberation of radioactive CO_2 from glucuronate.
Curve (*a*): 19% (w/w) aq. hydrochloric acid
Curve (*b*): 12% (w/w) aq. hydrochloric acid

The apparatus, reaction conditions, and reagents were all as previously described.[20] The stream of carrier-gas + reaction products was passed *via* the counting cell into the standard titration-cell.[20] Experiments were made with both 12% and 19% (w/w) aq. hydrochloric acid. For convenience, the time at which the heater was switched on was taken as arbitrary zero.

RESULTS

Typical results for the decarboxylation of potassium-D-glucuronate-6-^{14}C (7·10 mg) in 19% (w/w) hydrochloric acid are shown in Table II; the counts quoted are corrected for initial background and for detector dead-time. Table III shows the results obtained when a sample (6·05 mg) was decarboxylated in 12% (w/w) aq. hydrochloric acid.

When the total corrected counts given in Tables II and III are plotted against the quoted values for time, (see Fig. 1) extrapolation of the reaction-curves shows the true zero-time of the reactions to occur at 14 and 18 min respectively. (This arises from two factors: (*a*) Reflux began about 10 min after the heater was switched on; evolution of carbon dioxide begins, however, before reflux temperature is reached. (*b*) For a flow rate of 15 ml per min and a decarboxylation apparatus of volume approx. 150 ml, about 10 min must elapse before the first traces of carbon dioxide liberated are swept through the counting apparatus.)

TABLE II. DECARBOXYLATION IN 19% (W/W) HYDROCHLORIC ACID

Time,* *min*	True count† per min	Total corrected count†	% moles CO_2 per mole glucuronate
0	54 (background)	0	
10	150	455	
15	6,960	15,472	
20	14,301	76,061	
30	13,821	225,346	45
40	10,089	342,793	
50	6,741	420,687	
60	1,161	470,760	
70	2,176	501,370	86
80	1,064	516,229	
90	518	523,367	90
120	173	532,227	
150	96	536,038	92
180	86	538,698	
210	56	540,660	93
240	52	542,442	
270	54	544,220	—

* As in Table I.
† Deduced as shown in Table I.

After correction of the times shown in Table II to the true zero-time of the reaction, calculation of the rate constant by Guggenheim's method[25] gave $k_1 = 7{\cdot}4 \times 10^{-4}$ sec^{-1} for 19% acid. After corresponding correction of the times shown in Table III, subsequent calculation[25] gives the rate constant for 12% acid as $k_1 = 1{\cdot}6 \times 10^{-4}$ sec^{-1}.

DISCUSSION

The barium carbonate experiments indicated that recovery of the carbon dioxide *liberated in a rapid reaction* is quantitative, to within 1%, in 1 hr. This period was

TABLE III. DECARBOXYLATION IN 12% (W/W) HYDROCHLORIC ACID

Time,* *min*	True count† per min	Total corrected count†	% moles CO_2 per mole glucuronate
0	70 (background)	0	
15	291	1,599	
30	3,724	36,680	
45	3,409	88,128	
60	3,204	137,183	40
90	2,472	221,931	
120	2,163	289,088	
150	1,752	344,723	
180	1,140	387,774	
210	421	407,041	
240	623	427,498	88
270	482	444,000	91
300	361	456,309	93
330	220	464,885	
360	180	470,887	97
390	80	473,653	

*, †, As in Table II.

independent of the sample-weight, and there was no prolonged retention of carbon dioxide in the apparatus.

Table II shows that, for 19% acid, the true count per minute reaches a low steady value after 210 min (200 min from start of reflux). This is therefore the time required *for complete decarboxylation* with the present apparatus and reaction conditions. In terms of the total carbon dioxide evolved, however, (*i.e.* labelled + unlabelled) the yield equivalent to the percentage purity of the labelled material is obtained in 140–160 min from start of reflux (*cf.* ref. 20). This is the correct analytical reaction time; although decarboxylation is only 98–99% complete, the total yield of carbon dioxide includes >1% from side reactions. Generally, the correct reaction-time may vary within small limits (say ±10 min) depending on the apparatus design and also, to a small extent, on the flow-rate.

This may explain *small* differences from the reaction-times quoted by certain previous workers. It will not, however, explain the wide range of reaction-times covered when all previous recommendations are considered. The undesirable use of an oil-bath, maintained at 145°, to sustain a reflux temperature of 109° has already received comment.[20]

A similar consideration of the results in Table III shows that, under our conditions, the analytically correct time with 12% acid is 270–290 min from start of reflux. At that time, which agrees well with that proposed by several previous authors,[18,26,27] 97% of the total carbon dioxide comes from decarboxylation and 3% from side-reactions. It now appears that the widely differing times proposed by other authors[11,12,14,28] must be regarded as being in error.

Although the rate of decarboxylation is much slower in 12% than in 19% acid, the rate of evolution of carbon dioxide from the side-reactions involving decarboxylation by-products is little altered (*cf.* ref. 18). Since the reflux period for 19% acid is only half that for 12%, the amount of spurious carbon dioxide formed is proportionately less. This finding is supported by results obtained[21] for the decomposition of non-uronic acid carbohydrate materials. It has been suggested[11,29] that the use of 19% acid introduces errors by increasing the amount of carbon dioxide produced in side-reactions: our results directly oppose these opinions.

In carbohydrate materials, uronic acid groups are generally present in the form of aldobiuronic acids. In these the glycosidic linkage is particularly stable to hydrolysis; a similar effect occurs in polyuronides. Experiments with 2-α-D-galacturonosido-L-rhamnose (isolated[30] from *Plantago arenaria*) and with trigalacturonic acid and alginic acid showed, however, that their decarboxylation curves in 19% hydrochloric acid were closely similar to that of the labelled glucuronate. It is therefore believed that the reaction-times established in this work are generally applicable to heterogeneous polysaccharide systems.

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Zusammenfassung—Die Reaktionszeiten zur vollständigen (99%) Decarboxylierung von Glucuronsäure, die am C_6 mit C-14 markiert ist sind 200 und 360 Minuten im 19 bzw. 12% ige (Gewichtsprozent) Salzsäure, vom Beginn des Rückflusses gerechnet. Die entsprechenden Geschwindigkeitskonstanten erster Ordnung sind $7{\cdot}4 \times 10^{-4}$ und $1{\cdot}6 \times 10^{-4}$ sec^{-1}. Da jedoch Kohlendioxyd durch gleichzeitig ablaufende Nebenreaktionen erzeugt wird, werden analytisch korrekte Ergebnisse (durch Fehlerkompensation) in kürzerer Zeit erhalten, nämlich 140–160 und 270–290 Minuten für die beiden oben genannten Säurekonzentrationen. Die in 19% iger Säure erhaltenen Resultate sind zuverlässiger, da Kohlendioxyd aus unkontrollierbaren Nebenreaktionen zu geringerem Grade entwickelt wird. Dies ist in Gegensatz zu manchen Publikationen.

Résumé—Les temps de réaction, pour la décarboxylation complète (99%) de l'acide glucoronique marqué en C_6 avec du carbone 14, sont respectivement 200 et 360 minutes, au commencement du reflux, pour des solutions aqueuses d'acide chlorhydrique à 19% et à 12% en poids. Les constantes de vitesse du premier ordre correspondantes sont $7{,}4 \times 10^{-4}$ sec^{-1} et $1{,}6 \times 10^{-4}$ sec^{-1}. A cause de la réduction simultanée de l'anhydride carbonique par réactions secondaires, des résultats analytiques corrects sont donnés, par compensation des erreurs, dans des temps plus courts: 140–160 minutes pour l'acide à 19% et 270–290 minutes pour l'acide à 12%. Les résultats indiquent aussi que l'utilisation de l'acide à 19% réduit au minimum l'erreur provenant de la production d'anhydride carbonique dans les réactions secondaires, contrairement aux opinions publiées.

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29.

1033. *Studies on Uronic Acid Materials. Part IV.*[1] *Aqueous Decarboxylation of Uronic Acids, and the Decarboxylation of Pectic Materials during Extraction.*

By D. M. W. ANDERSON, A. M. BEWS, S. GARBUTT, and N. J. KING.

Rate-constants for the decarboxylation of some uronic acids in de-ionised water have been determined. The extent of the decarboxylation of pectic materials when heated with (*a*) aqueous 70% ethanol, (*b*) water, (*c*) aqueous ammonium oxalate and (*d*) aqueous ammonium oxalate–oxalic acid has been investigated. The results are discussed with respect to the extraction procedures now used in structural studies; in particular, extraction with ammonium oxalate–oxalic acid solutions at 85—90° should be avoided.

EXTRACTION is an all-important step in structural studies of polysaccharides: fractionation, degradation, chemical modification, and the creation of artefacts [2] are possible consequences of carelessly designed extraction schemes. In initial investigations, one can only use extraction conditions which are, on the basis of previous experience with some similar material, apparently the mildest available, consistent with extraction of the desired polysaccharide in reasonable yield. Poor yields may imply that only a non-representative fraction has been obtained.[3] Preliminary studies establishing an approximation to the composition of a new material may therefore be necessary before the optimum method of extracting a particular component can be devised; [4] even the cation composition can be important.[5] Often, however, as in starch chemistry (cf. ref. 6), less degraded products of higher molecular weight are obtained only after a continued series of studies has refined the method of extraction.

Studies assessing the degradation suffered by the product during extraction should be complementary to structural studies. All too often, structural studies reported on materials claimed to have been extracted " by the mildest possible means " when, in fact, no experiments were carried out to establish the validity of this statement.

Some polysaccharides, *e.g.*, pectic materials, can at present only be extracted or subsequently purified by methods which degrade and/or chemically modify the material present in the plant. It is then even more important to assess the extent of (*a*) the degradation and (*b*) the modification, such as demethylation and decarboxylation; degradation and chemical modification may be quite independent effects for some polysaccharides. Much is known of the degradation of pectic materials, which can occur in cold alkaline solution,[7,8] buffered (pH 7) " Versene " solution, or, at elevated temperatures, in weakly acidic solution (cf. ref. 9). Vollmert [7] concluded that depolymerisation of pectic materials in alkali ceased when concurrent de-esterification became complete; recent work [10] has shown that only about one de-esterification in 80 leads to cleavage of a glycosidic linkage.

Less is known, however, of the possible modification suffered by pectic materials during extraction. Exhaustive extraction with hot 70% aqueous ethanol [11] and hot water usually precede the extraction of the pectic complex with hot oxalate solutions. This paper shows that some decarboxylation occurs during each of these stages.

In studies of the various methods of decarboxylating uronic acids,[12] refluxing with de-ionised water caused complete decarboxylation of galacturonic acid in about 80 hours in a vessel continuously swept with carbon dioxide-free nitrogen. Kinetic studies of the evolution of carbon dioxide from some uronic acids were then made; these gave the results in Table 1 (it is there assumed that 1 mole of carbon dioxide resulted from decarboxylation of 1 mole of uronic acid and that all the carbon dioxide evolved came from carboxyl groups; under the experimental conditions the amounts of carbon dioxide liberated in

TABLE 1.

Decarboxylation (%) of uronic acids in boiling conductivity-grade water.

Time (10^3 sec.)	Glucurone	Galact-uronic acid	Trigalact-uronic acid	Ca L-sorburonate	Alginic acid
10	5·6	7·8	12·8	35·0	3·7
20	11·1	15·1	24·2	50·9	7·3
30	16·6	21·9	33·6	60·2	11·0
40	21·8	28·3	41·4	66·4	14·6
60	31·5	39·8	54·2	73·3	21·7
80	40·4	50·0	64·1	78·3	28·5
100	48·7	59·2	72·2	87·4	35·1
150	66·3	78·2	87·2	90·2	50·7
200	79·4	91·4	96·0	96·4	65·6
250	88·4	97·8	99·4	98·3	79·4
Hence $10^6 k_1$ (sec.$^{-1}$) =	5·18	6·56	12·5	57·6	1·42

side reactions or from general degradation or decomposition would be expected to be very small; cf. ref. 1).

The rate constants (k_1) were calculated by Guggenheim's method.[13] (The results reported were obtained by using conductivity-grade water; traces of metal ions catalyse decarboxylation,[14] and results obtained by adding salts and other substances will be given elsewhere.)

Alginic acid was the most stable of the materials refluxed with water, yet 7% decarboxylation occurred in 5 hours (*i.e.*, the original uronic anhydride content was reduced from 97 to 90%). A total reflux period of 5 hours would not be excessive if exhaustive aqueous extraction were required; it is difficult to remove starch completely by aqueous extraction,[15] and the contamination of pectins by starch is well known.[16] The study of aqueous decarboxylation was therefore extended to a commercial apple pectin, pectic acids from *N. translucens* [17] and *C. australis*,[18] ammonium pectates from *N. translucens*,[17] and lucerne.[19] Reflux in aqueous solution for several hours caused significant decarboxylation, as shown by the percentages quoted in Table 2. The same Table shows also that some decarboxylation occurs on prolonged refluxing with 70% aqueous ethanol.

After exhaustive extraction with 70% ethanol and with hot water, pectic substances are usually extracted by repeated treatments at 80—90° with 0·5% aqueous ammonium oxalate.[11,19,20] The percentage decarboxylation caused by this reagent is shown in Table 2. In recent years, extraction with hot water containing 0·25% of oxalic acid and 0·25% of ammonium oxalate has been preferred for certain materials.[11,21] Our experiments have shown, however (see Table 2), that this extractant causes extensive decarboxylation. (Control determinations showed that the carbon dioxide evolved did not result from decomposition of the oxalate solution.)

The pectic acid and ammonium pectate samples studied had all been isolated by hot oxalate extraction of material which had been pretreated with 70% ethanol and with hot water, yet carbon dioxide was evolved from each sample on further treatment with these extractants. In view of the experimental conditions, it seems reasonable to postulate that this carbon dioxide arose from decarboxylation, although there may be a small contribution from general decomposition or degradation. Clearly, the samples must therefore have undergone decarboxylation to at least a similar extent during their original extraction from the plant material. The present results indicate that the customary extraction solutions cause decarboxylation of pectic materials to the following extent: (*a*) ~0·2% per hr. in refluxing 70% aqueous ethanol; (*b*) 0·4—0·6% per hr. in refluxing hot water; (*c*) 0·3—0·9% per hr. on extraction at 85—90° with 0·5% aqueous ammonium oxalate; (*d*) 1·4% per hr. on extraction with water containing 0·25% each of ammonium oxalate and oxalic acid at 85—90° for three laboratory-prepared specimens and 0·6—0·7% per hr. for commercial apple pectin. Contact with each hot extractant for 4 hours in the presence of nitrogen will cause a total of 3—6% decarboxylation if 0·5% aqueous ammonium

TABLE 2.

The apparent percentage of decarboxylation * of some pectic materials when treated with aqueous solutions used in extraction.

Samples ‡ decarb-oxylated	Extractant and temperature	Period of extraction † (hours) 2	4	5	10	12	20	24	32	40	50	60
1	De-ionised	2·7	4·1	—	—	5·0	—	6·1	—	—	—	—
2	water,	1·8	2·6	—	—	5·1	—	7·6	—	—	—	—
3	b. p.	0·9	1·6	—	—	4·2	—	9·4	—	—	—	—
4		1·4	3·2	—	—	6·9	—	10·9	—	—	—	—
2	70% aq.	—	1·0	—	—	—	1·5	—	—	—	3·0	—
3	ethanol,	—	1·5	—	—	—	2·3	—	—	—	4·1	—
5	b. p.	—	1·6	—	2·4	—	4·5	—	7·0	—	11·9	—
6		—	1·8	—	3·4	—	6·2	—	9·1	—	14·6	—
7		—	1·9	—	3·9	—	6·8	—	9·6	—	15·2	—
1	0·25% aq.	—	4·9	—	—	—	—	28·0	34·2	—	—	—
2	ammonium	5·9	7·9	—	—	—	—	31·0	36·0	—	—	—
3	oxalate	2·7	3·8	—	5·9	—	—	14·4	—	24·0	28·0	—
4	+	2·2	6·0	—	—	—	—	32·0	38·4	—	—	—
5	0·25% aq.	—	—	4·6	7·5	—	14·3	—	—	26·6	—	36·2
6	oxalic acid	—	—	5·0	8·4	—	15·9	—	—	29·2	—	40·2
7	85—90°	—	—	5·8	10·1	—	19·4	—	—	36·1	—	48·8
1	0·5% aq.	1·0	—	—	4·8	5·7	—	11·5	—	—	—	—
2	ammonium	5·0	6·6	—	—	10·0	12·0	—	—	—	—	—
3	oxalate,	1·4	—	—	—	4·3	—	5·9	—	—	—	—
4	85—90°	1·6	2·6	—	—	6·1	—	9·6	—	—	—	—

* Values shown are the average of duplicate determinations made on each material under the stated conditions; agreement in the duplicate determinations was within ±10%.

† In presence of nitrogen.

‡ Origin of samples: 1, ammonium pectate from N. *translucens*; [17] 2, pectic acid from *C. australis*; [18] 3, commercial apple pectin; 4, ammonium pectate from lucerne; [19] 5, alginic acid; [23] 6, glucurone; 7, galacturonic acid.

oxalate is used; the total may reach 8% if the ammonium oxalate–oxalic acid treatment is employed. Increasing the extraction times increases the extent of the decarboxylation.

It is of interest that, in both the oxalate extractants, the commercial apple pectin (MeO = 5·1%) was more stable than the laboratory-prepared pectic acids, which contained no methoxyl. The closely related behaviour of the products from lucerne, *N. translucens*, and *C. australis* is interesting since the absence of methoxyl groups in the lucerne product was a consequence of de-esterification during isolation; [19] the products from *Nitella* and *Chara* came from algae whose cells contained no methoxyl.[17,18]

Previous workers [9,10] have established that the alkali-sensitivity of the 1,4-bonds in pectic materials depends on the presence of ester groups at position 6, the de-esterified product being practically stable to further degradation.[7,10] A second effect can now apparently be distinguished; de-esterified or initially non-esterified pectic materials are less stable to chemical modification than are esterified products.

Clearly, contact with any hot extractant must be as brief as possible if significant decarboxylation of pectic materials is to be avoided: in particular, hot oxalate–oxalic acid solutions cause considerable decarboxylation. If use of hot extraction solutions is unavoidable, the extent of the chemical modification suffered by the pectic material should be assessed, before structural studies, by decarboxylation experiments on the extracted material.

EXPERIMENTAL AND RESULTS

Origin of Samples.—(*a*) D(+)-Galacturonic acid monohydrate (Roche Biochemicals Ltd.) (Found: C, 34·8; H, 5·7. $C_6H_{12}O_8$ requires C, 34·0; H, 5·7%) had 96% purity [decarboxylation [22] in 19% (w/w) hydrochloric acid for 2½ hr.]; paper chromatography showed a trace of galactose. (*b*) D-Glucurone (Roche Biochemicals Ltd.) (Found: C, 40·8, 40·7; H,

4·6, 4·6. $C_6H_8O_6$ requires C, 40·9; H, 4·6%) had 97·2% purity. (*c*) Alginic acid was the sample prepared by cold extraction by Chanda *et al.*,[23] which had 97·4% purity. (*d*) Tri-galacturonic acid and calcium 5-oxo-D-gluconate (calcium L-sorburonate) were kindly provided by Dr. W. W. Reid.[24] The former was free from mono- and di-galacturonic acid but contained a small quantity of a polygalacturonic acid (detected by chromatography); it had 95·0% purity. The 5-oxo-D-gluconate was 93% pure. (*e*) Commercial apple pectin (B.D.H. Ltd., 240 grade) had uronic anhydride [22] 58%, OMe [25] 5·1%. (*f*) Ammonium pectate from *Nitella translucens* [17] had no methoxyl content,[25] uronic anhydride [22] 51%. (*g*) Ammonium pectate from lucerne [19] had no methoxyl and 51% of uronic anhydride. (*i*) Pectic acid from *Chara australis* [18] had no methoxyl and 52% of uronic anhydride.

Apparatus.—Anderson's decarboxylation apparatus and reaction conditions [22] were used. A reaction flask, having a thermometer-pocket sealed into the flask-wall, was used in experiments where temperatures other than reflux temperature were used. Kinetic measurements were facilitated by fitting a two-way stop-cock after T_2 (see ref. 22) so that the gas stream could be switched from one absorption trap to a second identical trap. A matched pair of traps was used; in these, the sintered discs had closely matching porosity so that identical back pressures, and hence constant reflux temperatures, were maintained. The nitrogen flow-rate was stabilised by a capillary-buffer system, needle valves, and a Rotameter.

Decarboxylation of Uronic Acids in Water.—Conductivity-grade water from a laboratory de-ioniser was refluxed in the decarboxylation apparatus, then allowed to cool in an atmosphere of carbon dioxide-free nitrogen. The weighed sample of uronic acid was added to the reaction flask. The carbon dioxide evolved was determined at intervals during the refluxing; the results are shown in Table 1. Control experiments confirmed that carbon dioxide was not evolved when water alone was refluxed. The decarboxylation of uronic acids in acid solution has been shown to be of the first order; [12] on the assumption that this applies also to aqueous solutions, the rate constants shown in Table 1 were calculated by Guggenheim's method,[13] which does not require a knowledge of the precise purity of the substrates.

Decarboxylation of Pectic Materials in Hot Water.—Samples (110—190 mg.) of commercial apple pectin, ammonium pectates from *N. translucens* and lucerne, and pectic acid from *C. australis* were refluxed with conductivity-grade water; the carbon dioxide evolved was determined at intervals up to 24 hr. from the start of refluxing. It has been shown [1] that 98% of the carbon dioxide liberated when uronic acid materials are refluxed in acid solution comes from the carboxyl groups. On the assumption that this will also hold for aqueous solutions, yields of carbon dioxide were expressed as percentage decarboxylation. The results from the hot-water experiments are shown in Table 2. Each result is the average of duplicate runs made with the substance named; agreement to within $\pm 10\%$ was always obtained.

Decarboxylation in Aqueous 70% Ethanol.—Samples (120—200 mg.) of galacturonic acid, glucurone, alginic acid, commercial apple pectin, and pectic acid from *C. australis* were refluxed with 70% aqueous ethanol, and the carbon dioxide liberated was determined at intervals up to 50 hr. from the start of refluxing. A very small amount of carbon dioxide was evolved when 70% aqueous ethanol was refluxed alone ($\ngtr 5\%$ of that evolved when substrates were present) and the yields of carbon dioxide obtained were corrected for this. On the assumption that the corrected yield of carbon dioxide came from carboxyl groups, the results are shown in Table 2.

Decarboxylation in 0·25% Aqueous Ammonium Oxalate + 0·25% Oxalic Acid Solution at 85—90°.—Samples (100—150 mg.) of galacturonic acid, glucurone, alginic acid, commercial pectin, pectic acid from *C. australis*, and ammonium pectates from lucerne and *N. translucens* were treated with water containing 0·25% each of ammonium oxalate and oxalic acid, maintained at 85—90°. The carbon dioxide liberated was determined at intervals up to 60 hr. The yields of carbon dioxide were corrected for the very small amounts of carbon dioxide detected when the hot oxalate solution alone was maintained at 90°. The percentages shown in Table 2 were obtained from calculations based on the corrected yields of carbon dioxide.

Decarboxylation with Aqueous 0·5% Ammonium Oxalate at 85—90°.—Samples (100—150 mg.) of commercial apple pectin, ammonium pectate from lucerne and *N. translucens*, and pectic acid from *C. australis* were treated at 85—90° with 0·5% aqueous ammonium oxalate. The carbon dioxide evolved was determined at intervals up to 24 hr. from the start of refluxing. Very small amounts of carbon dioxide were detected when the ammonium oxalate solution alone was kept at 90°. The percentages shown in Table 2 were calculated from the corrected yields of carbon dioxide.

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30.

STUDIES ON URONIC ACID MATERIALS—V*

THE THERMAL DECARBOXYLATION METHOD OF ANALYSIS

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Summary—The thermal decarboxylation method of determining uronic acids, proposed by Perlin, has been investigated. A specially designed oven, having good temperature stability, enabled the thermal decomposition of various compounds to be studied kinetically, with good reproducibility. Comparatively large errors can arise in the determination of the carbon dioxide evolved; these errors originate in the nature of the other volatile reaction products. Typical results are presented, and it is concluded that thermal decarboxylation does not compare favourably with the 150-min acid decarboxylation as a general analytical method.

VARIOUS aspects of the acid decarboxylation of uronic acid groups have been investigated[1,2,3] in attempts to improve the accuracy and reliability of this analytical reaction,[4] which is important in carbohydrate chemistry. The formation of potentially acidic vapours, such as methyl chloride and methyl formate, gives a source of error; a knowledge of these products is also important in studies of the reaction mechanism. A comprehensive study[5] was therefore made of the volatile reaction products from some 80 carbohydrate materials and related compounds: a vapour-phase infrared method[6] showed that the products included furan, 2-methylfuran, acetone, methylethylketone, diethylketone, acetaldehyde, propionaldehyde, chloral, methyl formate, methyl chloride and dimethylformal, although not all of these are formed from any one sugar residue. Acetone was known to be an acid decomposition product of rhamnose[7] and other sugars,[8] but the formation of some of the other compounds was unexpected. They have, however, been found subsequently[9] as products of the thermal decomposition of various carbohydrate materials. Under other experimental conditions the formation of other volatiles has also been reported, *e.g.*, methyl glyoxal, acetol and diacetyl from reducing sugars,[10] maltol and isomaltol[11] from the pyrolysis of starch.

There have been many studies of the formation of pyrodextrins[12,13] and the production of volatile organic compounds during thermal decomposition is, of course, well known[14] (*cf.* ref. 9). This formation of organic vapours by chemical reaction must be distinguished from the desorption of physically retained organic solvents. Although the latter effect has long been recognised,[15] and further examples continue to be reported,[16] it is now known[17] that solvent-retention continues up to the temperature at which the carbohydrate concerned begins to decompose. A knowledge of the pretreatment of the sample is therefore important in thermal decomposition studies.

Differential thermal analysis has been applied to starches[18] and other polysaccharides;[19] it has been suggested that decarboxylation causes a small endothermic

*Part IV: D. M. W. Anderson, A. M. Bews, S. Garbutt and N. J. King, *J. Chem. Soc.*, 1961, 5230.

reaction observed for uronic acid materials at 145–155°. Although these temperatures are only slightly higher than those at which carbohydrate decomposition was observed to begin,[17] they are some 100° lower than the decomposition temperatures proposed for the thermal decarboxylation method[20] of estimating uronic acids.

Since it appeared that Perlin's method[20] has not been widely adopted or studied further (*cf.* ref. 20), an examination of its applicability appeared to be a logical extension of our studies. This paper presents some results which show that thermal decarboxylation is inferior, for several practical reasons, to the acid decarboxylation reaction as a quantitative method[2,3] for the analysis of uronic acid contents.

EXPERIMENTAL

Design of apparatus

In Perlin's experiments,[20] the method of heating involved the refluxing of vapours. Whilst this method offers the advantage that over-heating cannot possibly occur, it is restrictive, since a continuous range of temperatures cannot be studied. Moreover, the absolute reaction temperature could not be established:[20] the reaction conditions are critical[13] in this type of investigation.

Preliminary investigations[5] revealed that careful experimental design would be required to give minimum temperature equilibration times with run-to-run reproducibility at absolute temperatures. Successive refinements with different designs of apparatus showed that, after the required stability of oven temperature was achieved, the following factors were also critical: (*a*) stabilisation of rate of flow-gas, (*b*) pre-heating of flow-gas to the required reaction temperature, (*c*) reproducible location of samples at the same position within the oven, (*d*) measurement of the actual sample temperature during the decomposition.

The apparatus finally satisfying all these requirements is represented diagramatically in Fig. 1. With its use, the rate of decomposition of a particular sample was reproducible to within ±2%.

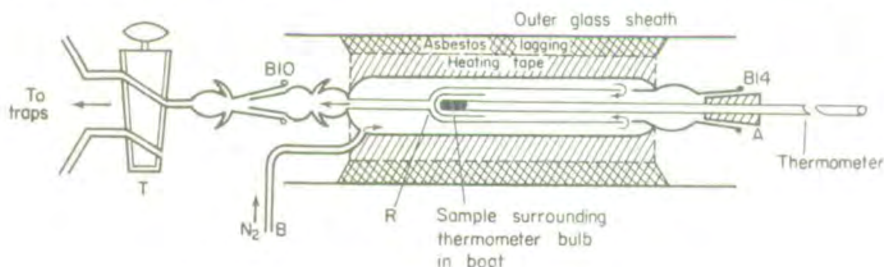

FIG. 1.—Construction of thermal decomposition apparatus.

Constructional details: The internal dimensions are a function of the size and shape of the sample-boat which, in turn, is dictated by the weight of sample necessary for the production of suitable quantities of carbon dioxide. Suitable sample weights were 30 mg for uronic acids and polyuronides, 100 mg for non-uronides. The boat must be sufficiently deep to contain the increased volume occupied by the samples upon decomposition: porcelain boats, 35 × 12 × 6 mm, were suitable.

By grinding off one end (the "hook" end) of a boat, a form of long-handled weighing-spoon was made, with a thermometer, bound to the boat by several turns of platinum wire, acting as the handle; samples were added carefully so as to surround the mercury bulb. A tightly-fitting rubber stopper was positioned on the thermometer stem at a part of the scale where readings were not required. The position chosen dictated the effective length of the "weighing spoon" handle; the constriction in the bore of the oven was positioned during manufacture so as to provide a locating device against which samples could be positioned centrally and reproducibly within the oven at its hottest part.

Heating: The external dimensions of the oven, 17 cm × 2.3 cm, were chosen so that its whole surface could be wound with a heating tape, 30″ long, 1″ broad ("Electrothermal" type HT 350). The heating tape was lagged with a layer of asbestos tape, and enclosed within an open-ended tightly-fitting glass sleeve, 25 cm long and 4 cm in diameter, which enclosed the whole assembly from A to B. Temperatures could be varied within the range 140–300° by adjustment of the Variac which supplied the continuous heating voltage. For particular temperatures, long- and short-period temperature variation did not exceed ±1° after several hours had been allowed for the establishment of equilibrium conditions.

Flow-gas: Cylinder nitrogen ("N.O.F." grade), dried and freed of traces of carbon dioxide by passage through scrubbing towers, was used. The flow-rate was established at 15 ml per min by means of two needle valves, a capillary-tube buffer system, and a calibrated Rotameter. For some experiments, residual traces of oxygen (approx. 20 ppm) in the cylinder gas were also removed by passage through a "Nilox" purifier (Southern Instruments Ltd.). Inclusion of the nitrogen inlet system within the jacketed heating assembly ensured that the flow-gas was correctly pre-heated.

Use of apparatus

Determination of the carbon dioxide evolved: Kinetic measurements were facilitated by a two-way stop-cock system and matched pairs of traps, as described.[21] Determinations were made (*a*) titrimetrically using a titration-cell designed[2] for carbon dioxide titrations, (*b*) gravimetrically, using conventional analytical adsorption tubes, (*c*) by infrared spectroscopy,[6] using the manometric calibration method.

Use of sulphuric acid bubblers: In some experiments, as indicated below, the gas-stream from the oven was led through a conventional gas-bubbler before determination of the carbon dioxide.

Detection of carbon monoxide: Standard colorimetric reactions[22] were used. The molybdenum blue method could not be used when sulphur dioxide was also present (*cf.* ref. 22, p. 347), and the palladous chloride method was therefore employed.

Identification of the volatile products: For each compound studied separate runs, using samples of 500 mg, were made so that the volatile products (before and after passage through the sulphuric acid bubbler) could be trapped in liquid nitrogen[23] and subsequently examined by the vapour-phase infrared technique.[6] (*Cf.* ref. 17).

Procedure: With the nitrogen flow-rate stabilised at 15 ml per min and with a thermometer-handled weighing-spoon in position, the oven temperature was allowed to equilibrate (usually overnight) at the required temperature. The weighing-spoon was withdrawn and allowed to cool, stop-cock T (Fig. 1) being closed so that the flow of nitrogen, emerging at A, prevented air from entering the oven. The sample, weighed by transfer from a suitable weighing-scoop, was added to the thermometer-spoon so as to surround the thermometer-bulb. The thermometer-spoon was replaced carefully in the oven, and stop-cock T opened. Preliminary experiments with water had established the internal volume (V ml) of the system from restriction R to T; $V/15$ min after opening T was taken as the zero-time for the kinetics. The sample reached the pre-established equilibrium temperature of the oven after about 5 min (*cf.* ref. 20). Thus results quoted below for "20 min at 255°" are, in effect, for 5 min heating over the range 0–255° + 15 min heating at 255°.

Origin of samples

The samples of glucurone and galacturonic acid monohydrate had 97% and 96% purity, respectively, by acid decarboxylation,[3] and have been described.[21] The alginic acid, pectic acid and pectin were commercial samples, for which acid decarboxylation[3] gave 79%, 61% and 57% respectively: the pectic acid and pectin contained 0.1% and 6.5% of methoxyl, respectively. The sample of *Acacia seyal*[24] gum contained 12% of glucuronic acid, and 1% of methoxyl.

The non-uronic samples studied were of commercial origin and were chromatographically pure.

RESULTS AND DISCUSSION

1. *Uronic acids and polyuronides*

Fig. 2 (dotted curves) shows the results obtained titrimetrically for decomposition at 255°; for temperatures between 255° and 280° the initial rate of evolution of carbon dioxide was faster, but the curves levelled off more quickly, so that the total yields were not significantly greater. Above 280°, however, the decomposition mechanism apparently changes, since the yields of carbon dioxide were considerably increased. Thus, after decomposition for 1 hr at 300°, titrimetric determinations indicated apparent uronic acid contents of 96%, 107% and 87% for glucurone, galacturonic acid and alginic acid respectively.

The titrimetric values for the yield and rate of evolution of carbon dioxide at 255° were much less than were reported by Perlin.[20] The experiments were therefore repeated, the carbon dioxide being determined gravimetrically. The apparent yields of carbon dioxide, as shown in Fig. 2 (full lines) agreed much more closely with Perlin's

values, the acid decarboxylation values for galacturonic acid and alginic acid—but not for glucurone—now being attained in approx. 20 min. Reasons for the large discrepancy between the titrimetric and gravimetric results had therefore to be found.

Investigation of the volatile reaction products: Carbon monoxide was detected colorimetrically, in agreement with Perlin.[20] Vapour-phase infrared spectroscopy showed that, after removal of carbon dioxide and water, the volatile products included acetone and other carbonyl compounds. Although these were formed in considerable quantity (*cf.* Perlin's "distillable oils") they were evidently of low volatility and their identification lay outside the applicability of the vapour-phase spectroscopic method.

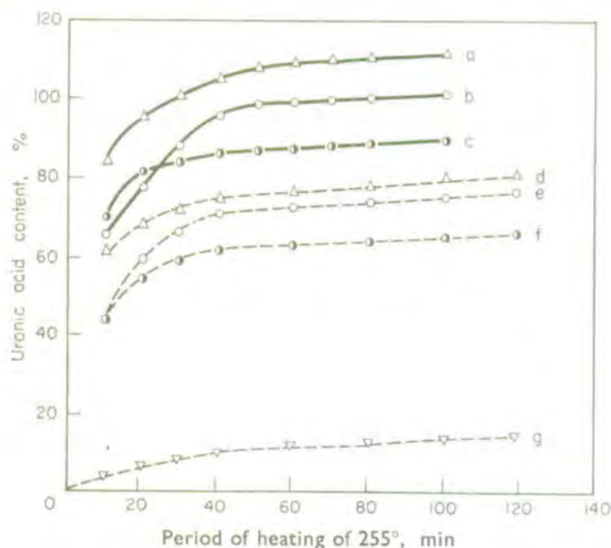

FIG. 2.—Rate of evolution of carbon dioxide from uronic compounds at 255°

Curve (*a*) galacturonic acid, (*b*) glucurone, (*c*) alginic acid — gravimetric determination of carbon dioxide

Curve (*d*) galacturonic acid, (*e*) glucurone, (*f*) alginic acid, (*g*) *Acacia seyal* gum — titrimetric detetermination of carbon dioxide

When these reaction products were passed through a sulphuric acid bubbler, spectroscopy showed that although the carbonyl compounds had been removed, sulphur dioxide and increased quantities of carbon monoxide, together with traces of carbon dioxide, were now present in the carrier-gas emerging from the bubbler. Evolution of these acidic gases continued for several hours after the end of a run. The thermal decomposition products must therefore include significant quantities of formic acid and other organic acids (*cf.* ref. 22, p. 347).

Infrared estimations of the amounts of carbon dioxide present in the mixture of acidic decomposition products indicated that direct titrimetric determinations gave the most reliable estimation of the actual carbon dioxide content. Titrimetric results are considerably higher if a sulphuric acid bubbler is used, as shown in Table I; the increased apparent uronic acid content results from the acidic gases evolved from the scrubber.

Fig. 1 also shows the corresponding figures for gravimetric determinations. Without a sulphuric acid bubbler, the result is virtually the total of all the volatile products: insertion of the bubbler gives lower results, which, however, are still high as a result of the acidic vapours released from the bubbler. A measure of these is given in Table I, column (*c*). When this is applied as a correction to column (*e*), the values expressed in columns (*a*) and (*f*) are, for experiments of this kind, in reasonable agreement with the estimations given by the specific infra-red method.

TABLE I. DEPENDENCE OF THE APPARENT PERCENTAGE URONIC ACID CONTENT ON THE METHOD OF ESTIMATING THE CARBON DIOXIDE EVOLVED*

Compound	Titrimetric			Gravimetric			Spectroscopic
	(*a*) Without H_2SO_4 scrubber	(*b*) With H_2SO_4 scrubber	(*c*) Difference (*b*–*a*)	(*d*) Without H_2SO_4 scrubber	(*e*) With H_2SO_4 scrubber	(*f*) Corrected Value (*e*–*c*)	
Glucurone	73	84	11	99	88	77	70–75
Galacturonic acid	77	91	14	109	97	83	75–80
Alginic acid	64	72	8	87	77	69	65–70

* All results quoted refer to decarboxylation at 255° for 1 hr.

TABLE II. APPARENT PERCENTAGE URONIC ACID CONTENT OF SOME SUGARS

Sugars	Thermal Decarboxylation at 255°				Decarboxylation in 19% HCl		
	30 min	60 min	90 min	120 min	50 min	100 min	150 min
Glucose	3·2	5·8	7·1	8·6	1·0	1·6	1·9
Galactose	2·5	5·8	6·2	8·4	1·4	2·2	2·9
Mannose	3·2	6·6	7·3	8·9	2·1	3·0	3·5
Arabinose	3·6	6·0	6·3	6·7	0·9	1·4	1·8
Xylose	4·7	6·6	8·5	9·7	1·1	1·4	1·6
Rhamnose	4·1	6·4	7·5	8·3	1·9	2·7	3·2
Fucose	5·2	6·7	7·4	8·2	1·2	2·1	2·8

TABLE III. APPARENT PERCENTAGE URONIC ACID CONTENT OF GLUCOSE ON THERMAL DECOMPOSITION AT DIFFERENT TEMPERATURES

Temp., °C	Period of heating, *min*			
	30	60	90	120
245	0·6	2·5	4·1	5·7
255	3·2	5·8	7·1	8·6
265	4·1	7·6	9·8	11·0

2. *Non-uronic, non-methylated sugars*

Direct titrimetric determinations of the carbon dioxide evolved at 255° gave the results shown in Table II. These results are similar to those reported by Perlin,[20] who found apparent uronic acid contents of "about 3% in 15 min" for reducing sugars.

When the values found are compared in Table II with values obtained by the 150-min acid decarboxylation method,[3] however, it is apparent that the thermal decomposition method gives relative errors which are considerably greater for most of the common sugars studied.

Temperature-dependence of the decomposition: Table III lists the results found by direct titration for glucose at 245°, 255° and 265°.

Volatile decomposition products other than carbon dioxide and water: (*a*) For glucose, galactose and mannose, only trace amounts of volatile products were formed, the main component being acetaldehyde. Gravimetric experiments, with and without sulphuric acid scrubbing, showed that the results were only marginally greater than those quoted in Table II.

(*b*) For arabinose, rhamnose and fucose, however, much larger amounts of acetaldehyde were formed, and gravimetric results were up to 50% higher than those shown in Table II, *e.g.*, for 1 hr reaction at 255°, arabinose gave 9·4% and rhamnose gave 10% apparent uronic acid content.

TABLE IV. COMPARISON OF THE APPARENT PERCENTAGE URONIC ACID CONTENTS OF METHYLATED COMPOUNDS AS FOUND BY DIFFERENT METHODS

Compound	Thermal decomposition for 1 hr at 255°			Decarboxylation for 2·5 hr in 19% HCl		Spectroscopy
	Volatile decompn. products	Titrimetric detn.	Grav. detn.	Volatile decompn. products	Titrimetric detn.	
2:3:4:6-tetra-*O*-methyl glucose	carbon dioxide methyl formate	*	*	carbon dioxide methyl chloride	1·7	0·8
3-*O*-methyl-glucose	carbon dioxide methyl formate	*	*	carbon dioxide methyl formate methyl chloride	2·4	0·8
α-Methyl-glucoside	carbon dioxide methyl formate	*	*	carbon dioxide methyl formate methyl chloride	3·1	1·4
Pectic Acid	carbon dioxide acetaldehyde	59	68	carbon dioxide furan methyl formate	61·8	60–61
Pectin	carbon dioxide methyl chloride methyl formate	54·0	63·0	carbon dioxide furan methyl formate methyl chloride	57·0	55–56

* Determinations not possible on these compounds, which volatilise before extensive decomposition begins.

3. *Methylated sugars and polysaccharides*

Although 2,3,4,6-tetramethyl-D-glucopyranose is more stable than D-glucose in acid solution,[25] methylated sugars yield acidic decomposition products on acid decarboxylations.[1] Table IV shows some typical results for 150-min acid decarboxylation,[3] infrared experiments showing that >50% of the apparent acidity is through volatile products other than carbon dioxide.

As a result of the effects already discussed, gravimetric determinations of methylated polysaccharides give high results.

4. *Minimum temperature required for effective thermal decarboxylation*

Our results have indicated that thermal decomposition is markedly temperature-sensitive in the range 245°–265°, with a distinct change in the decomposition mechanism above 280°. Carbohydrate materials begin to decompose[17] at about 130°, and the suggestion[19] that decarboxylation of uronic acid groups occurs at 145–155° made us investigate if any analytically useful, more specific, decarboxylation reaction would be given at lower decomposition temperatures.

For *Acacia seyal* gum, heating for 2.5-hr periods at each of 165°, 180° and 200° gave apparent uronic contents of 0·7%, 1·0% and 2·8%, respectively. For alginic acid evolution of carbon dioxide became detectable at 140° and estimable on heating for 2 hr at 145°; the results of various experiments are shown in Table V.

These experiments were sufficient to show that (*a*) the extent of decomposition depends on the uronic material studied, and (*b*) the time required for complete thermal decarboxylation would be much greater than the 150-min required for acid decarboxylation. The investigations were therefore discontinued, although it appears that the very small endothermic reaction observed by Chesters and Thomson[19] at 145–155° cannot result directly from "decarboxylation".

TABLE V. THERMAL DECOMPOSITION OF ALGINIC ACID

Decomposition temp., °C	Period of heating, *hr*	Apparent uronic acid, %
165	2·5	11·2
165	16	41·6
180	2·5	40·0
200	2·5	51·0

CONCLUSIONS

Decomposition at 255° for 20 min gave quantitative results for galacturonic and alginic acids if a *gravimetric* finish was used. The results are distinctly time-dependent (see Fig. 2) and considerable over-decarboxylation can occur. Furthermore, the rate of evolution of carbon dioxide varies for different compounds; the required yield of carbon dioxide is only given after 40 min for glucurone and after 60 min for *Acacia seyal* gum. In no case was a quantitative yield obtained in 15 min at 255°, as was reported by Perlin.[20] The reason for this is difficult to understand. The time taken for samples to attain the oven temperature was very similar to that reported by Perlin: the temperatures reported here were actual *sample* temperatures, not nominal oven or retort temperatures; the zero-time used for the kinetic measurements was corrected to allow for the internal volume of the oven, which had no "dead-space".

In investigations of this kind, the reaction kinetics and the results may be dependent on the apparatus design, on the surface area of the sample, and on its rate of increase in temperature; in agreement with Perlin,[20] all that can be done is to ensure, by careful apparatus design, that all the reaction conditions and variables are closely reproducible from run to run for different compounds.

The amounts of carbon monoxide detected agreed with those reported.[20] The nitrogen flow-gas used in most of our experiments contained approx. 20 ppm of

oxygen; when this was removed the results, as reported,[20] were not significantly altered.

It is of fundamental importance that when only the acidity from carbon dioxide is measured, thermal decarboxylation is not complete in 2 hr at 255°. The considerable differences between the gravimetric and titrimetric results are a consequence of the volatile reaction products which accompany the carbon dioxide; these products affect the gravimetric determination to a large extent. It is of interest that the volatilisable reaction products react slowly in cold concentrated sulphuric acid, liberating acidic vapours which give an additional source of error.

These facts must be considered together with the marked temperature- and time-dependence of the reaction, the differing reaction-times required for different compounds, and the relatively larger errors caused by side-reactions and the decomposition of non-uronic materials. Thermal decarboxylation, in our experience, does not compare favourably with acidic decarboxylation as a reliable reaction of general applicability for the analysis of materials containing uronic acid groups.

Acknowledgements—We thank Professor E. L. Hirst, C.B.E., F.R.S., for his interest in these studies and the Department of Scientific and Industrial Research for maintenance grants (to S. G. and J. F. S.).

Zusammenfassung—Die von Perlin vorgeschlagene Methode zur Bestimmung von Uronsäure durch thermische Decarboxylierung wurde untersucht. Ein speziell konstruierter Ofen, mit besonders hoher Temperaturkonstanz, gestattete die thermische Zersetzung verschiedener Verbindungen mit guter Reproduzierbarkeit kinetisch zu studieren. Verhältnismässig grosse Fehler können bei der Bestimmung der entwickelten Kohlensäure auftreten. Resultate werden mitgeteilt und es wird geschlossen, dass die thermische Decarboxylierung mit der 150-Minuten-Säuredecarboxylierung als allgemeine analytische Methode nicht konkurrieren kann.

Résumé—Les auteurs ont étudié la méthode de décarboxylation thermique des acides uroniques, proposée par Perlin. Un four conçu spécialement, ayant une bonne stabilité de température, permettait d'étudier cinétiquement la décomposition thermique de différents composés, avec une bonne reproductibilité. Des erreurs comparativement grandes peuvent se produire dans le dosage de l'anhydride carbonique dégagé; ces erreurs dépendent de la nature des autres produites de réaction volatils. Des résultats caractéristiques sont donnés et les auteurs concluent que la décarboxylation thermique ne peut être comparée favorablement à la décarboxylation acide pendant 150 minutes comme méthode analytique générale.

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31.

1. *Studies on Uronic Acid Materials. Part VI.* The Variation in Composition and Properties of Gum Nodules from* Acacia seyal *Del.*

By D. M. W. Anderson and M. A. Herbich.

As a preliminary to studies of the chemical structure of *Acacia seyal* gum, individual nodules of authenticated origin have been examined as crude gum and after purification by (i) precipitation, (ii) electrodialysis, and (iii) ion-exchange. Chemical composition and physical behaviour vary from nodule to nodule. The low natural nitrogen content is not decreased by any of the purification methods used. Passage through a column of diethylaminoethylcellulose shows that the purified gum from individual nodules is heterogeneous, two components being present. The proportion of component A in different nodules varies from 34% to 41%; components A and B contain 12·5% and 15·3%, respectively, of glucuronic acid.

As a result of specific immunological reactions [1] and electrophoresis studies,[2,3] it is now accepted [4-6] that gum arabic (*Acacia senegal* syn. *verek*) is a mixture of polysaccharides of similar composition; [1] no single over-all formula has significance,[5] and only general features can be indicated.[4] Early studies [7] have been criticised [5,6,8] on the grounds that composite commercial samples, inadequately authenticated, were used. Although it had been suspected,[1,9] despite some evidence to the contrary,[10] that different samples of certain plant gums varied in chemical constitution, the possible range of variation was not known until single nodules of *Combretum leonense* gum were studied.[11] The results implied that fine-structural differences exist from nodule to nodule, so that alcoholic precipitation of bulk material from an aqueous solution of many nodules produces a complex mixture of closely similar polymeric systems. Whenever sample size permits, it is therefore desirable to assess the extent of inter-nodule variation and to make structural studies on the simplest form of the polymer available, *i.e.*, that given by a single nodule, which itself may be polymolecular and/or polydisperse (terminology as in ref. 12).

Before studying the chemical structure of the components of *A. seyal* gum, we have investigated the extent to which a number of authenticated nodules vary in properties, in composition, and in heterogeneity (cf. ref. 6, p. 54).

Experimental

Collection and Origin of Specimens.—We are grateful to Mr. P. Vidal-Hall, Gum Research Officer to the Sudan Government, who collected suitable gum nodules from the red-barked *A. seyal* Del. (a close variant, *A. seyal* var. *fistula*, has a grey bark). *A. seyal* is not normally "tapped," and the nodules originated from "natural exudation." The nodules, taken only from trees which could be authenticated, were packed individually and despatched in sealed tins. Nodules I—VI were collected at Umm Ruaba Forest Reserve, Eastern Kordofan, on March 9th, 1960; nodules VII and VIII from El Ain Forest Reserve, Central Kordofan (700 miles distant from Umm Ruaba), on January 9th, 1961. Sample IX was a representative bulk sample of first quality commercial "gum talh" (*A. seyal*). Nodules I—VIII ranged in weight from 40 to 80 g.; their colour varied from pale yellow to dark brown. Nodules I—IV, VII, and VIII were clear and glassy, of spherical shape. Nodules V and VI were elongated and had a characteristic glazed appearance, which, we have since observed, results when nodules plasticise slightly at 90—100°. It therefore appears that nodules V and VI had been subjected to more vigorous natural drying conditions than the others; it is unlikely that they were products of an earlier season, since *A. seyal* nodules (unlike *A. senegal*) become brittle through dehydration and fall from the branches within a few months.

* Part V, Anderson, Garbutt, and Smith, *Talanta*, 1962, **9**, 689.

Analytical Methods.—The standard methods,[11] were used, namely: paper partition chromatography; determination of sugars liberated on hydrolysis; autohydrolysis; electrophoresis; and viscosity experiments. The suspended-level dilution viscometer had a water flow-time of 218 sec. at 25°. Methoxyl contents were found by the vapour-phase infrared method,[13] which distinguishes yields of methyl iodide from other volatile products arising from solvent retention, decomposition, etc. Results were corrected for moisture content. Optical rotations were found for 1% aqueous solutions.

Studies on Crude Material.—The nodules, individually crushed to pass a 30-mesh sieve, gave the results shown in Table 1.

TABLE 1.

Determinations on crude samples.

	I	II	III	IV	V	VI	VII	VIII	IX
Moisture (%)	13·5	13·6	13·3	14·3	11·0	11·0	16·1	15·9	11·4
	13·6	13·6	13·2	14·4	11·0	11·2	16·0	16·0	11·4
Ash (%)	3·42	2·81	2·89	3·31	2·04	1·94	2·90	2·70	3·55
	3·38	2·98	2·94	3·33	2·10	2·10	2·94	2·80	3·61
Nitrogen (%)	0·14	0·09	0·14	0·12	0·09	0·10	0·18	0·17	0·19
	0·15	0·10	0·14	0·13	0·10	0·10	0·19	0·18	0·19
Uronic anhydride (%)	12·4	12·2	12·1	11·2	9·0	9·2	16·4	12·1	11·6
	12·7	12·4	12·0	11·4	9·1	9·2	16·8	11·9	11·9
Methoxyl (%)	n.d.	n.d.	0·60	0·72	1·36	1·53	1·00	0·82	0·55
Limiting flow-time no.	8·7	8·7	12·7	14·7	8·2	9·8	15·3	19·0	15·6
$[\alpha]_D^{20}$	+52°	n.d.	+56°	+51°	n.d.	+48°	n.d.	+50°	+44°

n.d. = Not determined.

Autohydrolysis at 85—90° of 1% solutions of nodules III, V, and VIII gave arabinose, together with traces of galactose and an oligosaccharide. As was observed for *C. leonense* gum,[11] the increase in reducing power (see Fig. 1) varies from nodule to nodule. The acidity of the autohydrolysis solutions (pH 4·6) did not increase appreciably with time of heating (cf. *C. leonense*,[11] which had an appreciable acetyl content) and decomposition of the liberated sugars was not extensive. The viscosity of the solutions fell rapidly during autohydrolysis.

Purification of Crude Gum.—A portion of each crushed nodule was shaken with cold distilled water, to give a 2% solution, which was filtered through acid-hardened filter-paper. The solutions were acidified (0·1N in hydrochloric acid); addition of acetone (4 volumes) gave a white curdy precipitate which was removed by centrifugation. Further precipitation did not occur when the clear supernatant liquid was poured into acetone. This purification process was carried out a further 3 times: the purified gum was then dialysed against distilled water and freeze-dried.

Studies on Samples Purified by Precipitation.—The results obtained are compared in Table 2. For the determinations of the limiting flow-time number, 4% saline was found to give adequate suppression of the electroviscous effect. Although the uronic anhydride content of each sample was greater than that in the crude gum, indicating the elimination of some 5—10% of non-uronide contaminants, the precipitation processes had not reduced the nitrogen content, and the ash contents were not significantly reduced. Other purification methods were therefore investigated. It is well-known that bi- and ter-valent ions can cause gel-formation and cross-linking;[5] ash-free samples are therefore required for some analyses (cf. ref. 12).

TABLE 2.

Determinations on samples purified by precipitation.

	I	II	III	IV	V	VI	VII	VIII	IX
Ash (%)	2·38	1·81	2·71	2·49	0·91	n.d.	n.d.	n.d.	2·48
	2·41	1·82	2·78	2·51	0·92				2·52
Nitrogen (%)	0·14	0·09	0·13	0·16	0·07	0·10	0·18	0·17	0·20
	0·15	0·10	0·13	0·17	0·07	0·10	0·19	0·18	0·21
Uronic anhydride (%)	13·1	12·8	12·5	12·8	10·4	10·9	16·6	12·9	12·4
	12·9	12·9	12·7	12·6	10·6	10·8	16·4	12·7	12·6
Methoxyl (%)	n.d.	1·1	1·3	0·70	1·1	n.d.	n.d.	1·0	0·94
Limiting flow-time no.	11·4	9·1	12·0	12·8	7·4	11·6	13·8	17·4	13·2
$[\alpha]_D^{20}$	+58°	n.d.	+59°	n.d.	n.d.	n.d.	n.d.	+64°	n.d.

Purification by Electrodialysis.—A portion of each crushed nodule was electrodialysed [7] (as 2% aqueous solution), ion-exchange membranes being used.[14] Cooling coils in the electrodialysis compartments prevented the temperature of the gum from rising above 30°. Trial experiments (on sample IX) showed that electrodialysis for 6 hr. (cf. ref. 7) was required to achieve the low ash values shown in Table 3. Since ash determinations at the 0·01% level consume relatively large amounts of material, determinations were not made on all samples.

After electrodialysis for several hours, the gum solutions separated into a clear, colourless upper layer and a viscous, slightly coloured lower layer. The upper layer was removed by pipette and found to contain only traces of gum. This effect may be worthy of further examination, since disintegration of a complex coacervate may be involved (cf. ref. 5).

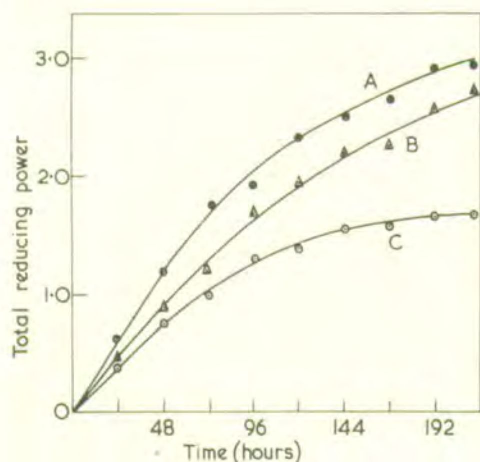

FIG. 1. Increase of reducing-power on autohydrolysis of (A) nodule (VII), (B) nodule III, and (C) nodule V. Reducing power is expressed as mg. of arabinose per 2 ml. of 1% solutions.

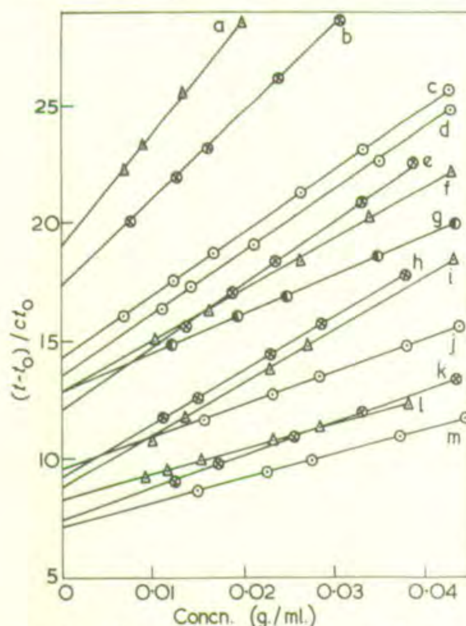

FIG. 2. Viscosity plots (in 4% aq. NaCl) of crude and purified samples. (a) VIII, crude. (b) VIII, pptd. (c) VIII, electrodialysed. (d) III, electrodialysed. (e) III, pptd. (f) III, crude. (g) III, ion-exchange. (h) II, pptd. (i) III, crude. (j) II, electrodialysed. (k) V, pptd. (l) V, crude. (m) V, electrodialysed.

Studies on Electrodialysed Samples.—The results obtained are compared in Table 3. Although the ash content had been effectively reduced, no elimination of nitrogen was achieved.

TABLE 3.

Determinations on electrodialysed samples.

	I	II	III	IV	V	VI	VII	VIII	IX
Ash (%)	0·02	0·01	n.d.	n.d.	0·02	0·02	0·03	n.d.	0·05
	0·02	0·02			0·03	0·03	0·04		0·05
Nitrogen (%)	0·15	0·11	0·16	0·17	0·10	0·11	0·19	0·17	0·17
	0·16	0·11	0·16	0·17	0·10	0·10	0·19	0·18	0·17
Uronic anhydride (%)	13·6	13·0	13·5	13·8	12·1	12·5	16·8	13·4	13·8
	13·7	13·3	13·6	13·6	12·2	12·3	16·6	13·5	13·8
Limiting flow-time no.	12·0	9·5	13·5	n.d.	7·0	12·4	13·6	14·2	n.d.

Potentiometric titrations showed that the ash-free gum behaved as a strong acid [7] (pH of a 1% aqueous solution = 2·9), and the values obtained for the neutralisation equivalent indicated that all the acidity arose from the uronic acid groups (*e.g.*, Found, for sample VIII: Neut.

equiv., 1340; uronic anhydride = 13·5%. Required; Neut. equiv., 1300 if all acidity is due to uronic acid groups).

Purification by Ion-exchange.—A dilute aqueous solution of nodule III was filtered, then de-ionised [15] by passage through a column of the cation-exchange resin "ZeoKarb 225." Analysis of the freeze-dried eluate gave: ash 2·4%, nitrogen 0·14%, uronic anhydride 13·5%, $[\alpha]_D$ +59°. Viscosity determinations gave the plot shown in Fig. 2g. This ion-exchange method was not applied to the other samples since the purification achieved did not approach that given by electrodialysis.

Comparison of the Viscosity Behaviour of Samples before and after Purification.—Samples were examined carefully to assess the extent of inter-nodule variation and the effect on each nodule of the various purification procedures. The viscosity plots for the crude and the purified samples of nodules II, III, V, and VIII are shown in Fig. 2; these curves are typical and represent the range of behaviour observed.

Fractionation Experiments on Aqueous Solutions of the Gum.—(1) *Chemical precipitation methods.* No useful fractionation resulted from (*a*) graded addition of ethanol, (*b*) addition of iodine–potassium iodide reagent (cf. ref. 16), or (*c*) addition of cetyltrimethylammonium bromide [17] at pH 4, 7, or 9.

(2) *Electrophoresis.* Several experiments were made with glass-fibre paper in 2M-sodium hydroxide at 1000 v for 6—18 hr. Movements of several cm. resulted, but there was no distinct separation of components (cf. ref. 3).

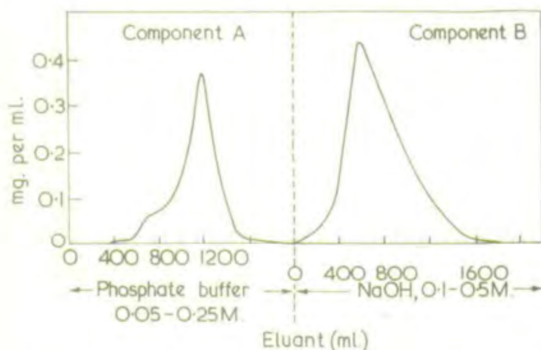

FIG. 3. Elution pattern from fractionation of a single nodule on diethylaminoethylcellulose. The broken line denotes change of solvent.

(3) *Chromatography on diethylaminoethylcellulose.*[18] A solution of sample I (electrodialysed, 360 mg.) was treated on a column (40 × 5 mm.) of diethylaminoethylcellulose; gradient elution with phosphate buffer (pH 4·6, 0·05M ⟶ 0·25M) was used, followed by gradient elution with aqueous sodium hydroxide (0·1M ⟶ 0·5M). The average flow-rate was ~40 ml. per hr. Fractions (40 ml.) were screened by the phenol method.[19] Fig. 3 shows the elution pattern observed. The total recovery from the column was 331 mg.: component A (117 mg., 35%) and component B (214 mg., 65%) contained 12·5% and 15·4% of uronic anhydride, respectively.

Sample II (electrodialysed, 220 mg.) gave an elution pattern similar to that shown in Fig. 3. Component A (66 mg., 34%) and component B (126 mg., 66%) had uronic anhydride contents of 12·4% and 14·9%, respectively.

Sample III (electrodialysed, 460 mg.) similarly gave 180 mg. (41%) of component A (uronic anhydride, 12·6%) and 258 mg. (59%) of component B (uronic anhydride, 15·2%).

Hydrolysis: Percentages of Sugars Present.—Treatment with 2N-sulphuric acid at 90—95° for 8 hr. completely hydrolysed the gum. Sample IX gave galactose 38%, arabinose 46%, rhamnose 3%, and glucuronic acid 12·5% (expressed as approximate percentages; cf. ref. 20). The method of determining the sugar ratios involves the separate stages of hydrolysis, neutralisation, reduction in volume, chromatographic separation, elution, and estimation of reducing power: it is considered that the results cannot be more accurate than ±5—10% of the actual percentage present.

For samples I—VIII, however, the results, particularly for the rhamnose content, varied by amounts which are considered to be outside the possible experimental error. The two most widely differing nodules were samples V and VII. Sample V gave glucuronic acid 11%,

galactose 42%, arabinose 47% and rhamnose 1%: sample VII gave glucuronic acid 16%, galactose 34%, arabinose 42%, and rhamnose 8%.

Discussion

The gum from *A. seyal* is similar to the *Acacia* gums previously studied in containing glucuronic acid, galactose, arabinose, and rhamnose. The presence of acid-labile residues and the marked decrease in viscosity detected on mild hydrolysis indicate that *A. seyal* probably further resembles other *Acacia* gums in having a main chain, resistant to hydrolysis, to which is attached acid-labile side-chains. Of the *Acacia* gums studied to date, all have given negative optical rotations with the exception of *A. karroo*,[20] to which must now be added *A. seyal*. The methoxyl content of the *A. seyal* nodules examined varied from 0·5% to 1·5%; only *A. mollissima* [21] has previously been reported to have a methoxyl content (0·35%). A methoxyl content of 1% has been found [22] to be significant in *Khaya grandifolia* gum.

The results presented in Tables 1—3 indicate that the inter-nodule variation in composition is greater than can be explained on the basis of possible analytical error. The variation is similar in extent to that previously found [11] for nodules of C. *leonense* gum.

The nodules examined were collected and authenticated by an expert on the identification of *Acacia* species. It may otherwise have been suggested that nodules V and VI (from their appearance), nodule VII (uronic acid content), and nodule VIII (viscosity) originated from some species other than *A. seyal*. However, the data for each nodule, taken as a whole, leave little basis for doubting the authenticity of the samples. Taken jointly, the nitrogen content and the optical rotation of an *Acacia* gum are strongly indicative of its species: preliminary studies of other Sudanese *Acacia* species such as *A. arabica, A. laeta, A. dealbata, A. drepanolobium*, and *A. campylacantha* (which have not been studied previously) have shown that the nitrogen content of *A. seyal* is characteristically low, and, moreover, is not reduced by any of the methods of purification used. The mechanism of gum formation is still far from clear,[6] and further knowledge of the nature of the nitrogen content in plant gums would be of value in assessing the relative importance of the enzymic polymerisation theory [5] in relation to the alternative theories [6,23] that gum formation results from (*a*) normal plant metabolism or (*b*) pathological reactions to resist invading micro-organisms or to avoid loss of moisture.[5]

Although it has been reported that the ash content of some species of gum can be eliminated [10,22] by precipitation methods, our experiments with *Acacia* species have shown that their ash content cannot be reduced by more than about 50%, even after 4 re-precipitations. The results reported for *A. seyal* are typical in this respect. Electrodialysis is the most effective method of reducing the ash content to a very low value; as shown in Fig. 2, the most viscous nodule (VIII) showed a marked decrease in viscosity on purification, although the other nodules were not affected to a comparable extent. In general, the purification methods studied do not appear to alter significantly the physical properties of the gum.

Fractionation of *A. seyal* gum on diethylaminoethylcellulose gave two components having uronic anhydride contents of 12·5% and 15·3%, respectively; the close similarity of the elution patterns suggested that different nodules contained the same two components in slightly varying proportions. Conclusive evidence of heterogeneity is often difficult to achieve. Indeed, conflicting results may be given by different techniques; trypsin is electrophoretically heterogeneous, although only one component was evident on examination by the ultracentrifuge.[24] For gum arabic,[1] chemical fractionation has been less successful than immunochemical experiments. Our failure to separate the components of *A. seyal* by electrophoresis (cf. ref. 3) may therefore be explained by the fact that, in single nodules, the two components do not differ sufficiently in uronic acid content, upon which electrophoretic movement must depend to a large extent.[25] Studies of the chemical structure of the two components of this gum are in progress.

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32.

597. *Studies on Uronic Acid Materials. Part VII.*[1] *The Kinetics and Mechanism of the Decarboxylation of Uronic Acids.*

By D. M. W. Anderson and S. Garbutt.

Mechanisms for the decarboxylation of uronic acids in mineral acid solution are discussed with reference to the dependence of the rate of decarboxylation on the uronic acid concentration and on the Hammett acidity function. The experimental values of the Arrhenius activation energies and the entropies of activation are compared with those for other reactions of known mechanism; in consequence, an S_E2 decarboxylation mechanism, which explains both the kinetic results and the nature of the products, is proposed.

The significance, in structural studies, of the possible decarboxylation of uronic acids in hot aqueous solutions has been discussed,[2] and the first-order rate constants for the acidic decarboxylation of glucuronic acid, labelled with ^{14}C at the carboxyl group, have been published.[3]

Although decarboxylation mechanisms for many carboxylic acids have been established,[4] the acidic decarboxylation of uronic acids clearly proceeds by a multi-step process [3] and none of the decarboxylation mechanisms [5,6] proposed to date has explained all the facts. This paper presents the results of studies which were undertaken in order to extend previous knowledge of the decarboxylation mechanism.

Experimental and Results

Origin of Samples.—The purity and origin of the samples of D(+)-galacturonic acid monohydrate, D(+)-glucurone, alginic acid, trigalacturonic acid, and calcium L-sorburonate (calcium 5-keto-D-gluconate) have been described.[2] 2-Keto-L-galactonic acid and calcium 2-keto-D-gluconate were kindly provided by Dr. W. W. Reid. L(−)-Ascorbic acid (B.D.H. Ltd.) gave 1·0 mol. of carbon dioxide after 2·5 hr. in refluxing 19% w/w aqueous hydrochloric acid.[3,7] "AnalaR" mineral acids were used, the appropriate dilutions being made with conductivity-grade distilled water.

Kinetic Measurements.—Anderson's decarboxylation apparatus [7] was used; the modifications facilitating reproducible kinetic measurements have been described.[2,3]

Order of Reaction with Respect to Uronic Acid Concentration.—The rate of decarboxylation of widely different weights of galacturonic acid monohydrate (30·0 mg., 322 mg., 999 mg.) in boiling 3·8% w/w aqueous hydrochloric acid was investigated. The averaged results, obtained from duplicate runs for each weight, are shown in Table 1. Since the molar yield is independent

Table 1.

The decarboxylation of galacturonic acid monohydrate in boiling 3·8% w/w hydrochloric acid. (Results expressed as % moles of carbon dioxide per mole of uronic acid.) (*a*) 30·0, (*b*) 322·2, (*c*) 999·3 mg. of uronic acid.

Time (sec.)	Carbon dioxide (% mole/mole) (*a*)	(*b*)	(*c*)
2000	7·9	8·0	8·2
4000	15·0	15·2	15·7
6000	21·0	21·3	22·0
10,000	32·5	33·1	34·0
15,000	46·2	46·7	48·1
20,000	57·4	58·3	60·1
30,000	98·2	99·8	102·9

of the sample weight, the rate-constant is of the first order with respect to the uronic acid concentration; calculation, by Guggenheim's method,[8] gives $k_1 = 4\cdot03 \times 10^{-5}$ sec.$^{-1}$.

Dependence of the Reaction Rate on Temperature.—Samples (30·0 mg.) of galacturonic acid monohydrate were decarboxylated in 3·8% w/w aqueous hydrochloric acid, the temperature

being maintained, in successive runs, at 103°, 100°, and 90°. Table 2 shows the values found for the first-order rate-constant. The graph of log k_1 against $1/T$ is shown in Fig. 1 (curve a), from which the values shown in Table 2 for the Arrhenius activation energy (E_A) and for the unimolecular Arrhenius factor A_1 ($k_1 = A_1 \exp - E_A/\boldsymbol{R}T$) were calculated.

TABLE 2.

Rate-constants, k_1 (10^{-4} sec.$^{-1}$), Arrhenius activation energies (E_A), and entropies of activation ($\Delta S^{\ddagger}$) for the decarboxylation of various uronic acids.

Compound in 3·8% w/w HCl	Temp. 112°	103°	100°	95°	90°	E (kcal. mole^{-1})	log A_1	$\Delta S^{\ddagger}$ (e.u.)
D(+)-Galacturonic acid monohydrate	—	0·40	0·31	—	0·12	25·5	10·43	−13·4
Compound in 19% w/w HCl	**112°**	**105°**	**100°**	**95°**	**90°**			
D(+)-Galacturonic acid monohydrate	12·0	5·7	3·6	2·2	1·3	27·9	12·9	−5·2
D(+)-Glucurone	6·5	3·7	2·6	1·6	1·0	23·8	10·34	−17·1
Trigalacturonic acid	13·0	6·6	3·8	2·8	1·7	28·0	13·08	−4·6
Alginic acid	4·6	2·4	1·5	0·99	0·57	26·5	11·73	−10·7
Ca L-sorburonate	37·0	21·0	13·0	8·2	5·0	26·4	11·57	−11·5
2-Keto-L-galactonic acid	6·8	3·6	2·7	1·6	—	23·8	10·34	−17·1
Ca 2-keto-D-gluconate	8·1	4·2	2·9	1·9	—	27·1	12·30	−8·1
Ascorbic acid	10·0	4·6	2·8	1·7	1·2	26·1	12·34	−7·9
					Mean	26·1		−10·6

The experiments were repeated for galacturonic acid monohydrate (30·0 mg.) in 19% w/w aqueous hydrochloric acid at 112°, 105°, 100°, 95°, and 90°. The values obtained for k_1 are shown in Table 2; plotting log k_1 against $1/T$ gave line (b) in Fig. 1, from which the values for E_A and A_1 shown in Table 2 were calculated. The close agreement in the values of E_A given by the 3·8% and the 19% acid indicates that the same decarboxylation mechanism probably operates at both these acid concentrations.

Similar experiments in the 19% acid were then conducted for glucurone, trigalacturonic acid, alginic acid, calcium L-sorburonate, 2-keto-L-galactonic acid, calcium 2-keto-D-gluconate, and ascorbic acid. The results for k_1, E_A, and A_1 are shown in Table 2, the plots of log k_1 against $1/T$ being shown in Fig. 1 (curves c—h). The values for E_A are in good agreement with those previously found,[9] by different methods, for the 2-keto-acids and for ascorbic acid.

Calculation of the Entropy of Activation, $\Delta S^{\ddagger}$.—A correlation has been found [10] between the entropy of activation and reaction mechanism, the values of $\Delta S^{\ddagger}$ varying between +5 and +9 e.u. for A-1 mechanisms, and between −20 and −25 e.u. for A-2 mechanisms.[10] Values for $\Delta S^{\ddagger}$ can be found from the Eyring equation, $\Delta S^{\ddagger} = 2{\cdot}303\boldsymbol{R} \log (A_2h/e\boldsymbol{k}T)$, where $\boldsymbol{k}$ is Boltzmann's constant. (For substitution in this expression, our values for A_1 were corrected to bimolecular values for the appropriate acid concentrations, the units for A_2 then being l. mole^{-1} sec.$^{-1}$.) The values obtained for $\Delta S^{\ddagger}$ are shown in Table 2; values approximately 1 unit less negative are obtained when the activity of the mineral acid solution is taken into account.

Dependence of Reaction Rate on the Mineral Acid Concentration.—First-order rate constants were obtained for the decarboxylation of uronic acids in boiling aqueous mineral acids of various concentrations. The values obtained were corrected to 100° (on the basis of the observed reflux temperatures and the activation energies already obtained from Fig. 1). These corrected values are shown in Table 3; Fig. 2 shows that they have a linear dependence [11] on the Hammett acidity function, H_0, when the values quoted for $-H_0$ at 25° by Long and Paul [12] are used. This suggests [12] that the reaction follows either an A-1 or an A-S_E2 mechanism. Values of the gradient (S') of the lines in Fig. 2 are shown in Table 3. (For $-H_0$ values greater than ~0·5, the plots of molarity against H_0 at various temperatures are parallel; [13] the values of H_0 at 25° may therefore be used for values observed at other temperatures, only the intercept on the ordinate of Fig. 2 being altered.)

As shown in Fig. 2, the rate constants for the decarboxylation of galacturonic acid in sulphuric and hydrochloric acid are identical over a limited range of H_0 values. Above a certain concentration, however, the plot for sulphuric acid tends to a limiting value (Fig. 2),

suggesting that a change in mechanism occurs. A similar effect has been reported [14] for aromatic carboxylic acids and also for galacturonic acid in phosphoric acid media.

The Effect of Solvation of Hydrogen Ions on the Kinetics.—Various workers [12] have extended the Zucker–Hammett hypothesis [11] to include the effect of solvation of hydrogen ions on reaction kinetics and mechanism. It has been suggested [15] that four water molecules are involved in proton solvation in acidic solution. For a decarboxylation reaction, Pedersen [16] obtained a linear relation between log k_1 and $(-H_0 + 4 \log a_{H_2O})$, where a_{H_2O} is the activity of the water. In a similar approach, Bunnett [17] plotted $(\log k_1 + H_0)$ against log a_{H_2O},

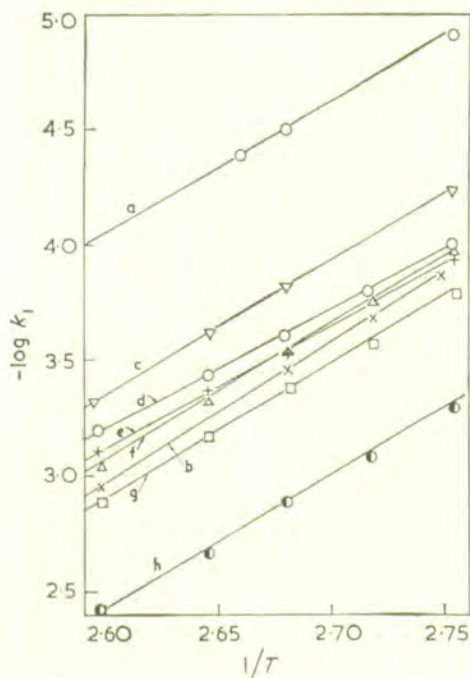


FIG. 1. Plots of log k_1 against $1/T$.

Galacturonic acid monohydrate (a) in 3·8% w/w HCl and (b) in 19% w/w HCl. c, Alginic acid. d, Glucuronic and 2-keto-L-galactonic acid. e, Calcium 2-keto-D-gluconate. f, Ascorbic acid. g, Trigalacturonic acid. h, Calcium 5-keto-D-gluconate. (c—h) in 19% w/w HCl.

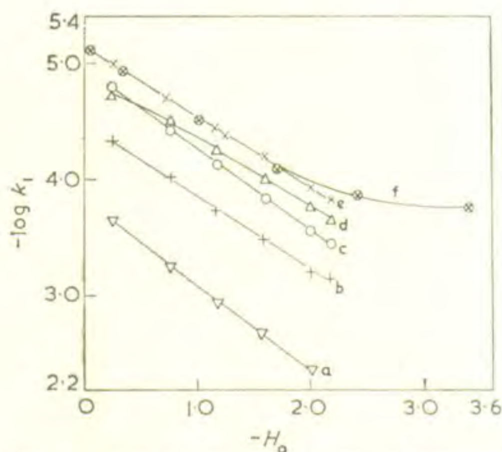


FIG. 2. Plots of log k_1 against the Hammett acidity function, H_0.

a, Calcium 5-keto-D-gluconate. b, Trigalacturonic acid. c, D(+)-Glucurone and 2-keto-L-galactonic acid. d, Alginic acid. e, D(+)-Galacturonic acid monohydrate in HCl. f, D(+)-Galacturonic acid monohydrate in H_2SO_4.

TABLE 3.

Values of the first-order rate-constants (as log k_1) for the decarboxylation of some uronic acids in mineral acids at 100°.

	Log k at the following Hammett acidity functions (H_0):							Slope
Uronic acid in HCl	−2·15	−2·01	−1·57	−1·22	−1·16	−0·74	−0·24	S'
D(+)-Glucurone	−3·47	−3·58	−3·84	−4·09	−4·13	−4·45	−4·78	−0·70
D(+)-Galacturonic acid monohydrate	−3·82	−3·92	−4·19	−4·40	−4·45	−4·66	−4·99	−0·61
Trigalacturonic acid	−3·17	−3·21	−3·48	—	−3·76	−4·02	−4·34	−0·62
Alginic acid	−3·66	−3·76	−4·00	—	−4·25	−4·51	−4·76	−0·60
Ca L-sorburonate	—	−2·35	−2·67	—	−2·97	−3·26	−3·65	−0·73

	Log k at the following Hammett acidity functions (H_0):						Slope
Uronic acid in H_2SO_4	−3·38	−2·41	−1·72	−1·01	−0·31	0·02	S'
D(+)-Galacturonic acid monohydrate	−3·75	−3·86	−4·10	−4·49	−4·93	−5·11	−0·61

suggesting that if the slope w of this plot is zero or less than unity, then water molecules are not involved in the reaction; for $w \approx +2$, water acts as a nucleophile; for $w \approx +5$ to $+7$, water acts as a proton-transfer agent. Bunnett's values [17] of log a_{H_2O} were therefore used in conjunction with the results in Table 3 to obtain the typical, slightly curved, Bunnett plots shown in Fig. 3 (data in Table 4). In hydrochloric acid, the limiting slopes vary between

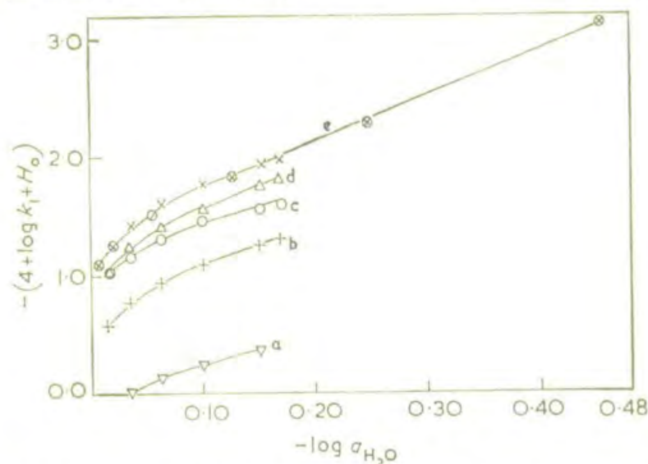


FIG. 3. Bunnett plots of $-\log a_{H_2O}$ against $-(4 + \log k_1 + H_0)$.
a, Calcium 5-keto-D-gluconate. b, Trigalacturonic acid. c, D(+)-Glucurone. d, Alginic acid. e, D(+)-Galacturonic acid monohydrate in HCl. f, D(+)-Galacturonic acid monohydrate in H_2SO_4.

+2·5 and +3·5 (mean +2·9), suggesting that water is acting as a nucleophile; in sulphuric acid, the value of w is more positive, as is generally found.[17] Bunnett [17] also plotted $\Delta S^\ddagger$ against w: the average values of w and of $\Delta S^\ddagger$ from Table 2 (−10·6 e.u.) agree well with Bunnett's correlations.

TABLE 4.

Values of $(4 + \log k_1 + H_0)$ and $\log a_{H_2O}$ for the decarboxylation of some uronic acids.

In hydrochloric acid at 100°.								
$-\log a_{H_2O}$	0·169	0·152	0·102	0·069	0·064	0·036	0·016	
$-H_0$	2·15	2·01	1·57	1·22	1·16	0·74	0·24	Slope
Uronic acid			$-(4 + \log k_1 + H_0)$					(w)
D(+)-Glucurone	1·60	1·56	1·43	1·33	1·30	1·17	1·02	+2·5
D(+)-Galacturonic acid monohydrate	1·97	1·93	1·76	1·62	1·60	1·43	1·23	+2·8
Trigalacturonic acid	1·29	1·24	1·09	—	0·92	0·76	0·57	+3·0
Alginic acid	1·79	1·75	1·57	—	1·40	1·23	1·02	+3·5
Ca L-sorburonate	—	0·36	0·24	—	0·12	0·01	−0·13	+2·5
In sulphuric acid at 100°.								
$-\log a_{H_2O}$	0·455	0·249	0·128	0·056	0·020	0·008		
$-H_0$	3·38	2·41	1·72	1·01	0·31	0·02		Slope
			$-(4 + \log k_1 + H_0)$					(w)
D(+)-Galacturonic acid monohydrate	3·13	2·27	1·82	1·50	1·24	1·09		+4·0

DISCUSSION

The kinetic results show that the acidic decarboxylation of uronic acids is bimolecular, depending on the first power of both the uronic acid and the mineral acid concentration. The dependence of log k_1 on the Hammett acidity function suggests that the decarboxylation follows either an A-1 or an A-S_E2 course: [12] the entropies of activation are not sufficiently negative to be correlated with values previously reported [10,18] for A-2 reactions.

An S_E2 mechanism was proposed [19] for the decarboxylation of polycyclic carboxylic

acids, and further examples have been reported;[14,20] the kinetic data published for some of these are listed in Table 5, together with values calculated for $\Delta S^{\ddagger}$ (with the aid of bimolecular A factors computed from the unimolecular values given in the references cited). The variation in $\Delta S^{\ddagger}$ (from $+0{\cdot}7$ to $-25{\cdot}2$; mean $= -11{\cdot}2$) for these S_E2 reactions is greater than the variation in $\Delta S^{\ddagger}$ found (see Table 2) for the uronic acid decarboxylations studied ($-4{\cdot}6$ to $-17{\cdot}1$; mean $-10{\cdot}6$); in the present state of knowledge, the observed kinetics are therefore compatible with the results expected from an A-S_E2 reaction.

TABLE 5.

Activation energies (E_A) and entropies of activation ($\Delta S^{\ddagger}$) for established A-S_E2 decarboxylation reactions.

Acid decarboxylated	Acid used	concn. % w/w	E_A (kcal. mole^{-1})	$\Delta S^{\ddagger}$ (cal. °c^{-1} mole^{-1})
Mesitoic acid (ref. 14)	H_2SO_4	83·3	27·4	+0·7
2,4,6-Trimethoxybenzoic acid (ref. 19)	$HClO_4$	10·3	18·4	−13·6
	$HClO_4$	59·7	20·6	−6·5
$Me_3Si{\cdot}[CH_2]_2{\cdot}CO_2H$ (ref. 20*a*)	H_2SO_4	90	20·5	−12·0
$Me_3Si{\cdot}[CH_2]_3{\cdot}CO_2H$ (ref. 20*a*)	H_2SO_4	90	19·3	−19·7
2,4,6-Trihydroxybenzoic acid (ref. 20*c*)	HCl	0·44	15·2	−25·2
2,4,6-Trihydroxybenzoic acid anion (ref. 20*c*)	HCl	0·44	21·5	−1·1

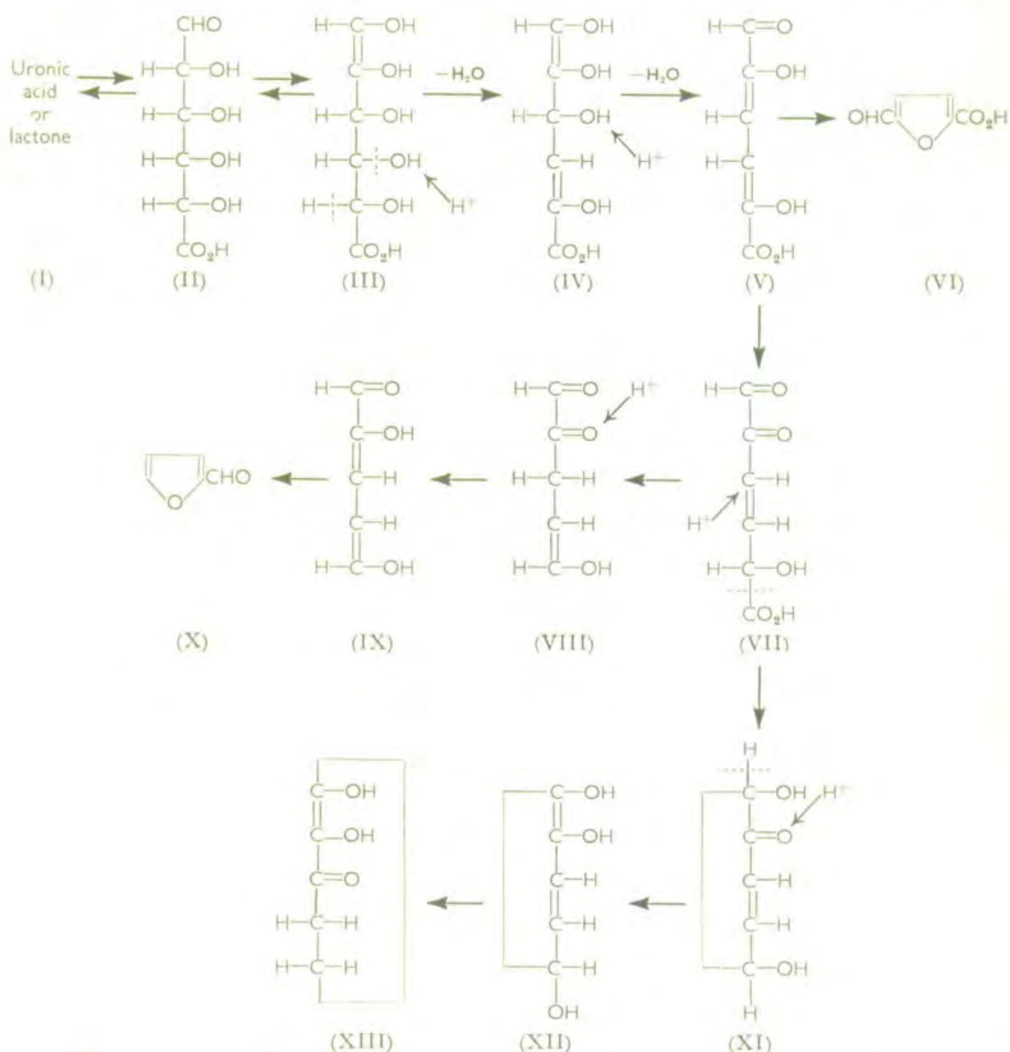
Open-chain Formation as the Initial Step of the Reaction Mechanism.—The activation energy (~17 kcal. mole^{-1}) for the mutarotation of simple sugars,[21] the kinetics of which may be explained by regarding ring-opening as the rate-controlling step, is considerably less than that for uronic acid decarboxylations (mean value from Table 2 = 26·1 kcal. mole^{-1}). A further indication that ring-opening is not rate-controlling in uronic acid decarboxylation is given by the similar reaction kinetics given by keto-aldonic acids, uronic acids, and lactones.

The acid hydrolysis of various glucopyranosides is believed to occur [22] *via* a closed-ring carbonium ion intermediate; the slowest rate reported for this reaction is, however, much faster than that of the fastest uronic acid decarboxylation. Furthermore, for uronic acid decarboxylations the rate dependence on the Hammett acidity function is the same in both hydrochloric and sulphuric acid, which have different ionic strengths, and this indicates that the formation of a carbonium ion intermediate is unlikely to be the rate-determining step.

There is considerable evidence [23] that sugars exist mainly in the straight-chain ene-diol form in widely different acid concentrations, the activation energy for the polarographic reduction of the straight-chain form being 20—25 kcal. mole^{-1}.[24] In the absence of direct evidence to the contrary, it appears to be a reasonable assumption that uronic acids, in acidic solution, also exist in the straight-chain ene-diol form, the formation of which is not rate-determining.

Significance of the Reaction Products.—Any proposed mechanism must explain the formation of the products observed under differing experimental conditions. Decarboxylation of galacturonic acid in concentrated sulphuric acid yields 5-formylfuroic acid;[25] 100 mole % of carbon dioxide, 32 mole % of furfuraldehyde, and 19 mole % of reductic acid were isolated [6] after decarboxylation in 3·5N-hydrochloric acid for 4 hours. Significant amounts of pentoses have not been isolated from the acidic solutions used to decarboxylate uronic acids.[6,26] The activation energy for the formation of furfuraldehyde from xylose in mineral acid is 14 kcal. mole^{-1}, with a slightly positive entropy of activation [27]—values which differ widely from those found for uronic acid decarboxylations. Further, the yield of furfuraldehyde from uronic acids is considerably less than that isolable from pentoses;[6,28] different mechanisms are therefore strongly indicated, although both may involve the formation of ene-diol intermediates.

After consideration of the kinetic results and other evidence which has been discussed, a reaction pathway which appears to explain the known facts is presented in the chart.



Proposed Reaction Mechanism.—Structures (I—III) are in equilibrium; the formation of (II) and (III) is unlikely to be rate-determining, since similar kinetics were observed for ring structures and linear chains. In general, the attack of a proton on a hydroxyl group will be fast: although elimination of water from (III) could be rate-determining, an A-$S_{E}2$ reaction, as indicated by the kinetics, would not result. Consideration shows that proton attack on the 4-hydroxyl group is required to explain the formation of the observed products. Elimination of water from (III) may be *cis* (galacturonic acid) or ***trans*** (glucuronic, mannuronic acid), so that the observed small differences in activation energy and entropy of activation would be expected to occur. Further elimination of water from (IV) leads to (V), which can either cyclise directly to give 5-formylfuroic acid (VI), or can give an intermediate (VII), which was readily decarboxylated.[29] Proton attack on (V) may be the rate-determining A-$S_{E}2$ reaction. The acid (VI) will be preferentially formed in concentrated sulphuric acid, where the change in bisulphate ion concentration may become important; this is consistent with the limiting value observed for log k_1 at high sulphuric acid concentrations (Fig. 2). Lower concentrations of mineral acid will favour

the rapid formation of the material (VII) which on decarboxylation gives either (VIII) or (XI).

The observed production of reductic acid (XIII) requires the formation of a carbon–carbon bond to be explained, and the mechanism proposed should also explain the non-formation of reductic acid from pentoses. It is suggested that the necessary carbon–carbon bond formation occurs when (VII) gives rise to (XI) upon decarboxylation, and not as the result of cyclisation between an aldehydic and a hydroxyl group, as has been suggested.[6] Cyclisation of (VII) to (XI) is clearly less favourable than the formation of (VIII) from (VII) by the normal βγ-unsaturated decarboxylation mechanism, and it can be deduced, from the relative yields of the products (X) and (XIII) reported, that the rate of formation of (VIII) from (VII) is about three times that of formation of (XI) from (VII); under mildly dehydrating conditions the yield of (XIII) increases[6] relative to that of (X). In the formation of furfuraldehyde, cyclisation of structures prior to (IX) would involve sterically strained systems.

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33.

STUDIES ON URONIC ACID MATERIALS

PART VIII[1]. A COMPARATIVE STUDY OF SOME COLORIMETRIC METHODS OF ESTIMATING THE URONIC ACID CONTENTS OF POLYSACCHARIDES

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Colorimetric methods are widely used in carbohydrate chemistry, being particularly useful for comparative analyses of large numbers of samples and for routine estimations of well-characterised materials.

Determination of the uronic acid content is a key analysis in studies of many carbohydrate materials, *e.g.* gums, mucilages, pectins, hemi-celluloses, mucopolysaccharides, glycoproteins and polysaccharides of marine origin. Such natural products are complex and often heterogeneous: they are usually closely associated with pigments, lipids, protein and inorganic materials. Elimination of these may not be possible without resultant degradation or modification (physical or chemical) of the carbohydrate material; careful choice of extraction conditions may be important[2].

Every carbohydrate material therefore presents a unique set of analytical requirements, and it is consequently difficult to formulate general analytical methods, particularly for colorimetric procedures. Methods developed and tested only with respect to synthetic mixtures of purified simple materials may give misleading results when applied to more complex natural materials. Colorimetric methods are susceptible to interferences by minor impurities, which need not be of natural origin. It is often difficult to remove *all* traces of the reagents added during purification stages; residual traces of inorganic ions can modify the chromophoric stages of reactions, causing inaccurate results.

For uronic acid determinations, the accepted reference procedures — which may themselves be subject to methodic errors — are (*a*) titration methods, which are not universally applicable, and (*b*) decarboxylation methods, which require up to 50 mg of material per determination in reactions of at least 2.5-h duration[3-5]. It is therefore not surprising that a large number of colorimetric methods, which are more rapid and require less material, have been proposed. These are based on non-stoichiometric reactions[6,7], and some of the reagents used are naphthoresorcinol[8-10], phloroglucinol[11], thioglycolic acid[12], 2-thiobarbituric acid[13,14], 2-methyl-indole[15], concentrated sulphuric acid[16], alkaline hydroxylamine + iron (III)[17], anthrone [18-20], carbazole[21-23] and carbazole + borate ions[24,25].

Conflicting reports regarding the specificity and validity of such colorimetric methods have been published[8,9]. The non-specific nature of the anthrone reaction for uronic acid groups is well known, and the effect of nitrogenous materials on the

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reaction has been studied in a number of papers by HELBERT AND BROWN[20]. The carbazole reaction[21] has been reported[26,27] to be unreliable in the presence of protein, but this was to be expected after DISCHE's initial report[21] that proteins suppressed colour development, causing large errors. DISCHE further stated[21] that the carbazole reaction "cannot be used for accurate determinations of absolute values for hexuronic acids in polysaccharides". In illustration of this, large discrepancies between colorimetric and decarboxylation values have been reported; for a seaweed polysaccharide, MCKINNELL AND PERCIVAL[28] found a uronic acid content of 18.3% (by decarboxylation) and 6.0% (by colorimetry)[22], the desulphated material giving 23.5% (decarboxylation) and 10.2% (colorimetry).

To discover the extent of the variation in values given by different colorimetric methods, comparative studies have been made on a variety of carbohydrate materials; such a study does not appear to have been reported previously. Results given by the methods most frequently used for colorimetric estimations of uronic acids — the carbazole[21], anthrone[18], and hydroxamic acid[17] methods — are presented and compared with the corresponding results obtained by decarboxylation[5].

EXPERIMENTAL

Reference compounds

The samples of galacturonic acid monohydrate and glucuronolactone as standards were the specimens described in an earlier investigation[2]. In addition to the analytical data already quoted[2], determinations of the neutralization equivalent gave values for the purity of the glucuronolactone and galacturonic acid of 97.2 and 96.0%, respectively.

The reference standard sample of galacturonic acid was used to make up the mixtures referred to in Tables I–III, and is listed as "Sample 1" in Table IV.

Acidic decarboxylation method

ANDERSON's apparatus and reaction conditions[4] were used. For this particular modification of LEFÈVRE AND TOLLEN's[3] reaction, a reaction time of 2.5 h has been confirmed[5]. In heterogeneous polysaccharides, pentoses and hexoses do not increase by more than 2–3% the yield of carbon dioxide from the uronic carboxyl groups[4] (*cf.* TRACEY[29]); amino sugars, peptides and proteins cause no interference[30].

Colorimetric methods

In all cases, absorption measurements were made against "blank" solutions prepared concurrently with the unknown sample.

(*a*) *Anthrone.* YEMM AND WILLIS' method[18] was used, absorption being measured at 540 mμ after colour development for 15 min.

(*b*) *Carbazole.* DISCHE's method[21] was used, absorption in 10-mm silica cells being measured at 530 mμ after colour development for 2 h at 22°.

(*c*) *Alkaline hydroxylamine.* KAYE AND KENT's procedure[17] was followed in initial determinations, absorption being measured at 505 mμ. The colour was observed to fade rapidly: a solution giving an optical density of 0.65 immediately after addition of iron(III) had later optical densities of 0.64 (10 min), 0.62 (20 min), 0.56 (30 min), 0.48 (40 min), 0.37 (50 min), and 0.25 (60 min).

KAYE AND KENT proposed[17] that the absorption curve given by glucurone (line D,

Fig. 1) should be corrected by the amount of absorption given by glucose (line B, Fig. 1), so giving the "corrected glucurone" curve (line C). The uronic acids in natural polymeric materials are not, however, generally present as esters or lactones, and esterification must therefore be effected (methanolic hydrogen chloride, sealed

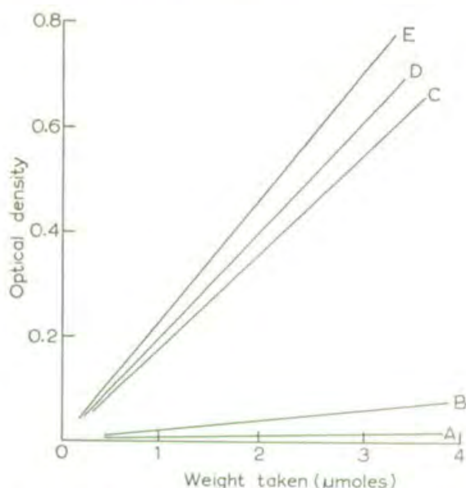

Fig. 1. Calibration curves for the alkaline hydroxylamine method. Curve (A) "esterified" hexoses and α-methyl hexoses, (B) glucose, (C) glucurone, corrected for glucose absorption, (D) glucurone, (E) "esterified" glucurone and galacturonic acid.

tube, 100°) before reaction with hydroxylamine. Hexoses thus treated gave line A in Fig. 1, whilst glucurone and galacturonic acid both gave line E. It is suggested that, if uronic acid X is to be estimated in a polymeric material, then the calibration curve should be obtained by subjecting pure X to the same analytical procedure as the polymer.

This method was found to be subject to four distinct modes of interference. (*a*) Some polysaccharides are not completely soluble in methanolic 1% hydrogen chloride, and incomplete esterification results. (*b*) Some esterified polysaccharides are not water-soluble, so that incomplete colour development is given. (*c*) Some polysaccharides give precipitates or gel formation on the addition of iron(III), so giving incomplete colour formation. (*d*) The presence of protein leads to high results. Materials subject to the interferences are correspondingly labelled A, B, C, or D in Table IV.

RESULTS

Interferences in the hydroxylamine method

The effect of protein on estimations by the hydroxylamine method is shown in Table I. Estimations were made on synthetic mixtures of edestin and galacturonic acid (reference standard sample).

The effect of hexoses and pentoses on estimations by the hydroxylamine method is shown in Table II. The use of galactose in place of glucose gave slightly greater positive errors; pentoses gave slightly smaller positive errors than glucose. The

TABLE I

EFFECT OF PROTEIN ON ESTIMATIONS

Composition of mixture		*% Galacturonic acid indicated by the hydroxylamine method*
% Protein	*% Galacturonic acid*	
17.7	82.3	88.7
50.3	49.7	130.5
90.6	9.4	190

TABLE II

EFFECT OF HEXOSES AND PENTOSES ON ESTIMATIONS

Composition of mixture		*% Galacturonic acid indicated by the hydroxylamine method*
% Glucose	*% Galacturonic acid*	
13.3	86.7	86.8
52.0	48.0	50.5
96.0	4.0	34.1

presence of hexoses and pentoses in a heterogeneous polysaccharide containing minor amounts of uronic acid may therefore lead to large errors in the colorimetric estimation of the uronic acid.

Interferences in the carbazole method

DISCHE[21] found a 20% error with 0.1% protein in solution; colour suppression has been confirmed by recent investigations[26,27]. DISCHE also reported[21] that correction should be applied for the colour given by hexoses: the extent of the errors which can arise is shown in Table III.

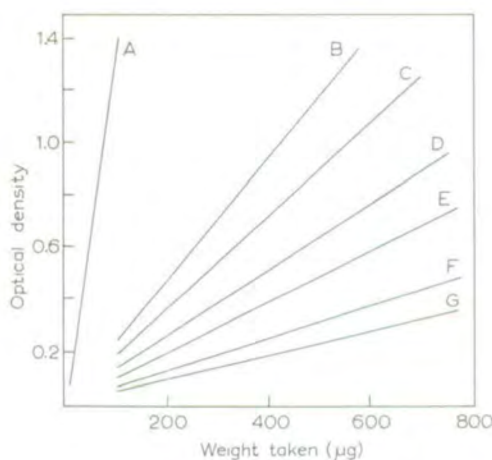

Fig. 2. Calibration curves for the anthrone method. Curve (A) pentoses, (B) glucose, (C) galacturonic acid, (D) galactose, (E) glucosamine hydrochloride, (F) glucurone, (G) glucurono-δ-lactone.

TABLE III

ERRORS ARISING IN THE CARBAZOLE METHOD

Composition of mixture		*% Galacturonic acid indicated by the carbazole method*
% Glucose	*% Galacturonic acid*	
15.1	84.9	86.0
48.0	52.0	58.3
90.0	10.0	30.1

TABLE IV

COMPARISON OF RESULTS[a]

Sample	*Uronic acid content (%) found by*			
	Acidic decarboxylation	*Hydroxylamine + iron(III)*	*Carbazole*	*Anthrone*
1. Galacturonic acid, reference specimen	96.0	98.2	98.0	99.0
2. Galacturonic acid, specimen A	75.7	70.5	70.2	71.4
3. Galacturonic acid, specimen B	88.4	86.4	85.8	87.1
4. Galacturonic acid, specimen C	80.5	76.6	76.2	77.8
5. Trigalacturonic acid	92.4	87.9	87.5	88.0
6. Glucurone, specimen A	78.9	119.8	118.7	119.9
7. Glucurone, specimen B	90.9	117.2	116.6	117.3
8. Glucurone, specimen C	91.1	101.1	100.1	100.5
9. Glucurono-δ-lactone	9.9	51.7	51.4	62.1
10. Melezitose	1.6	14.8	15.0	17.0
11. Hyaluronic acid	31.6	42.2 (A,D)	34.8	57.6
12. Bone gelatin No. 112	2.3	12.2 (D)	12.3	27.6
13. Bone gelatin No. 188	3.5	14.0 (D)	—	—
14. Gum ghatti	14.6	8.5 (A)	—	—
15. *Combretum leonense* gum	20.0	15.8 (A,C)	—	—
16. Alginic acid	97.4	65.7 (A,C)	—	—
17. Seaweed polysaccharide	17.0	24.4 (A,B,D)	—	—
18. Ammonium pectate A	55.0	48.0 (A,C)	—	—
19. Ammonium pectate B	74.1	51.4 (A,C)	77.4	77.8
20. Pectin, 240 grade	57.9	42.5 (A,B)	60.2	67.8
21. Plant roots, specimen A	6.6	7.6	8.2	25.4
22. Plant roots, specimen B	7.1	8.3	9.7	27.6
23. Plant roots, specimen C	6.3	6.4	7.6	24.3
24. Plant roots, specimen D	7.2	6.8	8.0	24.2

[a] (A) Polysaccharide incompletely soluble in methanolic 1% HCl.
(B) Esterified polysaccharide not water-soluble.
(C) Precipitation on addition of iron(III).
(D) Protein present.

Calibration curves

Calibration curves obtained for the reaction of some carbohydrate materials with anthrone are shown in Fig. 2. Different uronic acids give different absorptions. Interference by amino acids depends on the temperature, period of heating and acid

concentration[20]; some amino acids enhance the colour, some have no effect, and some depress colour formation.

Comparison of results

Comparisons of the results obtained by the three colorimetric methods and by acidic decarboxylation are shown in Table IV. The origin of the samples was as follows: specimen 1 was the reference standard sample; specimen 2–4, 6–10, and 20 were commercial materials; 5 – given by Dr. W. W. REID (University of Bristol); 11 – prepared from umbilical cord by Dr. N. J. KING; 12 and 13 – given by Dr. G. STAINSBY, British Glue and Gelatin Research Association; 14 – *J. Chem. Soc.*, (1955) 1160; 15 – *Talanta*, 3 (1959) 118; 16 – *J. Chem. Soc.*, (1952) 1833; – 17 *J. Chem. Soc.*, (1959) 2168; 18 – *J. Chem. Soc.*, (1961) 5333; 19 – *J. Chem. Soc.*, (1958) 4020; 21–24 – oat rootlets grown for cell-wall studies, provided by Dr. W. M. CROOKE, Macaulay Institute, Aberdeen.

DISCUSSION

The results presented show that large relative errors may be given by the colorimetric methods studied; both high and low values may occur, and there appears to be little basis for prediction or correction of any bias. For those specimens giving high colorimetric values, the anthrone method consistently gave the highest results of all. The methods studied gave more self-consistent results for galacturonic acid specimens than for the samples of glucurone investigated; the most satisfactory results were given for the specimen of highest purity, and this may have some significance. As has been pointed out recently[31], colour reactions which give satisfactory estimations of pure substances, generally lack specificity when applied to biological specimens. In consequence, natural products containing small percentages of uronic acids cannot be analysed successfully by colorimetric methods: this has been found recently for mucopolysaccharides[32] and for dextran products, on which colorimetry failed[33] at uronic acid/neutral sugar ratios of less than 1:10.

Uronic acid values obtained by colorimetric methods can clearly be subject to such large and unpredictable errors that it must be considered imprudent to place any reliance on values which cannot be substantiated (*cf.* ref. 34) by decarboxylation, which must be regarded as the best available reference method. It is not subject to interference from amino acids or amino sugars, and a correction can be applied for the carbon dioxide liberated from non-uronic acid residues if the sugar composition of the heterogeneous polysaccharide is known. Such amounts of carbon dioxide rarely contribute to the apparent uronic acid content by more than 2–3% absolute[30].

Colorimetry sometimes permits qualitative distinction to be made between uronic acids, and colorimetric results are most powerfully used in conjunction with decarboxylation data. Thus sodium heparinate gives substantially greater colorimetric absorptions than would be expected from its decarboxylation value, and this has led to speculation regarding the nature of the uronic acid present (*cf.* refs. 20, 24, 35). Similar deductions[36] from colorimetric results led to the identification of iduronic acid in chondroitin sulphate B, and may prove to be useful in investigations of the uronic acids present in alginic acid (*cf.* refs. 37, 38).

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SUMMARY

Values for the uronic acid content of a variety of polysaccharide materials were obtained by colorimetric methods and by acidic decarboxylation; very large differences were found, particularly for heterogeneous biological materials. Colorimetric methods are useful for "screening" many samples rapidly, and for routine or comparative measurements on characterised products. For materials of unknown structure, however, colorimetric results should be interpreted with caution, especially when shortage of material does not permit corroborative decarboxylation or titration values to be obtained.

RÉSUMÉ

Les auteurs ont effectué une étude comparative sur la détermination de le teneur en "acide uronique" des polysaccharides; les valeurs obtenues à l'aide des méthodes colorimétriques sont très différentes de celles obtenues par décarboxylation, en particulier avec les substances biologiques hétérogènes. Les méthodes colorimétriques peuvent être utiles lors d'essais en série ou pour des mesures comparatives de produits connus. Pour les produits dont on ne connait pas la structure, les résultats devraient être interprétés avec prudence.

ZUSAMMENFASSUNG

Bei der Bestimmung des Uronsäuregehaltes von Polysacchariden nach colorimetrischen Methoden und durch Decarboxylierungsreaktionen werden besonders bei heterogenem biologischen Material stark schwankende Werte erhalten. Colorimetrische Methoden eignen sich nur für Serienbestimmungen und Vergleichsmessungen bei gut charakterisierten Produkten. Bei Substanzen unbekannter Struktur sind colorimetrische Messwerte vorsichtig zu interpretieren, falls sie nicht durch eine Decarboxylierungsreaktion oder Titration gestützt werden können.

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34.

STUDIES ON URONIC ACID MATERIALS

PART IX[1]. THE SIMULTANEOUS DETERMINATION OF URONIC ACID AND ALKOXYL GROUPS IN POLYSACCHARIDES BY REFLUX WITH HYDRIODIC ACID

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Although colorimetric methods of estimating uronic acid groups are useful under certain conditions[1], they do not give absolute values and are subject to interferences when determinations on heterogeneous materials are required; particularly serious errors can arise when proteinaceous matter is present[1]. The random and individual nature of the interferences makes it difficult for their effect to be predicted and for suitable corrections to be applied.

Acidic decarboxylation is therefore much preferred analytically[2], since the side-reactions given by uronic materials, and the liberation of carbon dioxide from non-uronic residues, lead to comparatively small errors. These rarely exceed 3% absolute, and are always positive; their probable extent can be calculated once the proximate composition of the polysaccharide is known. In particular, interference from proteinaceous matter is negligible.

The kinetics and mechanism of the acidic decarboxylation of uronic acids have been investigated[3]. Although the analytical reaction[4] based on reflux with 19% (w/w) hydrochloric acid for 2.5 h[5] has given satisfactory results for several years, it became apparent that the following modifications would improve the method: (*a*) reduction of the required reaction time (*b*) increase in the specificity and sensitivity of determining the carbon dioxide evolved, and (*c*) development of apparatus simpler than that previously described[4], preferably eliminating specialised glass-blowing (this suggestion was made by workers in several other laboratories).

This paper describes how each of these advantages has been achieved. The modification involve (*a*) the use of constant-boiling hydriodic acid (55% w/w) as the decarboxylating medium, (*b*) the use of vapour-phase infra-red determination[6] of the carbon dioxide evolved, and (*c*) the use of a combined reaction-flask and reflux condenser (commercially available) as the reaction vessel. The assembled apparatus is therefore identical to that already described[7] for alkoxyl determinations, and it is possible to determine the alkoxyl and uronic content of a sample simultaneously.

This analytical method combines parts of the procedures of two earlier workers. BUSTON[8] used a modified Zeisel apparatus for uronic acid determinations with hydrochloric acid, and VOLLMERT[9] used hydriodic acid (57%) as the decarboxylating

medium. VOLLMERT recommended a reaction period of "1–2 h"; our experiments with characterised reference materials have shown that constant-boiling hydroidic acid gives complete decarboxylation in 1.5 h.

EXPERIMENTAL

Apparatus

The combined reaction flask and condenser [B.S. 1428: part C1: 9154 (part 2 apparatus)], delivery tube, and vapour trap have been described[7]. The apparatus is assembled as shown in ref. 7, Fig. 1, except that soda asbestos and Anhydrone are not added to the delivery tube. The omission of Anhydrone was found to give more complete and more reproducible recoveries of carbon dioxide: the traces of water vapour collected and subsequently transferred to the gas-cell do not interfere with the spectroscopic determinations required and do not cause significant "fogging" of the cell windows. Nitrogen (6–8 ml per min) is used as flow-gas; the methods of pretreatment and of stabilising the flow-rate have been described[7].

Spectroscopic determination of carbon dioxide and alkyl iodides

The collection of reaction products, and their quantitative transfer to a gas-cell[10] for determination, have been described previously[6] as have all the aspects of the determination of alkoxyl groups by this method[11].

Construction of calibration curves for carbon dioxide

Calibration is based on the very strong absorption by carbon dioxide at 2350 cm^{-1}; very few other gases give absorption in this part of the spectrum. Calibrations can be achieved manometrically[6], or by collecting the carbon dioxide given by known weights of sodium carbonate (M.A.R.) when reacted with acid in the decarboxylation apparatus. Fig. 1 shows a calibration curve obtained in this way, together with the calibration curve, for the same cell, for methyl iodide. After the known weight of carbon dioxide has been transferred to the gas-cell, dry air should be admitted so that the cell contents are at atmospheric pressure[6]. This minimises pressure broadening effects, and the trace amount of carbon dioxide so introduced is compensated by the double-beam operation of the spectrometer.

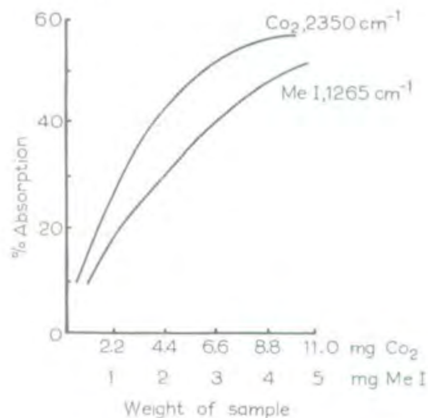

Fig. 1. Calibration curves for carbon dioxide (at 2350 cm^{-1}) and methyl iodide (at 1265 cm^{-1}). Length of gas-cell = 12.5 cm, volume = 56 ml.

Reagents and compounds

Analar hydriodic acid (about 55%, B.D.H. Ltd.) gives satisfactory results. Batches (250 ml) of the acid are preconditioned[7] before use by refluxing, with continuous passage of nitrogen at 6–8 ml per min, for 1 h.

The standard samples of glucurone, galacturonic acid, and alginic acid have been described[12]. The origin of other specimens is shown in footnotes to the Tables.

Procedure

The weight of sample taken should be sufficient to give a yield of carbon dioxide which falls within the range of sensitive response for the calibrated gas-cell: with the cell normally used (length 12.5 cm; internal volume = 56 ml) in our experiments, the suitable range of carbon dioxide is 1–6 mg. (Gas-cells giving similar sensitivities can be used with the less expensive bench-type spectrometers now available.)

The sample is refluxed for 1.5 h with 6 ml of the hydriodic acid. Some materials may be added to the reaction flask in a suitable long-handled weighing-spoon, but this is not possible with bulky, freeze-dried specimens, or with materials (*e.g.* gums, pectins) which "cake" or gel on the addition of aqueous solutions. The following technique is therefore recommended for all samples. The sample is weighed in a small weighing tube, in which dissolution is achieved by the addition of hydriodic acid (2 ml). The solution (or suspension in atypical cases) is transferred quantitatively to the reaction flask with the aid of a further four 1 ml portions of hydriodic acid.

Aqueous solutions may be analysed as follows. The concentration of the solution is adjusted so that 1 ml will yield a suitable weight of carbon dioxide. A 1-ml aliquot is transferred to the reaction flask, and then just under 5 ml of hydriodic acid (s. g. 1.94; about 66%) is added. The final volume is therefore approximately 6 ml and the acid is virtually of azeotropic composition. This procedure often eliminates drying stages if the isolation of solid material (*e.g.* in intermediate stages of a process) is not otherwise required.

RESULTS

Determination of the reaction-time required for quantitative results

Kinetic experiments with well-characterised samples[12] of glucurone, galacturonic acid and alginic acid showed that reflux for 1.5 h gave quantitative decarboxylation. Reflux for longer periods gave very little "over-production" of carbon dioxide, in marked contrast to previous experiences[4] with hydrochloric acid. Table I shows the agreement given by the hydrochloric and hydriodic acid reactions.

The "apparent uronic content" given by non-uronic compounds

All carbohydrates suffer some decomposition when refluxed with strong mineral acids, the carbon dioxide evolved indicating an apparent uronic acid content. In typical heterogeneous materials containing, say, 20% uronic acid + 30% hexose + 50% pentose, decomposition of the pentose and hexose will increase the yield of carbon dioxide originating from decarboxylation of the uronic residues. It is important to know the extent to which this occurs. The effect was investigated fully[13] for the hydrochloric acid reaction, and some of the values obtained are compared in Table II with the corresponding values given by reflux for 1.5 h in 55% hydriodic acid (*cf.* ref. 14, 15).

TABLE I

THE URONIC ACID CONTENT (%) OF SOME MATERIALS

Specimen	*By decarboxylation with hydriodic acid (55%) for 1.5 h*	*By decarboxylation with hydrochloric acid (19%) for 2.5 h*
Glucurone[a]	96.7	97.2
Galacturonic acid[a]	97.2	96.0
Alginic acid[a]	96.6	97.1
Acacia senegal gum[b]	16.1	15.9
Acacia seyal gum (Sample VIII)[c]	12.0	11.9, 11.6
Pectic acid[b]	62.8	61.8

[a] See ref. 12.
[b] Bulk commercial samples.
[c] ANDERSON AND HERBICH, *J. Chem. Soc.*, (1963) 1.

TABLE II

THE "APPARENT URONIC ACID" CONTENT OF SOME NON-URONIC MATERIALS[a]

Specimen	*Apparent uronic acid content from*	
	Reflux with 19% HCl for 2.5 h	*Reflux with 55% HI for 1.5 h*
Melezitose	1.6	1.8
Sucrose	2.6	3.2
Lactose	1.7	2.0
Glucoheptose	4.2	3.2
Mannose	3.5	3.4
Glucose	2.0	2.8
Galactose	2.8	2.5
Xylose	1.9	2.4
Arabinose	2.1	2.9
Ribose	2.2	3.4
Rhamnose	3.5	3.1
Fucose	3.0	2.2
Erythrose	4.8	3.2
Erythritol	0.1	0.4
2-Deoxy-glucose	1.2	2.4
Potato starch	—	2.5
Glycogen	—	3.1
Agar	—	6.4
Carrageenan	—	3.7
Inulin	3.0	3.1
Glucurosamine hydrochloride	0.3	1.0
Bone gelatin No. 112*	2.3	1.7
No. 188*	3.5	3.5
Edestin	—	0.4
1, 5-Gluconolactone	9.9	14.8
Ascorbic acid	99.4	99.2

[a] All samples were of commercial origin, except those marked* (research specimens from B.G.G.R.A.).

Identification of other volatile reaction products

The volatile reaction products evolved in reflux periods of up to 24 h were investigated for reactions in 19% hydrochloric acid and 55% hydriodic acid. With both these mineral acids, hexuronic acids and pentoses gave small amounts of furan, acetone and acetaldehyde; hexoses and methyl pentoses gave some 2,5-dimethylfuran and some 2-methylfuran, respectively. As indicated in Table III, however, the relative yields of these products were dependent on the mineral acid used.

TABLE III

THE VOLATILE PRODUCTS GIVEN BY SOME CARBOHYDRATES[a]

Compound	Period of reflux (h)	Volatile products from reflux with 19% HCl				Volatile products from reflux with 55% HI			
		Carbon dioxide	Furan	Acetaldehyde	Acetone	Carbon dioxide	Furan	Acetaldehyde	Acetone
Glucurone	0–3	+++	++	—	+	+++	tr	—	++
	3–9	+	tr	tr	tr	+	—	—	+
	9–12	+	+	+	+	tr	—	+	—
	12–24	tr	+	++	tr	tr	—	+	—
Galacturonic acid	0–6	+++	++	—	+	+++	tr	—	++
	6–9	+	+	tr	+	+	—	tr	tr
	9–12	+	+	+	tr	tr	—	tr	—
	12–24	tr	tr	tr	tr	tr	—	tr	—
Arabinose	0–12	+	++	—	—	+	++	tr	+
	12–24	+	++	+	—	tr	—	—	—
Ribose	0–24	+	+	+	—	+	—	tr	++

[a] +++ large amount, ++ small amount, + minor amount, tr just detectable.

The simultaneous determination of uronic and methoxyl contents

In spectroscopy, the most reliable results are generally obtained from absorptions of 30–70%. Under the conditions described, however, absorption at 2350 cm^{-1} is most sensitive to change in concentration for small amounts of carbon dioxide, *i.e.* those giving 15–50% absorption. As shown in Fig. 1, the gas-cell gives its most sensitive response for 1–6 mg quantities of carbon dioxide. The range of sample weights required is therefore: 4–24 mg for pure uronic acids or polyuronides, and 20–120 mg for materials containing 20% uronic acid.

This gas-cell also gives a sensitive response to 1–4 mg methyl iodide; nominal 50-mg samples therefore allow accurate simultaneous determinations of uronic acid and methoxyl contents in the range 8–48% and 0.5–2%, respectively. Methoxyl contents outwith this range can be determined by transferring the cell contents to a more sensitive or less sensitive gas-cell, as necessary. Simple procedures facilitating quantitative recovery from gas-cell to cold trap for transfer to a different cell have been described [6]. Although reflux periods in excess of 1 h during Zeisel determinations on carbohydrates can lead to error if a volumetric finish is used[16], longer reflux periods are not detrimental when the specific spectroscopic method is used to determine alkyl halides collected in a cold trap.

DISCUSSION

The amounts of carbon dioxide evolved from non-uronic materials in this rapid hydriodic acid reaction differ slightly from the amounts released by hydrochloric acid: on balance, however, results by either method are unlikely to be high by more than 3% absolute, even if nitrogenous materials are present.

The relative yields of other volatile products also differ from those evolved in hydrochloric acid decarboxylation.

This does not influence the analytical determination of carbon dioxide, but indicates that slight changes occur in the mechanism of the decomposition stages which follow the decarboxylation step. In particular, decarboxylation with hydriodic acid produces larger quantities of acetone (*cf.* ref. 17, 18).

The ability to determine alkoxyl and uronic groups simultaneously in a 1.5-h reaction is of value in routine determinations (economy of time) and in research investigations (economy of material). The method is particularly useful for determinations on pectins (50–70% uronic acids, 1–5% methoxyl) and plant gums (5–50% uronic acid, 0–2% methoxyl). Methoxyl contents of 1% are of structural significance[19]; recent studies on a number of *Acacia* gums have shown the presence of methoxyl groups, of which earlier investigators, particularly those dealing with gum arabic (*Acacia senegal*), appear to have been unaware.

A further advantage of the present method is its ability to differentiate between methoxyl and ethoxyl groups[7]. This is useful in investigations of artefacts arising from solvent retention [20,21] or from attempted reductions with potassium borohydride or diborane.

For complex multi-stage reactions, spectroscopic determination of carbon dioxide has considerable advantages over the conventional chemicals method. The spectroscopic method is specific and sensitive. Errors caused by the evolution of other acidic products are eliminated; the possibility of traces of the decarboxylating acid being carried over by the flow-gas is no longer an inherent source of high results. Other investigators have recently discussed the limitations of conventional methods of determining carbon dioxide in reactions which release other acidic vapours; as a result, the use of non-aqueous solvents[22], carbonic anhydrase[23], gas chromatography[24] and infra-red absorption[25–27] have all been recommended. Non-aqueous solvent methods may require purification of the carbon dioxide before its absorption[28].

The sensitivity of the present method may be increased by the use of more sensitive gas-cells[10,29], but the following simple technique is of value when a determination is required on an inadequate amount of sample which, by itself, would give an infrared absorption (say $< 15\%$ absorption) too small for accurate measurement. The weighed sample is pre-dissolved in the usual way and added to the reaction flask, to which had been added a known weight (sufficient to give 20–30% absorption) of the carbonate used in constructing the calibration curve. The weight of carbon dioxide from the polysaccharide is obtained as the difference between the weight recovered and the weight expected from the amount of carbonate taken.

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SUMMARY

A reaction period of 2.5 h is required for the decarboxylation of uronic acid groups with 19% (w/w) hydrochloric acid, but 55% (w/w) hydriodic acid gives complete decarboxylation in 1.5 h. This rapid reaction can be carried out in a standard Zeisel reaction flask and condenser. Vapour phase infra-red spectroscopy gives a specific determination of the carbon dioxide evolved, and facilitates simultaneous determinations of any alkoxyl groups present. The proposed method is particularly useful for pectins and plant gums; it gives greater sensitivity and reproducibility than previous methods.

RÉSUMÉ

La durée de décarboxylation de groupes uroniques peut être considérablement réduite, en utilisant l'acide iodhydrique à 55%, à la place de l'acide chlorhydrique à 19% (1 h. 30, au lieu de 2 h. 30). La spectroscopie infra-rouge en phase gazeuse permet de doser l'anhydride carbonique dégagé et facilite des déterminations simultanées de n'importe quel groupe alcoxyle présent. La méthode proposée est particulièrement utile pour les pectines et gommes végétales. Elle permet d'obtenir une sensibilité et une reproductibilité supérieures à celles des autres méthodes.

ZUSAMMENFASSUNG

Die Geschwindigkeit der Decarboxylierungsreaktion von Uronsäuren kann durch Verwendung von 55%iger Jodwasserstoffsäure an Stelle von 19%iger Salzäure beträchtlich erhöht werden. Das gebildete Kohlendioxyd wird IR-spektroskopisch bestimmt, wobei gleichzeitig die Bestimmung etwa vorhandener Alkoxygruppen möglich ist. Die beschriebene Methode eignet sich besonders zur Untersuchungen von Pektinen und Pflanzengummi. Die Empfindlichkeit und Reproduzierbarkeit ist grösser als bei den älteren Methoden.

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35.

The analytical importance of the methoxyl content of *Acacia* gum exudates

SIR,

Methoxyl groups occur frequently in plant gums as 4-methoxyglucuronic acid (*e.g.*, in *Albizzia*[1] and *Khaya*[2] species) or as ester groups (*e.g.*, in *Sterculia*[3] and *Astragalus*[4] gums). To date, however, the possibility of the presence of methoxyl groups in *Acacia* gum exudates appears largely to have been ignored, *e.g.*, in studies of *A. senegal*,[5] *A. pycnantha*,[6] *A. karroo*,[7] *A. cyanophylla*[8] and *A. sundra*,[9] although Stephen reported (without comment) a value of 0·35% for *A. mollissima*,[10] and Hulyalkar *et al.* found no methoxyl content in *A. catechu*.[11]

Recently, Anderson and Herbich observed[12] that the methoxyl content of a number of nodules of the gum from *A. seyal* ranged from 0·7–1·3%, and this has led us to analyse specimens of the gum from 12 further *Acacia* species, not hitherto studied chemically. We have also re-investigated three different specimens of *A. senegal syn. Verek* (gum arabic) and a sample of *A. karroo*. An infrared method,[13] specific for methoxyl groups, was used to analyse purified samples, prepared from authenticated single nodules of each species by electrodialysis so that artifacts arising from solvent retention[14] could not occur (*cf.* ref. 1).

The results shown in Table I indicate that the presence of methoxyl groups in *Acacia* gums is a more general occurrence than hitherto believed. In addition, the range of values found (0·75 to 1·44%) for 9 nodules of *A. nilotica* substantiates recent evidence[12,15] for inter-nodule variation in the composition of plant gums.

The viscosity of samples of gum tragacanth and of pectins is known[16] to be related to their methoxyl content. The limiting flow-time numbers for some of our *Acacia* samples, determined under standardised conditions, are also shown in the Table: a plot of methoxyl content *versus* limiting flow-time number gives a smooth curve.

TABLE I

Acacia species	Methoxyl, %*	Limiting flow-time number†
A. giraffae Burch	2·40	
A. nilotica (L.) Willd. ex Del.	1·14[a]	10·4
A. mellifera (Vahl) Benth.	1·06	
A. seyal Del.	1·02[b]	12·1
A. seyal Del. var. fistula	0·90	
A. arabica (Lam) *Willd.*	0·88	12·5
A. tortilis (Forsk.) *Hayne.*	0·57	
A. mearnsii De Wild.	0·45	
A. campylacantha Hochst. ex A. Rich.	0·42	16·0
A. drepanolobium Harms ex Sjöstedt.	0·40	16·6
A. senegal (L.) *Willd.*	0·36[c]	19·2
A. dealbata Link.	0·35	21·5
A. laeta R. Br. ex Benth.	0·33	23·0
A. nubica Benth.	0·15	
A. karroo Hayne.	0·13	

* Electro-dialysed, freeze-dried samples, corrected for trace residual moisture and ash content.
† In aq. 4% NaCl solution at 25°.
[a] Average of results for 9 nodules (range 0·75–1·44%).
[b] Average of results for 6 nodules (range 0·70–1·30%).
[c] Average of results for 3 nodules (range 0·34–0·37%).

It is therefore suggested that the methoxyl content of *Acacia* gums has some structural significance, and that greater analytical attention should be given to this in future studies. We do not subscribe to the view, recently expressed[17] in a study of gum Jeol, that a methoxyl content of 0·51% can readily be dismissed as being very low and not structurally significant.

It is of interest that our re-examination of *A. senegal* and *A. karroo* has revealed the presence of methoxyl groups. Re-examination of other species, *e.g.*, *A. pycnantha*, may well provide an explanation for the complex behaviour observed[6] during examination of the aldobiuronic acid fraction.

Acknowledgements—We thank Professor Sir Edmund Hirst, F.R.S. for his interest, and record our indebtedness to (the late) Mr. M. P. Vidal-Hall, Gum Research Officer, El Obeid, and to Dr. Dyer, Botanical Research Institute, Pretoria, for collecting authenticated specimens of *Acacia* gums for our studies. We thank Samuel Jones and Co., Ltd. (Camberwell); James Laing, Son and Co., Ltd. (Manchester); Rowntree and Co., Ltd. (York); and the Director of Forests, Sudanese Government, for financial support.

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STUDIES ON URONIC ACID MATERIALS

PART XI[1]. THE CARBOHYDRATE COMPONENT OF THE OLEORESIN FROM *Boswellia Papyrifera* (DEL.) HOCHST

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INTRODUCTION

The genus *Boswellia* (family *Burseraceae*) contains some 25 species; some of these [*e.g.*, *B. serrata* Roxb. (syn. *B. thurifera*; syn. *B. glabra*)] are indigenous to Central India, and others to Africa [*e.g.*, *B. carteri* Birdw. (syn. *B. sacra*) and *B. papyrifera* (Del.) Hochst.] The oleoresin sold commercially as Frankincense ("gum olibanum") is largely the exudate from *B. carteri*, but this may be adulterated with *B. papyrifera*, which is not so strongly aromatic, and with exudates from species of *Commiphora*. Studies on commercial samples should therefore be avoided; if samples cannot be authoritatively verified during collection, they ought to be backed by suitable botanical specimens of the leaves and inflorescence[2].

The only *Boswellia* species to have been studied chemically to date are *B. serrata* and *B. carteri*. No attention has been given to the exudate from *B. serrata* since Malandkar's preliminary report[3] in 1925, despite the fact that no information was given regarding the identity or amount of the uronic acid present (*cf.* ref. 4). Two investigations of the polysaccharide from *B. carteri* oleoresin have, however, been reported. Unfortunately, these conflict regarding the nature of the uronic acid. Jones and Nunn[5] found D-galactose, L-arabinose, and 4-*O*-methylglucuronic acid, in the ratio 7:1:4, together with traces of L-rhamnose and L-fucose; in contrast, El Khadem and Megahed[6] reported D-galactose, L-arabinose, and D-galacturonic acid, in the ratio 5:1:2.

It is most unusual for the uronic acid to differ markedly from species to species within any botanical genus. Further studies were clearly required to clarify the position, and our current interest[1] in convenient sources of 4-*O*-methylglucuronic acid prompted us to consider *Boswellia* exudates. Our attempts to secure authenticated specimens of *B. carteri* or *B. serrata* have not yet proved successful, but we report here the results of an analytical study of the exudate from *B. papyrifera* (Del.) Hochst, secured for us by the Sudanese Gum Research Officer.

EXPERIMENTAL AND RESULTS

Analytical Methods

Analyses were carried out using the standard methods described previously[7],

except that (*i*) the methoxyl and uronic anhydride contents were determined by infrared methods, after reaction with hydriodic acid[8], and (*ii*) paper chromatography was carried out with the following solvent systems: (*a*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); (*b*) butan-1-ol–ethanol–water (4:1:5); (*c*) butan-1-ol–pyridine–water–benzene (5:3:3:1); (*d*) acetone–ethanol–propan-2-ol–borate buffer, 0.05M, pH10 (3:1:1:2).

Examination of the crude oleoresin

The small, pale yellow-white "tears" were free from any contaminating adherents. The oleoresin had a pleasant, characteristic odour, and was completely insoluble in water. (Found: moisture, 5.2%. On a dry-weight basis: ash, 0.7%; nitrogen, 0.28%.

Isolation of the polysaccharide

The oleoresin, crushed to a fine powder, was refluxed with ethanol for 24 h. The white polysaccharide residue was collected by centrifugation, and carefully washed with small portions of ethanol until free of waxy and resinous matter; the carbohydrate material was then completely soluble in cold water. A solution was filtered, and electrodialysed in a grease-free perspex cell at 20° until current ceased to flow under an applied potential of 330 v. On being freeze-dried, the purified polysaccharide was obtained in the free-acid form as a white powder; yield, 21% of the crude oleoresin (dry-weight basis).

Determinations on the purified polysaccharide

Found: moisture, 5.0%; ash, nil; nitrogen, 0.78; uronic anhydride, 19; methoxyl, 3.28%; $[\alpha]_D^{25} -14°$ (*c* 1, water); limiting flow-time number, 16.8 (Ubbelohde suspended level dilution viscometer at 25.0°, in M aqueous sodium chloride). The specific infrared method[8] showed that the electrodialysis treatment had removed all traces of the ethanol used in the extraction stage (*cf.* ref. 9). Furthermore, attempted saponification (dilute NaOH) did not decrease the methoxyl content of the polysaccharide, which therefore has no methyl ester groups.

Attempted fractionation

In view of El Khadem and Megahed's claim[6] to have fractionated the carbohydrate from *B. carteri* into acidic ($[\alpha]_D -9.2°$) and neutral ($[\alpha]_D -14.7°$) polysaccharides, fractionation with cetyltrimethylammonium bromide ("Cetavlon") was attempted. Cetavlon (20% solution) was added to an aqueous solution of the electrodialysed material, until precipitation was complete; after removal at the centrifuge, the supernatant contained no polysaccharide (phenol–sulphuric acid test[10]).

Hydrolysis and paper chromatography

Hydrolysis was effected with sulphuric acid (2N) on a boiling-water bath for 8 h. After neutralisation (barium carbonate), filtration, and reduction in volume at 30°

(reduced pressure), the resulting pale-brown syrup was chromatographed against reference sugars on Whatman 3MM paper in each of the solvents (*a*)–(*d*).

Solvents (*a*) and (*d*) showed that the main uronic acid present was 4-*O*-methylglucuronic acid, with a trace of glucuronic acid. There was no galacturonic acid present (*cf.* ref. 6). Development for 48 h in solvent (*a*) gave two aldobiouronic acids having R_{gal} values of 0.28 and 0.56. The faster-moving acid was chromatographically identical to 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)D-galactose; hydrolysis of the material eluted from the thick paper gave 4-*O*-methylglucuronic acid and galactose. This aldobiouronic acid was also present in *B. carteri*[5].

The separations achieved in solvents (*a*), (*b*) and (*c*) showed that the neutral sugars present were galactose, arabinose, rhamnose, and fucose; by the standard analytical methods (*cf.* ref. 11), these were present in the ratio 14:2:1:trace. Calculation then gives the essential composition of the polysaccharide to be uronic acid 19%, galactose 66%, arabinose 10%, and rhamnose 5%, with a trace of fucose.

DISCUSSION

The results of these analyses indicate that the acid polysaccharide from *B. papyrifera* contains the same component sugars as that from *B. carteri*[5]. Indeed, the two species have close similarities. In view of this, the results do not support El Khadem and Megahed's suggestion[6] that *Boswellia* species contain galacturonic acid; these authors, unfortunately, investigated a commercial sample of unknown origin, and their claim to have effected a fractionation may also have arisen from this fact. This aspect of their work therefore requires re-investigation.

Jones and Nunn[5] reported a neutralisation equivalent of 545 for *B. carteri*. Assuming that all the titratable acidity arises from the uronic acid groups, calculation shows that the methoxyl content found (5.4%) is sufficient for virtually all of the uronic acid groups to be present in the 4-methoxy form. Our analytical values for *B. papyrifera* lead to a similar conclusion; the traces of free glucuronic acid observed chromatographically may have resulted from some demethylation during hydrolysis.

It is evident that the oleoresins from both *B. carteri* (yield of polysaccharide, 12%; uronic acid content, 32%) and *B. papyrifera* (yield of polysaccharide, 21%; uronic acid content, 19%) are convenient sources of 4-*O*-methylglucuronic acid. Further analytical and structural investigations are, however, necessary to supplement the present work on *B. papyrifera*, and the incomplete study[5] of *B. carteri*. It is hoped that this report will stimulate interest in the chemistry of *Boswellia* species.

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SUMMARY

An acidic polysaccharide is easily extracted from the oleoresin exuded by *B. papyrifera*. The results of an analytical study of the composition of the polysaccharide are given; they support an earlier report of the presence, in *Boswellia* exudates, of 4-*O*-methylglucuronic acid in appreciable quantity.

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Studies on Uronic Acid Materials. Part XII.[1] The Composition of *Acacia* Gum Exudates

By D. M. W. Anderson and K. A. Karamalla

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Studies on Uronic Acid Materials. Part XII.[1] The Composition of *Acacia* Gum Exudates

By D. M. W. Anderson and K. A. Karamalla

The exudates from seven *Acacia* species, not hitherto studied chemically, have been examined. As a result, variation in the characteristic features of the *Acacia* group of plant gums can now be discussed from a more representative viewpoint. Species considered previously to have atypical features (*e.g.*, positive optical rotation or low rhamnose content) are now seen to occur almost as frequently as those with negative rotations and unit correspondence between the rhamnose and uronic acid content.

ALTHOUGH several hundred *Acacia* species are known botanically, only *Acacia senegal*[2] (syn. *verek*), *A. mollissima*,[3] *A. pycnantha*,[4] *A. cyanophylla*,[5] *A. karroo*,[6] *A. catechu*,[7] *A. sundra*,[8] and *A. seyal*[9] have been studied chemically to any extent; a few further species, *e.g.*, *A. leucophloea*,[10] *A. decurrens*,[11] *A. arabicum*,[11] *A. farnesiana*,[12] and *A. sieberiana*[12] have been examined in minor detail, from some particular aspect. Table 1 summarises the general information available to date for comparative purposes.

In the belief that general conclusions regarding the typical features of this large genus could not reasonably be based on such limited information, we sought authenticated specimens of the exudate from further *Acacia* species.

This Paper presents the data obtained from comparative studies of seven species not investigated previously.

EXPERIMENTAL AND RESULTS

Collection and Origin of Specimens.—We are grateful to the late Mr. M. P. Vidal-Hall, formerly Gum Research Officer, Republic of the Sudan, for identifying and collecting nodules of natural exudation gum from (a) *Acacia nilotica* (L.) Willd. ex Del., (b) *A. arabica* (Lam.) Willd., (c) *A. campylacantha* Hochst ex A. Rich. [syn. *A. suma* (non Kurz), syn. *A. polyacantha* Willd., subsp. *campylacantha* (Hochst ex A. Rich.) Brenan]; (d) *A. fistula* [syn. *A. seyal* Del., var.

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fistula (Schweinf.) Oliv.] (e) *A. nubica* Benth. Details of their collection are as follows: *A. nilotica*, from a single tree, Hawata, Kassala Province, 3 Feb., 1963; *A. arabica*, from small trees, Baruki Rahad, Kordofan Province, Mar., 1961; *A. campylacantha*, from a single tree, Umm Shuheita, Kadugli District, 9 Jan., 1961; *A. fistula*, from a single tree, Goz el Ganzara, 15 Dec., 1962.

TABLE 1

Data for species studied prior to this Paper

Acacia species	[α]D	Equiv.	Approx. sugar composition (%): Glucuronic acid *	D-Gal	L-Ara	L-Rha
1. *A. senegal* [2,4]	−28°	1400	13	37	37	13
2. *A. mollissima* [3]	−49°	1880	9	38	45	7
3. *A. pycnantha* [4]	−8°	—	1—5 †	65	27	1—2
4. *A. cyanophylla* [5]	−20°	740	24	46	8	21
5. *A. karroo* [6]	+54°	1660	12	50	36	2
6. *A. catechu* [7]	−32°	1025	18	50	17	14
7. *A. sundra* [8]	−30°	980	18	42	28	14
8. *A. seyal* [9]	+58°	1340	13	38	46	3

* Decarboxylation values where quoted, or calculated from Equiv. and/or sugar ratios. † The summary of ref. 4 states 5% uronic acid, although a decarboxylation value (corrected) of 1·2% and a titrimetric value of 0·8% were quoted.

The other specimens studied are: (f) *A. drepanolobium* Harms ex Sjöstedt, collected by Mr. W. M. C. Bagshaw, Provincial Forest Officer, at Tabora, Western Province, Tanganyika, July, 1961; (g) *A. dealbata* Link, collected by Mr. R. L. Willans, Government Silviculturalist, Lupembe Forest Reserve, Southern Highlands Province, Tanganyika, 9th Nov., 1960.

Analytical Methods.—All the results quoted are corrected for moisture content. The methods used for determinations of moisture, ash, and nitrogen contents have been described.[9] Rotations were found at 20° for 3% aqueous solutions. Viscosity measurements were made at 25·0° with suspended-level dilution viscometers, using aqueous solutions 3% with respect to the gum and 4% with respect to sodium chloride. Vapour-phase infrared methods were used to determine (a) methoxyl, by modified Zeisel reaction,[13] (b) uronic anhydride, by acidic decarboxylation with hydriodic acid,[13] (c) rhamnose, by release of acetaldehyde on periodate oxidation of a hydrolysate.[14] Standard methods were used for complete hydrolyses, paper partition chromatography, and sugar determinations after elution from the chromatograms (cf. ref. 9). The solvent systems were: butan-1-ol–pyridine–water–benzene (5 : 3 : 3 : 1 :, top layer); ethyl acetate–acetic acid–formic acid–water (18 : 3 : 1 : 4).

Studies on Native Material.—A limited number of preliminary analyses on the specimens of native gum should always be made (cf. ref. 15) when studying new species, so that any anomalous effects, formation of artefacts, and fractionations occurring during the purification stages can be detected. Table 2 presents the results of analyses of the native specimens.

TABLE 2

Determinations on crude samples

	A. nilotica	*A. arabica*	*A. campylacantha*	*A. drepanolobium*	*A. dealbata*	*A. fistula*	*A. nubica*
Insoluble * (%)	0·25	5·2	0·7	18·6	6·9	0·12	1·4
Ash (%)	2·48	2·38	2·92	2·52	2·94	2·91	1·54
Nitrogen (%)	0·08	0·08	0·34	1·25	0·65	0·07	0·20
Methoxyl (%)	1·05	0·87	0·2	0·4	0·26	0·9	0·1
Uronic acid (%)	9·6	10·0	10·4	9·0	7·7	9·3	6·4
Flow-time (sec.) †	360	421	389	426	765	520	348
$[\alpha]_D^{20}$ ‡	+106°	+97°	−3°	+74°	−24°	+60°	+98°

* % insoluble after shaking in cold water (sufficient to give a 5% solution) for 24 hr. † Ubbelohde suspended-level viscometer at 25°; water = 212 sec., A. *senegal* = 450—700 sec., dependent upon origin of specimen. ‡ *c*, 3% in water.

Studies on Electrodialysed Material.—Each gum was purified by exhaustive electrodialysis (cf. ref. 9) against distilled water. Table 3 gives the results obtained from analyses of purified specimens.

TABLE 3

Determinations on electrodialysed samples

	A. nilotica	*A. arabica*	*A. campylacantha*	*A. drepanolobium*	*A. dealbata*	*A. fistula*	*A. nubica*
Ash (%)	0·02	0·03	0·05	0·00	0·03	0·00	0·03
Nitrogen (%)	0·02	0·07	0·28	1·12	0·61	0·06	0·21
Methoxyl (%)	0·96	0·88	0·42	0·40	0·35	0·90	0·15
Flow-time (sec.) †	285	382	328	417 *	534	402	294
Limiting flow-time no.	9·5	12·5	16·0	16·6 *	21·5	19·4	9·8
$[\alpha]_D^{20}$ (*c*, 3% in water)	+108°	+100°	−3°	+75° *	−25°	+61°	+100°
Equiv.	1890	2370	2020	2060	1840	1530	3030
Hence uronic anhydride (%)	9·3	7·4	8·7	8·6	9·5	11·5	5·8
Uronic anhydride (%)(decarbox.)	9·2	7·8	8·2	9·0	7·8	9·3	7·3
D-Galactose (%)	44	36	54	26	41	37	33
L-Arabinose (%)	46	54	27	56	41	50	58
L-Rhamnose (%)	0·4	0·4	7	1·0	6	$\ngtr$0·4	0·6

* Values for soluble material; this gum does not dissolve completely in cold water. † Water = 186 sec.

DISCUSSION

Since the values quoted in Tables 2 and 3 were obtained from replicate analyses of single nodule specimens they should be regarded as representative rather than as absolute values; previous studies [9,16] indicated that appreciable inter-nodule variation occurs in gum exudates, and the extent of this effect in other *Acacia* species is under examination. Of the *Acacia* species studied prior to 1962 (*i.e.*, species 1—7 in

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Table 1), all except *A. karroo* had negative rotations and glucuronic acid : rhamnose ratios approximating to unity. The overall picture now differs. Of the fifteen *Acacia* species for which data now exist (Tables 1 and 3), eight have negative rotations (range −3 to −49°) and seven have positive rotations (range +54 to +108°). Clearly, some major structural differences must exist to account for these results.

The rhamnose content of *Acacia* species has long been of interest. Heidelberger's original evidence for heterogeneity in *A. senegal* was based on the observation that the rhamnose content of a fraction, obtained in small yield, was depleted; a recent study [17] established that some of the L-rhamnose present in *A. senegal* is glycosidically linked to D-glucuronic acid residues. The small amounts of rhamnose in *A. pycnantha* [4] (1—2%) and in *A. karroo* [6] (≈2%) could not be determined with confidence by paper chromatography: when, therefore, five of the seven species under investigation were observed to contain only chromatographic traces of rhamnose, an infrared method [14] was developed to determine rhamnose when present at less than the 2% level.

A. seyal [9] (Table 1) and all the species reported in Table 3 contain methoxyl groups. Of species studied earlier, only *A. mollissima* was reported to have a methoxyl content; re-examination [18] of *A. senegal* and *A. karroo* has, however, shown that these also contain methoxyl groups. In this laboratory, recent experiments have indicated that the physical properties of gum solutions are dependent on both the methoxyl and the nitrogen content,[19] and that *Acacia* gums contain aldobiouronic acids in which 4-*O*-methylglucuronic acid is present.[20] To date, only *A. karroo* [6] has been reported to contain any aldobiouronic acid other than the ubiquitous 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose.

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38.

STUDIES ON URONIC ACID MATERIALS

PART XIII[1]. THE COMPOSITION OF GUM EXUDATES FROM *Albizia sericocephala* AND *Albizia glaberrima* WITH AN APPENDIX ON BOTANICAL NOMENCLATURE IN THE GENUS *Albizia*

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INTRODUCTION

The genus *Albizia* (family *Leguminosae*, sub-family *Mimosoideae*) contains 100–150 species[2]; although it is distinguishable botanically from the closely related genera *Inga* and *Acacia*, considerable confusion existed between these three genera in the past. Consequently, complex series of synonyms exist for many *Albizia* species (see APPENDIX). It is unfortunate that "*A.*" is used to abbreviate both "*Albizia*" and "*Acacia*", and it is important that confusion between these genera be avoided. Although the spelling "*Albizzia*" has been used consistently by chemical authors to date, the correct botanical form[2] is *Albizia*.

Although Adriaens[3] reported the exudation of gum by *Albizia fastigiata* and *Albizia gummifera*, a structural study of *Albizia zygia*[4] and a note on the composition of *Albizia glaberrima*[5], published simultaneously in September 1961, appear to have been the first significant studies of *Albizia* exudates. These contributions were followed by notes on the composition of the gums from *Albizia lebbeck*[6] and *Albizia procera*[7], and on the aldobiouronic acids in *A. procera*[8]. It is therefore clear that Drummond and Percival were incorrect in stating[4] "the genus *Albizia* contains some twenty-six species, of which only two, *A. zygia* and *A. sassa*, produce gum".

This paper primarily presents results from an analytical study of the composition of the exudate from *Albizia sericocephala* Benth., now known[2] to be an African subspecies of *Albizia amara* (Roxb.) Boiv., which occurs almost exclusively in Asia (see APPENDIX).

Comparison of our results with those reported previously for *A. zygia*[4], *A. glaberrima*[5], *A. lebbeck*[6], and *A. procera*[7] (see Tables I and II) indicated that some of the analytical data for *A. glaberrima*[5] were inconsistent with the broad characteristic features of the other species studied to date. Thus, Torto reported[5] *A. glaberrima* gum:

(*a*) to be insoluble;

(*b*) to have a methoxyl content of 3.5%: this value is unusually high–more than would be present, indeed, if *all* of its glucuronic acid (*ca.* 16%, by calculation from the neutralisation equivalent reported[5]) were present in the 4-*O*-methyl form;

(*c*) to contain galactose, arabinose, and rhamnose, in the ratio 3:1:2–allowing

for 16% of uronic acid, it follows that the gum contained *ca.* 28% of rhamnose, an unusually high value for a plant gum.

Re-investigation of the composition of *A. glaberrima* gum was therefore desirable, and, in Tables I and II, we compare our results for an authenticated specimen with those reported[5] by Torto.

EXPERIMENTAL AND RESULTS

Analytical methods

The methods used have been described[1], except that paper chromatography was carried out with the following solvent systems(v/v): (*a*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); (*b*) ethyl acetate–pyridine–water (10:4:3); (*c*) butan-1-ol–pyridine–water–benzene (5:3:3:1, top layer); (*d*) butanone–acetic acid–water, saturated with boric acid (9:1:1).

Origin of specimens

Gum from *Albizia sericocephala* (clean, pale-yellow nodules, closely similar to commercial grades of gum arabic (*Acacia senegal*) in appearance) was collected at El Obeid in February 1965 by the Gum Research Officer, Republic of the Sudan. *Albizia glaberrima* gum (a brown, semi-plastic mass) was collected, near Entebbe in June 1965, by the Conservator (Research) of Forests, Uganda.

Examination of the crude gums

Both specimens dissolved when shaken in cold water for 36 h. *A. sericocephala* gave a very viscous solution ($[\eta] = 92.6$; *cf. Acacia senegal*, $[\eta] = 15$–25). In contrast, *A. glaberrima* gave a very low viscosity, $[\eta] = 4.7$.

Table I presents the results of other analyses and gives comparisons with the corresponding data available for *A. zygia*[4], *A. lebbeck*[6], and *A. procera*[7].

TABLE I

ANALYTICAL DATA FOR CRUDE *Albizia* GUMS

	A. sericocephala	*A. glaberrima*	*A. zygia*[4]	*A. lebbeck*[6]	*A. procera*[7]
Moisture, %[a]	11	27	17	11	12
Ash, %	4.4	5.7	5.8	4.6	5.7
Limiting flow-time number[b]	92.6	4.7	n.d[c]	n.d	n.d
Nitrogen, %	0.74	0.50	0[d]	0.26	0.2
Methoxyl, %	0.69	0.83	ca. 1	n.d	n.d
Uronic anhydride, %	17.4	20.6	n.d	n.d	n.d
Solubility	Complete in water	Complete in water	Partial in N NaOH	Complete in 2% NaOH	Complete in water

[a]Other analyses are corrected for these values. [b]In 4% saline at 25.0°. [c]n.d, not determined. [d]Our reference specimen of *A. zygia* gives N, 0.89%.

TABLE II

THE COMPOSITION OF PURIFIED *Albizia* GUMS

	A. sericocephala	*A. glaberrima*	*A. glaberrima*[5]	*A. zygia*[4]		*A. lebbeck*[6]	*A. procera*[7]	
Method of Purification[a]	e	e	p		p	p	p	
Yield, %	70	67	60	58[b]	16[c]	55	65	
Ash, %	nil	nil	nil	0.25	0.22	n.d[d]	trace	
Nitrogen, %	0.57	0.39	nil	n.d	n.d	n.d	n.d	
Methoxyl, %	0.65	0.58	3.5	1.3	n.d	n.d	1.5	
Neutralisation equivalent,	840	896	1100	723	n.d	1500	n.d	
Hence, uronic anhydride, %[e]	21	20	16	24	n.d	12	n.d	
Uronic anhydride, % (decarboxylation)	18	22	n.d	23.5	32.6	n.d	11.1	
Specific rotation (water)	$[\alpha]_D^{17}$ −12° (c 0.7)	$[\alpha]_D^{17}$ −11° (c 1.0)	$[\alpha]_D^{17}$ −12° (c 0.3, NaOH)	+21° (c 0.49)	+38.7° (c 0.32, 0.1N NaOH)	n.d	$[\alpha]_D^{30}$ +15° (c 0.2)	
Limiting flow-time No.[f]	57.5	4.7	n.d	n.d	n.d	n.d	n.d	
Sugar composition[g]			calc.	calc.	calc.	i	calc. Ref. 7	calc. Ref. 8
Galactose, %	30	34	42	27	40	+	47	48
Mannose, %	10	6	trace	10	13		trace	trace
Arabinose, %	30	30	14	40	16	+	31	32
Rhamnose, %	12	8	28	trace	—	+	11	8
Glucuronic acid, %	14	19	16 (combined)	15	21		11 (combined)	11 (combined)
4-*O*-Methylglucuronic acid, %	4	3		8	11			

[a]e, electrodialysis; p, precipitation by alcohol.
[b]Material extracted by cold, dilute alkali.
[c]Material extracted by hot water.
[d]n.d, not determined.
[e]Assuming all titratable acidity arises from uronic acid groups.
[f]In 4% saline at 25°.
[g]Calc., calculated from sugar ratios reported; i, insufficient information available for calculation.

Purification

The crude gums were each shaken for 36 h with sufficient cold water to give 5% (w/v) solutions. After filtration through several layers of fine muslin, followed by filtrations through acid-hardened paper, the gum solutions were electrodialysed at 330 volts, in a grease-free, perspex cell fitted with cooling coils, until current ceased to flow.

During the electrodialysis of *A. glaberrima*, a fine, brown precipitate was deposited; this material (yield, 4%) was proteinaceous (N, 2.06%) and has not yet been investigated.

The free gum-acids were isolated by freeze-drying. *A. sericocephala* gave a white product (yield, 70%), and *A. glaberrima* a pale-brown product (yield, 67%).

Analysis of the purified gums

Table II presents the results obtained for *A. sericocephala* and *A. glaberrima*, and compares these with the data reported for *A. glaberrima*[5], *A. zygia*[4], *A. lebbeck*[6], and *A. procera*[7].

Examination of aldobiouronic acids in A. sericocephala *and* A. glaberrima

Samples were partially hydrolysed (N sulphuric acid, 100°, 8 h), neutralised (barium carbonate), filtered, de-ionised [Amberlite IR-120 (H^+ form) resin)], and concentrated at 35°. Comparison of the partial hydrolysates on the same chromatogram [Whatman No. 1 paper, solvent (*a*)] revealed that each gum contained three aldobiouronic acids, having the mobilities R_{Gal} = 0.21, 0.42, and 0.58. A mixed-indicator spray[9], specific for the detection of acidic saccharides, was used to locate the separated components after the chromatogram had been dried in a current of air to remove all traces of the acidic solvent. The acid having R_{Gal} = 0.42 was present in trace amount only in each gum, and its identity has not been investigated.

For each gum, the aldobiouronic acids having R_{Gal} = 0.21 and 0.58 were separated [Whatman 3MM paper, solvent (*a*)], located (by spraying side strips), and isolated by elution from the paper. The aldobiouronic acids from each gum were hydrolysed (2N sulphuric acid, 100°, 6 h), neutralised (barium carbonate), filtered, de-ionised [IR-120 (H^+ form) resin], concentrated at 35°, and examined in solvents (*a*), (*b*), and (*c*). The aldobiouronic acid having R_{Gal} = 0.21 gave glucuronic acid, glucurone, and galactose; the acid having R_{Gal} = 0.58 gave 4-*O*-methylglucuronic acid and galactose. The faster-moving acid was then shown to be identical chromatographically in solvent (*a*) with 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose, and distinct from 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose. The slower-moving aldobiouronic acid was chromatographically identical with 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose.

DISCUSSION

The exudates from *A. lebbeck*[6] and *A. procera*[7] are used as substitutes for,

and adulterants of, gum arabic (*Acacia senegal*). *A. sericocephala* is found in those parts of the Sudan at which gum cultivation is concentrated; its exudate is so similar in appearance to commercial grades of *A. senegal* that admixture of the two gums would be extremely difficult to detect. Studies on any commercial sample of gum must always be treated with reserve.

A. sericocephala is not normally tapped; our specimen originated from natural exudation. Its exceptional viscosity, in comparison with the *Acacia* gums, makes it of possible commercial interest. We hope to secure, for study, a specimen of the gum exuded by *A. sericocephala* in response to tapping.

Although both specimens of *A. glaberrima* have the same rotation, there are considerable differences between some of the other results found by Torto[5] and by ourselves. Despite a careful check on our results, the differences remain. It is difficult to find an explanation for this, although *A. glaberrima* is now known (see APPENDIX) to exist in different varieties, and the two specimens involved originated from widely separated parts of Africa. Torto's specimen was obtained from trees heavily infested with moth larvae, and this may be significant; in view of the complete solubility of other *Albizia* species, it is interesting that both Torto's specimen and the other species originating from Ghana, *A. zygia*, were virtually insoluble in water.

The rhamnose content reported by Torto[5] is three times our value, and does not align with the trend set by the other species studied to date. The same comment applies to Torto's methoxyl content of 3.5%, which is so high, in relation to the uronic acid content of *A. glaberrima*, as to suggest that this species has some structural feature which is not typical of the other *Albizia* species studied so far. We found no support for this in our analysis, and we suggest that Torto's high value may be due to solvent retention[10] of the methanol used to effect purification by precipitation. In order to detect such possible artifacts, we have always considered it essential[11,12] to report a limited number of exploratory analyses on any gum prior to attempting its purification.

A. procera has now been reported[8] to contain the same two aldobiouronic acids identified in *A. zygia*[4] gum. Although *A. zygia* gum was fractionated, *A. procera* gum is claimed to be a homogeneous polysaccharide. We have confirmed Torto's report[5] that *A. glaberrima* contains residues of three aldobiouronic acids; *A. sericocephala* appears to contain the same three acids, and chromatographic evidence indicates that the two major aldobiouronic acids in *A. sericocephala* and *A. glaberrima* are different from those in *A. zygia* and *A. procera*.

The *Albizia* exudates therefore have a number of interesting features, although two of the distinctions made by Drummond and Percival[4] are no longer correct; rhamnose is not a major constituent[13] in all *Acacia* gums, and the presence of two uronic acids is not an unusual feature[14] in the *Mimosoideae* family. Structural investigations on *A. sericocephala* will be carried out, and analytical studies of further *Albizia* species are required to give a broader view of the characteristics of this genus.

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SUMMARY

The compositions of the gum exudates from *Albizia sericocephala* and *Albizia glaberrima* have been investigated. The results of analyses are compared with data available for the other *Albizia* species studied previously. The main aldobiouronic acids present in *A. sericocephala* and *A. glaberrima* differ from those found in *A. zygia* and *A. procera*. The analytical results obtained for *A. glaberrima* differ in a number of respects from those reported previously for this species by Torto.

An APPENDIX lists several *Albizia* species for which a number of botanical synonyms exist, often reflecting earlier confusion with the genus *Acacia*.

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APPENDIX

NOMENCLATURE OF SOME GUM-FORMING *Albizia* SPECIES

(a) *Albizia procera* (Roxb.) Benth.
syn. *Albizia elata* Benth.
Acacia procera Willd.

(b) *Albizia lebbeck* (L.) Benth.
syn. *Albizia latifolia* Boiv.
Albizia speciosa Benth.
Acacia lebbeck Willd.
Acacia speciosa Willd.
Acacia sirissa Roxb.
Acacia macrophylla Bunge.

(c) *Albizia zygia* (D.C.) Macbride
syn. *Albizia brownei* (Walp.) Oliv.
Zygia brownei Walp.
Inga zygia D.C.
Acacia zygia (D.C.) Baill.
Acacia welwitschioides (Schweinf.) ex Bak.

(d) *Albizia adianthifolia* (Schumach.)
syn. *Mimosa adianthifolia* Schumach.
Zygia fastigiata E. Mey.
Albizia fastigiata (E. Mey.) Oliv.
Albizia sassa of various authors, *not* (Willd.) Chiov.
Albizia gummifera of various authors, *not* (J. F. Gmel.) C. A. Smith.

(e) *Albizia amara* (Roxb.) Boiv.
typical subspecies *amara* (Asia, India)
syn. *Mimosa amara* Roxb.
Albizia gracilifolia Harms.
Albizia nellyrenza Grah.
Albizia affinis Fourn.
subspecies *sericocephala* (Benth.) Brenan
syn. *Albizia sericocephala* Benth.
Inga sericocephala A. Rich.
Albizia struthiophylla Milne-Redhead.

(f) *Albizia ferruginea* (Guill. and Perr.) Benth.
syn. *Inga ferruginea* Guill. and Perr.

(g) *Albizia gummifera* (J. F. Gmel.) C. A. Smith
syn. *Albizia sassa* (Willd.) Chiov.
Inga sassa Willd.
Sassa gummifera J. F. Gmel.

(h) *Albizia glaberrima* (Schumach. and Thonn.) Benth.
Var. *glaberrima* (typical)
syn. *Mimosa glaberrima* Schumach. and Thonn.
Albizia warneckii Harms.
Albizia eggelingii Bak. f.
Var. *mpwapwensis* Brenan
Var. *glabrescens* (Oliv.) Brenan
syn. *Albizia glabrescens* Oliv.

(i) *Albizia lophantha*
syn. *Acacia speciosa* Hort. ex Stend.
Acacia lophantha

(j) *Albizia stipulata*
syn. *Acacia smithiana* Roxb. ex Wall.

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STUDIES ON URONIC ACID MATERIALS

PART XIV*. METHYLATION WITH THE SODIUM HYDRIDE–METHYL IODIDE–DIMETHYL SULPHOXIDE SYSTEM

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INTRODUCTION

Recently, there has been renewed interest in methods for methylating polysaccharides. Attempts have been made to improve yields and also to reduce the number of repetitive treatments required with the classical Haworth[2] and Purdie[3] techniques. Kuhn[4] and his co-workers used *N,N*-dimethylformamide as solvent with silver oxide and methyl iodide for permethylations and have since proposed[5] the use of *N,N*-dimethylformamide and dimethyl sulphoxide, separately or in admixture, as solvents for methylations with barium hydroxide and methyl iodide or dimethyl sulphate.

Our attempts to methylate *Acacia* gum polysaccharides, using barium salts, gave reasonable methoxyl contents, but emulsions, which were extremely difficult to break, tended to form. The use of dimethyl sulphoxide, with powdered sodium hydroxide and dimethyl sulphate, has been reported[6] to give high yields of almost fully methylated, neutral polysaccharides, but, in our experience, this technique is less successful with acidic materials.

We have investigated the use of sodium hydride and methyl iodide for the methylation of acidic *Acacia* polysaccharides dissolved in dimethyl sulphoxide. (A similar technique has been used to methylate glycoproteins and neutral polysaccharides[7], but its use with acidic materials has not, to our knowledge, been reported previously). We have also used this reaction for the rapid methylation of mono- and di-saccharides; sodium hydride has been used in the methylation of monosaccharide derivatives in ether-type solvents[8,9] or in *N,N*-dimethylformamide[9].

In the presence of acidic groups, the sodium hydride reaction might lead to the following side-reactions, with the formation of artifacts: (*a*) condensation of ester groups with the methylsulphinyl carbanion to give a sulphoxide; (*b*) in the presence of ester groups, β-elimination could occur to give 4,5-unsaturated acids[10] (esters of both glucuronic and galacturonic acids can undergo[11] such eliminations); (*c*) the product from (*b*) could react further to give a variety of products.

The methylated products were therefore examined to ascertain whether such artifacts had arisen.

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*For Part XIII see ref. 1

EXPERIMENTAL

Paper chromatography was carried out on Whatman No. 1 paper with the following solvent systems (v/v): (*a*) butan-1-ol–ethanol–water (4:1:5, upper layer); (*b*) butan-1-ol–acetic acid–water (4:1:5, upper layer); (*c*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4).

Gas–liquid chromatography (g.l.c.) was carried out on columns (3 ft × 0.25 in) of polyethyleneglycol adipate (15% by weight on acid-washed Celite, 80–100 mesh) at 150°; the carrier gas was nitrogen at a flow rate of 100 ml/min. The chromatograph (Model S3A, Gas Chromatography Ltd, Maidenhead) was fitted with flame-ionisation detectors. Retention times are given relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside.

Weights recorded are those corrected for moisture content (by drying to constant weight at 105°). Hydrolyses and methanolyses were effected with N sulphuric acid for 8 h at 100° and with 5% methanolic hydrogen chloride for 6 h at 100° (sealed tube), respectively. Reagent-grade dimethyl sulphoxide and methyl iodide were redistilled before use. Methoxyl contents were determined by a specific, vapour-phase, infrared method[12]. Absorption spectra were taken with a Perkin–Elmer 137 UV Spectrophotometer.

Experimental precautions. Powdered sodium hydride was used without difficulty throughout our experiments; due precautions were observed when handling this reagent. Where preferred, the commercial dispersion of sodium hydride in oil may be used. A convenient method is to add the dispersion to dry dimethyl sulphoxide; the layer containing sodium hydride dissolved in dimethyl sulphoxide may then be added to a solution, in dimethyl sulphoxide, of the material to be methylated.

RESULTS

(a) Acidic polysaccharides

Methylation of the gum from Acacia nubica *(Benth.)*

The gum (4.64 g) was dissolved in dimethyl sulphoxide (250 ml), and powdered sodium hydride (2 g) was added in small portions, with gentle stirring, during 1 h. The solution turned yellow and, ultimately, became semi-solid. Methyl iodide (5 ml) was added dropwise with stirring during 2 h. The solution was stirred overnight, and one drop then gave a neutral reaction when added to water. A further three additions of sodium hydride and methyl iodide were made to the reaction mixture on successive days, as described for the first addition. The mixture was then poured into water (1.5 l) to precipitate the methylated polysaccharide, and any excess of methyl iodide was removed by aspiration. The precipitate was collected (centrifuge) and dissolved in chloroform. The supernatant solution (aqueous dimethyl sulphoxide) was extracted with chloroform, and the extract was combined with the solution of precipitated gum. The chloroform solution was washed with water to remove dimethyl sulphoxide,

dried ($MgSO_4$), concentrated (rotary evaporator at less than 30°), and then added to light petroleum (b.p. 60–80°) to precipitate the methylated polysaccharide, which was removed by centrifugation and dried at room temperature under diminished pressure: yield, 3.98 g (85%) (Found: OMe, 40.8%).

Single-step methylation of a degraded gum from Acacia nubica

Degraded gum (1.86 g), prepared from *A. nubica* gum by autohydrolysis, was dissolved in dimethyl sulphoxide (50 ml). Sodium hydride (2 g) was added in small portions, followed by methyl iodide (5 ml), as for the whole gum. The mixture was stirred overnight, water (200 ml) was added, and the mixture was extracted with chloroform. The isolation of the methylated, degraded gum (1.62 g, 87%) then followed the sequence already described for the whole gum (Found: OMe, 37.1%).

Tests for the formation of artifacts

The following tests were made on the methylated whole-gum and methylated, degraded gum.

The methylated gum, dissolved in spectroscopic grade ethanol, showed no absorption at 235 mμ, indicating the absence of β-elimination products.

The methylated gum was tested for the presence of unsaturation by the thiobarbituric acid method[10]. Methylated gum (10 mg) was shaken with water (1 ml), and hydrochloric acid (5 ml, 0.5M) and thiobarbituric acid (10 ml, 0.01M) were then added. The solution was immersed in a boiling water-bath for 30 min and cooled, and the absorption spectrum was examined. There was no absorption at 547 mμ, indicating the absence of 4,5-unsaturated derivatives of D-glucuronic acid.

Small-scale methylation of degraded A. nubica *gum*

To degraded gum (30 mg) in dimethyl sulphoxide (10 ml) was added, as described above, sodium hydride (500 mg) followed by methyl iodide (1.3 ml), and the mixture was stirred gently overnight. A second addition of reagents was then made, the mixture was stirred overnight, and the methylated product (20 mg) was isolated as described for the methylation of whole gum (Found: OMe, 40.0%).

A half-portion of the product was dissolved in chloroform and examined by g.l.c. The remainder of the product was hydrolysed and then examined by paper chromatography in solvents (*a*) and (*b*). In all of these examinations, the chromatograms were identical with those obtained from the product of the large-scale methylation of degraded *A. nubica* gum.

(b) Acidic mono- and di-saccharides

Methylation of D-*glucuronic acid.*

D-Glucuronic acid [200 mg, chromatographically homogeneous in solvent (*c*) and containing no D-glucurone] was dissolved in dimethyl sulphoxide (10 ml), and sodium hydride (500 mg) was added in small portions with gentle stirring during 1 h. Methyl iodide (1.4 ml) was added dropwise, and the mixture was stored overnight.

The methylated product was then isolated by pouring into water, followed by extraction with chloroform at room temperature.

The solution of the product in chloroform was divided into four parts. Portion 1 was examined directly by g.l.c. and gave only three peaks, having relative retention times of 0.17, 2.14, and 2.81. The first component had the same retention time as dimethyl sulphoxide. The other two peaks had the same retention times as were given by an authentic specimen of methyl (methyl 2,3,4-tri-*O*-methyl-αβ-D-glucopyranosid)-uronate.

Portion 2 was concentrated to dryness and subjected to methanolysis, and the neutralised (silver carbonate) solution was concentrated to dryness. A solution of the residue in chloroform was examined by g.l.c. as for portion 1. The same peaks were obtained, but with a decreased proportion of dimethyl sulphoxide.

Portion 3 was concentrated to dryness and hydrolysed. The product was neutralised (barium carbonate) and examined by paper chromatography in solvents (*a*) and (*b*). Only one component, chromatographically identical with 2,3,4-tri-*O*-methyl-D-glucuronic acid, was detected.

Portion 4 was concentrated to dryness and warmed with water (1 ml), and then hydrochloric acid (5 ml, 0.5M) and thiobarbituric acid (10 ml, 0.01M) were added. The solution was kept for 30 min at 100° and then cooled; there was no absorption at 547 mμ.

Methylation of methyl α-D-glucopyranoside and methyl α-D-galactopyranoside

Quantities and procedure were as for D-glucuronic acid, except that the mixtures were stirred for only 1 h after addition of the methyl iodide. Examination by g.l.c. of the products from each glycoside showed that only the fully methylated sugars were present.

Methylation of an aldobiouronic acid

6-*O*-(β-D-Glucopyranosyluronic acid)-D-galactose (60 mg) was dissolved in dimethyl sulphoxide (10 ml). Reaction with sodium hydride (500 mg) and methyl iodide (1.4 ml) was then carried out as described above for the small-scale methylation of the degraded gum from *A. nubica.*

A half-portion of the product was hydrolysed and examined by paper chromatography in solvents (*a*) and (*b*). Only two components, chromatographically identical with reference samples of 2,3,4-tri-*O*-methyl-D-glucuronic acid and 2,3,4-tri-*O*-methyl-D-galactose, were detected. The remainder of the product was hydrolysed in methanolic hydrogen chloride and neutralised (silver carbonate), and the methanol was removed by evaporation. The product was dissolved in dry chloroform and examined by g.l.c. Components having retention times identical with those of the methyl glycosides of 2,3,4-tri-*O*-methyl-D-galactose and of the methyl ester of 2,3,4-tri-*O*-methyl-D-glucuronic acid were found*.

**Note added in proof.* In some reactions, it has been observed that 2,3,5-tri-*O*-methyl-D-galactose may also be formed, presumably through reaction of the disaccharide in the furanose form.

DISCUSSION

The methylation technique described gives complete methylation of monosaccharides and acidic disaccharides in one stage, and methoxyl contents exceeding 36% have consistently been achieved for acidic polysaccharides by making 2–4 additions of the reagents. Careful tests have failed to indicate the formation of artifacts. The products are given in high yield, and satisfactory results have been obtained with 30-mg samples. Yields of only 20–30% were obtained when classical methods were used, in multi-stage processes, to methylate acidic polysaccharides from *A. seyal*[13] and *A. nilotica*[14]. In the light of this direct comparison, the sodium hydride method clearly offers distinct advantages.

It is hoped that this communication will lead other investigators to assess the performance of the method with a wider range of polysaccharides.

ACKNOWLEDGEMENT

We are grateful to Sir Edmund Hirst, C.B.E., F.R.S., for his interest in these studies, and we thank the Science Research Council for the award of a maintenance grant (to G.M.C.). We acknowledge the generous gift by Messrs. I.C.I. Ltd. of the gas chromatograph and its ancillary equipment.

SUMMARY

Reaction of acidic poly-, mono-, and di-saccharides with sodium hydride and methyl iodide, in dimethyl sulphoxide as solvent, requires very few repetitive treatments to give highly methylated products in very good yield. Artifacts could not be detected in the products.

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STUDIES ON URONIC ACID MATERIALS

PART XVI[1]. INTER-NODULE VARIATION AND THE ACIDIC COMPONENTS IN *Acacia nilotica* GUM

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INTRODUCTION

In a preliminary study of the gum exudates from several *Acacia* species, *Acacia nilotica* (L.) Willd. ex Del. was found[2] to differ in a number of interesting respects from those species studied prior to 1963. Thus, *A. nilotica* gum gave a high, positive specific rotation (+106°), a high methoxyl content (1.05%), and contained only traces of rhamnose that could not be estimated satisfactorily by paper chromatography.

In addition, *A. nilotica* gum gave solutions of low viscosity, and its unusually low nitrogen content (0.08%) was decreased to 0.02% on electrodialysis (corresponding decreases are not shown by other *Acacia* gums[2,3]). *A. nilotica* could, therefore, constitute a useful limiting case in investigations into the extent of the dependence of the physico-chemical properties of *Acacia* gum solutions on their natural nitrogenous content, and it was therefore selected for study in preference to other available species[2].

In view of the inter-nodule variation observed in *A. seyal* gum[3], a preliminary analytical survey of ten nodules of *A. nilotica* gum was undertaken.

EXPERIMENTAL

Origin of Specimens. Ten large nodules of gum from *Acacia nilotica* were collected by (the late) M.P. Vidal-Hall, formerly Gum Research Officer to the Republic of the Sudan, at Hawata, Kassala Province, Eastern Sudan, on February 3rd, 1963. All of the nodules originated by natural exudation; nodules (**1**), (**2**), (**3**), and (**4**) were taken from individual small trees, nodules **5–10** from individual large trees, all growing in close proximity.

Analytical Methods. The general methods have been described[2], except that paper chromatography was carried out with the following solvent systems (v/v): (*A*) butan-1-ol–ethanol–water (4:1:5, top layer); (*B*) butan-1-ol–pyridine–water–benzene (5:3:3:1, top layer); (*C*) ethyl acetate–pyridine–water (10:4:3); (*D*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); (*E*) ethyl acetate–acetic acid–water (9:2:2); (*F*) butan-1-ol–acetic acid–water (4:1:5, top layer).

TABLE I

ANALYTICAL DATA [a] FOR PURIFIED NODULAR SPECIMENS OF *Acacia nilotica* GUM

	Nodules										*Average*	
	1	2	3	4	5	6	7	8	9	10	*All nodules*	*omitting* 5
Ash, %	0.02	—	—	—	0.04	—	—	—	—	—	—	—
Nitrogen, %	0.02	0.02	0.02	0.03	0.02	0.01	0.03	0.01	0.01	0.01	0.02	0.02
Methoxyl, %	0.96	0.75	1.25	1.18	1.44	1.30	1.25	1.03	1.13	1.28	1.16	1.12
Reducing power[b]	0.07	0.05	0.13	0.05	0.16	0.13	0.07	0.35	0.20	0.10	0.13	0.13
Flow time[c], sec	304	312	305	307	333	305	307	306	304	309	309	307
Limiting flow time number[c]	9.5	—	—	—	10.4	—	—	—	—	—	—	—
$[\alpha]_D^{20}$ (*c* 3.0, water)	+108°	+109°	+108°	+108°	+54°	+107°	+108°	+106°	+106°	+106°	102°	107°
Neutralisation equivalent	1890	1890	1860	1930	1645	1960	1860	1880	1860	1850	1860	1890
Hence, uronic anhydride, %[d]	9.3	9.3	9.5	9.1	10.7	9.0	9.5	9.4	9.5	9.5	9.5	9.3
Uronic anhydride (decarbox.)	9.2	9.2	9.4	9.3	10.9	9.3	9.2	9.3	9.1	9.3	9.4	9.3
D-Galactose	44	43	41	43	33	40	43	42	43	39	41	42
L-Arabinose	46	47	46	47	55	46	45	47	46	49	47	46

[a] All results corrected to a dry-weight basis. [b] Expressed as apparent % of free pentose. [c] In 4% saline at 25.0°; water = 200 sec. [d] If all acidity arises from uronic acid residues.

RESULTS

Studies on the crude gum. The results of analyses of nodule (**1**) have been published[2] under the heading "*A. nilotica*" in Table II therein.

Studies on gum samples purified by electrodialysis. Each of the ten nodules of gum was purified separately by electrodialysis[4], and the free gum acids were isolated by freeze-drying; analytical data are given in Table I. Particular care was taken with duplicate analyses when it was observed that the results for nodule (**5**) differed significantly from the others; the results quoted are average values for satisfactory replicates.

Partial hydrolysis of the gum acid, and separation of acidic sugars. Electrodialysed specimen (**1**) (16 g) was hydrolysed with N sulphuric acid (300 ml) for 12 h at 100°. After neutralisation (barium carbonate), de-ionisation [Amberlite IR-120 (H^+ form)], and concentration, paper chromatography (solvents *B* and *D*) revealed the presence of galactose, arabinose, glucuronic acid, and 4-*O*-methylglucuronic acid. Two acidic disaccharides were also indicated (these were, in fact, two mixtures of acidic disaccharides).

The neutral and acidic components were separated by passage through Duolite A4 (formate form). Elution with water gave a neutral syrup (10.7 g) which contained galactose and arabinose; this fraction was not examined further. The acidic components were eluted with dilute formic acid to give a syrup (4.4 g) after the necessary isolation stages.

Fractionation of the acidic sugars. The acidic syrup (4.4 g) was added to a cellulose column (80×4 cm) and eluted with solvent *E*. Fractions (10 ml) were collected; the contents of every third tube were examined in solvents *D* and *E*. Five main fractions were obtained: fraction *a* (tubes 24–28), 216 mg; fraction *b* (30–61), 1498 mg; fraction *c* (62–100), 931 mg; fraction *d* (134–307), 941 mg; fraction *e* (492–600), 309 mg. The total recovery was therefore 88%.

Examination of the acidic fractions. (*a*) Fraction *a* had R_{Gal} 3.0 (solvent *E*) and crystallised readily. Recrystallisation from water gave D-glucopyranurono-6→3-lactone, m.p. and mixed m.p. 177°, $[\alpha]_D^{20}$ +19° (*c* 1.0, water).

(*b*) Fraction *b* gave a single chromatographic spot having R_{Rha} 0.91 (solvent D) and R_{Gal} 0.39 (solvent *B*). It had $[\alpha]_D^{20}$ +37° (*c* 1.5, water) (Found: OCH_3, 14.6. Calc. for a monomethylhexuronic acid: OCH_3, 14.9%).

The pale syrup (0.4 g) was converted into the methyl ester methyl glycoside (0.34 g) with dry, 2% methanolic hydrogen chloride, reduced with potassium borohydride, and hydrolysed. The product (0.24 g) had R_{Rha} 0.94 in solvent *A* and gave a single spot, having R_{Gal} 1.94 and 2.1, respectively, in solvents *B* and *D*. This behaviour was identical with that of 4-*O*-methylglucose. After purification on Whatman No. 3MM

paper in solvent *A*, the product had OCH_3, 15.0% (calc. for $C_7H_{14}O_6$: OCH_3, 15.6%), and the crystalline phenylosazone had m.p. 156° (lit., 157–160°), after two recrystallisations from hot water. Fraction *b* was thus 4-*O*-methyl-D-glucuronic acid; the amide of methyl 4-*O*-methyl-α-D-glucopyranuronoside was obtained as large, colourless plates, m.p. 231° (lit., 232–236°), $[\alpha]_D^{20}$ +140° (*c* 0.5, water).

(*c*) Fraction *c* gave a single spot corresponding to D-glucuronic acid in solvent *D*: examination in solvent *B* showed the presence of a trace of galactose. In view of the identity of fraction (*a*), this fraction was not examined further.

(*d*) Fraction *d* had $[\alpha]_D^{20}$ +55° (*c* 1.0, water), and partially crystallised. Examination in solvents *D* and *E* gave spots (brown with aniline oxalate spray) having R_{Gal} 0.69 and 0.66, respectively. Hydrolysis (2N sulphuric acid, 6 h) and subsequent examination (solvent *D*) showed the presence of galactose and 4-*O*-methylglucuronic acid in equal proportions (visual examination). A small portion of the fraction (80 mg) was treated with methanolic hydrogen chloride, reduced with potassium borohydride, hydrolysed, and fractionated on 3MM paper in solvent *A*, to give 4-*O*-methyl-D-glucose [28 mg; phenylosazone, m.p. 158° (lit., 159°)], $[\alpha]_D^{20}$ +58° (*c* 0.1, water); and D-galactose (31 mg; m.p. and mixed m.p. 156°).

Fraction *d* (500 mg) was methylated with dimethyl sulphate and sodium hydroxide, followed by methyl iodide and silver oxide, to give a product (286 mg; OCH_3, 52.1%) which was reduced with lithium aluminium hydride. The methylated, neutral product (213 mg) was hydrolysed (N hydrochloric acid, 4 h); the hydrolysate gave 3 spots (R_G 0.84, 0.72, 0.69) in solvent *A*. The three components were separated on a cellulose column (2×38 cm) using butan-1-ol–light petroleum (7:3), saturated with water, as eluant. The first component (68 mg) had R_G 0.85 (solvent *A*), was identical with 2,3,4-tri-*O*-methyl-D-glucose in solvents *A*, *B*, and *F*, and gave an anilide, m.p. 152° (lit., 145–150°). The second component (18 mg) had R_G 0.71 (solvent *A*), was identical with 2,3,6-tri-*O*-methyl-D-galactose in solvents *A* and *F*, and was oxidised with bromine to 2,3,6-tri-*O*-methyl-D-galactonolactone, m.p. 96–97° (lit., 98°). The third component (17 mg) was identical with 2,3,4-tri-*O*-methyl-D-galactose in solvents *A*, *B*, and *F*, and gave an anilide, m.p. and mixed m.p. 166° (lit., 167–170°).

Separation of the aldobiouronic acids in Fraction d

Fraction *d* was suspected to be a mixture of monomethylaldobiouronic acids when the rotation for the corresponding fraction from the other specimens of *A. nilotica* was observed to vary significantly from +48 to +71°. Separation of fraction *d* into two aldobiouronic acids was achieved on strips (4″ wide) of 3MM paper, in solvent *D* for 96 h. The chromatograms were freed from acidic solvent by air-drying for 48 h, followed by heating for 5 min at 150°. The located zones were eluted with cold water to give aldobiouronic acids *A* (101 mg) and *B* (95 mg).

Examination of aldobiouronic acids A *and* B

Acid *A* (101 mg) had R_{Gal} 0.79 (solvent *D*) and $[\alpha]_D^{20}$ +93° (*c* 1.0, water).

A sample (40 mg) was converted into the methyl ester methyl glycoside; one half-portion of the product was reduced with potassium borohydride and hydrolysed. The products were chromatographically identified (solvent *A*) as galactose and 4-*O*-methylglucose. The second half-portion reduced 1.8 mol. of sodium periodate. In duplicate experiments, the aldobiouronic acid gave, on periodate oxidation, 1.01 and 1.04 mol. of formaldehyde, indicating that C-6 of the D-galactose residue was not involved in a linkage. These experiments, considered in conjunction with the methylation evidence reported above for acidic fraction (*d*), led to identification of acid *A* as 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose.

Acid *B* (yield 95 mg) had R_{Gal} 0.68 (solvent *D*) and $[\alpha]_D^{20}$ $+6°$ (*c* 0.95, water). Methanolysis, followed by potassium borohydride reduction and subsequent hydrolysis, gave only galactose and 4-*O*-methylglucose (solvent *D*). Periodate oxidation of the aldobiouronic acid at pH 8 gave no formaldehyde. The methyl ester methyl glycoside, on oxidation with sodium periodate in darkness for 2 days at room temperature consumed 3.1 mol. of periodate. These experiments, considered in conjunction with the methylation evidence reported above for acidic fraction (*d*), led to the identification of acid *B* as 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose.

(*e*) Fraction *e* (309 mg) had $[\alpha]_D^{20}$ $+31°$ (*c* 1.2, water) and gave, after hydrolysis, only galactose and glucuronic acid (solvents *B* and *D*). Methanolysis, followed by potassium borohydride reduction and hydrolysis, gave only galactose and glucose. Chromatographic separation in solvent *D* for 120 h was required to reveal the presence of two components having R_{Gal} 0.21 (major component) and 0.28. Fraction *e* was then fractionated on 3MM paper (4" wide) in solvent *D* for 160 h to give aldobiouronic acids *C* and *D*.

Examination of aldobiouronic acids C *and* D

Acid *C* (103 mg) had R_{Gal} 0.21 (solvent *D*) and gave $[\alpha]_D^{20}$ $-5°$ (*c* 0.4, water). It was chromatographically homogeneous and identical in solvents *D*, *E*, and *F* with 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose. Reduction of the methyl ester methyl glycosides with potassium borohydride, followed by hydrolysis, gave only galactose and glucose. Methylation of a portion (80 mg) by the Haworth and the Purdie methods, followed by reduction (lithium aluminium hydride), gave a product (38 mg) which, after hydrolysis, was fractionated on 3MM paper in solvent *A* to give 2,3,4-tri-*O*-methyl-D-glucose (14 mg) [R_{Gal} 0.85 (solvent *A*); anilide, m.p. $+148°$ (lit., 145–150°)] and 2,3,4-tri-*O*-methyl-D-galactose (11 mg) [R_{Gal} 0.67 (solvent *A*), chromatographically identical with an authentic specimen in solvents *A*, *D*, and *F*; attempted preparation of the anilide did not yield a crystalline product].

Acid *D* (18 mg) had R_{Gal} 0.28 and 0.32 on Whatman No. 1 and 3MM papers, respectively, in solvent *D*, and had $[\alpha]_D^{21}$ $+107°$ (*c* 0.1, water). Hydrolysis gave only galactose and glucuronic acid; periodate oxidation at pH 8 for 24 h gave 0.93 mol. of formaldehyde. This acid has subsequently been obtained from other *Acacia* species, and has been more rigorously characterised[5] as 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose.

DISCUSSION

The analytical results in Table I supplement the limited data of this type extant[3,6]; the additional work involved in such an approach is justified for the following reasons.

For such complex polysaccharides, it is reasonable to expect that some variation in composition and properties may exist between gum specimens exuded by different trees of a particular species (it is becoming apparent that the inter-nodule variation for one species may be greater than that for another). This investigation has shown that a knowledge of the inter-nodule variation is useful whenever an aspect of heterogeneity, or the possibility of fractionation, is involved.

In this study of *A. nilotica*, the natural exudates, collected on the same day, from ten different trees growing in close proximity, have been examined. This approach can be extended to examine (*i*) the seasonal variation for a species, (*ii*) the variation between specimens from geographical locations differing in climate and type of soil, and (*iii*) the variation between exudations resulting from different stimuli, *e.g.*, from tapping, from natural processes, from diseased trees, and from trees attacked by ants or borer beetles. Such studies could provide useful evidence regarding the mode of biosynthesis of gum and the nature of the carbohydrate systems serving as gum precursors in the tree.

The available analytical data substantiate the view[3] that a single, gum nodule is itself complex and offers the simplest system available for structural investigation. Recent developments in analysis make such an approach possible if the collection of reasonably large nodules can be arranged. A preliminary analytical survey of several nodules is, nevertheless, required, to select the most representative nodule of the species for structural study, and to establish the extent to which it varies from other specimens. The value of this approach is seen in Table I, from which specimen (**5**) must be regarded as atypical (in some respects) of the *A. nilotica* species. On the basis of present knowledge, there is, however, no evidence that specimen (**5**) did not originate from *A. nilotica*. The ten specimens studied were collected by an accepted authority on the Sudanese *Acacias*, whose undertaking was, in the research collaboration between the Sudanese Department of Forests and this laboratory, to collect, personally, only specimens which could be authenticated beyond doubt. *Acacia nilotica* is, moreover, distinctly characteristic to a trained fieldsman; the species resembling it most closely is *A. arabica*. Inspection of the available analytical data for *Acacia* species[2] indicates that although *A. pycnantha*, *A. arabica*, and *A. fistula* each have *some* feature in common with specimen (**5**) in Table I, its analytical characteristics, *taken as a whole*, do not suggest that it would be more correctly assigned to any other species for which information exists. Although this view may require alteration in the future, the present reserve associated with this specimen further justifies a limited, preliminary, analytical survey of the nodules of any gum species under structural investigation.

Determinations of the traces of rhamnose in *A. nilotica* could only be achieved

by a spectroscopic method developed[7] for the purpose. Several *Acacia* species are now known to contain <1% of rhamnose, and this cannot now be considered a major constituent in all *Acacia* gums (*cf.* ref. 8) Furthermore, polysaccharides containing both D-glucuronic acid and its 4-*O*-methyl ether are no longer unusual (*cf.* ref. 8); these acids occur conjointly in species of the *Albizia*[4,8], *Khaya*[10] and, now, of the *Acacia* genera.

Methoxyl groups were recently reported[9] to occur in *Acacia* species; the relatively high methoxyl content in *A. nilotica* has facilitated the present confirmation that the methoxyl groups are structurally significant[9]. Prior to this investigation, only *A. karroo*[11] and *A. senegal*[1] had been reported to contain two aldobiouronic acids; on re-examination, those species previously reported to contain only 6-*O*-(*β*-D-glucopyranosyluronic acid)-D-galactose may be found to be more complex.

The optical rotation data presented for acid fraction (*d*) and its components give an indication of the extent to which heteropolymolecularity[1] is displayed by *A. nilotica* gum. Calculation shows that the proportions of the monomethylaldobiouronic acids *A* and *B* vary, in the nodules examined, from approximately 1:1 to 3:1. Furthermore, with the recovery of these monomethyl acids accounting for the observed methoxyl content, and with no evidence of alternative locations for the methoxyl groups in this or other *Acacia* species[1,5], the inter-nodule variations in methoxyl content suggest that the heteropolymolecularity extends to differences in the proportions of the monomethyl acids ($A + B$) and the non-methylated acids ($C + D$).

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SUMMARY

Inter-nodule variations in the composition and properties of *Acacia nilotica* gum have been investigated. The results confirm the value of this type of analytical survey of a species, prior to structural studies on a single, representative nodule.

The acidic components of the gum have been examined in detail. For the first time in the genus *Acacia*, four aldobiouronic acids, two of which contain 4-*O*-methyl-D-glucuronic acid, were present and were identified as 4-*O*-(4-*O*-methyl-*α*-D-glucopyranosyluronic acid)-D-galactose (*A*), 6-*O*-(4-*O*-methyl-*β*-D-glucopyranosyluronic acid)-D-galactose (*B*), 6-*O*-(*β*-D-glucopyranosyluronic acid)-D-galactose (*C*), and 4-*O*-(*α*-D-glucopyranosyluronic acid)-D-galactose (*D*).
The analytical data indicate that there is an inter-nodule variation in the proportions of acids *A* and *B*, and, further, in the proportions of the monomethyl acids ($A + B$) and the unsubstituted acids ($C + D$).

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Studies on Uronic Acid Materials. Part XVII.[1] Some Structural Features of *Acacia Senegal* Gum (Gum Arabic)

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Studies on Uronic Acid Materials. Part XVII.[1] Some Structural Features of *Acacia Senegal* Gum (Gum Arabic)

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Acacia senegal gum is subjected to seven successive Smith degradations. The *O*-methyl derivatives of each of the polysaccharides obtained from the first five degradations are analysed, after methanolysis, by gas–liquid partition chromatography. After the fifth degradation, the *O*-methyl sugars characterised from the methylated polysaccharide are 2,3,4,6-tetra-, 2,4,6- and 2,3,4-tri-, and 2,6- and 2,4-di-*O*-methyl-D-galactose; partial acid hydrolysis of this galactan affords 6-*O*-β-D-galactopyranosyl-D-galactose, 3-*O*-β-D-galactopyranosyl-D-galactose, and the β1,3-linked galactotriose.

The same sample of *A. senegal* gum is degraded by autohydrolysis; the methylated autohydrolysed gum contains 2,3,4,6-tetra-, 2,4,6- and 2,3,4-tri-, and 2,4-di-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-glucuronic acid, and trace amounts of 2,3,5-tri-*O*-methyl-L-arabinose.

As a result of these investigations, modifications to the accepted structural features of the gum are proposed.

EARLIER investigations on the primary chemical structure of *Acacia senegal* gum have been the subject of several reviews.[2-6] The gum has been recognised as an acidic polysaccharide containing D-galactose, L-arabinose, L-rhamnose, and D-glucuronic acid. Smith[7] deduced several important structural features from a methylation study of the degraded gum resulting from the

[1] Part XVI, D. M. W. Anderson and K. A. Karamalla, *Carbohydrate Res.*, in the press.
[2] F. Smith and R. Montgomery, "The Chemistry of Plant Gums and Mucilages," Reinhold Publ., Inc., New York, 1959.
[3] E. L. Hirst, "Plant Gums," 4th Internl. Congress of Biochem., Vienna, 1958.
[4] E. L. Hirst, *Proc. Roy. Soc.*, 1959, *A*, **252**, 287.
[5] E. L. Hirst and J. K. N. Jones, "The Gums of Mucilages and Plants," in "Encyclopedia of Plant Physiology," ed. Ruhland, Springer-Verlag, Berlin, 1958.
[6] M. Glicksman and R. E. Schachat, "Gum Arabic," in "Industrial Gums," ed. Whistler, Academic Press, New York, 1959.
[7] F. Smith, *J. Chem. Soc.*, 1939, 744, 1724; 1940, 1035; J. Jackson and F. Smith, *ibid.*, 1940, 74, 79.

removal of the acid-labile arabinofuranose and rhamnopyranose residues by autohydrolysis. Partial acid hydrolyses have shown that 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose,[8] 3-*O*-α-D-galactopyranosyl-L-arabinose,[7,9] 3-*O*-β-D-galactopyranosyl-D-galactose,[7] and 3-*O*-β-L-arabinopyranosyl-L-arabinose,[10] are constituent units of the gum.

The structural interpretations from Smith's experiments were limited to those based on branched frameworks of 1,3- and 1,6-linked D-galactose residues. Evidence for the mode of distribution of the 1,3- and 1,6-linkages between these residues in the " core " of the gum was obtained by Dillon, O'Ceallachain, and O'Colla,[11] who subjected the gum to three successive Barry degradations; [12] further treatment of the degraded polysaccharide with periodate and phenylhydrazine gave a product in high yield. This indicated that little degradation had taken place during the fourth Barry degradation; it was concluded that the gum contained a fundamental chain of D-galactose units, exclusively involving 1,3-linkages.

A previous investigation [13] showed that the sample of *Acacia senegal* gum used in the present studies contained D-galactose (39%), L-arabinose (28%), L-rhamnose (14%), D-glucuronic acid (17·5%), and 4-*O*-methyl-D-glucuronic acid (1·5%). (All percentages are for anhydro sugars and are corrected for moisture and protein contents.)

The methylated gum was subjected to methanolysis and the mixture of methyl glycosides was analysed by gas–liquid partition chromatography; [14] the methyl glycosides of 2,3,4-tri-*O*-methyl-L-rhamnose, 2,3,5- and 2,3,4-tri-, and 2,5-di-*O*-methyl-L-arabinose, 2,3,4,6-tetra-, 2,4,6- and 2,3,4-tri-, and 2,4-di-*O*-methyl-D-galactose, and 2,3,4-tri- and 2,3-di-*O*-methyl-D-glucuronic acid were identified. With the exception of 2,3,4-tri-*O*-methyl-D-galactose, all these *O*-methyl sugars were present in the methylated gum studied by Aspinall, Charlson, Hirst, and Young; [15] 2,3,4-tri-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-L-arabinose, and 2,4,6-tri-*O*-methyl-D-galactose were not reported in the methylated sample investigated by Smith.[7] The presence of 2,3,4-tri-*O*-methyl-L-arabinose was not unexpected, since graded acid hydrolysis of the gum had yielded 3-*O*-β-L-arabinopyranosyl-L-arabinose.[10] The identification of small amounts of 2,4,6-tri-*O*-methyl-D-galactose in the methylated whole gum indicated that a re-examination of the methylated degraded gum, obtained after autohydrolysis and methylation, was necessary.

In a previous investigation,[13] electrodialysed whole gum (2% solution; pH, 2·8) was heated for 50 hours on a boiling-water bath; the autohydrolysed gum, isolated after exhaustive dialysis, contained uronic acid (21%), galactose (77%), and arabinose (2%). Smith [7] reported that hydrolysis of the methylated autohydrolysed gum yielded 2,3,4,6-tetra-, 2,3,4-tri- and 2,4-di-*O*-methyl-D-galactose, and 2,3,4-tri-*O*-methyl-D-glucuronic acid in the approximate molar proportions 1 : 5 : 3 : 3. Our re-examination of the methylated autohydrolysed gum has, however, shown the presence of 2,3,4,6-tetra-(++), 2,4,6-(++) and 2,3,4-tri-(+++), 2,4-di-(+++) and 2-*O*-methyl-D-galactose (+), 2,3,4-tri-*O*-methyl-D-glucuronic acid (+++), and trace amounts of 2,3,5-tri-*O*-methyl-L-arabinose. The approximate relative molar proportions indicated were estimated from the peak areas obtained for the methyl glycosides by gas–liquid partition chromatography, and from the intensities of the spots on paper chromatograms given by the *O*-methyl sugars after hydrolysis of their methyl glycosides. The presence of increased proportions of 2,4,6-tri-*O*-methyl-D-galactose in the methylated autohydrolysed gum suggests that some of the residues in the chains of β1,3-linked galactose units are 6-*O*-substituted with acid-labile arabinofuranose units.

During autohydrolysis, extensive degradation of the gum macromolecule ($\bar{M}_n$ *ca.* 250,000) [16] occurs to yield a polysaccharide of relatively low molecular weight ($\bar{M}_n = 4800$).[13] This degradation is much greater than can result from the simple removal of arabinose and rhamnose from the periphery. The postulate [2] that blocks of degraded units might have been interconnected by labile arabinofuranose residues has been rejected.[13] Moreover, there is no evidence from methylation studies for the presence of any galactofuranose residues; this implies that certain galactopyranosidic bonds must be unusually reactive under the mild conditions of autohydrolysis.

A sample of the whole gum was subjected to successive degradations using the procedure, devised by Smith and his co-workers,[17] involving periodate oxidation, borohydride reduction, and controlled acid hydrolysis. The first periodate oxidation was stopped after 48 hours; after borohydride reduction, and hydrolysis of acetal linkages with N-sulphuric acid at room temperature for 2 days, polysaccharide A was isolated. This contained galactose (69%), arabinose (27%), and glucuronic acid (4%); acid hydrolysis yielded a small amount of 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose. Thus, the first Smith degradation removed all the rhamnose and 4-*O*-methylglucuronic acid residues, together with some of the galactose, arabinose, and glucuronic acid. From the structural features accepted to date, non-reducing end-group arabinose and galactose, as well as 6-*O*-substituted galactose residues and 4-*O*-substituted glu-

[8] S. W. Challinor, W. N. Haworth, and E. L. Hirst, *J. Chem. Soc.*, 1931, 258.
[9] J. K. N. Jones, *J. Chem. Soc.*, 1953, 1672.
[10] P. Andrews and J. K. N. Jones, *J. Chem. Soc.*, 1955, 583.
[11] T. Dillon, D. F. O'Ceallachain, and P. O'Colla, *Proc. Roy. Irish Acad.*, 1953, **55B**, 331; 1954, **57B**, 31.
[12] V. C. Barry, *Nature*, 1943, **152**, 537.
[13] D. M. W. Anderson and J. F. Stoddart, *Carbohydrate Res.*, 1966, **2**, 104.
[14] G. O. Aspinall, *J. Chem. Soc.*, 1963, 1676.
[15] G. O. Aspinall, A. J. Charlson, E. L. Hirst, and R. Young, *J. Chem. Soc.*, 1963, 1696.
[16] H. B. Oakley, *Trans. Faraday Soc.*, 1935, **31**, 136; 1936, **32**, 1360; 1937, **33**, 372.
[17] I. J. Goldstein, G. W. Hay, B. A. Lewis, and F. Smith, *Abs. Papers Amer. Chem. Soc.*, 1959, 135, 3D.

curonic acid residues, should be cleaved during the first Smith degradation. Methylation evidence for the whole gum indicates that there are some non-reducing end-group galactopyranose, arabinopyranose, and arabinofuranose residues, together with a few C-6-substituted galactopyranose units; most of the glucuronic acid residues are substituted at C-4, with very few apparently present as non-reducing end-groups. The isolation and characterisation, after deacetylation, of 4-*O*-α-L-rhamnopyranosyl-D-glucose from acetolysis of diborane-reduced acetylated gum established that some L-rhamnopyranose residues are glycosidically linked to C-4 of D-glucuronic acid.[15] Complete oxidation of all glucuronic acid residues would be expected during the first treatment with periodate; the incomplete oxidation observed may result from steric hindrance.[18]

There is an additional problem with acidic polysaccharides; difficulty is encountered when attempting to hydrolyse acetal linkages involving acidic fragments, since they tend not to be cleaved by cold dilute acid.[19] Such fragments should, however, be removed during subsequent Smith degradations, since glucuronic acid residues are glycosidically linked to the C-6 positions of galactose residues, which are also usually 3-*O*-substituted with short side-chains containing galactopyranose, arabinopyranose, and arabinofuranose residues. Removal of these side-chains by successive Smith degradations will leave those galactose residues carrying acidic fragments on C-6 vulnerable to attack by periodate; any persistent acidic fragments will, therefore, be eliminated subsequently.

On methanolysis, the *O*-methyl derivative of polysaccharide A gave the methyl glycosides of 2,3,5-tri- and 2,5-di-*O*-methyl-L-arabinose, 2,3,4,6-tetra-, 2,4,6- and 2,3,4-tri-, and 2,6- and 2,4-di-*O*-methyl-D-galactose, and 2,3,4-tri-*O*-methyl-D-glucuronic acid. A methylation study by Dillon *et al.*[11] on the polysaccharide obtained after one Barry degradation gave, as hydrolytic cleavage fragments, 2,3,5- and 2,3,4-tri-, and 2,5- and 2,3-di-*O*-methyl-L-arabinose, and 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-*O*-methyl-D-galactose. Our results show that all the rhamnopyranose and arabinopyranose residues are removed by one Smith degradation. The presence of some 2,6-di-*O*-methyl-D-galactose may result from under-methylation, at the C-4 position, of certain galactose residues.

A second Smith degradation yielded polysaccharide B, which contained galactose (89%) and arabinose (11%). On methanolysis, the *O*-methyl derivative of polysaccharide B gave the methyl glycosides of 2,3,5-tri- and 2,5-di-*O*-methyl-L-arabinose, and 2,3,4,6-tetra-, 2,4,6- and 2,3,4-tri-, and 2,6- and 2,4-di-*O*-methyl-D-galactose. A Smith degradation of polysaccharide B gave polysaccharide C, which contained galactose (98%) and arabinose (2%). Methylation and methanolysis of this polysaccharide gave the methyl glycosides of 2,3,5-tri-*O*-methyl-L-arabinose and 2,3,4,6-tetra-, 2,4,6- and 2,3,4-tri-, and 2,6- and 2,4-di-*O*-methyl-D-galactose. Successive Smith degradations then gave polysaccharide D and E, which were examined by methylation and partial acid hydrolysis; on methylation and methanolysis, both polysaccharides D and E gave 2,3,4,6-tetra-, 2,4,6- and 2,3,4-tri-, and 2,6- and 2,4-di-*O*-methyl-D-galactose as their methyl glycosides. The ratio of the amounts of 2,4,6-tri-*O*-methyl-D-galactose to 2,3,4,6-tetra- and 2,4-di-*O*-methyl-D-galactose, as judged from the peak areas obtained for their methyl glycosides on gas–liquid partition chromatography, was greater for polysaccharide E than for D. Partial acid hydrolysis of polysaccharides D, E, F, and G afforded 3-*O*-β-D-galactopyranosyl-D-galactose, 6-*O*-β-D-galactopyranosyl-D-galactose (in small amounts), the β1,3-linked galactotriose, and higher oligosaccharides. The absence of the α or β 1,4-linked galactobiose, and the fact that the degraded polysaccharide proved difficult to methylate, has led us to believe that the small amounts of 2,6-di-*O*-methyl-D-galactose arose from under-methylation. The presence of some 2-*O*-methyl-D-galactose in the *O*-methyl derivatives of the whole gum and autohydrolysed gum may also be ascribed to incomplete methylation; this occurs[20-22] with structurally related polysaccharides of the arabinogalactan type that have a high proportion of β1,3-linked galactose units.

Molecular-sieve chromatography[13] of the whole gum and of polysaccharides A—E gave the elution patterns shown in Figure 2. The number-average molecular weights, $\bar{M}_n$, have been estimated from the calibration curve shown in Figure 1; without confirmation by some more fundamental method, these values must be regarded as approximate. For a commercial sample of gum arabic, Oakley[16] obtained a value of *ca.* 250,000 for $\bar{M}_n$ by osmometry. Assuming a similar value for the present sample, there is a significant drop in $\bar{M}_n$ to *ca.* 96,000 for polysaccharide A after the first Smith degradation, and subsequently to 17,000 for polysaccharide E after the fifth Smith degradation. (A few glycosidic bonds may, however, be cleaved[9] during hydrolysis of acetal linkages with cold dilute acid.)

Partial acid hydrolysis of polysaccharide E′ (obtained after five successive Smith degradations in a duplicate experiment on a larger scale) led to the isolation of 6-*O*-β-D-galactopyranosyl-D-galactose. Proof of its structure followed from the value of the specific rotation, and methanolysis of the *O*-methyl derivative to give the methyl glycosides of 2,3,4,6-tetra- and 2,3,4-tri-*O*-methyl-D-galactose. The β1,3-linked galactobiose[7,23] and galactotriose[23] have been characterised previously in the whole gum.

[18] H. Klosterman and F. Smith, *J. Amer. Chem. Soc.*, 1952, **74**, 5336.
[19] G. O. Aspinall, V. P. Bhavanandan, and T. B. Christensen, *J. Chem. Soc.*, 1965, 2677.
[20] J. K. N. Jones and P. E. Reid, *J. Polymer Sci.*, 1963, **2**, C, 63.
[21] G. O. Aspinall and R. M. Fairweather, *Carbohyd. Res.*, 1965, **1**, 83.
[22] M. L. Wolfrom and D. L. Patin, *J. Org. Chem.*, 1965, **30**, 4060.
[23] G. O. Aspinall and R. Young, *J. Chem. Soc.*, 1965, 3003.

On the basis of the available evidence, several structural interpretations for polysaccharide E are possible; these must, however, take into consideration the limitations involved in applying Smith degradations in stepwise fashion to polysaccharides. It is unlikely that

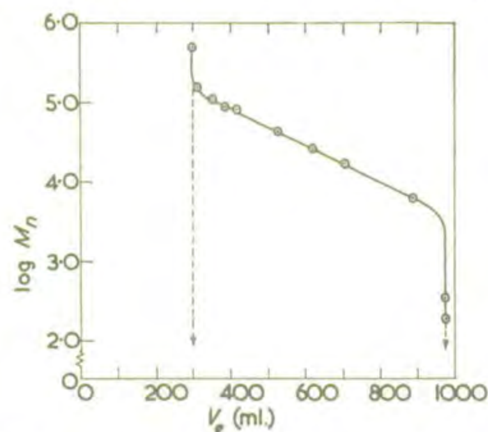

FIGURE 1 Plot of elution volume, V_e, against log $\bar{M}_n$ for dextran fractions of known $\bar{M}_n$ values. "Bio-Gel P300" column (4·8 × 50 cm.): elution with M-sodium chloride. (The arrows correspond with those on Figure 2)

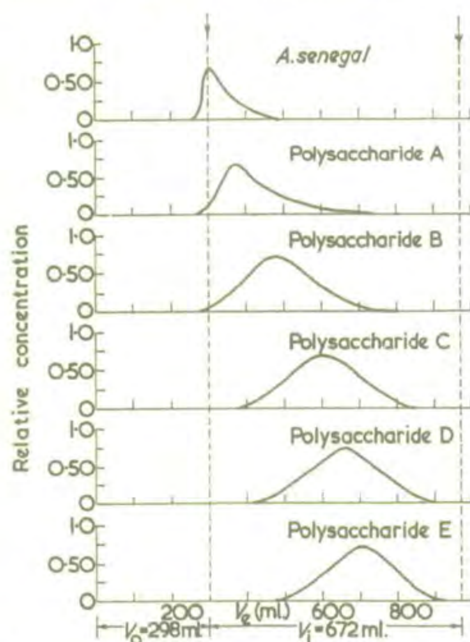

FIGURE 2 Elution patterns for *Acacia senegal* gum and for polysaccharides A—E. "Bio-Gel P300" column (4·8 × 50 cm.): elution with M-sodium chloride

complete reaction is achieved at every stage in the degradation scheme. The methylation evidence indicates that polysaccharide E is not a simple linear β1,3-galactan. Whilst 2,4,6-tri-*O*-methyl-D-galactose is the most predominant *O*-methyl sugar obtained from the methylated polysaccharide, the identification of some 2,4-di-*O*-methyl-D-galactose and 2,3,4,6-tetra-*O*-methyl-D-galactose indicate occasional branching at the C-3 and C-6 positions. The presence of small amounts of the β1,6-galactobiose in the partial acid hydrolysate of polysaccharide E is further support for a branched structure; moreover, this disaccharide is also present in the partial acid hydrolysates of polysaccharides F and G. If there is a "main chain" of β1,3-linked galactose units in polysaccharide E, it must carry short side-chains of β1,3-linked galactose residues; the branch points for these side-chains would be through β1,6-linkages (Figure 3, I). Alternatively, there could be occasional β1,6-linkages along a "main chain" of β1,3-linked galactose units; in this case, the branch points for short side-chains would be through β1,3-linkages to the β1,6-linked residues in the "main chain" (Figure 3, II). A randomly branched structure of β1,3-linked

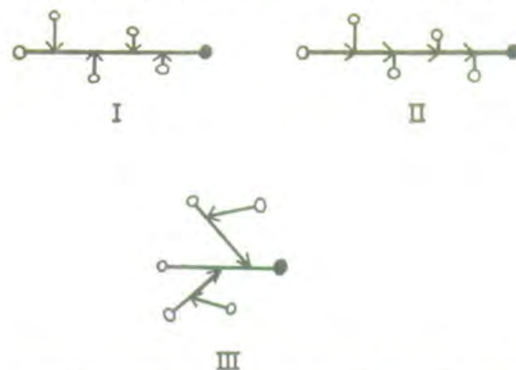

FIGURE 3 Diagrammatic representation of some of the possible structures for the "periodate-resistant" galactan framework (polysaccharide E) of *Acacia senegal* gum; —— = chains of β1,3-linked D-galactopyranose residues; ↓ = β1,6-linkage at branch point; ● = reducing end group; ○ = terminal non-reducing group

chains with β1,6 branch points would also satisfy the available structural evidence (Figure 3, III).

The polysaccharide entities of which the gum is comprised are probably polymer systems which have, in addition to a molecular weight distribution, a variation in monosaccharide composition as well as a distribution in the mode of linking and branching of certain monosaccharide units.[13] A possible structural fragment from the internal chains of one of these entities is represented by the polysaccharide array shown in Figure 4. At present, any of the three galactan frameworks shown in Figure 3 may be considered to form the basis for fragments of this general type. The implication is that the polysaccharide entities are more highly branched, and consequently more globular in shape, than has previously been recognised.

There is some evidence from physical measurements, particularly from viscosity studies, to support this view. The gum from *A. senegal* is almost unique amongst commercially important gums in forming water-soluble solutions over a very wide range of concentrations. Moreover, it has a low viscosity [6] when compared on an equal concentration basis with polysaccharides known to have substantially linear structures and approximately the same number-average molecular weights, *e.g.*, locust bean gum and guaran. If the polysaccharide entities of *A. senegal* gum are in the form of stiff coils, as has been suggested by Veis and Eggenberger [24]

[24] A. Veis and D. N. Eggenberger, *J. Amer. Chem. Soc.*, 1954, **76**, 1560.

from light scattering measurements, these should unfold in solution, as a result of inter-coulombic repulsions between the ionised carboxyl groups, to give elongated entities which would be expected to show non-Newtonian behaviour. Recently, however, Warburton [25] has observed that a 5% (w/v) gum solution shows ideal Newtonian flow, up to a shear rate of 100 reciprocal seconds, in a Couette concentric cylinder viscometer. This has led him to suggest that in solution the gum gives "a suspension of non-entangling spheroids." A similar conclusion was reached by Smith and Montgomery,[2] who noted that a 30% gum solution showed no

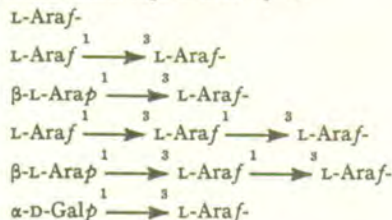

FIGURE 4 Possible structural fragment in *Acacia senegal* gum

structure viscosity, *i.e.*, behaved as a Newtonian solution.

Although it is widely accepted that *A. senegal* gum and structurally related polysaccharides have a "main chain" or "backbone," unequivocal proof for this hypothesis is still lacking.

EXPERIMENTAL

The analytical methods have been described by Anderson and Stoddart.[13]

Paper chromatography was carried out on Whatman Nos. 1 and 3MM papers using the following solvent systems (v/v); (*a*) benzene–butan-1-ol–pyridine–water (1 : 5 : 3 : 3, upper layer); (*b*) ethyl acetate–acetic acid–formic acid–water (18 : 3 : 1 : 4); (*c*) butan-1-ol–ethanol–water (4 : 1 : 5, upper layer); (*d*) butan-1-ol–acetic acid–water (4 : 1 : 5, upper layer); (*e*) ethyl acetate–pyridine–water (10 : 4 : 3); (*f*) butan-2-one–water–ammonia (*d* 0·880) (200 : 17 : 1); (*g*) benzene–ethanol–water (169 : 47 : 15, upper layer). R_G values of methylated sugars refer to distances moved relative to 2,3,4,6-tetra-*O*-methyl-D-glucose in solvent (*c*). Gas–liquid partition chromatography (Chromatograph type S3A, fitted with flame ionisation detectors, supplied by Gas Chromatography Ltd.) of mixtures of *O*-methyl sugars was carried out at nitrogen flow rates of *ca.* 100 ml./min. on columns of (i) 15% by weight of butane-1,4-diol succinate polyester on Celite (120 × 0·5 cm.) at 175°, (ii) 15% by weight of ethylene glycol adipate polyester on Celite (75 × 0·5 cm.) at 160°. Retention times (*T*) are quoted relative to methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside as standard. Polysaccharides were methylated successively with dimethyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide. Unless otherwise stated,

[25] B. Warburton, "The Rheology and Physical Chemistry of some *Acacia* Systems," Society of Chemical Industry Monograph No. 24, pp. 118–130.

methanolyses were carried out under reflux for 6 hr. with methanolic 5% hydrogen chloride.

Methylation of A. senegal *Gum*.—The whole gum (400 mg.) was methylated to give a product (340 mg.), $[\alpha]_D$ $-46°$ (*c* 1·0 in $CHCl_3$) (Found: OMe, 41·9%). Methanolysis followed by g.l.c. examination of the mixture of methyl glycosides gave the results shown in Table 1.

TABLE 1

Examination of methanolysis products from methylated *Acacia senegal* gum

O-Methyl sugars	Relative retention times (*T*) of methyl glycosides Column (i)	Column (ii)	R_G
2,3,4-Tri-*O*-methyl-L-rhamnose	0·49	0·44	1·01
2,3,5-Tri-*O*-methyl-L-arabinose	0·58, 0·72	0·51, 0·64	0·98
2,3,4-Tri-*O*-methyl-L-arabinose	0·96	0·84	0·78
2,5-Di-*O*-methyl-L-arabinose	1·78, 3·29	1·27 (2·21) *	0·80
2,3,4,6-Tetra-*O*-methyl-D-galactose	1·68	1·67	0·87
2,4,6-Tri-*O*-methyl-D-galactose	3·74, 4·18	3·05, 3·48	0·73
2,3,4-Tri-*O*-methyl-D-galactose	6·42	5·27	0·73
2,4-Di-*O*-methyl-D-galactose	14·6, 16·5	9·8, 11·0	0·53
2,3,4-Tri-*O*-methyl-D-glucuronic acid †	2·23, 2·96	(2·21), 2·73	—
2,3-Di-*O*-methyl-D-glucuronic acid †	7·8, 9·3	6·2, 7·1	—
2-*O*-Methyl-D-galactose	—	—	0·32
Unknown sugars	1·17, 1·44	0·98, 1·15, 2·48	—

* Figures in parentheses indicate *T* values of components that were not completely resolved. † As methyl ester methyl glycoside.

TABLE 2

Examination of methanolysis products from methylated autohydrolysed degraded gum

O-Methyl sugars	Approx. relative molar proportions	Relative retention times (*T*) of methyl glycosides Column (i)	Column (ii)	R_G
2,3,5-Tri-*O*-methyl-L-arabinose	Trace	0·58, 0·72	0·46, 0·64	—
2,3,4,6-Tetra-*O*-methyl-D-galactose	++	1·67	1·64	0·88
2,4,6-Tri-*O*-methyl-D-galactose	++	3·73, 4·18	2·99, 3·46	0·72
2,3,4-Tri-*O*-methyl-D-galactose	+++	6·42	5·22	0·72
2,4-Di-*O*-methyl-D-galactose	+++	14·7, 15·9	9·9, 11·2	0·52
2,3,4-Tri-*O*-methyl-D-glucuronic acid *	+++	2·31, 2·94	2·09, 2·68	—
2-*O*-Methyl-D-galactose	+	—	—	0·34

* As methyl ester methyl glycoside.

TABLE 3

Formic acid released (mmoles/g.) on periodate oxidations

Time (hr.)	1	3	4	6	11	12	24	48	72	96
A. senegal gum	0·73	0·99	—	1·14	—	1·44	1·49	1·58	—	—
Polysaccharide A	0·80	1·21	—	1·55	1·74	—	2·12	2·74	3·17	3·26
Polysaccharide B	1·18	1·19	—	—	—	1·22	1·23	—	—	—
Polysaccharide C	1·10	—	1·18	1·21	—	—	1·23	—	—	—

TABLE 4

O-Methyl sugars present in methylated polysaccharides A—E

O-Methyl sugars	A	B	C	D	E	Approx. relative molar proportions in E
2,3,5-Tri-*O*-methyl-L-arabinose	+	+	+			
2,5-Di-*O*-methyl-L-arabinose	+	+				
2,3,4,6-Tetra-*O*-methyl-D-galactose	+	+	+	+	+	1
2,4,6-Tri-*O*-methyl-D-galactose	+	+	+	+	+	12
2,3,4-Tri-*O*-methyl-D-galactose	+	+	+	+	+	Trace
2,6-Di-*O*-methyl-D-galactose	+	+	+	+	+	3
2,4-Di-*O*-methyl-D-galactose	+	+	+	+	+	1
2,3,4-Tri-*O*-methyl-D-glucuronic acid	+					

Methylation of Autohydrolysed Gum.—The preparation of this degraded material has been described.[13] The autohydrolysed gum (200 mg.) was methylated to give a product (82 mg.), $[\alpha]_D$ $-56°$ (*c* 1·0 in $CHCl_3$) (Found: OMe, 42·1%). The methylated polysaccharide (50 mg.) was heated under reflux for 8 hr. with methanolic 5% hydrogen chloride. After neutralisation with silver carbonate, and filtration, the methanolic solution was divided into two approximately equal parts which were concentrated to syrups under reduced pressure. Examination of one portion for methyl glycosides by g.l.c. showed the *O*-methyl sugars listed in Table 2; retention times on columns (i) and (ii) were comparable with those for methyl glycosides from authentic sugars. The other portion was hydrolysed with N-sulphuric acid for 3 hr. at 100°; the hydrolysate was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120(H), and reduced to a small volume. Paper

chromatographic examination in solvents (c) and (f) showed the presence of 2-*O*-methylgalactose in addition to those *O*-methyl sugars already characterised as methyl glycosides by g.l.c.

Preparation and Methylation of Polysaccharide A.—Whole gum (20·0 g.) was dissolved in water (500 ml.) and 0·25M-sodium metaperiodate solution (500 ml.) was added. The oxidation (in darkness at room temperature) was followed by measuring the release of formic acid with time; the results, obtained by titrating aliquots (1 ml.) with 0·0099N-sodium hydroxide, using Methyl Red as indicator, are shown in Table 3. After 48 hr., the reaction was stopped by addition of ethylene glycol (10 ml.); the solution was dialysed against running tap water for 2 days. Sodium borohydride (5 g.) was added and the mixture kept at room temperature for 30 hr., then dialysed for a further 2 days. The polyalcohol was hydrolysed in N-sulphuric acid at room temperature for 2 days, after which the solution was neutralised (barium carbonate), filtered, deionised [Amberlite resin IR-120(H)], reduced in volume to *ca*. 250 ml., and dialysed against water (1 l.). The syrup obtained from the dialysate was shown by paper chromatography to contain glycerol and glycollic aldehyde as the main components, together with trace amounts of arabinose, ethylene glycol, and some slower-moving non-reducing glycosides. After dialysis against running tap water for 2 days, polysaccharide A (13·6 g., yield, 69%) $[\alpha]_D$ −28° (*c*, 1·0) was isolated as the freeze-dried product. Analysis showed it to contain uronic acid (4%, by decarboxylation), galactose (69%), and arabinose (27%). Hydrolysis of polysaccharide A with N-sulphuric acid for 7 hr. on a boiling water-bath gave a trace of the aldobiouronic acid, 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose [R_{gal} 0·21 in solvent (*b*)] in addition to galactose and arabinose. Molecular-sieve chromatography [13] of polysaccharide A (20 mg.) on a previously calibrated Bio-Gel P 300 " column (50 × 4·8 cm.) gave $\bar{M}_n \approx 96{,}000$.

Polysaccharide A (300 mg.) was methylated to give a product (213 mg.), $[\alpha]_D$ −41° (*c* 1·0 in $CHCl_3$) (Found: OMe, 39·4%, not raised on further methylation). Methanolysis of a sample of this product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table 4. Examination of a hydrolysate of the methyl glycosides by paper chromatography showed the presence of 2-*O*-methylgalactose, in addition to those *O*-methyl sugars already characterised by g.l.c. of their methyl glycosides.

Preparation and Methylation of Polysaccharide B.—Borohydride reduction of periodate-oxidised polysaccharide A (12·0 g.), followed by controlled hydrolysis at room temperature with N-sulphuric acid for 2 days, gave polysaccharide B (6·4 g., yield, 54%), $[\alpha]_D$ −9° (*c* 1·0), which contained galactose (89%) and arabinose (11%). The release of formic acid on periodate oxidation of polysaccharide A is shown in Table 3. Molecular-sieve chromatography [13] indicated that polysaccharide B had $\bar{M}_n \approx 59{,}000$.

Polysaccharide B (412 mg.) was methylated to give a product (296 mg.), $[\alpha]_D$ −31° (*c* 1·0 in $CHCl_3$) (Found: OMe, 39·0%, not raised on further methylation). Methanolysis of a sample of this product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table 4.

Preparation and Methylation of Polysaccharide C.—Borohydride reduction of periodate-oxidised polysaccharide B (5·0 g.), followed by controlled hydrolysis with N-sulphuric acid for 2 days at room temperature, gave polysaccharide C (3·1 g., yield, 63%, $[\alpha]_D$ +14° (*c* 1·0) which contained galactose (98%) and arabinose (2%). Results for release of formic acid on periodate oxidation of polysaccharide B are shown in Table 3. Molecular-sieve chromatography [13] indicated that polysaccharide C had $\bar{M}_n \approx 29{,}000$.

Polysaccharide C (204 mg.) was methylated to give a product (104 mg.), $[\alpha]_D$ −12° (*c* 1·0 in $CHCl_3$) (Found: OMe, 38·1%, not raised on further methylation). Methanolysis of a sample of this product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table 4.

Preparation, Partial Acid Hydrolysis, and Methylation of Polysaccharide D.—Borohydride reduction of periodate-oxidised polysaccharide C (2·5 g.), followed by controlled hydrolysis with N-sulphuric acid for 2 days at room temperature, gave polysaccharide D (1·55 g., yield, 64%), $[\alpha]_D$ +25° (*c* 1·0), which contained only galactose. Results for release of formic acid on periodate oxidation of polysaccharide C are shown in Table 3. An estimated value of 21,000 for the $\bar{M}_n$ of polysaccharide D was obtained from molecular-sieve chromatography.[13]

Polysaccharide D (200 mg.) was hydrolysed with 0·5N-sulphuric acid (10 ml.) for 1 hr. on a boiling water-bath, and the cooled hydrolysate was neutralised with barium carbonate, filtered, treated with Amberlite resin IR 120(H), and concentrated. Paper chromatographic examination of the syrup in solvent (e) indicated the presence of galactose, two neutral disaccharides with the mobilities of 3-*O*-β-D-galactopyranosyl-D-galactose (R_{gal} 0·54, major component) and 6-θ-β-D-galactopyranosyl-D-galactose (R_{gal} 0·37, minor component), and higher neutral oligosaccharides including the β1,3-linked galactotriose (R_{gal} 0·25).

Polysaccharide D (93 mg.) was methylated to give a product (42 mg.) $[\alpha]_D$ −1° (*c* 0·84 in $CHCl_3$) (Found: OMe, 36·7%, not raised on further methylation). Methanolysis of a sample of this product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table 4.

Preparation, Partial Acid Hydrolysis, and Methylation of Polysaccharide E.—Borohydride reduction of periodate-oxidised polysaccharide D (500 mg.) followed by controlled hydrolysis with N-sulphuric acid for 2 days at room temperature, yielded a galactan, polysaccharide E (322 mg. yield, 67%), $[\alpha]_D$ +30° (*c* 1·0). The amount of formic acid released on periodate oxidation of polysaccharide D over 24 hr. was 0·57 mmoles/g. An estimated value of 17,000 for the $\bar{M}_n$ of polysaccharide E was obtained from molecular-sieve chromatography.[13]

Partial acid hydrolysis of polysaccharide E (10 mg.), followed by paper chromatographic examination of the hydrolysate indicated the presence of galactose and the same neutral oligosaccharides found in polysaccharide D.

Polysaccharide E (89 mg.) was methylated to give a product (38 mg.) $[\alpha]_D$ −1° (*c* 0·76 in $CHCl_3$) (Found: OMe, 36·5% not raised on further methylation). Methanolysis, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table 4.

Preparation of Polysaccharides F and G.—Borohydride reduction of periodate-oxidised polysaccharide E (100 mg.), followed by controlled acid hydrolysis yielded polysaccharide F (70 mg., yield, 70%). The amount of formic acid released on periodate oxidation of polysaccharide E after 24 hr. was 0·30 mmoles/g. During a similar degradation on

polysaccharide F (50 mg.) to yield polysaccharide G (36 mg., yield, 72%), the amount of formic acid released after 24 hr. was 0·35 mmoles/g.

Partial Acid Hydrolysis of Polysaccharides F and G.—Hydrolysis of these two polysaccharides with 0·5N-sulphuric acid for 1 hr. on a boiling-water bath, and paper chromatographic examination of the hydrolysate in solvent (e), showed galactose, 3-*O*-β-D-galactopyranosyl-D-galactose (R_{gal} 0·52), trace amounts of 6-*O*-β-D-galactopyranosyl-D-galactose (R_{gal} 0·36), and the β1,3-linked galactotriose (R_{gal} 0·23).

Preparation and Partial Acid Hydrolysis of Polysaccharide E′.—A sample of gum (60 g.) was subjected to five successive Smith degradations, without isolation of the intermediate polysaccharides, to obtain polysaccharide E′ (2 g.). This polysaccharide (1·5 g.) was hydrolysed with 0·5N-sulphuric acid (500 ml.) for 1 hr. on a boiling water-bath, and the cooled hydrolysate was neutralised (barium hydroxide and barium carbonate), filtered, deionised [Amberlite resin IR 120(H)], and concentrated. The syrup was chromatographed on Whatman No. 3 MM paper, previously treated with 1% (v/v) acetic acid, and two pure disaccharides and one trisaccharide were isolated.

Fraction 1 (11·2 mg.) had $[\alpha]_D$ +35° (*c* 0·22) and yielded galactose on acid hydrolysis. It had the same chromatographic mobility [R_{gal} 0·31 in solvent (a), 0·20 in solvent (b), and 0·37 in solvent (e)] as an authentic sample of 6-*O*-β-D-galactopyranosyl-D-galactose. A portion (8 mg.) was methylated successively with dimethyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide. G.l.c. examination of the methanolysis product revealed peaks with retention times corresponding to the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-galactose (i, *T* 1·68; ii, *T* 1·67) and 2,3,4-tri-*O*-methyl-D-galactose (i, *T* 6·33; ii, *T* 5·27).

Fraction 2 (60 mg.) had $[\alpha]_D$ +61° (*c* 1·20) and yielded galactose on acid hydrolysis. It had the same chromatographic mobility [R_{gal} 0·49 in solvent (a), 0·27 in solvent (b), and 0·53 in solvent (e)] as an authentic sample of 3-*O*-β-D-galactopyranosyl-D-galactose. The disaccharide was crystallised from aqueous acetone to give needles of 3-*O*-β-D-galactopyranosyl-D-galactose monohydrate, which had m. p. 156—159°.

Fraction 3 (50 mg.) had $[\alpha]_D$ +52° (*c* 1·00) and yielded galactose and the β1,3-linked galactobiose as the only disaccharide on partial acid hydrolysis. The trisaccharide was crystallised from aqueous ethanol, m. p. 238—242° (decomp.) and had R_{gal} values of 0·22 in solvent (a) and 0·24 in solvent (e).

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42.

STUDIES, ON URONIC ACID MATERIALS

PART XVIII. LIGHT-SCATTERING STUDIES ON SOME MOLECULAR-WEIGHT FRACTIONS FROM *Acacia senegal* GUM

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INTRODUCTION

Despite the industrial importance of *Acacia senegal* gum (gum arabic), there have been few studies to date of its molecular size and shape.

From osmotic pressure measurements, Thomas and Murray[2] found gum arabic solutions to be isotonic over the range pH 6–9, and Oakley[3] obtained values of 191,000 and 217,000 for the number-average molecular weight in 0.1N and 0.5N sodium chloride, respectively. Ultracentrifugal studies by Säverborn[4] indicated values in the range of 256,000–326,000. More recently, two light-scattering results have been reported; Veis and Eggenberber[5] found a weight-average molecular weight ($\overline{M}_w$) of $(1.00 \pm 0.05) \times 10^6$ in 0.02N hydrochloric acid, whereas Deb and Mukherjee[6] found $\overline{M}_w = 0.58 \times 10^6$ in a solution 0.02 N with respect to a mixture of potassium, calcium, and magnesium chlorides. Contrary to the opinion expressed by Deb and Mukherjee[6], these values cannot be regarded as being in satisfactory agreement; the reason for this requires investigation.

Electrolyte concentrations of only 0.02N were used in the light-scattering studies[5,6] but, in our experience (*cf.* Oakley[7]), considerably higher concentrations are required to suppress ionisation of the carboxyl groups in the gum. Furthermore, Deb and Mukherjee[6], without explanation, used a mixed solvent containing divalent cations. The reason for this is difficult to understand in view of the cross-linking tendencies of calcium ions in acidic polysaccharide systems. A more fundamental factor may also be involved. The investigators to date appear to have used commercial samples, without giving adequate attention to their characterisation. It is now recognised[8,9] that early samples of gum arabic were mixtures of the exudates from various *Acacia* species. The composition[10] and physical properties[11] of *Acacia* gums vary from species to species and, furthermore, within a particular species[12,13]. The commercial gum arabic produced at the present time originates almost exclusively from *A. senegal*, but admixture with other species, particularly *A. seyal* and *A. laeta*, or with other genera, *e.g.*, *Albizia sericocephala*[14], can never be discounted. Commercial samples should not be used in fundamental studies.

Recently, an authenticated specimen of *A. senegal* gum was characterised care-

fully, and it was reported[15] that an arbitrary number of molecular-weight fractions could be obtained by fractional precipitation with sodium sulphate. Preliminary experiments with molecular-sieve chromatography[16] indicated that the gum had a broad molecular-weight distribution, with considerable contributions from high molecular-weight material[17], in agreement with Veis and Eggenberger[5]. In view of these indications, it appeared that light scattering was preferable to osmometry as a means of obtaining a self-consistent series of reliable measurements of the whole gum and of its fractions.

EXPERIMENTAL

Characterisation and purification of the whole gum.— Details of the characterisation of the specimen of *A. senegal* gum have been given[15]. After dissolution in cold distilled water, the gum was filtered, dialysed against tap water, exhaustively electrodialysed against distilled water[12], and then freeze-dried.

Fractionation of the gum. — Fractionation was achieved by the procedure described by Anderson and Stoddart[15]. The purified gum (40 g, in the free-acid form) was dissolved in water (400 ml) and maintained at 28–30°. Anhydrous sodium sulphate was added in small amounts to the solution with continuous stirring. When precipitation began, the rate of stirring was decreased to enable the precipitate to rise to the surface and coagulate; it was then transferred quickly to a warm Buchner funnel (15 cm), and the excess solution was removed. The remaining mother-liquor was filtered through a similar funnel; the residue on both funnels gave Fraction 1. By repetition of the above procedure, Fractions 2 and 3 were obtained (the number of fractions isolated at this stage is arbitrary). Eventually, the solution contained gum which could not be precipitated with sodium sulphate, and this gave Fraction 4. The fractions were dialysed, exhaustively electrodialysed, and freeze-dried. On a dry-weight basis, the yields of Fractions 1–4 were 16, 8, 8, and 0.5 g, respectively; the total recovery of gum was 81%.

Preparation of the sodium salt of the whole gum and each of its fractions.—The sodium salt of the whole gum, and of its fractions, was prepared by neutralisation, followed potentiometrically, with sodium hydroxide solution. The neutralised products were dialysed to remove any excess of alkali, and the sodium salts were finally recovered by freeze-drying.

Solutions for light-scattering experiments were made by direct weighing, allowance being made for the pre-determined moisture content of each of the products. The weighed amounts were then dissolved in molar sodium chloride.

Light-scattering photometer.— Light-scattering experiments were carried out at 25.00 ±0.02° by using an instrument based on the design of McIntyre and Doderer[18], and constructed in the Procter Department, University of Leeds. Details of this instrument and its calibration have been described[19]. In use, one galvanometer (G_1) monitors the main beam, whilst a second galvanometer (G_2) receives the light scattered at any angle in the range 20–160° and also the main beam (at 0°) after suitable

attenuation. By using the ratio of the galvanometer readings, compensations for the inevitable fluctuations (of the order of $\pm 5\%$) in the intensity of the main beam are possible: the "reduced intensity-of-scatter", G'_θ, for a solution at $\theta°$ is given by

$$G'_\theta = \frac{(G_2/G_1)_{\bar{\theta}}}{(G_2/G_1)_0}.$$

Unpolarised blue light (4358 Å) was used, and was selected from the mercury spectrum by a combined 47B and 2E Wratten filter.

Clarification of solutions. — Each solution was clarified by successive passage through 5- and 1.2-μ "Millipore" filters, using a stainless-steel filter holder attached to a 50-ml syringe. The filtrate ran directly into the cylindrical light-scattering cell, which had been cleaned by steaming followed by rinsing with condensing acetone vapour. The solvent was treated in a similar manner and its reduced intensity-of-scatter, G''_θ, was also determined from the ratios of galvanometer readings.

The scatter due to the gum at $\theta°$, G_θ, was then taken as equal to $(G'_\theta - G''_\theta)$, and the usual corrections for volume viewed and partial polarisation, which both vary with θ, were applied[20]. The use of the difference $G'_\theta - G''_\theta$ implies that the solution and the solvent each contain identical amounts of scattering impurities. Ideally, each should be free from such scatter, but this is rarely achieved in practice, particularly for aqueous solutions [21].

Ultracentrifugation is the alternative technique to ultrafiltration for clarifying macromolecular solutions, and the two methods were compared for effectiveness by centrifuging a sample at 17,000 r.p.m. (30,000 g) in a Spinco Model L ultracentrifuge for 3 h. The upper half of the solution from each centrifuge tube was transferred to the light-scattering cell, and the scattering envelope was determined. A portion of the solvent was similarly treated; in neither case was there any significant difference between the envelopes resulting from clarification by centrifugation or by filtration. Since the "Millipore" filters are much more rapid and convenient to use than the centrifuge, all subsequent clarifications were achieved by ultrafiltration.

In the centrifugal clarification, sedimentation did not occur under the conditions used. The level-of-scatter did not change on subsequent passage through the "Millipore" filters, which therefore do not retain any of the gum molecules. Colorimetric experiments did not detect differences from the initial concentration after clarification. The use of the initial concentration of gum therefore appears to be justified, although, ideally, the concentration after clarification should be used in calculating results. Any concentration difference is unlikely to exceed 1μg/ml; this does not introduce any serious error into the molecular-weight calculation.

RESULT AND DISCUSSION

Fig. 1 shows how the reciprocal, reduced intensity-of-scatter, G_θ^{-1}, varies with the angle-of-scatter θ (plotted as $\sin^2(\theta/2)$ as suggested by Zimm[22]) for the whole gum and Fractions 1, 2, and 3. The curves bend downwards steeply at the lower angles.

If the observed scatter arose solely from the gum, these curves would indicate that the gum molecules behave as stiff rods in solution. It is well known, however, that gum arabic solutions have a comparatively low viscosity, even when fairly concentrated, whereas rod-like molecules give solutions of high viscosity, even at low concentrations, and the viscosity rises sharply with increasing concentration. It is suggested, therefore, that the curvature seen in Fig. 1 is not a reflection of the shape of the gum molecules, but an indication of incomplete clarification of the solutions from dust and suspended impurities despite the care taken with clarification processes. Unwanted scatter from such sources would be manifest most particularly at the lower angles and be of less importance at the higher angles. Repeated passage of the solutions through the filters failed to reduce the scatter, which was independent of time over several hours. More reliable figures for the angular variation of scatter could not, therefore, be obtained for the present fractions. This illustrates the need for caution and for consideration of all relevant information when interpreting the data obtained from such experiments.

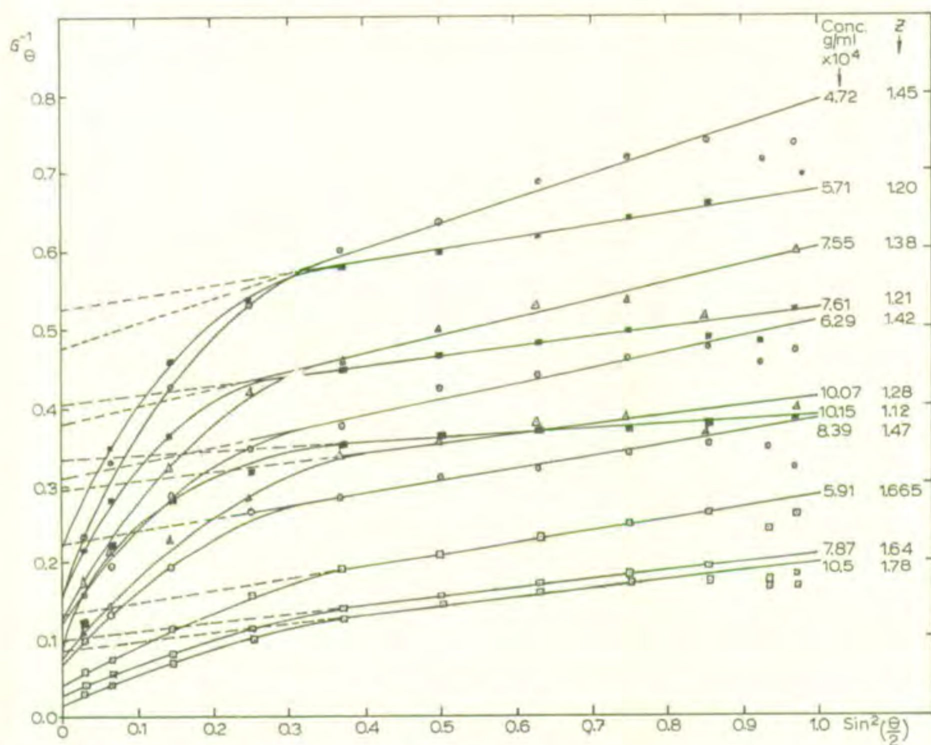

Fig. 1. Plot of the reciprocal, reduced intensity-of-scatter, G_θ^{-1}, *versus* $\sin^2(\theta/2)$ for *A. senegal* gum (○) and Fractions 1 (□), 2 (△), and 3 (■) obtained therefrom.

In order to obtain estimates of molecular weight from the data in Fig. 1, it is assumed that the linear parts of the curves are due to scatter from the gum molecules. In this region, contributions from unwanted impurities will be minimal, and almost independent of angle. Thus, the whole of the linear region most nearly represents

scatter from the gum molecules, although the level of scatter will be in error by an amount equal to the contribution from residual impurities. (At the present time, the magnitude of this contribution is indeterminate, but it is possibly 10% of the observed total scatter). Since the linear portion is not parallel to the $\sin^2(\theta/2)$ axis, the effective size of the scattering units in solution exceeds $\lambda/20$ (*i.e.* exceeds 160 Å).

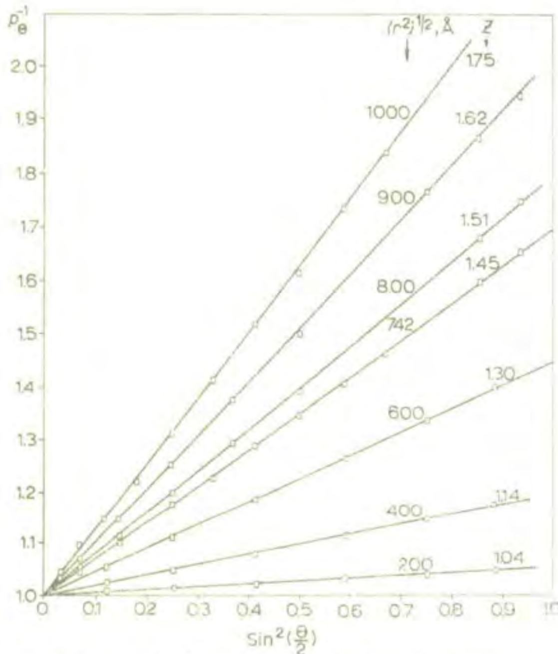

Fig. 2. Plot of P_θ^{-1} *versus* $\sin^2(\theta/2)$ for polydisperse coils of various root-mean-square end-to-end distances $(r^2)^{\frac{1}{2}}$. The dissymmetry (Z) is shown on each curve.

It is therefore necessary to extrapolate to $\sin^2(\theta/2) = 0$, using the intercept to compute the molecular weight from expression (1),

$$\frac{1}{M_w} = \frac{KC}{R_o} \tag{1}$$

where $R_o = G_o \times$ the instrument constant (which depends only on geometrical factors, and on the galvanometer and photo-tube sensitivities).

In this way, molecular weights are obtained without reference to any model for the scattering units in solution. The assumption made above means that the calculated values are likely to be somewhat higher than the true values. The experimental data from which Fig. 1 was plotted, together with the intercepts and the corresponding values of $\overline{M}_w$, are given in Table I.

The use of equation (1) to obtain $\overline{M}_w$ from R_o also presumes that C/R_o is independent of C, *i.e.*, that virial coefficients are zero. This presumption appeared to be reasonable in the present experiments, since the use of molar sodium chloride as solvent minimises the interionic forces between the charged molecules[7]. Moreover,

any small variations of pH with dilution will not affect the total charge carried by each gum molecule[2], since totally neutralised gums have been used. In such circumstances, the gum may be treated as a neutral polymer. The work of Veis and Eggenberger[5],

TABLE I

RECIPROCAL SCATTERED INTENSITIES OF GUM FRACTIONS AT VARIOUS CONCENTRATIONS AND ANGLES

Angle	$G_\theta^{-1} \times 10$										
	Fraction 1			*Fraction 2*		*Fraction 3*			*Whole gum*		
$\theta°$	*Conc. (g/ml × 10⁴)*			*Conc. (g/ml × 10⁴)*		*Conc. (g/ml × 10⁴)*			*Conc. (g/ml × 10⁴)*		
	10.5	*7.87*	*5.91*	*10.07*	*7.55*	*10.15*	*7.61*	*5.71*	*8.39*	*6.29*	*4.72*
20	0.3	0.4	0.6	1.0	1.7	1.2	2.2	2.2	1.0	1.6	2.3
30	0.4	0.6	0.8	1.4	2.1	2.2	2.8	3.5	1.4	2.0	3.3
45	0.7	0.8	1.1	2.3	3.2	2.8	3.6	4.6	2.0	2.9	4.3
60	1.0	1.2	1.6	2.8	4.2	3.1	4.2	5.3	2.7	3.5	5.4
75	1.3	1.4	1.9	3.4	4.5	3.5	4.5	5.8	2.8	3.7	6.0
90	1.5	1.6	2.1	3.5	5.0	3.6	4.6	5.9	3.2	4.3	6.4
105	1.6	1.7	2.3	3.8	5.2	3.7	4.7	6.1	3.2	4.4	6.9
120	1.7	1.8	2.5	3.8	5.3	3.7	4.9	6.4	3.4	4.6	7.2
135	1.7	1.9	2.6	3.6	5.1	3.8	4.8	6.6	3.5	4.7	7.4
150	1.6	1.8	2.4	3.5	4.8	—	4.8	7.2	3.4	4.5	7.1
160	1.6	1.8	2.6	3.9	6.0	3.8	5.2	9.0	3.2	4.7	7.4
G_θ^{-1}	0.09	0.10	0.14	0.29	0.37	0.33	0.40	0.52	0.23	0.31	0.48
Z	1.78	1.65	1.67	1.28	1.38	1.12	1.21	1.20	1.47	1.42	1.43
$M_w \times 10^5$	10.74	12.35	12.45	3.50	3.63	2.99	3.26	3.36	5.42	6.22	5.77

using concentrations 10 to 100 times greater than our own, shows that the value of the virial coefficient, B, decreases sharply with increasing ionic strength, μ, and is approximately 4×10^{-5} for $\mu = 2 \times 10^{-2}$ for un-ionised arabic acid in 0.02M hydrochloric acid (the most appropriate parallel available[5] to that studied here). At the highest concentration of whole gum used in the present study, neglect of the term $2BC$ in the full expression[22] (2), where P_θ is the particle scattering factor, is equivalent to an

$$\frac{KC}{R_\theta} = \frac{P_\theta^{-1}}{M_w} + 2BC \tag{2}$$

error of about 4% in $\overline{M}_w$, in the opposite direction to the error in $\overline{M}_w$ arising from the inclusion of scatter by impurities. Whilst it would be of interest to determine the correct value of B, this cannot be done from the results given in Table I because of the unsystematic variation of $\overline{M}_w$ with C. Indeed, it is difficult to estimate the probable correctness of the values of B and $\overline{M}_w$ reported by Veis and Eggenberger[5], as they recorded scatter at only three angles. Their use of more concentrated solutions implies, however, that the contribution from unwanted scatter was quite low.

It is possible to use the molecular weights found for the fractions to derive a "reconstituted weight-average molecular weight", which should be given if all the

fractions were recombined. This test provides a guide to the reliability of the molecular weights relative to one another, and indicates whether significant degradation or aggregation, or loss of material of high or low molecular weight, occurred during fractionation. A value is required, however, for the molecular weight of Fraction 4; this could not be obtained by light scattering on account of the high degree of unwanted scatter. A value of 100,000 was, however, estimated for $\overline{M}_w$ by molecular-sieve chromatography[16,17], the column being calibrated with dextrans of known $\overline{M}_w$. Calculation then gives the reconstituted molecular weight as 7.2×10^5, compared with the value of 5.8×10^5 found for the original whole gum. This indicates that there may have been some loss, during fractionation, of material of low molecular weight; this seems reasonable in view of the extensive dialysis procedures necessary after the sodium sulphate precipitations. Table I shows, however, that the largest experimental uncertainty is associated with Fraction 1; the relatively high proportion of this fraction of high molecular weight means that any error in its $\overline{M}_w$ will contribute significantly to the error in the "reconstituted" $\overline{M}_w$.

Although there is some uncertainty regarding the extent to which the scatter at higher angles represents scatter from the gum alone, it is, nevertheless, worthwhile to obtain an estimate of the dimensions of the gum in solution using the observed scatter-envelope. This necessitates the choice of a model for the dissolved gum (no choice would be required if the observed envelopes arose solely from the gum). We have chosen the "polydisperse* random coil" system, which best fits Veis and Eggenberger's results[5], although it may not be in keeping with recent chemical evidence[1] that the gum is very highly branched.

The particle scattering factor, P_θ, for a system of polydisperse random coils varies linearly with $\sin^2(\theta/2)$. The plots shown in Fig. 2 have been calculated from equation[23] (3) where $x = k^2s^2\bar{r}^2/6$; $k = 2\pi/\lambda'$, (λ' being the wavelength of light in

$$P_\theta = \frac{2}{x^2}\,[e^{-x} - (1-x)] \tag{3}$$

solution); $s = 2\sin(\theta/2)$; $(\bar{r}^2)^{\frac{1}{2}}$ = root-mean-square of the separation of the ends of the coil. The slope of the line relating P_θ, or P_θ^{-1}, to $\sin^2(\theta/2)$ increases with increase in $(\bar{r}^2)^{\frac{1}{2}}$. Since G_θ^{-1}, plotted in Fig. 1, equals $(G_0^{-1})(P_\theta^{-1})$, it follows that polydisperse coils would give a linear plot of G_θ^{-1} *versus* $\sin^2(\theta/2)$, G_0^{-1} being a constant for each solution examined. The straight lines in Fig. 1, each extrapolated from scatter at the larger angles, can thus be used to determine $(\bar{r}^2)^{\frac{1}{2}}$. The most convenient way of doing this is to calculate the dissymmetry ${}_{45}Z_{135}$ (*i.e.*, the ratio of G_{45}/G_{135}) and to read off the corresponding value of $(\bar{r}^2)^{\frac{1}{2}}$ from the theoretical graph of ${}_{45}Z_{135}$ *versus* $(\bar{r}^2)^{\frac{1}{2}}$ (Fig. 3). The values of ${}_{45}Z_{135}$ and the corresponding values of $(\bar{r}^2)^{\frac{1}{2}}$ are shown in Table II. Unlike the values of $\overline{M}_w$, these values of $(\bar{r}^2)^{\frac{1}{2}}$ are unlikely to be in error even if there is a small amount of scatter from impurities.

*The significance of the term "polydisperse" in this context follows the usage of Veis and Eggenberger[5], and differs from the convention used in an earlier part of this series[15].

TABLE II

SUMMARY OF RESULTS OBTAINED BY LIGHT SCATTERING

Fraction Number	$\overline{M}_w \times 10^5$	Z	$(\bar{r}^2)^{\frac{1}{2}}$, Å	$(\bar{r}_g^2)^{\frac{1}{2}}$, Å
1	11.85	1.70	948	387
2	3.56	1.33	633	258
3	3.20	1.18	455	186
4	1.00[a]			
Whole gum	5.8	1.44	739	339

[a]Estimated by molecular-sieve chromatography[17].

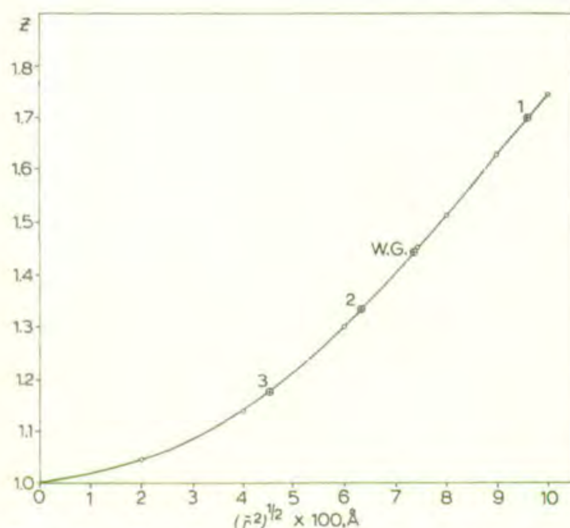

Fig. 3. Plot (○) of Z *versus* $(\bar{r}^2)^{\frac{1}{2}}$ from Fig. 2. Experimental points shown as ⊗ for whole gum (W.G.) and Fractions 1, 2, and 3.

The radius-of-gyration $(\bar{r}_g^2)^{\frac{1}{2}}$, calculated from $(\bar{r}^2)^{\frac{1}{2}}$ by the expression $\bar{r}^2 = 6\bar{r}_g^2$, is included in Table II. These data may be compared with that of Veis and Eggenberger[5], who concluded that for the whole gum in its un-ionised form $\overline{M}_w = 1 \times 10^6$ and that Z (for blue light) was 1.65. These values agree closely with our values for Fraction 1, but there is a discrepancy in the corresponding calculated values of $(\bar{r}^2)^{\frac{1}{2}}$. Veis and Eggenberger[5] apparently used the relation of Z to $(\bar{r}^2)^{\frac{1}{2}}$ for monodisperse coils in this instance [$(\bar{r}^2)^{\frac{1}{2}}$ for monodisperse coils and polydisperse coils = 1035 and 920, respectively, for $Z = 1.65$], although later in their paper the polydisperse coil model was used for ionised arabic acid. Veis and Eggenberger also give much larger values of $(\bar{r}^2)^{\frac{1}{2}}$ for fully ionised arabic acid [$(\bar{r}^2)^{\frac{1}{2}} = 2400$ Å for $Z = 2.7$], but their solutions were of low ionic strength, and it is reasonable to expect the polyelectrolyte to stretch out in these conditions. We deliberately maintained the ionic strength at a high level to avoid a sharp dependence of Z on C at the low concentrations used.

Thus, our results are to be compared with the results for un-ionised arabic acid in dilute hydrochloric acid rather than with the results for the ionised gum[5]. The close agreement of our value of $\overline{M}_w$ for the whole gum with that of Deb and Mukherjee[6] is interesting in view of the solvent system they used.

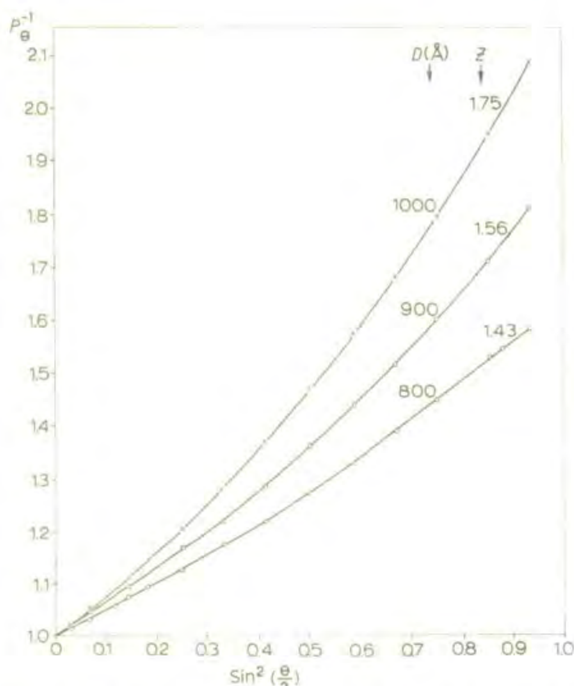

Fig. 4. Plot of P_θ^{-1} *versus* $\sin^2(\theta/2)$ for spherical molecules having diameters D of 800, 900, 1000 Å, and dissymmetries of 1.43, 1.56, and 1.75, respectively.

In view of the recent suggestions that gum arabic molecules are more globular in shape than previously recognised[1], and that they behave as a suspension of non-entangling spheroids in solution[24], it is of interest, finally, to consider the form of the scatter envelope for the highly branched gum if it were to behave as equivalent to a sphere, since this molecular form, like the coil, would impart low viscosity to a solution. The scatter envelope for spheres has a slight upward curvature (see Fig. 4) which has been calculated according to equation[25] (4), where $x = ksD/2$, D being the

$$P_\theta = \frac{3}{x^3}[(\sin x - x \cos x)]^2 \qquad (4)$$

diameter of the sphere. The present results, do not, however, enable a significant distinction to be drawn between the spherical and the polydisperse coil models in view of the very slight curvature for the envelope due to spheres and the uncertainties arising from the difficulty of obtaining ideally clear solutions.

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SUMMARY

Light-scattering studies on the sodium salt of *A. senegal* gum, in molar sodium chloride solutions, give $\overline{M}_w = 580{,}000$. The $\overline{M}_w$ values obtained for three molecular-weight fractions, obtained from the whole gum by sodium sulphate precipitation, confirm previous observations that the gum has a very broad molecular-weight distribution. The results obtained are compared with those of earlier investigators, and reasons for some of the differences are discussed. Studies of the shape of the gum molecules in solution did not distinguish between the spheroidal and the polydisperse coil models.

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43.

STUDIES ON URONIC ACID MATERIALS

PART XIX*. THE COMPOSITION OF THE GUM FROM *Acacia laeta* VAR. *hashab*

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(Received August 17th, 1966; in revised form, November 17th, 1966)

INTRODUCTION

Some samples of gum arabic (*Acacia senegal*) tend to form mucilaginous gels, rather than the desired, viscous, aqueous solutions; this behaviour remains unexplained. The gel formation is, however, a property of only a small proportion of nodules[2,3], with the actual proportion determining the commercial acceptability of a particular sample. *Acacia laeta* (previously referred to, erroneously, as *A. lacta*[4]) differs[2-4] from other *Acacia* species in the extent to which its exudate forms gels at low concentrations. As suggested[4], it is necessary to ascertain how the properties and structure of *A. laeta* differ from those of other *Acacia* exudates[5].

There is a further reason for investigating this species. *Acacia laeta* R. Br. ex Benth. is a natural hybrid of *A. senegal* (L.) Willd. and *A. mellifera* (Vahl.) Benth., and it occurs in two varieties: *A. laeta* var. *hashab* (which resembles *A. senegal*) and *A. laeta* var. *mellifera* (which resembles *A. mellifera*). Intercomparisons of the properties of the exudates from these four species may, in due course, contribute to the present meagre knowledge of the mechanism of gum formation.

This paper deals with the composition of the gum from *A. laeta* var. *hashab*; preliminary experiments[3,6] had indicated a marked, inter-nodule variation, and also some close similarities to *A. senegal* gum. To give the desired basis for comparison, it became important to establish the possible range of variation and the average values for the composition and properties of the gum from *A. laeta* var. *hashab*.

EXPERIMENTAL

Origin of specimens. — Samples of the gum exudate from *Acacia laeta* var. *hashab* were collected by (the late) M. P. Vidal-Hall, formerly Gum Research Officer to the Republic of Sudan, from authenticated, numbered trees in El Ain Forest Reserve, near El Obeid. Twelve samples, each comprising a small number (5–8) of pale-brown nodules exuded from a single tree in response to tapping, were obtained as detailed in Table I.

One of the samples contained an unusually high number (14) of nodules.

*For Part XVIII, see Ref. 1.

TABLE I

ORIGIN OF SPECIMENS

Sample	*Tree number*	*Date of tapping*	*Number of days between tapping and collection*
1	124	6 Nov., 1960	40
2	285	6 Nov., 1960	40
3	251	15 Nov., 1960	40
4	133	15 Nov., 1960	40
5	133	15 Nov., 1960	82
6	251	15 Nov., 1960	82
7	84	13 Nov., 1962	40
8	84	13 Nov., 1962	56
9	84	13 Nov., 1962	71
10	90	13 Nov., 1962	40
11	90	13 Nov., 1962	56
12	90	13 Nov., 1962	71
N1–N6	124	6 Nov., 1960	40

Several of these were crushed to form Sample 1, and six of the nodules (designated Nl–N6) were examined individually after purification; three further nodules (N7–N9) were examined individually in the native form.

Analytical methods. — The general analytical methods and chromatographic systems used have been described[7]. Gas–liquid partition chromatography (g.l.c.) was performed with a chromatograph (Model S3A, Gas Chromatography Ltd.) fitted with flame ionisation detectors. The carrier gas was nitrogen at 100 ml/min. The columns used were: (*A*) 15% by weight of poly(ethylene glycol adipate) on Celite (75 × 0.5 cm) at 160°; (*B*) 15% by weight of poly(butane-1,4-diol succinate) on Celite (120 × 0.5 cm) at 175°. Retention times (*T*) are given relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside.

Purification of samples. — Each gum sample was ground to a coarse powder, and a portion (10 g) was dissolved in water (300 ml). The insoluble residue was removed by filtration, and the solution was dialysed against distilled water (4 l) for 24 h (this dialysate was examined as described below). Purification was then completed by electrodialysis[8]. In the first series of purifications, the solutions were concentrated and freeze-dried. On storage over a period of 3–6 months, these freeze-dried samples gradually became insoluble; this effect was most marked where the samples had been freeze-dried from fairly concentrated solutions. In subsequent purifications, therefore, the electrodialysed solutions were not concentrated before freeze-drying. In confirmation of earlier reports[2–4], solutions for which the concentration exceeded 5% (w/v) were liable, if allowed to stand for several days, to turn into insoluble, mucilaginous gels.

RESULTS

The free sugars in A. laeta *gum.* — The dialysate from the preliminary stage of

purification was concentrated, and examined chromatographically[7] in solvents *B* and *D*. Glucuronic acid, galactose, arabinose, and rhamnose were detected.

Analyses of the crude gum. — Analyses (*cf.* Ref. 5) were made on the crude gum samples. The following values are the average results: moisture, 14.9% (range, 13.2–15.6%); insoluble material, 1.1% (range, 0.53–2.05%); ash, 3.3% (range, 2.8–3.8%); $[\alpha]_D^{18}$ (*c* 2.0, M sodium chloride), $-35°$ (range, $-30°$ to $-42°$); limiting-viscosity number (in M sodium chloride), 21.3 cm^3g^{-1} (range, 19.1–23.4); N, 0.69% (range, 0.48–0.85%); OMe, 0.41% (range, 0.30–0.53%); uronic anhydride (by decarboxylation), 14% (range, 12–16%).

Analyses of electrodialysed samples. — Table II gives the data obtained for the purified, bulk samples from single trees (specimens 1–12), and for the purified, single nodules (specimens Nl-N6, all from tree 124). Average values are also shown for specimens 1–12, for specimens Nl–N6, and for all of the 18 specimens examined.

Viscosity characteristics of the gum. — The loss of solubility that occurred on storage of electrodialysed specimens precluded a reliable comparison of the viscosity of each specimen in the purified form. Fig. 1 gives the viscosity behaviour of the crude specimens dissolved in molar sodium chloride, an electrolyte concentration sufficient to swamp any variations in the viscosity arising from the natural cation content associated with the uronic acid groups in the gum. Checks with two of the samples confirmed earlier indications (*cf.* Ref. 9) that, for comparative purposes, there is little difference in the viscosity behaviour of crude and purified samples of gum, provided that an adequate concentration of salt is present.

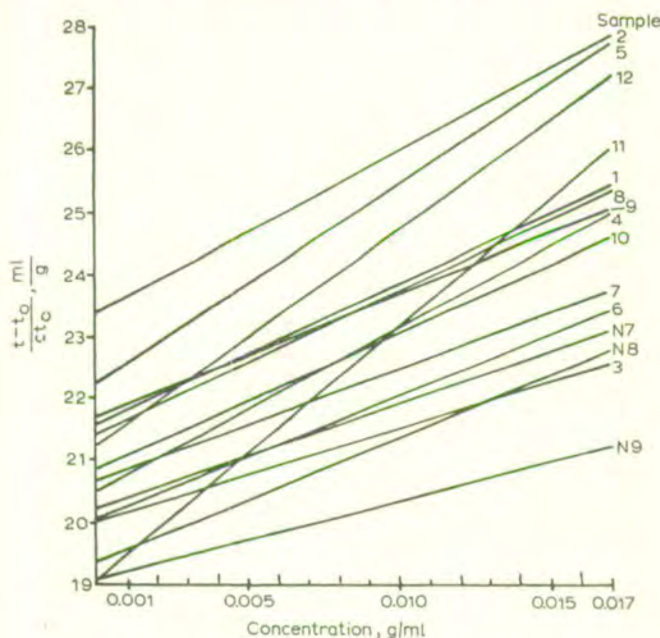

Fig. 1. The viscosity–concentration relationship for different specimens of the gum from *A. laeta* var. *hashab*.

TABLE II

ANALYTICAL DATA[a] FOR PURIFIED SPECIMENS OF GUM FROM ***Acacia laeta*** VAR. ***hashab***

Specimen	*Nitrogen* (%)	*Methoxyl* (%)	*Reducing power*[b]	$[\alpha]_D^{18}$ (c 1.0, *water*)	*Neutralisation equivalent*[c]	*Uronic anhydride*[d], %	*Molar ratios of neutral sugars* *Gal*	*Ara*	*Rha*
1	0.56	0.52	0.72	−32	1240	14.2	10	9.0	2.9
2	0.61	0.34	0.67	−36	1430	12.2	10	8.4	3.7
3	0.74	0.37	0.71	−30	1400	12.6	10	9.1	3.1
4	0.60	0.32	0.70	−39	1180	14.9	10	9.8	3.9
5	0.78	0.56	0.63	−41	1260	14.0	10	10.1	3.2
6	0.79	0.27	0.80	−36	1090	16.1	10	8.3	3.2
7	0.65	0.34	0.71	−42	1250	14.0	10	6.7	3.0
8	0.79	0.36	0.65	−31	1260	14.0	10	7.4	3.3
9	0.94	0.42	0.64	−41	1440	12.2	10	7.9	3.4
10	0.59	0.37	0.86	−29	1300	13.4	10	7.6	3.8
11	0.80	0.39	0.88	−32	1580	11.1	10	7.7	4.1
12	0.93	0.39	0.87	−30	1440	12.2	10	8.6	3.3
N1	0.54	0.52	0.69	−35	1210	14.5	10	8.4	2.5
N2	0.55	0.49	0.73	−32	1240	14.3	10	8.5	2.5
N3	0.47	0.54	0.71	−37	1230	14.3	10	8.3	2.3
N4	0.49	0.46	0.73	−39	1250	14.0	10	8.8	2.5
N5	0.56	0.50	0.70	−35	1320	13.4	10	8.5	2.6
N6	0.49	0.53	0.71	−34	1270	13.9	10	8.1	2.6
Av. 1–18	0.66	0.43	0.73	−35	1300	13.6	10	8.4	3.1
Av. 1–12	0.73	0.38	0.74	−35	1320	13.4	10	8.4	3.4
Av. N1–N6	0.52	0.51	0.71	−35	1250	14.1	10	8.4	2·5

[a] All data are corrected to an ash-free, dry-weight basis. [b] Expressed as apparent % of free pentose. [c] By direct titration with alkali to phenolphthalein end-point; potentiometric titration gave results in close agreement. [d] Calculated from neutralisation equivalent, assuming that all acidity arises from the uronic acid groups.

The sugar components of the gum (from complete hydrolysis). — Electrodialysed gum (sample 3, 5 g) was dissolved in sulphuric acid (2N, 500 ml) and heated under reflux for 12 h. The hydrolysate was cooled, neutralised (barium hydroxide and barium carbonate), centrifuged, and then treated with Amberlite IR-120 (H^+) resin. The product was fractionated on a column of Duolite A4 resin (20 × 2 cm) in the formate form. The neutral fraction was eluted with water (2 l); the acidic fraction was eluted with formic acid [5% (v/v), 2 l]. After evaporation, the neutral fraction gave a syrup (3.3 g); paper chromatography (solvent *B*) indicated galactose, arabinose (R_{Gal} 1.34), rhamnose (R_{Gal} 2.11) and, between the arabinose and rhamnose, a diffuse trace of material giving a pink colour with aniline hydrogen oxalate. The acidic fraction was taken to dryness several times from aqueous solution to eliminate formic acid; paper chromatography (solvent *D*) indicated the presence of D-glucuronic acid (R_{Gal} 1.18), D-glucurono-6,3-lactone (R_{Gal} 3.1), and 4-*O*-methyl-D-glucuronic acid (R_{Gal} 2.62).

The neutral fraction was chromatographed on a cellulose column (60 × 3 cm) with solvent *B*. Fractions (20 ml) were collected; every fifth fraction was concentrated and examined chromatographically (solvent *B*). The appropriate fractions were combined, and concentrated; the respective syrups of D-galactose, L-arabinose, and L-rhamnose crystallised on standing. After recrystallisation from water, the identities of the sugars were confirmed as follows:

Fraction (*a*): D-galactose monohydrate (900 mg); m.p. and mixed m.p. 118–119°; $[\alpha]_D^{18}$ +80° (equilibrium, *c* 4.0, water). The sugar was characterised by oxidation to galactaric acid, m.p. 213–214° (lit.[10], 213°).

Fraction (*b*): L-arabinose (1040 mg); m.p. and mixed m.p. 159–160°; $[\alpha]_D^{18}$ +105° (equilibrium, *c* 3.0, water); R_{Gal} 1.36 (solvent *B*). The X-ray diffraction pattern was identical to that of an authentic sample of L-arabinose. The phenylosazone had m.p. 164–165° (lit.[10], 165°).

Fraction (*c*): L-rhamnose monohydrate (275 mg); m.p. and mixed m.p. 93–94°; $[\alpha]_D^{18}$ +9° (equilibrium, *c* 3.0, water); R_{Gal} 2.10 (solvent *B*). The X-ray diffraction pattern was identical to that of an authentic sample of L-rhamnose. The phenylosazone had m.p. 181–182° (lit.[10], 182°).

Partial acid hydrolyses: trace neutral sugars and aldobiouronic acids. — After trial partial hydrolyses with acid and with an acidic resin, the gum (sample 7, 30 g) was hydrolysed successively at 100° with sulphuric acid under the following, graded conditions: (*a*) 0.01N, 500 ml, 60 h; (*b*) 0.2N, 500 ml, 4 h; (*c*) 0.5N, 250 ml, 2 h; (*d*) 2N, 250 ml, 1 h; (*e*) 2N, 250 ml, 4 h; (*f*) 2N, 250 ml, 2 h. At each stage after (*a*), the hydrolysate was neutralised (barium carbonate), centrifuged, de-ionised (IR-120 resin), evaporated, and added to ethanol. The degraded gum was removed by centrifugation, and the centrifugate was evaporated to a syrup, which was separated into acidic and neutral fractions on a column (15 × 2.5 cm) of DEAE-Sephadex G-25 (formate form). The neutral fraction was eluted with water (1.5 l), and the acidic fraction with formic acid (3% v/v, 1.5 l).

When the sequence of graded hydrolyses was complete, the neutral fractions

obtained from each stage were combined, treated with charcoal, and evaporated to a syrup (2.6 g). Similarly, the acidic fractions were combined, evaporated to dryness several times to eliminate formic acid, treated with charcoal, and evaporated to a syrup (310 mg). A subsequent Paper will discuss the detailed examination of the degraded gums and of the oligosaccharides in the combined, neutral fractions. In this Paper, we report on the trace monosaccharides in the combined, neutral fractions, and on the identification of the aldobiouronic acids in the combined, acidic fractions.

The trace, neutral sugars travelled as a single band between arabinose and rhamnose (solvent *B*). When the band was re-chromatographed on Whatman No. 1 paper in solvent *C*, two fractions were obtained. Fraction *(a)* (6 mg) had R_{Gal} 1.28 (solvent *D*) and R_{Gal} 1.95 (solvent *C*); it was identical with xylose in both these solvents. After conversion into the methyl glycosides, and methylation[11], the product was examined by g.l.c. and by paper chromatography, together with the product obtained by similar treatment of authentic xylose. In each case, two major components were detected *T* 0.41, 0.51 (column *A*); *T* 0.49, 0.60 (column *B*)], and, after hydrolysis, both products gave methylated, reducing sugars having R_G 0.83, 0.92, and 0.49 (minor component) in solvent *A*. This fraction was therefore tentatively identified as xylose. Fraction *(b)* (3.1 mg) had R_{Gal} 1.52 (solvent *D*) and R_{Gal} 2.32 (solvent *C*); it was identical with ribose in both these solvents, and also in the comparative tests outlined above for fraction *(a)*.

Identification of the aldobiouronic acids. — The combined acidic fraction from the partial, acid hydrolysates was resolved into components *A* and *B* by chromatography on 3 MM paper in solvent *B*.

Component A: (189.5 mg) had R_{Gal} 0.20 (solvent *D*); $[\alpha]_D^{18}$ 0° (*c* 3.8, water); (lit.[7,12], − 5°; + 1.7°, − 3°, − 7.8°). The aldobiouronic acid was isolated as a chromatographically pure syrup. Hydrolysis gave only galactose and glucuronic acid. Reduction of the methyl ester methyl glycosides with potassium borohydride, followed by hydrolysis, gave only galactose and glucose; the glucose was isolated by chromatography on 3MM paper, (solvent *B*), and had $[\alpha]_D^{18}$ + 50° (equilibrium, *c* 0.25, water); the phenylosazone had m.p. 204–205° (lit.[10], 205°). The methanolysis product of the fully methylated aldobiouronic acid was examined by g.l.c. and found to contain the methyl glycosides of 2,3,4-tri-*O*-methyl-D-glucuronic acid [*T* 2.14, 2.66 (column *A*); *T* 2.41, 3.08 (column *B*)]; 2,3,4-tri-*O*-methyl-D-galactose [*T* 5.36 (column *A*); *T* 6.44 (column *B*)]; 2,3,5-tri-*O*-methyl-D-galactose (trace only) [*T* 3.27, 4.32 (column *A*); *T* 4.02, 5.37 (column *B*)]. Reduction of the methyl ester methyl glycosides (lithium aluminium hydride) gave a product, which, after hydrolysis, was examined in solvent *A*; components identical with 2,3,4-tri-*O*-methyl-D-glucose (R_{Gal} 0.86) and 2,3,4-tri-*O*-methyl-D-galactose (R_{Gal} 0.70) were revealed. Component *A* was thereby identified as 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose.

Component B: (75 mg) had R_{Gal} 0.68 (solvent *D*); $[\alpha]_D^{18}$ + 4° (*c* 1, water) [lit., − 1°, + 6° (ref. 12); + 6° (ref. 7)]. The aldobiouronic acid was isolated as a chromatographically pure syrup. Hydrolysis gave only galactose and 4-*O*-methylglucuronic acid [(R_{Gal} 2.64 (solvent *D*)]. Methanolysis of the methylated[11] aldobiouronic acid gave the same

components as identified for component *A*. Methanolysis of the aldobiouronic acid, followed by potassium borohydride reduction and subsequent hydrolysis, gave only galactose and 4-*O*-methylglucose (solvent *D*). The 4-*O*-methylglucose was isolated by chromatography on 3 MM paper, and had $[\alpha]_D^{18}$ +55°; R_{Gal} 1.62 (solvent *D*), 2.63 (solvent *A*), and 2.23 (solvent *C*). The phenylosazone had m.p. 157° (lit.[13], 158–159°). Component *B* was therefore identified as 6-*O*-(4-*O*-methyl-β-D-glucopyranosyl-uronic acid)-D-galactose.

DISCUSSION

The exudate from *A. laeta* var. *hashab* gives a further interesting illustration of the occurrence of inter-nodule variation; by calculation, the data obtained give average values for the parameters expressing the composition and properties of the acidic gum polysaccharide. The variations from the average values for this species are similar in extent to those found for *A. seyal*[9], and more marked than those for *A. nilotica*[7].

Of the *Acacia* species studied to date, *A. laeta* and *A. seyal* are unusual in occurring in varieties distinguishable by their external, botanical characteristics. It will be of interest to discover the differences that occur in the constitution and properties of the gums exuded by these varieties. In *A. laeta*, for example, the assignment of a particular tree to the *hashab* variety or to the *mellifera* variety may be a botanical oversimplification; a range of variation between two extreme forms of the hybrid may exist. Studies of the exudates from *A. mellifera* and *A. laeta* var. *mellifera* are therefore necessary.

The specimens available gave the opportunity to ascertain that the variation between six, single nodules from one tree (samples Nl–N6) is considerably less than the variation between "bulk" samples (*i.e.*, a mixture of a few nodules) from different, individual trees (samples 1–12). The same conclusion was reached[14] in similar experiments with single nodules of *A. senegal* gum. Although the only consistent variation in the present results involves an increase in the nitrogen content of the nodules with their period of formation relative to the date of tapping, the danger of drawing premature conclusions from the data must be avoided: the work involved in examining a statistically useful number of specimens appears at present to be prohibitive. The present results are sufficient, however, to indicate the direction that further experiments should pursue.

The variation in the limiting-viscosity numbers for the specimens (Fig. 1) suggests that significant variations in the molecular size of the gum may exist from sample to sample. The significance of the variation in slope of the viscosity plots (Fig. 1) is not clear; similar variations were reported[9] for *A. seyal* gum. The possibility that these variations in slope reflect changes in the typical molecular shape, or in molecular composition, will be investigated. The extent of the inter-nodule variations further suggests that attempts to effect fractionation (*cf.* Ref. 15) must be made prior to general structural studies, and studies of fine structure involving consecutive Smith degradations[16].

The analytical data presented do not indicate any unusual features for an *Acacia* species now that the presence of methoxyl groups has become expected[17]. Of the other *Acacia* species examined to date[5,7], *A. senegal*[5,16] most closely resembles *A. laeta* var. *hashab*. The trace components of the partial acid hydrolysates, identified as xylose and ribose, may have been artefacts formed during the hydrolysis procedure. If these sugars do exist in the gum, they are present in such small proportions that they are unlikely to have structural significance. Previous workers have reported traces of xylose in *A. sundra*[18], of xylose and fucose in *A. sieberiana*[19], and of ribose in *A. seyal*[9].

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SUMMARY

Inter-nodule variations in the composition and properties of the gum from *Acacia laeta* var. *hashab* have been investigated. Six nodules from one tree showed less variation than samples from other trees. Average values for the analytical parameters expressing the composition of the gum have been calculated from analytical data for eighteen samples. Of the other *Acacia* gums investigated to date, *A. senegal* is the most closely similar in composition.

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4.4.

STUDIES ON URONIC ACID MATERIALS

PART XX*. THE VISCOSITY–MOLECULAR WEIGHT RELATIONSHIP FOR *Acacia* GUMS

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INTRODUCTION

Since the classical investigations of Kern[2], there have been significant advances in the understanding of polyelectrolyte behaviour, particularly of those synthetic polymers in which structure, and distributions of molecular weight can be controlled. Corresponding advances have not been made with natural polymers, such as plant gums. In particular, studies of molecular weight in the genus *Acacia* appear to have been confined to *A. cyanophylla*[3] gum and commercial samples of *A. senegal* gum[4].

The observation[5] that molecular weight fractions can be obtained from *A. senegal* gum by fractional precipitation with sodium sulphate led to a light-scattering study[4] of a specimen of that gum and three fractions obtained from it. The viscosity characteristics of these four materials have now been studied, and, in consequence, the constants involved in the Mark–Houwink[6] modification of Staudinger's viscosity–molecular weight relationship can be calculated.

In view of the indication[7] that the exudates from some *Acacia* species differ extensively from *A. senegal* gum in composition and properties, it was necessary to test whether the Staudinger constants for *A. senegal* gum would be valid for other *Acacia* species.

Weight-average molecular weights for specimens of *A. arabica* gum and *A. nubica* gum have therefore been determined by light-scattering. These particular species were selected as test cases for the following reasons: (*a*) their limiting-viscosity numbers are[7,8] lower than the values typical of *A. senegal* gum; (*b*) preliminary experiments[8] with molecular-sieve chromatography[9,10] indicate that *A. arabica* gum has a higher molecular weight than *A. senegal* gum; (*c*) structural studies of the gums from *A. arabica*[8] and *A. nubica*[11] indicate significant differences in structure from that of *A. senegal* gum.

EXPERIMENTAL AND RESULTS

The origin of the gum specimens from *A. arabica* and *A. nubica* have been given[7], and the fractionation procedure[5] with sodium sulphate has been described. Light-

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scattering data have been published[4] for the specimen of *A. senegal* gum, and for the particular set of fractions used in the present study. The gum, and its fractions, were in the fully neutralised, sodium salt, form.

Viscosity measurements. — These were made at 25.00 ± 0.01° in suspended-level, Ubbelohde, dilution viscometers having solvent flow-times greater than 170 sec, and negligible, kinetic energy correction. The data obtained for *A. senegal* gum and its fractions, as sodium salts in M sodium chloride, are given in Table I; preliminary

TABLE I

VISCOSITY DATA FOR *A. senegal* GUM AND ITS FRACTIONS IN MOLAR SODIUM CHLORIDE SOLUTION AT 25.00°

Fraction 1		*Fraction 2*		*Fraction 3*		*Whole gum*	
Conc × 10^3 (*g/ml*)	$\frac{\eta Sp}{c}$ (*ml/g*)	*Conc* × 10^3 (*g/ml*)	$\frac{\eta Sp}{c}$ (*ml/g*)	*Conc* × 10^3 (*g/ml*)	$\frac{\eta Sp}{c}$ (*ml/g*)	*Conc* × 10^3 (*g/ml*)	$\frac{\eta Sp}{c}$ (*ml/g*)
17.83	38.4	19.63	14.6	18.81	14.0	19.99	20.5
12.74	31.9	14.02	13.8	13.45	13.5	14.29	19.4
8.91	30.3	9.82	13.1	9.41	13.3	10.00	18.5
5.94	28.3			6.27	13.1	6.67	17.7
By graphical extrapolation:							
lim ($c \rightarrow 0$)	25.4	lim ($c \rightarrow 0$)	12.9	lim ($c \rightarrow 0$)	12.5	lim ($c \rightarrow 0$)	16.4

experiments[12] had shown that the limiting-viscosity number was constant for concentrations of sodium chloride ranging from 2–8% w/v. Independent investigations, under comparable conditions to those described above, established limiting-viscosity numbers (ml/g) of 9.8 for *A. nubica*[7] gum, and 12.5 (ref. 7) and 9.9 (ref. 8) for different batches of the *A. arabica* gum (a large, bulk sample from many trees).

Calculation of the Staudinger constants for A. senegal *gum.* — The values of K' and a (following Flory's symbols[13]) in the Mark–Houwink[6] equation,

$$[\eta] = K' M_w^a \tag{1}$$

were found from the plot of log $[\eta]$ *versus* log M_w for each fraction and for the whole gum (Fig. 1). The values deduced are $K' = 1.3 \times 10^{-2}$ and $a = 0.54$.

The experimental data were also used to obtain the additional parameters given by the Flory–Fox[14] equation (2), where K and the molecular expansion factor (α)

$$[\eta] = K M_w^{\frac{1}{2}} \alpha^3 \tag{2}$$

$$K = \phi \left[\frac{(\bar{r}_o)^2}{M_w} \right]^{1.5} \tag{3}$$

$$\alpha = \frac{(\bar{r}^2)^{\frac{1}{2}}}{[(\bar{r}_o)^2]^{\frac{1}{2}}} \tag{4}$$

are given by equations 3 and 4. For equation 4, $(\bar{r}^2)^{\frac{1}{2}}$ is the root-mean-square, end-to-end distance of the molecule, and $[(\bar{r}_o)^2]^{\frac{1}{2}}$ its unperturbed dimension; ϕ is a universal constant, equal to 2.1×10^{23} when $[\eta]$ is expressed in ml/g. The expressions of Kurata

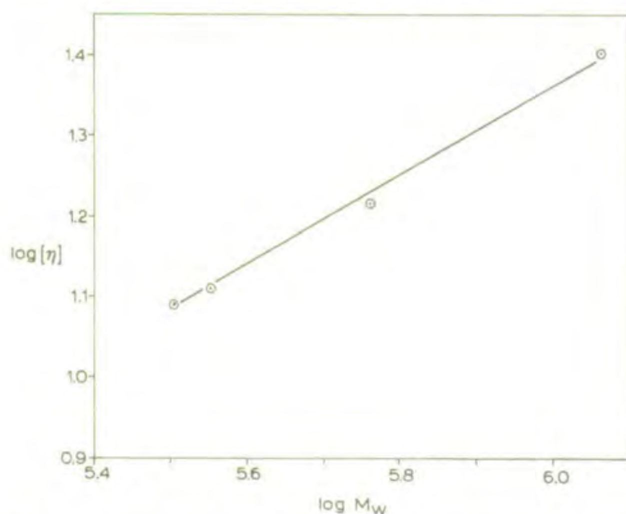

Fig. 1. Log $[\eta]$ *versus* log M_w for *Acacia senegal* gum and its fractions (in the sodium salt form).

and Stockmayer[15], and Stockmayer and Fixman[16], provide a graphical method of evaluating K from equation 5.

$$[\eta] = KM_w^{\frac{1}{2}} + 0.51\ \phi\ BM \tag{5}$$

By plotting $[\eta]M_w^{-\frac{1}{2}}$ *versus* $M_w^{\frac{1}{2}}$, a value of 1.96×10^{-2} was found for K by extrapolation, and substitution in equation 2 gave $\alpha = 1.042$. Values for $[(\bar{r}_o)^2]^{\frac{1}{2}}$ were then calculated from equation (3), and the theoretical values for $(\bar{r}^2)^{\frac{1}{2}}$ from equation (4) are compared in Table II with those determined experimentally.

TABLE II

M_w, $[\eta]$, $[(\bar{r}_o)^2]^{\frac{1}{2}}$, $(\bar{r}^2)^{\frac{1}{2}}$ FOR GUM FRACTIONS FROM *Acacia senegal*, AS THE SODIUM SALTS IN MOLAR SODIUM CHLORIDE

		Fractions			*Whole Gum*
		1	*2*	*3*	
$M_w \times 10^{-5}$		11.85	3.56	3.20	5.80
$[\eta]$ (ml/g)		25.4	12.9	12.5	16.4
$[(\bar{r}_o)^2]^{\frac{1}{2}}$ (Å),	theory[a]	494	271	257	345
$(\bar{r}^2)^{\frac{1}{2}}$ (Å),	theory[b]	514	282	267	360
	experimental	930	635	460	745
$\phi \times 10^{-23}$	theory	2.1	2.1	2.1	2.1
	experimental[c]	0.34	0.18	0.41	0.23

[a]Calculated from equation 3; [b]calculated from equation 4; [c]deduced from the experimental value for $(\bar{r}^2)^{\frac{1}{2}}$ by means of equations 2, 3, and 4.

Light-scattering measurements. — These were made (through the courtesy of Dr. G. Stainsby) in the Procter Department, Leeds University. The photometer, and the experimental procedure used to determine the values of M_w for *A. senegal* gum and its fractions, have been described[4]. The same experimental conditions were used to determine molecular weights for the gums from *A. arabica* and *A. nubica* in the form of their sodium salts in M sodium chloride. The plots of the reciprocal, reduced intensity-of-scatter (G_θ^{-1}) *versus* $\sin^2(\theta/_2)$ for *A. arabica* gum are given in Fig. 2; Fig. 3 gives the corresponding data for *A. nubica* gum. Table III compares the data derived from these plots with those obtained previously[4] for *A. senegal* gum.

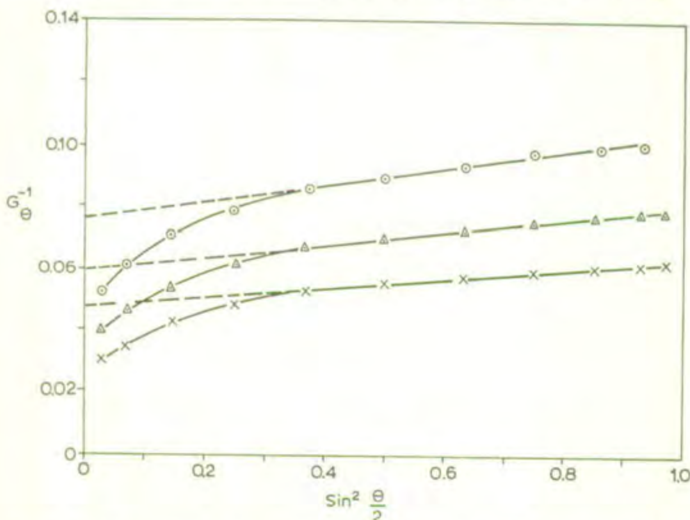

Fig. 2. The reciprocal, reduced intensity-of-scatter (G_θ^{-1}) *versus* $\sin^2(\theta/_2)$ for *A. arabica* gum as the sodium salt in M sodium chloride.

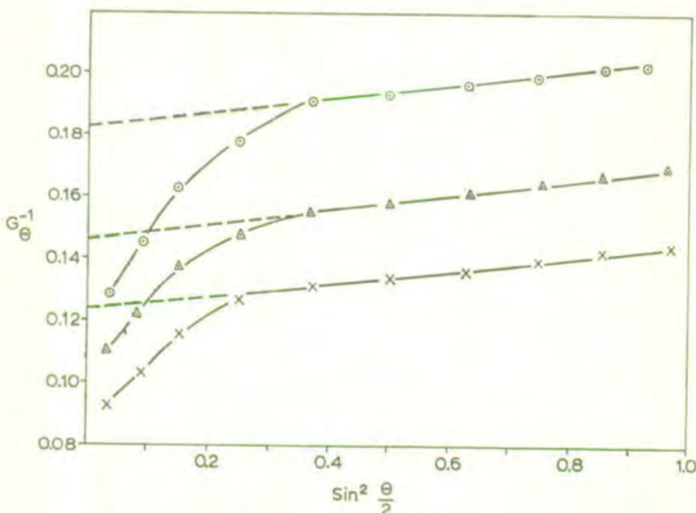

Fig. 3. The reciprocal, reduced intensity-of-scatter (G_θ^{-1}) *versus* $\sin^2(\theta/_2)$ for *A. nubica* gum as the sodium salt in M sodium chloride.

DISCUSSION

The data obtained for *A. senegal* gum obey the Mark–Houwink relationship, and the value obtained for the modified Staudinger constant a falls within the usual range[13]. Although a linear plot of log $[\eta]$ *versus* log M_w (Fig. 1) was obtained from the four points available, two of these points are not widely separated. In any fractional

TABLE III

DATA FOR GUMS FROM *A. senegal*, *A. arabica*, AND *A. nubica*, AS SODIUM SALTS IN MOLAR SODIUM CHLORIDE

Acacia senegal			Acacia arabica			Acacia nubica		
Conc $\times 10^4$ *g/ml*	*Z*	$M_w \times 10^{-5}$	*Conc* $\times 10^4$ *g/ml*	*Z*	$M_w \times 10^{-5}$	*Conc* $\times 10^4$ *g/ml*	*Z*	$M_w \times 10^{-5}$
8.39	1.47	5.42	11.0	1.21	18.6	9.5	1.12	8.42
6.29	1.41	6.22	8.8	1.23	19.2	7.6	1.12	8.87
4.72	1.43	5.77	7.0	1.25	18.3	6.1	1.10	8.85
Mean Values:								
Z	1.44			1.23			1.11	
$M_w \times 10^{-5}$	5.80			18.9			8.71	
$(\overline{r^2})^{\frac{1}{2}}$, Å	739			550			340	
$[\eta]$, ml/g	16.4			9.9 (ref. 8) 12.5 (ref. 7)			9.8 (ref. 7)	

precipitation procedure, the number of fractions isolated is arbitrary[5], and it is intended to repeat the fractional precipitation procedure to obtain, for the present specimen of gum, an increased number of fractions separated as widely as possible within the molecular weight range. Although analyses have established[17] that the sample used in the present study is not atypical in terms of empirical composition, *A. senegal* is known[18] to be very variable botanically, and the values obtained for K' and a may not hold accurately for all specimens of *A. senegal* gum. Fractionation experiments with other specimens of the gum are therefore also necessary.

Further factors also require consideration. The equations relating M_w to $[\eta]$ presume that the different fractions involved are identical chemically, and differ only in molecular size. The composition of three fractions isolated from *A. senegal* gum in a separate experiment has been investigated[5]. Although small variations in chemical composition were detectable, particularly for the ratio of galactose to arabinose, the periodate oxidation data indicated that there are unlikely to be extensive differences in the general pattern of branching. In the fractionations of *A. senegal* gum that have been effected with sodium sulphate, a correlation has been noted[5,12] between the nitrogen content and the limiting-viscosity number of each fraction. The nitrogen content is known to arise from proteinaceous material. Although aggregation of the polysaccharide by the protein appears to be unlikely during viscosity determinations when M sodium chloride is used as solvent[5], further examination of the effect is desirable.

Caution must also be exercised in the application of these constants to degraded forms of the gum. It is clearly established (*cf.* refs. 19, 20, 5, and references cited therein) that the extent of the degradation that occurs under mild conditions, such as autohydrolysis, is much greater than would be expected to result from the removal of the available, labile residues at the periphery of what is now believed to be a relatively highly branched molecule[21]. Some deep-seated form of decomposition affecting the branched "core" (a term preferable[5] to "main chain") of the polymer must accompany modifications at its periphery. The values of K' and a for the whole gum may, therefore, not be valid for any of its degraded forms. Studies of the changes in size and shape of gum molecules during degradative processes are necessary, and molecular-sieve chromatography[5,9,10] may facilitate these.

The experimental values for $(\bar{r}^2)$ and ϕ differ markedly from the theoretical values (Table II) obtained from relationships developed for essentially linear macromolecules. These differences support the view that the polysaccharide entities in *A. senegal* gum are more highly branched[21] than previously believed. Veis and Eggenberger[22] also obtained higher experimental values for $(\bar{r}^2)^{\frac{1}{2}}$ than were predicted from theoretical relationships.

In conclusion, the data in Table III indicate that values of the modified Staudinger constants for *A. senegal* will not generally be applicable to other *Acacia* species: although less viscous, the gums from *A. nubica* and *A. arabica* are of higher molecular weight than *A. senegal*. These observations are supported by the light-scattering data for Z and $(\bar{r}^2)^{\frac{1}{2}}$, which indicate that the gum molecules in *A. nubica* and *A. arabica* are more compact and more highly branched (therefore tending to be even more spherical) than in *A. senegal*. These results are further supported by the results of chromatographic experiments with molecular sieves, and structural studies, currently in progress[8].

In Figs. 2 and 3, linear extrapolations have been used, consistent with the treatment previously adopted[4] (in preference to that of Benoit, Holtzer, and Doty[23]) for the corresponding data for *A. senegal* gum.

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SUMMARY

The relationship between the viscosity and molecular weight of a sample of *Acacia senegal* gum has been investigated, and values for the modified Staudinger constants have been calculated. Reasons for exercising caution in the use of these

constants are discussed. Determinations of the molecular weight of the gums from *Acacia nubica* and *Acacia arabica* have shown that the Staudinger constants for *Acacia senegal* gum will not be of general applicability within the *Acacia* genus.

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Studies on Uronic Acid Materials. Part XXI.[1] Some Structural Features of *Acacia arabica* Gum

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Studies on Uronic Acid Materials. Part XXI.[1] Some Structural Features of *Acacia arabica* Gum

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The polysaccharide exuded by *Acacia arabica* trees has a high positive specific rotation and contains residues of D-galactose, L-arabinose, L-rhamnose, D-glucuronic acid, and 4-*O*-methyl-D-glucuronic acid. Linkage analysis affords 3-*O*-β-L-arabinopyranosyl-L-arabinose and 3-*O*-β-L-arabinofuranosyl-L-arabinose. An examination of the *O*-methyl derivative of the gum yields 2,3,5- and 2,3,4-tri-, and 2,5-, 3,5-, and 3,4-di-*O*-methyl-L-arabinose, 2,4-di-*O*-methyl-D-galactose, and 2,3,4-tri-*O*-methyl-D-glucuronic acid together with small amounts of 2,3,4-tri-*O*-methyl-L-rhamnose, 4-*O*-methyl-L-arabinose, and 2,3,4,6-tetra-, 2,3,6-, 2,4,6-, and 2,3,4-tri-, and 2-*O*-methyl-D-galactose. The degraded gum obtained after controlled acid hydrolysis is examined by linkage and methylation analysis. Partial acid hydrolysis affords 3-*O*-β-D-galactopyranosyl-D-galactose and 6-*O*-β-D-galactopyranosyl-D-galactose. An examination of the *O*-methyl derivative of the degraded gum yields 2,3,4,6-tetra-, 2,3,6-, 2,4,6-, and 2,3,4-tri-, 2,6- and 2,4-di-, and 2-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-glucuronic acid, and trace amounts of 2,3,4-tri-*O*-methyl-L-arabinose. The degraded gum is submitted to a Smith degradation and the whole gum to four successive Smith degradations. The products are examined by linkage and methylation analysis. The structural evidence suggests that *A. arabica* gum molecules possess highly branched galactan frameworks to which are attached uronic acid residues and arabinose-containing side-chains. This conclusion is supported by the results of viscosity and light-scattering measurements carried out on aqueous solutions of the gum.

THE results of a re-investigation[2,3] of *Acacia senegal* gum have led to the development of earlier ideas concerning its molecular structure to include a structural type in which the galactan framework is not necessarily based on a linear "main chain" or "backbone" of β1,3-linked D-galactose residues. This conclusion has prompted further investigations into molecular structure within the *Acacia* group of plant gums. The results of a detailed examination of *Acacia arabica* gum are reported in this Paper, and the main structural features of this gum are compared with those of *A. senegal* gum.[2-4] Preliminary investigations[5,6] on *A. arabica* gum showed that it was composed of galactose, arabinose, uronic acid, and a trace of rhamnose. The value obtained[5] for the limiting viscosity number was lower than that found[4,5] for *A. senegal* gum. The gum has also been shown to have a high positive specific rotation[5] and a high methoxy-content.[6,7] The presence of uronic acid units linked α1,4 to galactose residues has been indicated[8] and chromatographic evidence for the presence of the four aldobiouronic acids, 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose, 6-*O*-(4-*O*-methyl-β-D-glucuronpyranosyluronic acid)-D-galactose, 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose, and 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose has been reported.[9]

The gum nodules from *A. arabica* (Lam.) *Willd.* used in the present investigation were collected by (the late) Mr. M. P. Vidal-Hall, formerly Gum Research Officer, Republic of the Sudan, from small trees at Baruki Rahad, Kordofan Province, in March 1961. After purification, the gum was shown to be an acidic polysaccharide containing residues of D-galactose (32%), L-arabinose (57%), L-rhamnose (0·4%), D-glucuronic acid (4%), and 4-*O*-methyl-D-glucuronic acid (6%). Although it proved impracticable to isolate sufficient quantities of rhamnose to enable a value for its specific rotation to be determined, the sugar was assumed to be present in the L-form because of its occurrence in this series in other *Acacia* gums.[10] The low rhamnose content of *A. arabica* gum and the high positive value of +112° for its specific rotation are not now considered to be atypical features within the *Acacia* group of plant gums.[5]

Samples of *A. arabica* gum were examined by zone electrophoresis on glass-fibre paper,[11] filter paper,[3,12] and cellulose acetate film,[3,12] and by ion-exchange chromatography on DEAE-cellulose.[13] Since no sharp discontinuities in the properties of the molecular species were indicated by these experiments, it seems probable that *A. arabica* gum exhibits the same kind of heterogeneity as *A. senegal* gum.[2,4] If this is the case, then *A. arabica* gum may be considered to contain a continuous spectrum of related molecular species.[14] Despite the fact that solutions of *A. arabica* gum have a lower limiting viscosity number ($[\eta] = 9·9$ ml./g.) than *A. senegal* gum solutions[3,4] ($[\eta] = 20·0$ ml./g.), the gum

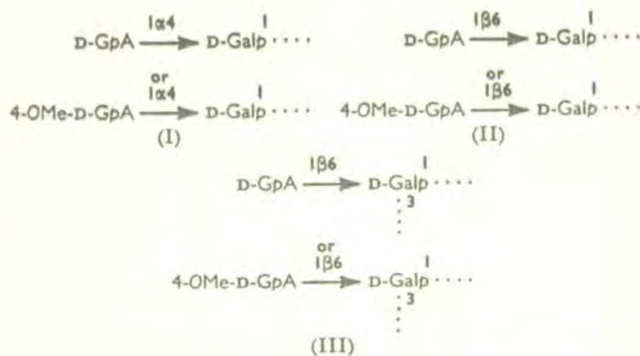
[1] Part XX, D. M. W. Anderson and S. Rahman, *Carbohydrate Res.*, 1967, **4**, 298.
[2] D. M. W. Anderson, Sir Edmund Hirst, and J. F. Stoddart, *J. Chem. Soc. (C)*, 1966, 1959.
[3] J. F. Stoddart, Ph.D. Thesis, 1966, University of Edinburgh.
[4] D. M. W. Anderson and J. F. Stoddart, *Carbohydrate Res.*, 1966, **2**, 104.
[5] D. M. W. Anderson and K. A. Karamalla, *J. Chem. Soc. (C)*, 1966, 762.
[6] D. M. W. Anderson and J. F. Stoddart, "Proceedings of the SAC Symposium at Nottingham," ed. P. W. Shallis, Heffer, Cambridge, 1965, 232.
[7] D. M. W. Anderson, G. M. Cree, M. Herbich, K. A. Karamalla, and J. F. Stoddart, *Talanta*, 1964, **11**, 1559.
[8] A. M. Stephen and E. A. C. L. E. Schelpe, *S. African Ind. Chemist*, 1964, **18**, 12.
[9] G. M. Cree, Ph.D. Thesis, 1966, University of Edinburgh.
[10] F. Smith and R. Montgomery, "The Chemistry of Plant Gums and Mucilages," Reinhold, New York, 1959.
[11] B. A. Lewis and F. Smith, *J. Amer. Chem. Soc.*, 1957, **79**, 3929.
[12] D. M. W. Anderson, A. C. Munro, and J. F. Stoddart, unpublished results.
[13] M. A. Jermyn, *Austral. J. Biol. Sci.*, 1962, **15**, 789.
[14] E. L. Hirst, "Plant Gums," 4th International Congress of Biochemistry, Vienna, 1958; *Proc. Roy. Soc.*, 1959, *A*, **252**, 287; *Biochem. J.*, 1961, **79**, 16P.

from *A. arabica* has a higher weight-average molecular-weight ($\bar{M}_w = 2{\cdot}3 \times 10^6$) than *A. senegal* gum [3] ($\bar{M}_w =$ 600,000). A comparison of the physicochemical properties of *A. arabica* gum solutions with those of *A. senegal* gum solutions suggests that there are important differences in the molecular architecture of the two gums in addition to fine structural variations, which are predictable from a knowledge of the differences in chemical composition of the two gums.

Hydrolysis of the gum under controlled conditions with 0·01N-sulphuric acid resulted in the release of almost all the arabinose together with small amounts of galactose. Degraded gum A, isolated after dialysis, contained galactose (86%), arabinose (2%), and uronic acid (11%). The value of +33° for the specific rotation of degraded gum A is somewhat different from the value of −11° obtained [4] for autohydrolysed *A. senegal* gum. The differences may be partly explained by the configurations of the linkages involving uronic acid residues to galactose units. Degraded gum A contains some uronic acid residues linked α1,4 to galactose units. These linkages are absent in autohydrolysed *A. senegal* gum.[2] On the other hand, autohydrolysed *A. senegal* gum contains a higher proportion of uronic acid residues linked β1,6 to galactose units. Differences in the arrangement of galactose residues within the galactan frameworks of the two degraded gums may also contribute to the difference in specific rotation. Molecular-sieve chromatography [2-4,15] gave an estimated value of 5400 for the number-average molecular-weight of degraded gum A. If the whole gum is based on a galactan framework, then obviously considerable degradation of this framework has occurred during the mild conditions of controlled acid-hydrolysis. This degradation does not appear to have been caused by the presence of any internal acid-labile arabinofuranose residues, since acid hydrolysis of borohydride-reduced degraded gum A yielded only galactitol and no arabinitol (cf. ref. 4). Moreover, there is no evidence for the presence of any galactofuranose residues in the gum. This implies that, just as in *A. senegal* gum,[2,4] certain galactopyranosidic bonds must be unusually sensitive towards mild conditions of acid hydrolysis. Smidsrød *et al.* [16] have drawn attention to the fact that, when the overall proton concentration is low, the negative charge associated with acidic polysaccharides will cause the proton concentration in the region of the macromolecules to be higher than that in the bulk of the solution. This effect may explain the unexpectedly high rates of acid hydrolysis of acidic polysaccharides above pH values of 1.

Partial acid hydrolysis of degraded gum A yielded two galactobioses. The first had the same paper chromatographic mobility as 3-*O*-β-D-galactopyranosyl-D-galactose and was characterised as its crystalline monohydrate. Methylation analysis confirmed that a galactose unit was 3-*O*-substituted by a galactopyranose residue in the disaccharide. Methylation evidence for the second galactobiose indicated that a galactose unit was 6-*O*-substituted by a galactopyranose residue. The paper chromatographic behaviour of the disaccharide confirmed that it was identical to 6-*O*-β-D-galactopyranosyl-D-galactose.

An examination of methylated degraded gum A showed the presence of 2,3,4-tri-*O*-methyl-L-arabinose (a trace), 2,3,4,6-tetra-(++), 2,3,6- (+/2), 2,4,6- (++), and 2,3,4-tri- (+++), 2,6- (+) and 2,4,-di- (+++), and 2-*O*-methyl-D-galactose (+), and 2,3,4-tri-*O*-methyl-D-glucuronic acid (++). These *O*-methyl sugars indicate the presence of terminal non-reducing L-arabinopyranose, D-galactopyranose, and (4-*O*-methyl)- D-glucopyranosyluronic acid residues, and 4-*O*-, 3-*O*-, 6-*O*-, and 3,6-di-*O*-substituted D-galactopyranose units. The presence of some 2,6-di- and 2-*O*-methyl-D-galactose is ascribed to undermethylation. An examination of the methylated reduced product, obtained after reduction of methylated degraded gum A with lithium aluminium hydride [17] followed by re-methylation, showed the presence of 2,3,4,6-tetra-*O*-methyl-D-glucose in addition to the neutral *O*-methyl sugars already mentioned. This confirms that (4-*O*-methyl)-D-glucuronic acid residues are present as non-reducing end-groups in degraded gum A. Since aldobiouronic acids involving uronic acid units linked α1,4 to galactose residues have been identified,[9] the presence of some 4-*O*-substituted galactose units in degraded gum A is to be expected. Likewise, the presence of some of the 6-*O*- and 3,6-di-*O*-substituted galactose units is in accord with the identification [9] of aldobiouronic acids involving uronic acid units linked β1,6 to galactose residues. Partial methanolysis of methylated degraded gum A has indicated the presence of the methylated aldobiouronic acids, 4-*O*-(2,3,4-tri-*O*-methyl-α-D-glucopyranosyluronic acid)-2,3,6-tri-*O*-methyl-D-galactose, 6-*O*-(2,3,4-tri-*O*-methyl-β-D-glucopyranosyluronic acid)-2,3,4-tri-*O*-methyl-D-galactose together with small amounts of 6-*O*-(2,3,4-tri-*O*-methyl-β-D-glucopyranosyluronic acid)-2,4-di-*O*-methyl-D-galactose. It follows that (I), (II), and (III) are possible



structural fragments for residues attached to the galactan framework of degraded gum A. In addition to (4-*O*-methyl)-D-glucopyranosyluronic acid residues in

[15] D. M. W. Anderson and J. F. Stoddart, *Analyt. Chim. Acta*, 1966, **34**, 401; *Lab. Practice*, 1967, **16**, 841.

[16] O. Smidsrød, A. Haug, and B. Larsen, *Acta Chem. Scand.*, 1966, **20**, 1026.

[17] M. Abdel-Akher and F. Smith, *Nature*, 1950, **166**, 1037.

such fragments as (I), (II), and (III), L-arabinopyranose and D-galactopyranose residues occupy terminal non-reducing positions. The occurrence of a small proportion of L-arabinopyranose residues attached directly to the galactan framework constitutes a feature that has not been exhibited by any other *Acacia* gum studied to date. The remainder of the 6-*O*- and 3,6-di-*O*-substituted galactose units, together with the 3-*O*-substituted galactose residues, are present in a highly branched framework of β1,6- and β1,3-linked D-galactose residues. Some idea of the distribution of β1,3- and β1,6-linkages within this galactan framework was obtained by linkage and methylation analysis of the degraded gum B, isolated after a Smith degradation [18] of degraded gum A. Partial acid hydrolysis of degraded gum B gave both the β1,3- and β1,6-galactobioses. An examination of methylated degraded gum B indicated the presence of 2,3,4,6-tetra- (++), 2,4,6- (+++++) and 2,3,4-tri- (+), 2,6- (+) and 2,4-di- (++), and 2-*O*-methyl-D-galactose (+), and 2,3,4-tri-*O*-methyl-D-glucuronic acid (a trace). Since all the uronic acid residues in degraded gum A are present as terminal non-reducing groups, the identification of a small amount of uronic acid residues in degraded gum B is probably explained by incomplete oxidation of a few uronic acid units by periodate during the Smith degradation of degraded gum A. Undermethylation is probably responsible for the presence of some 2,6-di- and 2-*O*-methyl-D-galactose. The proportions of 2,3,4,6-tetra- and 2,4-di- to 2,4,6- and 2,3,4-tri-*O*-methyl-D-galactose is evidence that degraded gum B is composed of highly branched galactan fragments. The fact that these fragments are sufficiently large in molecular size to be retained by cellophane dialysis tubing suggests that there is in degraded gum A a significant proportion of mutually β1,3-linked D-galactose residues which are resistant to Smith degradation. The occurrence of 2,4,6-tri-*O*-methyl-D-galactose as the main *O*-methyl sugar component obtained from methylated degraded gum B is evidence for a high proportion of 3-*O*-substituted D-galactose residues in degraded gum B. The identification of some 2,3,4-tri-*O*-methyl-D-galactose from methylated degraded gum B indicates that (IV) is a possible structural fragment of degraded gum A.

```
   3       1β6           1β3       1
···· D-Galp ──────► D-Galp ──────► D-Galp ····
                      ▲3
                      │
                      │1
                      R

where R =
     1          1               4   1               6   1
L-Arap ····, D-Galp ····, ···· D-Galp ····, ···· D-Galp ····
```

(IV)

The galactan framework of *A. arabica* gum appears to be based on a branched structure of β1,3- and β1,6-linked D-galactose residues. Two possible structures may be proposed. In the first, a "main chain" or "backbone" of blocks of β1,3- and β1,6-linked D-galactose units carries short side-chains of D-galactose residues containing both β1,3- and β1,6-linkages. The second structure is based on a branched framework (in which no "main chain" appears) of β1,3- and β1,6-linked D-galactose residues, and it is suggested that *A. arabica* gum molecules possess galactan frameworks of this second type.

The disaccharides obtained from the diffusate of the mild acid hydrolysates of the whole gum give some idea of the possible modes of linkage of units in the arabinose-containing side-chains, which are attached to the galactan framework. Two arabinobioses were separated on thick paper. The first was identical on paper chromatography to 3-*O*-β-L-arabinopyranosyl-L-arabinose. Acid hydrolysis gave only arabinose, and methylation analysis indicated that an arabinose unit was 3-*O*-substituted by an arabinopyranose residue. The disaccharide was characterised as its crystalline phenylosazone. The second arabinobiose had the same paper chromatographic mobility as 3-*O*-β-L-arabinofuranosyl-L-arabinose. Acid hydrolysis gave only arabinose, and methylation analysis indicated that an arabinose unit was 3-*O*-substituted by an arabinofuranose residue. The fact that both arabinobioses are released by relatively mild acid hydrolysis conditions suggests that in each case the reducing arabinose residue was initially present in the furanose form in the arabinose-containing side-chains.

An examination of the *O*-methyl derivative of *A. arabica* gum indicated the presence of 2,3,5- and 2,3,4-tri-, and 2,5-, 3,5-, and 3,4-di-*O*-methyl-L-arabinose, 2,4-di-*O*-methyl-D-galactose, and 2,3,4-tri-*O*-methyl-D-glucuronic acid together with small amounts of 2,3,4-tri-*O*-methyl-L-rhamnose, 4-*O*-methyl-L-arabinose, and 2,3,4,6-tetra-, 2,3,6-, 2,4,6-, and 2,3,4-tri-, and 2-*O*-methyl-D-galactose. Small amounts of galactose and arabinose are not thought to be structurally significant. The structural significance of 4-*O*-methyl-L-arabinose and 2-*O*-methyl-D-galactose is also doubtful. They probably arise from either incomplete methylation or demethylation during hydrolysis. Although the presence of a high proportion of 2,4-di-*O*-methyl-D-galactose indicates that most of the D-galactose residues in *A. arabica* gum are 3,6-di-*O*-substituted, the identification of trace amounts of 2,3,4,6-tetra-, 2,3,6-, 2,4,6-, and 2,3,4-tri-*O*-methyl-D-galactose is evidence for a few terminal non-reducing D-galactose residues and some 4-*O*-, 3-*O*-, and 6-*O*-substituted D-galactose units, respectively. The identification of 2,3,4-tri-*O*-methyl-D-glucuronic acid suggests that D-glucuronic acid and 4-*O*-methyl-D-glucuronic acid residues occur as terminal non-reducing units in the whole gum. The identification of trace amounts of 2,3,4-tri-*O*-methyl-L-rhamnose confirms the presence of small amounts of L-rhamnose residues as non-reducing end-groups in *A. arabica* gum.[6] However, the rhamnose content is so small that

[18] I. J. Goldstein, G. W. Hay, B. A. Lewis, and F. Smith, "Methods in Carbohydrate Chemistry," "General Polysaccharides," ed. R. L. Whistler, Academic Press, New York, 1965, vol. V, p. 361.

these residues must have little structural significance. The presence of 2,3,5- and 2,3,4-tri-*O*-methyl-L-arabinose indicates that the arabinose-containing side-chains are terminated in some cases by L-arabinofuranose and in other cases by L-arabinopyranose residues. The identification of some 2,5-di-*O*-methyl-L-arabinose is evidence for some 3-*O*-substituted L-arabinofuranose residues in the arabinose-containing side-chains. The major dimethyl ether of arabinose to be isolated and characterised was, however, the 3,5-di-*O*-methyl isomer. Previous to its isolation from methylated *A. arabica* gum, methylated *A. pycnantha* gum had been the only other *O*-methyl derivative of an *Acacia* gum to yield 3,5-di-*O*-methyl-L-arabinose.* [19] Its isolation from methylated *A. arabica* gum suggests that there is a proportion of 2-*O*-substituted L-arabinofuranose residues in the arabinose-containing side-chains. If the small amounts of 3,4-di-*O*-methyl-L-arabinose * do not arise from undermethylation or from demethylation, then there must be a small proportion of 2-*O*-substituted L-arabinopyranose residues in the arabinose-containing side-chains as well. Methylation evidence for the whole gum and for degraded gum A suggests that the arabinose-containing side-chains are attached to the galactan framework at the C-3 and/or C-6 positions of certain D-galactose residues.

A sample of *A. arabica* gum was submitted to four successive Smith degradations.[18] The percentage yields of Smith-degraded products are low compared with those obtained during successive Smith degradations on *A. senegal* gum.[2,3] Molecular-sieve chromatography,[2-4,15] however, indicates that there is pronounced cleavage of the macro-molecule during successive Smith degradations. This cleavage of internal D-galactose residues is particularly drastic during the second and subsequent Smith degradations. The loss of a large number of small fragments during dialysis probably accounts for the low percentage yields of polysaccharides (III) and (IV). The complete fragmentation of the macromolecule during successive Smith degradations shows that long chains of periodate-resistant β1,3-linked D-galactose residues are not such an important structural feature of *A. arabica* gum as they are of *A. senegal* gum.[2,3] In *A. arabica* gum, blocks of periodate-immune β1,3-linked D-galactose units must be interspersed by blocks of periodate-vulnerable β1,6-linked D-galactose residues in a highly branched galactan framework. Fragmentation of this framework, which contains 6-*O*-substituted D-galactose residues, is to be expected as the arabinose-containing side-chains are progressively removed from the C-3 positions of 6-*O*-substituted D-galactose units.

Four successive Smith degradations were required to remove all the arabinose residues from *A. senegal* gum.[2,3] In contrast, all arabinose residues were not eliminated from the product [polysaccharide (IV)] obtained from *A. arabica* gum after four successive Smith degradations. This means that the arabinose-containing side-chains in *A. arabica* gum contain more arabinose residues on the average than are present in the arabinose-containing side-chains of *A. senegal* gum. During successive Smith degradations, the specific rotation decreases from +112° for *A. arabica* gum to +46° for polysaccharide (III). Linkages associated with the arabinose-containing side-chains and their attachment to the galactan framework may be partly responsible for the high positive specific-rotation of *A. arabica* gum.

On methanolysis, the *O*-methyl derivative of polysaccharides (I), (II), (III), and (IV) gave the methyl

FIGURE 1 Diagrammatic representation of the high degree of branching exhibited by part of the galactan framework of an *Acacia arabica* gum molecule. Uronic acid residues and arabinose-containing side-chains are attached to this framework. ○ = D-Galactopyranose residues; —— = β1,3-linkages; —▶— = β1,6-linkages

glycosides of 2,3,5-tri-, 2,5- and 3,5-di-*O*-methyl-L-arabinose, and 2,3,4,6-tetra-, 2,4,6- and 2,3,4-tri-, and 2,4-di-*O*-methyl-D-galactose. The identification of some 2,5- and 3,5-di-*O*-methyl-L-arabinose from methylated polysaccharide (IV) indicates that some of the arabinose-containing side-chains in *A. arabica* gum must contain at least six 1,3- and/or 1,2-linked L-arabinose units. The absence of any evidence for arabinopyranose residues in polysaccharide (I) confirms that the 4-*O*-methyl-L-arabinose characterised from methylated *A. arabica* gum is not structurally significant.

The structural evidence therefore strongly suggests that *A. arabica* gum molecules possess branched galactan frameworks to which are attached uronic acid residues and arabinose-containing side-chains. This structural feature is represented diagramatically in Figure 1. The high degree of branching of the galactan framework implies that not all the uronic acid and arabinose residues are to be found on the periphery of the gum

* Note added in proof. Since the submission of this manuscript two Papers have been published by Stephen and his collaborators (M. Kaplan and A. M. Stephen, *Tetrahedron*, 1967, **23**, 193; A. M. Stephen and D. C. Vogt, *ibid.*, p. 1473) in which the *O*-methyl derivatives of gums from *A. mearnsii*, *A. karroo*, *A. cyanophylla*, *A. decurrens*, *A. podalyriaefolia* (?), and *A. giraffae* have been reported to afford 3,5-di-*O*-methyl-L-arabinose. The *O*-methyl derivatives of gums from *A. karroo* and *A. podalyriaefolia* have also been reported to yield 3,4-di-*O*-methyl-L-arabinose.

[19] G. O. Aspinall, E. L. Hirst, and A. Nicolson, *J. Chem. Soc.*, 1959, 1697; A. Nicolson, Ph.D. Thesis, 1959, University of Edinburgh.

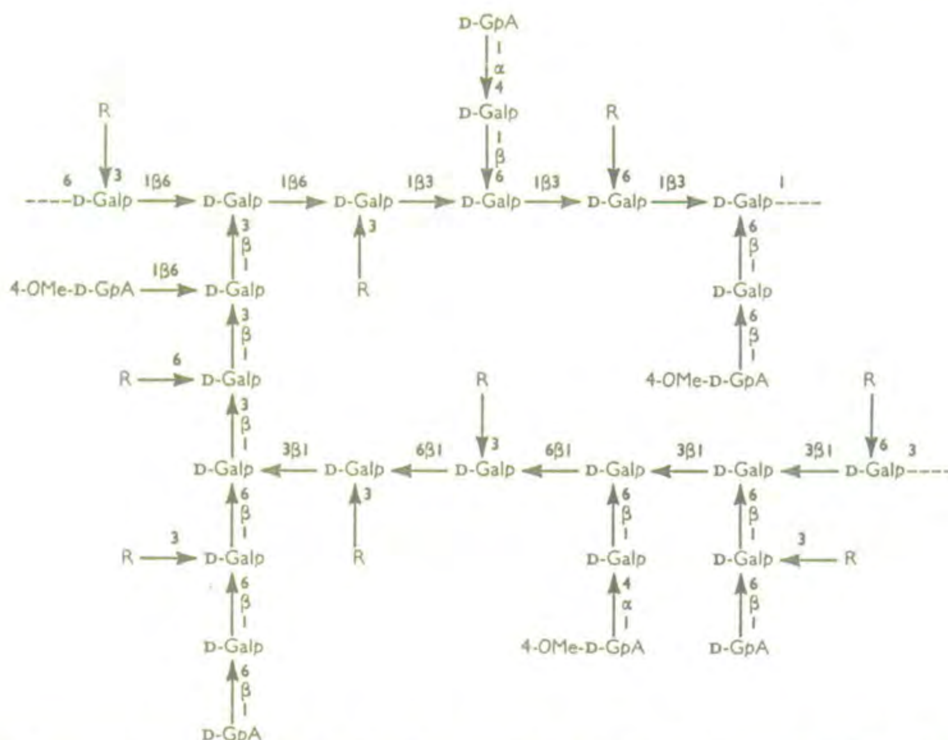


FIGURE 2 Possible structural fragment from an *Acacia arabica* gum molecule. R represents L-Ara*p*- and L-Ara*f*-containing side-chains. Some of these side-chains are at least 6 units long and contain ···· ^{3}L-Ara*f*^{1} ····, ···· ^{2}L-Ara*f*^{1} ····, and ···· ^{2}L-Ara*p*^{1} ···· residues. They may be terminated by L-Ara*f*^{1} ···· or L-Ara*p*^{1} ···· residues

molecules. A possible structural fragment from one of these gum molecules is represented by the polysaccharide array shown in Figure 2. The galactan framework of *A. arabica* gum is more highly branched than that of *A. senegal* gum, and the arabinose-containing side-chains are longer in *A. arabica* gum than in *A. senegal* gum. These observations imply that the molecules of *A. arabica* gum have a more compact structure than those of *A. senegal* gum. This conclusion is supported by the results of physicochemical measurements made on solutions of the two gums. The fact that *A. arabica* gum has a lower limiting viscosity number than *A. senegal* gum, despite its higher weight-average molecular-weight, is evidence that *A. arabica* gum molecules are more densely packed with sugar residues, and consequently more compact, than *A. senegal* gum molecules.

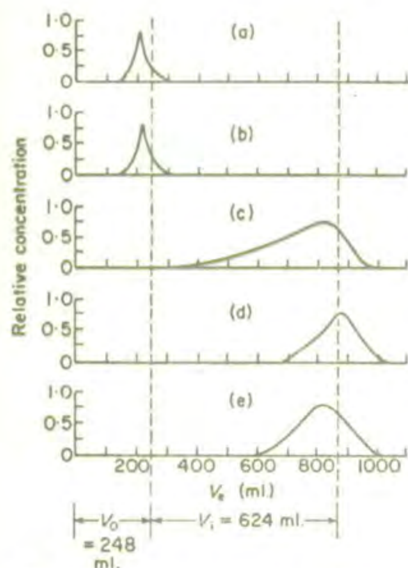


FIGURE 3 Elution patterns for *Acacia arabica* gum (a), for polysaccharides (I)—(III) (b)–(d), and for degraded gum A (e) from a " Bio-Gel P300 " column (50 × 4·8 cm.): elution with M-sodium chloride solution; V_0 = elution volume of " blue dextran "; $V_0 + V$ = elution volume of sucrose

From a comparison of the structural features of *A. arabica* gum with those of *A. senegal* gum,[2-4] it is clear that there are important structural differences associated, not only with the nature and mode of attachment to the galactan frameworks of uronic acid residues and arabinose-containing side chains,but also with the branching patterns of the galactan frameworks themselves. Indeed, it is becoming evident that structural variations between gums of different species within the *Acacia* genus are more pronounced than was at one time supposed.[19]

EXPERIMENTAL

The analytical methods have been described elsewhere [2-4] in more detail. Paper chromatography was carried out on Whatman Nos. 1 and 3MM papers using the following solvent systems (v/v): (a) benzene–butan-1-ol–pyridine–water (1 : 5 : 3 : 3, upper layer); (b) ethyl acetate–acetic acid–formic acid–water (18 : 3 : 1 : 4); (c) butan-1-ol–

ethanol–water (4 : 1 : 5, upper layer); (d) butan-1-ol–acetic acid–water (4 : 1 : 5, upper layer); (e) ethyl acetate–pyridine–water (10 : 4 : 3); (f) butanone–water–ammonia (*d* 0·880) (200 : 17 : 1); (g) butanone–acetic acid–water (9 : 1 : 1, satd. with boric acid). R_{gal} Values of sugars refer to distances moved relative to that of galactose. R_G values of *O*-methyl sugars refer to distances moved relative to that of 2,3,4,6-tetra-*O*-methyl-D-glucose. Zone electrophoresis of sugars on Whatman No. 1 papers was carried out in 0·05M-borate buffer (pH 9·2). M_G Values refer to the true migration of the sugar relative to that of glucose. Gas–liquid partition chromatography (Chromatograph type S3A, fitted with flame ionisation detectors, supplied by Gas Chromatography Ltd.) of mixtures of *O*-methyl sugars was carried out at nitrogen flow-rates of *ca.*, 100 ml./min. on columns of (i) 15% by weight of butan-1,4-diol succinate polyester on 60—70 mesh Celite (5 ft. × ¼ in.) at 175° and (ii) 15% by weight of ethylene glycol adipate polyester on 60—70 mesh Celite (3 ft. × ¼ in.) at 160°. Retention times (*T*) are quoted relative to methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside as standard. Gas–liquid partition chromatography of mixtures of *O*-trimethylsilyl sugars was carried out at nitrogen flow-rates of *ca.*, 100 ml./min. on a column of 3% by weight of SE53 on 60—70 mesh Celite (5 ft. × ¼ in.) at 130°. Proportions of galactose to arabinose in polysaccharide hydrolysates were estimated [20] from peak areas obtained on g.l.c. of their *O*-trimethylsilyl derivatives. Unless otherwise stated, polysaccharides were methylated successively with dimethyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide, and methanolyses were carried out under reflux for 6 hr. with methanolic 5% hydrogen chloride.

Purification of A. arabica *Gum*.—The gum (160 g.) was dissolved in water (3 l.), filtered, dialysed, and electrodialysed. The polysaccharide was isolated as the freeze-dried product (152 g.), $[\alpha]_D$ +112° (*c* 1·24) (Found: ash, 0·02; N, 0·07; OMe, 0·88%; Equiv. wt., 1880; uronic acid, 10; galactose, 32; arabinose, 57; rhamnose, 0·4%; all sugar percentages are for the anhydro-forms and are corrected for moisture and protein contents).

The gum migrated as a single band on electrophoresis on glass-fibre paper in 2N-sodium hydroxide solution.[11] With strips (18 × 5 cm.) of Whatman GF/A paper, a potential of *ca.* 300 v across a 16 cm. length was applied *via* wicks of double thickness Whatman No. 3MM papers for 24 hr. Strips were dried and sprayed [21] with 0·5% (w/v) potassium permanganate in N-sodium hydroxide solution. The gum also migrated as single bands on electrophoresis on strips (36 × 5 cm.) of Whatman No. 1 paper in 0·1M-ammonium carbonate buffer (pH 8·9) at field strengths of 5 v/cm. for 2 hr., and on strips (18 × 5 cm.) of cellulose acetate film in both 0·1M-ammonium carbonate buffer (pH 8·9) and 0·1M-acetate buffer (pH 4·7) at field strengths between 15 and 20 v/cm. for 2—4 hr. Polysaccharide bands on both paper and cellulose acetate strips were either stained in saturated ethanolic solutions of Alcian Blue or by a modification [3] of the periodate–rosaniline hydrochloride method.[22] The gum was also chromatographed [23] on a DEAE-cellulose column (30 × 1·5 cm.). Gradient elution with sodium chloride solution (0·0 ⟶ 0·3M) in 0·02M-acetate buffer (pH 4·1) yielded a single, slightly asymmetric peak for its elution pattern.

Viscosity determinations gave a limiting viscosity number of 9·9 ml./g. Light-scattering measurements, carried out in the Procter Department, University of Leeds, through the kind co-operation of Dr. G. Stainsby, yielded a value of 2·3 × 10⁶ for the weight-average molecular-weight of the gum.

Separation and Characterisation of Neutral Sugars.—The gum (1 g.) was hydrolysed with N-sulphuric acid (50 ml.) for 10 hr. on a boiling-water bath, and the cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and concentrated to a syrup (921 mg.). The sugars were separated on Whatman No. 3MM papers in solvent (a) to give three pure monosaccharides.

Fraction 1 (212 mg.) had $[\alpha]_D$ +80° (equil.) (*c* 1·0) and had the same paper chromatographic mobility as D-galactose, m. p. 162°, and *X*-ray diffraction pattern identical to that of an authentic specimen.

Fraction 2 (506 mg.) had $[\alpha]_D$ +104° (equil.) (*c* 1·0) and had the same paper chromatographic mobility as L-arabinose, m. p. 159°, and *X*-ray diffraction pattern identical to that of an authentic specimen.

Fraction 3 (2 mg.) was paper chromatographically identical to rhamnose in solvents (a), (b), (c), and (e).

Preparation of Degraded Gum A.—The gum (20 g.) was hydrolysed with 0·01N-sulphuric acid (800 ml.) for 100 hr. on a boiling-water bath. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and dialysed against water (2·5 l.). Dialysis was completed against running tap-water, and freeze-drying gave degraded gum A (4 g.), $[\alpha]_D$ +33° (*c* 1·04) (Found: uronic acid, 11%; galactose, 86%; arabinose, 2%).

Degraded gum A (50 mg.) was dissolved in water (10 ml.) and reduced with sodium borohydride (50 mg.). After dialysis, the freeze-dried product was hydrolysed. Paper chromatographic examination of the hydrolysate in solvent (g) indicated the presence of galactitol.

The diffusate from the dialysis of degraded gum A was concentrated to a syrup (11 g.), a portion (2 g.) of which was chromatographed on Whatman No. 3MM papers in solvent (b) to give two pure disaccharides.

Fraction 1 (89 mg.) had $[\alpha]_D$ +193° (*c* 1·78) and yielded arabinose on acid hydrolysis. It had the same paper chromatographic mobility [R_{gal} 0·83 in solvent (a), 0·74 in solvent (b), and 0·92 in solvent (e)] as 3-*O*-β-L-arabinopyranosyl-L-arabinose. A portion (21 mg.) of this disaccharide was methylated with methyl iodide and silver oxide in *NN*-dimethylformamide.[24] Gas–liquid chromatographic examination of the methanolysis products revealed peaks with retention times corresponding to the methyl glycosides of 2,3,4-tri- [(i), *T* 1·02; (ii), *T* 0·83], and 2,5- [(i), *T* 1·79, 3·30; (ii), *T* 1·25, 2·21] and 2,4-di-*O*-methyl-L-arabinose [(i), *T* 2·07, 2·17; (ii), *T* 1·42, 1·50]. Hydrolysis of the methyl glycosides followed by paper chromatography in solvent (c) confirmed the presence of 2,3,4-tri- (R_G 0·81), and 2,5-(R_G 0·85) and 2,4-di-*O*-methyl-L-arabinose

[20] C. J. Ludlow, T. M. Harris, and F. T. Wolf, *Phytochemistry*, 1966, **5**, 251.
[21] D. R. Briggs, E. F. Garner, and F. Smith, *Nature*, 1956, **178**, 154.
[22] A. B. S. Conacher and D. I. Rees, *Analyst*, 1966, **91**, 55.
[23] D. M. W. Anderson, Sir Edmund Hirst, S. Rahman, and G. Stainsby, *Carbohydrate Res.*, 1967, **3**, 308.
[24] R. Kuhn, H. Trischmann, and I. Löw, *Angew. Chem.*, 1955, **67**, 32; O. Perila and C. T. Bishop, *Canad. J. Chem.*, 1961, **39**, 815.

(R_G 0·65). The phenylosazone of the disaccharide was prepared. After recrystallisation from ethanol, it had m. p. 229—232° (decomp.).

Fraction 2 (28 mg.) was re-chromatographed on Whatman No. 3MM papers in solvent (f) to give a syrup (8·7 mg.), which had $[\alpha]_D$ +90° (*c* 0·17) and yielded arabinose on acid hydrolysis. It had the same paper chromatographic mobility [R_{gal} 1·23 in solvent (b) and 1·41 in solvent (f)] as 3-*O*-β-L-arabinofuranosyl-L-arabinose. A portion (2 mg.) of the disaccharide was methylated with methyl iodide and silver oxide in *NN*-dimethylformamide.[24] Gas–liquid chromatographic examination of the methanolysis products revealed peaks with retention times corresponding to the methyl glycosides of 2,3,5-tri- [(i), *T* 0·58, 0·72; (ii), *T* 0·51, 0·63], and 2,5-[(i), *T* 1·78, 3·29; (ii), *T* 1·27, 2·22] and 2,4-di-*O*-methyl-L-arabinose [(i), *T* 2·07, 2·17; (ii), *T* 1·41 ,1·49].

Partial Acid Hydrolysis of Degraded Gum A.—Degraded gum A (2 g.) was hydrolysed with 0·5N-sulphuric acid (500 ml.) for 1 hr. on a boiling-water bath. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and concentrated to a syrup (1·9 g.). The syrup was chromatographed on Whatman No. 3MM papers in solvent (a) to give two pure disaccharides.

Fraction 1 (39 mg.) had $[\alpha]_D$ +64° (*c* 0·78) and yielded galactose on acid hydrolysis. It had the same paper chromatographic mobility [R_{gal} 0·47 in solvent (a) and 0·51 in solvent (e)] as an authentic sample of 3-*O*-β-D-galactopyranosyl-D-galactose. A portion (10 mg.) of the disaccharide was methylated with methyl iodide and silver oxide in *NN*-dimethylformamide.[24] Gas–liquid chromatographic examination of the methanolysis products revealed peaks with retention times corresponding to the methyl glycosides of 2,3,4,6-tetra-[(i), *T* 1·67; (ii), *T* 1·64], and 2,4,6-[(i), *T* 3·72, 4·17; (ii), *T* 2·98, 3·45] and 2,5,6-tri-*O*-methyl-D-galactose [(i), *T* 3·99; (ii), *T* 3·27]. Hydrolysis of the methyl glycosides followed by paper chromatography in solvent (c) confirmed the presence of 2,3,4,6-tetra- (R_G 0·90), and 2,4,6-(R_G 0·72) and 2,5,6-tri-*O*-methyl-D-galactose (R_G 0·88). The disaccharide was crystallised from aqueous acetone to give needles of 3-*O*-β-D-galactopyranosyl-D-galactose monohydrate, m. p. and mixed m. p. 156—159°.

Fraction 2 (55 mg.) had $[\alpha]_D$ +31° (*c* 1·10) and yielded galactose on acid hydrolysis. It had the same paper chromatographic mobility [R_{gal} 0·29 in solvent (a) and 0·35 in solvent (e)] as an authentic sample of 6-*O*-β-D-galactopyranosyl-D-galactose. A portion (10 mg.) of the disaccharide was methylated with methyl iodide and silver oxide in *NN*-dimethylformamide.[24] Gas–liquid chromatographic examination of the methanolysis products revealed peaks with retention times corresponding to the methyl glycosides of 2,3,4,6-tetra-[(i), *T* 1·69; (ii), *T* 1·67], and 2,3,5-[(i), *T* 4·03, 5·37; (ii), *T* 3·33, 4·36] and 2,3,4-tri-*O*-methyl-D-galactose [(i), *T* 6·47; (ii), *T* 5·29]. Hydrolysis of the methyl glycosides followed by paper chromatography in solvent (c) confirmed the presence of 2,3,4,6-tetra-(R_G 0·89), and 2,3,5-(R_G 0·87) and 2,3,4-tri-*O*-methyl-D-galactose (R_G 0·70).

Methylation of Degraded Gum A.—Degraded gum A (1·050 g.) was methylated to give a product (926 mg.), $[\alpha]_D$ +14° (*c* 1·18 in $CHCl_3$) (Found: OMe, 40·4%, not raised on further attempted methylation). Methanolysis of a sample of this product followed by g.l.c. examination of the mixture of methyl glycosides gave the results shown in Table 1. Examination of a hydrolysate of the methyl glycosides in solvents (c) and (f) indicated the presence of 2,6-di- and 2-*O*-methyl-D-galactose, in addition to those *O*-methyl sugars already characterised by g.l.c. of their methyl glycosides (Table 1).

TABLE 1

Examination of methanolysis and hydrolysis products from methylated degraded gum A

O-Methyl sugars	Relative retention times (*T*) of methyl glycosides: Column (i)	Column (ii)	R_G in solvent (*c*)	Approx. relative molar props.
2,3,4-Tri-*O*-methyl-L-arabinose	0·97	0·85	0·80	Trace
2,3,4,6-Tetra-*O*-methyl-D-galactose	1·67	1·66	0·87	++
2,3,6-Tri-*O*-methyl-D-galactose	(2·88) * (3·70) (4·12)	(2·30) (3·04) (3·46)	0·72	+/2
2,4,6-Tri-*O*-methyl-D-galactose	(3·70) (4·12)	(3·04) (3·46)	0·72	++
2,3,4-Tri-*O*-methyl-D-galactose	6·38	5·27	0·72	+++
2,6-Di-*O*-methyl-D-galactose			0·54	+
2,4-Di-*O*-methyl-D-galactose	14·4, 16·3	9·8, 11·4	0·50	+++
2-*O*-Methyl-D-galactose			0·33	+
2,3,4-Tri-*O*-methyl-D-glucuronic acid †	2·25 (2·88)	2·14, 2·74		++

* Figures in parentheses indicate *T* values of components not completely resolved. † As methyl ester methyl glycoside.

Methylated degraded gum A (750 mg.) was heated under reflux for 8 hr. with methanolic 5% hydrogen chloride (75 ml.). The resulting mixture of methyl glycosides was hydrolysed with N-sulphuric acid (100 ml.) on a boiling-water bath for 4 hr. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and concentrated to a syrup (700 mg.). The mixture of *O*-methyl sugars was separated on Whatman No. 3MM papers by double development (personal communication from Drs. N. S. Anderson and D. A. Rees) in solvent (f) to give eight fractions.

Fraction 1 (52 mg.) had $[\alpha]_D$ +116° (*c* 1·04). It had the same paper chromatographic mobility [R_G 0·87 in solvent (c) and 0·72 in solvent (f)] as an authentic sample of 2,3,4,6-tetra-*O*-methyl-D-galactose. It was characterised by conversion into *N*-phenyl-2,3,4,6-tetra-*O*-methyl-D-galactosylamine, which, after recrystallisation from ethyl acetate, had m. p. 190—192°.

Fraction 2 (59 mg.) had $[\alpha]_D$ +102° (*c* 1·18). Demethylation [25] gave galactose and arabinose. Gas–liquid chromatographic examination of the mixture of derived methyl glycosides indicated the presence of 2,3,4,6-tetra-[(i), *T*

[25] L. Hough, J. K. N. Jones, and W. H. Wadman, *J. Chem. Soc.*, 1950, 1702.

1·65; (ii), *T* 1·67] and 2,3,6-tri-*O*-methyl-D-galactose [(i), *T* 2·81, 3·79, 4·17; (ii), *T* 2·36, 3·06, 3·47], and 2,3,4-tri-*O*-methyl-L-arabinose [(i), *T* 0·97; (ii), *T* 0·83].

Fraction 3 (48 mg.) had $[\alpha]_D$ +98° (*c* 0·96). Gas–liquid chromatographic examination of the derived methyl glycosides indicated the presence of a mixture of approximately equimolar proportions of 2,4,6-[(i), *T* 3·73, 4·17; (ii), *T* 3·04, 3·47] and 2,3,4-tri-*O*-methyl-D-galactose [(i), *T* 6·41; (ii), *T* 5·27]. The mixture of *O*-methyl sugars was re-chromatographed in solvent (f) to obtain a syrup (12 mg.) of almost pure 2,4,6-tri-*O*-methyl-D-galactose. It was characterised by conversion into *N*-phenyl-2,4,6-tri-*O*-methyl-D-galactosylamine, which, after recrystallisation twice from ethyl acetate and once from acetone–ether–light petroleum [1 : 1 : 1 (v/v)] gave needle-shaped crystals, m. p. 163—165°.

Fraction 4 (54 mg.) had $[\alpha]_D$ +119° (*c* 1·08). It had the same paper chromatographic mobility [R_G 0·70 in solvent (c)] as 2,3,4-tri-*O*-methyl-D-galactose and was characterised by conversion into *N*-phenyl-2,3,4-tri-*O*-methyl-D-galactosylamine, which, after recrystallisation from ethyl acetate, had m. p. 163—165°. Gas–liquid chromatographic examination of the derived methyl glycosides indicated the presence of 2,3,4-tri-*O*-methyl-D-galactose [(i), *T* 6·38; (ii), *T* 5·26].

Fraction 5 (44·5 mg.) had $[\alpha]_D$ +80° (*c* 0·89). It had the same paper chromatographic mobility [R_G 0·50 in solvent (c) and 0·18 in solvent (f)] and paper electrophoretic mobility [M_G 0·26, 10 v/cm., 0·5 mA/cm.] as 2,6-di-*O*-methyl-D-galactose. A portion (2 mg.) of the fraction was submitted to periodate oxidation.[26] Paper chromatographic examination of the oxidation products in solvent (c) revealed a brilliant yellow spot (*p*-anisidine hydrochloride spray) with the same mobility (R_G 0·20) as 2-*O*-methylmalondialdehyde.

Fraction 6 (69·5 mg.) had $[\alpha]_D$ +87° (*c* 1·39). It had the same paper chromatographic mobility [R_G 0·46 in solvent (c) and 0·12 in solvent (f)] as an authentic sample of 2,4-di-*O*-methyl-D-galactose. Recrystallisation of the *O*-methyl sugar from acetone containing 1% of water gave 2,4-di-*O*-methyl-D-galactose monohydrate, m. p. 100—102° (*X*-ray powder photograph identical to that obtained for an authentic sample). The derived *N*-phenyl-2,4-di-*O*-methyl-D-galactosylamine, after recrystallisation from acetone, had m. p. 214—216°.

Fraction 7 (20 mg.) had $[\alpha]_D$ +83° (*c* 0·40). It had the same paper chromatographic mobility [R_G 0·29 in solvent (c) and 0·05 in solvent (f)] and paper electrophoretic mobility [M_G 0·41, 10 v/cm., 0·5 mA/cm.] as an authentic sample of 2-*O*-methyl-D-galactose. Recrystallisation of the *O*-methyl sugar from glacial acetic acid gave crystals with m. p. 156—158° (*X*-ray powder photograph identical to that obtained for an authentic sample).

Fraction 8 (43 mg.) had $[\alpha]_D$ +50° (*c* 0·86). It had the same paper chromatographic mobility [R_G 0·88 in solvent (d)] as 2,3,4-tri-*O*-methyl-D-glucuronic acid. Gas–liquid chromatographic examination of the derived methyl glycosides indicated the presence of the methyl ester of 2,3,4-tri-*O*-methyl-D-glucuronic acid [(i), *T* 2·23, 2·80; (ii), *T* 2·08, 2·66].

Reduction of Methylated Degraded Gum A.—Lithium aluminium hydride (60 mg.) was added to methylated degraded gum A (60 mg.), dissolved in dry tetrahydrofuran (10 ml.), and the mixture was heated under reflux for 3 hr. On cooling, the excess of hydride was destroyed by addition of ethyl acetate and water, and the mixture was reduced to dryness and exhaustively extracted with chloroform. The chloroform extract was concentrated to a syrup, which was methylated [24] with methyl iodide (1 ml.) and silver oxide (1 g.) in *NN*-dimethylformamide (1 ml.) to give a product (41 mg.), $[\alpha]_D$ +16° (*c* 0·82 in $CHCl_3$). Methanolysis of this product followed by g.l.c. examination of the mixture of methyl glycosides showed the presence of 2,3,4,6-tetra-*O*-methyl-D-glucose [(i), *T* 1·00, 1·39; (ii), *T* 1·00, 1·37] in addition to those neutral *O*-methyl sugars already identified from methylated degraded gum A (Table 1). Acid hydrolysis of the methyl glycosides, followed by paper chromatographic examination of the free sugars, confirmed these results.

Partial Methanolysis of Methylated Degraded Gum A.—Methylated degraded gum A (100 mg.) was heated under reflux with methanolic 2% hydrogen chloride (15 ml.) for 2 hr. The cooled methanolic solution was neutralised with silver carbonate, treated with hydrogen sulphide, filtered, concentrated to a syrup, which was hydrolysed with 0·5N-sulphuric acid (10 ml.) for 5 hr. on a boiling-water bath. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and adsorbed on to a column (25 × 2·5 cm.) of Duolite A4 resin in the formate form. After elution of the neutral *O*-methyl sugars with water (3 l.), the acidic *O*-methyl sugars were eluted with aqueous 5% formic acid (500 ml.). Water and formic acid were removed, and paper chromatography in solvents (c) and (f) of the resulting syrup indicated that it was free of neutral *O*-methyl sugars. Methanolysis (methanolic 5% hydrogen chloride under reflux for 16 hr.) of the syrup followed by g.l.c. examination of the products revealed peaks with retention times corresponding to the methyl glycosides of 2,3,4-tri-*O*-methyl-D-glucuronic acid methyl ester (+++), [(i), *T* 2·22, (2·82); (ii), *T* 2·06, 2·65], 2,3,6- (+) [(i), *T* (2·82), 3·80, 4·17; (ii), *T* 2·35, 3·07, 3·47] and 2,3,4-tri-(++) [(i), *T* 6·40; (ii), *T* 5·26], and 2,4-di-*O*-methyl-D-galactose (+/2)[(i), *T* 14·2, 16·3; (ii), *T* 9·9, 11·2]. Examination of a hydrolysate of the methyl glycosides by paper chromatography in solvents (c), (d), and (f) confirmed the presence of these *O*-methyl sugars.

Smith Degradation of Degraded Gum A.—Degraded gum A (1 g.) was dissolved in water (25 ml.) and 0·5M-sodium metaperiodate solution (25 ml.) was added. The amount of periodate consumed after 96 hr. was 6·35 mmoles/g. and the amount of formic acid released in this time was 2·9 mmoles/g. After 96 hr., the reaction was stopped by addition of ethylene glycol (2 ml.). The solution was dialysed against running tap-water for 2 days. Sodium borohydride (500 mg.) was added and the mixture kept at room temperature for 30 hr. After dialysis for a further 2 days, the polyalcohol was hydrolysed in N-sulphuric acid at room temperature for 2 days. The solution was then neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and dialysed against water (500 ml.). The syrup obtained from the dialysate was shown by paper chromatography in solvents (b), (c), and (g) to contain glycerol and glycollic aldehyde as the main components, together with small amounts of some slower moving non-reducing glycosides. After further dialysis against running tap water for 2 days, degraded gum B

[26] R. U. Lemieux and H. F. Bauer, *Canad. J. Chem.*, 1953, **31**, 814.

(85 mg., yield, 8·5%), $[\alpha]_D$ +20° (*c* 0·85) was isolated as the freeze-dried product.

Partial Acid Hydrolysis and Methylation of Degraded Gum B.—Degraded gum B (10 mg.) was hydrolysed with 0·5N-sulphuric acid (5 ml.) for 1 hr. on a boiling-water bath. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and concentrated. Paper chromatographic examination of the syrup in solvent (a) indicated the presence of galactose, two neutral disaccharides with the mobilities of 3-*O*-β-D-galactopyranosyl-D-galactose (R_{gal} 0·47) and 6-*O*-β-D-galactopyranosyl-D-galactose (R_{gal} 0·29), and higher neutral oligosaccharides.

Degraded gum B (50 mg.) was methylated successively with dimethyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide in *NN*-dimethylformamide.[24] Methanolysis followed by g.l.c. examination of the mixture of methyl glycosides gave the results shown in Table 2.

TABLE 2

Examination of methanolysis products from methylated degraded gum B

O-Methyl sugars	Relative retention times (*T*) of methyl glycosides Column (i)	Column (ii)	Approx. relative molar props.
2,3,4,6-Tetra-*O*-methyl-D-galactose	1·68	1·67	++
2,4,6-Tri-*O*-methyl-D-galactose	3·72, 4·16	2·99, 3·45	++++
2,3,4-Tri-*O*-methyl-D-galactose	6·40	5·26	+
2,4-Di-*O*-methyl-D-galactose	14·6, 16·1	9·8, 11·2	++
2,3,4-Tri-*O*-methyl-D-glucuronic acid *	2·32, 2·96	2·08, 2·67	Trace
Unknown sugars	0·21, 0·32	0·20, 0·30	—

* As methyl ester methyl glycoside.

Examination of a hydrolysate of the methyl glycosides by paper chromatography in solvents (c) and (f) indicated the presence of 2,6-di-(+) and 2-*O*-methylgalactose(+), in addition to those *O*-methyl sugars already characterised by g.l.c. of their methyl glycosides.

Methylation of A. arabica *Gum.*—The gum (10 g.) was methylated to give a product (5·6 g.), $[\alpha]_D$ +77° (*c* 0·93 in $CHCl_3$) (Found: OMe, 38·2%, not raised on further attempted methylation). The methylated gum (5 g.) was suspended in 2N-sulphuric acid (75 ml.) and kept at room temperature until dissolution was almost complete (4 days). Water (75 ml.) was added and the solution warmed for 2 hr. at 50° before being heated on a boiling-water bath for 12 hr. The cooled solution was neutralised with barium carbonate, filtered, and treated with Amberlite resin IR-120 (H). Separation of neutral *O*-methyl sugars from acidic *O*-methyl sugars was accomplished by ion-exchange chromatography on a column (30 × 2·5 cm.) of Duolite A4 resin in the formate form. Elution of the column with water (2 l.) gave the neutral *O*-methyl sugar fraction. Elution with 5% formic acid (500 ml.) gave the acidic *O*-methyl sugar fraction.

Concentration of the acidic *O*-methyl sugar fraction yielded a syrup (180 mg.), which was heated under reflux with methanolic 2% hydrogen chloride overnight. After neutralisation with silver carbonate, filtration, and concentration to a syrup, the methyl ester methyl glycosides were reduced by heating under reflux in dry tetrahydrofuran (40 ml.) with lithium aluminium hydride (300 mg.) for 2 hr. The excess of lithium aluminium hydride was destroyed by careful addition of water to the cooled reaction mixture. Tetrahydrofuran and water were removed and the residue extracted exhaustively with chloroform and acetone. Concentration of the extract gave a syrup, which was hydrolysed with 0·5N-sulphuric acid (10 ml.) for 4 hr. on a boiling-water bath. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H), and concentrated to a syrup (80 mg.). Paper chromatographic examination of this product indicated the presence of 2,3,4-tri-*O*-methyl-D-glucose [R_G 0·88 in solvent (c) and 0·69 in solvent (f)] as the main component. Gas–liquid chromatographic examination of the derived methyl glycosides confirmed the presence of 2,3,4-tri-*O*-methyl-D-glucose [(i), *T* 3·43, 3·38].

Concentration of the neutral *O*-methyl sugars yielded a syrup (3·9 g.). A portion (3·5 g.) of this syrup was chromatographed on a cellulose column (100 × 3·5 cm.) with light petroleum (b. p. 100—120°)–butan-1-ol (7 : 3, satd. with water) as eluant, to give five fractions, and with light petroleum (b. p. 100—120°)–butan-1-ol (1 : 1, satd. with water) as eluant, to give a further five fractions.

Fraction 1 (360 mg.) had $[\alpha]_D$ −18° (*c* 2·14). It had the same paper chromatographic mobility [R_G 0·99 in solvent (c) and 1·0 in solvent (f)] as 2,3,5-tri-*O*-methyl-L-arabinose. It was characterised by conversion into 2,3,5-tri-*O*-methyl-L-arabonamide, which, after recrystallisation from acetone, gave needle-shaped crystals, m. p. 135—136°.

Fraction 2 (65 mg.) had $[\alpha]_D$ +31° (*c* 1·30). Demethylation [25] gave galactose and arabinose. Gas–liquid chromatographic examination of the derived methyl glycosides indicated the presence of 2,3,4,6-tetra-*O*-methyl-D-galactose [(i), *T* 1·67] and 2,3,5-tri-[(i), *T* 0·58, 0·72], and 2,5-[(i), *T* 1·75, 3·25] and 3,5-di-*O*-methyl-L-arabinose [(i), *T* 1·05, 2·32].

Fraction 3 (685 mg.) had $[\alpha]_D$ +25° (*c* 1·06). Gas–liquid chromatographic examination of the derived methyl glycosides indicated that 2,5-[(i), *T* 1·78, 3·27] and 3,5-di-*O*-methyl-L-arabinose [(i), *T* 1·07, 2·33] were the main components of the mixture. A portion (500 mg.) of the syrup was chromatographed on Whatman No. 3MM papers in solvent (c) to obtain a syrupy mixture (313 mg.) of 2,5- and 3,5-di-*O*-methyl-L-arabinose. Paper electrophoretic examination confirmed the presence of 2,5-(M_G 0·00, 10 v/cm., 0·5 mA./cm.) and 3,5-di-*O*-methyl-L-arabinose [M_G 0·70, 10 v/cm., 0·5 mA./cm., brown spot (yellow under u.v.) with aniline oxalate]. These components were separated from the syrupy mixture (150 mg.) by continuous electrophoresis at 600 v on a sheet of Whatman No. 54 paper in 0·05M-borate buffer (pH 9·2) for 24 hr. Migration towards the anode of 3,5-di-*O*-methyl-L-arabinose as its borate complex permitted separation of this *O*-methyl sugar from its 2,5-di-*O*-methyl isomer into tubes at the bottom of the paper curtain. Tubes containing 2,5-di-*O*-methyl-L-arabinose were combined, borate was removed as methyl borate by repeated evaporation with methanol, and the sugar (35 mg.) characterised by conversion into 2,5-di-*O*-methyl L-arabonamide, which, after recrystallisation from ethyl acetate, had m. p. 124—126°. Tubes containing 3,5-di-*O*-methyl-L-arabinose were combined, borate was removed, and the sugar (27 mg.) characterised by conversion into 3,5-di-*O*-methyl-L-arabonamide, which, after recrystallisation from ethyl acetate, had m. p. 143—144°.

Fraction 4 (346 mg.) had $[\alpha]_D$ +95° (*c* 1·23). Paper chromatographic examination indicated the presence of 2,3,4-tri-*O*-methyl-L-arabinose [R_G 0·78 in solvent (c) and

0·55 in solvent (f)] in addition to 2,5- and 3,5-di-*O*-methyl-L-arabinose.

Fraction 5 (116 mg.) had $[\alpha]_D$ +93° (*c* 1·12). Gas–liquid chromatographic examination of the derived methyl glycosides indicated that 2,4,6- [(i), *T* (3·70), (4·10)], 2,3,6- [(i), *T* 2·88, (3·70), (4·10)] and 2,3,4-tri-*O*-methyl-D-galactose [(i), *T* 6·30] were the main components.

Fraction 6 (93 mg.) had $[\alpha]_D$ +100° (*c* 1·86). Conversion of a small portion (4 mg.) to the methyl glycosides followed by g.l.c. examination showed the presence of 3,4-di-*O*-methyl-L-arabinose [(i), *T* 1·91]. The remainder of the syrup was chromatographed on Whatman No. 3MM papers in solvent (c) to give a product (23 mg.). Demethylation [25] gave arabinose. On paper chromatographic and paper electrophoretic examination, it had R_G values of 0·55 in solvent (c) and 0·20 in solvent (f) and a M_G value of 0·21 (10 v/cm., 0·5 mA/cm.). A portion (5 mg.) was converted into the methyl glycosides and methylated with methyl iodide (0·5 ml.) and silver oxide (500 mg.) in *NN*-dimethylformamide (0·5 ml.). Gas–liquid chromatographic examination of the reaction mixture showed the presence of the methyl glycoside(s) of 2,3,4-tri-*O*-methyl-L-arabinose [(i), *T* 0·97].

Fraction 7 (146 mg.) had $[\alpha]_D$ +90° (*c* 0·80). Paper chromatographic examination in solvents (c) and (f) showed the presence of 2,4-di-*O*-methyl-D-galactose as the main component of the mixture.

Fraction 8 (56 mg.) had $[\alpha]_D$ +111° (*c* 1·12). This fraction was chromatographed on Whatman No. 3 MM papers in solvent (c) to give a product (19 mg.). Demethylation [25] gave arabinose. The *O*-methyl sugar had the same paper chromatographic mobility [R_G 0·35 in solvent (c) and 0·08 in solvent (f)] as an authentic specimen of 4-*O*-methyl-L-arabinose (kindly supplied by Dr. C. T. Bishop). It also had the same paper electrophoretic mobility [M_G 0·29, 10 v/cm., 0·4 mA/cm.] as the authentic sample. A portion (5 mg.) was converted to the methyl glycosides and methylated (cf. Fraction 6). Gas–liquid chromatographic examination of the reaction mixture indicated the presence of the methyl glycoside(s) of 2,3,4-tri-*O*-methyl-L-arabinose [(i), *T* 0·96].

Fraction 9 (66 mg.) had $[\alpha]_D$ +91° (*c* 1·32). Paper chromatographic examination in solvents (c) and (f) indicated the presence of 2-*O*-methyl-D-galactose as the main component of the mixture.

Fraction 10 (31 mg.) was shown by paper chromatography in solvents (a) and (b) to contain galactose and arabinose.

Methanolysis of a sample of methylated *A. arabica* gum, followed by g.l.c. examination of the mixture of methyl glycosides, indicated the presence of small amounts of 2,3,4-tri-*O*-methyl-L-rhamnose [(i), *T* 0·49], in addition to those *O*-methyl sugars already characterised from the cellulose column.

TABLE 3

Formic acid released (mmoles/g.) on periodate oxidations

Time (hr.):	1	3	6	12	24	48	72	96
A. arabica gum ...	0·14	0·20	0·25	0·32	0·36	0·49	0·60	0·69
Polysaccharide (I)	0·37	0·40	0·40	0·42	0·43			
Polysaccharide (II)	0·79	0·80	0·84	0·91	0·95			

Preparation, Partial Acid Hydrolysis, and Methylation of Polysaccharide (I).—Borohydride reduction of periodate-oxidised whole gum (40·0 g.), followed by controlled acid hydrolysis at room temperature for 2 days, gave polysaccharide (I) (23·4 g., yield, 59%), $[\alpha]_D$ +63° (*c* 1·04) (Found: galactose, 41%; arabinose, 59%). Results for the release of formic acid with time on periodate oxidation of whole gum are shown in Table 3. The amount of periodate consumed by whole gum in 96 hr. was 3·01 mmoles/g.

Partial acid hydrolysis of polysaccharide (I) (20 mg.), followed by paper chromatographic examination of the hydrolysate indicated the presence of galactose, arabinose, two neutral disaccharides with the mobilities of 3-*O*-β-D-galactopyranosyl-D-galactose [R_{gal} 0·49 in solvent (a), 0·29 in solvent (b), and 0·53 in solvent (e), minor component] and 6-*O*-β-D-galactopyranosyl-D-galactose [R_{gal} 0·30 in solvent (a), 0·20 in solvent (b), and 0·38 in solvent (e), major component], and higher neutral oligosaccharides.

Polysaccharide (I) (308 mg.) was methylated to give a product (222 mg.), $[\alpha]_D$ +43° (*c* 1·48 in $CHCl_3$) (Found: OMe, 39·9%, not raised on further attempted methylation). Methanolysis of a sample of this product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table 4. Acid hydrolysis of the methyl glycosides, followed by paper chromatographic examination of the free sugars, confirmed these results.

Preparation, Partial Acid Hydrolysis, and Methylation of Polysaccharide (II).—Borohydride reduction of periodate-oxidised polysaccharide (I) (22·5 g.), followed by controlled acid hydrolysis at room temperature for 2 days, gave polysaccharide (II) (9·8 g., yield, 44%), $[\alpha]_D$ +51° (*c* 0·86) (Found: galactose, 50%; arabinose, 50%). Results for the release of formic acid with time on periodate oxidation of polysaccharide (I) are shown in Table 3. The amount of periodate consumed by polysaccharide (I) in 24 hr. was 2·44 mmoles/g.

Partial acid hydrolysis of polysaccharide (II) (20 mg.), followed by paper chromatographic examination of the hydrolysate indicated the presence of galactose, arabinose, two neutral disaccharides with the mobilities of 3-*O*-β-D-galactopyranosyl-D-galactose [R_{gal} 0·45 in solvent (a) and 0·53 in solvent (e), minor component] and 6-*O*-β-D-galactopyranosyl-D-galactose [R_{gal} 0·26 in solvent (a) and 0·40 in solvent (e), major component], and higher neutral oligosaccharides.

TABLE 4

O-Methyl sugars present in methylated polysaccharides (I)—(IV)

O-Methyl sugar	(I)	(II)	(III)	(IV)
2,3,5-Tri-*O*-methyl-L-arabinose	++	++	+	+
2,5-Di-*O*-methyl-L-arabinose ...	++	+	+	+
3,5-Di-*O*-methyl-L-arabinose ...	+++	++	+	+
2,3,4,6-Tetra-*O*-methyl-D-galactose	Trace	Trace	+	+
2,4,6-Tri-*O*-methyl-D-galactose	+	+	++	++
2,3,4-Tri-*O*-methyl-D-galactose	Trace	+	+	+
2,4-Di-*O*-methyl-D-galactose ...	++	++	+	+

Polysaccharide (II) (258 mg.) was methylated to give a product (152 mg.), $[\alpha]_D$ +30° (*c* 1·14 in $CHCl_3$) (Found: OMe, 42·1%, not raised on further attempted methylation). Methanolysis of a sample of this product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table 4. Acid hydrolysis of the methyl glycosides, followed by paper chromatographic examination of the free sugar, confirmed these results.

Preparation, Partial Acid Hydrolysis, and Methylation of Polysaccharide (III).—Borohydride reduction of periodate-oxidised polysaccharide (II) (8·50 g.), followed by controlled acid hydrolysis at room temperature for 2 days gave polysaccharide (III) (1·40 g., yield, 17%), $[\alpha]_D$ +46° (*c* 1·06) (Found: galactose, 72%; arabinose, 28%). Results for the release of formic acid with time on periodate oxidation of polysaccharide (II) are shown in Table 3. The amount of periodate consumed by polysaccharide (II) in 24 hr. was 3·28 mmoles/g.

Partial acid hydrolysis of polysaccharide (III) (20 mg.), followed by paper chromatographic examination of the hydrolysate indicated the presence of galactose, arabinose, two neutral disaccharides with the mobilities of 3-*O*-β-D-galactopyranosyl-D-galactose [R_{gal} 0·56 in solvent (e), minor component] and 6-*O*-β-D-galactopyranosyl-D-galactose [R_{gal} 0·43 in solvent (e), major component], and higher neutral oligosaccharides.

Polysaccharide (III) (110 mg.) was methylated to give a product (76 mg.), $[\alpha]_D$ +26° (*c* 0·90 in $CHCl_3$) (Found: OMe, 42·9%, not raised on further attempted methylation). Methanolysis of a sample of this product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table 4. Acid hydrolysis of the methyl glycosides, followed by paper chromatographic examination of the free sugars, confirmed these results.

Preparation and Methylation of Polysaccharide (IV).—Borohydride reduction of periodate-oxidised polysaccharide (III) (1·00 g.), followed by controlled acid hydrolysis at room temperature for 2 days, gave polysaccharide (IV) (25 mg., yield, 2·5%). Acid hydrolysis yielded galactose and arabinose. The amount of formic acid released on periodate oxidation of polysaccharide (III) in 48 hr. was 0·99 mmoles/g. The corresponding amount of periodate consumed was 3·58 mmoles/g.

Polysaccharide (IV) (20 mg.) was dissolved in *NN*-dimethylformamide (5 ml.) and methylation was carried out by shaking with methyl iodide (5 ml.) and silver oxide (2 g.). Methanolysis of the methylated product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table 4.

Molecular-sieve Chromatography.—Figure 3 shows the elution patterns obtained for *A. arabica* gum, polysaccharides (I), (II), and (III), and degraded gum A. The elution volumes for both *A. arabica* gum and polysaccharide (I) precede the " exclusion volume " of the column as indicated by the elution volume for " blue dextran." This behaviour is indicative of very high molecular-weights for these two polysaccharides. The elution pattern for polysaccharide (II) indicates the presence of a high proportion of low molecular-weight material, while the elution volume for polysaccharide (III) suggests an estimated value [2-4,15] for the number-average molecular-weight of less than 2000. An estimated value [2-4,15] of 5400 was obtained for the number-average molecular-weight of degraded gum A.

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46.

STUDIES ON URONIC ACID MATERIALS

PART XXII*. THE COMPOSITION OF THE GUM FROM *Acacia drepanolobium* HARMS EX SJŐSTED

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INTRODUCTION

A preliminary study[2] showed that *Acacia drepanolobium* is a further example (*cf. A. nilotica*[3], *A. nubica*[4], and *A. arabica*[1]) of an *Acacia* gum having a positive specific rotation and a low content of L-rhamnose that does not bear a unit molar correspondence to the uronic acid content. It was also noted that *A. drepanolobium* gum does not dissolve completely in cold water, giving about 20% of a water-insoluble gel that can be dispersed in cold, dilute, alkaline solution.

The solubility difficulty associated with some commercial specimens of gum arabic (*A. senegal* syn. *verek*) has received comment recently[5]. The reason for the occasional formation of mucilaginous gels by some *Acacia* species is not known. We have examined many specimens of *A. senegal* gum, but they have not given sufficient quantities of the insoluble gel to facilitate its study. In contrast, *A. drepanolobium* conveniently provides considerable proportions of water-insoluble material, and we now present the results of an analytical study of the differences in composition between the water-soluble gum, a fraction soluble in dilute sodium chloride solution, and the insoluble gel.

EXPERIMENTAL

Origin of specimens. — Nodules of the gum from *Acacia drepanolobium* Harms ex Sjősted were collected by Mr. W. M. C. Bagshawe, Provincial Forest Officer, at Tabora, Western Province, Tanganyika, in July, 1961.

Analytical methods. — The standard methods involved have been described previously[6].

Fractionation procedure. — Two portions of crushed, bark-free, *A. drepanolobium* gum (*ca.* 20 g) were each stirred with distilled water (500 ml) for 12 h; the soluble fraction (*A*) was collected by filtration (muslin, and then paper), dialysed against tap water, and then electrodialysed. The residue was stirred with M sodium chloride solution (500 ml) for 12 h; the salt-soluble fraction (*B*) was collected by filtration, dialysed against tap water, and then electrodialysed. The residual insoluble gel

*For Part XXI, see Ref. 1.

(Fraction *C*) was dialysed against tap water. The three fractions were freeze-dried from aqueous solutions or dispersions. The percentage yields of the three fractions are shown in Table I.

Viscosity measurements. — Determinations on Fractions *A* and *B* were made in M sodium chloride solution in an Ubbelohde dilution viscometer at 25° (solvent flow-time, 193.6 sec). Fraction *C* was studied in M sodium hydroxide solution at 25° (solvent flow-time, 215.2 sec).

Specific rotations. — Fractions *A* and *B* were studied in distilled water and in M sodium hydroxide; Fraction *C* was studied in M sodium hydroxide. Specific rotations of sugars were measured in aqueous solutions. All measurements were made at $19 \pm 1°$.

Periodate oxidations. — These were performed at room temperature in darkness by mixing equal volumes of 4% polysaccharide solutions and 0.25M sodium metaperiodate. The formic acid released was titrated (Methyl Red) with standard sodium hydroxide. The reduction of periodate was determined with standard arsenite solution. Periodate oxidation of oligosaccharides was performed at room temperature in darkness, using excess of sodium metaperiodate. Formaldehyde was estimated colorimetrically with chromotropic acid[7]. Periodate oxidations of *O*-methyl sugars were carried out by the method of Lemieux and Bauer[8].

Paper chromatography. — Whatman No. 1 and 3MM papers were used with the following solvent systems (v/v):(*a*) benzene–butyl alcohol–pyridine–water (1:5:3:3, upper layer); (*b*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); (*c*) ethyl acetate–acetic acid–formic acid–water (18:8:3:9); (*d*) butyl alcohol–ethanol–water (4:1:5, upper layer); (*e*) ethyl acetate–pyridine–water (10:4:3); (*f*) butanone–acetic acid–water (9:1:1, saturated with boric acid); (*g*) butanone–water–ammonia (sp. gr. 0.880) (200:17:1). Chromatograms were developed as described previously[6], except that D-glucose was detected by the following enzymic method. Chromatograms were sprayed, in succession, with 0.5M phosphate buffer (pH 7.0), 0.05% peroxidase, 0.05% glucose oxidase, and 0.1% *o*-dianisidine in 90% aqueous ethanol. Glucose oxidase specifically oxidises D-glucose, giving hydrogen peroxide, which is coupled with *o*-dianisidine by peroxidase, giving a purple spot. R_{Gal}, R_G, and R_{Rha} values refer to distances moved relative to D-galactose, 2,3,4,6-tetra-*O*-methyl-D-glucose, and L-rhamnose, respectively.

Gas–liquid chromatography. — G.l.c. of mixtures of *O*-methyl sugars was performed on a chromatograph (Type S3A, Gas Chromatography Ltd.) fitted with flame-ionisation detectors, at nitrogen flow-rates of *ca.* 100 ml/min on columns of (*i*) 15% by weight of poly(butane-1,4-diol succinate) on Celite (120 × 0.5 cm) at 175°, (*ii*) 15% by weight of poly(ethylene glycol adipate) on Celite (75 × 0.5 cm) at 160°. Retention times (*T*) are quoted relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside.

*Conversion of 4-*O*-methyl-D-glucuronic acid into 4-*O*-methyl-D-glucose.* — 4-*O*-Methyl-D-glucuronic acid (29 mg) was converted into the methyl ester methyl glycoside by treatment for 8 h with boiling, 5% methanolic hydrogen chloride. The resulting solution was neutralised with silver carbonate and filtered, and the residue was washed

TABLE I

ANALYTICAL DATA[a] FOR *Acacia drepanolobium*, FRACTIONS *A*, *B*, AND *C*

	First series of extractions			*Second series of extractions*		
	A	B	C	A	B	C
Recovery, %	79	8	13	80	8	12
Ash, %	0.00	0.00	2.78	0.00	0.00	2.56
Nitrogen, %	1.11	1.21	0.58	1.18	1.24	0.61
$[\eta]$, cm^3g^{-1}	17.8	19.5	22.8	15.0	17.2	20.2
Methoxyl, %	0.43	0.43	0.41	0.42	0.42	0.42
$[\alpha]_D$ (*c* 1.0, water)[b]	+77.8°	+77.2°	—	+82.2°	+82.6	—
$[\alpha]_D$ (*c* 1.0, N sodium hydroxide)[b]	+77.2°	+78.2°	+78.0°	+81.5°	+81.9°	+80.0°
Equivalent weight [b,c]	1976	1997	—	1985	1980	—
Uronic anhydride, %[b,d]	8.9	8.8	—	8.8	8.8	—
Uronic anhydride, %[b,e]	9.6	9.9	9.7	9.5	9.8	9.8
Formic acid released on periodate oxidation (moles/g × 10^3)[b]	1.07	1.05	1.02	1.06	1.08	1.05
Periodate reduced (moles/g × 10^3)[b]	3.96	3.97	3.93	3.95	3.98	3.96
L-Arabinose, %[b]	52	51	49	53	53	51
D-Galactose, %[b]	38	39	41	37	37	39
L-Rhamnose, %[b,f]	1	1	1	1	1	1
D-Glucuronic acid, %[b,g]	7	7	7	7	7	7
4-*O*-Methyl-D-glucuronic acid, %[b,g]	2	2	2	2	2	2

[a]All data corrected to a dry-weight basis; [b]data corrected for all non-carbohydrate material; [c]direct titration (potentiometric) with 0.02N sodium hydroxide; [d]assuming that all acidity arises from uronic acid groups; [e]by acid decarboxylation (Ref. 14); [f]by acetaldehyde released on periodate oxidation (Ref. 15); [g]assuming that all the methoxyl content arises from 4-*O*-methyl-D-glucuronic acid.

with warm methanol. The extract was evaporated to dryness, and dissolved in distilled water (10 ml). This solution was mixed with sodium borohydride (0.25 g in 25 ml of water) and kept overnight. Excess of borohydride was destroyed by the addition of Amberlite IR-120 (H^+) resin. The resin and the solvent were removed, and the borate was removed as methyl borate. The product (4-*O*-methyl-D-glucose) was purified on sheets of filter paper with solvent (*a*).

Borohydride reduction of aldobiouronic acids. — Sodium borohydride (25 mg in 5 ml of water) was added dropwise to the aldobiouronic acid (5–10 mg), and the mixture was kept overnight. Excess of borohydride was destroyed by the addition of Amberlite IR-120 (H^+). The resin and the solvent were removed, and the borate was volatilised as methyl borate. The product was boiled with 5% methanolic hydrogen chloride for 4 h, the solution was neutralised (silver carbonate) and filtered, and the residue was washed with warm methanol. The extract was evaporated to dryness, the residue was dissolved in distilled water (10 ml), and sodium borohydride (25 mg in 5 ml of water) was added. After storage overnight, excess of borohydride was destroyed, and borate was removed as before; the reduced product, (glucopyranosylgalactitol and/or 4-*O*-methylglucopyranosylgalactitol) was isolated as a syrup.

Methylation of aldobiouronic acids. — The micro Kuhn method developed by Perila and Bishop[9] was used. The aldobiouronic acid (10 mg) was shaken with methyl iodide (1 ml), *N,N*-dimethylformamide (1 ml), and silver oxide (1 g) at room temperature, in darkness, for 18 h. The mixture was filtered, the residue washed with chloroform, and the filtrate evaporated to dryness. The product was boiled with 5% methanolic hydrogen chloride for 8 h. The resulting methyl glycosides were analysed by g.l.c. A portion of the methanolysate was evaporated to dryness, and reduced with sodium borohydride (25 mg in 5 ml of water) for 12 h. The methyl glycosides were hydrolysed with N sulphuric acid for 4 h at 100°, and the methylated reducing sugars were examined by paper chromatography in solvents (*d*) and (*g*).

RESULTS

Comparative study of Fractions A, B, *and* C. — Hydrolysis (N sulphuric acid, 7 h) of each fraction, followed by paper-chromatographic examination, showed the presence of galactose, arabinose, and acidic disaccharides. Paper chromatograms, examined in ultraviolet light after spraying with aniline hydrogen oxalate, revealed that a trace of rhamnose was present in each fraction. After more drastic hydrolysis (2N sulphuric acid, 7 h), examination in solvent (*b*) showed that D-glucuronic acid, D-glucurono-6,3-lactone, and 4-*O*-methyl-D-glucuronic acid were present in each fraction.

Analytical data for the fractions are shown in Table I. Methylated sugars were not detected in the gum hydrolysates; if the methoxyl content of the gum is assumed to be located solely in residues of 4-*O*-methyl-D-glucuronic acid, calculation shows that this acid is present in each fraction to the extent of 2%.

Periodate oxidation of each fraction was followed by simultaneous determination

(2-ml aliquots) of formic acid and sodium metaperiodate. Oxidation of all fractions was complete after 48 h. For Fraction *C*, the oxidation mixtures did not become homogeneous until after 18 h.

The intrinsic viscosities shown in Table I were measured within a few days after isolation of the fractions. When the measurements were repeated after 2 months, it was found that the intrinsic viscosity of each fraction had increased, and a further increase was apparent after storage for 6 months in the free-acid form. This phenomenon is under investigation.

Study of Fraction A. — *A. drepanolobium* gum (160 g) was extracted with cold water, as described above, to yield Fraction *A* (130 g), which contained 10% moisture and 7% protein.

The neutral sugars of Fraction A. — The polysaccharide (0.75 g) was hydrolysed (N sulphuric acid, 7 h). The hydrolysate was fractionated on Whatman 3MM sheets for 36 h with solvent (*a*); crystalline D-galactose and L-arabinose were isolated.

D-Galactose (114 mg) had $[\alpha]_D^{19}$ +81° (equil., *c* 1.0) and, after recrystallisation from ethanol, m.p. and mixed m.p. 168°. The X-ray diffractogram was identical with that of an authentic specimen.

L-Arabinose (180 mg) had $[\alpha]_D^{19}$ +105° (equil., *c* 1.0) and, after recrystallisation from aqueous ethanol, m.p. and mixed m.p. 159°. The X-ray diffractogram was identical with that of an authentic specimen. After recrystallisation from water, the phenylosazone had m.p. and mixed m.p. 165°.

Fraction *A* (3 g) was hydrolysed (N sulphuric acid, 7 h). The hydrolysate was fractionated on Whatman 3MM sheets for 16 h with solvent (*a*). The position of rhamnose was located by spraying side-strips on which L-rhamnose had been run as a marker. Elution of the appropriate zone with water gave L-rhamnose (21 mg) having $[\alpha]_D^{19}$ +6° (*c* 0.21). After recrystallisation from aqueous ethanol, the L-rhamnose had m.p. and mixed m.p. 93–94°.

The acidic sugars of Fraction A. — The polysaccharide (10 g) was hydrolysed for 7 h with N sulphuric acid. Paper chromatography of the hydrolysate in solvent (*b*) indicated the presence of galactose, arabinose, and two acidic fractions (I) and (II) having R_{Gal} values of 0.26 and 0.59: these acidic fractions were isolated as syrups from filter sheets developed in solvent (*b*).

Fraction I (147 mg) was examined by paper chromatography in solvent (*b*) for 72 h; two acidic components were observed, having R_{Gal} 0.23 and 0.29, respectively. Similarly, paper chromatography in solvent (*c*) for 24 h gave two acidic components (aldobiouronic acids *D* and *E*) having R_{Gal} 0.56 and 0.61. Fraction I was hydrolysed (2N sulphuric acid, 7 h); paper chromatography of the hydrolysate in solvent (*b*) showed *ca.* equal amounts of galactose and glucurono-6,3-lactone, accompanied by traces of glucuronic acid and unhydrolysed aldobiouronic acids *D* and *E*. Crystalline D-galactose and D-glucurono-6,3-lactone were isolated from Whatman 3MM sheets developed in solvent (*b*). D-Galactose (50 mg) had $[\alpha]_D^{19}$ +80° (equil., *c* 1.0) and m.p. and mixed m.p. 167°, after recrystallisation from ethanol. D-Glucurono-6,3-lactone (45 mg) had $[\alpha]_D^{19}$ +19° (equil., *c* 0.9); after recrystallisation from

aqueous ethanol, it had m.p. and mixed m.p. 176°, with an X-ray diffractogram identical with that of authentic material.

Fraction II (78 mg) was examined by paper chromatography in solvent (*b*) for 72 h; two acidic components were observed, having R_{Gal} 0.56 and 0.66. Similarly, paper chromatography in solvent (*c*) for 24 h gave two acidic components (aldobiouronic acids *F* and *G*) having R_{Gal} 0.90 and 0.94. Fraction II was hydrolysed (2N sulphuric acid, 7 h); paper chromatography of the hydrolysate in solvent (*b*) showed *ca.* equal amounts of galactose and 4-*O*-methylglucuronic acid, accompanied by traces of unhydrolysed aldobiouronic acids *F* and *G*. Crystalline D-galactose and pure 4-*O*-methylglucuronic acid were isolated from 3MM sheets developed in solvent (*b*). D-Galactose (25 mg) had $[\alpha]_D^{19}$ +80° (equil., *c* 0.5) and m.p. and mixed m.p. 168°. 4-*O*-Methylglucuronic acid (31 mg) had $[\alpha]_D^{18}$ +36° (*c* 0.62); borohydride reduction of the methyl ester gave 4-*O*-methylglucose, which was purified on filter sheets with solvent (*a*) and isolated as a syrup. 4-*O*-Methylglucose had R_{Gal} 1.92 and R_{Rha} 0.90 in solvent (*a*), R_{Gal} 2.19 and R_{Rha} 0.84 in solvent (*b*), and R_{Gal} 1.74 and R_{Rha} 0.93 in solvent (*d*). After recrystallisation from aqueous acetone, the phenylosazone had m.p. 156–158° (lit[10], 158–159°).

The aldobiouronic acids of Fraction A. — The hydrolysate from Fraction *A* was fractionated [solvent (*b*), 96 h] on 3MM filter sheets; four aldobiouronic acids *D*,*E*,*F*, and *G* were isolated as syrups, and identified as follows.

Acid D (102 mg) had R_{Gal} 0.23 [solvent (*b*)] and R_{Gal} 0.56 [solvent (*c*)], and was chromatographically identical with 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose in these solvents. The syrup had $[\alpha]_D^{19}$ −3° (*c* 1.02), and this suggests the presence of a β-D-linkage. The syrup (5 mg) was hydrolysed (2N sulphuric acid), and paper chromatography [solvent (*b*)] revealed *ca.* equal amounts of D-galactose and D-glucurono-6,3-lactone. Acid *D* (10 mg) was reduced, using borohydride, as described above. The resulting hydrolysate was examined chromatographically [solvent (*f*)]; *ca.* equal amounts of glucose and galactitol were detected. The oxidation of known weights (3–5 mg) of acid *D* with excess of sodium metaperiodate did not give formaldehyde. Acid *D* (10 mg) was methylated by the Kuhn method, and the methyl glycosides produced on methanolysis of the product were examined by g.l.c.; the products were 2,3,4-tri-*O*-methyl-D-glucuronic acid [column (*i*), *T* 2.33, 2.96; column (*ii*), *T* 2.08, 2.70]; 2,3,4-tri-*O*-methyl-D-galactose [column (*i*), *T* 6.59; column (*ii*) *T* 5.25]; and a trace of 2,3,5-tri-*O*-methyl-D-galactose [column (*i*), *T* 4.09, 4,50; column (*ii*), *T* 3.44, 4.14]. A portion of the methanolysate was reduced with sodium borohydride; after removal of methyl glycosides by mild hydrolysis with acid, the products were examined chromatographically in solvent (*d*). This revealed 2,3,4-tri-*O*-methyl-D-galactose (red spot, R_G 0.72) and a red–black spot (R_G 0.84) corresponding to 2,3,4-tri-*O*-methyl-D-glucose and 2,3,5-tri-*O*-methyl-D-galactose. Double development (solvent (*g*)] separated the 2,3,4-tri-*O*-methyl-D-glucose (red spot) from 2,3,5-tri-*O*-methyl-D-galactose (black spot). These experiments led to the identification of acid *D* as 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose.

Acid E (91 mg) had R_{Gal} 0.29 [solvent (*b*)] and R_{Gal} 0.61 [solvent (*c*)], and

was chromatographically identical with 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose. The syrup had $[\alpha]_D^{20}$ +106° (*c* 0.91) and this suggests an α-D linkage. The syrup (5 mg) was hydrolyzed (2N sulphuric acid), and paper chromatography [solvent (*b*)] showed *ca.* equal amounts of D-galactose and glucurono-6,3-lactone. Acid *E* (5–10 mg) was reduced, using borohydride, as described above. The resulting glucosylgalactitol was hydrolysed (N sulphuric acid), and the hydrolysate was examined chromatographically [solvent (*f*)]; *ca.* equal amounts of glucose and galactitol were detected. The oxidation of known weights (3–5 mg) of acid *B* with excess of sodium metaperiodate gave 1.01 moles of formaldehyde per mole of acid *E*. Acid *E* (10 mg) was methylated by the Kuhn method, and the methyl glycosides produced on methanolysis of the product were examined by g.l.c.; the products were 2,3,4-tri-*O*-methyl-D-glucuronic acid [column (*i*), *T* 2.40, (2.99); column (*ii*), *T* 2.04, 2.70] and 2,3,6-tri-*O*-methyl-D-galactose [column (*i*), *T* (2.99), 3.88, and 4.23; column (*ii*), *T* 2.42, 3.07, and 3.41]. A portion of the methanolysate was reduced with sodium borohydride; after removal of methyl glycosides by mild hydrolysis with acid, the products were examined chromatographically in solvent (*d*). This revealed 2,3,6-tri-*O*-methyl-D-galactose (R_G 0.76) and 2,3,4-tri-*O*-methyl-D-glucose (R_G 0.84). Double development [solvent (*g*)] revealed the same *O*-methyl sugars. These experiments led to the identification of acid *E* as 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose.

Acid F (42 mg) had R_{Gal} 0.56 [solvent (*b*)] and R_{Gal} 0.90 [solvent (*c*)], and was chromatographically identical with 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose. The syrup had $[\alpha]_D^{19}$ +3° (*c* 0.42) (for lit. values, see Ref. 5); this suggests a β-D linkage. The syrup (5 mg) was hydrolysed; paper chromatography [solvent (*b*)] showed *ca.* equal amounts of D-galactose and 4-*O*-methylglucuronic acid. Acid *F* (5–10 mg) was reduced with borohydride to give a 4-*O*-methylglucosylgalactitol, and the hydrolysate was examined chromatographically [solvents (*a*) and (*f*)]; *ca.* equal amounts of galactitol and 4-*O*-methylglucose were detected. The remainder of the hydrolysate was oxidised by the method of Lemieux and Bauer[8], and the products were examined in solvent (*a*). Spraying with aniline hydrogen phthalate revealed 2-*O*-methylerythrose, a characteristic product of 4-*O*-methylglucose, as a yellow-green spot (R_F 0.55). Known weights of acid *F* (3–5 mg) did not give formaldehyde when oxidised with excess of metaperiodate. On methylation, acid *F* gave the same methylated sugars as acid *D*. These experiments led to the identification of acid *F* as 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose.

Acid G (31 mg) had R_{Gal} 0.66 [solvent (*b*)] and R_{Gal} 0.94 [solvent (*c*)], and was chromatographically identical with 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose. The syrup (5 mg) was hydrolysed; paper chromatography [solvent (*b*)] showed *ca.* equal amounts of D-galactose and 4-*O*-methyl-D-glucuronic acid. Acid *G* (5–10 mg) was reduced with borohydride to a 4-*O*-methylglucosylgalactitol; the hydrolysate in solvents (*a*) and (*f*) showed *ca.* equal amounts of 4-*O*-methylglucose and galactitol. The hydrolysate was periodate oxidised[8], and the aniline hydrogen phthalate spray revealed the presence of a 2-*O*-methylerythrose. Periodate oxidation led to the production of 0.96 moles of formaldehyde per mole of acid *G*. On methylation,

acid *G* gave the same methylated sugars as acid *E*. These experiments led to the identification of acid *G* as 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose.

The aldobiouronic acids of fractions B *and* C. — After hydrolysis under the same conditions as for Fraction *A*, fractions *B* and *C* were examined chromatographically and were found to contain similar proportions of the four aldobiouronic acids identified in Fraction *A*.

DISCUSSION

Acacia drepanolobium gum has been shown to contain the same sugars, and the same four aldobiouronic acids, as other gums of the genus *Acacia* having high, positive rotations (*cf.*, *A. nilotica*[3], *A. arabica*[1], *A. nubica*[4], and *A. seyal*[4]).

A study of the analytical data presented in Table I shows that there are no striking differences in the composition of the gum fractions *A* (water-soluble), *B* (salt-soluble) and *C* (water- and salt-insoluble, but soluble to the extent of 1% in molar sodium hydroxide). Fractions *A* and *B* have an arabinose–galactose ratio of $52 \pm 1{:}38 \pm 1$, and this must be compared with the ratio of $50 \pm 1{:}40 \pm 1$ for Fraction *C*; although this may not be analytically significant, some small differences may be sufficient to account for the solubility differences exhibited by Fractions *A*, *B*, and *C*. It is possible that the differences may involve some variation in fine structure or degree of branching, as in the case with soluble and insoluble laminaran[11]. Although the sugar composition and periodate oxidation data for Fractions *A*, *B*, and *C* are in good agreement, structural differences between these fractions cannot be discounted until a full structural study has been made.

The most significant analytical differences between Fractions *A*, *B*, and *C* lie in the ash and protein contents of the purified fractions. The high ash content of Fraction *C* may reflect the difficulty encountered in purifying an aqueous dispersion, as opposed to a true solution. It is possible, however, that the gel formation shown by Fraction *C* is a consequence of crosslinking effects arising from the presence of polyvalent cations, although no foundation for this theory was found in experiments with *A. senegal* gum[12].

It is of interest that the nitrogen content of Fraction *C* is only *ca.* 50% of that present in Fractions *A* and *B*. Protein–carbohydrate interaction has been offered in the past as a possible explanation of gel formation; the jelly isolated from *Acacia pycnantha*[13] contained three times as much nitrogen as the water-soluble gum.

A further explanation of the physical differences between Fractions *A*, *B*, and *C* may involve the molecular size and/or shape of the polysaccharides involved: Table I shows that the intrinsic viscosity of Fractions *A*, *B*, and *C* increases in the same order as the increase in insolubility. Physico-chemical studies of Fractions *A*, *B*, and *C* will be carried out in due course.

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SUMMARY

The gum polysaccharide from *Acacia drepanolobium* is not completely water-soluble, and has been subjected to graded extractions giving Fractions *A* (cold-water soluble, 80%), *B* (salt-soluble, 8%), and *C* (a gel soluble in dilute alkali, 12%). Fraction *A* contains L-arabinose (52%), D-galactose (38%), L-rhamnose (1%), D-glucuronic acid (7%), and 4-*O*-methyl-D-glucuronic acid (2%). Fractions *A*, *B*, and *C* each contain four aldobiouronic acids, *viz.*, 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose; 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose; 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose; and 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose.

The analytical differences between fractions *A*, *B*, and *C* have been investigated, and the factors that may account for their physical differences are discussed.

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Studies on uronic acid materials
Part XXIII.[1] Possible degradations with the sodium hydride–methyl iodide–methyl sulphoxide methylation system

Anderson and Cree[2] have described the potential use of the sodium hydride–methyl iodide–methyl sulphoxide system for methylating acidic mono- and disaccharides, and also for some acidic polysaccharides. The method appeared to offer advantages when compared with the classical Haworth and Purdie methods, but it was suggested[2] that the method should be evaluated with a wider range of polysaccharides before general conclusions could be reached.

Following a communication[3] that the sodium hydride system caused undesirable degradation of dextrans with the formation of abnormally high yields of 2,3,4,6-tetra-*O*-methyl-D-glucose, we have now compared the methylation products, obtained with sodium hydride, of a potato starch and of three different *Acacia* gum polysaccharides, with their respective methylated products obtained by treatments with the Haworth and Purdie reagents.

EXPERIMENTAL

Methylations with the sodium hydride–methyl iodide–methyl sulphoxide system, and the analyses by paper and gas chromatography, were carried out by the procedures and apparatus described previously[2].

RESULTS

Experiments with *Acacia nubica* gum showed that two additions of sodium hydride and methyl iodide were required to give complete methylation (OMe, 40.3%); the methoxyl content after 1 addition was only 16.3%. Lithium hydride was less effective (OMe after 1 treatment, 11.5%) than sodium hydride. Calcium hydride was ineffective when used to replace the sodium hydride, and stable emulsions were formed (*cf.* the effect of barium salts[2]).

Potato starch. — A laboratory sample of potato starch (var. Kerr's Pink) was methylated exhaustively with (*a*) the sodium hydride–methyl iodide–methyl sulphoxide system, and (*b*) several additions of the Haworth and the Purdie reagents. After methanolysis, the methyl glycosides of products (*a*) and (*b*) were examined by g.l.c.; the chromatograms were identical, except that product (*a*) showed a small peak caused by a trace of residual methyl sulphoxide.

Acacia drepanolobium *gum.* — This gum polysaccharide contains[1] D-galactose (38%), L-arabinose (52%), L-rhamnose (1%), D-glucuronic acid (7%), and 4-*O*-methyl-D-glucuronic acid (2%). The gum was methylated by methods (*a*) and (*b*), as above. After methanolysis of each product, the chromatograms for products (*a*) and (*b*) were identical, except for the presence in (*a*) of a trace of methyl sulphoxide.

Acacia campylacantha *gum.* — This gum polysaccharide contains[4] D-galactose (56%), L-arabinose (28%), L-rhamnose (7%), D-glucuronic acid (7%), and 4-*O*-methyl-D-glucuronic acid (2%). The chromatograms of the methanolysed, fully methylated gum obtained by methods (*a*) and (*b*) were similar in all respects other than that the proportion of 2,3,4-tri-*O*-methyl-L-rhamnose in product (*a*) was slightly less than in product (*b*).

Acacia senegal *gum.* — This gum polysaccharide contains[5] D-galactose (39%), L-arabinose (28%), L-rhamnose (14%), D-glucuronic acid (17.5%), and 4-*O*-methyl-D-glucuronic acid (1.5%). The gum was methylated by methods (*a*) and (*b*), as above. When the chromatograms of the methanolysate were compared, the proportion of 2,3,4 tri-*O*-methyl-L-rhamnose in product (*a*) was only about 33% of that in product (*b*). The proportion of 2,3-di-*O*-methyl-D-glucuronic acid also appeared to be less in product (*a*) than in (*b*).

DISCUSSION

Base-catalysed reactions proceed at greatly increased rates in methyl sulphoxide, in which the strongly basic methylsulphinyl carbanion is readily formed[6]; the methyl sulphoxide is a reactant, as well as being the solvent. There is no doubt that the sodium hydride–methyl iodide–methyl sulphoxide system is much more strongly alkaline in reaction[7] than a well-conducted Haworth methylation.

Hamilton and Thompson[8] have shown that alkaline hydrolysis of gum arabic (*Acacia senegal*) leads to the loss of residues of L-rhamnose and D-glucuronic acid. At least some of the L-rhamnopyranose residues in gum arabic were shown[9] to be

(1→4)-linked to D-glucuronic acid residues; subsequent structural studies[5,10] have confirmed earlier indications[11] that all of the L-rhamnopyranose residues are present as non-reducing end-groups.

It appears that those *Acacia* gum exudates (*e.g.*, *A. nubica* and *A. drepanolobium*) having low L-rhamnose and low methoxyl contents (and therefore having the majority of their D-glucuronic acid residues as non-reducing end-groups) are reasonably stable under the conditions described[2] for methylation by the sodium hydride–methyl sulphoxide–methyl iodide system. In contrast, those *Acacia* gum polysaccharides having significant proportions of L-rhamnopyranose residues as end groups attached to position 4 of D-glucuronic acid residues (*e.g.*, *A. senegal*) appear to be much less stable; loss of 2,3,4-tri-*O*-methyl-L-rhamnose, and also probably 2,3-di-*O*-methyl-D-glucuronic acid, occurs. This may be explained in terms of a β-elimination at C-4 of the glucuronic acid; such an elimination did not occur[2] with *A. nubica* gum.

Although our results show that the sodium hydride–methyl sulphoxide methylation system does offer certain advantages for some polysaccharides, we must repeat here the suggestion[2] that its performance should be assessed with care on a wider range of polysaccharides. All too frequently, structural studies are based on the product from only one methylation system; methylation by more than one method should be attempted, so that the results can be compared.

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STUDIES ON URONIC ACID MATERIALS

PART XXIV*. AN ANALYTICAL STUDY OF DIFFERENT FORMS OF THE GUM FROM *Acacia senegal* WILLD.

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ABSTRACT

Some of the analytical parameters for *Acacia senegal* gum have been determined for (*a*) twelve "bulk" samples of tapped gum from three different districts of the Sudan, and (*b*) thirteen, different, single-nodule specimens of "natural exudate" gum. These samples show the same range of analytical variation that has been noted previously for samples of gum from other *Acacia* species. In addition, the following atypical variants of *Acacia senegal* gum have been studied: (*a*) three specimens of "non-nodular "gum, (*b*) a sample from a tree infested with wood-boring beetles, and (*c*) a sample of "Hennawi" gum, a dark-colored, sweet-tasting gum from the main stem of trees.

The viscosity data obtained indicate that values of $\overline{M}_w$ for the samples fall in the range 0.26×10^6 to 1.16×10^6; this explains the lack of agreement for $\overline{M}_w$ of *A. senegal* gum samples studied by earlier investigators.

The atypical, non-nodular and beetle-borer specimens of gum do not show any striking analytical differences from the mean values obtained for the typical samples. The "Hennawi" variant differs from typical, tapped samples in two important respects: the uronic acid residues present in the free acid form are fewer in number, and the rhamnose content is considerably less.

INTRODUCTION

Earlier investigators[2,3] considered that different samples of gum arabic (*Acacia senegal* Willd., syn. *verek*) did not vary in properties to any great extent, but this is not the current industrial view. Botanically, *A. senegal* is very variable[4], and the Sudanese consider that the gum viscosity of a sample is dependent on the age of the tree involved. A sa result of empirical, technological observations, some manufacturers restrict their purchases to gum originating from a certain district; others decline a current season's crop ("green gum"), preferring gum stored in the Sudan since the previous season.

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It is now clearly established that distinct internodule variations occur in the gums from *A. seyal*[5], *A. nilotica*[6], and *A. laeta*[7], but there are no modern analytical values for specimens of *A. senegal* gum. Widely differing values for the molecular weight (both for $\overline{M}_n$ and $\overline{M}_w$) of *A. senegal* gum have been reported, and these have been summarised[8]; the implication that different samples of *A. senegal* gum may vary widely in $\overline{M}_n$ or $\overline{M}_w$ must therefore be considered, and the possible range of variation requires evaluation. Information on the viscosity behaviour of a wide selection of specimens would therefore indicate those likely to display the widest extremes when studied more fundamentally by the light-scattering technique.

Accordingly, authentic specimens of *A. senegal* gum from different geographical locations and different types of soil, from different seasons, and from different times of collection within a season, have been investigated. In addition, the variation from tree to tree has been investigated for normal specimens of the gum obtained (*i*) by "tapping" and (*ii*) by natural exudation. We have also examined three unusual forms of natural-exudate gum from *A. senegal*: (*a*) non-nodular forms of the gum, exuded as thin, spiral filaments or ribbons, rather than as oval-shaped nodules; (*b*) "Hennawi" gum, which is a very dark-colored, sweet-tasting form of *A. senegal* gum exuded from the main stems of trees, as opposed to the upper branches usually tapped; (*c*) the gum exuded by trees infested with wood-boring beetles. This exudate was in the form of hard, dark-brown masses of wood borings and debris, bound together with a gum exudate comprising about 40% of the masses by weight.

EXPERIMENTAL

Analytical methods. — The standard methods involved have been described[9] previously.

Origins of specimens. — All of the specimens, from *A. senegal* trees that could be identified botanically beyond all doubt, were collected by (the late) Mr. M. P. Vidal-Hall, formerly Gum Research Officer to the Republic of the Sudan. Details of their collection are as follows:

1. "*QN*" *samples*. These were bulk samples from many trees, growing on heavy clay soil at Qala en Nahal, Kassala Province, Eastern Sudan: 1960 crop, first (QN1), second (QN2), third (QN3), and final collection (QN4); 1962 crop, first collection (QN5).

2. "*UR*" samples. These were bulk samples from many trees, growing on sandy soil at Umm Ruaba, Kordofan Province, Central Sudan: 1960 crop, representative sample (UR1), final collection (UR2).

3. "*GG*" samples. These were bulk samples from many trees, growing on sandy soil at Goz el Ganzara, Kordofan Province, Central Sudan: 1960 crop, representative sample (GG1); 1962 crop, first (19 January, GG2), second (4 February, GG3), third (19 February, GG4), and final collection (5 March, GG5).

All of the QN, UR, and GG samples were normal samples of top commercial-quality gum from *A. senegal*, pale straw in colour, and virtually free from sand

and bark. Exudation was in response to tapping. Each sample (*ca.* 1500 g) 'was crushed and sieved to give material representative of the complete sample.

4. Single-nodule samples of natural exudate gum from individual trees of *A. senegal*, collected at Umm Ruaba (sandy soil) on 9 March, 1960:
Tree *A* (age 25 years) gave 3 nodules (samples A1, A2, and A3); *B* (12 years), 2 nodules (B1 andB2); *C* (8 years), 2 nodules (C1 and C2); *D* (15 years), 1 nodule (D); *E* (12 years 1 nodule (E); *F* (17 years), 1 nodule (F); *G* (20 years), 1 nodule (G); *H* (10 years), 1 nodule (H); *I* (15 years), 1 nodule (I). Each of these samples (15–30 g) was crushed to a fine powder and investigated individually.

5. "*Hennawi*" *sample*. This very dark brown variant of *A. senegal* gum has a sweet taste and is exuded naturally at wounds to the lower, main stem of the tree. Such gum is not usually tapped. It is known in the Sudan as "*Hennawi*" gum, eagerly sought by native children as a sweetmeat. The specimen (HW) of *Hennawi* gum studied was collected at Goz el Ganzara on 13 March 1963.

6. "*NN*" *samples*. Occasionally, *A. senegal* trees exude gum that differs from the customary oval nodules in forming long filaments, spirals, and ribbons. Three non-nodular (NN) forms of the gum were recognised: broad, opaque, flat ribbons (NN1); thin, circular, opaque filaments (NN2); and thin, circular, strongly refractive filaments (NN3). These samples were collected at Goz el Ganzara on 11 February 1961.

7. "*BB*" *samples*. Trees infested with wood-boring longhorn (*Cerambycidae*) or jewel (*Buprestidae*) beetles exude hard, dark-brown masses of a gum mixed with wood borings and other debris. The sample (180 g) of beetle-borer gum was collected at Goz el Ganzara in January 1965. One half-portion of the exudate (90 g) was stirred with cold water (750 ml) for 48 h, filtered, dialysed against tap-water, and then exhaustively electrodialysed. The gum polysaccharide, sample BB(*a*), was isolated as the freeze-dried product (33.4 g, 37%). The remainder of the gum exudate (90 g) was stirred with cold water (750 ml) for 24 h, and this extraction was processed in the same way as for BB(*a*) to give sample BB(*b*) (27.7 g, 31%). The residue from the extraction yielding BB(*b*) was then further exhaustively extracted with cold water (500 ml) and, after processing as before, this extraction gave sample BB(*c*) (8.9 g, 10%).

Purification of samples. — All of the QN, UR, GG, HW, and NN samples, and, the single-nodule samples A-I, were purified by dissolution in cold water, filtration dialysis, and exhaustive electrodialysis.

RESULTS

Table I gives the analytical data obtained for the various bulk samples QN, UR, GG, NN, and HW. Table II gives the analytical data for the single-nodule samples from individual trees. Table III gives more-detailed analytical information for samples QN1, HW, BB(*a*), BB(*b*), and BB(*c*). Figure 1 gives the viscosity plots from which the limiting-viscosity numbers, [η], recorded in Tables II and III were found.

Since the modified Staudinger constants K' and a are now known[10] to have

TABLE I

ANALYTICAL DATA FOR BULK SAMPLES OF *Acacia senegal* GUM

Sample	*Moisture* %	*Ash* %	*Nitrogen* %	*Insoluble*[a] %	*Flow-time number* ($cm^3 g^{-1}$) *Crude gum*[b]	*Purified gum*[c,d]
QN1	12.8	3.64	0.33	0.4	57	25 (19.9)
QN2	13.4	3.37	0.34	0.3	47	18
QN3	13.3	3.07	0.36	0.4	43	20
QN4	13.1	2.91	0.35	0.2	37	17
QN5	12.7	3.62	0.34	0.6	62	30
UR1	13.2	3.05	0.39	0.4	35	21
UR2	13.5	3.52	0.39	1.0	34	22
GG1	12.4	3.68	0.39	1.5	54	36
GG2	12.4	4.16	0.42	1.6	64	43 (24.5)
GG3	12.9	3.98	0.41	1.3	67	36
GG4	13.0	3.93	0.38	0.5	79	38
GG5	13.2	3.81	0.38	0.2	71	32
NN1	13.4	3.88	0.40	0.4	83	36
NN2	13.2	3.70	0.34	0.2	62	36
NN3	13.1	3.97	0.39	0.3	70	35
HW	13.6	5.67	0.37	0.5	39	35 (20.6)
Average	13.1	3.75	0.37	0.6	56	30
Range	12.4–13.6	2.91–5.67	0.33–0.42	0.2–1.6	34–83	17–43
Standard deviation	0.36	0.63	0.028	0.47		

[a]Insoluble after 24 h in cold water; [b]0.03 g/ml in water at 25.0°; [c]0.03 g/ml in 4% sodium chloride solution at 25.0°; [d]values in brackets are the corresponding limiting (C→O) flow-time numbers.

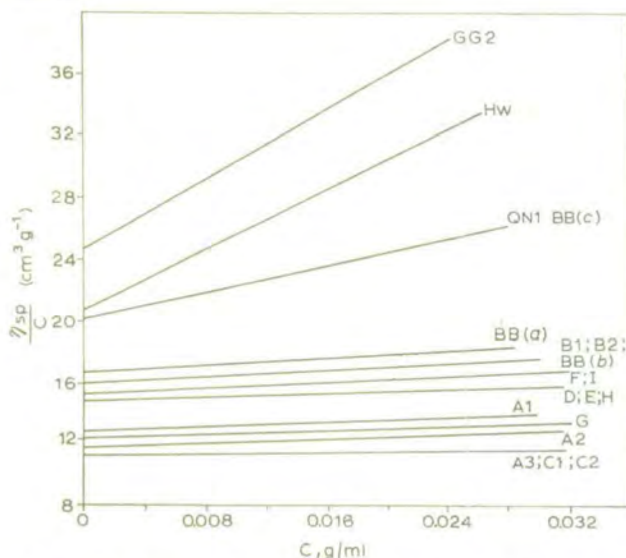

Fig. 1. The viscosity-concentration relationship for different specimens of *Acacia senegal* gum. The following limiting-viscosity numbers and M_w values (from $[\eta]=K'M_w^a$, where[10] $K'=1.3\times10^{-2}$, and $a=0.54$) were obtained for samples GG2, 24.6, 1.16×10^6; HW, 20.6, 0.84×10^6; QN1 and BB(*c*), 19.9, 0.79×10^6; BB(*a*), 16.5, 0.55×10^6; A1, 12.4, 0.33×10^6; A3, C1, C2, 11.0, 0.26×10^6.

TABLE II

ANALYTICAL DATA FOR SINGLE-NODULE NATURAL EXUDATE *Acacia senegal* GUM

Sample	*Age of tree (years)*	*Crude material*		*Electrodialysed material*			
		Moisture %	*Ash* %	*Nitrogen* %	$[\alpha]_D$ *(degrees)*	*Limiting-viscosity number* ($cm^3 g^{-1}$)	$\bar{M}_w$[a]
A1	25	12.6	3.72	0.24	−29	12.5	334,000
A2		11.6	3.78	0.23	−31	11.6	291,000
A3		11.1	3.80	0.24	−32	11.0	264,000
B1	12	11.7	4.43	0.23	−28	15.6	504,000
B2		12.9	4.44	0.23	−29	15.8	516,000
C1	8	10.4	3.40	0.27	−29	11.0	264,000
C2		9.8	3.66	0.26	−31	11.0	264,000
D	15	15.6	3.88	0.32	−30	14.1	418,000
E	12	12.8	4.64	0.34	−32	14.5	440,000
F	17	13.3	4.01	0.35	−29	15.1	474,000
G	20	15.4	4.15	0.23	−31	12.2	320,000
H	10	14.7	3.97	0.45	−29	14.4	434,000
I	15	12.1	3.27	0.33	−27	15.0	468,000
Average		12.6	3.93	0.29	−30	13.4	
Range		9.8–15.6	3.27–4.64	0.23–0.45	−27–−32	11.0–15.8	
Standard deviation		1.8	0.40	0.06	1.5	1.9	

[a]From $[\eta] = K'M_w^a$, where $K' = 1.3 \times 10^{-2}$ and $a = 0.54$ (see Ref. 10).

values of 1.30×10^{-2} and 0.54, respectively, values for $\bar{M}_w$ can be calculated from the viscosity data by the relationship $[\eta] = K'M_w^a$, and these values are shown in Fig. 1.

DISCUSSION

From Fig. 1 and the viscosity data in Tables I, II, and III, it is clear that $\bar{M}_w$ for *Acacia senegal* gum varies widely from sample to sample; the variation, for the samples studied, is from 1.16×10^6 (for GG2) to 0.26×10^6 (for nodules A3, C1, and C2). This explains the lack of agreement[8] between the values for $\bar{M}_w$ reported by earlier investigators, *viz.* 0.58×10^6 (Deb and Mukherjee[11]) and 1.00×10^6 (Veis and Eggenberger[12]). From our experiments, a value of 0.58×10^6 (Ref. 8) appears to be a more representative value than 1.00×10^6, although values higher than the latter may yet be found. *A. senegal* gum has a broad, skew, molecular-weight distribution, and fractions having $\bar{M}_w$ ranging from 1.18×10^6 to 0.1×10^6 have been obtained[10] for sample QN1. It should be emphasised that the values deduced for $\bar{M}_w$ in this paper are based on the modified Staudinger constants obtained from fractionations[10] of only one *A. senegal* gum sample: these values for K' and a do not hold for other *Acacia* species[10], and, indeed, they may not be generally applicable to all specimens of such a complex polymer system as *A. senegal* gum.

TABLE III

COMPARATIVE ANALYTICAL DATA[a] FOR SOME ELECTRODIALYSED TYPICAL (QN1) AND ATYPICAL (HW, BB) SPECIMENS OF *Acacia senegal* GUM

	QN1	*HW*	*BB(a)*	*BB(b)*	*BB(c)*
Ash, %	0.07	0.01	0.02	0.01	0.02
Nitrogen, %	0.33	0.35	0.25	0.21	0.28
$[\eta]$, cm^3g^{-1}	19.9	20.6	16.4	15.8	20.1
$[\alpha]_D$ (*c* 1.0, water)[b]	−31.0°	−27.6°	−30.5°	−31.3°	−31.5°
Methoxyl[b,c], %	0.25	0.25	0.25	0.25	0.25
Equivalent weight[b]	1085	1047	1066	1065	1060
Hence, uronic anhydride[b,d], %	16.2	16.8	16.5	16.5	16.5
Uronic anhydride [b,e], %	17.2	17.5	17.4	17.3	17.5
Formic acid released on periodate oxidation[b] (mole/g) × 10^3	1.62	1.69	1.76	1.85	1.90
Periodate consumed[b] (mole/g) × 10^3	5.07	5.18	5.23	5.34	5.42
Hence, $\frac{\text{periodate consumption}}{\text{formic acid released}}$	3.13	3.06	2.97	2.89	2.85
Rhamnose[b,c], %	14.0	6.2	13.0	12.6	12.8
Galactose[b], %	40	46	43	43	44
Arabinose[b], %	28	30	27	27	26
Glucuronic acid[b], %	16.0	16.5	15.5	15.5	15.5
4-*O*-methylglucuronic acid, %	1.5	1.5	1.5	1.5	1.5

[a]All data corrected to a dry-weight basis; [b]corrected for all non-carbohydrate material; [c]determined by vapour-phase, infrared methods; [d]assuming that all acidity arises from uronic acid groups; [e]assuming that all methoxyl content arises from 4-*O*-methylglucuronic acid.

Many factors influence[13] the viscosity of *Acacia* gum solutions. We have observed that electrodialysed specimens, after storage in the freeze-dried state for several months, give increased limiting-viscosity numbers when solutions are made up in a standardised way to facilitate comparison; for sample QN1, $[\eta]$ increased from 19.9 to 29.0, and for sample GG2, $[\eta]$ increased from 24.5 to 35.1. Other *Acacia* species give the same effect, and this is under investigation. The viscosity data presented in Tables I and II were obtained for 3% solutions immediately after dissolution. Table II gives some support for the belief that gum viscosity is dependent on the age of the tree; the oldest and youngest trees studied gave gum of low viscosity.

The "bulk" samples, representative of many nodules, show distinct analytical differences, although they must be expected to show "averaged" values. The differences shown in Table II for the single-nodule specimens are perhaps more fundamental in significance. Table I shows that the GG samples are more viscous than the QN or UR samples; it is interesting (*cf.* Ref. 5) that the most-viscous samples have higher than average nitrogen contents, although a high nitrogen content does not necessarily imply high viscosity, as shown by the UR samples. Insufficient data are available for any conclusions regarding seasonal effects to be reached; there is a suggestion, however, that the viscosity of the QN samples decreases as the season continues, whereas, in contrast, the viscosity of the GG samples increases.

The results for trees *A*, *B*, and *C* in Table II indicate that each tree tends to

produce a characteristic form of the gum, the different nodules from a particular tree being strikingly similar to each other in comparison to those from other trees.

The "Hennawi" variant of the gum has two distinctive features. It contains only 6% of rhamnose, *i.e.*, less than 50% of that present normally. A solution of "Hennawi" gum has pH 6.25; all of the other samples studied fell within the range pH 4.49 to 4.66, and the electrodialysed specimens fell within the range pH 2.47 to 2.60. The comparatively sweet taste of "Hennawi" gum is not due to the presence of free sugars; its reducing power is closely similar to that of the other samples. The explanation appears to involve the fact that "Hennawi" gum has very few, if any, free uronic carboxyl groups, with many more than usual being involved in salt formation with cations. The fact that the ash content of clean "Hennawi" gum is much higher than usual [5.67%, compared with an average (Table I) of 3.75%] lends support to this explanation.

When the data given in Tables I and II were obtained, it became apparent that a structural study of the HW and BB samples offered an opportunity to acquire information on the biosynthesis of *A. senegal* gum. The effect of different external stimuli to gum production (*e.g.*, natural exudation, tapping wounds, beetle infestation) is not yet known. Accordingly, it was decided to compare the structural features of samples HW, BB(*a*), BB(*b*), and BB(*c*) with those of a typical tapped-gum sample QN1. Table III shows the analytical differences between these samples; their structural differences will be discussed in a subsequent paper.

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STUDIES ON URONIC ACID MATERIALS

PART XXV*. SOME UNUSUAL FORMS OF THE GUM FROM *Acacia senegal* WILLD.

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ABSTRACT

A comparative study, involving classical methylation and Smith-degradation techniques, has been carried out on samples of normal "tapped" gum from *Acacia senegal*, natural exudate gum ("Hennawi" gum) from the main trunk of the tree, and the gum exuded by a tree infested with wood-boring beetles. The studies have shown that the beetle-borer specimen was virtually identical to the specimen of normal exudate gum; the specimen of "Hennawi" gum was basically similar, but differed in two important aspects concerning some of the peripheral end-group positions involving rhamnose and glucuronic acid residues. Theories of the origin, function, precursors, and mode of biosynthesis of plant gums are examined critically in terms of the results obtained.

INTRODUCTION

In the preceding part of this series, we reported the results of an analytical study of some different forms of the gum exudate from *Acacia senegal* Willd. Two of the specimens were of particular interest; these were (*a*) the gum exuded by a tree infested with wood-boring beetles, and (*b*) "Hennawi" gum, exuded from the lower, main trunk of the tree, and found[1] to contain less than 50% of the customary proportion of rhamnose. There is, at present, a lack of knowledge regarding the biosynthetic mechanism of gum production, and the nature of the external injury or physiological stimulus necessary to cause the formation of gum. It was therefore decided to carry out structural studies of the "Hennawi" and beetle-borer variants of *Acacia senegal* gum, and, for comparison, of a normal specimen of "tapped" gum.

EXPERIMENTAL

Origins of specimens. — These have been described[1]. The sample of "Hennawi" gum is designated HW; the beetle-borer sample is designated BB [extraction into three

*For Part XXIV, see Ref. 1.

fractions BB(*a*), BB(*b*), and BB(*c*) has been described[1]]. These atypical specimens of gum have been compared, in this study, with specimen QN1, which is representative[1] of the best quality, tapped gum from the first collection of the 1960 gum season at Qala en Nahal, Kassala Province, Eastern Sudan.

Analytical methods. — The standard methods involved have been described previously[2]. Paper chromatography was carried out on Whatman No. 1 paper with the following solvent systems (v/v): (*a*) benzene–butyl alcohol–pyridine–water (1:5:3:3, upper layer); (*b*) ethyl acetate–pyridine–water (10:4:3); (*c*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); (*d*) butyl alcohol–ethanol–water (4:1:5, upper layer); (*e*) butyl alcohol–acetic acid–water (4:1:5, upper layer); (*f*) butanone–water–ammonia (sp. gr. 0.880) (200:17:1). R_G values of methylated sugars refer to distances moved relative to that of 2,3,4,6-tetra-*O*-methyl-D-glucose in solvent (*d*). Chromatograms were developed with aniline hydrogen oxalate, alkaline silver nitrate, or the periodate–permanganate reagent.

Gas–liquid partition chromatography (chromatograph Type S3A, fitted with flame-ionisation detectors, Gas Chromatography Ltd.) was carried out at nitrogen flow-rates of *ca.* 100 ml/min. on columns of (*i*) 15% of poly(butane-1,4-diol succinate) on Celite (120 × 0.5 cm) at 175°, (*ii*) 15% of poly(ethylene glycol adipate) on Celite (75 × 0.5 cm) at 160°. Retention times (T) are quoted relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside as standard.

Polysaccharides were methylated successively with methyl sulphate and sodium hydroxide solution, and with methyl iodide and silver oxide. Methanolyses were carried out under reflux for 7 h with 5% methanolic hydrogen chloride.

Periodate oxidations were carried out at room temperature in darkness by mixing equal volumes of 4% polysaccharide solutions and 0.25M periodate. The formic acid released was determined by titration with standard sodium hydroxide solution. Periodate consumption was determined by titration with standard arsenite solution[3].

Attempted fractionations. — Samples HW, BB(*a*), BB(*b*), BB(*c*), and QN1 each migrated as a single band on electrophoresis on cellulose acetate film in (*a*) 0.1M ammonium carbonate buffer (pH 8.9), or (*b*) 0.1M acetate buffer (pH 4.7), and all the samples had the same mobility when run under comparable conditions at 18.8 volts/cm.

Samples HW, BB(*a*), BB(*b*), BB(*c*), and QN1 were examined chromatographically on a DEAE-cellulose column (45 × 1.5 cm). On gradient elution with sodium chloride solutions (0.0 → 0.3M) in 0.02M acetate buffer (pH 4.1), each sample gave a slightly asymmetric peak.

RESULTS

The analytical data for samples HW, BB(*a*), BB(*b*), BB(*c*), and QN1 have been reported[1].

Methylation study of the samples. — Each (300 mg) of the five samples HW, BB(*a*), BB(*b*), BB(*c*), and QN1 was methylated with the classical Haworth and Purdie reagents. Yields, specific rotations, and methoxyl contents of the methylated products

are shown in Table I; methoxyl contents were not raised on further attempted methylation. On methanolysis, and examination of the mixture of methyl glycosides by g.l.c., the methylated product of each sample gave identical chromatograms, except

TABLE I

DATA FOR *O*-METHYL DERIVATIVES OF SAMPLES QN1, HW, BB(*a*), BB(*b*), AND BB(*c*)

	QN1	*HW*	*BB*(a)	*BB*(b)	*BB*(c)
Yield (mg)[a]	250	240	250	260	240
$[\alpha]_D$ (*c* 1.0, chloroform)	−46°	−42°	−45°	−46°	−46°
Methoxyl, %	41.5	42.0	42.1	41.6	41.5

[a]Weight of sample methylated = 300 mg.

that the methylated product from HW gave less 2,3,4-tri-*O*-methyl-L-rhamnose and more 2,3,4-tri-*O*-methyl-D-glucuronic acid than did the other methylated products. Table II gives the results of the chromatographic examinations; retention times on columns (*i*) and (*ii*) were comparable with those for methyl glycosides from the authentic *O*-methyl sugars. A portion of each methanolysate was hydrolysed (N sulphuric acid, 4 h) on a boiling water-bath. The cooled solutions were neutralised (barium carbonate), filtered, treated with Amberlite resin IR-120 (H^+), and concentrated. Paper chromatography of each hydrolysate [solvents (*d*) and (*f*)] revealed the presence of some 2-*O*-methylgalactose in addition to those *O*-methyl sugars already identified by g.l.c. of the methyl glycosides (Table II).

TABLE II

CHROMATOGRAPHIC EXAMINATIONS OF METHANOLYSIS PRODUCTS FROM SAMPLES QN1, HW, BB(*a*), BB(*b*), AND BB(*c*)

Relative retention times (T) *on g.l.c.*[a] *Column* (i)	*Column* (ii)	*Paper chromatography*, R_G *in solvent* (d)	O-*Methyl sugars identified*
0.48	(0.51)	1.01	2,3,4-Tri-*O*-methyl-L-rhamnose
0.58; 0.72	(0.51); 0.64	0.97	2,3,5-Tri-*O*-methyl-L-arabinose
1.00	0.85	0.79	2,3,4-Tri-*O*-methyl-L-arabinose
1.79; 3.16	1.29; (2.21)	0.82	2,5-Di-*O*-methyl-L-arabinose
1.68	1.64	0.88	2,3,4,6-Tetra-*O*-methyl-D-galactose
3.77; 4.22	3.01; 3.51	0.74	2,4,6-Tri-*O*-methyl-D-galactose
6.35	5.14	0.74	2,3,4-Tri-*O*-methyl-D-galactose
14.6; 16.4	9.7; 11.0	0.53	2,4-Di-*O*-methyl-D-galactose
2.35; 2.94	(2.21); 2.65	—	2,3,4-Tri-*O*-methyl-D-glucuronic acid[b]
7.9; 9.3	5.8; 6.9	—	2,3-Di-*O*-methyl-D-glucuronic acid[b]
		0.34	2-*O*-Methyl-D-galactose
1.18; 1.44	0.98; 1.13; 2.50	—	Unknown sugars

[a]Incompletely resolved components shown in brackets. [b]As methyl ester methyl glycoside.

Smith degradations[4] *of samples.* — Each of the samples HW, BB(*a*), BB(*b*), BB(*c*), and QN1 was subjected to the Smith degradation process as follows. The sample (2.0 g) was dissolved in water, and 0.25M sodium metaperiodate (50 ml) was added. The oxidation mixtures were left in darkness for 48 h at room temperature. The reactions were stopped by the addition of ethylene glycol (2 ml), and the solutions were dialysed against tap water for 48 h. Sodium borohydride (0.5 g) was added to the solution, and the solutions were kept for 30 h at room temperature and were then dialysed for 48 h. The resulting polyalcohol was hydrolysed at room temperature (N sulphuric acid; 48 h), followed by dialysis against tap water for 48 h to remove sulphuric acid and small carbohydrate fragments. The Smith-degraded polysaccharides were then isolated as the freeze-dried products (yields and analytical data shown in Table III), and the values of $\bar{M}_n$ were determined by molecular-sieve chromatography[2] on a previously calibrated "Bio-Gel P300" column.

TABLE III

ANALYTICAL DATA[a] FOR THE SMITH-DEGRADED POLYSACCHARIDES

	Sample				
	QN1	*HW*	*BB*(a)	*BB*(b)	*BB*(c)
Yield, %	67	63	67	66	65
Nitrogen, %	0	0	0	0	0
$[\alpha]_D$ (*c* 1.0, water)	−28.2°	−28.0°	−29.1°	−28.0°	−28.5°
M_n[b]	98,000	96,000	93,000	96,000	93,000
Uronic acid[c], %	3.7	1.6	4.4	3.8	4.3
Galactose, %	70	71	70	70	69
Arabinose, %	26	27	26	26	27
Formic acid released on periodate oxidation (moles/g) × 10^3	1.85	1.80	1.83	1.78	1.75
Periodate consumed (moles/g) × 10^3	5.53	5.45	5.31	5.29	5.42

[a]All data corrected to dry-weight basis. [b]Estimated by molecular-sieve chromatography. [c]By acidic decarboxylation.

Hydrolysis (N sulphuric acid) of the Smith-degraded product from sample HW, followed by paper chromatography of the hydrolysate, showed the presence of galactose and arabinose. Hydrolyses of the Smith-degraded products from samples BB(*a*), BB(*b*), BB(*c*), and QN1, followed by paper chromatography, showed the presence of galactose, arabinose, and a trace of an aldobiouronic acid having the mobility of 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose [R_{Gal} 0.23, solvent (*c*)]. Mild hydrolysis of the Smith-degraded polysaccharides (0.5N sulphuric acid, 1 h, on a boiling water-bath), followed by paper chromatography, showed the presence of galactose, arabinose, and two neutral disaccharides having the chromatographic mobilities of 3-*O*-β-D-galactopyranosyl-D-galactose [R_{Gal} 0.49, solvent (*a*); R_{Gal} 0.53, solvent (*b*)] and 6-*O*-β-D-galactopyranosyl-D-galactose [R_{Gal} 0.29, solvent (*a*); R_{Gal} 0.38, solvent (*b*)].

Methylation of the Smith-degraded polysaccharides. — Each of the Smith-

degraded polysaccharides obtained respectively from samples HW, QN1, BB(*a*), BB(*b*), and BB(*c*) was methylated successively with the Haworth and Purdie reagents. Yields, specific rotations, and methoxyl contents of the methylated products are shown in Table IV; methoxyl contents were not raised on further attempted methylation. On methanolysis and g.l.c. of the resulting mixtures of methyl glycosides, the methylated products from each of the Smith-degraded polysaccharides gave identical traces, with the exception that the methylated product from Smith-degraded sample HW gave no detectable amounts of 2,3,4-tri-*O*-methyl-D-glucuronic acid. The results of the g.l.c. analyses are shown in Table V. A portion of each methanolysate was hydrolysed (N sulphuric acid, 4 h, boiling water-bath). The cooled solutions were neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H^+), and concentrated. Paper-chromatographic examination of the hydrolysates [solvents (*d*) and (*f*)] showed the presence of 2-*O*-methylgalactose, in addition to those *O*-methyl sugars already identified by g.l.c. of their methyl glycosides (Table V).

TABLE IV

DATA FOR *O*-METHYL DERIVATIVES OF THE SMITH-DEGRADED POLYSACCHARIDES FROM SAMPLES QN1, HW, BB(*a*), BB(*b*), AND BB(*c*)

	QN1	*HW*	*BB*(a)	*BB*(b)	*BB*(c)
Yield (mg)[a]	220	212	220	215	225
$[\alpha]_D$ (*c* 1.0, chloroform)	−41°	−40°	−41°	−41°	−42°
Methoxyl, %	40.5	40.9	41.1	40.9	40.5

[a]Weight of sample taken = 300 mg.

TABLE V

CHROMATOGRAPHIC EXAMINATIONS OF METHANOLYSIS PRODUCTS FROM METHYLATED SMITH-DEGRADED POLYSACCHARIDES FROM SAMPLES QN1, BB(*a*), BB(*b*), AND BB(*c*)[a]

Relative retention times (T) *on g.l.c.*[b]		*Paper chromatography, R_G in solvent* (d)	O-*Methyl sugars identified*
Column (i)	*Column* (ii)		
0.57; 0.73	0.50; 0.64	0.97	2,3,5-Tri-*O*-methyl-L-arabinose
1.79; 3.15	1.30; (2.21)	0.82	2,5-Di-*O*-methyl-L-arabinose
1.68	1.65	0.88	2,3,4,6-Tetra-*O*-methyl-D-galactose
3.78; 4.22	2.99; 3.50	0.74	2,4,6-Tri-*O*-methyl-D-galactose
6.34	5.14	0.74	2,3,4-Tri-*O*-methyl-D-galactose
9.1	6.5; 6.9; 7.7	0.59	2,6-Di-*O*-methyl-D-galactose
14.6; 16.4	9.7; 11.0	0.53	2,4-Di-*O*-methyl-D-galactose
2.35; 2.95	(2.21); 2.66	—	2,3,4-Tri-*O*-methyl-D-glucuronic acid[c]
—	—	0.34	2-*O*-Methyl-D-galactose
1.08; 1.52	0.86; 2.48	—	Unknown sugars

[a]Identical results for sample HW, except that 2,3,4-tri-*O*-methyl-D-glucuronic acid was absent. [b]Incompletely resolved peaks shown in brackets. [c]As methyl ester methyl glycoside.

DISCUSSION

The results presented in this paper indicate that, structurally, the three fractions from the beetle-borer gum exudate are very similar to normal, tapped gum from *Acacia senegal*. Although the sample of "Hennawi" gum is basically similar as far as the interior structure is concerned, it differs in two important aspects that concern some of the peripheral end-group positions of the gum molecule.

It is now some time since theories[5-9] concerning the origin and function of plant gums were considered, and there has been little agreement on either of these points to date. It has also been debated whether gum exudates are formed at the site of a wound, or whether they are generated elsewhere in the plant and then transported to the site of exudation. It has been suggested that gum exudates may be a product of normal plant metabolism[6,7], that they may arise from a pathological condition of the tree[6], or that they may arise from some infection or invasion[7] by micro-organisms which may be fungal[6] or bacterial [7,10,11] in nature. These theories were advanced much earlier this century; and there has been no modern work to substantiate or refute them. Some of these theories are discussed in non-critical reviews that contain factual errors *e.g. A. verek* is not a variety of *A. arabica*[11,12], and gum arabic does not contain galacturonic acid[11,12]. Misprints and such errors in reviews tend to be perpetuated (*cf.* Ref. 13).

It has been suggested[5,9] that starch may undergo transformation into gum, but the enzyme systems necessary to transform a polyglucan into a highly branched polysaccharide containing galactose, arabinose, rhamnose, glucuronic acid, and 4-*O*-methylglucuronic acid appear to be impossibly complex; starch was not found[14] in the wood tissues of excised branches from *A. senegal* trees. It seems more reasonable that the gum acid has, as its precursor, some highly branched arabino-galactan of a hemi-cellulosic type, to which is added rhamnose-, glucuronic acid-, and 4-*O*-methylglucuronic acid-terminated side-chains in the final stages of gum production. The enzyme systems probably differ at different parts of the tree (*cf.* "Hennawi" gum).

Although modern commercial samples of good quality, Sudanese gum arabic originate almost entirely from *A. senegal*, contamination or adulteration with other genera[15] and with other *Acacia* species is quite possible, since the various nodules cannot be distinguished by colour, shape, or size. Commercial samples should never be used for fundamental studies. Early specimens of gum arabic were undoubtedly mixtures from various *Acacia* species[8,16], which are now known (*cf.* earlier parts of this series) to differ considerably in sugar composition and structure. The reason why no rhamnose was detected in certain samples of gum arabic[17] is now quite clear, if the term "gum arabic" was taken to apply to the exudate from any *Acacia* species[8]; some *Acacia* species contain 12–14% of rhamnose, others 6–7% of rhamnose, and some less than 1% of rhamnose[18]. In addition to this variation between species, our results now show that authentic nodules from *A. senegal* can have different amounts of rhamnose. Much of the evidence to date regarding the chemical heterogeneity of gum arabic was based on the depleted rhamnose content of certain fractions from initially hetero-

geneous, commercial samples; the point was made some time ago[7] that the classical work of Heidelberger and his colleagues should be repeated on specimens from a single *Acacia* tree or at least from one variety of *Acacia*.

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STUDIES ON URONIC ACID MATERIALS

PART XXVI*. THE ALDOBIOURONIC ACIDS IN GUMS FROM *Acacia* SPECIES

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ABSTRACT

Acacia seyal gum contains four aldobiouronic acids that have been characterised as 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose (*A*), 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose (*B*), 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose (*C*), and 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose (*D*). With these acids as chromatographic standards, the aldobiouronic acids present in a further seventeen species of *Acacia* gum have been investigated. Gums having positive rotations and significant methoxyl contents give aldobiouronic acids *A*–*D*, whereas gums having positive rotations and low methoxyl contents do not give significant proportions of acids *C* and *D*. Gums having negative rotations and significant methoxyl contents give acids *A* and *C*; one gum having a negative rotation and a low methoxyl content did not give a significant proportion of acid *C*.

INTRODUCTION

Although *Acacia* gums have been studied for many years, the possibility of the presence of methoxyl groups was largely ignored in early work. Recently, however, it was suggested[2] that a re-examination of the aldobiouronic acid fraction of species studied earlier (*e.g.*, *A. pycnantha*) might reveal structurally significant features, and it was reported[3] that *A. nilotica* gum contains four aldobiouronic acids. We have therefore investigated the nature of the aldobiouronic acids present in eighteen different species of *Acacia* gum, in order to ascertain whether the presence of four aldobiouronic acids is a general feature within the *Acacia* group. Structural investigations undertaken simultaneously with the present study have shown that *A. senegal*[4] gum and *A. laeta*[5] gum each contain two aldobiouronic acids and that *A. arabica*[6] gum contains four aldobiouronic acids. Stephen and Vogt[7] have also reported the result of a re-investigation of *A. karroo* gum, which contains two aldobiouronic acids.

EXPERIMENTAL

Origins of specimens. — The origins of the following specimens of gum have

*For Part XXV, see Ref. 1.

been given elsewhere: *Acacia nilotica*[3,8], *A. arabica*[6,8], *A. campylacantha*[8], *A. fistula*[8], *A. nubica*[8], *A. senegal*[4], *A. laeta*[5], *A. drepanolobium*[8], *A. dealbata*[8], and *A. seyal*[9]. The following specimens were collected by the Sudanese Gum Research Officer: *A. multijuga, A. mellifera, A. tortilis, and A. adansoniana.* The following specimens were collected by officers of the Botanical Research Institute, Pretoria: *A. giraffae* Burch., *A. karroo* Hayne, *A. mearnsii* De Willd. (syn. *A. mollissima* and *A. decurrens* of some authors). The gum from *A. pycnantha* was collected by Mr. Bednall, Conservator of Forests, Adelaide.

Analytical methods. — The standard methods involved have been described[5]. Gas–liquid chromatography was performed as described previously[4]. Paper chromatography was performed as described[3], except that solvent *E* was ethyl acetate–acetic acid–formic acid–water (18:8:3:9), and solvent *G* was butanone–water–conc. ammonia (200:17:1).

RESULTS

Location of methoxyl groups. — A report[10] that *A. seyal* gum probably contained a mono-*O*-methylpentose (R_G 0.55; solvent *A*) could not be confirmed, and a report[11] that *A. mollissima* gum contained 4-*O*-methylglucuronic acid led to the following experiments to discover if the methoxyl content of *A. senegal* gum is associated with its acidic components. A sample of *A. senegal* gum (3% aqueous solution) was autohydrolysed at 100°. Samples were taken at intervals, and dialysed, and the non-dialysable material was freeze-dried. The results of analyses of the uronic anhydride and methoxyl content are shown in Table I.

TABLE I

AUTOHYDROLYSIS OF *A. senegal* GUM

Time of hydrolysis, h	*Uronic anhydride (UA), %*	*Methoxyl (OMe), %*	*OMe × 100/UA*
0	15.5	0.24	1.6
10	18.0	0.29	1.6
24	20.2	0.31	1.5
36	21.5	0.34	1.5
50	21.7	0.37	1.6

To ascertain whether the methoxyl content was present to any extent as the methyl ester group of a uronic acid, a 5% solution of the sodium salt of *A. seyal* gum (preferred to *A. senegal* gum because of its higher methoxyl content[2]) in 0.05N sodium hydroxide was kept at 60°, and samples were taken at intervals. After neutralisation and dialysis, the freeze-dried samples were analysed as shown in Table II. A similar constancy in the ratio of methoxyl to uronic anhydride content was also obtained for samples of the gum from *A. senegal, A. nilotica,* and *A. tortilis.*

The aldobiouronic acids of A. seyal. — A sample of the gum was hydrolyzed (N sulphuric acid, 100°, 8 h). Paper chromatography (solvent *D*) showed four acidic

components *A*, *B*, *C*, and *D* having R_{Gal} values of 0.26, 0.32, 0.60, and 0.69, respectively. Components *A* and *B* were separated from *C* and *D* by chromatography on Whatman No. 3MM paper (solvent *D*). Hydrolysis (2N sulphuric acid, 6 h) of components *A* and

TABLE II

SAPONIFICATION OF *A. seyal* GUM

Time of saponification, h	*Methoxyl (OMe), %*	*Uronic anhydride (UA), %*	*OMe × 100/UA*
0	0.86	9.9	8.7
12	0.82	9.7	8.2
24	0.84	9.3	9.0
32	0.71	8.8	8.1
48	0.72	7.8	9.2
56	0.65	8.0	8.1
72	0.66	7.8	8.5

B gave, on paper-chromatographic fractionation, D-galactose {$[\alpha]_D$ +81° (*c*, 1.0); m.p. and mixed m.p. 169°} and D-glucurono-6,3-lactone {$[\alpha]_D$ +18° (*c*, 1.0); m.p. and mixed m.p. 177°}. A portion (*ca.* 40 mg) of the mixture of acids *A* and *B* was dissolved in methyl sulphoxide (10 ml), and methylated[12] with sodium hydride (500 mg) and methyl iodide (1.4 ml). After methanolysis, the products were identified (g.l.c.) as 2,3,4-tri-*O*-methyl-D-glucuronic acid, 2,3,4-, 2,3,5-, and 2,3,6-tri-*O*-methyl-D-galactose. A portion of the mixture of methyl glycosides was hydrolysed to the free sugars; paper chromatography (solvents *F* and *G*) indicated the presence of 2,3,4-tri-*O*-methyl-D-glucuronic acid, and 2,3,4-tri- and 2,3,6-tri-*O*-methyl-D-galactose.

Hydrolysis of the mixture of acids *C* and *D* gave galactose and 4-*O*-methylglucuronic acid (examination in solvents *D* and *E*). A portion (60 mg) of the mixture was methanolysed and neutralised (Ag_2CO_3). The filtered solution was evaporated to give a syrup that was dissolved in ammoniacal methanol (10%) and kept at 0°. After some months, crystals of the amide of methyl 4-*O*-methyl-α-D-glucopyranosiduronic acid were obtained, which, after recrystallisation from ethanol, had m.p. 231–233° (lit.[13], 234–236°). After removal of the crystalline amide, the solution was concentrated, and the residue was hydrolysed. Paper chromatography showed the presence of galactose and 4-*O*-methylglucuronic acid; D-galactose {m.p. and mixed m.p. 169–170°; $[\alpha]_D$ +80° (*c* 1.0)} was isolated by chromatography on 3MM paper.

A portion of the mixture of acids *C* and *D* was methylated in methyl sulphoxide[12], methanolysed, and examined by g.l.c. and by paper chromatography, as described for the mixture of acids *A* and *B*. Acids *C* and *D* gave the same products as acids *A* and *B*.

The acids *A*, *B*, *C*, and *D* were then separated by chromatography on thick paper with solvent *D*. As the zones moved closely together, they were detected by dipping the chromatograms (after careful drying to remove all of the acetic and formic acids) in a mixed-indicator system containing Thymol Blue (50 mg), Methyl Red

(250 mg), and Bromothymol Blue (600 mg) in ethanol (1 litre), to which N sodium hydroxide solution had been added until the colour became blue-green; acidic sugars appeared as red spots on a yellow-green background. The sugar acids were eluted from the paper with water, and the aqueous solutions (at mildly acidic pH) were extracted with chloroform to remove the indicators.

Acid *A* {R_{Gal} 0.26 (solvent *D*), $[\alpha]_D$ $-3°$ (*c* 2.5)} was chromatographically identical with authentic 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose in solvents *D* and *E*. Formaldehyde was not evolved on attempted periodate oxidation. A portion (200 mg) of the acid was methylated by four additions of the Haworth reagents, the methylated sugars were extracted with chloroform, the extract was concentrated, and the residue was hydrolysed. Paper chromatography (solvents *A* and *E*) revealed the presence of 2,3,4-tri-*O*-methyl-D-glucuronic acid and 2,3,4-tri-*O*-methyl-D-galactose. The uronic acid had already been characterised in the unmethylated, acidic disaccharide, and was not investigated further. The 2,3,4-tri-*O*-methyl-D-galactose (80 mg) was isolated by chromatography on thick paper (solvent *A*), and had R_G 0.63 (solvent *A*); $[\alpha]_D$ $+110°$ (*c* 1.0); the aniline derivative had m.p. 168°.

Acid *B* {R_{Gal} 0.32 (solvent *D*), $[\alpha]_D$ $+101°$ (*c* 0.2)} was chromatographically identical with authentic 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose in solvents *D* and *E*. Hydrolysis gave only galactose and glucuronic acid; periodate oxidation at pH 8 for 24 h gave formaldehyde. A portion (150 mg) of the acid was methylated as described for acid *A*, and the methyl glycosides produced on methanolysis of the product had the same retention times in g.l.c. as the glycosides of 2,3,4-tri-*O*-methyl-D-glucuronic acid and 2,3,6-tri-*O*-methyl-D-galactose. The latter compound was isolated by chromatography on thick paper with solvent *A* {yield, 50 mg; R_G 0.70 (solvent *A*); $[\alpha]_D$ $+84°$ (*c* 0.5)}; the product was oxidised with bromine to give 2,3,6-tri-*O*-methyl-D-galactonolactone, m.p. 96°.

Acid *C* {R_{Gal} 0.60 (solvent *D*); $[\alpha]_D$ $+4°$ (*c* 0.2)} was chromatographically identical with authentic 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose in solvents *D* and *E*. Hydrolysis gave *ca.* equal amounts of 4-*O*-methylglucuronic acid and galactose. Formaldehyde was not produced on attempted oxidation with periodate. A portion (200 mg) of the acid was methylated and then hydrolysed, and the resulting methylated sugars were the same as those given by acid *A*. The 2,3,4-tri-*O*-methyl-D-galactose was characterised as 2,3,4-tri-*O*-methyl-*N*-phenyl-D-galactosylamine, m.p. 164°.

Acid *D* {R_{Gal} 0.69 (solvent D), $[\alpha]_D$ $+95°$ (*c* 0.1)} was chromatographically identical with authentic 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose. Hydrolysis gave *ca.* equal amounts of galactose and 4-*O*-methylglucuronic acid. Formaldehyde was released on periodate oxidation. A portion (120 mg) of the acid was methylated, and the methyl glycosides produced on methanolysis of the product were the same as those given by acid *B*; the 2,3,6-tri-*O*-methyl-D-galactose was characterised as 2,3,6-tri-*O*-methyl-D-galactonolactone, m.p. 94°.

Identification of the aldobiouronic acids in other Acacia *species.* — Each of the remaining seventeen species of *Acacia* gum was subjected to partial hydrolysis (N sul-

phuric acid, 100°, 8 h), and the hydrolysates were examined by paper chromatography in solvents *D* and *E*, with acids *A–D* as reference standards. The results are given below.

Specific rotations of the Acacia *species containing aldobiouronic acids* A, B, C, *and* D. — *A. adansoniana* (+83°); *A. arabica* (+100°); *A. drepanolobium* (+75°); *A. fistula* (+61°); *A. multijuga* (+40°); *A. nilotica* (+108°); *A. seyal* (+58°); *A. tortilis* (+96°); and *A. giraffae* (water-soluble portion, positive rotation). Independent investigations, carried out simultaneously with this study, have confirmed the results for *A. nilotica*[3], *A. arabica*[6], *A. drepanolobium*[14], and *A. seyal*[15]. *A. karroo* (+54°) and *A. nubica* (+100°) gave isolable amounts of acids *A* and *B*, but only chromatographic traces of acids *C* and *D*; the methoxyl content of these species is low, and the proportions of acids *C* and *D*, if present, must be very small.

Specific rotations of the Acacia *species containing aldobiouronic acids* A *and* B C *only*. — *A. campylacantha* (−3°); *A. dealbata* (−25°); *A. laeta* (−36°); *A. mearnsii* (−49°); *A. mellifera* (−51°); *A. senegal* (−31°); and *A. pycnantha* (− 8°; this species gave only a chromatographic trace of acid *C*). These conclusions have been verified independently for *A. laeta*[5] and *A. senegal*[4].

DISCUSSION

The results in Tables I and II indicate that the methoxyl content in an *Acacia* gum polysaccharide is associated with the uronic acid residues, but is not present as methyl ester. Searches to date have failed to confirm the presence of any methylated sugars in the gums studied.

The four aldobiouronic acids present in *A. seyal* gum are 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose (*A*), 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose (*B*), 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose (*C*), and 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose (*D*). These acids have been used as reference standards in a chromatographic examination of the aldobiouronic acids in seventeen further *Acacia* species of gum.

Of the species studied to date, those having positive specific rotations contain the four aldobiouronic acids (*A–D*); the exceptions are *A. karroo* and *A. nubica* gums, which have low methoxyl contents and consequently small proportions (if any) of the 4-*O*-methyl acids *C* and *D*. The *Acacia* species having negative specific rotations contain only aldobiouronic acids (*A* and *C*) that have β-D linkages; the presence of acid *C* in *A. pycnantha* gum is doubtful, however, since this species has a low methoxyl content.

From the species studied to date, it therefore appears that, provided the species in question has a significant methoxyl content, *Acacia* gums having positive specific rotations contain aldobiouronic acids *A–D*, whereas *Acacia* species having negative specific rotations contain only acids *A* and *C*.

The uronic acid content of the majority of *Acacia* gums is only of the order of 10%, and it is therefore unlikely that the presence of the aldobiouronic acids having α-D linkages (acids *B* and *D*) is alone sufficient to convert a negative rotation into a

strongly positive one. The explanation is more likely to be found in fundamental structural differences involving the arabinose side-chains and/or the branched framework of galactose residues.

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STUDIES ON URONIC ACID MATERIALS

PART XXVII*. THE STRUCTURE OF THE GUM FROM *Acacia nubica* BENTH.

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ABSTRACT

The polysaccharide exuded by *Acacia nubica* trees has a high, positive specific rotation, has low methoxyl and L-rhamnose contents, and contains D-galactose, L-arabinose, and D-glucuronic acid, which is present in two aldobiouronic acids, 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose and 4-*O*-(α-D-glucopyranosyluronic acid-D-galactose. Autohydrolysis experiments gave 3-*O*-β-L-arabinofuranosyl-L-arabinose, 3-*O*-β-L-arabinopyranosyl-L-arabinose, β-(1 → 3)-linked-L-arabinose trisaccharides, and a degraded gum *A* of molecular weight 5,730, which was studied by linkage and methylation analysis. Partial hydrolysis with acid gave 3-*O*-β-D-galactopyranosyl-D-galactose and 6-*O*-β-D-galactopyranosyl-D-galactose. An examination of the *O*-methyl derivative of degraded gum *A* gave 2,3,4,6-tetra-, 2,3,4-, 2,3,6-, and 2,4,6-tri-, and 2,4-di-*O*-methyl-D-galactose; 2,3,4-tri-*O*-methyl-L-arabinose; and 2,3,4-tri-*O*-methyl-D-glucuronic acid. Degraded gum *A* was subjected to a Smith degradation, and the product was examined by linkage and methylation analysis.

The *O*-methyl derivative of the whole gum gave 2,3,4- and 2,3,5-tri-, and 2,5- and 3,5-di-*O*-methyl-L-arabinose; 2,3,4,6-tetra-, 2,4,6-, 2,3,6-, and 2,3,4-tri-, 2,6- and 2,4-di-, and 2-*O*-methyl-D-galactose; and 2,3,4-tri-*O*-methyl-D-glucuronic acid. The whole gum was subjected to five successive Smith-degradations, and the Smith-degraded polysaccharides *S1*–*S5* were each examined by linkage and methylation analysis.

The structural evidence suggests that *A. nubica* gum molecules possess highly branched D-galactan frameworks, to which are attached D-glucuronic acid residues and L-arabinose-containing side-chains, some of which are at least six units long. The gum contains the largest proportion of L-arabinose in any of the *Acacia* gum exudates studied to date.

INTRODUCTION

In a preliminary study of the gum exudates from several *Acacia* species, *Acacia*

*For Part XXVI, see Ref. 1.

nubica Benth. was found[2] to differ in several interesting respects from the *Acacia* species studied previously. Thus, *A. nubica* gum gave a high, positive specific rotation (+100°), very low methoxyl and rhamnose contents, and the highest arabinose content of any *Acacia* species examined to date. A structural study of the gum from *A. nubica* was therefore undertaken, since these analytical parameters indicated that the gum might provide an example of one extreme structure-type within the genus *Acacia*. Recent studies[1,3] have indicated that structural variations between gums of different *Acacia* species are more pronounced than was at one time supposed.

RESULTS

The crude gum from *Acacia nubica* is dextrorotatory ($[\alpha]_D$ +100°) and is composed (see Table I) of D-galactose, L-arabinose, D-glucuronic acid, and L-rhamnose in the proportions 33:59:7:1. There is a very small methoxyl content (0.05–0.1% for

TABLE I

ANALYTICAL DATA[a] FOR THREE SAMPLES OF *Acacia nubica* GUM

	Sample A		*Sample* B		*Sample* C	
	Crude	*Purified*	*Crude*	*Purified*	*Crude*	*Purified*
Moisture, %	10.4	5.2	11.1	5.0	9.2	5.1
Ash, %	1.54	0.02	1.52	0.01	1.54	0.01
Nitrogen, %	0.20	0.21	0.23	0.21	0.21	0.16
Uronic anhydride, %[b]	6.4	7.3	7.5	7.6	7.6	7.2
Cold-water insoluble, %	1.4	—	1.5	—	1.9	—
pH, 3% solution	4.70	2.5	4.74	—	4.85	—
Free, titratable acidity[c]	0.49	—	0.64	—	0.60	—
$[\alpha]_D^{20}$ (*c* 3.0)	+98°	+101°	+100°	+100°	+99°	+100°
Methoxyl, %[b]	—	0.05	—	0.05	—	0.06
D-Galactose, %	33	29	—	28	—	28
L-Arabinose, %	59	63	—	64	—	64
L-Rhamnose, %[b]	1	0.6	—	trace	—	trace

[a]All data corrected to dry-weight basis. [b]By vapour-phase infrared methods. [c]As ml of 0.02N NaOH per 10 ml of 3% solution.

different specimens), and a small proportion (0.3%) of the uronic acid is probably present as 4-*O*-methyl-D-glucuronic acid. The gum contains nitrogenous material (N, 0.21%) of which some, at least, was shown to be proteinaceous. The crude gum gave a positive test for peroxidase. Paper-chromatographic examination of a solution of crude gum showed that material of low molecular weight was present; after isolation by dialysis, it was shown that free arabinose, galactose, rhamnose, and 3-*O*-β-L-arabinopyranosyl-L-arabinose were present. The proportion of rhamnose appeared (visual examination of chromatograms) to be greater than that obtained on complete hydrolysis of the purified gum polysaccharide.

Autohydrolysis experiments. — Reasonably complete autohydrolysis of the gum (5% solution) required 120 h at 98° [*cf.*, autohydrolysis of *A. senegal* gum (uronic acid, 16%) which requires only 50 h at 98°]. During autohydrolysis, the pH decreased from 2.5 to 1.9, and the specific rotation increased from +100 to +108° (24 h) and then decreased to +87° (144 h) as shown in Table II, which also indicates the nature of the reducing sugars liberated. Three arabinose oligomers were detected: 3-*O*-β-L-arabinopyranosyl-L-arabinose(*X*),3-*O*-β-L-arabinofuranosyl-L-arabinose(*Y*), and a trisaccharide (*Z*), identified tentatively as *O*-β-L-arabinopyranosyl-(1 → 3)-*O*-β-L-arabinofuranosyl-(1 → 3)-L-arabinose. Disaccharide *X* has been detected previously in several *Acacia* gums, and disaccharide *Y* has been detected in the gums from *A. pycnantha* and *A. senegal*; trisaccharide *Z* has not been reported previously. The disaccharide, 3-*O*-α-D-galactopyranosyl-L-arabinose, was not detected; it has been reported to be present in the gums from *A. senegal*, *A. cyanophylla*, and *A. karroo*.

Degraded gum *A*, isolated after autohydrolysis (yield 24%; methoxyl, 0.1%; $[\alpha]_D$ +44°), contained glucuronic acid (12%), galactose (83%), and arabinose (5%); its reducing end-group was shown to be a galactose unit, and its degree of polymerisation[4] was found to be 33 (M_w = 5,730). This value must be compared with the value of 4,800 obtained for a sample of *A. senegal* gum degraded by autohydrolysis[5]. Mild hydrolysis of degraded gum *A* gave 3-*O*-β-D-galactopyranosyl-D-galactose, 6-*O*-β-D-galactopyranosyl-D-galactose, the β-D-(1 → 3)- and β-D-(1 → 6)-linked galactose trisaccharides, and a fraction which was probably a mixture of tri- and tetra-saccharides. The β-D-(1 → 3)-linked trisaccharide has been detected in *A. senegal* gum, but this is the first report of the β-D-(1 → 6)-linked trisaccharide in an *Acacia* gum. In contrast to *A. senegal* gum, the *A. nubica* degraded gum *A* gave more of the β-D-(1 → 6)- than of the β-D-(1 → 3)-linked disaccharides.

Graded hydrolysis experiments. — Hydrolysis of the whole gum (N-sulphuric acid, 100°, 8 h), followed by cellulose-column chromatography, gave L-rhamnose, L-arabinose, D-galactose, D-glucurono-6,3-lactone, and 4-*O*-methylglucuronic acid. The aldobiouronic acids were shown to be 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose and (in minor proportion) 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose.

Methylation studies. — Degraded gum *A* was methylated[6] by the sodium hydride–methyl iodide–methyl sulphoxide system, and cellulose-column chromatography of the methylated products showed the presence of 2,3,4,6-tetra-*O*-methyl-D-galactose; 2,3,4-tri-*O*-methyl-L-arabinose; 2,3,4-, 2,4,6-, and 2,3,6-tri-*O*-methyl-D-galactose; 2,4-di-*O*-methyl-D-galactose; and 2,3,4-tri-*O*-methyl-D-glucuronic acid.

After methylation[6] of the whole gum, cellulose-column chromatography showed the presence of 2,3,5-tri-*O*-methyl-L-arabinose; 2,3,4,6-tetra-*O*-methyl-D-galactose; 2,5- and 3,5-di-*O*-methyl-L-arabinose; 2,3,4-tri-*O*-methyl-L-arabinose; 2,3,4-, 2,4,6-, and 2,3,6-tri-*O*-methyl-D-galactose; 2,6- and 2-4-di-*O*-methyl-D-galactose; 2-*O*-methyl-D-galactose; and 2,3,4-tri-*O*-methyl-D-glucuronic acid. About 50% of the arabinose was present as 2,3,5-tri-*O*-methyl-L-arabinose, with a major proportion of di-*O*-methyl-L-arabinose, and a small proportion of 2,3,4-tri-*O*-methyl-

TABLE II

THE CHANGES OCCURRING ON AUTOHYDROLYSIS

Period of autohydrolysis (h)	$[\alpha]_D$	*pH*	*Reducing sugars*[a]	*Reducing sugars liberated*[b]				*Galactose disaccharides*	
				Arabinose	*Galactose*	*Arabinose oligomers*[c]	*Arabinose disaccharide*[d]	β-D-$(1\rightarrow6)$[e]	β-D-$(1\rightarrow3)$[f]
0	+100°	2.5	—	—	—	—	—	—	—
6	+104°	2.3	10.1	++	—	+	—	—	—
12	+105°	2.2	16.4	+++	—	+	—	—	—
24	+108°	2.2	27.7	+++	trace	+	trace	—	—
48	+98°	2.1	46.2	+++	+	+	+	—	—
72	+90°	2.1	55.8	+++	+	+	+	trace	—
96	+89°	2.0	63.7	+++	++	+	+	+	trace
120	+88°	1.9	65.2	+++	++	trace	+	+	trace
144	+87°	1.9	65.9	+++	++	trace	++	+	+
177	—	—	—	+++	++	trace	++	+	+

[a]Expressed as mg of free arabinose per ml. [b]Chromatographic solvent (*b*); aniline oxalate spray. [c]Oligomers, pink streak R_{Gal}-0.4 to 0.65. [d]R_{Gal} 0.79: 3-*O*-β-L-arabinopyranosyl-L-arabinose. [e]R_{Gal} 0.32. [f]R_{Gal} 0.47.

L-arabinose. The di-*O*-methylarabinose fraction consisted of 2,5-di-*O*-methyl-L-arabinose (one part) and the less-common 3,5-di-*O*-methyl-L-arabinose (four parts). L-Arabinofuranosyl-(1 → 2)-L-arabinose units must therefore be present in the gum, and arabinopyranose units appear to occur only as non-reducing end-groups. The major galactose-containing fraction from the methylated whole gum (about 60% of the total galactose) was present as a mixture of 2,4,6-tri-*O*-methyl-D-galactose (5 parts), 2,3,4-tri- (one part), and 2,3,6-tri-*O*-methyl-D-galactose (trace). Most of the remaining galactose occurred as 2,4-di-*O*-methyl-D-galactose, with relatively little 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,6-di-*O*-methyl-D-galactose, or 2-*O*-methyl-D-galactose. Under-methylation of O-4 of galactose units has been reported[5] in *A. senegal* gum; the occurrence of 2,6-di- and 2-*O*-methylgalactose in *A. nubica* gum may also arise from under-methylation. The acidic residues in the gum are present as end groups. When the methylation results are compared, the most striking feature is the very small proportion of galactopyranose end-groups in the whole gum, when compared with the degraded gum. The whole gum contains proportionately more 2,4,6-tri-*O*-methyl-D-galactose and 2,4-di-*O*-methyl-D-galactose. It therefore appears that the point of attachment of the arabinose-containing units in the whole gum is to C-3 of (1 → 6)-linked galactose units and otherwise unsubstituted galactose units. Some arabinose may be linked to C-6 of galactose residues, but this cannot occur to any great extent as the most abundant, methylated galactose from the methylated whole gum would then be 2,3,4-tri-*O*-methyl-D-galactose.

Smith degradations. — Five successive Smith-degradations were carried out on the whole gum, and the five degraded polysaccharides (*S1–S5*) isolated were examined by hydrolysis and methylation. Every stage of the sequence of Smith degradations gave the same methylated sugars, *viz.*, 2,3,5-tri-*O*-methyl-L-arabinose; 2,5- and 3,5-di-*O*-methyl-L-arabinose; 2,3,4,6-tetra-*O*-methyl-D-galactose; 2,3,4- and 2,4,6-tri-*O*-methyl-D-galactose; and 2,4-di-*O*-methyl-D-galactose. Although it is unlikely that complete reaction is obtained at each stage of such a sequence of Smith degradations, the following conclusions appear to be reasonable. (*a*) Product *S1* did not contain rhamnose, glucuronic acid, or arabinopyranose units. This confirms the evidence from the methylation study, which indicated that acidic and arabinopyranose units are present exclusively as end groups. The rhamnose residues are oxidised by periodate and are probably present in peripheral, end-group positions. (*b*) Only the first Smith-degradation gave ethylene glycol and threitol as low molecular-weight products. Thus, all of the arabinopyranose end-groups, and oxidisable galactose residues linked through C-4, are eliminated at the first stage. (*c*) Yields from the degradations were low at every stage, compared with the yields obtained[5] in a study of *A. senegal* gum. This indicates a higher proportion of end groups in *A. nubica* and suggests a higher degree of branching than in *A. senegal*, which is itself extensively branched[5]. (*d*) Arabinose was the major sugar present in all the Smith-degraded polysaccharides *S1–S5*: this suggests that most of the arabinose-containing side-chains are at least 6 arabinose residues in length, and some may be considerably longer.

DISCUSSION

The core of *A. nubica* gum appears to be a highly branched galactan framework; some branches are terminated by D-glucuronic acid (and a very small proportion of its 4-*O*-methyl derivative) linked mainly β-(1 → 6) to D-galactose, but with some α-(1 → 4) links also. The only linkages detected between the D-galactose residues were β-(1 → 3)- and β-(1 → 6), with the latter type preponderating. Blocks of three contiguous β-(1 → 6)-linked D-galactose residues occur. There is no evidence for a "backbone" of (1 → 3)-linked D-galactose residues; this is at variance with early studies of *Acacia* gum exudates, but is in agreement with recent investigations of gums from *A. senegal*[5] and *A. arabica*[3].

Chains of L-arabinose residues are attached to the branched galactan framework, mainly at C-3 of the D-galactose residues; the L-arabinose chains are, on average, at least six units long, and some are terminated by L-arabinofuranose and L-arabinopyranose residues linked β-(1 → 3) to L-arabinose. The chains contain only (1 → 2)-linked L-arabinofuranose residues, with a smaller proportion of (1 → 3)-linkages. It is not known whether chains occur which are exclusively (1 → 2)- or (1 → 3)-linked, or whether both types of linkage occur in one chain.

A. nubica and *A. arabica*[3] exudates are the first strongly dextrorotatory *Acacia* gums to be studied structurally, and it is of interest to consider the nature of the major structural differences between these species and the *Acacia* species that give laevorotatory gums, *e.g.*, *A. senegal*[5]. Although the (1 → 4)-linked aldobiouronic acid present has a high positive rotation, it is not sufficiently abundant to make a decisive contribution to the overall rotation. Indeed, when the (1 → 4)-aldobiouronic acid linkage was destroyed by periodate oxidation, the residual polysaccharide was even more strongly dextrorotatory, and this effect persisted for Smith-degradation products *S*2 and *S*3. The origin of the highly positive rotation is clearly a deep-seated feature of the gum molecule.

A. nubica gum resembles other *Acacia* gums in giving a degraded gum consisting mainly of D-galactose residues linked β-(1 → 3) and β-(1 → 6). Unlike the gums from *A. senegal*[5] and *A. pycnantha*[7], β-D-(1 → 6)-linkages preponderate in *A. nubica* gum. It has been deduced[5] that *A. senegal* gum has a branched, tree-like core of D-galactose residues; there is evidence that the core of *A. nubica* gum is even more highly branched. As in *A. senegal*[5] gum, the L-arabinose chains are attached to C-3 of D-galactose; in *A. pycnantha*[7], they are attached to C-6 of D-galactose, and in *A. seyal*[8] they are attached to both C-3 and C-6 of D-galactose. The L-arabinose chains in *A. nubica* gum are much longer, however, than in *A. senegal*[5] gum (where four Smith-degradations eliminated all of the L-arabinose). The L-arabinose chains also differ in the respect that they consist largely of (1 → 2)-linked L-arabinofuranose units. Until recently, 3,5-di-*O*-methyl-L-arabinose had been reported to occur in only one species, *A. pycnantha*[7], but from studies carried out at the same time as this work[9], this sugar is now known to occur in *A. arabica* gum[3], and also in gums from several other *Acacia* species[10].

It therefore appears that *A. nubica* gum has a markedly different structure to that of *A. senegal* gum. It is not known whether *A. nubica* and *A. arabica* are typical of the dextrorotatory species that have low contents of L-rhamnose, or whether *A. senegal* is typical of the laevorotatory species that have relatively high contents of L-rhamnose; further studies of a wider range of *Acacia* gum exudates are required.

EXPERIMENTAL

The standard analytical methods have been described[5,11]. Paper chromatography was carried out on Whatman No. 1 and 3MM papers with the following solvent systems (v/v): (*a*) butyl alcohol–ethanol–water (4:1:5, upper layer); (*b*) butyl alcohol–pyridine–water–benzene (5:3:3:1, upper layer); (*c*) ethyl acetate–pyridine–water (10:4:3); (*d*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); (*e*) ethyl acetate–acetic acid–formic acid–water (18:8:3:9); (*f*) butyl alcohol–acetic acid–water (4:1:5, upper layer); (*g*) acetone–ethanol–propan-2-ol–borate buffer (0.05M, pH 10) (3:1:1:2); (*h*) butanone–acetic acid–water, saturated with boric acid (9:1:1); (*i*) butanone–water–conc. ammonia (200:17:1). R_{Gal} values refer to distances moved relative to galactose; R_G values of *O*-methyl sugars refer to distances moved relative to 2,3,4,6-tetra-*O*-methyl-D-glucose. Zone electrophoresis of sugars on Whatman No. 1 paper was carried out in 0.05M borate buffer (pH 10); M_G values refer to the true migration of the sugar relative to that of glucose. G.l.c. [chromatograph Type S3A, fitted with flame-ionisation detectors (Gas Chromatography Ltd.)] of mixtures of *O*-methyl sugars was carried out at nitrogen flow-rates of *ca.* 100 ml/min on columns of (*i*) 15% by weight of poly(butane-1,4-diol succinate) on 60–70 mesh Celite (5 ft × 0.25 in) at 175°, and (*ii*) 15% by weight of poly(ethylene glycol adipate) on 60–70 mesh Celite (3 ft × 0.25 in) at 160°. Retention times (T) are quoted relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside. Unless otherwise stated, methylations were carried out by the sodium hydride–methyl iodide–methyl sulphoxide system[6].

Origins of specimens. — Three samples of the exudate from *A. nubica* Benth. were available; they were collected by (the late) M.P. Vidal-Hall, formerly Gum Research Officer, El Obeid, from a single tree at Goz el Ganzara, Kordofan Province, Republic of the Sudan, on 15 December 1962 (sample *A*), 30 December 1962 (sample *B*), and 25 January 1963 (sample *C*).

Analytical data for the crude samples. — The results of analyses of samples *A*, *B*, and *C* are shown in Table I.

Purification and analysis of the purified gum. — Aqueous solutions of samples *A*, *B*, and *C* were filtered, dialysed, and then exhaustively electrodialysed. The results of analyses are shown in Table I. All further studies were made on Sample *A*.

Separation and characterisation of neutral sugars. — A series of experiments established that hydrolysis with N sulphuric acid for 8 h at 100° cleaved all glycosidic linkages (excepting those of aldobiouronic acids), without causing significant degradation of the sugars released. Gum sample *A* (5 g) was hydrolysed, and the hydrolysate

was neutralised ($BaCO_3$), deionised [IR-120(H^+) resin], and concentrated to a syrup that was applied to a cellulose column (60 × 4 cm), which was then developed with solvent (*b*). Fractions (25 ml) were collected; arabinose first appeared in tube 50. *Fraction 1:* the contents of tubes 11 to 47 were combined and evaporated to a syrup (15 mg). Chromatography [solvents (*a*), (*b*), (*c*), and (*d*)] revealed the presence of rhamnose. The syrup was taken up in ethanol–water. On standing, crystals were obtained: these gave an X-ray diffractogram identical to that of authentic rhamnose. *Fraction 2* had m.p. 158°, $[\alpha]_D^{20}$ +105° (*c* 0.5, water), and was identical chromatographically to L-arabinose in solvents (*a*), (*b*), and (*c*). The sugar was characterised as its phenylosazone, m.p. 163–164°; the X-ray diffractogram was identical to that of authentic L-arabinose. *Fraction 3* had m.p. 171°, $[\alpha]_D^{20}$ +81° (*c* 0.5, water) and was identical chromatographically to D-galactose in solvents (*a*), (*b*), and (*c*). This sugar was characterised as its phenylosazone, m.p. 200°; the X-ray diffractogram was identical to that of authentic D-galactose.

Separation and characterisation of acidic sugars. — The acidic sugars, virtually immobile in solvent (*b*), were eluted with water from the cellulose column described above, and isolated as a syrup (600 mg): chromatography [solvents (*b*) and (*d*)] revealed the presence of galactose, glucuronic acid, and glucurono-6,3-lactone. The syrup (200 mg) was hydrolysed with sulphuric acid (2N, 100°, 8 h), and the neutralised hydrolysate ($BaCO_3$) was filtered and deionised. The products were separated into neutral and acidic components on a column (40 × 3 cm) of Duolite A4 resin. The neutral sugars, with traces of glucurono-6,3-lactone, were eluted with water and not investigated further. The acidic fractions were eluted with formic acid (5%), and isolated as a syrup (95 mg) that was chromatographed [solvent (*d*)] on thick paper; five fractions were obtained. *Fraction 1* was D-glucurono-6,3-lactone, R_{Gal} 3.49, $[\alpha]_D$ +19° (*c* 2.0, water); re-crystallisation from water gave crystals, m.p. and mixed m.p. 177°. *Fraction 2* (3 mg) was syrupy 4-*O*-methyl-D-glucuronic acid, R_{Gal} 2.58, $[\alpha]_D$ +33° (*c* 0.1, water), and was chromatographically identical to authentic material [solvents (*d*) and (*e*)]. Reduction (borohydride) of the methyl ester methyl glycoside gave a trace of material that was chromatographically identical [solvent (*a*)] to 4-*O*-methyl-D-glucose. *Fraction 3* was chromatographically identical to D-glucuronic acid, in solvents (*d*) and (*e*), and since glucurono-6,3-lactone had been characterised from Fraction 1, this material was not examined further. *Fraction 4* was chromatographically identical to D-galactose in solvents (*a*), (*b*), and (*c*). *Fraction 5* consisted of a trace of unhydrolysed aldobiouronic acid, R_{Gal} 0.23, identical chromatographically in solvent (*d*) to 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose.

The aldobiouronic acids. — The aldobiouronic acid fraction was obtained by partial hydrolysis of the gum with N sulphuric acid for 8 h. The reaction product was neutralised ($BaCO_3$), filtered, concentrated to a syrup, and added to ethanol. The precipitated barium aldobiouronates were removed at the centrifuge, deionised, and purified by paper chromatography on thick paper [solvent (*d*)]. Traces of material [R_{Gal} 0.61, solvent (*d*)] could not be isolated in sufficient quantity for characterisation. The major component was a syrup [R_{Gal} 0.28, solvent (*d*)], $[\alpha]_D$+ 11° (*c* 0.5, water).

Since this did not correspond to any of the aldobiouronic acids commonly found[1] in the *Acacia* group, the product was re-examined; prolonged paper chromatography [solvent (*d*)] resolved two components having R_{Gal} 0.26 (major) and R_{Gal} 0.32 (minor component). The syrupy major component was chromatographically identical to authentic 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose, and had $[\alpha]_D$ −4° (*c* 1.0, water); the minor component was identical chromatographically to 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose, $[\alpha]_D$ +87° (*c* 1.0, water). These two aldobiouronic acids were then rigorously characterised by the same sequences of reactions already described[1] for the identification of the aldobiouronic acids in *A. seyal* gum.

Autohydrolysis experiments. — Solutions (5%) of purified samples *A* and *B* were heated at 98°; portions (5 ml) were withdrawn at various intervals and analysed. The results for solution *A* are shown in Table II; solution *B* gave virtually identical results.

Degraded gum *A* was obtained by autohydrolysis of a solution of sample *A* (29.14 g in 600 ml of water) for 100 h at 98°. The solution was cooled, filtered, and dialysed against distilled water for 5 days; the dialysates were concentrated, and retained for further study. The solution of degraded gum *A* was freeze-dried (yield, dry weight basis, 24.5%); $[\alpha]_D$ +44° (*c* 1.9, water); methoxyl, 0.10; uronic acid, 12; galactose, 83; arabinose, 5%.

Examination of degraded gum A. — Degraded gum *A* was hydrolysed at 100° in sulphuric acid (0.5N); aliquots were removed at 0.5, 1, 1.5, 2, 3, 4, and 5 h, neutralised, and examined chromatographically in solvents (*a*) and (*b*). The yield of disaccharides, estimated visually, was greatest after 1 h. Accordingly, degraded gum *A* (1.793 g) was dissolved in sulphuric acid (0.5N, 100 ml) and hydrolysed for 1 h. The solution was neutralised, filtered, concentrated, and examined by paper chromatography in solvents (*a*), (*b*), and (*c*). Five fractions were obtained. *Fraction 1*, R_{Gal} 0.54 [solvent (*a*)], 0.47 (*b*), 0.51 (*c*), was chromatographically identical to 3-*O*-β-D-galactopyranosyl-D-galactose. Crystallisation from acetone–water gave a product having $[\alpha]_D$ +61° (*c* 1.1, water); m.p. and mixed m.p. 161°. *Fraction 2*, R_{Gal} 0.36 (*a*), 0.31 (*b*), and 0.44 (*c*), did not crystallise; it had $[\alpha]_D^{17}$ +33° (*c* 0.12, water), and the phenylosazone had m.p. 198–199°. The fraction was chromatographically identical to 6-*O*-β-D-galactopyranosyl-D-galactose; it was methylated[6], and, after methanolysis, the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-galactose [T = 1.67 (*i*); 1.66 (*ii*)]; 2,3,4-tri-*O*-methyl-D-galactose [T = 5.06 (*i*); 6.22 (*ii*)]; and 2,3,5-tri-*O*-methyl-D-galactose [T = 3.16, 4.12 (*i*); 3.92, 5.30 (*ii*)] were identified by g.l.c. Fraction 2 was about three times more abundant than Fraction 1. *Fraction 3*, R_{Gal} 0.22, (*a*) and (*b*), present in trace amount only, was chromatographically identical to *O*-β-D-galactopyranosyl-[1 → 3)-*O*-β-D-galactopyranosyl-(1 → 3)-D-galactose, with a degree of polymerisation[12] = 2.91. Partial hydrolysis gave only galactose and 3-*O*-β-D-galactopyranosyl-D-galactose. *Fraction 4*, R_{Gal} 0.14 (*a*), 0.16 (*b*); degree of polymerisation[12] 2.80; was a syrup having $[\alpha]_D$ +18° (*c* 0.11, water). Partial hydrolysis with acid gave galactose and 6-*O*-β-D-galactopyranosyl-D-galactose. After methylation[6] and methanolysis, the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-galactose, and 2,3,4- and 2,3,5-tri-*O*-methyl-D-galactose, were identified by g.l.c. The mixture of methyl glycosides

was hydrolysed, and double development of the resulting sugars in solvent (*j*) gave spots corresponding to 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,3,4-tri-*O*-methyl-D-galactose. It was concluded that the trisaccharide was *O*-β-D-galactopyranosyl-(1 → 6)-*O*-β-D-galactopyranosyl-(1 → 6)-D-galactose. *Fraction 5* had R_{Gal} 0.08 (*a*), 0.09 (*b*); degree of polymerisation[12] = 3.6. Partial hydrolysis by acid, with and without prior reduction of the reducing end-group, showed the presence of 3-*O*-β-D-galactopyranosyl-D-galactose and 6-*O*-β-D-galactopyranosyl-D-galactose. It was concluded that this fraction was a mixture of tetra- and tri-saccharides.

Degraded gum *A* (415 mg) was dissolved in water (100 ml), and sodium borohydride (400 mg) was added. After 1 day, further sodium borohydride (100 mg) was added. After dialysis, the reduced, degraded gum was freeze-dried; yield, 91%. A portion of the reduced, degraded gum was hydrolysed (N sulphuric acid, 100°, 7 h). After neutralisation and concentration, chromatographic analysis (solvent *h*) showed galactose, traces of arabinose, and galactitol. A portion of the reduced, degraded gum (200 mg) was hydrolysed, neutralised, filtered, deionised, and concentrated to *ca.* 25 ml. The solution was made 0.1N with respect to ammonia, and IRA-400 resin (OH^-) was added[13]. The solution was heated (100°, 6 h), cooled, filtered, and taken to dryness. Paper chromatography [solvent (*b*)] of a solution of the residue revealed galactitol and immobile material. With solvent (*d*), galactitol and slow-moving material were found: galactose was not detected with either solvent. On evaporation, a semi-crystalline mass was obtained; this was treated with acetic anhydride containing a trace of sulphuric acid. After 24 h, water was added; hexa-*O*-acetylgalactitol (6 mg) was precipitated, and recrystallisation from ethanol gave crystals, m.p. 168°.

Molecular weight of degraded gum A. — Reduced, degraded gum *A* (41.83 mg) was dissolved in *p*-hydroxybenzaldehyde solution (0.1%, 10 ml), and degraded gum *A* (44.23 mg) was likewise dissolved. After complete oxidation with sodium metaperiodate, 9.946 μg of formaldehyde was liberated from each mg of the degraded gum, and 16.734 μg/mg was obtained from the reduced, degraded gum, corresponding to a molecular weight of 5,730 for the degraded gum *A*.

Investigation of other autohydrolysis products. — (*a*) *Proteinaceous material.* Filtration of the solution of autohydrolysed gum gave a brown residue which was suspended in hydrochloric acid (6N) and hydrolysed for 12 h (sealed tube). Concentration under diminished pressure gave a brown syrup which was chromatographed in solvent (*a*). On detection with ninhydrin, blue spots were revealed, indicative of the proteinaceous nature of the residue. (*b*) *Carbohydrate material.* The dialysate of the autohydrolysate contained arabinose and galactose (in the ratio 4:1), a mixture of oligosaccharides, and some 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose.

The yield of arabinose oligomers was, however, very low, and attempts were made to improve the yield by carrying out the autohydrolysis in a dialysis sac so that material of low molecular weight was not, in turn, subjected to continued hydrolytic conditions. After trial kinetic experiments to establish the conditions that gave the maximum yield of oligosaccharides, a 5% solution of the purified whole gum was

autohydrolysed for 16 h at 95°. The dialysate was concentrated, and four fractions were obtained by chromatography in solvent (*d*). *Fraction 1* was identical to arabinose in solvents (*a*), (*b*), (*c*), and (*d*), and was not examined further. *Fraction 2*; R_{Gal} 1.23 (*d*), 1.28 (*b*) (*cf*., L-arabinose, 1.39), 1.24 (*c*) (*cf*., L-arabinose, 1.38); was a syrup having $[\alpha]_D$ +88° (*c* 0.1, water). Hydrolysis gave only arabinose, and the phenylosazone had m.p. 201–203°. It was suspected that this disaccharide was 3-*O*-β-L-arabinofuranosyl-L-arabinose (lit.[14], $[\alpha]_D$ +89° and +94°; phenylosazone, m.p. 200°). After methylation[6] and methanolysis, the products were found (g.l.c.) to be the methyl glycosides of 2,3,5-tri-*O*-methyl-L-arabinose [T = 0.52, 0.67 (*i*); 0.54, 0.73 (*ii*)]; 2,3,4-tri-*O*-methyl-L-arabinose (trace) [T = 0.82 (*i*); 0.96 (*ii*)]; 2,5-di-*O*-methyl-L-arabinose [T = 1.30, 2.23 (*i*); 1.77, 3.16 (*ii*)]; and a trace of 2,4-di-*O*-methylarabinose [T = 1.47, 1.60 (*i*); 2.19, 2.29 (*ii*)]. *Fraction 3* had R_{Gal} 0.70 [solvent (*d*)], 0.88 (*c*), and was chromatographically identical to authentic 3-*O*-β-L-arabinopyranosyl-L-arabinose. Hydrolysis gave only arabinose, and the phenylosazone had m.p. 229–230° and mixed m.p. 230° (with an authentic sample[14] of m.p. 233°). After methylation[6] and methanolysis, the products were found (g.l.c.) to be the methyl glycosides of 2,3,4-tri-*O*-methyl-L-arabinose [T = 0.83 (*i*); 0.98 (*ii*)]; 2,5-di-*O*-methyl-L-arabinose [T = 1.30, 2.21 (*i*); 1.78, 3.17 (*ii*)]; and a trace of 2,4-di-*O*-methyl-L-arabinose [T = 1.47, 1.61 (*i*); 2.20, 2.28 (*ii*)]. *Fraction 4* had R_{Gal} 0.54 [solvent (*d*)], and hydrolysis gave only arabinose. Mild hydrolysis (sulphuric acid, 0.01N; 100°, 2 h), followed by neutralisation (Deacidite FF resin), gave arabinose and 3-*O*-β-L-arabinopyranosyl-L-arabinose as the major products, with traces of 3-*O*-β-L-arabinofuranosyl-L-arabinose. Methylation[6], methanolysis, and g.l.c. examination of the products showed the following glycosides to be present: 2,3,4- and 2,3,5(trace only)-tri-*O*-methylarabinose; 2,5- and 2,4(trace only)-di-*O*-methylarabinose. This fraction was therefore identified as *O*-β-L-arabinopyranosyl-(1 → 3)-*O*-β-L-arabinofuranosyl-(1 → 3)-L-arabinose, although the presence of the homologous β-(1 → 3)-linked L-arabinofuranosyl trisaccharide cannot be excluded.

Methylation studies. — The methylations of *A. nubica* gum and of degraded *A. nubica* gum A have been described[6].

Examination of methylated, degraded gum A. — The methylated, degraded gum had $[\alpha]_D$ +10° (*c* 0.1, chloroform); methoxyl, 41.8%. Methanolysis, followed by g.l.c. examination of a portion of the mixture of methyl glycosides, gave the results shown in Table III. Hydrolysis of the major portion, with examination of the free *O*-methyl sugars by paper chromatography in solvents (*a*), (*f*), and (*j*), gave the results in Table III. The mixture of methylated sugars was then fractionated on a cellulose column [65 × 4 cm, solvent (*a*)]; when necessary, sub-fractions were obtained by thick-paper chromatography [solvent (*j*)]. When all of the neutral sugars had been eluted, acidic components were eluted with water. *Fraction 1* (193 mg), which had R_{Gal} 0.91 [solvent (*a*)], $[\alpha]_D$ +111° (*c* 1.9, water), was chromatographically identical to 2,3,4,6-tetra-*O*-methyl-D-galactose in solvents (*a*), (*f*), and (*j*). It was characterised by conversion into 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine, m.p. 195–196° (from ethyl acetate). *Fraction 2* (12 mg), R_G 0.84 [solvent (*a*)], was chromatographically

TABLE III

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED, DEGRADED GUM *A*

Relative retention times (T) *of methyl glycosides*[a]		R_G *values in*			O-*Methyl sugars identified*
Column (i)	*Column* (ii)	*Solvent*(a)	*Solvent*(f)	*Solvent*(j)[b]	
0.82	1.04	0.85	—	0.73	2,3,4-tri-*O*-methyl-L-arabinose
1.65	1.65	0.92	0.91	0.87	2,3,4,6-tetra-*O*-methyl-D-galactose
2.03, 2.61	2.31, (2.89)	0.10	0.80	0	2,3,4-tri-*O*-methyl-D-glucuronic acid[c]
2.40, (2.93), (3.35)	(2.89), (3.71), (4.18)	0.73	0.72	0.67	2,3,6-tri-*O*-methyl-D-galactose
(2.93), (3.35)	(3.71), (4.18)	0.73	0.72	0.56	2,4,6-tri-*O*-methyl-D-galactose
4.99	6.39	0.73	0.72	0.51	2,3,4-tri-*O*-methyl-D-galactose
9.4, 10.7	14.6, 16.4	0.49	0.50	0.21	2,4-di-*O*-methyl-D-galactose

[a]Values in parentheses indicate incompletely resolved components. [b]Using double development. [c]As methyl ester.

identical, in solvents (*a*) and (*j*), to 2,3,4-tri-*O*-methyl-L-arabinose. *Fraction 3* (341 mg) was a mixture [solvent (*j*)] of 2,3,4-, 2,4,6-, and 2,3,6-tri-*O*-methyl-D-galactose. Since the 2,3,4- and 2,4,6-tri-*O*-methyl-D-galactose could not be separated satisfactorily, the procedure of Dutton and Unrau[15] was applied to Fraction 3. As a result, galactitol, arabinitol, and threitol were obtained in the proportions 16:9:1, and hence the proportions of 2,4,6-, 2,3,4-, and 2,3,6-tri-*O*-methyl-D-galactose must have been 11:7:1. *Fraction 4* (266 mg), R_G 0.48 [solvent (*a*)], $[\alpha]_D$ $+84°$ (*c* 2.5, water), was chromatographically identical to 2,4-di-*O*-methyl-D-galactose in solvents (*a*) and (*j*). Demethylation gave D-galactose, and the sugar was characterised as 2,4-di-*O*-methyl-*N*-phenyl-D-galactosylamine, m.p. 214–215° (from ethyl acetate). *Fraction 5* (130 mg), R_G 0.80 [solvent (*f*)], $[\alpha]_D$ $+55°$ (*c* 2.9, water), was identical chromatographically to 2,3,4-tri-*O*-methyl-D-glucuronic acid in solvent (*f*). The methyl ester methyl glycoside was reduced (lithium aluminium hydride) to give 2,3,4-tri-*O*-methyl-D-glucose, which was purified by thick-paper chromatography [solvent (*a*)]; yield, 52 mg. This sugar was characterised as 2,3,4-tri-*O*-methyl-*N*-phenyl-D-glucosylamine, m.p. 147°.

Examination of methylated, whole gum. — The methylated[6] gum (methoxyl, 41.0%) was methanolysed, and g.l.c. examination of a portion of the mixture of methyl glycosides gave the results shown in Table IV. The major portion was hydrolysed, and the free *O*-methyl sugars were examined by paper chromatography in solvent (*a*) (results in Table IV). The mixture of methylated sugars was then fractionated on a cellulose column (65 × 4 cm); the initial eluant was light petroleum–butyl alcohol (7:3), saturated with water. When most of the tri-*O*-methylgalactoses had been eluted, the eluant was changed to light petroleum–butyl alcohol (1:1), saturated with water. When necessary, sub-fractions were obtained by thick-paper chromatography in solvents (*a*) and (*j*). After the neutral sugars had been eluted from the column, the acidic components were eluted with water to give the following fractions. *Fraction 1* (942 mg, syrup) had $[\alpha]_D$ $-29°$ (*c* 1.0, water), and was identical chromatographically to 2,3,5-tri-*O*-methyl-L-arabinose. Demethylation gave arabinose only. The sugar was characterised by conversion into 2,3,5-tri-*O*-methyl-L-arabinonamide, m.p. 136° (from ethyl acetate). *Fraction 2* (33 mg) had $[\alpha]_D$ $+110°$ (*c* 0.7, water), and was identical chromatographically to 2,3,4,6-tetra-*O*-methyl-D-galactose. The sugar was characterised by conversion into 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine, m.p. and mixed m.p. 195–196°. *Fraction 3* (750 mg, syrup) was a mixture of 2,5- and 3,5-di-*O*-methyl-L-arabinose. Paper-electrophoretic examination (7.3 volts/cm, 0.6 mamps/cm, Whatman 3 MM paper, 6 h) in 0.05M borate buffer (pH 10) confirmed the presence of 2,5-(M_G 0.00) and 3,5-di-*O*-methylarabinose (M_G 0.70). Continuous electrophoresis on a paper curtain at 600 volts allowed the two components to be separated, the 3,5-di-*O*-methylarabinose migrating towards the anode as the borate complex. After elution of the sugars with water, the solutions were deionised and taken to dryness, and borate was removed by several distillations of 1% methanolic hydrogen chloride from the residue. The resulting methyl glycosides were hydrolysed to the free sugars as subfractions (*a*) and (*b*). Subfraction (*a*) (101 mg), $[\alpha]_D$ $-37°$ (*c* 0.10, water), had the same mobility as 2,5-di-*O*-methyl-L-arabinose,

TABLE IV

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED *A. nubica* GUM

Relative retention times (T) *of methyl glycosides*[a]		R_G *value in solvent* (a)	*Colour with aniline oxalate*	O-*Methyl sugars identified*
Column (i)	*Column* (ii)			
0.51, 0.64	0.58, 0.74	0.97	black	2,3,5-tri-*O*-methyl-L-arabinose
(0.78)	(1.03)	0.78	pink	2,3,4-tri-*O*-methyl-L-arabinose
(0.78), (1.67)	(1.03), (2.39)	0.81	dark brown	3,5-di-*O*-methyl-L-arabinose
1.29, (2.14)	1.77, 3.16	0.84	black	2,5-di-*O*-methyl-L-arabinose
(1.67)	1.66	0.91	red–brown	2,3,4,6-tetra-*O*-methyl-D-galactose
(2.14), 2.65	(2.39), (2.93)	*ca.* 0.1	red	2,3,4-tri-*O*-methyl-D-glucuronic acid[b]
2.39, (3.02), (3.39)	(2.93), (3.81), (4.23)	0.71	red–brown	2,3,6-tri-*O*-methyl-D-galactose
(3.02), (3.39)	(3.81), (4.23)	0.71	red–brown	2,4,6-tri-*O*-methyl-D-galactose
5.09	6.52	0.71	red–brown	2,3,4-tri-*O*-methyl-D-galactose
6.13	9.35	0.54	red–brown	2,6-di-*O*-methyl-D-galactose
9.15, **10**.18	15.0, 17.0	0.52	red–brown	2,4-di-*O*-methyl-D-galactose
—	—	0.36	red–brown	2-*O*-methyl-D-galactose

[a]Figures in parentheses denote unresolved components. [b]As methyl ester methyl glycoside.

and gave the same colour with the aniline oxalate spray: it was immobile on paper electrophoresis in borate buffer. The sugar was characterised by conversion into 2,5-di-*O*-methyl-L-arabinonamide, m.p. 129°, (from ethyl acetate). Subfraction (*b*) (392 mh), $[\alpha]_D$ −29°, was identical to 3,5-di-*O*-methyl-L-arabinose on paper chromatography[16], and paper electrophoresis[17] in borate buffer. The sugar was characterised by conversion into 3,5-di-*O*-methyl-L-arabinonamide, m.p. 143° (from ethyl acetate). *Fraction 4* (70 mg), $[\alpha]_D$ +112° (*c* 1.3, water), was identical chromatographically to 2,3,4-tri-*O*-methyl-L-arabinose, and was characterised by conversion into 2,3,4-tri-*O*-methyl-L-arabinonamide, m.p. 103°. *Fraction 5* (505 mg) was shown by paper chromatography and g.l.c. to be a mixture of 2,4,6-, 2,3,4-, and 2,3,6-tri-*O*-methyl-D-galactose in the proportions 5:1:trace. These sugars had already been characterised during the examination of the degraded gum, and they were not investigated further. *Fraction 6* (10 mg) gave galactose on demethylation, and was chromatographically identical to 2,6-di-*O*-methyl-D-galactose in solvents (*a*) and (*j*). *Fraction 7* (254 mg) had $[\alpha]_D$ +86° (*c* 2.5, water), and crystallised spontaneously, m.p. 86–87°. The sugar was identical chromatographically to 2,4-di-*O*-methyl-D-galactose, and was characterised as 2,4-di-*O*-methyl-*N*-phenyl-D-galactosylamine, m.p. and mixed m.p. 214°. *Fraction 8* (14 mg), $[\alpha]_D$ +77°, gave only galactose on demethylation, and was identical chromatographically to 2-*O*-methyl-D-galactose in solvents (*a*) and (*j*). *Fraction 9* (173 mg) had $[\alpha]_D$ +53° (*c* 1.0, water), and was identical in solvent (*f*) to 2,3,4-tri-*O*-methyl-D-glucuronic acid. Reduction of the methyl ester methyl glycoside gave 2,3,4-tri-*O*-methyl-D-glucose. This sugar had already been characterised in the degraded gum and was not examined further.

Smith degradation of degraded gum A. — Degraded gum *A* (1.782 g) was dissolved in water (100 ml), and 0.25M sodium metaperiodate solution (100 ml) was added. The amount of formic acid released (mmoles/g) was 6.93 (23 h), 7.14 (29 h), and 7.19 (33 h). After 36 h, the reaction was stopped by addition of ethylene glycol. The solution was dialysed (36 h), and then sodium borohydride (2 g) was added. After 48 h, the solution was dialysed for 100 h, and the dialysate was shown [solvent (*a*)] to contain glycolic acid, glycerol, and threitol. The polyalcohol was hydrolysed in N sulphuric acid for 48 h at room temperature. After neutralisation ($BaCO_3$), and concentration to 15 ml, the solution was added to ethanol (1 litre). The precipitate was re-dissolved, and the solution was neutralised, filtered, and freeze-dried to give degraded gum *B* (yield, 16.2%).

Partial acid hydrolysis and methylation of degraded gum B. — Degraded gum *B* (10 mg) was hydrolysed (0.5N sulphuric acid, 1 h), and the products were examined chromatographically in solvents (*b*), (*c*), and (*d*). Galactose, traces of arabinose and glycerol, and three oligosaccharides, *viz.*, 6-*O*-β-D-galactopyranosyl-D-galactose, 3-*O*-β-D-galactopyranosyl-D-galactose, and the β-(1 → 3)-linked D-galactose trisaccharide were identified by comparison with authentic samples; the trisaccharide was tentatively identified from its reported chromatographic mobility.

Degraded gum *B* (82 mg) was methylated[6]; methanolysis of the product, followed by g.l.c. examination of the mixture of glycosides, gave the results shown

in Table V. A portion of the mixture of methylated glycosides was hydrolysed, and the free sugars were examined in solvents (*a*) and (*j*); the sugars listed in Table V were identified.

TABLE V

EXAMINATION OF METHANOLYSIS PRODUCTS FROM METHYLATED DEGRADED GUM *B*

Relative retention times (T) *of methyl glycosides*		*Approx. relative proportions*	O-*Methyl sugar identified*
Column (i)	*Column* (ii)		
1.67	1.67	+ + +	2,3,4,6-tetra-*O*-methyl-D-galactose
2.97, 3.40	3.74, 4.21	+ + +	2,4,6-tri-*O*-methyl-D-galactose
5.06	6.49	+	2,3,4-tri-*O*-methyl-D-galactose
9.59, 10.9	14.6, 16.4	+	2,4-di-*O*-methyl-D-galactose
0.54, 0.67	0.57, 0.73	trace	2,3,5-tri-*O*-methyl-L-arabinose

Successive Smith-degradations of the whole gum. — Borohydride reduction of periodate-oxidised whole gum (20.6 g), followed by controlled acid hydrolysis of the polyalcohol for 48 h at room temperature, gave polysaccharide *S1* (yield, 54%; analytical data as shown in Tables VI and VII). The dialysate from the controlled acid hydrolysis of the polyalcohol was concentrated to a syrup; chromatographic examination in solvent (*b*) revealed the presence of glycolaldehyde (R_F 0.67, major product); glycerol (R_F 0.53, major product); threitol (R_F 0.40, minor product), and arabinose (R_F 0.34, trace). Examinations were also made in solvents (*a*), (*c*), (*d*), and (*h*), with similar conclusions.

TABLE VI

FORMIC ACID RELEASED[a] ON PERIODATE OXIDATIONS

Time (h)	*3*	*6*	*9*	*12*	*24*	*27*	*30*	*48*	*54*	*96*	*142*
A. nubica gum	0.39	0.50	0.58	0.66	0.83		0.89	1.0			
Polysaccharide *S1*	0.63	0.66	0.68		0.74	0.74					
Polysaccharide *S2*	0.46	0.47	0.48		0.48						
Polysaccharide *S3*	0.32	0.34		0.37	0.41		0.42	0.48			
Polysaccharide *S4*					0.10		0.14			0.40	0.46

[a]As mmoles of formic acid per g of polysaccharide.

Examination of Smith-degraded polysaccharide S1. — Hydrolysis, followed by chromatographic examination [solvents (*b*), (*c*), and (*d*)], showed only galactose and arabinose (35:65). Partial hydrolysis (0.5N sulphuric acid, 2 h), followed by chromatographic examination [solvents (*b*) and (*c*)], showed arabinose, galactose, 3-*O*-β-D-galactopyranosyl-D-galactose, and 6-*O*-β-D-galactopyranosyl-D-galactose to be present.

TABLE VII

RESULTS OF SMITH DEGRADATIONS

	Whole gum	*Smith-degraded polysaccharides*				
		S1	*S2*	*S3*	*S4*	*S5*
Yield from preceding polysaccharide, %	—	54	37	29	25	11
$[\alpha]_D$	+100°	+102°	+117°	+112°	+98°	+89°
Formic acid released on periodate oxidation[a]	1.01	0.74	0.48	0.49	0.46	—
Sugar ratios, %: galactose	33	35	35	30	26	31
arabinose	59	65	65	70	74	69
rhamnose	0.7	—	—	—	—	—
glucuronic acid	7	—	—	—	—	—

[a]As mmoles of formic acid per g of polysaccharide.

Polysaccharide *S1* (350 mg) was methylated[6] (yield, 300 mg; methoxyl, 39.7%). A portion of the methylation product was methanolysed, and half of the product was examined by g.l.c.; the remainder was hydrolysed to the free sugars and examined by paper chromatography [solvents (*a*) and (*j*)]. The methyl glycosides and methylated sugars listed in Table VIII were identified. The 2,3,4- and 2,4,6-tri-*O*-methyl-D-galactose were resolved in solvent (*j*); by g.l.c., the ratio of 2,3,4- to 2,4,6-tri-*O*-methyl-D-galactose was 1:2.

TABLE VIII

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED, DEGRADED GUMS *S1–S5*.

Relative retention times (T) *of methyl glycosides*[a]		R_G *in solvent* (*a*)	*O-Methyl sugars identified*
Column (i)	*Column* (ii)		
0.51, 0.64	0.58, 0.74	0.97	2,3,5-tri-*O*-methyl-L-arabinose
0.78, (1.67)	1.05, 2.40	0.81	3,5-di-*O*-methyl-L-arabinose
1.29, 2.18	(1.80), 3.19	0.84	2,5-di-*O*-methyl-L-arabinose
(1.67)	(1.67)	0.92	2,3,4,6-tetra-*O*-methyl-D-galactose
3.00, 3.45	3.78, 4.26	0.73	2,4,6-tri-*O*-methyl-D-galactose
5.11	6.53	0.73	2,3,4-tri-*O*-methyl-D-galactose
9.62, 11.01	14.9, 16.9	0.48	2,4-di-*O*-methyl-D-galactose

[a]Parentheses denote sugars that were incompletely resolved.

Preparation, partial and complete hydrolyses, and methylation of degraded polysaccharide S2. — The second Smith-degradation product, *S2*, was obtained from *S1* (10.6 g) in a manner similar to that described above for the preparation of polysaccharide *S1* from the whole gum. The yield and analytical data for polysaccharide *S2* are shown in Tables VI and VII. Hydrolysis, and partial hydrolysis, gave the same sugars, in similar proportions to those identified in polysaccharide *S1*. A portion

(326 mg) of polysaccharide *S2* was methylated[6] (yield, 120 mg; methoxyl, 40.0%). Paper-chromatographic and g.l.c. examination of the methanolysis and hydrolysis products from methylated polysaccharide *S2* gave the methyl glycosides and *O*-methyl sugars shown in Table VIII. In this instance, however, the ratio of 2,3,4- to 2,3,6-tri-*O*-methyl-D-galactose was 2:5.

Preparation, partial and complete hydrolysis, and methylation of degraded polysaccharide S3. — The third Smith-degradation product, *S3*, was obtained from *S2* (3.26 g) by the method outlined above for *S1*. The yields and analytical data for polysaccharide *S3* are shown in Tables VI and VII. Partial and complete hydrolyses showed the same sugars as identified in polysaccharides *S1* and *S2*. A portion (346 mg) of polysaccharide *S3* was methylated (methoxyl, 40.1%). Methanolysis and hydrolysis of the product was followed by g.l.c. and paper chromatography; the methyl glycosides and *O*-methyl sugars shown in Table VIII were identified. The proportion of 2,3,4,6-tetra-*O*-methyl-D-galactose was, however, much smaller, and the ratio of 2,3,4- to 2,4,6-tri-*O*-methyl-D-galactose was 1:5.

Preparation, hydrolyses, and methylation of degraded polysaccharide S4. — In view of the low yield of *S3* (0.856 g from 20 g of *A. nubica* gum), the first three Smith-degradations were repeated on a larger scale (90 g of *A. nubica* gum). These were carried out serially as described for the preparation of polysaccharides *S1*, *S2*, and *S3*, with the exception that *S1* and *S2* were not isolated. The product (3.34 g), *S3*, from the third degradation, was freeze-dried and analysed: it had the same ratio of galactose to arabinose as *S3* described above, and this preparation of *S3* (3.05 g) was used to prepare *S4*.

The percentage yield and analytical data for *S4* are shown in Tables VI and VII. Partial and complete hydrolyses showed arabinose, galactose, 6-*O*-β-D-galactopyranosyl-D-galactose, 3-*O*-β-D-galactopyranosyl-D-galactose (and its homologous trisaccharide) to be present. A portion of *S4* (61 mg) was methylated: the product was shown by g.l.c. and paper chromatography to contain the methylated sugars shown in Table VIII. The ratio of 2,3,4- to 2,4,6-tri-*O*-methyl-D-galactose was 1:12.

Preparation, hydrolysis, and methylation of degraded polysaccharide S5. — The fifth Smith-degraded product, *S5*, was obtained from *S4* (487 mg). The yield and analytical data are shown in Tables VI and VII. Total hydrolysis showed only galactose and arabinose to be present. The remaining material (30 mg) was methylated[6]. The product was methanolysed, and 50% of the solution obtained was retained for g.l.c. examination; the remainder was hydrolysed to the free sugars and examined by paper chromatography. The glycosides and sugars shown in Table VIII were identified although only a trace of 2,3,4,6-tetra-*O*-methyl-D-galactose was present; the ratio of 2,3,4- to 2,4,6-tri-*O*-methyl-D-galactose was 1:5. In view of the small amount of, *S5* obtained (48 mg), the results must be considered with caution. Perhaps the most significant result from the preparation of this degraded polysaccharide is the small yield (11%). Considering the Smith-degradation sequence overall, the significant factors are: (*a*) the progressively smaller yields at each stage, (*b*) the steady decrease in the amount of 2,3,4-6-tetra-*O*-methyl-D-galactose detected, and (*c*) the preponder-

ance of 3,5-di-*O*-methyl-L-arabinose as a major product in all of the polysaccharides; the proportion of 2,5- relative to 3,5-di-*O*-methyl-L-arabinose decreased as the degradation sequence proceeded from *S1* to *S5*.

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STUDIES ON URONIC ACID MATERIALS

PART XXVIII. SOME STRUCTURAL FEATURES OF *Acacia drepanolobium* GUM.

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ABSTRACT

Linkage analysis of Fraction *A* of *A. drepanolobium* gum yielded 3-*O*-β-L-arabinopyranosyl-L-arabinose, 3-*O*-β-L-arabinofuranosyl-L-arabinose, 3-*O*-β-D-galactopyranosyl-D-galactose, 6-*O*-β-D-galactopyranosyl-D-galactose, and the β-D-(1→3)- and β-D-(1→6)-linked trisaccharides. The *O*-methyl derivative of degraded gum *A* was analysed, after methanolysis, by gas–liquid partition chromatography. After paper-chromatographic separation of a hydrolysate of methylated, degraded gum *A*, the resulting *O*-methyl sugars were characterised. Degraded gum *A* was subjected to a Smith degradation, and the product was examined by linkage and methylation analysis. Degraded gum *A* was subjected to a controlled Smith degradation, and the product was shown to contain arabinitol. This shows that at least some of the reducing galactose residues of degraded gum *A* are substituted at both C-3 and C-6. The structural evidence obtained shows that, although *A. drepanolobium* gum Fraction *A* and *A. arabica* gum are very similar, some differences do exist between these two gums.

INTRODUCTION

The gum polysaccharide from *Acacia drepanolobium* Harms ex Sjösted is not completely water-soluble. In a previous paper[2], the gum was subjected to a graded extraction procedure whereby three fractions were obtained: (*A*) water-soluble, 80%; (*B*) salt-soluble, 8%; (*C*) a gel soluble in dilute alkali, 12%. Analytical data for these fractions have been given[2], and the identity of the four aldobiouronic acids present in each fraction has been established[2]. This Paper presents the results of a structural study of the water-soluble fraction of *Acacia drepanolobium* gum.

EXPERIMENTAL

The origin of the gum[2], the preparation[2] of the electrodialysed, freeze-dried, sample of the cold water-soluble fraction of the gum, and the standard analytical

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methods[3] have been described. Paper chromatography was carried out on Whatman No. 1 and 3MM papers with the following solvent system (v/v): (*a*) benzene–butyl alcohol–pyridine–water (1:5:3:3, upper layer); (*b*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); (*c*) ethyl acetate–pyridine–water (10:4:3); (*d*) butyl alcohol–ethanol–water (4:1:5, upper layer); (*e*) butanone–water–ammonia (sp. gr. 0.88) (200:17:1); (*f*) butanone–acetic acid–water (9:1:1, saturated with boric acid). R_{Gal} and R_G values refer to distances moved relative to D-galactose and 2,3,4,6-tetra-*O*-methyl-D-glucose, respectively.

Gas–liquid partition chromatography (g.l.c.) of mixtures of methyl glycosides was carried out on a Chromatograph Type S3A (Gas Chromatography Ltd.), at fitted with flame-ionisation detectors nitrogen flow-rates of *ca.* 100 ml/min on columns of (*i*) 15% by weight of poly- (butane-1,4-diol succinate) on Celite (120 × 0.5 cm) at 175°; (*ii*) 15% by weight of poly(ethylene glycol adipate) on Celite (75 × 0.5 cm) at 160°. Retention times (*T*) are given relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside.

Degraded gums *A* and *B* were methylated successively with methyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide. The reduced (lithium aluminium hydride), methylated, degraded gum *A* was methylated by Kuhn's method[4]. Oligosaccharides were methylated by the method of Perila and Bishop[5]. Methanolyses were carried out under reflux for 7 h with dry 5% methanolic hydrogen chloride.

RESULTS

Determination of conditions for autohydrolysis. — The time required for complete autohydrolysis of an *Acacia* gum differs from species to species, since it is dependent on such features as the proportion of uronic acid groups present; at 98°, *A. senegal* gum requires 50 h, whereas *A. nubica* gum requires[1] 120 h. In a trial experiment, a solution (4%) of soluble *A. drepanolobium* gum was heated on a boiling waterbath for 120 h. Aliquots (2 ml) were withdrawn at intervals, filtered, and diluted to 10 ml. The changes in the specific rotation and in free reducing power (alkaline ferricyanide method[6]) are shown in Table I. In a parallel experiment, a 4% solution of the soluble gum was also heated on a boiling water-bath for 120 h. At intervals, aliquots (6 ml) were withdrawn and filtered, and portions (5 ml) were then added to 2M sodium chloride (5 ml). The resulting gum solution in M sodium chloride was used to obtain the values for the limiting-viscosity number [η] shown in Table I. From the kinetic data (Table I), it was deduced that a period of 84 h was required for complete autohydrolysis of a 4% solution of the gum.

Autohydrolysis of water-soluble A. drepanolobium *gum to give degraded gum* A. — A solution of the gum (50 g in 1.25 l water) was heated on a boiling water-bath for 84 h. The solution was then cooled, filtered, and dialysed against distilled water (4 l), the distilled water being changed daily over a period of 4 days. Freeze-drying gave degraded gum *A* (11 g, 22%), $[\alpha]_D$ +31° (*c* 1.0, water) (Found: uronic acid, 17;

TABLE I

THE CHANGES OCCURRING DURING AUTOHYDROLYSIS OF *A. drepanolobium* gum

Time (h)	$[\alpha]_D$ *(degrees)*	*Reducing power*[a]	$[\eta]$ *(ml/g)*
0	+82	1.56	25.1
2	+84	5.20	—
5	—	—	18.4
9	+89	12.7	—
10	—	—	13.2
24	+91	22.2	7.5
48	+92	29.7	5.2
72	+92	32.6	3.6
96	+92	32.8	3.5
120	+90	33.0	—

[a] Expressed as mg of arabinose per ml of autohydrolysate.

galactose, 80; arabinose, 3; methoxyl, 0.73%). Hydrolysis (N-sulphuric acid, 7 h) of degraded gum *A* indicated the presence of the four aldobiouronic acids identified previously[2], namely 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose, 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose, 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose, and 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose, having, respectively, R_{Gal} 0.22, 0.27, 0.59, and 0.65 in solvent (*b*).

The dialysates (4 × 4 l) from the autohydrolysate were concentrated to a syrup (30 g). Chromatographic examination showed the presence of galactose, arabinose, rhamnose (trace), and two neutral disaccharides having the mobilities of (*1*) 3-*O*-β-L-arabinopyranosyl-L-arabinose [R_{Gal} 0.77, solvent (*a*); 0.71, solvent (*b*)] and (*2*) 3-*O*-β-L-arabinofuranosyl-L-arabinose [R_{Gal} 1.25, solvent (*b*); 1.47, solvent (*e*)]. Disaccharide (*1*) was isolated by fractionation of a portion of the dialysate on 3MM papers in solvent (*b*). The product (50 mg) had $[\alpha]_D$ +195° (*c* 1.0), gave only arabinose on acid hydrolysis, and had the same paper-chromatographic mobility [R_{Gal} 0.77, solvent (*a*); 0.71, solvent (*b*)] as an authentic specimen of 3-*O*-β-L-arabinopyranosyl-L-arabinose. A portion (15 mg) was methylated: g.l.c. examination of the methanolysis products revealed the methyl glycosides of 2,3,4-tri-*O*-methyl-L-arabinose [(*i*) *T* 1.01; (*ii*) *T* 0.82], 2,5-di-[(*i*) *T* 1.81, 3.25; (*ii*) *T* 1.26, 2.18] and 2,4-di-*O*-methyl-L-arabinose [(*i*) *T* 2.08, 2.18; (*ii*) *T* 1.48, 1.56]. Hydrolysis of the methyl glycosides, followed by paper chromatography in solvent (*d*), confirmed the presence of 2,3,4-tri-*O*-methyl-L-arabinose (R_G 0.79) and 2,5- and 2,4-di-*O*-methyl-L-arabinose (R_G 0.84 and 0.65, respectively). The phenylosazone of the disaccharide had m.p. 233–234° (decomp.) (from water).

Disaccharide (*2*) was isolated by fractionation of a portion of the dialysate on 3MM papers in solvent (*e*). The product (32 mg) had $[\alpha]_D$ +89.7° (*c* 0.64), gave only arabinose on acid hydrolysis, and had the same paper-chromatographic mobility [R_{Gal} 1.25, solvent (*b*); 1.47, solvent (*e*)] as an authentic specimen of 3-*O*-β-L-arabinofuranosyl-L-arabinose. A portion (10 mg) was methylated: g.l.c. examination of the

methanolysis products revealed the methyl glycosides of 2,3,5-tri[(*i*) T 0.56, 0.73; (*ii*) T 0.47, 0.63] and 2,5-di-[(*i*) T 1.82, 3.24; (*ii*) T 1.27, 2.20] and 2,4-di-*O*-methyl-L-arabinose [(*i*) T, 2.07, 2.17; (*ii*) T 1.46, 1.57]. Hydrolysis of the methyl glycosides, followed by paper chromatography in solvent (*d*), confirmed the presence of 2,3,5-tri-(R_G 0.98), 2,5-di-(R_G 0.84), and 2,4-di-*O*-methyl-L-arabinose (R_G 0.65). The phenylosazone of the disaccharide had m.p. 196–198° (from water).

Partial hydrolysis of degraded gum A. — Degraded gum A was subjected to partial, acid hydrolysis with 0.5N sulphuric acid (1 h, boiling water-bath). After neutralisation (barium carbonate), filtration, deionisation [Amberlite IR-120 (H^+) resin], and concentration, the resulting syrup (4.8 g) was fractionated on Whatman 3MM papers to give four fractions.

Fraction 1 (62 mg) had $[\alpha]_D$ +60° (*c* ,0.2), gave only galactose on acid hydrolysis, and had the same paper-chromatographic mobility as an authentic specimen of 3-*O*-β-D-galactopyranosyl-D-galactose [R_{Gal} 0.48, solvent (*a*); 0.53, solvent (*e*)]. A portion (15 mg) was methylated; g.l.c. showed the methanolysis products to be the methyl glycosides of 2,3,4,6-tetra- [(*i*) T 1.68; (*ii*) T 1.66], 2,4,6-tri- [(*i*) T 3.76, 4.23; (*ii*) T 3.00, 3.45] and 2,5,6-tri-*O*-methyl-D-galactose [(*i*) T 3.98; (*ii*) T 3.26]. Hydrolysis of the methyl glycosides, followed by paper chromatography [solvent (*d*)], showed the presence of 2,3,4,6-tetra-, 2,4,6-tri- and 2,5,6-tri-*O*-methyl-D-galactose (R_G 0.90, 0.72, and 0.88, respectively). The disaccharide, recrystallised from aqueous acetone, gave needles of 3-*O*-β-D-galactopyranosyl-D-galactose monohydrate, m.p. and mixed m.p. 156–159°.

Fraction 2 (94 mg) had $[\alpha]_D$ +29° (*c* 0.94), gave only galactose on acid hydrolysis and was identical with an authentic specimen of 6-*O*-β-D-galactopyranosyl-D-galactose [R_{Gal} 0.29, solvent (*a*); 0.37, solvent (*c*)]. A portion (20 mg) was methylated; g.l.c. showed the methanolysis products to be the methyl glycosides of 2,3,4,6-tetra- [(*i*) T 1.68; (*ii*) T 1.66], 2,3,5-tri-[(*i*) T 4.05, 5.39; (*ii*) T 3.28, 4.37], and 2,3,4-tri-*O*-methyl-D-galactose [(*i*) T 6.45; (*ii*) T 5.30]. Hydrolysis of the methyl glycosides, followed by paper chromatography [solvent (*d*)], showed the presence of 2,3,4,6-tetra-, 2,3,5-tri- (R_G 0.86) and 2,3,4-tri-*O*-methyl-D-galactose (R_G 0.71). After recrystallisation from water, the phenylosazone of 6-*O*-β-D-galactopyranosyl-D-galactose had m.p. and mixed m.p. 200–203°.

Fraction 3 (9 mg) had $[\alpha]_D$ +46° (*c* 0.18), gave D-galactose and 3-*O*-β-D-galactopyranosyl-D-galactose on partial hydrolysis with acid, and had[7] D.P. = 2.90. On paper chromatography, the trisaccharide was identical [R_G 0.22, solvent (*a*); 0.23, solvent (*c*)] with an authentic specimen of the β-(1→3)-linked D-galactotrisaccharide. A portion (4 mg) was methylated; g.l.c. showed the presence of the methyl glycosides of 2,3,4,6-tetra-, 2,4,6-tri- and 2,5,6-tri-*O*-methyl-D-galactose (retention times as for Fraction 1).

Fraction 4 (19 mg) had $[\alpha]_D$ +20° (*c*, 0.38), gave galactose and 6-*O*-β-D-galactopyranosyl-D-galactose on partial hydrolysis with acid, and had[17] D.P. = 2.95. On paper chromatography, the trisaccharide was identical [R_G 0.15, solvent (*a*); 0.18, solvent (*b*)] with an authentic specimen of the β-(1→6)-linked D-galactose-

trisaccharide. A portion (8 mg) was methylated; g.l.c. showed the presence of the methyl glycosides of 2,3,4,6-tetra-, 2,3,5-tri, and 2,3,4-tri-*O*-methyl-D-galactose (*T*-values as for Fraction 2). Paper chromatography of the hydrolysate showed the presence of the same three methylated sugars (R_G values as for Fraction 2).

Borohydride reduction of degraded gum A. — Degraded gum *A* (500 mg) was dissolved in water (100 ml), and sodium borohydride (400 mg) was added. After 24 h, further sodium borohydride (100 mg) was added. After 6 h, the solution was dialysed for 2 days. The freeze-dried product was hydrolysed to yield galactose, arabinose, and the same four aldobiouronic acids that were obtained from unreduced, degraded gum *A*. In addition, paper chromatography [solvent (*f*)] showed the presence of galactitol; arabinitol was absent.

Degraded gum *A* no formaldehyde on periodate oxidation. The yield of formaldehyde from reduced, degraded gum *A* (40.4 mg) was 180 μg (0.25 h), 230 μg (0.5 h), 235 μg (1 h), 245 μg (2 h), 245 μg (6 h), 250 μg (24 h); calculation gives a value of 4,800 for the $\overline{M}\eta$ of degraded gum *A*. Molecul-sieve chromatography[3,8], with a calibrated Bio-Gel P300 column, indicated a value of 5,200 for the $\overline{M}\eta$ of degraded gum *A*.

Methylation of degraded gum A. — Degraded gum *A* (1.34 g) was methylated to give a product (974 mg), $[\alpha]_D$ +18° (*c* 1.00, chloroform) (Found: methoxyl, 40.1%; not raised on further attempted methylation). Methanolysis of a portion of this product, followed by g.l.c., gave the results shown in Table II. The methyl glycosides

TABLE II

THE METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED, DEGRADED GUM A

Relative retention time (T) *of methyl glycosides*[a]		R_G *in solvent* (d)	*Relative molar propns.*	O-*Methyl sugars identified*
column (i)	*column* (ii)			
0.56, 0.73	0.49, 0.63	0.98	tr	2,3,5-tri-*O*-methyl-L-arabinose
1.01	0.82	0.79	tr	2,3,4-tri-*O*-methyl-L-arabinose
1.68	1.66	0.90	++	2,3,4,6-tetra-*O*-methyl-D-galactose
(3.00), (3.76), (4.25)	2.45, (3.04), (3.50)	0.72	±	2,3,6-tri-*O*-methyl-D-galactose
(3.76), (4.25)	(3.04), (3.50)	0.72	++	2,4,6-tri-*O*-methyl-D-galactose
6.45	5.30	0.72	+++	2,3,4-tri-*O*-methyl-D-galactose
14.5, 16.3	9.9, 11.3	0.53	+++	2,4-di-*O*-methyl-D-galactose
2.38, (3.00)	2.20, 2.75	—	++	2,3,4-tri-*O*-methyl-D-glucuronic acid[b]
—	—	0.59	+	2,6-di-*O*-methyl-D-galactose
—	—	0.34	+	2-*O*-methyl-D-galactose

[a]Figures in parentheses indicate incompletely resolved components. [b]As methyl ester methyl glycoside.

were hydrolysed, and paper chromatography in solvents (*d*) and (*e*) indicated the presence of 2,6-di- and 2-*O*-methyl-D-galactose, in addition to the *O*-methyl sugars shown in Table II.

Methanolysis of methylated, degraded gum A. — Methylated, degraded gum *A* (790 mg) was heated under reflux for 8 h with 5% methanolic hydrogen chloride (80 ml). The resulting mixture of methyl glycosides was hydrolysed with sulphuric acid (N, 100 ml) on a boiling water-bath for 4 h. After being cooled, the solution was neutralised (barium carbonate), filtered, deionised [Amberlite resin IR-120 (H^+)], and concentrated to a syrup (750 mg). The mixture of *O*-methyl sugars was fractionated on Whatman 3MM papers by double development in solvent (*e*) to give seven fractions.

Fraction 1 (58 mg) had $[\alpha]_D$ +117° (*c* ,0.58), and was chromatographically identical with 2,3,4,6-tetra-*O*-methyl-D-galactose [R_G 0.89, solvent (*d*); 0.72, solvent (*e*)]. The derived 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine had m.p. 192–195° (lit.[9], 192-197°), after recrystallisation from ethyl acetate.

Fraction 2 (39 mg) had $[\alpha]_D$ +100° (*c*, 0.39); demethylation gave galactose and arabinose. A portion (5 mg) was heated under reflux for 7 h with 5% methanolic hydrogen chloride, and g.l.c. indicated the presence of 2,3,5-tri- (+) [(*i*) *T* 0.56, 0.73; (*ii*) *T* 0.48, 0.63], 2,3,4-tri-*O*-methyl-L-arabinose (+ +) [(*i*) *T* 1.00, (*ii*) *T* 0.82], and 2,3,4,6-tetra-*O*-methyl-D-galactose (+ + + +) [(*i*) *T* 1.68; (*ii*) *T* 1.66].

Fraction 3 contained three components when examined in solvent (*c*). The fraction was therefore refractionated on 3MM papers by double development in solvent (*e*) to give Fractions 3(*a*), (*b*), and (*c*).

Fraction 3(a) (26 mg) had $[\alpha]_D$ +98° (*c* 0.26), and paper chromatography indicated the presence of 2,3,6-tri- (+ +) and 2,4,6-tri-*O*-methyl-D-galactose (+). After reflux with methanolic hydrogen chloride, g.l.c. indicated the presence of the methyl glycosides of these two *O*-methyl sugars.

Fraction 3(b) (54 mg) had $[\alpha]_D$ +95° (*c* 0.54). A portion (5 mg) was refluxed with methanolic hydrogen chloride, and g.l.c. indicated the presence of 2,4,6-tri- with a trace of 2,3,4-tri-*O*-methyl-D-galactose. The derived 2,4,6-tri-*O*-methyl-*N*-phenyl-D-galactosylamine, after recrystallisation from ethyl acetate (twice) and from acetone–ether–light petroleum (1:1:1), had m.p. 163–165° (lit.[9], 170°).

Fraction 3(c) (31 mg) had $[\alpha]_D$ +110° (*c* 0.31) and was identical (R_{Gal} 0.71) with 2,3,4-tri-*O*-methyl-D-galactose in solvent (*d*). This was confirmed by g.l.c. examination of the methyl glycoside. 2,3,4-Tri-*O*-methyl-*N*-phenyl-D-galactosylamine was prepared, and, after recrystallisation from ethyl acetate, had m.p. 164–167° (lit.[9], 164–169°).

Fraction 4 (22 mg) had $[\alpha]_D$ +80° (*c* 0.44), and was identical [R_G 0.54, solvent (*d*); 0.19, solvent (*e*)] with 2,6-di-*O*-methyl-D-galactose. After periodate oxidation, paper chromatography in solvent (*d*) revealed a bright-yellow spot (*p*-anisidine hydrochloride spray) having the same mobility (R_G 0.20) as 2-methoxymalonaldehyde.

Fraction 5 (42 mg) had $[\alpha]_D$ +88° (*c* 0.42) and behaved identically [R_G 0.49, solvent (*d*); 0.12, solvent (*d*)] with 2,4-di-*O*-methyl-D-galactose. Recrystallisation

from acetone containing 1% of water gave 2,4-di-*O*-methyl-D-galactose monohydrate, m.p. 96–97°. The X-ray diffractogram was identical with that of an authentic specimen. The derived 2,4-di-*O*-methyl-*N*-phenyl-D-galactosylamine had, after recrystallisation from acetone, m.p. 216–219° (lit.[9], 214–228°).

Fraction 6 (28 mg) had $[\alpha]_D$ +81° (*c* 0.56) and behaved identically in solvent (*d*) with an authentic specimen of 2-*O*-methyl-D-galactose (R_G 0.31). Recrystallisation from glacial acetic acid gave crystals, m.p. 151–154° (lit.[9], 145–148°).

Fraction 7 (76 mg) had $[\alpha]_D$ +53° (*c* 0.76). After reflux with methanolic hydrogen chloride, g.l.c. indicated the presence of the methyl ester methyl glycoside of 2,3,4-tri-*O*-methyl-D-glucuronic acid. This was reduced (sodium borohydride, 200 mg) for 12 h; excess of borohydride was destroyed by the addition of Amberlite resin IR-120 (H^+), and borate was removed by distillation as methyl borate. Thick-paper chromatography yielded 2,3,4-tri-*O*-methyl-D-glucose (26 mg). A portion (5 mg) was refluxed with methanolic hydrogen chloride for 7 h; g.l.c. indicated the presence of the methyl glycoside of 2,3,4-tri-*O*-methyl-D-glucose.

Reduction of methylated, degraded gum A. — Lithium aluminium hydride (50 mg) was added to methylated, degraded gum *A* (50 mg) in tetrahydrofuran (10 ml), and the mixture was refluxed for 3 h. After cooling, the excess hydride was destroyed by the addition of ethyl acetate and water; the mixture was taken to dryness and extracted with chloroform. This extract was concentrated to a syrup which was methylated with methyl iodide (1 ml) and silver oxide (1 g) in *N*,*N*-dimethylformamide (1 ml) to give a product (36 mg), $[\alpha]_D$ +19° (*c* 0.72, chloroform). After conversion into the methyl glycosides, g.l.c. gave the results shown in Table III. Examination of the hydrolysate of the methyl glycosides by paper chromatography [solvents (*d*) and (*e*)] indicated the presence of 2,6-di- and 2-*O*-methyl-D-galactose, in addition to the *O*-methyl sugars shown in Table III.

Partial methanolysis of methylated, degraded gum A. — Methylated, degraded gum *A* (100 mg) was refluxed with 2% methanolic hydrogen chloride (15 ml) for 2 h. The methanolysate was neutralised (silver carbonate), filtered, and evaporated to a syrup that was hydrolysed with sulphuric acid (0.5N, 10 ml) for 5 h on a boiling water-bath. After being cooled, the solution was neutralised (barium carbonate), filtered, deionised [Amberlite IR-120 (H^+)], and then fractionated on a column (3 × 33 cm) of Duolite A-4 resin in the formate form. Neutral *O*-methyl sugars were eluted with water (3 l); this fraction was not investigated further. Acidic *O*-methyl sugars were then eluted with 5% aqueous formic acid (500 ml); after removal of the formic acid and water, paper chromatography of the resulting syrup in solvent (*d*) indicated that there were no neutral *O*-methyl sugars present. This syrup was treated with 5% methanolic hydrogen chloride for 7 h; the product was examined by g.l.c., and this indicated that the methyl glycosides of 2,3,4-tri-*O*-methyl-D-glucuronic acid, 2,3,6-tri-, 2,3,4-tri-, 2,6-di-, and 2,4-di-*O*-methyl-D-galactose were present. Examination of the hydrolysate of these methyl glycosides by paper chromatography in solvents (*d*) and (*e*) confirmed these results.

Smith degradation[10] *of degraded gum* A. — Degraded gum *A* (1.0 g) was dissolved

TABLE III

THE METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED REDUCED DEGRADED GUM A

Relative retention time (T) *of methyl glycosides*[a]		R_G *in solvent* (d)	*Relative molar propns.*	O-*Methyl sugars identified*
column (i)	*column* (ii)			
0.57, 0.73	0.49, 0.63	0.98	tr	2,3,5-tri-*O*-methyl-L-arabinose
(1.00)	0.82	0.79	tr	2,3,4-tri-*O*-methyl-L-arabinose
(1.00), 1.40	1.00, 1.39	1.00	++	2,3,4,6-tetra-*O*-methyl-D-glucose
1.68	1.66	0.90	++	2,3,4,6-tetra-*O*-methyl-D-galactose
2.96, (3.75), (4.25)	2.45, (3.04), (3.50)	0.72	+/2	2,3,6-tri-*O*-methyl-D-galactose
(3.75), (4.25)	(3.04), (3.50)	0.72	++	2,4,6-tri-*O*-methyl-D-galactose
6.45	5.30	0.72	++++	2,3,4-tri-*O*-methyl-D-galactose
14.6, 16.5	9.9, 11.3	0.53	+++	2,4-di-*O*-methyl-D-galactose
—	—	0.59	+	2,6-di-*O*-methyl-D-galactose
—	—	0.34	+	2-*O*-methyl-D-galactose

[a]As for Table II.

in water (25 ml) and 0.5M sodium metaperiodate solution (25 ml) was added; 6.8 mmoles of periodate was reduced, and 4.0 mmoles of formic acid was released after 96 h, when the reaction was stopped by the addition of ethylene glycol (2 ml). The solution was dialysed for 48 h, sodium borohydride (0.5 g) was then added, and the mixture was kept at room temperature for 30 h. After dialysis for 48 h, the polyalcohol was hydrolysed (N sulphuric acid, room temperature, 48 h). The solution was neutralised (barium carbonate), filtered, deionised [Amberlite IR-120 (H^+)], and dialysed. The syrup (390 mg) obtained from the dialysate was shown by paper chromatography in solvents (*a*), (*b*), and (*c*) to contain glycerol and glycolaldehyde as the main components, together with small proportions of slower-moving, non-reducing glycosides. A portion of the dialysate was hydrolysed (N sulphuric acid, 7 h), and paper chromatography [solvents (*a*) and (*b*)] showed a trace of galactose to be present. After further dialysis (48 h), degraded gum *B* [93 mg, yield 9%], $[\alpha]_D$ +9° (*c* 0.93), was isolated as the freeze-dried product.

Mild hydrolysis (0.5N sulphuric acid, 1 h) of degraded gum *B* (10 mg) followed by paper chromatography [solvent (*a*)] showed the presence of galactose and two neutral disaccharides having the chromatographic mobilities of 3-*O*-*β*-D-galactopyranosyl-D-galactose (R_{Gal} 0.48) and 6-*O*-*β*-D-galactopyranosyl-D-galactose (R_{Gal} 0.29).

Degraded gum *B* was exhaustively methylated. Methanolysis, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in

Table IV. Paper-chromatographic examination of a hydrolysate of the methyl glycosides [solvents (*d*) and (*e*)] indicated the presence of 2,6-di- and 2-*O*-methyl-D-galactose, in addition to the *O*-methyl sugars identified as methyl glycosides by g.l.c.

TABLE IV

THE METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED, DEGRADED GUM B

Relative retention time (T) *of methyl glycosides*[a]		R_G *in solvent* (d)	*Relative molar propns.*	O-*Methyl sugars identified*
column (i)	*column* (ii)			
1.68	1.66	0.90	++	2,3,4,6-tetra-*O*-methyl-D-galactose
3.76, 4.25	3.00, 3.45	0.72	+++++	2,4,6-tri-*O*-methyl-D-galactose
6.45	5.29	0.72	++	2,3,4-tri-*O*-methyl-D-galactose
14.6, 16.4	9.9, 11.3	0.53	++	2,4-di-*O*-methyl-D-galactose
2.38, 3.00	2.20, 2.75	—	tr	2,3,4-tri-*O*-methyl-D-glucuronic acid[b]
—	—	0.59	+	2,6-di-*O*-methyl-D-galactose
—	—	0.34	+	2-*O*-methyl-D-galactose

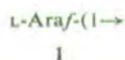
[a]Figures in parentheses indicate incompletely resolved components; [b]As methyl ester methyl glycoside.

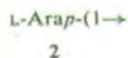
Controlled Smith degradation[10] *of degraded gum* A. — Degraded gum *A* (1.0 g) was treated in the same way as described for the Smith degradation, with the exception that the periodate oxidation was carried out at 2°, giving degraded gum *C* (105 mg, 10%) which was isolated by freeze-drying. A portion of this product (20 mg) was hydrolysed, and paper chromatography in solvent (*f*) indicated the presence of galactose and arabinitol; galactitol was absent.

DISCUSSION

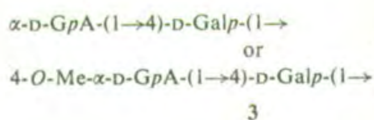
Autohydrolysis of Fraction *A* of *A. drepanolobium* effected the release of all of the rhamnose, most of the arabinose, and some galactose. Degraded gum *A*, isolated after dialysis, contained D-galactose (80%), L-arabinose (3%), and uronic acid (17%). Molecular-sieve chromatography[3,8] gave estimated values of 5,200 and over 200,000 for the number-average molecular-weights of degraded gum *A* and *A. drepanolobium* gum Fraction *A*, respectively. Degradation on autohydrolysis is much greater than can result from the simple removal of arabinose and rhamnose from the periphery. It does not appear to have been caused by the presence of any internal, acid-labile arabinofuranose residues, since acid hydrolysis of borohydride reduced, degraded gum *A* yielded only galactitol. There is no evidence of any galactofuranose residues in the polysaccharide, although, under the experimental circumstances, traces of di- and tri-*O*-methyl-D-galactofuranoses would be difficult to detect by g.l.c. and paper chromatography. The possibility that some galactopyranosidic bonds may be unusually sensitive to mild conditions of autohydrolysis has already been proposed for other plant gums[3,12,13].

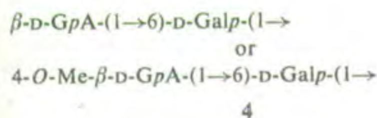
An examination of methylated, degraded gum *A* showed the presence of 2,3,4,6-tetra-(+ +), 2,4,6-(+ +), 2,3,6-(±), and 2,3,4-tri-(+ + +), 2,4-(+ + +), and 2,6-di-(+), and 2-*O*-methyl-D-galactose (+), 2,3,4-tri-*O*-methyl-D-glucuronic acid (+ +), and traces of 2,3,5- and 2,3,4-tri-*O*-methyl-L-arabinose. The presence of 2-*O*-methyl-D-galactose is probably due to under-methylation. This shows that the units **1** and **2** are structural fragments of degraded gum *A*. Methylation has shown traces of terminal, non-reducing L-arabinofuranose residues to be present in the

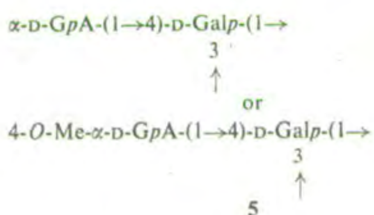


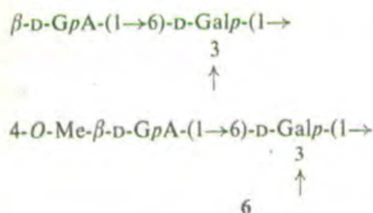


autohydrolysed gum of *A. senegal*[11], and terminal, non-reducing L-arabinopyranose residues to be present in degraded gum *A* of *A. arabica*[12]. Lithium aluminium hydride reduction of methylated, degraded gum *A*, followed by a Kuhn methylation[4], yielded 2,3,4,6-tetra-*O*-methyl-D-glucose after methanolysis. This confirms that D-glucuronic acid, and its 4-*O*-methyl analogue, are terminal, non-reducing end-groups in degraded gum *A*. Partial methanolysis was carried out on methylated, degraded gum *A*; after hydrolysis of the methyl glycosides, the methylated aldobiouronic acids were separated from the neutral *O*-methyl sugars. Methanolysis of the methylated aldobiouronic acids, followed by g.l.c. examination of the mixture of methyl glycosides, showed the presence of 2,3,4-tri-*O*-methyl-D-glucuronic acid, and 2,3,6- and 2,3,4-tri-, and 2,4- and 2,6-di-*O*-methyl-D-galactose. This indicates that units **3–6** are structural fragments of degraded gum *A*.









Smith degradation of degraded gum *A* yielded degraded gum *B*. Partial, acid hydrolysis of degraded gum *B* gave 3-*O*-β-D-galactopyranosyl-D-galactose and 6-*O*-β-D-galactopyranosyl-D-galactose. An examination of methylated, degraded gum *B* indicated the presence of 2,3,4,6-tetra-(+ +), 2,4,6-(+ + + + +) and 2,3,4-tri-(+ +), 2,6-(+) and 2,4-di-(+ +), and 2-*O*-methyl-D-galactose (+), with a trace of 2,3,4-tri-*O*-methyl-D-glucuronic acid. The presence of a trace of glucuronic acid in degraded gum *B* may be explained by incomplete oxidation of degraded gum *A*, and the presence of 2,6-di- and 2-*O*-methyl-D-galactose may arise from under-methyl-

ation. The proportion of the *O*-methylgalactoses obtained from methylated degraded gum *B* is evidence that degraded gum *B* is a galactan composed predominantly of β-D-(1→3) glycosidic linkages, with a few β-D-(1→6) branch points. The identification of some 2,3,4-tri-*O*-methyl-D-galactose from methylated, degraded gum *B* indicates that unit **7** is a possible structural feature of degraded gum *A*.

→3)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→3)-D-Gal*p*-(1→
(3)
↑
(1)
R

where R = L-Ara*f*-; L-Ara*p*-; D-Gal*p*-; →4)-D-Gal*p*-; or →6)-D-Gal*p*-

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A controlled Smith degradation of degraded gum *A* yielded degraded gum *C*. Periodate oxidation of degraded gum *A* did not yield formaldehyde, so it may be concluded that the reducing galactose residue is substituted at C-6. The fact that degraded gum *C* contains arabinitol is evidence that at least some of the reducing galactose residues of degraded gum *A* are substituted[14] at C-3 (see Fig. 1).

Fig. 1

Mild, acid hydrolysis of degraded gum *A* afforded 3-*O*-β-D-galactopyranosyl-D-galactose, 6-*O*-β-D-galactopyranosyl-D-galactose, and two trisaccharides, which were shown by methylation analysis and polarimetry to be the β-D-(1→3)- and β-D-(1→6) linked galactose trisaccharides. Characterisation of these oligosaccharides shows that units **8** and **9** are structural fragments of degraded gum *A*.

-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-D-Gal*p*-(1→ **8**

-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-D-Gal*p*-(1→ **9**

Two disaccharides were obtained from the diffusate of the autohydrolysate of Fraction *A*. Methylation and preparation of crystalline phenylosazones showed these to be 3-*O*-β-L-arabinofuranosyl-L-arabinose and 3-*O*-β-L-arabinopyranosyl-L-arabinose. The fact that both disaccharides are released under autohydrolysis conditions suggests that, in each case, the reducing L-arabinose residue was initially present in the furanose form. Thus units **10** and **11** are structural fragments of Fraction *A* of *A. drepanolobium* gum.

On the basis of the available evidence, Fraction *A* of *A. drepanolobium* gum is similar to *A. arabica* gum[12]. Both gums have high positive specific rotations, and the

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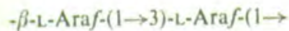

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yields and properties of the degraded gums obtained from them are strikingly similar. There are, however, two important differences; degraded gum *A* from Fraction *A* of *A. drepanolobium* gum contains traces of terminal, non-reducing L-arabinopyranosyl and L-arabinofuranosyl residues; degraded gum *A* from *A, arabica* gum contains only terminal, non-reducing L-arabinopyranosyl residues[12]. Partial methanolysis of methylated, degraded gum *A* from *A. drepanolobium* gum has shown units that **3–6** are structural fragments of degraded gum *A*. Partial methanolysis of methylated, degraded gum *A* of *A. arabica* gum[12] has shown that only units **3**, **4**, and **6** are structural fragments of degraded *A. arabica* gum.

ACKNOWLEDGMENTS

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53.

STUDIES ON URONIC ACID MATERIALS

PART XXIX*. SOME STRUCTURAL FEATURES OF *Acacia laeta* GUM

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ABSTRACT

The gum polysaccharide exuded by *Acacia laeta* trees contains D-galactose, L-arabinose, L-rhamnose, D-glucuronic acid, and 4-*O*-methyl-D-glucuronic acid. Linkage analysis has yielded 3-*O*-β-L-arabinopyranosyl-L-arabinose, 3-*O*-β-L-arabinofuranosyl-L-arabinose, 3-*O*-α-D-galactopyranosyl-L-arabinose, 3-*O*-β-D-galactopyranosyl-D-galactose, and 6-*O*-β-D-galactopyranosyl-D-galactose. The *O*-methyl derivatives of *A. laeta* gum and autohydrolysed *A. laeta* gum were analysed, after methanolysis, by g.l.c. The autohydrolysed gum was subjected to a Smith degradation, and the product examined by linkage and methylation analysis. *A. laeta* gum was subjected to eight successive Smith-degradations; the *O*-methyl derivatives of each of the polysaccharides obtained from the first six degradations and from the eight degradation were analysed, after methanolysis, by g.l.c. The structural evidence obtained does not give unequivocal proof that the gum contains a "main chain" of β-(1→3)-linked D-galactose residues.

INTRODUCTION

A. laeta R. Br. ex Benth. is a natural hybrid of *A. senegal* (L.) Willd. and *A. mellifera* (Vahl.) Benth., and it occurs in two varieties: *A. laeta* var. *hashab* (which resembles *A. senegal*) and *A. laeta* var. *mellifera* (which resembles *A. mellifera*). The results of a detailed examination of *A. laeta* var. *hashab* gum are reported in this Paper, and the structural features of this gum are compared with those of *A. senegal* gum (gum arabic). The characterisation of two aldobiouronic acids, 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose and 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose, from *A. laeta* var. *hashab* gum has been reported[2], together with the results of an analytical study of the extensive inter-nodule variation shown by this species, from which average values for the analytical parameters expressing the composition of the gum were calculated.

RESULTS AND DISCUSSION

The gum nodules from *A. laeta* var. *hashab* were collected, by the Gum Research Officer to the Republic of Sudan, from botanically authenticated trees at the El

*For Part XXVIII, see Ref. 1.

Ain Forest Reserve, near El Obeid, in November 1962. After purification, the gum polysaccharide was shown to have the specific, rotation and sugar composition shown in Table I. Samples of the gum were examined by zone electrophoresis on cellulose-acetate film, and by ion-exchange chromatography on DEAE-cellulose[3]. No sharp discontinuities in the properties of the molecular species were indicated by these experiments. It therefore seems probable that *A. laeta* var. *hashab* gum exhibits the same kind of heterogeneity as *A. senegal* gum[4,5], and may be considered to contain a continuous spectrum of related molecular species.

Two disaccharides were isolated by thick-paper chromatography of the combined neutral syrups obtained from the graded hydrolysis of the gum outlined in Fig. 1. The first disaccharide had the same paper-chromatographic mobility as 3-*O*-β-D-galactopyranosyl-D-galactose and was characterised as its crystalline monohydrate. Methylation evidence confirmed the presence of a $(1\rightarrow3)$ link. Methylation data for the second disaccharide and preparation of the phenylosazone confirmed that the disaccharide was 6-*O*-β-D-galactopyranosyl-D-galactose. Partial hydrolyses with acid have shown that 3-*O*-β-D-galactopyranosyl-D-galactose[6] and 6-*O*-β-D-galactopyranosyl-D-galactose[4] are constituent units of *A. senegal* gum.

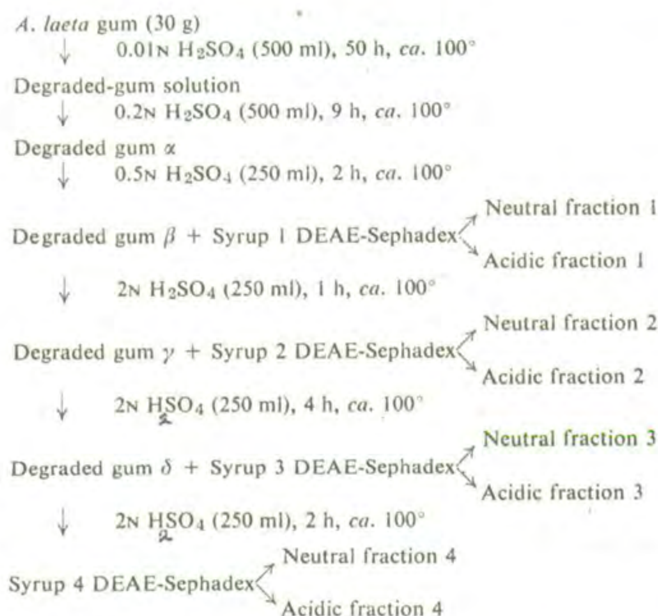


Fig. 1. Diagramatic representation of stepwise hydrolysis of *A. laeta* gum.

A disaccharide, obtained by thick-paper chromatography of the diffusate from an autohydrolysis (80° for 6 h) in a dialysis bag placed in distilled water, had the same paper-chromatographic mobility as 3-*O*-β-L-arabinofuranosyl-L-arabinose. Confirmation of this structure was obtained by acid hydrolysis, methylation data, and preparation of the phenylosazone. The fact that the disaccharide is released under

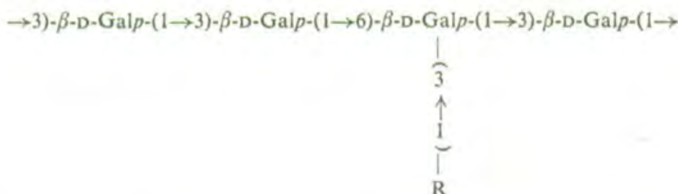
such mild conditions suggests that the reducing L-arabinose residue was initially present (in the gum) in the furanose form. This disaccharide has been characterised from the gums of *A. arabica*[7], *A. drepanolobium*[1], and *A. nubica*[8]. Paper chromatography of a mild, acid hydrolysate has indicated that it is a constituent unit of *A. senegal* gum[9].

Autohydrolysis (60 h on a boiling-water bath) of a 4% gum solution released all of the rhamnose, nearly all of the arabinose, and some galactose. Degraded-gum *A*, isolated after dialysis, contained galactose (76%), arabinose (3%), and uronic acid (21%). The specific rotations of the whole gum and degraded-gum *A* from *A. laeta* are $-42°$ and $-28°$, respectively; *cf.* values of $-31.5°$ and $-11°$, respectively, for *A. senegal*[5]. Molecular-sieve chromatography[4,5,10,11] gave estimated values of 5,700 and >200,000, respectively, for the number-average molecular weights of degraded-gum *A* and *A. laeta* gum. This degradation is much greater than can result from the simple removal of arabinose and rhamnose from the periphery, and does not appear to have been caused by the presence of any internal, acid-labile residues of arabinofuranose, since acid hydrolysis of borohydride-reduced, degraded-gum *A* yielded only galactitol and no arabinitol. There is no evidence for the presence of any galactofuranose residues in the gum; thus, as in the case of *A. senegal* gum[4,5], certain galactopyranosidic bonds must be unusually sensitive towards the mild conditions of autohydrolysis.

An examination of methylated, degraded-gum *A* showed the presence of 2,3,4,6-tetra-(++), 2,4,6-(++), 2,3,4-tri-(++), 2,4-di-(+++), and 2-*O*-methyl-D-galactose (+), 2,3,4-tri-*O*-methyl-D-glucuronic acid (+++), and trace amounts of 2,6-di-*O*-methyl-D-galactose and 2,3,4- and 2,3,5-tri-*O*-methyl-L-arabinose. The presence of 2,6-di- and 2-*O*-methyl-D-galactose is ascribed to undermethylation. Methylation has revealed the presence of traces of terminal, non-reducing L-arabinopyranose and L-arabinofuranose residues in the degraded-gum *A* of *A. drepanolobium*[1]. Only terminal, non-reducing L-arabinopyranose residues are present in degraded-gum *A* of *A. arabica*[7], and only terminal, non-reducing L-arabinofuranose residues in the autohydrolysed gum of *A. senegal*[4]. The proportions of the other *O*-methyl sugars obtained from methylated, degraded-gum *A* of *A. laeta* are similar to those obtained from methylated, autohydrolysed gum of *A. senegal*[4], except that the former contains a larger proportion of 2,4,6-tri-*O*-methyl-D-galactose. This suggests that degraded-gum *A* of *A. laeta* has a larger proportion of β-D-(1→3)-glycosidic links than autohydrolysed gum of *A. senegal*. This could be the reason for the lower specific rotation of degraded-gum *A* of *A. laeta*.

Smith degradation[12] of degraded-gum *A* yielded degraded-gum *B*. Partial, acid hydrolysis of degraded-gum *B* gave 3-*O*-β-D-galactopyranosyl-D-galactose and a trace of 6-*O*-β-D-galactopyranosyl-D-galactose. An examination of methylated, degraded-gum *B* indicated the presence of 2,3,4,6-tetra-(+), 2,4,6-(++++++), 2,3,4-(trace) and 2,3,6-tri-(trace), 2,6-(+) and 2,4-di-(++), and 2-*O*-methyl-D-galactose (+). The presence of 2,3,6-tri-, 2,6-di-, and 2-*O*-methyl-D-galactose is probably due to undermethylation. The proportions of the *O*-methyl-D-galactose is

evidence that degraded-gum *B* is a galactan composed predominantly of β-D-(1→3)-glycosidic linkages, with a few β-D-(1→6) branch-points. The identification of a trace of 2,3,4-tri-*O*-methyl-D-galactose from methylated, degraded-gum *B* indicates that *X* is a possible structural fragment of degraded-gum *A*.



where *R* = L-Ara*p*-(1→, L-Ara*f*-(1→, D-Gal*p*-(1→, or →6)-D-Gal*p*-(1→

X

Two disaccharides were obtained from the diffusate of the autohydrolysate of *A. laeta* gum by thick-paper chromatography. The first was identified as 3-*O*-β-L-arabinopyranosyl-L-arabinose by paper chromatography, acid hydrolysis, methylation evidence, and preparation of the crystalline phenylosazone. The second disaccharide was identified as 3-*O*-α-D-galactopyranosyl-D-arabinose, by using the same methods. The fact that both disaccharides are released on autohydrolysis suggests that, in each case, the reducing arabinose residue was initially present in the furanose form. Partial hydrolyses with acid have shown that 3-*O*-β-L-arabinopyranosyl-L-arabinose[13] and 3-*O*-α-D-galactopyranosyl-L-arabinose[6,14] are constituent units of *A. senegal* gum.

The methylated gum was methanolysed, and analysed by g.l.c.; the methyl glycosides of 2,3,4-tri-*O*-methyl-L-rhamnose, 2,3,5- and 2,3,4-tri- and 2,5-di-*O*-methyl-L-arabinose, 2,3,4,6-tetra-, 2,4,6- and 2,3,4-tri-, and 2,4-di-*O*-methyl-D-galactose, and 2,3,4-tri- and 2,3-di-*O*-methyl-D-glucuronic acid were identified. The g.l.c. traces were virtually identical with those obtained[4] for a methanolysate of methylated *A. senegal* gum, and are indicative of a strong structural similarity between the gums of *A. senegal* and *A. laeta* var. *hashab*.

A sample of *A. laeta* gum was subjected to eight successive Smith-degradations[12]. The relative data are given in Table I. The first Smith-degradation removed all of the rhamnose and 4-*O*-methylglucuronic acid residues, together with some of the galactose, arabinose, and glucuronic acid. Complete oxidation of all of the glucuronic acid residues would be expected during the first treatment with periodate; the incomplete oxidation observed may result from steric hindrance[15].

On methanolysis, the *O*-methyl derivative of polysaccharide **1** gave the methyl glycosides of the methylated sugars detailed in Table II. Thus, all of the arabinopyranose and rhamnopyranose residues are removed by one Smith-degradation. The presence of 2,6-di-*O*-methyl-D-galactose may result from undermethylation at O-4 of certain D-galactose residues. On methanolysis, the *O*-methyl derivatives of polysaccharides **2** and **3** gave rise, with the exception of 2,3,4-tri-*O*-methyl-D-glucuronic acid, to the same *O*-methyl sugars. Similarly, the *O*-methyl derivative of

TABLE I

DATA FOR *A. laeta* GUM AND SMITH-DEGRADED POLYSACCHARIDES 1–8

Polysaccharide	$[\alpha]_D$ (*degrees*)	*Constituent sugars* (%)					*Recovery* (%)	*Periodate reduced* (*mmoles/g*)	*Formic acid released* (*mmoles/g*)
		Gal.	*Ara.*	*Rha.*	*Glu.A.*	*4-O-Methyl-Glu.A.*			
A. laeta gum	−42	44	29	13	10.5	3.5	—	5.24	1.40
1	−18	68	28	—	4	—	60	5.00	2.06
2	+4	91	9	—	—	—	53	4.14	1.62
3	+20	97	3	—	—	—	60	2.81	1.25
4	+26.5	97	3	—	—	—	65	1.47	0.69
5	+28	100	—	—	—	—	73	1.43	0.63
6	+28	100	—	—	—	—	77	1.44	0.67
7	—	100	—	—	—	—	79	0.95	0.43
8	+27	100	—	—	—	—	78	—	—

polysaccharide **4** gave the same methyl glycosides, with the exception of 2,5-di-*O*-methyl-L-arabinose. Polysaccharides **5**, **6**, and **8** contained only galactose, and, on methylation and methanolysis, gave rise to all of the methyl galactosides detected in the earlier methanolysates.

Acid hydrolysis of polysaccharide **1** yielded a small proportion of 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose. Partial, acid hydrolysis of each of the degraded polysaccharides gave 3-*O*-β-D-galactopyranosyl-D-galactose and 6-*O*-β-D-galactopyranosyl-D-galactose, together with the β-(1→3)-linked-D-galactose trisaccharide in the partial hydrolysates of polysaccharides **2**–**8**. In addition, the β-(1→6)-linked-D galactose trisaccharide was present in the hydrolysates of polysaccharides **2**, **3**, and **4**. The detected amounts of the β-(1→6)-linked D-galactose disaccharide decreased from polysaccharides **2** to **8**. The (1→3)-linked trisaccharide was isolated from a large-scale partial hydrolysate of polysaccharide **5′** (obtained after five successive Smith-degradations in a duplicate experiment) and was characterised by crystallisation from aqueous ethanol.

On molecular-sieve chromatography[4,5,10,11] with "Bio-Gel P300", *A. laeta* gum was eluted at the void volume. Since the viscosity properties of *A. laeta* gum are similar to those of *A. senegal* gum, its number-average molecular weight, $\bar{M}_n$, is probably close to the value of 250,000 which Oakley[16] obtained for *A. senegal* gum. Molecular-sieve chromatography on a previously calibrated "Bio-Gel P300" column enabled the approximate, number-average molecular-weights, $\bar{M}_n$, of polysaccharides **1**–**8** to be estimated. There is a significant drop in $\bar{M}_n$ to *ca.* 91,000 for polysaccharide **1** after the first Smith-degradation, and subsequently to 14,000, 12,000, and 9,500 for polysaccharides **6**, **7**, and **8**, respectively.

On the basis of the available evidence, the structural interpretations for polysaccharides **5**–**8** are those that have been put forward for polysaccharide *E*, the fifth Smith-degraded product of *A. senegal* gum[4]. Methylation evidence indicates that

polysaccharides **5–8** are not simple, linear β-D-(1→3)-linked galactans. Although 2,4,6-tri-*O*-methyl-D-galactose is the preponderant *O*-methyl sugar obtained from methylated polysaccharides **5–8**, the identification of some 2,4-di-*O*-methyl-D-galactose and 2,3,4,6-tetra-*O*-methyl-D-galactose indicates occasional branching at the C-3 and C-6 positions. The presence of small amounts of the β-(1→6)-linked-D-galactose disaccharide in the partial, acid hydrolysates of these polysaccharides, and the relatively, low intrinsic-viscosity of the gum, give further support for a branched structure. If, however, there is a "main chain" of β-(1→3)-linked D-galactose residues in polysaccharides **5–8**, it must carry short side-chains of β-(1→3)-linked D-galactose residues; the branch points for these would be through β-D-(1→6)-linkages (Fig. 2*A*). Alternatively, there could be occasional β-D-(1→6)-linkages along a "main chain" of β-D-(1→3)-linked D-galactose residues; here the branch points for the short side-chains would be through β-D-(1→3)-linkages to β-D-(1→6)-linked residues in the "main chain" (Figure 2*B*). A randomly branched structure of β-D-(1→3)-linked chains with β-D-(1→6) branch points would also satisfy the available structural evidence (Fig. 2*C*).

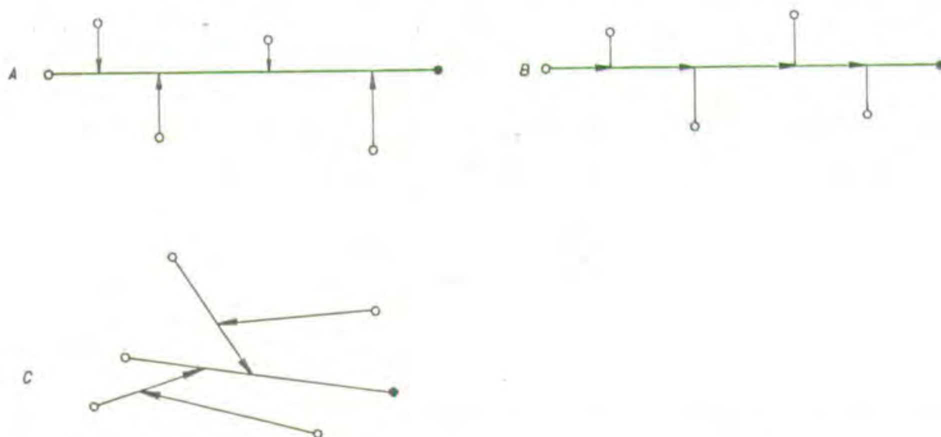


Fig. 2. Diagramatic representation of some of the possible structures for the galactan framework of *A. laeta* var. *hashab* gum.

— are chains of β-(1→3)-linked D-Gal*p* residues; ↓ are β-(1→6)-linkages at branch points; ● are reducing end-groups; ○ are terminal, non-reducing groups.

The ratio of the amounts of 2,4,6-tri-*O*-methyl-D-galactose to 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,4-di-*O*-methyl-D-galactose (g.l.c. of methyl glycosides) increased from polysaccharides **5** to **8**, indicative of a progressive decrease in branching. Similarly, the ratio of 2,4,6-tri-*O*-methyl-D-galactose to 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,4-di-*O*-methyl-D-galactose is greater for polysaccharide **5** than for polysaccharide *E* of *A. senegal* gum[4]. This suggests that the galactan framework of *A. laeta* gum has a higher proportion of β-D-(1→3)-linkages than *A. senegal* gum. This could be one of the factors contributing to the lower specific rotation of *A. laeta* gum.

Four Smith-degradations were required to remove all of the arabinose residues from *A. senegal* gum[4]. Thus, the longest arabinose-containing side-chains in *A. senegal* gum contain four residues. However, five Smith-degradations were required to remove all of the arabinose residues from *A. laeta* gum, and therefore some of the arabinose-containing side-chains of *A. laeta* gum must contain five residues. Methylation analysis of autohydrolysed *A. senegal* gum showed that arabinofuranose units were the only arabinose residues attached to the galactan framework[4]. In contrast, both arabinofuranose and arabinopyranose units have been shown to be attached to the galactan framework of *A. laeta* gum.

Apart from these differences, *A. laeta* and *A. senegal* gums are closely similar on an overall molecular basis. As in the case of *A. senegal* gum[4], there is no unequivocal proof for a "main chain" or "backbone" in *A. laeta* gum.

EXPERIMENTAL

The analytical methods have been described in detail[5]. Paper chromatography was carried out on Whatman Nos. 1 and 3MM papers with the following solvent systems (v/v); (*a*) benzene–butyl alcohol–pyridine–water (1:5:3:3, upper layer); (*b*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); (*c*) ethyl acetate–pyridine–water (10:4:3); (*d*) butyl alcohol–ethanol–water (4:1:5, upper layer); (*e*) butanone–acetic acid–water (9:1:1, saturated with boric acid); (*f*) butanone–water–ammonia (sp. gr. 0.88) (200:17:1); (*g*) ethyl acetate–acetic acid–water (9:2:2); (*h*) butanone–half saturated with water. R_{Gal} values refer to distances moved relative to that of galactose. R_G values of *O*-methyl sugars refer to distances moved relative to that of 2,3,4,6-tetra-*O*-methyl-D-glucose (see Table II). G.l.c. (Chromatograph Type S3A, flame-ionisation detection, Gas Chromatography Ltd.) of mixtures of *O*-methyl sugars was carried out at nitrogen flow-rates of ca. 100 ml/min on columns of (*i*) 15% by weight of poly(butane-1,4-diol succinate) on 60-70 mesh Celite (5 ft. × 0.25 in.) at 175° and (*ii*) 15% by weight of poly(ethylene glycol adipate) on 60–70 mesh Celite (3 ft. × 0.25 in.) at 160°. Retention times (T) (Table II) are quoted relative to methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside as standard. Unless otherwise stated, polysaccharides were methylated successively with methyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide, and methanolyses were carried out under reflux for 7 h with 5% methanolic hydrogen chloride. Disaccharides were methylated first by Haworth's method[18] and then by Kuhn's method[19].

Purification of A. laeta gum. — The gum (220 g) was dissolved in water (4 l), filtered, dialysed, and electrodialysed. The polysaccharide was isolated as the freeze-dried product (208 g), $[\alpha]_D$ −42° (*c* 1.0) (Found: limiting-viscosity number, 20.7; N, 0.65; OMe, 0.35%; equivalent weight, 1250; uronic acid, 14.0; galactose, 44; arabinose, 29; rhamnose, 13%. All sugar percentages are for the "anhydro" forms and are corrected for moisture and protein contents.

The gum migrated as a single band on electrophoresis on strips (18 × 5 cm) of cellulose-acetate film in both 0.1M ammonium carbonate buffer (pH 8.9) and 0.1M acetate buffer (pH 4.7) at field strengths between 15 and 20 volts/cm for 2–4 h.

Polysaccharide bands were stained by a modification of the periodate–rosaniline hydrochloride method[17]. The gum was also chromatographed[3] on a DEAE-cellulose column (35 × 1.5 cm). Gradient elution with sodium chloride solution (0.02→0.50M), buffered at pH 6, yielded a single, slightly asymmetric peak.

Separation and characterisation of neutral sugars. — The gum (5 g) was hydrolysed with 2N sulphuric acid (500 ml) for 12 h on a boiling-water bath, and the cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H^+), and concentrated to a syrup. The syrup was fractionated on a Duolite A-4 resin column (20 × 2 cm) in the formate form, the neutral sugars being eluted with water (2 l). The neutral fraction was concentrated to a syrup (3.3 g) and chromatographed on a cellulose column (60 × 3 cm) with solvent (*a*), to give three pure monosaccharides.

Fraction1 (900 mg) was D-galactose, m.p. and mixed m.p. 118–119°, $[\alpha]_D$ +80° (equil.) (*c* 4.0). It gave mucic acid, m.p. 213–214°.

Fraction 2 (1.04 g) was L-arabinose, m.p. (from aqueous ethanol) and mixed m.p. 159°, $[\alpha]_D$ +105° (equil.) (*c* 3.0). The X-ray diffraction pattern was identical with that of an authentic specimen of L-arabinose. The derived phenylosazone had m.p. 164–165°.

Fraction 3 (275 mg) was L-rhamnose, m.p. (from aqueous ethanol) and mixed m.p. 159°, $[\alpha]_D$ +9° (equil.) (*c* 3.0). The X-ray diffraction pattern was identical with that of an authentic specimen of L-rhamnose. The derived phenylosazone had m.p. 181–182° (from water).

Isolation and characterisation of D-*galactose disaccharides.* — The gum (30 g) was hydrolysed with 0.01N sulphuric acid (500 ml) for 60 h on a boiling-water bath. After dialysis against tap water for 48 h, the solution was adjusted to 0.2N with respect to sulphuric acid and hydrolysed for 4 h on a boiling-water bath. After neutralisation, degraded-gum α (6.5 g) was isolated by alcohol precipitation. Fig. 1 shows the stepwise hydrolysis of degraded-gum α. Syrups 1-4 were separated into a neutral fraction and an acidic fraction by ion-exchange chromatography on a column (15 × 2.5 cm) of DEAE-Sephadex G-25 in the formate form. The four neutral fractions were combined and concentrated to a syrup (2.6 g), which was chromatographed on Whatman 3MM papers in solvent (*a*) to give two pure disaccharides.

Fraction 1 (118 mg) had $[\alpha]_D$ +62° (*c* 2.36) and yielded galactose on acid hydrolysis. It had the same R_{Gal} values [0.47 in solvent (*a*) and 0.53 in solvent (*c*)] as 3-*O*-β-D-galactopyranosyl-D-galactose. A portion (50 mg) of the disaccharide was methylated. G.l.c. examination of the methanolysis products revealed peaks with retention times corresponding to the methyl glycosides of 2,3,4,6-tetra-, and 2,4,6- and 2,5,6-tri-*O*-methyl D-galactose [(*i*) *T* 3.99; (*ii*) *T* 3.22]. Hydrolysis of the methyl glycosides, followed by paper-chromatographic examination in solvent (*d*), confirmed the presence of 2,3,4,6-tetra-, and 2,4,6- and 2,5,6-tri-*O*-methyl-D-galactose (R_G 0.86). The disaccharide was crystallised from aqueous acetone to give needles of 3-*O*-β-D-galactopyranosyl-D-galactose monohydrate, m.p. and mixed m.p. 157°; its X-ray diffraction pattern was identical with that of authentic material.

Fraction 2 (87.5 mg) had $[\alpha]_D$ +28° (*c* 1.70) and yielded galactose on acid hydrolysis. It had the same R_{Gal} values [0.29 in solvent (*a*) and 0.38 in solvent (*c*)] as 6-*O*-β-D-galactopyranosyl-D-galactose. A portion (40 mg) was methylated and methanolysed to give products corresponding (g.l.c.) to the methyl glycosides of 2,3,4,6-tetra-, and 2,3,5- [(*i*) *T* 4.03, 5.37; (*ii*) *T* 3.32, 4.35] and 2,3,4-tri-*O*-methyl-D-galactose. Hydrolysis of the methyl glycosides, followed by paper chromatography in solvent (*d*), confirmed the presence of 2,3,4,6-tetra-, and 2,3,5- (R_G 0.83) and 2,3,4-tri-*O*-methyl-D-galactose. The phenylosazone had m.p. 201–203° (from water).

Isolation and characterisation of 3-O-β-L-arabinofuranosyl-L-arabinose. — Chromatography in solvent (*b*) and iodometric titration during a small-scale autohydrolysis at 100° showed that the reaction was essentially complete after 50 h. However, a disaccharide released at the beginning of the autohydrolysis had been almost totally broken down after 50 h. In order to isolate this disaccharide, the gum (5 g) in water (250 ml) was heated at 80° in a dialysis bag placed in distilled water (1 liter), which was changed at 2-h intervals. After 6 h, the autohydrolysate was cooled, and dialysed against distilled water (1 liter) for a further 24 h. The combined dialysates were concentrated to a syrup (1.3 g) which was chromatographed on Whatman 3MM papers in solvent (*b*) to give the disaccharide (28 mg), $[\alpha]_D$ +90° (*c* 0.56), which yielded arabinose on acid hydrolysis. It had the same paper-chromatographic mobility [R_{Gal} 1.25 in solvent (*b*)] as 3-*O*-β-L-arabinofuranosyl-L-arabinose. A portion (10 mg) was methylated and methanolysed to give products corresponding (g.l.c.) to the methyl glycosides of 2,3,5-tri-, and 2,5- and 2,4-di-*O*-methyl-L-arabinose [(*i*) *T* 2.19, 2.26; (*ii*) *T* 1.52, 1.63]. Hydrolysis of the methyl glycosides, followed by paper chromatography in solvent (*d*), confirmed the presence of 2,3,5-tri-, and 2,5- and 2,4-di-*O*-methyl-L-arabinose (R_G 0.66). The phenylosazone of the disaccharide had m.p. 198–199° (from water).

Preparation of degraded-gum A. — The gum (60 g) was dissolved in water (1.5 l) and autohydrolysed for 60 h on a boiling-water bath. The cooled solution was filtered, and dialysed against distilled water (8 l). Freeze-drying gave degraded-gum *A* (25 g), $[\alpha]_D$ −28° (*c* 1.0) (Found: uronic acid, 21; galactose, 76; arabinose, 3%).

Degraded-gum *A* (500 mg) was dissolved in water (100 ml) and reduced with sodium borohydride (500 mg). After dialysis, the freeze-dried product was hydrolysed. Paper-chromatographic examination of the hydrolysate in solvent (*e*) indicated a trace of galactitol, but no arabinitol.

The diffusate was concentrated to a syrup (33 g). A portion (5 g) was fractionated on a cellulose column (60 × 3 cm) with solvent (*i*) to give two disaccharides, which were purified by further fractionation on Whatman 3MM paper in solvent (*b*).

Fraction 1 (271 mg), $[\alpha]_D$ +148° (*c* 3.0,) yielded galactose and arabinose on acid hydrolysis. It had the same paper-chromatographic mobility [R_{Gal} 0.71 in solvent (*a*) and 0.51 in solvent (*b*)] as 3-*O*-α-D-galactopyranosyl-L-arabinose. A portion (50 mg) was methylated and methanolysed to give products corresponding (g.l.c.) to the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-galactose, and 2,5- and 2,4-di-*O*-methyl-L-arabinose [(*i*) *T* 2.20, 2.25; (*ii*) *T* 1.53, 1.61]. Hydrolysis of the methyl

glycosides, followed by paper chromatography in solvent (*d*), confirmed the presence of 2,3,4,6-tetra-*O*-methyl-D-galactose, and 2,5- and 2,4-di-*O*-methyl-L-arabinose (R_G 0.67). The phenylosazone of the disaccharide had m.p. 235° (decomp.) (from water).

Fraction 2 (35 mg), $[\alpha]_D$ +180° (*c* 0.70), yielded arabinose on acid hydrolysis. It had the same paper-chromatographic mobility [R_{Gal} 0.71 in solvent (*b*) and 0.86 in solvent (*c*)] as 3-*O*-β-L-arabinopyranosyl-L-arabinose. A portion (15 mg) was methylated and methanolysed to give products corresponding (g.l.c.) to the methyl glycosides of 2,3,4-tri-, and 2,5- and 2,4-di-*O*-methyl-L-arabinose [(*i*) *T* 2.18, 2.24; (*ii*) *T* 1.50, 1.59]. Hydrolysis of the methyl glycosides, followed by paper chromatography in solvent (*d*), confirmed the presence of 2,3,4-tri-, and 2,5- and 2,4-di-*O*-methyl-L-arabinose (R_G 0.68). The phenylosazone of the disaccharide had m.p. 233–234° (decomp.) (from ethanol).

Methylation of degraded-gum A. — Degraded-gum *A* (5 g) was methylated to give a product (3.8 g) (Found: OMe, 39.1%; not raised by further attempted methylation). A portion (50 mg) was methanolysed to give the *O*-methyl sugars listed in Table II. Hydrolysis of the methyl glycosides, followed by paper-chromatographic examination in solvents (*d*) and (*f*), indicated the presence of 2,6-di- and 2-*O*-methyl-D-galactose, in addition to those *O*-methyl sugars already characterised as their methyl glycosides.

TABLE II

EXAMINATION OF METHANOLYSIS PRODUCTS FROM METHYLATED, DEGRADED-GUM *A*

O-Methyl sugars	*Approximate, relative molar proportions*	*Relative retention times* (T) *of methyl glycosides*		R_G *in solvent* (f)
		Column (i)	*Column* (ii)	
2,3,5-Tri-*O*-methyl-L-arabinose	Trace	0.58; 0.72	0.46; 0.64	—
2,3,4-Tri-*O*-methyl-L-arabinose	Trace	0.98	0.85	—
2,3,4,6-Tetra-*O*-methyl-D-galactose	++	1.67	1.64	0.78
2,4,6-Tri-*O*-methyl-D-galactose	++	3.75; 4.20	2.98; 3.46	0.39
2,3,4-Tri-*O*-methyl-D-galactose	++	6.45	5.20	0.33
2,4-Di-*O*-methyl-D-galactose	+++	14.6; 16.4	9.9; 11.3	0.11
2,6-Di-*O*-methyl-D-galactose	Trace	—	—	0.18
2,3,4-Tri-*O*-methyl-D-glucuronic acid[a]	+++	2.21; 2.97	2.09; 2.70	—
2-*O*-Methyl-D-galactose	+	—	—	0.05

[a]As methyl ester methyl glycoside.

Smith degradation of degraded-gum A. — Degraded-gum *A* (15 g) was dissolved in 0.25M-sodium metaperiodate solution (1.5 l) and left in darkness. After 96 h, the reaction was stopped by addition of ethylene glycol (30 ml). The solution was dialysed against running tap-water for 2 days. Sodium borohydride (4 g) was added, and the mixture kept at room temperature for 30 h. After dialysis for a further 2 days, the

polyalcohol was hydrolysed in N sulphuric acid for 2 days at room temperature. The solution was then neutralised with barium carbonate, filtered, deionised with Amberlite resin IR-120 (H^+), and dialysed against distilled water (2 l). The syrup obtained from the dialysate was shown by paper chromatography in solvent (*d*) to contain glycerol and glycolaldehyde. After further dialysis against running tap-water for 2 days, degraded-gum *B* (1.25 g; 8.4%) was isolated as the freeze-dried product.

Partial acid hydrolysis and methylation of degraded-gum B. — Degraded-gum *B* (20 mg) was hydrolysed with 0.5N sulphuric acid (10 ml) for 1 h on a boiling-water bath. The solution was neutralised with barium carbonate, filtered, deionised with Amberlite resin IR-120 (H^+), and concentrated. Paper-chromatographic examination in solvent (*a*) indicated the presence of galactose, 3-*O*-β-D-galactopyranosyl-D-galactose, and a trace of 6-*O*-β-D-galactopyranosyl-D-galactose.

Degraded-gum *B* (500 mg) was methylated and methanolysed to give the methyl glycosides shown in Table III. Hydrolysis of the methyl glycosides, followed by paper chromatography in solvents (*d*) and (*f*), indicated the presence of 2,6-di- and 2-*O*-methyl-D-galactose, in addition to those *O*-methyl sugars already characterised by g.l.c. of their methyl glycosides.

TABLE III

EXAMINATION OF METHANOLYSIS PRODUCTS FROM METHYLATED, DEGRADED-GUM *B*

O-Methyl sugars	*Approximate relative molar proportions*	*Relative retention times* (T) *of methyl glycosides*		R_G *in solvent* (f)
		Column (i)	*Column* (ii)	
2,3,4,6-Tetra-*O*-methyl-D-galactose	+	1.68	1.67	0.78
2,4,6-Tri-*O*-methyl-D-galactose	++++++	3.72; 4.16	2.99; 3.44	0.39
2,3,6-Tri-*O*-methyl-D-galactose	Trace	—	—	0.49
2,3,4-Tri-*O*-methyl-D-galactose	Trace	6.40	5.26	0.33
2,4-Di-*O*-methyl-D-galactose	++	14.6; 16.4	9.8; 11.1	0.11
2,6-Di-*O*-methyl-D-galactose	+	—	—	0.18
2-*O*-methyl-D-galactose	+	—	—	0.05

Methylation of A. laeta *gum*. — The gum (500 mg) was methylated to give a product (410 mg), $[\alpha]_D$ −49° (*c* 1.0, chloroform) (Found: OMe, 42.1%). Methanolysis gave the methyl glycosides shown in Table IV. Hydrolysis of tke methyl glycosides, followed by paper chromatography in solvent (*d*), indicated the presence of 2-*O*-methylgalactose, in addition to those *O*-methyl sugars already characterised by g.l.c. of their methyl glycosides.

Preparation, partial acid hydrolysis, and methylation of polysaccharide 1. — The gum (28.3 g of polysaccharide) was dissolved in water (700 ml), mixed with 0.25M sodium metaperiodate (700 ml), and left for 48 h. The amount of formic acid released in this time was 1.40 mmoles/g, and the periodate consumed was 5.24 mmoles/g. The reaction was stopped by addition of ethylene glycol (15 ml), and the solution

was dialysed against running tap-water for 48 h. Sodium borohydride (7.5 g) was added, and the mixture kept for 30 h at room temperature, and then dialysed for a further 48 h. The polyalcohol was hydrolysed in N sulphuric acid for 48 h at room temperature.

TABLE IV

EXAMINATION OF METHANOLYSIS PRODUCTS FROM METHYLATED *A. laeta* GUM

O-*Methyl sugars*	*Relative retention times* (T) *of methyl glycosides*		R_G *in solvent* (d)
	Column (i)	*Column* (ii)	
2,3,4-Tri-*O*-methyl-L-rhamnose	0.50	0.45	1.01
2,3,5-Tri-*O*-methyl-L-arabinose	0.60; 0.72	0.52; 0.62	0.98
2,3,4-Tri-*O*-methyl-L-arabinose	1.03	0.82	0.78
2,5-Di-*O*-methyl-L-arabinose	1.81; 3.18	1.27;(2.16)[a]	0.80
2,3,4-6-Tetra-*O*-methyl-D-galactose	1.70	1.67	0.88
2,4,6-Tri-*O*-methyl-D-galactose	3.83; 4.27	2.92; 3.45	0.72
2,3,4-Di-*O*-methyl-D-galactose	6.66	5.09	0.72
2,4-Di-*O*-methyl-D-galactose	14.7; 16.6	9.8; 11.2	0.52
2,3,4-Tri-*O*-methyl-D-glucuronic acid[b]	2.34; 2.91	(2.16);2.70	—
2,3-Di-*O*-methyl-D-glucuronic acid[b]	7.8; 9.5	5.8; 6.8	—
2-*O*-methyl-D-galactose	—	—	0.32
Unknown Peaks	1.19; 1.45	0.98; 1.13	—

[a]Figures in parentheses indicate T values of components not completely resolved. [b]As methyl ester methyl glycoside.

After dialysis against running tap-water for 48 h, polysaccharide **1** was isolated as the freeze-dried product (17.0 g, 60%), $[\alpha]_D$ $-18°$ (c 0.82) (Found: uronic acid, 4; galactose, 68; arabinose, 28%). Hydrolysis of polysaccharide **1** with N sulphuric acid for 7 h on a boiling-water bath gave a trace of 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose [R_{Gal} 0.22 in solvent (*b*)] in addition to galactose and arabinose. Molecular-sieve chromatography[4,5,10,11] of polysaccharide **1** (20 mg) on a previously calibrated "Bio-Gel P 300" column (45×4.8 cm) gave $\bar{M}_n$ ~91,000.

Polysaccharide **1** (20 mg) was hydrolysed with 0.5N sulphuric acid (10 ml) for 1 h on a boiling-water bath. Paper-chromatographic examination of the hydrolysate indicated the presence of galactose, arabinose, and two neutral disaccharides having the mobilities of 3-*O*-β-D-galactopyranosyl-D-galactose [R_{Gal} 0.49 in solvent (*a*) and 0.54 in solvent (*c*), minor component] and 6-*O*-β-D-galactopyranosyl-D-galactose [R_{Gal} 0.31 in solvent (*a*) and 0.38 in solvent (*c*), major component].

Polysaccharide **1** (200 mg) was methylated to give a product (125 mg), $[\alpha]_D$ $-43°$ (c 1.0, chloroform) (Found: OMe, 39.5%, not raised on further methylation) (see Table VI). Methanolysis of a sample of this product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table V.

Preparation, partial acid hydrolysis, and methylation of polysaccharides **2–8**. — The following weights of polysaccharide were periodate oxidised, reduced with

borohydride, hydrolysed, and the corresponding degraded-polysaccharides were recovered as for the first Smith-degradation. Polysaccharide **1** (16.5 g) gave polysaccharide **2** (8.75 g); **2** (8.35 g) **3** (5.0 g); **3** (4.6 g) → **4** (3.0 g); **4** (2.6 g) → **5** (1.9 g); **5** (1.55 g) → **6** (1.2 g); **6** (0.86 g); → **7** (0.68 g); **7** (0.63 g) → **8** (0.49 g). The relevant data are given in Table I.

TABLE V

O-METHYL SUGARS PRESENT IN METHYLATED, SMITH-DEGRADED POLYSACCHARIDES 1–6 AND 8

O-Methyl sugars	1	2	3	4	5	6	8	*Approximate, relative molar proportions in* 5	6	8
2,3,5-Tri-*O*-methyl-L-arabinose	+	+	+	+						
2,5-Di-*O*-methyl-L-arabinose	+	+	+							
2,3,4,6-Tetra-*O*-methyl-D-galactose	+	+	+	+	+	+	+	1	1	1
2,4,6-Tri-*O*-methyl-D-galactose	+	+	+	+	+	+	+	14	16	18
2,3,4-Tri-*O*-methyl-D-galactose	+	+	+	+	+	+	+	Trace	Trace	Trace
2,6-Di-*O*-methyl-D-galactose	+	+	+	+	+	+	+	3	1	Trace
2,4-Di-*O*-methyl-D-galactose	+	+	+	+	+	+	+	1	1	1
2,3,4-Tri-*O*-methyl-D-glucuronic acid	+									

Partial, acid hydrolysis of polysaccharide **2** (20 mg), followed by paper-chromatographic examination of the hydrolysate, indicated the presence of equal amounts of 3-*O*-β-D-galactopyranosyl-D-galactose and 6-*O*-β-D-galactopyranosyl-D-galactose, and equal amounts of the β-(1→3)-linked [R_{Gal} 0.22 in solvent (*a*) and 0.23 in solvent (*c*)] and the β-(1→6)-linked D-galactose trisaccharide [R_{Gal} 0.15 in solvent (*a*) and 0.18 in solvent (*c*)]. Partial, acid hydrolysis of polysaccharides **3** and **4** gave the same oligosaccharides as obtained from polysaccharide **2**. However, the proportions of the β-(1→6)-linked di- and tri-saccharides decreased steadily from polysaccharides **2** to **4**. Partial, acid hydrolysis of polysaccharides **5** to **8** indicated the presence of the β-(1→3)-linked di- and tri-saccharides, and the β-(1→6)-linked disaccharide. The proportion of the latter disaccharide continued to fall from polysaccharides **5** to **8**; only a trace was obtained from polysaccharide **8**.

Polysaccharides **1**–**6** and **8** were methylated (Table VI). Methanolysis of a sample of the products, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table V.

Preparation, and partial acid hydrolysis of polysaccharide **5'**. — *A. laeta* gum (30 g) was subjected to five successive Smith-degradations, without isolation of the intermediate polysaccharides, to obtain polysaccharide **5'** (2.2 g). The polysaccharide was hydrolysed with 0.5N sulphuric acid (500 ml) for 1 h on a boiling-water bath. The hydrolysate was chromatographed on Whatman 3MM papers in solvent (*a*), and a pure trisaccharide was isolated.

The trisaccharide (44 mg), $[\alpha]_D$ +51° (*c* 0.88), yielded galactose and 3-*O*-β-D-galactopyranosyl-D-galactose, as the only disaccharide, on partial hydrolysis with

TABLE VI

METHYLATION OF SMITH-DEGRADED POLYSACCHARIDES

	Polysaccharide						
	1	2	3	4	5	6	8
Amount of polysaccharide used (mg)	200	193	165	158	184	180	250
Amount of product (mg)	125	120	80	85	76	65	81
$[\alpha]_D$ of product (degrees)	−43	−33	−11	−1	−1	+1	+1
OMe (%)	39.5	41.3	39.1	37.2	37.1	36.8	36.5

acid. The trisaccharide (crystallised from aqueous ethanol) had m.p. and mixed m.p. 237–242° (decomp.), and R_{Gal} values of 0.22 in solvent (*a*) and 0.23 in solvent (*c*).

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54.

STUDIES ON URONIC ACID MATERIALS

PART XXX*. EXAMINATION OF THREE FRACTIONS OBTAINED FROM ACACIA DREPANOLOBIUM GUM

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ABSTRACT

Dissolution of *A. drepanolobium* gum Fraction C (the water-insoluble gel) has been attempted using eight solvents. 1% Sodium borohydride solution was found to be the best solvent, giving a 69% yield of Soluble Fraction C. Molecular-sieve chromatography using Bio-Gel P300 indicated that Soluble Fraction C has a much higher molecular weight than the cold water-soluble gum(Fraction A) and this has been confirmed by light-scattering measurements. Smith-degradation and methylation analyses have indicated that Fractions A, B, and Soluble Fraction C are structurally very similar.

INTRODUCTION

In a previous paper[2], Fraction A (water soluble), Fraction B (soluble in M sodium chloride solution) and Fraction C (the insoluble gel) of *A. drepanolobium* gum were compared analytically. Four aldobiouronic acids were also characterised after N sulphuric

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* For Part XXIX, see Ref. 1.

acid hydrolysis of Fraction A. In this Paper, methods for dissolving Fraction C are explored. After dissolution in 1% sodium borohydride solution, soluble Fraction C is compared with Fractions A and B in terms of (a) Smith degradation and methylation analyses, (b) molecular weight measurements, in order to ascertain whether the insolubility of Fraction C arose from structural or from molecular weight differences.

MATERIALS AND METHODS

The gum nodules from A. drepanolobium Harms ex Sjöstedt were collected by Mr. W.M.C. Bagshaw, Provincial Forest Officer, at Tabora, Western Province, Tanganyika, in July 1961. Powdered A. drepanolobium gum (45 g) was fractionated as described previously[2] to give Fraction A (35 g), Fraction B (3.5 g) and Fraction C (5.0 g).

The standard analytical methods have been described previously[3]. Paper chromatography was carried out on Whatman No. 1 paper using the following solvent systems (v/v); (a) benzene - butyl alcohol - pyridine - water (1:5:3:3, upper layer); (b) ethyl acetate - acetic acid - formic acid - water (18:3:1:4); (c) ethyl acetate - pyridine - water (10:4:3); (d) butyl alcohol - ethanol - water (4:1:5, upper layer); (e) butyl alcohol - acetic acid - water (4:1:5, upper layer); (f) butan-2-one - water - conc. ammonia (200:17:1). R_G values of methylated sugars refer to distances moved relative to 2,3,4,6-tetra-O-methyl-D-glucose in

solvent (d). G.l.c. of mixtures of O-methyl sugars was carried out as described previously[4] on columns of (i) 15% by weight of poly(butane-1,4-diol succinate) on 60-70 mesh Celite (120 x 0.5 cm) at 175°, and (ii) 15% by weight of poly(ethylene glycol adipate) on 60-70 mesh Celite (75 x 0.5 cm) at 160°. Retention times (T) are quoted relative to methyl 2,3,4,6-tetra-O-methyl -β-D-glucopyranoside. Polysaccharides were methylated successively with dimethyl sulphate and sodium hydroxide solution, and with methyl iodide and silver oxide. Methanolyses were carried out under reflux for 7 h with methanolic 5% hydrogen chloride.

Light-scattering measurements were made with a SOFICA Model 42000 Photo Gonio Diffusometer, using unpolarised green light (5460 Å). Weight-average molecular weights, $\bar{M}_w$, were calculated as described previously[5,6].

RESULTS

Attempts to dissolve Fraction C. - It has already been shown that Fraction C is soluble in N sodium hydroxide solution, to the extent of 1% (w/v)[2]. This method of dissolution is not entirely satisfactory, since alkaline degradation of the polysaccharide will probably occur. Different methods of dissolution were therefore attempted. Samples of Fraction C (120 mg) were extracted with (i) distilled water; (ii) 3M sodium chloride solution; (iii) 3M magnesium chloride solution; (iv) 7M urea solution; (v) 2% disodium ethylenediaminetetra-

acetate solution (w/v); (vi) 2% sodium hexametaphosphate solution (w/v); (vii) 1% sodium borohydride solution (w/v); (viii) 0.1M phosphate buffer, pH 9.0; (ix) 3% sodium borate solution (w/v). Each extraction was filtered, dialysed against tap water for 48 h, and isolated as the freeze-dried product. Table I shows the percentage of Fraction C dissolved by these solvents. All the freeze-dried products were water-soluble.

The best solvent for Fraction C is undoubtedly 1% sodium borohydride solution (w/v). Fraction C (3.75 g) was therefore extracted with 1% sodium borohydride solution (400 ml), filtered, dialysed against tap water for 48 h, and electrodialysed to give Soluble Fraction C (2.25 g) as the freeze-dried product. The analytical data for Soluble Fraction C are shown in Table II. Hydrolysis (N sulphuric acid for 7 h at 100°) of Soluble Fraction C, followed by paper chromatography, indicated the presence of galactose, arabinose, rhamnose, and the same four aldobiouronic acids which were detected in Fractions A, B, and C[2].

In order to check whether Fraction C had been degraded by treatment with 1% sodium borohydride solution (w/v), Fractions A, B, and Soluble Fraction C were examined by molecular-sieve chromatography using a previously calibrated "Bio-Gel P300" column (45 x 4.5 cm) [3,7]. The elution volume of Soluble Fraction C was less than that of "Blue Dextran 2000"; fractions A and B were eluted at the same elution volume as "Blue Dextran" (Pharmacia, Uppsala, Sweden). This suggested that Soluble

Fraction C had a higher molecular weight than Fractions A and B. Light-scattering measurements subsequently gave weight average molecular weights of 9.5×10^5, 10.2×10^5, and 22.0×10^5 for Fractions A, B, and Soluble Fraction C respectively.

Methylation analysis of Fractions A, B, and Soluble Fraction C. - The three fractions (300 mg) were methylated exhaustively. Yields, specific rotations, and methoxyl contents of the methylated products are shown in Table III. Methoxyl contents were not raised on further attempted methylation. On methanolysis and g.l.c. examination of the mixture of methyl glycosides, the methylated products of each fraction gave identical traces using columns (i) and (ii). Results of g.l.c. examination are shown in Table IV; retention times are comparable with those for methyl glycosides from authentic O-methyl sugars. A portion of the methanolysate from each methylated product was hydrolysed with N sulphuric acid for 4 h on a boiling water bath. The cooled solutions were neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H^+) and concentrated. Paper chromatographic examination of the hydrolysates in solvents (d) and (f) showed the presence of 2-O-methylgalactose and 4-O-methylarabinose in addition to those O-methyl sugars already identified by g.l.c. of their methyl glycosides (Table IV).

Smith degradation[8] of Fractions, A, B, and Soluble Fraction C. The purified fractions (1.5 g polysaccharide) were dissolved in water (37.5 ml) and 0.25M sodium metaperiodate solution (37.5 ml)

and 0.25M sodium metaperiodate solution (37.5 ml) was added. The oxidation mixtures were left in darkness at room temperature for 48 h, when the reactions were stopped by addition of ethylene glycol (2 ml). The solutions were dialysed against tap water for 48 h. Sodium borohydride (0.5 g) was added to each solution, and the mixtures were kept at room temperature for 30 h, then dialysed for a further 48 h. The three polyalcohols were hydrolysed in N sulphuric acid at room temperature for 48 h, and the hydrolysates were neutralised with barium carbonate, filtered, and deionised with Amberlite resin IR-120 (H^+). The solutions were each dialysed against distilled water (1 l) for 24 h, and then against tap water for 48 h. The three Smith-degraded polysaccharides were isolated as freeze-dried products, and the dialysates were concentrated to syrups.

Yields and analytical data for the three Smith-degraded polysaccharides are shown in Table V. Hydrolysis of the Smith-degraded polysaccharides (N sulphuric acid for 7 h at 100°), followed by paper chromatography, indicated the presence of only galactose and arabinose. The Smith-degraded polysaccharides were hydrolysed with 0.5N sulphuric acid for 1 h on a boiling water bath. Paper chromatographic examination indicated the presence of galactose, arabinose, and two neutral disaccharides with the mobilities of 3-O-β-D-galactopyranosyl-D-galactose [R_{Gal} 0.49 in solvent (a), 0.54 in solvent (c)] and 6-O-β-D-galactopyranosyl-D-galactose [R_{Gal} 0.31 in solvent (a),

0.[illegible]3 in solvent (c),major component].

Chromatographic examination of the three dialysates in solvents (a) and (c) showed the presence of glycolaldehyde and glycerol, and traces of ethylene glycol and threitol. The traces of ethylene glycol would be expected from non-reducing arabinopyranosyl end-groups. The traces of threitol would be expected from D-galactopyranosyl residues with a substituent on C-4, as in the α1,4-linked aldobiouronic acids previously detected in the hydrolysates of Fractions A, B, and C. Glycerol from the dialysate of the Smith-degradation of Fraction A, after purification by thick paper chromatography in solvent (a), was characterised by preparation of its tribenzoate derivative, m.p. 75°-76°.

Methylation analysis of Smith-degraded products of Fractions A, B, and Soluble Fraction C. - The three degraded products (200 mg) were methylated exhaustively using the methods of Haworth and Purdie. Yields, specific rotations, and methoxyl contents of the methylated products are shown in Table VI. Methoxyl contents were not raised on further attempted methylation. On methanolysis and g.l.c. examination of the mixtures of methyl glycosides using columns (i) and (ii), the three methylated products gave identical results. Results of g.l.c. examination are shown in Table VII. A portion of each methanolysate was hydrolysed (N sulphuric acid for 4 h at 100°). The cooled solutions were neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H^{+}) and concentrated. Paper chromatographic

examination of the hydrolysates in solvents (d) and (f) showed the presence of 2,6-di- and 2-*O*-methylgalactose in addition to those *O*-methyl sugars already identified by g.l.c. of their methyl glycosides (Table VII).

DISCUSSION

A. drepanolobium gum Fraction C is sparingly soluble (1% w/v) in M sodium hydroxide solution. This method of dissolution is not satisfactory, since degradation may occur under such alkaline conditions. Eight different aqueous solvents for Fraction C were therefore investigated (Table I). 3M Sodium chloride solution, 3M magnesium chloride solution and 7M urea solution were unsuccessful solvents; hydrogen bonding between polysaccharide molecules is therefore unlikely to be the major reason for the insolubility of Fraction C. 3% Sodium borate solution and 0.1M phosphate buffer dissolved Fraction C to the extent of 7% and 10.5% respectively; these solvents have pH 9, and this may account for the slight solubilisation effected. Fraction C was dissolved to a greater extent by 2% disodium ethylenediaminetetra-acetate solution (17.5%) and by 2% sodium hexametaphosphate solution (47%). The action of these solvents is probably dependent on the chelation of calcium and magnesium[9,10] and cross-linking by polyvalent metal ions might contribute to the insolubility of Fraction C in aqueous solutions. The higher solubility in sodium hexametaphosphate solution is expected

since its optimum conditions[11] are at neutral pH and room temperature (i.e. the conditions of extraction); the optimum conditions for disodium ethylenediaminetetra-acetate solution[9] are at pH 10 and 60°-70°.

The best solvent found for Fraction C is 1% sodium borohydride solution. This has pH=9, and will contain borate ions, but these facts cannot account for the dissolution action of this solvent in view of the results reported above for sodium borate solution and for phosphate buffer. Dilute sodium borohydride solution has also been found[12] to be an effective solvent for water-insoluble gums from the genera Combretum, Lannea and Teclea.

Fraction C was extracted with 1% sodium borohydride solution in a large scale[#] experiment to yield Soluble Fraction C as the freeze-dried, electrodialysed product. The yield of solubilised gum was 69%, based on the estimated dry weight of the insoluble gel. This represents virtually complete recovery of the polysaccharide present; all the bark and other foreign matter in the gum sample had inevitably become concentrated in the gel (fraction C).

Analysis showed Soluble Fraction C to have the same sugar percentages as Fraction C; on periodate oxidation the two fractions released the same amount of formic acid and reduced the same amount of periodate.

Fractions A, B, and Soluble Fraction C were compared using methylation analysis. The methylated products were obtained in similar yields, and had the same specific rotations. On methanolysis and g.l.c. examination the three methylated products gave identical results. On Smith degradation, Fractions A, B, and Soluble Fraction C gave similar yields of Smith-degraded products. The analytical data (Table V) for the Smith-degraded polysaccharides are almost identical, and on methylation analysis the three degraded polysaccharides gave identical results. There is therefore a close structural similarity between Fractions A, B, and Soluble Fraction C.

Soluble Fraction C has a lower intrinsic viscosity than that obtained in M sodium hydroxide solution for Fraction C[2]. Molecular-sieve chromatography on a previously calibrated Bio-Gel P300 column was therefore carried out to determine if the decrease in viscosity was the result of degradation of the polysaccharide during the extraction with sodium borohydride solution. Soluble Fraction C was eluted before the elution volume of "blue dextran"; in contrast, Fractions A and B were eluted at the same elution volume as "blue dextran". Soluble Fraction C could therefore differ from fractions A and B in having <u>either</u> a much higher molecular weight, <u>or</u> a different molecular shape[13]. Differences in shape imply differences in molecular structure, such as the frequency or pattern of branching. Since the Smith degradation and methylation analyses showed that

the three Fractions are closely similar structurally, it seems unlikely that the differences between Fractions A, B, and Soluble Fraction C are due to differences in molecular shape. Light-scattering measurements confirmed that Soluble Fraction C has a much higher weight-average molecular weight (22.0×10^5) than Fraction A (9.5×10^5) and Fraction B (10.2×10^5). The precise reasons why sodium borohydride solution dissolves Fraction C are not known; the interactions which cause the polysaccharide molecules to aggregate seem to be counteracted irrevocably, since, after freeze drying, the product (Soluble Fraction C) remains water-soluble. Although it appears that gross degradation does not occur during the solubilising process, the possibility of some degradation occurring cannot be excluded. The true molecular weight of the gel (Fraction C) may be considerably higher than indicated by the experiments involving soluble Fraction C.

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Table I

Solubility of Fraction C in different solvents

Solvents	% Fraction C Dissolved
Distilled water	1
3M sodium chloride solution	1.5
3M magnesium chloride solution	1.5
7M urea solution	2
3% solution (w/v) of sodium borate	7
0.1M phosphate buffer, pH 9.0	10.5
2% solution (w/v) of sodium salt of ethylenediamine tetraacetate	17.5
2% solution (w/v) of sodium hexametaphosphate	47
1% solution (w/v) of sodium borohydride	69

TABLE II

Analytical data for Soluble Fraction C

	Soluble Fraction C
Moisture, %	10.2
Nitrogen, %	0.85
Intrinsic Viscosity, $cm^3\ g^{-1}$	11.1
$[\alpha]_D$ (C 1.0, water)	$+79.5^{\circ}$
Equivalent Weight	1955
Hence Uronic Acid Anhydride, %	9.0
Galactose, %	51
Arabinose, %	39
Rhamnose, %	1
Formic acid released on periodate oxidation (moles/g x 10^3)	1.05
Periodate reduced (moles/g x 10^3)	3.95

Table III

Data for Methylated Fractions A, B, and Soluble Fraction C

	Fraction A	Fraction B	Soluble Fraction C
Yield [a] (mg)	240	250	200
$[\alpha]_D$ (C 1.0, $CHCl_3$)	+60°	+61°	+59°
OMe, %	40.8	40.7	41.0

Footnote

[a] From 300 mg of each fraction

Table IV

Examination of methanolysis and hydrolysis products from methylated Fractions A and B, and Methylated Soluble Fraction C.

Relative retention times, T, of methyl glycosides on columns:- * (i)	(ii)	R_G in solvent (d)	O-methyl sugars
0.48	(0.50)		2,3,4-tri-O-methyl-L-rhamnose
0.55;0.73	(0.50);0.64	0.97	2,3,5-tri-O-methyl-L-arabinose
(1.05)	(0.82)	0.79	2,3,4-tri-O-methyl-L-arabinose
1.80;3.20	1.26;(2.20)	0.82	2,5-di-O-methyl-L-arabinose
(1.05);(2.36)	(0.82);1.76	0.82	3,5-di-O-methyl-L-arabinose
1.98	1.43	0.56	3,4-di-O-methyl-L-arabinose
1.68	1.65	0.88	2,3,4,6-tetra-O-methyl-D-galactose
(3.05);(3.89);(4.22)	2.44;(3.00);(3.44)	0.73	2,3,6-tri-O-methyl-D-galactose
(3.89);(4.22)	(3.00);(3.44)	0.73	2,4,6-tri-O-methyl-D-galactose
6.42	5.08	0.73	2,3,4-tri-O-methyl-D-galactose
14.6;16.4	9.9;11.4	0.52	2,4-di-O-methyl-D-galactose
(2.36);(3.05)	(2.20);2.75		2,3,4-tri-O-methyl-D-glucuronic acid †
		0.32	2-O-methyl-D-galactose
		0.35	4-O-methyl-L-arabinose

* Figures in parentheses indicate T values of components which are incompletely resolved.

† As methyl ester methyl glycoside.

Table V

Analytical data for the Smith-degraded polysaccharides

	Fraction A	Fraction B	Soluble Fraction C
Moisture,%	11.9	10.5	10.8
Nitrogen,%	1.01	0.98	0.32
Yield of polysaccharide (g)	1.11	1.14	1.12
Yield,%	55.5	57	56
Galactose,%	43	44	44
Arabinose,%	57	56	56
$[\alpha]_D$ (C 1.0, water)	$+45^\circ$	$+45^\circ$	$+45^\circ$
Formic acid released on periodate oxidation [mole/g] x 10^3	0.94	0.96	0.97
Periodate consumed on periodate oxidation [mole/g] x 10^3	3.38	3.47	3.45
Ratio of $\frac{\text{Periodate Consumption}}{\text{Formic Acid Released}}$	3.59	3.62	3.56

Table VI

Data for methylated Smith-degraded Fractions A, B, and Soluble Fraction C.

	Fraction A	Fraction B	Fraction C
Yield[a] (mg)	156	150	155
$[\alpha]_D$ (C 1.0, $CHCl_3$)	+39°	+39°	+38°
OMe%	40.1	40.0	40.1

Footnote:

[a] From 200 mg of each fraction.

Table VII

Examination of methanolysis and hydrolysis products from methylated Smith-degraded polysaccharides

Relative retention times, T, of methyl glycosides on columns:- (i)	(ii)	R_G in solvent (d)	Hence O-methyl sugars identified
0.56; 0.74	0.50; 0.63	0.97	2,3,5-tri-O-methyl-L-arabinose
1.06; 2.36	0.81; 1.76	0.82	3,5-di-O-methyl-L-arabinose
1.80; 3.22	1.26; 2.20	0.82	2,5-di-O-methyl-L-arabinose
1.68	1.65	0.88	2,3,4,6-tetra-O-methyl-D-galactose
3.90; 4.22	3.00; 3.45	0.73	2,4,6-tri-O-methyl-D-galactose
6.45	5.08	0.73	2,3,4-tri-O-methyl-D-galactose
14.6; 16.4	9.9; 11.4	0.52	2,4-di-O-methyl-D-galactose
		0.55	2,6-di-O-methyl-D-galactose
		0.32	2-O-methyl-D-galactose
1.53	1.05		Unknown Sugars

STUDIES ON URONIC ACID MATERIALS

PART XXXI*. SOME STRUCTURAL FEATURES OF THE WATER-SOLUBLE FRACTION OF ACACIA DREPANOLOBIUM GUM.

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ABSTRACT

The O-methyl derivative of the water-soluble fraction of the gum from Acacia drepanolobium Harms ex Sjösted yielded 2,3,5- and 2,3,4-tri-, and 2,5-, 3,5- and 3,4-di-O-methyl-L-arabinose, 2,4-di-O-methyl-D-galactose, 2,3,4-tri-O-methyl-D-glucuronic acid, and small amounts of 2,3,4-tri-O-methyl-L-rhamnose, 4-O-methyl-L-arabinose, 2,3,4,6-tetra-, 2,4,6-, 2,3,6- and 2,3,4-tri- and 2-O-methyl-D-galactose. The water-soluble gum was subjected to five successive Smith degradations: the O-methyl derivatives of the degraded polysaccharides were analysed, after methanolysis, by gas-liquid partition chromatography. The structural evidence obtained indicates that arabinose side-chains, some of which are considerably longer than in other Acacia gums studied to date, are attached to a compact, branched galactan framework.

INTRODUCTION

The gum exudate from A. drepanolobium Harms ex Sjösted is not completely soluble in cold distilled water[2]. Extraction of the gum with water gives

* For Part XXX, see Ref. 1.

Fraction A (ca. 80% yield) and extraction of the residue with M sodium chloride solution gives Fraction B (ca. 8% yield), leaving the insoluble gel, Fraction C (ca. 12% yield). A comparative examination of these three Fractions has been reported[1,3]; a partial acid hydrolysis study of Fraction A has indicated a close similarity with A. arabica gum[4]. In this Paper five successive Smith degradations, and methylation analyses, are utilized to establish the dominant features of the molecular structure of A. drepanolobium gum Fraction A.

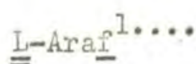
RESULTS AND DISCUSSION

The gum exudate from A. drepanolobium Harms ex Sjöstedt was collected by Mr. W. M. C. Bagshawe, Provincial Forest Officer, at Tabora, Western Province, Tanganyika, in July 1961. After aqueous extraction and isolation[3], the gum Fraction A was found to have the specific rotation and sugar composition shown in Table I. Samples of Fraction A were examined by zone electrophoresis on cellulose acetate film, and by ion-exchange chromatography on D.E.A.E.-cellulose[5]; no sharp discontinuities in properties were indicated by these experiments. It is therefore possible that A. drepanolobium gum Fraction A exhibits the same kind of heterogeneity as the gum exudates of A. arabica[6], A. laeta[7], and A. senegal[8], comprising a continuous spectrum of related molecular species.

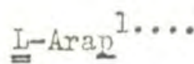
An examination of the O-methyl derivative of Fraction A showed the presence of 2,3,5- and 2,3,4-tri-, and 2,5-, 3,5- and 3,4-di-O-methyl-L-arabinose, 2,4-di-O-methyl-D-galactose, 2,3,4-tri-O-methyl-D-glucuronic acid, and small amounts of 2,3,4-tri-O-methyl-L-rhamnose, 4-O-methyl-L-arabinose, and 2,3,4,6-tetra-, 2,4,6-, 2,3,6- and 2,3,4-tri-, and 2-O-methyl-D-galactose (See Table II).

The small amounts of monomethyl-galactose and monomethyl-arabinose are not thought to be structurally significant; the presence of 2-O-methyl-D-galactose is ascribed to undermethylation, and Smith degradation studies suggest that the 4-O-methyl-L-arabinose arises similarly. The high proportion of 2,4-di-O-methyl-D-galactose shows that most of the D-galactose residues are 3,6-di-O-substituted. The small amounts of 2,3,4,6-tetra-, and 2,4,6-, 2,3,6- and 2,3,4-tri-O-methyl-D-galactose show that there are a few terminal non-reducing D-galactose residues, and some 3-O-, 4-O-, and 6-O-substituted D-galactose units. The identification of 2,3,4-tri-O-methyl-D-glucuronic acid indicates that D-glucuronic acid (and a small amount of its 4-O-methyl ether) occur as terminal non-reducing units. The presence of 2,3,4-tri-O-methyl-L-rhamnose indicates that most, if not all, of the L-rhamnose (1%) is present as non-reducing end-group, and, by analogy with A. senegal gum[9,10], it is possible that these residues are attached to some of the D-glucuronic acid units.

The presence of 2,3,5- and 2,3,4-tri-O-methyl-L-arabinose shows that the arabinose side-chains are terminated by either L-arabinofuranose (I) or L-arabinopyranose residues (II). The identification of some 2,5-di-O-methyl-

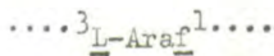


I



II

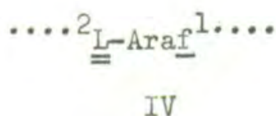
L-arabinose indicates the presence of some 3-O-substituted L-arabinofuranose

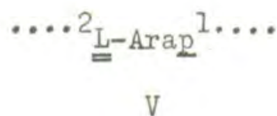


III

residues (III) in these arabinose chains. The major di-O-methyl ether of

arabinose present was 3,5-di-O-methyl-L-arabinose, which has previously been isolated from the methylated gums of A. pycnantha[11,12], A. arabica[6], A. karroo[13], and A. nubica[14], and detected in the methanolysates of the methylated gums from A. mearnsii, A. giraffae, A. decurrens, and A. cyanophylla[15]. The presence of 3,5-di-O-methyl-L-arabinose in methylated Fraction A shows that there is a large proportion of 2-O-substituted L-arabinofuranose residues (IV) in the arabinose chains; the detection of 3,4-di-O-methyl-L-arabinose indicates that these chains also contain a small proportion of 2-O-substituted L-arabinopyranosyl residues (V). 3,4-Di-O-methyl-L-arabinose has previously been isolated from the methylated gums from A. arabica[6] and A. karroo[13], and a trace of this O-methyl sugar has been detected in the methanolysate of the methylated gum from A. podalyriaefolia[15]. Methylation evidence for





A. drepanolobium gum Fraction A and autohydrolysed Fraction A[4] suggests that the arabinose chains are attached to a branched galactan framework at the C-3 and C-6 positions of certain D-galactose residues.

A sample of Fraction A was subjected to five successive Smith degradations; the percentage yields of the products (polysaccharides A - E) are shown in Table I. Although these yields are lower than those obtained from successive Smith degradations of A. senegal[8] and A. laeta gums[7], they are higher, particularly for the later degradations, than were obtained from A. arabica gum[6]. The relative decrease in molecular weight from Fraction A to its first

Smith-degraded product, polysaccharide A, is similar to that reported for A. senegal gum[8]. Molecular-sieve chromatography indicates a drastic decrease in molecular size during the second Smith degradation, i.e. from polysaccharide A ($\overline{Mn}$ ca. 95,500) to polysaccharide B ($\overline{Mn}$ ca. 9,200). The third, fourth, and fifth Smith degradations did not cause such relatively large decreases in $\overline{Mn}$, but the yields from these degradations are low. The ~~degradation~~ pattern of this sequence of Smith degradations differs from that reported for A. laeta[7], A. senegal[8], and A. arabica gums[6]. Four successive Smith degradations were required to remove all the arabinose units from A. senegal gum[8], and five degradations were required for A. laeta gum[7]. For A. arabica gum[6], four successive Smith degradations decreased the arabinose content, but the fourth Smith-degraded product contained a considerable proportion of arabinose: it was concluded[6] that the arabinose chains in A. arabica are at least six units long. A similar conclusion was reached for A. nubica gum[14]. In contrast, five successive Smith degradations of A. drepanolobium gum Fraction A led to an increase in the arabinose content, from 52% for the gum Fraction A to 71% for the fifth degradation product (polysaccharide E).

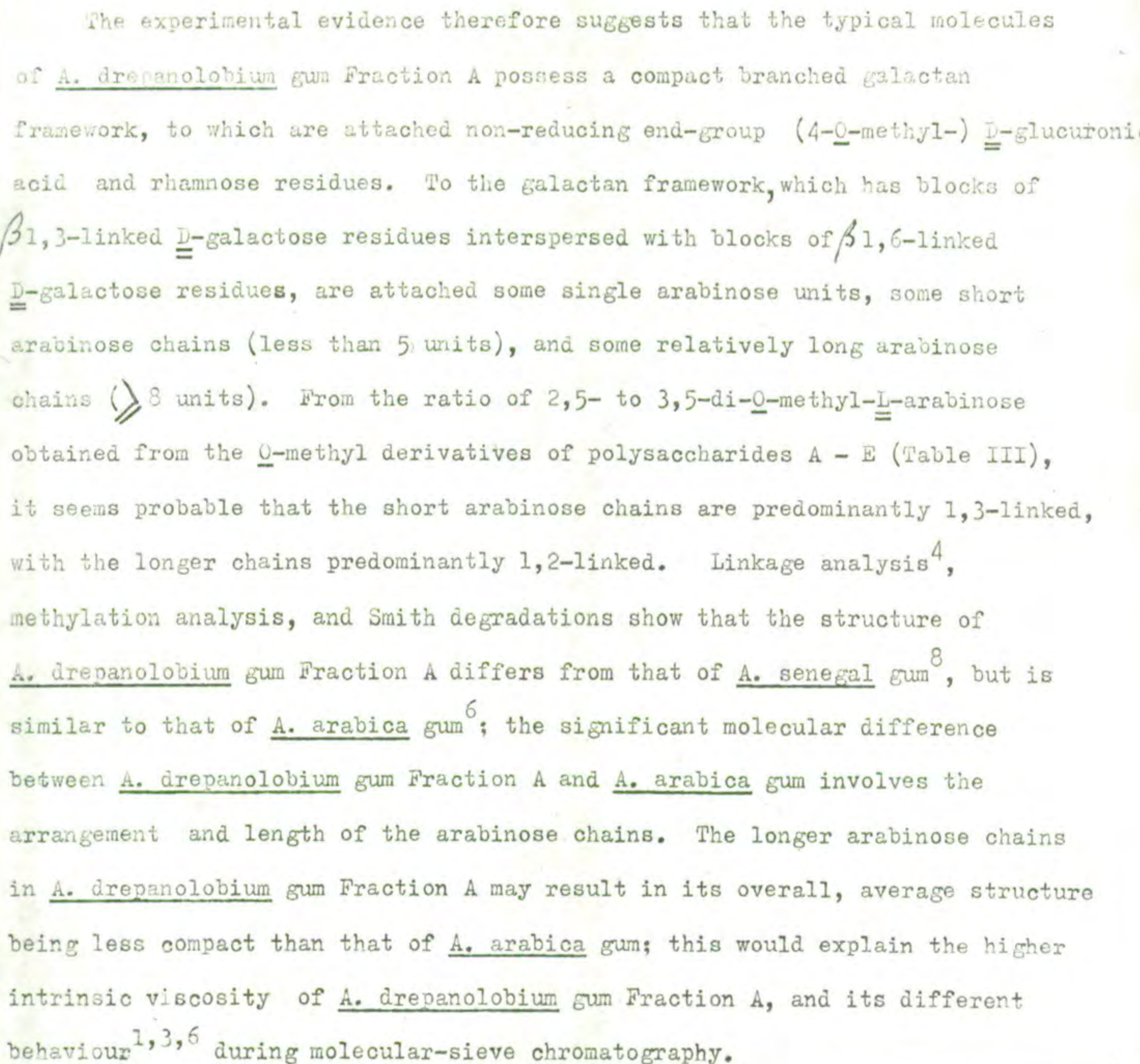
On methanolysis, the O-methyl derivatives of the Smith-degradation polysaccharides A - E gave the methyl glycosides of 2,3,5-tri-, 2,5- and 3,5-di-O-methyl-L-arabinose, and 2,3,4,6-tetra-, 2,4,6- and 2,3,4-tri-, and 2,4-di-O-methyl-D-galactose in the proportions shown in Table III. The absence of arabinopyranose residues in polysaccharide A suggests that the 4-O-methyl-L-arabinose obtained from methylated Fraction A is not structurally significant. 3,5-Di-O-methyl-L-arabinose is by far the most abundant O-methyl sugar in

polysaccharide E; this permits a calculation showing that gum Fraction A contains chains of at least eight arabinose residues, with the possibility that much longer arabinose chains may be present.

From the evidence obtained, some of the structural features of A. drepanolobium gum Fraction A can be deduced. Smith degradation and methylation analysis of the gum Fraction A and the autohydrolysed gum[4] indicate that long chains of β1,3-linked D-galactose residues are not such a dominating feature of A. drepanolobium gum Fraction A as they are of A. senegal gum[8] and A. laeta gum[7]. Smith degradation and methylation analysis of the autohydrolysed gum suggest that the branched galactan frameworks of A. drepanolobium gum are closely similar to A. arabica gum, being more compact and highly branched than those of A. senegal gum[4]. In these compact frameworks, blocks of periodate-resistant β1,3-linked D-galactose units must be interspersed with blocks of periodate-vulnerable β1,6-linked D-galactose residues. The first Smith degradation of A. drepanolobium gum Fraction A appears to proceed similarly to that of A. senegal gum[8], although arabinose residues must be removed from the C-3 positions of 6-O-substituted D-galactose units, since the second degradation causes a large drop in $\overline{M}n$. In the third, fourth, and fifth Smith degradations the $\overline{M}n$ of the degradation products falls only slightly, although the yields are in the 20-30% range. This implies that, at each of these stages, the majority of the polymer molecules are periodate-vulnerable to such an extent that drastic degradation, resulting in the formation of molecular fragments so small that they can escape during the dialysis process, takes place. In contrast, the minority (ca. 30%) of the molecular arrays at each stage are substantially stable, in their inner core, to periodate attack so that they suffer comparatively little degradation, although a majority of

these in turn become periodate-vulnerable at the ensuing stage. This indicates that although some of the arabinose chains in the whole gum are comparatively long, there are also some short chains of two, three or four arabinose units.

A scheme such as the following is necessary to explain the unusual increase in the relative arabinose content of the Smith-degraded polysaccharides B - E. If the first degradation removes single arabinose units and/or arabinose chains attached by 2-O-substituted L-arabinopyranose units to the branched galactan framework, some periodate-vulnerable 6-O-substituted D-galactose residues will be formed in the core. The second degradation fragments the molecule drastically; those parts of the galactan framework which resist this periodate oxidation bear a high proportion of the arabinose chains or single arabinose units. These will be progressively removed from the C-3 positions of 6-O-substituted D-galactose units in the subsequent degradations, leaving molecules that will be rapidly fragmented. On successive Smith degradations those structures having comparatively long arabinose chains attached to galactan frameworks consisting mainly of β1,3-linked D-galactose residues will tend to survive. Figure 1 shows one possible molecular array (based on the sugar ratios shown in Table I) that represents generally the broad spectrum of structures possible for the periodate-resistant portions of polysaccharide D which, in turn, give the Smith-degraded product, polysaccharide E, in small yield. This model illustrates the need to assign some unusually long arabinose chains to A. drepanolobium gum Fraction A; if, in Figure 1, polysaccharide D contained eight short chains of arabinose residues instead of four longer chains averaging at least eight units (some may be considerably longer) the arabinose content of polysaccharide E would be less than that of polysaccharide D.



The experimental evidence therefore suggests that the typical molecules of *A. drepanolobium* gum Fraction A possess a compact branched galactan framework, to which are attached non-reducing end-group (4-*O*-methyl-) *D*-glucuronic acid and rhamnose residues. To the galactan framework, which has blocks of β1,3-linked *D*-galactose residues interspersed with blocks of β1,6-linked *D*-galactose residues, are attached some single arabinose units, some short arabinose chains (less than 5 units), and some relatively long arabinose chains (≫ 8 units). From the ratio of 2,5- to 3,5-di-*O*-methyl-*L*-arabinose obtained from the *O*-methyl derivatives of polysaccharides A - E (Table III), it seems probable that the short arabinose chains are predominantly 1,3-linked, with the longer chains predominantly 1,2-linked. Linkage analysis[4], methylation analysis, and Smith degradations show that the structure of *A. drepanolobium* gum Fraction A differs from that of *A. senegal* gum[8], but is similar to that of *A. arabica* gum[6]; the significant molecular difference between *A. drepanolobium* gum Fraction A and *A. arabica* gum involves the arrangement and length of the arabinose chains. The longer arabinose chains in *A. drepanolobium* gum Fraction A may result in its overall, average structure being less compact than that of *A. arabica* gum; this would explain the higher intrinsic viscosity of *A. drepanolobium* gum Fraction A, and its different behaviour[1,3,6] during molecular-sieve chromatography.

EXPERIMENTAL

The analytical methods have been described elsewhere in more detail[8,16]. Paper chromatography was carried out on Whatman Nos. 1 and 3MM papers using the following solvent systems (v/v); (a) benzene - butyl alcohol - pyridine - water (1:5:3:3, upper layer); (b) ethyl acetate - acetic acid - formic acid - water (18:3:1:4); (c) ethyl acetate - acetic acid - formic acid - water (18:8:3:9); (d) butyl alcohol - ethanol - water (4:1:5, upper layer); (e) butyl alcohol - acetic acid - water (4:1:5, upper layer); (f) ethyl acetate - pyridine - water (10:4:3); (g) butan-2-one - water - ammonia (d. 0.880)(200:17:1). R_{Gal} values of sugars refer to distances moved relative to that of galactose. R_G values of O-methyl sugars refer to distances moved relative to that of 2,3,4,6-tetra-O-methyl-D-glucose. Gas-liquid partition chromatography (Chromatograph Type S3A, fitted with flame ionisation detectors, supplied by Gas chromatography Ltd.) of mixtures of O-methyl sugars was carried out at nitrogen flow rates of ca. 100 ml./min. on columns of (i) 15% by weight of poly(butane-1,4-diol succinate) on Celite (5 ft. x 1/4 in.) at 175°; and (ii) 15% by weight of poly(ethylene glycol adipate) on Celite (3 ft. x 1/4 in.) at 160°. Retention times (T) are quoted relative to methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside as standard. Polysaccharides were methylated successively with dimethyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide. Methanolyses were carried out under reflux for 7 h with methanolic 5% hydrogen chloride.

Purification of A. drepanolobium gum Fraction A.- The preparation of the electrodialysed, freeze-dried sample of A. drepanolobium gum Fraction A used in the methylation study has been described previously[3]. A portion of A. drepanolobium gum was extracted with cold water, as described previously[3], to

yield Fraction A (148 g from 180 g). The samples of Fraction A had $[\alpha]_D$ +78° (c, 1.0)(Found: L-arabinose, 52%; D-galactose, 38%; L-rhamnose, 1%; D-glucuronic acid, 7%; 4-O-methyl-D-glucuronic acid, 2%; all sugar percentages are for the anhydro forms and are corrected for moisture and protein contents).

Fraction A migrated as a single band in electrophoresis on strips (18 x 5 cm) of cellulose acetate film in both 0.1M ammonium carbonate buffer (pH, 8.9) and 0.1M acetate buffer (pH, 4.7) at field strengths between 15 and 20 volts/cm for 2 - 4 h. Polysaccharide bands were stained by a modification of the periodate-rosaniline hydrochloride method[17]. The gum was also chromatographed[5] on a D.E.A.E.-cellulose column (45 x 1.3 cm). Gradient elution with sodium chloride solution (0.0 - 0.5M) in 0.02M acetate buffer (pH, 4.1) yielded a single, slightly asymmetric peak for its elution pattern.

Methylation of A. drepanolobium gum Fraction A.- The purified gum (10 g polysaccharide) was methylated by the Haworth and Purdie proceedures to give a product (5.9 g), $[\alpha]_D$ +60° (c, 1.00 in $CHCl_3$)(Found: OMe, 39.5%, not raised on further attempted methylation). Methanolysis of a portion of this product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table II.

The methylated gum (5 g) was refluxed with methanolic 5% hydrogen chloride (500 ml) for 7 h. The methanolysate was cooled, neutralised with silver carbonate, filtered, and concentrated to a syrup. The methyl glycosides were removed by hydrolysis (N sulphuric acid for 4 h at 100°). The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H^+) and concentrated to a syrup. This syrup was applied to a column (33 x 3 cm) of Duolite A4 in the formate form. Elution of the column with water (3 l) gave the neutral O-methyl sugar fraction; elution with aqueous 5%

formic acid (500 ml) gave the acidic O-methyl sugar fraction.

The acidic O-methyl sugar fraction (165 mg) was refluxed with methanolic 5% hydrogen chloride for 7 h. The derived methyl ester methyl glycosides were reduced by refluxing in dry tetrahydrofuran (40 ml) with lithium aluminium hydride (300 mg) for 2 h. After cooling, the excess hydride was destroyed by addition of ethyl acetate and water. The mixture was evaporated to dryness, extracted exhaustively with chloroform, and concentrated to a syrup. The methyl glycosides were removed by hydrolysis (N sulphuric acid for 4 h at 100°). The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H^+) and concentrated to a syrup (85 mg). Fractionation of this product on Whatman 3MM papers in solvent (d) yielded pure 2,3,4-tri-O-methyl-D-glucose (35 mg). The derived N-phenyl-2,3,4-tri-O-methyl-D-glucosylamine, after recrystallisation from ethyl acetate, had m.p. 146° - 148° (Lit. m.p.[18] 145° - 150°).

A portion of the neutral O-methyl sugar fraction (3.5 g) was chromatographed on a cellulose column (100 x 3.5 cm) with light petroleum (b.p. 100°- 120°) - butyl alcohol (7:3, saturated with water) as eluant to give six fractions, and with light petroleum (b.p. 100°- 120°) - butyl alcohol (1:1, saturated with water) as eluant to give a further five fractions.

Fraction 1 (35 mg) from tubes 1 - 80. Paper chromatography in solvent (d) showed the presence of 2,3,4-tri-O-methyl-L-rhamnose (R_G 1.03) and 2,3,5-tri-O-methyl-L-arabinose (R_G 0.98). Demethylation gave arabinose and rhamnose. A portion (10 mg) was refluxed with methanolic 5% hydrogen chloride for 7 h. G.l.c. examination of the mixture of methyl glycosides indicated the presence of 2,3,4-tri-O-methyl-L-rhamnose [(i),T 0.48; (ii),T (0.50)] and 2,3,5-tri-O-methyl-L-arabinose [(i),T 0.56, 0.73; (ii),T (0.50), 0.64].

Fraction 2 (355 mg) from tubes 81 - 190 had $[\alpha]_D$ -19° (c, 1.00). It had the same paper chromatographic mobility [R_G 0.98 in solvent (d) and 1.00 in solvent (g)] as 2,3,5-tri-O-methyl-L-arabinose. It was characterized by conversion into 2,3,5-tri-O-methyl-L-arabonamide which, after recrystallisation from acetone, had m.p. 133° - 135° [Lit. m.p.[18] 134° - 138°].

Fraction 3 (93 mg) from tubes 191 - 280 had $[\alpha]_D$ +27° (c, 0.93). Demethylation gave galactose and arabinose. A portion was refluxed with methanolic 5% hydrogen chloride for 7 h. G.l.c. examination of the mixture of methyl glycosides indicated the presence of 2,3,4,6-tetra-O-methyl-D-galactose [(i),T 1.68; (ii),T 1.66] and 2,3,5-tri- [(i),T 0.56, 0.73; (ii),T 0.50, 0.64], and 2,5- [(i),T 1.75, 3.29; (ii),T 1.24, 2.20] and 3,5-di-O-methyl-L-arabinose [(i),T 1.04, 2.40; (ii),T 0.85, 1.76].

Fraction 4 (770 mg) from tubes 281 - 420 had $[\alpha]_D$ +26° (c, 1.00). A portion (10 mg) was refluxed with methanolic 5% hydrogen chloride for 7 h. G.l.c. examination of the mixture of methyl glycosides indicated that 2,5- [(i),T 1.76, 3.29; (ii),T 1.24, 2.20] and 3,5-di-O-methyl-L-arabinose [(i),T 1.04, 2.40; (ii),T 0.85, 1.76] were the main components of the mixture. A portion (600 mg) was fractionated on Whatman 3MM papers in solvent (d) to yield a mixture (390 mg) of 2,5- and 3,5-di-O-methyl-L-arabinose. Paper electrophoretic examination in 0.05M borate buffer confirmed the presence of 2,5- [M_G 0.00, 10 volts/cm, 0.5 ma/cm] and 3,5-di-O-methyl-L-arabinose [M_G 0.68, 10 volts/cm, 0.5 ma/cm, brown spot (yellow under u.v.) with aniline oxalate[19]]. These components were separated from the syrupy mixture (200 mg) by paper electrophoresis in borate buffer[20] on a preparative scale. Zones were detected by spraying side strips. Carbohydrate material and borate buffer were eluted from each zone; sodium ions were removed with Amberlite resin IR-120 (H^+) and borate was removed as methyl borate by

repeated evaporation with methanol. 2,5-Di-O-methyl-L-arabinose (47 mg) was characterized by conversion into 2,5-di-O-methyl-L-arabonamide which, after recrystallisation from ethyl acetate, had m.p. 130° - 131° [Lit. m.p.[18] 122° - 132°]. 3,5-Di-O-methyl-L-arabinose (39 mg) was characterized by conversion into 3,5-di-O-methyl-L-arabonamide which, after recrystallisation from ethyl acetate, had m.p. 139° - 141° [Lit. m.p.[18] 144° - 145°].

Fraction 5 (181 mg) from tubes 421 - 650 had $[\alpha]_D$ +100° (c, 1.81). Demethylation gave arabinose. Paper chromatographic examination indicated the presence of 2,3,4-tri-O-methyl-L-arabinose [R_G 0.78 in solvent (d) and 0.54 in solvent (g)] in addition to 2,5- and 3,5-di-O-methyl-L-arabinose.

Fraction 6 (123 mg) from tubes 651 - 1200 had $[\alpha]_D$ +98° (c, 1.23). Demethylation gave galactose and a trace of arabinose. A portion (20 mg) was refluxed with methanolic 5% hydrogen chloride. G.l.c. examination of the mixture of methyl glycosides indicated that 2,4,6- [(i),T (3.76), (4.20); (ii),T (3.05), (3.50)], 2,3,6- [(i),T 3.04, (3.76), (4.20); (ii),T 2.50, (3.05), (3.50)] and 2,3,4-tri-O-methyl-D-galactose [(i),T 6.45; (ii),T 5.25] were the main components. The identification of these three trimethylgalactoses was confirmed by paper chromatography involving double development in solvent (g).

Fraction 7 (56 mg) from tubes 1201 - 1520 had $[\alpha]_D$ +97° (c, 1.12). A portion (6 mg) was refluxed with methanolic 5% hydrogen chloride. G.l.c. examination of the mixture of methyl glycosides showed the presence of 3,4-di-O-methyl-L-arabinose [(i),T 1.90; (ii),T 1.38]. The remainder of the fraction was chromatographed on Whatman 3MM papers in solvent (d) to give a product (10 mg). Demethylation gave arabinose. It had the same paper chromatographic mobilities [R_G 0.56 in solvent (d) and 0.20 in solvent (g)] as an authentic specimen of 3,4-di-O-methyl-L-arabinose. A portion (4 mg) was converted into the methyl glycosides and methylated with methyl iodide (0.5 ml) and silver oxide (0.5 g) in

N,N-dimethylformamide (0.5 ml). G.l.c. examination of the reaction mixture showed the presence of the methyl glycoside(s) of 2,3,4-tri-O-methyl-L-arabinose [(i),T 1.00; (ii),T 0.81].

Fraction 8 (170 mg) from tubes 1521 - 1640 had $[\alpha]_D$ +89° (c, 0.85). Paper chromatographic examination in solvent (d) showed that 2,4-di-O-methyl-D-galactose was the main component of the mixture. After purification by thick paper chromatography, it was characterized by conversion into N-phenyl-2,4-di-O-methyl-D-galactosylamine which, after recrystallisation from acetone, had m.p. 214° - 216° [Lit. m.p.[18] 214° - 228°].

Fraction 9 (45 mg) from tubes 1641 - 1770 had $[\alpha]_D$ +108° (c, 0.45). Fractionation on Whatman 3MM papers in solvent (d) gave a product (15 mg). Demethylation gave arabinose. The O-methyl sugar had the same chromatographic mobility [R_G 0.35 in solvent (d) and 0.09 in solvent (g)] as an authentic specime of 4-O-methyl-L-arabinose. A portion (5 mg) was converted to the methyl glycosides and methylated with methyl iodide (0.5 ml) and silver oxide (0.5 g) in N,N-dimethylformamide (0.5 ml). G.l.c. examination of the reaction mixture indicated the presence of the methyl glycoside(s) of 2,3,4-tri-O-methyl-L-arabinose [(i),T 1.00; (ii),T 0.82].

Fraction 10 (46 mg) from tubes 1771 - 1820 had $[\alpha]_D$ +88° (c, 0.46). Paper chromatographic examination in solvents (d) and (g) indicated that 2-O-methyl-D-galactose was the main component of the mixture.

Fraction 11 (24 mg) was obtained from tubes 1821 - 2000. Paper chromatography in solvents (a), (b) and (f) showed the presence of galactose (major component) and arabinose (minor component).

Preparation, partial acid hydrolysis and methylation of polysaccharide A.- The gum (123 g polysaccharide) was dissolved in water (3.1 l), mixed with 0.25M

sodium metaperiodate solution (3.1 l) and left for 48 h. The amount of formic acid released in this time was 1.05 mmoles/g. The corresponding amount of periodate consumed was 3.96 mmoles/g. The reaction was stopped by addition of ethylene glycol (50 ml), and the solution dialysed against running tap water for 48 h. Sodium borohydride (32 g) was added, and the mixture kept at room temperature for 30 h, then dialysed for a further 48 h. The polyalcohol was hydrolysed in N sulphuric acid at room temperature for 48 h. After dialysis against running tap water for 48 h, polysaccharide A was isolated as the freeze dried product (68.2 g polysaccharide; yield 55.5%), $[\alpha]_D$ +45° (c, 1.63)(Found: galactose, 43%; arabinose, 57%). Molecular-sieve chromatography of polysaccharide A (20 mg) on a previously calibrated Bio-Gel P300 column[16,21,22] (45 x 4.8 cm) gave $\overline{M}_n$ ca. 95,500. (See Table I).

Polysaccharide A (20 mg) was hydrolysed with 0.5N sulphuric acid (10 ml) for 1 h on a boiling water bath. Paper chromatographic examination of the hydrolysate indicated the presence of galactose, arabinose, two neutral disaccharides with the mobilities of 3-O-β-D-galactopyranosyl-D-galactose [R_{Gal} 0.49 in solvent (a) and 0.54 in solvent (f), minor component] and 6-O-β-D-galactopyranosyl-D-galactose [R_{Gal} 0.31 in solvent (a) and 0.38 in solvent (f), major component], and higher neutral oligosaccharides.

Polysaccharide A (200 mg) was methylated to give a product (110 mg), $[\alpha]_D$ +39° (c, 0.50 in $CHCl_3$)(Found: OMe, 40.5%, not raised on further attempted methylation)(See Table IV). Methanolysis of a sample of this product followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table III. Hydrolysis of the methyl glycosides followed by paper chromatography in solvents (d) and (g) indicated the presence of 2,6-di- and 2-O-methyl-D-galactose in addition to those O-methyl sugars already characterized by g.l.c.

of their methyl glycosides.

Preparation, partial acid hydrolysis and methylation of polysaccharides B-E. The following weights of polysaccharide were periodate oxidised, borohydride reduced and hydrolysed, and the corresponding weights of degraded polysaccharides recovered as for the first Smith degradation. Polysaccharide A (67.4 g) gave polysaccharide B (28.4 g). This (27.7 g) gave polysaccharide C (7.4 g). Smith degradation of polysaccharide C (6.9 g) gave polysaccharide D (1.40 g). This (1.07 g) gave polysaccharide E (0.32 g). The amount of periodate consumed and formic acid released on periodate oxidation, and the percentage yields, specific rotations and constituent sugars of each degraded polysaccharide are given in Table I. Molecular-sieve chromatography of polysaccharides B - E indicated values for $\overline{M}_n$ of ca. 9,200, 8,000, 7,200 and 6,000 respectively.

Partial acid hydrolysis of polysaccharides B, C and D, followed by paper chromatographic examination of the hydrolysates, indicated the presence of 3-O-β-D-galactopyranosyl-D-galactose (minor component) and 6-O-β-D-galactopyranosyl-D-galactose (major component), and higher neutral oligosaccharides. The proportion of the β1,3-linked galactobiose increased steadily from polysaccharide B to D. Partial acid hydrolysis of polysaccharide E, followed by paper chromatographic examination of the hydrolysate, indicated the presence of equal amounts of 3-O-β-D-galactopyranosyl-D-galactose and 6-O-β-D-galactopyranosyl-D-galactose, and higher neutral oligosaccharides.

Polysaccharides B - E were methylated (Table IV). Methanolysis of a sample of the products, followed by g.l.c. examination of the mixtures of methyl glycosides, gave the results shown in Table III.

In the fourth and fifth Smith degradations the controlled acid hydrolysates were neutralised with barium carbonate, filtered, treated with Amberlite resin

IR-120 (H+), and dialysed against distilled water (2 l) for 24 h. After further dialysis against running tap water the respective degraded products were isolated. The dialysates from the fourth and fifth degradations were deionised and concentrated to syrups (1.74 g, and 0.35 g respectively). Paper chromatographic examination of the syrups indicated the presence of glycerol, glycolaldehyde, arabinose (a trace), and slower non-reducing glycosides. Hydrolysis of the syrups followed by paper chromatographic examination indicated the presence of glycerol, glycolaldehyde, arabinose, and galactose (a trace).

ACKNOWLEDGMENTS.

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```
           A
           A
           A                                 Gal1
           A                                    β
           A                                    6
           A      A                      A-Gal1
           A      A                             β
           A      A                             6
          Gal1β6Gal1β3Gal1β3Gal1β3 1Gal
                                   β
                                   6
                                   1Gal-A-A-A-A-A-A-A-A-A
                                   β
                                   3
                                   1Gal
                                   β
                                   6
Gal1β6Gal1β3Gal1β6Gal1β6Gal1β3Gal1β3Gal1β3Gal1β3Gal1β3Gal
 A     A                      A     A                  A
 A                            A     A                  A
                              A                        A
                              A                        A
                              A                        A
                              A                        A
                              A                        A
                              A
          A
          A
          A
          A
          A                                  Gal1
          A       A                             β
          A       A                             6
          Gal1β6Gal1β3Gal1β3Gal1β3 1Gal
                                   β
                                   6
                                   1Gal-A-A-A-A-A-A-A-A
                                   β
                                   3
                                   1Gal
                                   β
                                   6
                Gal1β3Gal1β3Gal1β3Gal1β3Gal
                 A     A                 A
                 A                       A
                 A                       A
                 A                       A
                 A                       A
                 A                       A
                 A
```

Gal represents D-galactose; A represents L-arabinose

Figure 1. Possible molecular structure of the periodate-resistant portions of polysaccharides D (above) and E.

Table I

ata for *A. drepanolobium* gum Fraction A and Smith-degraded polysaccharides A-E.

saccharide	$[\alpha]_D$	Constituent Sugars %					Yield %	mmoles/g IO_4^- reduced on periodate oxidation	mmoles/g Formic Acid released on periodate oxidation
		Gal	Ara	Rha	Gluc A	4-O-Me Gluc A			
raction A	+78°	38	52	1	7	2	-	3.96	1.05
A	+45°	43	57	-	-	-	55.5	3.38	0.94
B	+20°	46	54	-	-	-	42	4.18	1.47
C	-2°	33	67	-	-	-	26.7	3.78	1.15
D	-21°	32	68	-	-	-	20.3	1.87	0.53
E	-36°	29	71	-	-	-	29.5	1.05	0.35

Table II

Examination of methanolysis and hydrolysis products from methylated Fraction A

Relative retention times, T, of methyl glycosides on columns:- * (i)	(ii)	R_G in solvent (d)	O-methyl sugars
0.48	(0.50)		2,3,4-tri-O-methyl-L-rhamnose
0.55;0.73	(0.50);0.64	0.97	2,3,5-tri-O-methyl-L-arabinose
(1.05)	(0.82)	0.79	2,3,4-tri-O-methyl-L-arabinose
1.80;3.20	1.26;(2.20)	0.82	2,5-di-O-methyl-L-arabinose
(1.05);(2.3)	(0.82);1.76	0.82	3,5-di-O-methyl-L-arabinose
1.98	1.43	0.56	3,4-di-O-methyl-L-arabinose
1.68	1.65	0.88	2,3,4,6-tetra-O-methyl-D-galactose
(3.05);(3.89);(4.22)	2.44;(3.00);(3.44)	0.73	2,3,6-tri-O-methyl-D-galactose
(3.89);(4.22)	(3.00);(3.44)	0.73	2,4,6-tri-O-methyl-D-galactose
6.42	5.08	0.73	2,3,4-tri-O-methyl-D-galactose
14.6;16.4	9.9;11.4	0.52	2,4-di-O-methyl-D-galactose
(2.36);(3.05)	(2.20);2.75		2,3,4-tri-O-methyl-D-glucuronic acid †
		0.32	2-O-methyl-D-galactose
		0.35	4-O-methyl-L-arabinose

* Figures in parenthesis indicate T values of components which are not completely resolved.

† As methyl ester methyl glycoside.

Table III

Relative proportion of O-methyl sugars present in methylated polysaccharides A-E

O-methyl sugar	A	B	C	D	E
2,3,5-tri-O-methyl-L-arabinose	4	2	2	2	4
2,5-di-O-methyl-L-arabinose	4	2	2	1	1
3,5-di-O-methyl-L-arabinose	7	6	8	9	10
2,3,4,6-tetra-O-methyl-D-galactose	trace	trace	1	1	1
2,4,6-tri-O-methyl-D-galactose	3	4	2	2	2
2,3,4-tri-O-methyl-D-galactose	1	2	1	1	1/2
2,4-di-O-methyl-D-galactose	3	3	1	1	1

Table IV

Methylation of Smith-degraded polysaccharides.

	Polysaccharide				
	A	B	C	D	E
Amount of polysaccharide used (mg)	200	179	136	124	186
Amount of product obtained (mg)	110	85	60	53	65
$[\alpha]_D$ of product	+39°	+24°	+2°	-17°	-29°
OMe of product, %	40.5	41.2	42.1	41.5	41.0

56.

STUDIES ON URONIC ACID MATERIALS

PART XXXII*. SOME STRUCTURAL FEATURES OF THE GUM EXUDATE FROM ACACIA SEYAL DEL.

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(Received May , 1968)

ABSTRACT

The gum polysaccharide exuded by Acacia seyal trees contains D-galactose (38%), L-arabinose (45%), L-rhamnose (4%), D-glucuronic acid (7%) and 4-O-methyl-D-glucuronic acid (6%). Linkage analysis yields 3-O-β-L-arabinopyranosyl-L-arabinose, 3-O-β-L-arabinofuranosyl-L-arabinose, 3-O-β-D-galactopyranosyl-D-galactose and 6-O-β-D-galactopyranosyl-D-galactose. The O-methyl derivatives of A. seyal gum and degraded gum A (obtained by partial hydrolysis with 0.01 N acid) are analysed, after methanolysis, by gas-liquid partition chromatography. Degraded gum A is subjected to a Smith degradation, and A. seyal gum is subjected to four successive Smith degradations; the degradation products are examined by methylation and linkage analysis. Degraded gum A is also subjected to a controlled Smith degradation to establish the nature of the reducing end-group. The structural evidence obtained indicates a strong similarity between A. seyal gum and A. arabica gum[2].

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* For Part XXXI, see Ref. 1.

INTRODUCTION

It has already been shown[3] that A. seyal gum is not unusual in having a positive specific rotation and a low content of L-rhamnose that does not bear a unit molar correspondence to the uronic acid content. D-Glucuronic acid and its 4-O-methyl ether have been isolated from the gum and characterized[4]; acid hydrolysis, followed by preparative paper chromatography, led to the isolation and characterization[4] of four aldobiouronic acids, viz., 6-O-(β-D-glucopyranosyluronic acid)-D-galactose, 6-O-(4-O-methyl-β-D-glucopyranosyluronic acid)-D-galactose, 4-O-(α-D-glucopyranosyluronic acid)-D-galactose and 4-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-galactose. An inter-nodule analytical study of A. seyal gum has shown[5] that D-galactose, L-arabinose, and L-rhamnose are constituent sugars of the gum; for different nodules, the proportions of these sugars were found[5] to vary between 34 - 42%, 42 - 47%, and 1 - 8% respectively. The results of a detailed structural study of A. seyal gum are reported and discussed in this Paper.

RESULTS AND DISCUSSION

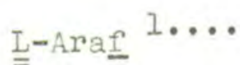
A single nodule of gum from A. seyal Del. was used; it was collected, by the Gum Research Officer to The Republic of Sudan, from a botanically authenticated tree at Umm Ruaba Forest Reserve, Eastern Kordofan, on 9th March 1960. After purification, the gum polysaccharide was found to have the specific rotation and sugar composition given in Table I. The

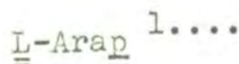
gum was examined by zone electrophoresis, and by ion-exchange chromatography on D.E.A.E.-cellulose[6]. Since no sharp discontinuities in the properties of the molecular species were indicated by these experiments, it seems probable that A. seyal gum exhibits the same kind of heterogeneity which has been suggested for the gums of A. senegal[7,8], A. arabica,[2] A. laeta[9] and A. drepanolobium[1,10]. If this is the case, then A. seyal gum may be considered to contain a continuous spectrum of related molecular species.

Mild, acid hydrolysis of the gum (0.01N sulphuric acid for 100 h at 100°) effected the release of all the rhamnose, most of the arabinose, and some galactose. Degraded gum A, isolated after dialysis, contained D-galactose (82%), L-arabinose (3%), and uronic acid (15%); molecular-sieve chromatography gave an estimated value of 4,900 for its number-average molecular weight, $\bar{M}n$. Light-scattering measurements gave a value of ca. 850,000 for the weight-average molecular weight, $\bar{M}w$, of A. seyal gum. The extensive degradation on mild acid hydrolysis is therefore much greater than can result from the simple removal of arabinose and rhamnose from the periphery of the molecule, nor can it have been caused by the presence of internal acid-labile arabinofuranose residues, since acid hydrolysis of borohydride -reduced degraded gum A yielded galactitol but not arabinitol. There is no evidence for any galactofuranose residues inthe polysaccharide, although traces of di- and tri-O-methyl-D-

galactofuranoses would be difficult to detect in the mixtures of methyl glycosides examined by g.l.c. Bouveng and Lindberg have observed that certain galacto-pyranosidic bonds in highly branched arabinogalactans are unusually sensitive to mild acid hydrolysis[11-15]. It therefore seems probable that certain galactopyranosidic bonds in *A. seyal* gum are unusually sensitive to mild acid hydrolysis, as has already been reported for *A. arabica*[2], *A. senegal*[7,8], *A. laeta*[9], and *A. drepanolobium*[1,10] gums.

An examination of methylated degraded gum A showed the presence of 2,3,4,6-tetra- (++), 2,4,6- (++), 2,3,6- (+) and 2,3,4-tri- (++++), 2,4- (+++) and 2,6-di- (+) and 2-O-methyl-D-galactose (+), 2,3,4-tri-O-methyl-D-glucuronic acid (++), and traces of 2,3,5- and 2,3,4-tri-O-methyl-L-arabinose (see Table II). ~~The presence of 2-O-methyl-D-galactose is probably due to undermethylation.~~ This shows that I and II are structural fragments of degraded gum A. Methylation analysis has shown the presence of traces of

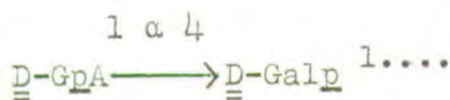


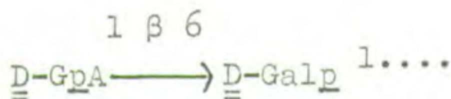


I II

terminal non-reducing L-arabinofuranose residues in the autohydrolysed gum from *A. senegal*[7,8], and terminal non-reducing L-arabinopyranose residues in *A. arabica* degraded gum A[2]. Traces of terminal non-reducing L-arabinofuranose and

L-arabinopyranose residues have been detected in the degraded gum A of A. laeta[9] and A. drepanolobium gum Fraction A[10]. Lithium aluminium hydride reduction of methylated degraded gum A, followed by a Kuhn methylation[16], yielded 2,3,4,6- tetra-O-methyl-D-glucose after methanolysis (see Table III). This confirms that D-glucuronic acid, and its 4-O-methyl ether, are non-reducing end-groups in degraded gum A. After partial methanolysis of methylated degraded gum A and hydrolysis of the methyl glycosides, the methylated aldobiouronic acids were separated from the neutral O-methyl sugars by ion-exchange chromatography. Methanolysis of the methylated aldobiouronic acids, followed by g.l.c. examination of the mixture of methyl glycosides, showed the presence of 2,3,4-tri-O-methyl-D-glucuronic acid, and 2,3,6- and 2,3,4-tri-, and 2,4- and 2,6-di-O-methyl-D-galactose. This indicates that III - VI are structural fragments of degraded gum A. Similar experiments on the methylated degraded gum A from A. arabica[2] and A. drepanolobium Fraction A[10] have shown these gums to contain fragments III, IV and VI, and III - VI respectively.





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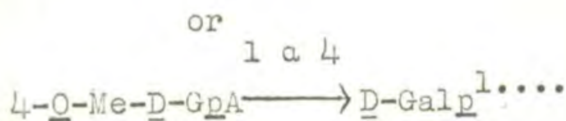

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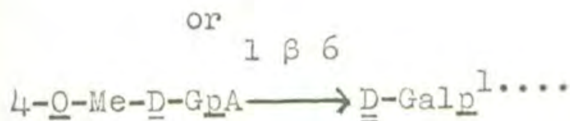


III

IV

D-GpA $\xrightarrow{1\ \alpha\ 4}$ D-Galp 1....
.3
:

or

4-O-Me-D-GpA $\xrightarrow{1\ \alpha\ 4}$ D-Galp 1....
.3
:

V

D-GpA $\xrightarrow{1\ \beta\ 6}$ D-Galp 1....
.3
:

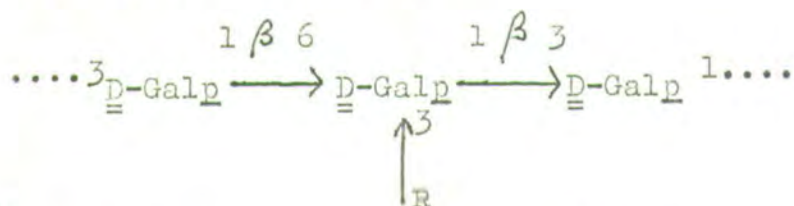
or

4-O-Me-D-GpA $\xrightarrow{1\ \beta\ 6}$ D-Galp 1....
.3
:

VI

Smith degradation of degraded gum A yielded degraded gum B, and partial acid hydrolysis gave 3-O-β-D-galactopyranosyl-D-galactose and 6-O-β-D-galactopyranosyl-D-galactose. An examination of methylated degraded gum B indicated the presence of 2,3,4,6-tetra (++), 2,4,6- (++++) and 2,3,4-tri- (+), 2,6- (+/2) and 2,4-di- (+), and 2-O-methyl-D-galactose (+/2), and a trace of 2,3,4-tri-O-methyl-D-glucuronic acid (see Table IV). The presence of a trace of glucuronic acid in degraded gum B could arise from incomplete oxidation of degraded gum A, and the 2,6-di- and 2-O-methyl-D-galactose probably result from undermethylation. The proportion of the O-methyl-D-galactoses obtained from methylated degraded gum B indicates that degraded gum B is a branched galactan composed predominantly of β1,3 glycosidic linkages with a few β1,6 branch points, and the identification of some 2,3,4-tri-O-methyl-D-galactose indicates

that VII is a possible structural unit of degraded gum A. A similar structural unit occurs in



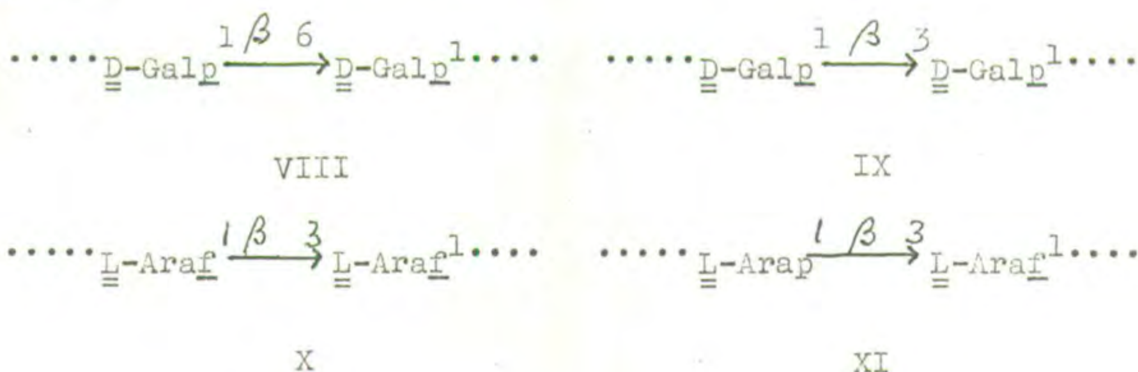
VII

the degraded gum A of A. arabica[2] and A. drepanolobium Fraction A[10].

A controlled Smith degradation[7] of degraded gum A gave degraded gum C. Periodate oxidation of degraded gum A did not yield formaldehyde, and the reducing galactose residue is therefore substituted at C-6. Degraded gum C contains arabinitol; at least some of the reducing end-group galactose residues of degraded gum A are therefore substituted at C-3[17].

Mild acid hydrolysis of degraded gum A gave two galactobioses, which were characterized as 3-O-β-D-galactopyranosyl-D-galactose and 6-O-β-D-galactopyranosyl-D-galactose. Two arabinobioses, obtained from the diffusate of the mild acid hydrolysis of A. seyal gum, were characterized as 3-O-β-L-arabinofuranosyl-L-arabinose and 3-O-β-L-arabinopyranosyl-L-arabinose; since

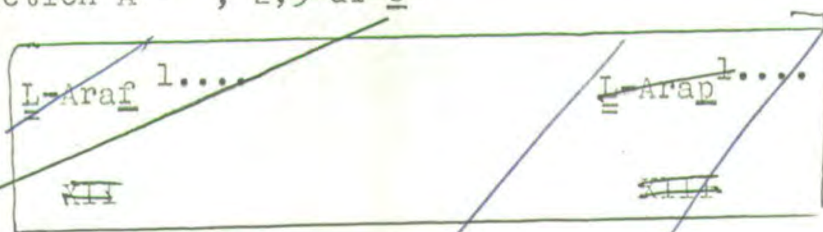
these arabinobioses are released under very mild acidic conditions, the reducing arabinose residues were probably present initially in the furanose form. Thus VIII, IX, X and XI are structural fragments



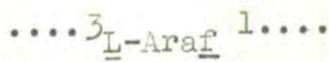
of A. seyal gum.

An examination of the O-methyl derivative of A. seyal gum indicated the presence of 2,3,4-tri-O-methyl-L-rhamnose (±½), 2,3,5- (++) and 2,3,4-tri- (+) and 2,5- (+++++), 3,5- (++) and 3,4-di-O-methyl-L-arabinose (+), 2,4-di-O-methyl-D-galactose (+++++), 2,3,4-tri-O-methyl-D-glucuronic acid (+++), and small amounts of 4-O-methyl-L-arabinose, 2,3,4,6-tetra-, 2,3,6-, 2,4,6- and 2,3,4-tri- and 2-O-methyl-D-galactose, and 2,3-di-O -methyl-D-glucuronic acid (see Table V). The presence of 2-O-methyl-D-galactose is ascribed to undermethylation, and subsequent Smith degradations of the gum suggested that the 4-O-methyl-L-arabinose probably arises similarly. The high proportion of 2,4-di-O-methyl-D-galactose indicates that most of the D-galactose residues in A. seyal gum are 3,6-di-O-substituted, although the identification of trace amounts of 2,3,4,6-tetra-, 2,3,6-, 2,4,6- and 2,3,4-

tri-O-methyl-D-galactose indicates that there are a few terminal non-reducing D-galactose residues, and some 4-O-, 3-O- and 6-O-substituted D-galactose units respectively. The identification of 2,3,4-tri-O-methyl-D-glucuronic acid and 2,3,4-tri-O-methyl-L-rhamnose suggests that D-glucuronic acid, 4-O-methyl-D-glucuronic acid, and L-rhamnose occur as terminal non-reducing units in the gum, and the 2,3-di-O-methyl-D-glucuronic acid suggests that the terminal non-reducing L-rhamnose is attached to D-glucuronic acid residues [18,19]. The presence of 2,3,5- and 2,3,4-tri-O-methyl -L-arabinose indicates that the arabinose chains are terminated either by L-arabinofuranose (I) or by L-arabinopyranose residues (II). In contrast to A. arabica gum[2] and A. drepanolobium gum Fraction A[1,20], 2,5-di-O-

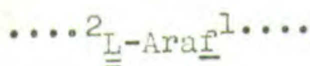


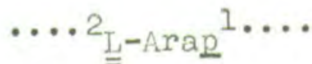
methyl-L-arabinose is the major di-O-methyl-L-arabinose obtained from methylated A. seyal gum, with a much smaller proportion of the 3,5 isomer. This shows that many L-arabinofuranose residues are 3-O-substituted (XII) in the arabinose chains, which also contain a few



XII

2-O-substituted-L-arabinofuranose residues (XIII). The identification of 3,4-di-O-methyl-L-arabinose indicates that there is a small proportion of 2-O-substituted-L-arabinopyranose residues (XIV) in the arabinose chains and the significance of this will be mentioned later. Methylation analysis of





XIII XIV

A. seyal gum and its degraded gum A suggests that the arabinose chains are attached to the branched galactan framework at either the C-3 or C-6 positions of certain D-galactose residues.

A. seyal gum was submitted to four successive Smith degradations which gave polysaccharides α, β, γ, and δ respectively. Although the yields of ~~the Smith-degraded~~ these products are low compared with those obtained during successive Smith degradations of A. senegal[8], A. laeta[9] and A. drepanolobium[1] gums, they are similar to those obtained from A. arabica gum[2]. Molecular-sieve chromatography indicates that there is pronounced fragmentation of the A. seyal gum macromolecule during Smith degradations; the cleavage of D-galactose residues is particularly drastic during the second and subsequent Smith degradations. The virtually complete fragmentation of the macromolecule during successive Smith degradations shows that long chains of periodate-resistant β1, 3-linked D-galactose residues are not such a dominant structural feature of A. seyal gum as they are of A. senegal[8] and A. laeta[9] gums. A. seyal gum contains blocks

of periodate-resistant β1,3-linked D-galactose residues interspersed with blocks of periodate-vulnerable β1,6-linked D-galactose residues in a highly branched galactan framework, fragmentation of which occurs as the arabinose chains are progressively removed from the C-3 positions of 6-O-substituted D-galactose units.

Arabinose residues were present in the product obtained from A. arabica gum[2] after four successive Smith degradations, and in that from A. drepanolobium gum[1] after five degradations. In contrast, four successive Smith degradations removed all the arabinose residues from A. seyal gum; it has already been pointed out that there are, however, some 2-O-substituted-L-arabinopyranose residues in the arabinose chains, some of which could therefore be longer than four units; for example, the model shown in Fig. 1 shows that periodate oxidation of the arabinose chain depicted, having a minimum length of five units, would yield the permissible arabinose chains of up to three units long in polysaccharide α.

As for A. drepanolobium gum Fraction A[1], the specific rotation of the Smith-degraded products from A. seyal gum decreases to a negative value after four successive degradations; during successive Smith degradations on A. arabica gum[2] the specific rotation decreases but remains positive. In contrast, A. senegal[8] and A. laeta[9] gums have negative specific rotations, but their Smith-degradation products have positive rotations. It is now clear that the structure of the branched galactan core may vary markedly from one gum species to another within the Acacia genus.

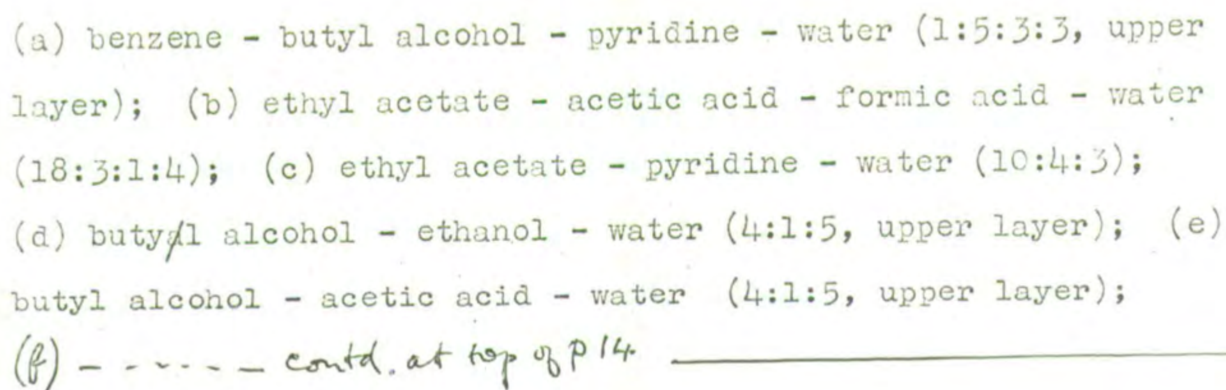
~~to a +ve value in the Smith degraded product.~~

On methanolysis, the O-methyl derivatives of the Smith-degraded polysaccharides α, β, and γ gave the results shown in Table VI. 2,3,5-Tri-O-methyl-L-arabinose was the only O-methyl-L-arabinose to be detected in the methanolysate of methylated polysaccharide γ. The absence of L-arabinopyranose residues in polysaccharide α suggests that the 4-O-methyl-L-arabinose identified in methylated *A. seyal* gum is not structurally significant.

The structural evidence therefore indicates that *A. seyal* gum is very similar to *A. drepanolobium*[1] and *A. arabica*[2] gums, having galactan frameworks that are more highly branched than in *A. senegal*[8] and *A. laeta*[9] gums. To these frameworks are attached uronic acid residues and arabinose chains which, on average, are shorter than those in *A. arabica* gum[2] and considerably shorter than those in *A. drepanolobium* gum Fraction A[1]. Molecular-sieve chromatography and viscometry further indicate that molecules of *A. seyal* gum are similar to those of *A. arabica* gum[2] in having a more compact structure than *A. senegal*[8] and *A. laeta*[9] gum molecules.

EXPERIMENTAL

The analytical methods have been described elsewhere in more detail[7]. Paper chromatography was carried out on Whatman Nos. 1 and 3MM papers using the following solvent systems (v/v);



(a) benzene - butyl alcohol - pyridine - water (1:5:3:3, upper layer); (b) ethyl acetate - acetic acid - formic acid - water (18:3:1:4); (c) ethyl acetate - pyridine - water (10:4:3); (d) butyl alcohol - ethanol - water (4:1:5, upper layer); (e) butyl alcohol - acetic acid - water (4:1:5, upper layer);

(f) - - - - - contd. at top of p 14

(f) butan-2-one - water - ammonia (d. 0.880)(200:17:1); (g) butan-2-one - acetic acid - water (9:1:1, saturated with boric acid). R_{Gal} values of sugars refer to distances moved relative to that of galactose. R_G values of O-methyl sugars refer to distances moved relative to that of 2,3,4,6-tetra-O-methyl-D-glucose (see Table V). Gas-liquid partition chromatography (Chromatograph Type S3A, fitted with flame ionisation detectors, supplied by Gas Chromatography Ltd.) of mixtures of O-methyl sugars was carried out at nitrogen flow rates of ca. 100 ml/min on columns of (i) 15% by weight of butan-1,4-diol succinate polyester on 60-70 mesh Celite (5 ft x $\frac{1}{4}$ in) at 175° and (ii) 15% by weight of ethylene glycol adipate polyester on 60-70 mesh Celite (3 ft x $\frac{1}{4}$ in) at 160°. Retention times (T) are quoted relative to methyl 2,3,4,6-tetra-O-methyl-D-glucopyranoside as standard. The retention times of the methylated sugars are given in Tables II - V. Unless otherwise stated, polysaccharides were methylated successively with dimethyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide, and methanolyses were carried out under reflux for 7 h with methanolic 5% hydrogen chloride. Disaccharides were methylated by the method of Perila and Bishop[22].

Purification of A. seyal gum.- The gum (91 g) was dissolved in water (2 l), filtered, and dialysed against running tap water. The polysaccharide was isolated as the freeze-dried product (87.5 g), $[\alpha]_D$ +59° (c, 0.50)(Found: moisture, 8.9%; N, 0.17%; protein, 1.1%; OMe, 0.70%, limiting viscosity number, 9.9; D-galactose, 38%; L-arabinose, 45%; L-rhamnose, 4%; D-glucuronic acid, 7%; 4-O-methyl-D-glucuronic acid, 6%; all sugar percentages are for the anhydro forms and are corrected for moisture and protein contents).

The gum was shown to migrate as a single band on electrophoresis on cellulose acetate film in both 0.1M ammonium carbonate buffer (pH 8.9) and 0.1M acetate buffer

(pH 4.7). The gum was chromatographed on a D.E.A.E.-cellulose column[6] (45 x 1.3 cm). Gradient elution with sodium chloride solution (0.0M → 0.5M) in 0.02M acetate buffer (pH 4.1) gave a single slightly asymmetric peak.

Preparation of degraded gum A.- The gum (20.0 g polysaccharide) was hydrolysed with 0.01N sulphuric acid (800 ml) for 100 h on a boiling water bath. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H^+), and dialysed against distilled water (3 l for 24 h). Dialysis was complete against running tap water. Degraded gum A was isolated as the freeze-dried product (4.4 g polysaccharide; yield , 22%); $[\alpha]_D$ +21° (c, 0.87), (Found: uronic acid, 15%; D-galactose, 82%; L-arabinose, 3%). Hydrolysis of degraded gum A (20 mg) gave galactose, arabinose, and the four aldobiouronic acids which have been characterized from the whole gum[4] [viz., 6-O-(β-D-glucopyranosyluronic acid)-D-galactose, 6-O-(4-O-methyl-β-D-glucopyranosyluronic acid)-D-galactose, 4-O-(α-D-glucopyranosyluronic acid)-D-galactose and 4-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-galactose]. Molecular-sieve chromatography using a previously calibrated "Bio-Gel P300" column[23-2] (45 x 5.0 cm) gave an estimated $\bar{M}n$ for degraded gum A of 4,900.

The dialysate from above (3 l) was concentrated to a syrup (11.6 g). Chromatographic examination of the syrup showed the presence of galactose, arabinose, rhamnose, and two neutral disaccharides with the mobilities of 3-O-β-L-arabinofuranosyl-L-arabinose [R_{Gal} 1.25 in solvent (b) and 1.43 in solvent (f)] and 3-O-β-L-arabinopyranosyl-L-arabinose [R_{Gal} 0.77 in solvent (a) and 0.71 in solvent (b)].

The first arabinobiose was isolated by fractionation of a portion of the diffusate on Whatman 3MM papers in solvent (f) (35 mg). This had $[\alpha]_D$ +90° (c, 0.70) yielded arabinose on acid hydrolysis. It had the same paper chromatographic mobility (R_{Gal} 1.25 in solvent (b) and 1.44 in solvent (f)) as an authentic specimen of 3-O-β-L-arabinofuranosyl-L-arabinose. A portion (15 mg) was methylated. G.l.c.

examination of the methanolysis products revealed peaks with retention times corresponding to the methyl glycosides of 2,3,5-tri- and 2,5- and 2,4-di-O-methyl-L-arabinose [(i), T 2.07, 2.13; (ii), T 1.46, 1.56]. Hydrolysis of the methyl glycoside followed by paper chromatography in solvent (d) confirmed the presence of 2,3,5-tri- and 2,5- and 2,4-di-O-methyl-L-arabinose (R_G 0.65).

The second arabinobiose was isolated by fractionation of a portion of the diffusate on Whatman 3MM papers in solvent (b) (63 mg). This had $[\alpha]_D$ +193° (c, 0.6) and yielded arabinose on acid hydrolysis. It had the same paper chromatographic mobility [R_{Gal} 0.77 in solvent (a) and 0.71 in solvent (b)] as an authentic specimen of 3-O-β-L-arabinopyranosyl-L-arabinose. A portion (15 mg) was methylated. G.l.c. examination of the methanolysis products revealed peaks with retention times corresponding to the methyl glycosides of 2,3,4-tri-, and 2,5- and 2,4-di-O-methyl-L-arabinose [(i), T 2.08, 2.19; (ii), T 1.47, 1.56]. Hydrolysis of the methyl glycosides followed by paper chromatography in solvent (d) confirmed the presence of 2,3,4-tri-, and 2,5- and 2,4-di-O-methyl-L-arabinose (R_G 0.65). The phenylosazone of the disaccharide was prepared and, after recrystallisation from water, had m.p. 232° - 234° (decomp.)(Lit. m.p.[26], 233° - 235°).

Partial acid hydrolysis of degraded gum A.- Degraded gum A (1.9 g polysaccharide) was hydrolysed with 0.5N sulphuric acid (500 ml) on a boiling water bath for 1 h. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H^+) and concentrated to a syrup (1.8 g). The syrup was chromatographed on Whatman 3MM papers in solvent (a) to give two pure disaccharides.

Fraction 1 (30 mg) had $[\alpha]_D$ +62° (c, 0.60) and yielded galactose on acid hydrolysis. It had the same paper chromatographic mobility [R_{Gal} 0.47 in solvent (a) and 0.53 in solvent (c)] as an authentic specimen of 3-O-β-D-galactopyranosyl-D-galactose. A portion (10 mg) was methylated. G.l.c. examination of the

methanolysis products revealed peaks with retention times corresponding to the methyl glycosides of 2,3,4,6-tetra-, and 2,4,6- and 2,5,6-tri-O-methyl-D-galactose [(i),T 3.98; (ii),T 3.25]. Hydrolysis of the methyl glycosides followed by paper chromatography in solvent (d) confirmed the presence of 2,3,4,6-tetra-, and 2,4,6- and 2,5,6-tri-O-methyl-D-galactose (R_G 0.88). The disaccharide was crystallised from aqueous acetone to give needles of 3-O-β-D-galactopyranosyl-D-galactose monohydrate, which had m.p. and mixed m.p. 157° - 159° (Lit. m.p.[26], 159° - 160°).

Fraction 2 (44 mg) had $[\alpha]_D$ +31° (c, 0.44) and yielded galactose on acid hydrolysis. It had the same paper chromatographic mobility [R_{Gal} 0.30 in solvent (a and 0.38 in solvent (c)] as an authentic specimen of 6-O-β-D-galactopyranosyl-D-galactose. A portion (15 mg) was methylated. G.l.c. examination of the methanolysis products revealed peaks with retention times corresponding to the methyl glycosides of 2,3,4,6-tetra-, and 2,3,5- [(i),T 4.05, 5.40; (ii),T 3.30, 4.3 and 2,3,4-tri-O-methyl-D-galactose. Hydrolysis of the methyl glycosides followed by paper chromatography in solvent (d) confirmed the presence of 2,3,4,6-tetra-, and 2,3,5- (R_G 0.86) and 2,3,4-tri-O-methyl-D-galactose.

Borohydride reduction of degraded gum A.- Degraded gum A (50 mg) was dissolved in water (10 ml), and sodium borohydride (100 mg) was added. The solution was left for 24 h at room temperature before further sodium borohydride was added (100 mg). After the solution had been stirred for 6 h, it was dialysed against running tap wat for 2 days and freeze-dried to yield the product (45 mg). A portion of the product (20 mg) was hydrolysed (N sulphuric acid for 7 h at 100°) to yield galactose, arabinose, and the same four aldobiouronic acids detected in the hydrolysate of degraded gum A. In addition paper chromatography in solvent (g) indicated the presence of galactitol. No arabinitol was detected. Degraded gum A gave no formaldehyde on periodate oxidation. Some formaldehyde was released on periodate oxidation of reduced degraded gum A.

Methylation of degraded gum A.- Degraded gum A (400 mg) was methylated successively with dimethyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide, to give a product (240 mg), $[\alpha]_D$ -14° (c, 0.83 in $CHCl_3$)(Found: OMe, 41.1%, not raised on further attempted methylation). Methanolysis of a sample of this product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table II. Examination of the hydrolysate of the methyl glycosides by paper chromatography in solvents (d) and (f) indicated the presence of 2,6-di- and 2-O-methyl-D-galactose in addition to those O-methyl sugars already characterized by g.l.c. of their methyl glycosides.

Reduction of methylated degraded gum A.- Lithium aluminium hydride (50 mg) was added to methylated degraded gum A (50 mg) dissolved in tetrahydrofuran (10 ml); the mixture was refluxed for 3 h. After cooling, the excess hydride was destroyed by addition of ethyl acetate and water. The mixture was reduced to dryness and extracted with chloroform. The chloroform extract was concentrated to a syrup. This was methylated with methyl iodide (1 ml) and silver oxide (1 g) in N,N-dimethyl formamide (1 ml) to give a product (35 mg), $[\alpha]_D$ -13° (c, 0.70 in $CHCl_3$). Methanolysis of the product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table III. Hydrolysis of the methyl glycosides followed by paper chromatography in solvents (d) and (f) indicated the presence of 2,6-di- and 2-O-methyl-D-galactose, in addition to those O-methyl sugars already identified by g.l.c. of their methyl glycosides.

Partial methanolysis of methylated degraded gum A.- Methylated degraded gum A (100 mg) was refluxed with methanolic 2% hydrogen chloride (15 ml) for 2 h. The methanolysate was neutralised with silver carbonate, filtered and evaporated to a syrup, which was hydrolysed with 0.5N sulphuric acid (10 ml) for 5 h on a boiling water bath . The cooled solution was neutralised with barium carbonate, filtered,

treated with Amberlite resin IR-120 (H^+), and adsorbed on to a column (33 x 3 cm) of Duolite A-4 in the formate form. After elution of the neutral O-methyl sugars with water (3 l), the acidic O-methyl sugars were eluted with aqueous 5% formic acid (500 ml). Water and formic acid were removed, and paper chromatography of the resulting syrup in solvent (d) indicated that it was free of neutral O-methyl sugars. The syrup was refluxed with methanolic 5% hydrogen chloride for 7 h. G.l.c. examination of the products revealed peaks with retention times corresponding to the methyl glycosides of 2,3,4-tri-O-methyl-D-glucuronic acid, and 2,3,6- and 2,3,4-tri-, and 2,6- and 2,4-di-O-methyl-D-galactose. Hydrolysis of the methyl glycosides followed by paper chromatography in solvents (d), (e) and (f) confirmed these results.

Smith degradation[17] of degraded gum A.- Degraded gum A (1.0 g polysaccharide) was dissolved in water (25 ml) and 0.5M sodium metaperiodate solution (25 ml) was added. After 96 h, 7.1 mmole of periodate was reduced and 3.2 mmole of formic acid was released. After 96 h, the reaction was stopped by addition of ethylene glycol (2 ml), and the solution was dialysed against running tap water for 48 h. Sodium borohydride (0.5 g) was added, and the mixture kept at room temperature for 30 h. After dialysis for a further 48 h, the polyalcohol was hydrolysed in N sulphuric acid at room temperature for 48 h. The solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H^+) and dialysed against distilled water (1 l) for 24 h. After further dialysis against running tap water for 48 h, degraded gum B (50 mg polysaccharide; yield, 5%), $[\alpha]_D$ +7.5° (c, 0.50), was isolated as the freeze-dried product.

The syrup obtained from the dialysate (400 mg) was shown by paper chromatography in solvents (a), (b) and (c) to contain glycerol and glycollic aldehyde as main components, together with small amounts of slower moving non-reducing glycosides. Hydrolysis of a portion of the dialysate (N sulphuric acid for 7 h at 100°) followed

by paper chromatography in solvents (a) and (b) showed a trace of galactose to be present.

Mild acid hydrolysis (0.5N sulphuric acid for 1 h at 100°) of degraded gum B (10 mg), followed by paper chromatography in solvent (a) showed the presence of galactose and two neutral disaccharides with the chromatographic mobilities of 3-O-β-D-galactopyranosyl-D-galactose (R_{Gal} 0.48) and 6-O-β-D-galactopyranosyl-D-galactose (R_{Gal} 0.31). Degraded gum B was exhaustively methylated. Methanolysis followed by g.l.c. examination of the mixture of methyl glycosides gave the results shown in Table IV. Hydrolysis of the methyl glycosides, followed by paper chromatography in solvents (d) and (f), indicated the presence of 2,6-di- and 2-O-methyl-D-galactose in addition to those O-methyl sugars already identified by g.l.c. of their methyl glycosides.

Controlled Smith degradation[27] of degraded gum A.- Degraded gum A (1.0 g polysaccharide) was treated in the same way as in the normal Smith degradation except that the periodate oxidation stage was carried out at 2°. Degraded gum C was isolate as the freeze-dried product (60 mg polysaccharide; yield, 6%). Hydrolysis of a porti of degraded gum C (20 mg), followed by paper chromatography in solvent (g), indicated the presence of galactose and arabinitol, but no galactitol.

Methylation of A. seyal gum.- The purified gum (300 mg polysaccharide) was methylated by the Haworth and Purdie proceedures to give a product (255 mg), $[\alpha]_D$ +17 (c, 1.00 in $CHCl_3$)(Found: OMe, 41.6%, not raised on further attempted methylation). Methanolysis of a portion, followed by g l.c. examination of the mixture of methyl glycosides, gave the results shown in Table V. The same O-methyl sugars were detected as were detected and characterized from methylated A. drepanolobium gum Fraction A[1]. However the major di-O-methyl-L-arabinose was 2,5-di-O-methyl-L-arabinose. Only a small amount of 3,4-di-O-methyl-L-arabinose was detected. Hydrolysis of the methyl

lycosides followed by paper chromatography in solvents (d), (e) and (f) indicated he presence of 4-O-methyl-L-arabinose and 2-O-methyl-D-galactose and confirmed the resence of 2,3,4-tri-O-methyl-L-arabinose.

Preparation, partial acid hydrolysis, and methylation of polysaccharide α.- ... (54 g polysaccharide) was dissolved in water (1.5 l), mixed with 0.25M sodium etaperiodate solution (1.5 l) and left for 72 h. The amount of formic acid released n this time was 0.76 mmoles/g. The corresponding amount of periodate reduced was .89 mmoles/g. The reaction was stopped by addition of ethylene glycol (20 ml), and he solution dialysed against running tap water for 48 h. Sodium borohydride (15 g) s added, and the mixture kept at room temperature for 30 h, then dialysed for a urther 48 h. The polyalcohol was hydrolysed in N sulphuric acid at room temperature or 48 h. After dialysis against running tap water for 48 h, polysaccharide α was solated as the freeze-dried product (27.5 g polysaccharide; yield, 51%), $[\alpha]_D$ 0° c, 0.50)(Found: galactose, 67%; arabinose, 33%).

Polysaccharide α (20 mg) was hydrolysed with 0.5N sulphuric acid (10 ml) for 1 h n a boiling water-bath. Paper chromatography of the hydrolysate indicated the resence of galactose, arabinose, and two neutral disaccharides with the mobilities of -O-β-D-galactopyranosyl-D-galactose [R_{Gal} 0.48 in solvent (a) and 0.54 in solvent (c inor component] and 6-O-β-D-galactopyranosyl-D-galactose [R_{Gal} 0.31 in solvent (a) nd 0.38 in solvent (c), major component], and higher neutral oligosaccharides.

Polysaccharide α (315 mg) was methylated with dimethyl sulphate and sodium ydroxide, and with methyl iodide and silver oxide, to give a product (274 mg), $[\alpha]_D$ -31.7° (c, 0.89 in $CHCl_3$)(Found: OMe, 41.1%, not raised on further attempted ethylation)(See Table VII). Methanolysis of a sample of this product, followed by .l.c. examination of the mixture of methyl glycosides, gave the results shown in able VI. Hydrolysis of the methyl glycosides, followed by paper chromatography in

solvents (d) and (f), indicated the presence of 2,6-di- and 2-O-methyl-D-galactose in addition to those O-methyl sugars already characterized by g.l.c. of their methyl glycosides.

Preparation, partial acid hydrolysis and methylation of polysaccharides α, β and γ. The following weights of polysaccharide were periodate-oxidised, borohydride-reduced and hydrolysed, and the corresponding degraded polysaccharides recovered as for the first Smith degradation. Polysaccharide α (26.6 g) gave polysaccharide β (7.7 g). This (7.1 g) gave polysaccharide γ (0.93 g). Smith degradation of polysaccharide γ (0.70 g) gave polysaccharide δ (50 mg). The amount of periodate reduced and formic acid released on periodate oxidation, and the percentage yields, specific rotations, and constituent sugars of each degraded polysaccharide are given in Table I.

Partial acid hydrolysis of polysaccharide β (20 mg), polysaccharide γ (5 mg) and polysaccharide δ (5 mg), followed by paper chromatographic examination of the hydrolysates, indicated the presence of 6-O-β-D-galactopyranosyl-D-galactose (major component) and 3-O-β-D-galactopyranosyl-D-galactose (minor component), and higher neutral oligosaccharides.

Polysaccharides β, γ and δ were methylated (Table VII). Methanolysis of a sample of each O-methyl derivative, followed by g.l.c. examination of the mixtures of methyl glycosides, gave the results shown in Table VI.

Molecular-sieve chromatography of A. seyal gum and the Smith-degraded products.- On molecular-sieve chromatography, using a previously calibrated Bio-Gel P300 column (45 x 5.0 cm), both A. seyal and polysaccharide α were eluted before the elution volume of "Blue Dextran 2000". This indicates that A. seyal gum and polysaccharide α have very high number-average molecular weights (perhaps as high as 500,000). Light-scattering measurements indicated that the weight-average molecular weight of A. seyal gum was ca. 850,000. Molecular-sieve chromatography indicated that polysaccharides β

and γ had number-average molecular weights of ca. 8,000 and less than 3,000 respectively.

ACKNOWLEDGMENTS

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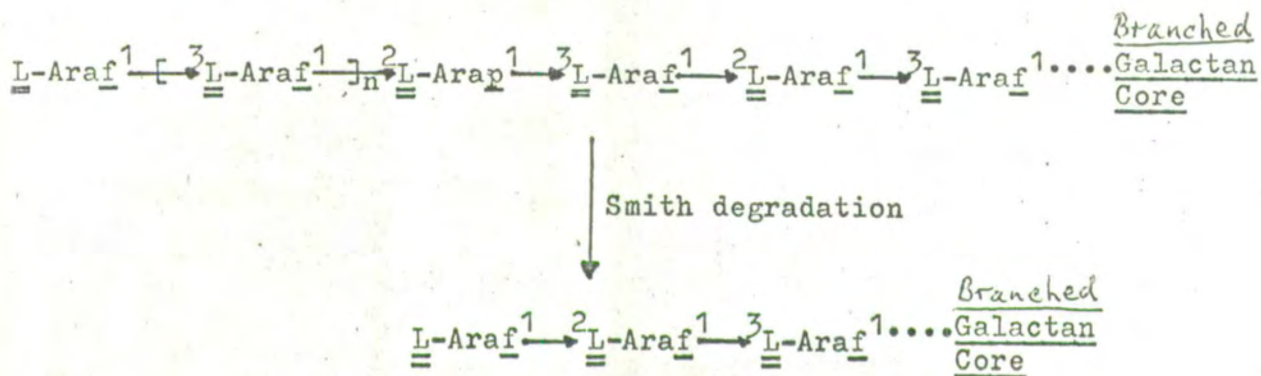

Figure 1.

Table I

ta for *A. seyal* gum and Smith-degraded polysaccharides α – δ.

lysaccharide	$[\alpha]_D$	Constituent sugars %					Recovery %	mmoles/g IO_4' reduced on periodate oxidation	mmoles/g formic Acid released on periodate oxidation
		Gal	Ara	Rha	Gluc. Acid	4-Me Gluc. Acid			
seyal gum	+59°	38	45	4	7	6	-	3.89	0.76
lysaccharide α	0°	67	33	-	-	-	51	3.89	1.00
lysaccharide β	-15°	89	11	-	-	-	29	5.58	1.99
lysaccharide γ	-24°	95	5	-	-	-	12.5	4.89	1.56
lysaccharide δ	-25°	100	-	-	-	-	7	-	-

Table II

Examination of Methanolysis and hydrolysis products from methylation degraded gum A

Relative retention times (T) of methyl glycosides on columns:- * (i)	(ii)	R_G in Solvent (d)	Approximate relative molar proportions	O-Methyl Sugar
0.56;0.73	0.50;0.63	0.98	Trace	2,3,5-tri-O-methyl-L-arabinose
1.02	0.82	0.79	Trace	2,3,4-tri-O-methyl-L-arabinose
1.69	1.66	0.90	++	2,3,4,6-tetra-O-methyl-D-galactose
(3.02);(3.80);(4.29)	2.50;(3.05);(3.51)	0.72	+	2,3,6-tri-O-methyl-D-galactose
(3.80);(4.29)	(3.05);(3.51)	0.72	++	2,4,6-tri-O-methyl-D-galactose
6.40	5.29	0.72	++++	2,3,4-tri-O-methyl-D-galactose
14.4;16.3	9.9,11.3	0.53	+++	2,4-di-O-methyl-D-galactose
2.40;(3.02)	2.21;2.72		++	2,3,4,-tri-O-methyl-D-glucuronic acid †
		0.59	+	2,6-di-O-methyl-D-galactose
		0.34	+	2-O-methyl-D-galactose

* Figures in parenthesis indicate T values of components which are not completely resolved.

† As methyl ester methyl glycoside.

Table III

Examination of methanolysis and hydrolysis products from methylated reduced degraded gum A

Relative retention times, (T) of methyl glycosides on columns:- * (i)	(ii)	R_G in Solvent (d)	Approximate relative molar Proportions	O-Methyl sugar
0.56;0.73	0.50;0.64	0.98	Trace	2,3,5-tri-O-methyl-L-arabinose
(1.01)	0.82	0.79	Trace	2,3,4-tri-O-methyl-L-arabinose
(1.01); .40	1.00;1.39	1.00	++	2,3,4,6-tetra-O-methyl-D-glucose
1.68	1.66	0.90	++	2,3,4,6-tetra-O-methyl-D-galactose
(3.02);(3.79);(4.29)	2.49;(3.05);(3.51)	0.72	+	2,3,6-tri-O-methyl-D-galactose
(3.79);(4.29)	(3.05);(3.52)	0.72	++	2,4,6-tri-O-methyl-D-galactose
6.40	5.29	0.72	++++	2,3,4-tri-O-methyl-D-galactose
14.4;16.4	9.9;11.3	0.53	+++	2,4-di-O-methyl-D-galactose
		0.59	+	2,6-di-O-methyl-D-galactose
		0.34	+	2-O-methyl-D-galactose

* Figures in parenthesis indicate T values of components which are not completely resolved.

Table IV

Examination of methanolysis and hydrolysis products from methylated degraded gum B.

Relative retention times (T) of methyl glycosides on columns: (i)	(ii)	R_G in Solvent (d)	Approximate relative molar Proportions	Hence O-Methyl Sugar identified
1.68	1.66	0.90	++	2,3,4,6-tetra-O-methyl-D-galactose
3.75;4.25	3.00;3.44	0.72	++++	2,4,6-tri-O-methyl-D-galactose
6.45	5.30	0.72	+	2,3,4-tri-O-methyl-D-galactose
14.6;16.4	9.9;11.3	0.53	+	2,4-di-O-methyl-D-galactose
2.57;3.00	2.21;2.74		Trace	2,3,4-tri-O-methyl-D-glucuronic acid †
		0.59	+/2	2,6-di-O-methyl-D-galactose
		0.33	+/2	2-O-methyl-D-galactose

† As methyl ester methyl glycoside.

Table V

Examination of methanolysis and hydrolysis products from methylated *A.seyal* gum

Relative retention times, T, of methyl glycosides on columns:- * (i)	(ii)	R_G in solvent (d)	Hence O-methyl sugar identified
0.48	(0.50)	1.01	2,3,4-tri-O-methyl-L-rhamnose
0.53;0.73	(0.50);0.64	0.98	2,3,5-tri-O-methyl-L-arabinose
(1.03)	(0.82)	0.79	2,3,4-tri-O-methyl-L-arabinose
1.81;3.20	1.25;2.21	0.82	2,5-di-O-methyl-L-arabinose
(1.03);(2.36)	(0.82);1.75	0.82	3,5-di-O-methyl-L-arabinose
1.99	1.44	0.56	3,4-di-O-methyl-L-arabinose
1.68	1.66	0.88	2,3,4,6-tetra-O-methyl-D-galactose
(3.01);(3.89);(4.22)	2.45;(3.00);(3.45)	0.72	2,3,6-tri-O-methyl-D-galactose
(3.89);(4.22)	(3.00);(3.45)	0.72	2,4,6-tri-O-methyl-D-galactose
6.40	5.20	0.72	2,3,4-tri-O-methyl-D-galactose
14.6;16.4	9.9;11.3	0.52	2,4-di-O-methyl-D-galactose
(2.36);(3.01)	(2.21);2.75	-	2,3,4-tri-O-methyl-D-glucuronic acid
		0.32	2-O-methyl-D-galactose
		0.36	4-O-methyl-L-arabinose
7.8;9.3	6.2;7.1	-	2,3-di-O-methyl-D-glucuronic acid †

* Figures in parentheses indicate T values of components which are not completely resolved.

† As methyl ester methyl glycoside.

Table VI

Relative proportions of O-methyl sugars present in methylated polysaccharides $\alpha - \delta$

O-methyl sugar	Methylated polysaccharides			
	α	β	γ	δ
2,3,5-tri-O-methyl-L-arabinose	2	2	trace	-
2,5-di-O-methyl-L-arabinose	2	1/2	-	-
3,5-di-O-methyl-L-arabinose	1/2	trace	-	-
2,3,4,6-tetra-O-methyl-D-galactose	trace	1	1	2
2,4,6-tri-O-methyl-D-galactose	6	6	4	4
2,3,4-tri-O-methyl-D-galactose	2	3	2	2
2,4-di-O-methyl-D-galactose	3	2	1	1

Table VII

Methylation data for Smith-degraded polysaccharides.

	Polysaccharides			
Amount of polysaccharide used (mg)	315	232	85	30
Amount of product obtained (mg)	274	190	21	7
$[\alpha]_D$ of product	-31.7°	-38.6°	-39.5°	-
OMe of product, %	41.1	40.8	41.4	-