ANALYTICAL AND STRUCTURAL STUDIES ON GUM EXUDATES FROM THE *LANNEA* AND *AZADIRACHTA* GENERA

by

A. HENDRIE B.Sc.

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TO MY WIFE AND PARENTS

CONTENTS

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ABSTRACT

An analytical study of gum samples from three species of the genus Lannea showed them to have similar chemical properties, suggesting that interspecies variation in this genus is unlikely to be large. Molecular-sieve chromatography indicated a broad molecular weight distribution for these gums.

A sample of Lannea humilis gum was subjected to hydrolysis, Smith degradation, and methylation studies, and these revealed its molecular structure to be based on a branched galactan framework of short chains of β-1,3-linked D-galactose residues joined by β-1,3- and β-1,6-linkages. To this framework, at suitable C-3 and C-6 positions, are attached short D-galactose and L-arabinose side chains, and three different uronic acids. The mode of attachment of L-rhamnose is uncertain. From an acid hydrolysate of the gum, the unusual aldobiouronic acid 4-O-(α-D-galactopyranosyluronic acid)-D-galactose, and the more common 6-O-(β-D-glucopyranosyluronic acid)-D-galactose and 6-O-(4-O-methyl-β-D-glucopyranosyluronic acid)-D-galactose, were isolated and characterised.

Two samples of Azadirachta indica gum, from different botanical sources, were found to contain an amino sugar (ca. 2%) and much proteinaceous material (ca. 35%). Attempts to remove protein, using common denaturing agents, were unsuccessful, but better results were achieved by enzymic hydrolysis. The amino acid composition of each sample was determined. Molecular-sieve chromatography of Azadirachta indica gum, using a denaturing solvent, revealed degradation of one of the peaks in its elution

diagram; a portion of gum (2 g) was fractionated by molecular-sieve chromatography, and the two samples of Azadirachta indica gum and these fractionation products were compared.

An automated colorimetric method of molecular-sieve chromatography was developed and evaluated. It was found to be inexpensive, rapid and to give reproducible results, although some difficulty over relative peak heights was encountered.

Preliminary studies were made on a comparison of two methylation methods and on the electrophoresis of dyed polysaccharides.

SECTION I

GENERAL INTRODUCTION

Plant gums, which are exuded by some tropical and subtropical trees and shrubs, are high molecular weight, acidic polysaccharides, existing as nearly-neutral salts in the natural state. They have been employed for a wide variety of functions, such as adhesion and colloid stabilisation, for many years, and have been considered for centuries to be therapeutic agents by the natives of Africa, India and parts of Asia.

In the past 7-10 years, there has been much chemical investigation of both analytical and structural aspects of gum exudates; this is the subject of a recent review[1]. These investigations have been facilitated by the development of sequential periodate degradation (Smith degradation) studies[2], which provide information about the structural framework of the gum molecule, and by further developments in technique and materials for all forms of chromatography. The emergence of molecular-sieve chromatography[3], a technique of column or thin-layer chromatography in which fractionation is based primarily on molecular size, is of importance, as it is well suited to the study of macromolecules.

The botanical classification of gum-producing plants is well established, and attempts have been made to relate plant taxonomy to results of chemical analyses of the gum exudates[4]. It has been found that taxonomy can give useful indications of gums likely to prove chemically interesting, but the importance of studying only botanically authenticated specimens has been stressed[5,6], commercial mixtures and unauthenticated samples yielding results of little permanent scientific value.

Some recent studies have been focussed on the protein component of plant gums, a feature of the molecules hitherto largely ignored, and preliminary surveys of amino acids present in the protein component of gums from two *Araucaria*[7], one *Acacia*[7] and one *Combretum*[8] species have been made. Molecular-sieve chromatographic studies of *Acacia campylacantha* gum[7] have shown the bonding between protein and carbohydrate components to be complex, involving hydrogen bonds and other mechanisms of aggregation, but probably not involving covalent protein-polysaccharide linkages.

Most of the work performed on gum exudates thus far has centred on the (admittedly commercially important) *Acacia* gums, with some revival of interest in the genus *Prunus*[9]. Other chemically interesting genera have meanwhile remained on the sidelines. Good, authenticated samples of gums from the *Lannea* and *Azadirachta* genera have come into our possession, so the opportunity was taken to investigate these: interest in these samples was stimulated by confusion over chemical results reported in the literature for a *Lannea* gum, and by the unusually high nitrogen content of an *Azadirachta* gum.

This thesis comprises two main studies. Samples from three species of the genus *Lannea* are compared (Section III A) and one of these samples, *Lannea humilis* gum, is subjected to a detailed structural examination (Section III B). *Azadirachta indica* gum is found (Section IV) to possess an amino sugar, and a high content of proteinaceous material; the proteinaceous component is studied.

In Section V, a description is given of a subsidiary study, involving the development and evaluation of an automated colorimetric method of molecular-sieve chromatography.

SECTION II

EXPERIMENTAL METHODS

2.1 GENERAL METHODS

Weighings. All accurate weighings were made within the range of the graticule scale (range, 0-100 mg) of a Stanton Unimatic Model CL 1 single-pan balance, having an accuracy of ±0.1 mg.

Dialyses of polysaccharides, to remove low molecular weight material, were carried out in cellophane tubing (Kalle Aktiengesellschaft, Wiesbaden) against running tap-water unless otherwise stated.

Electrodialyses of polysaccarides were carried out in a three-compartment cell fitted with cellophane membranes; the polysaccharide solution in the middle compartment was stirred continuously, and cooled by passage of cold water through a cooling coil.

Reductions in volume were carried out on a rotary evaporator at temperatures below 40°C.

Moisture contents were determined by heating to constant weight at 105°C.

Ash contents were determined by heating to constant weight in a muffle furnace at 550°C.

Nitrogen contents were determined by a semi-micro Kjeldahl method.

Carbon, hydrogen and nitrogen contents were determined using a Perkin-Elmer 240 Elemental Analyzer. Correction was made for moisture content of the gum samples.

Methoxyl contents were determined by a vapour-phase infrared method[10,11].

Equivalent weight determinations on electrodialysed polysaccharides were carried out by direct titration with standard (ca 0.01N) sodium hydroxide solution, using phenolphthalein indicator.

Uronic acid contents were determined, after acidic decarboxylation, by a vapour-phase infrared method[12], using a spectrometer calibrated for known weights of carbon dioxide by the sodium carbonate method[11]. Values for uronic acid content are expressed as the anhydride.

Quantitative estimation of sugars. Sugars were separated by chromatography on previously washed Whatman No. 3MM papers. After elution from the paper, sugars were estimated colorimetrically by the phenol-sulphuric acid method[13]. The optical density was read on a Unicam SP1300 spectrophotometer using filter 2. Calibration curves were constructed for known weights of sugars.

Melting points were observed on a Kofler hot stage microscope (thermometers corrected).

2.2 PHYSICAL METHODS

Specific rotations of aqueous or chloroform solutions were measured using the sodium D-line, with a Perkin-Elmer Model 141 Polarimeter at 20 ±2°C.

Viscosity determinations were carried out in M-sodium chloride solution in an Ubbelohde suspended-level dilution viscometer at 25.00 ±0.01°C. Solutions were filtered carefully before additions

were made to the viscometer. Flow-times were measured to within 0.1 sec by means of a stop-watch. The isoionic dilution technique was used; a solution of the gum (5 ml) was placed in the viscometer and the flow-time measured. Flow-times were also obtained for successive dilutions with M-sodium chloride solution (two additions of 1 ml each, followed by two of 2 ml). Since preliminary experiments had indicated that any loss of gum from M-sodium chloride solution on filtering was negligible, concentration values were estimated from the dry weight of gum dissolved in a known volume.

For the low concentrations of gum employed, the densities of M-sodium chloride and gum solutions were assumed to be equal, and the results expressed in terms of the limiting viscosity number, $[\eta]$.

$$[\eta] = \lim_{c \to 0} \frac{\eta_{sp}}{c} = \lim_{c \to 0} \frac{t-t_0}{ct_0}$$

where η_{sp} is the specific viscosity, η_{sp}/c the viscosity number, t_0 and t the flow-times in sec for solvent and solution respectively, and c is the concentration of gum (g/ml).

Light-scattering measurements were carried out at 27.00 ±0.02°C using a SOFICA photogoniodiffusometer Model 4200. Unpolarised green light (546 nm) was selected from a mercury lamp spectrum by use of a Wratten Kodak N61 filter.

Using M-sodium chloride solution as solvent, gum solutions (approximately 0.1% and 0.2%) were accurately prepared; the molecular weight was calculated as the average for these solutions. Chloroform was used as solvent for methylated polysaccharides. Solutions were clarified by passage through filters of average pore size 0.45 μm and 0.22 μm (Millipore, Ltd., Bedford, Mass., U.S.A.)

using a stainless-steel filter holder attached to a 20 ml syringe. Concentrations of gum solutions were assumed to be unaltered by ultrafiltration[14].

For each concentration, the intensity of scattered light at various angles between 30° and 150° was recorded, and corrected scale readings, I_θ, for angle θ, were calculated[15] from the equation:-

$$I_\theta = \frac{(I_p - I_0)\sin\theta}{1+\cos^2\theta} \qquad \ldots\ldots (1)$$

where I_p and I_0 are the scale readings for polymer solution and solvent respectively. In an ideal solution, the molecular weight, M, is obtained from the equation:-

$$\frac{1}{M} = \frac{2\pi^2 n_0^2}{\lambda^4 NR} \cdot I_B \cdot [dn/dc]^2 \cdot [c/I_\theta]_{c=0}^{\theta=0} \qquad \ldots (2)$$

where n_0 = refractive index of solvent

n = refractive index of solution

N = Avogadro's Number

λ = wavelength of incident light (546 nm)

R = Rayleigh constant of standard benzene (16.3×10^{-6} for λ = 546 nm)

c = concentration in g/ml

I_B = intensity diffused, selected for standard benzene

dn/dc = refractive index increment.

An extrapolation method introduced by Zimm[16] for derivation of molecular weight from this equation, without making any assumptions about the shape of the macromolecule, has been found to give unsymmetrical Zimm plots and was not used[17].

If it is assumed that M in equation (2) is independent of c, then:-

$$\frac{1}{M} = \frac{2\pi^2 n_o^2}{\lambda^4 NR} \cdot [dn/dc]^2 \cdot I_B \cdot c \cdot [1/I_\theta]_{\theta=0} \quad \ldots (3)$$

$$\text{i.e.,} \quad M = \frac{R}{\frac{2\pi^2 n_o^2}{\lambda^4 N} \cdot [dn/dc]^2 \cdot I_B \cdot c \cdot [1/I_\theta]_{\theta=0}} \quad \ldots (4)$$

The reciprocal corrected scale reading $1/I_\theta$, is plotted against $\sin^2 \theta/2$. Extrapolation of the linear portion of this graph to $\theta = 0$ gives a value for $[1/I_\theta]_{\theta=0}$. The downward curvature of these graphs at low angles is thought to be caused by scatter from dust particles suspended in solution [17].

Refractive index increments, dn/dc, were determined using a Brice-Phoenix Differential Refractometer [18], for green light (546 nm) at 28°C. The average of three determinations, for polysaccharide concentrations of 2%, 3% and 4% in M-sodium chloride solution, was taken.

The instrument scale readings r_0 and r_{180}, for positions of the differential cell at 0° and 180°, respectively, are obtained for solvent and solution. This gives:-

$$\Delta r = (r_{180}-r_0)_{\text{solution}} - (r_{180}-r_0)_{\text{solvent}} \quad \ldots . (5)$$

The refractive index difference, Δn, is calculated from:-

$$\Delta n = k.\Delta r \quad \ldots . (6)$$

where k is the instrument calibration constant. The refractive index increment, dn/dc, is then obtained from:-

$$\frac{dn}{dc} = \frac{k.\Delta r}{c} \quad \ldots . (7)$$

The constant k was determined by application of equation (7) to standard sucrose solutions.

Infrared spectroscopy was carried out using a Hilger-Watts H.1200 double-beam grating spectrophotometer.

Ultracentrifugation was carried out using a Beckman-Spinco Model E Analytical Ultracentrifuge. Polysaccharide solutions (0.5% in 0.5M-sodium chloride solution) were examined at 44,770 r.p.m. After the ultracentrifuge had attained this speed, the boundary patterns, obtained by the Schlieren optical system, were photographed at 16 min intervals.

2.3) CHEMICAL METHODS

Small-scale polysaccharide hydrolyses were carried out with N-sulphuric acid for 7.5 hours on a boiling water bath, unless otherwise stated. These conditions do not cause any extreme hydrolysis of uronosyl linkages in the polysaccharides studied in this investigation; this was taken into account when determining proportions of galactose. Hydrolysate solutions were neutralised with barium carbonate, filtered, deionised with Amberlite IR-120(H) resin, and concentrated on a rotary evaporator.

Small-scale polysaccharide methylations.

(a) Haworth[19]. Methylations were carried out in an atmosphere of nitrogen at room temperature. Dimethyl sulphate (2 ml) and sodium hydroxide solution [2 ml, 30% (w/v)] were added dropwise with stirring to the polysaccharide (100-500 mg) in water (10 ml) over a period of 1 hour. Acetone (5 ml) was added to the reaction

mixture, and six further additions of dimethyl sulphate (12 ml) and sodium hydroxide (17 ml) were made, allowing 3 hours for each addition. After stirring for 12 hours, the reaction was stopped by heating at 60°C for 30 min, with nitrogen bubbling vigorously through the solution. The reaction mixture was cooled, then neutralised with 4N-sulphuric acid and made slightly acid (pH 4.0). The methylated product was extracted into chloroform (4 × 100 ml extractions) and the extract shaken with saturated sodium chloride solution (ca. 100 ml). The chloroform layer was separated, dried over anhydrous sodium sulphate, concentrated on a rotary evaporator and poured, with stirring, into light petroleum (b.p. 60°-80°, ca. 400 ml). The precipitated, methylated polysaccharide was isolated, after filtration and drying, as a white amorphous powder.

(b) Purdie and Irvine[20]. The partially methylated product (100-400 mg) was dissolved in methanol (10 ml) and methyl iodide (10 ml). Silver oxide (1 g) was added in four batches of ca. 250 mg every 1.5 hours; the mixture was refluxed for 6 hours in the dark in a dry flask fitted with a water condenser and a calcium chloride tube. The mixture was cooled, filtered through sintered glass and the residue was extracted six times with hot chloroform (ca. 50 ml). Any dissolved silver ions were removed by passing hydrogen sulphide through the combined filtrate and extracts. The solution was re-filtered, concentrated and poured, with stirring, into light petroleum (b.p. 60°-80°, ca. 400 ml). The precipitated methylated polysaccharide was isolated, after filtration and drying, as a white amorphous powder.

(c) Anderson and Cree[21]. The polysaccharide (100-400 mg) was

dissolved in dimethylsulphoxide (25 ml). The solution was stirred and sodium hydride (200 mg) was added in small portions over 1 hour, followed by methyl iodide (0.5 ml) added over 2 hours. The solution was stirred overnight. Three further additions of sodium hydride and methyl iodide were made on successive days as described for the first addition. The resulting solution was poured into water (ca. 150 ml), excess methyl iodide removed by aspiration, then the methylated product extracted into chloroform (4 x 100 ml extractions). The chloroform extract was washed with water, dried over anhydrous sodium sulphate, concentrated and poured, with stirring, into light petroleum (b.p. 60°-80°, ca. 400 ml). The precipitated, methylated polysaccharide was isolated, after filtration and drying, as a white amorphous powder.

Small-scale oligosaccharide methylations[22,23]. The oligosaccharide (0.5-2.0 mg) was shaken with methyl iodide (0.2 ml), N,N-dimethylformamide (0.2 ml) and silver oxide (0.2 g) at room temperature in the dark for 18 hours. The mixture was filtered and the residue washed with chloroform. The combined filtrate and washings were concentrated to a syrup on a rotary evaporator.

Methanolyses. Unless otherwise stated, methanolyses were carried out under reflux for 6 hours with methanolic 5% hydrogen chloride. After cooling, solutions were neutralised with silver carbonate and filtered. The residue was washed with methanol, and any dissolved silver ions were removed by passing hydrogen sulphide through the combined filtrate and washings. After re-filtration, the solution was concentrated to a syrup on a rotary evaporator.

Periodate oxidations of polysaccharides were carried out in

the dark at room temperature unless otherwise stated.

(a) Consumption of periodate. The amount of periodate consumed by a polysaccharide was estimated by back-titration of excess periodate. To a portion (1 ml) of the periodate solution was added excess potassium iodide and the iodine liberated was titrated, after addition of sodium bicarbonate (ca. 200 mg), with standard sodium arsenite solution (ca. 0.05N) using "Thyodene" as indicator[24].

(b) Formic acid released was estimated titrimetrically[25] with standard sodium hydroxide (ca. 0.1N) for portions (1 ml) of the solution. Methyl red was used as indicator.

Molecular weight of polysaccharides by end-group analysis was obtained by periodate oxidation of polysaccharide followed by colorimetric estimation of formaldehyde released using chromotropic acid[26]. The polysaccharide (30-50 mg) was dissolved in p-hydroxybenzaldehyde solution [10 ml, 0.1% (w/v)]. This solvent prevents recombination of formaldehyde with oxypolysaccharide[27]. To the solution was added a portion (1 ml) of sodium metaperiodate solution such that a slight excess of sodium metaperiodate was present. At suitable time intervals, samples (1 ml) were transferred to centrifuge tubes, treated with 0.5M-sodium sulphite solution (1 ml) to destroy excess periodate, and with ethanol (4 ml) to precipitate the oxypolysaccharide. The tubes were stored for 2 days at 2°C then centrifuged. Portions (1 ml) of the supernatant were treated with 9 ml chromotropic acid reagent [2 g of the sodium salt of chromotropic acid (B.D.H., "for formaldehyde determinations") dissolved in a solution of AnalaR sulphuric acid (566 ml) and water (320 ml)[28]]

on a boiling water bath for 30 min. After cooling, thiourea solution [2 ml, 4.6% (w/v)] was added, and the optical density was measured on a Unicam SP1300 spectrophotometer using filter 4. A calibration curve for formaldehyde was constructed by periodate oxidation of solutions of AnalaR glucose, 0.5M with respect to sodium bicarbonate. Assuming production of one molecule of formaldehyde per average polymer unit, a value for number-average molecular weight, $\bar{M}_n$, of the polysaccharide may be calculated.

2.4) CHROMATOGRAPHIC AND ELECTROPHORETIC SEPARATIONS

Ion-exchange chromatography on diethylaminoethylcellulose (DEAE-cellulose)[29]. DEAE-cellulose powder (Whatman DE32, microgranular form, 10 g) was treated with 0.5N-hydrochloric acid (250 ml) for 30 min. After filtration and washing until the effluent pH was ca. 4, the exchanger was treated with 0.5N-sodium hydroxide solution (250 ml) for 30 min. After further filtration and washing until the effluent was neutral, the exchanger was equilibrated with 0.02M-acetate buffer (pH 4.1). Columns (45 × 1.3 cm) were packed by continuous addition of a slurry of the exchanger (the column outlet being opened after the first 2 cm of exchanger had been packed) and allowed to settle. Samples (5-10 mg) of polysaccharides in buffer (1 ml) were washed into the columns with excess of buffer; elution of the acidic polysaccharides was performed by application of a sodium chloride concentration gradient (0→0.5M) in 0.02M-acetate buffer (pH 4.1) with total elution volume of 250 ml. Fractions (2.1 ml), collected using an automatic collector, were screened by

the phenol-sulphuric acid method[13]. The optical density of each fraction was read on a Unicam SP1300 spectrophotometer using filter 2.

Paper chromatography of sugars was carried out on Whatman No. 1 papers, using the following solvent systems (v/v):-

(a) benzene, butan-1-ol, pyridine, water (1:5:3:3, upper layer)

(b) ethyl acetate, pyridine, water (10:4:3)

(c) acetic acid, ethyl acetate, formic acid, water (3:18:1:4)

(d) acetic acid, ethyl acetate, formic acid, water (8:18:3:9)

(e) butan-1-ol, ethanol, water (4:1:5, upper layer)

(f) ammonia (d. 0.88), butan-2-one, water (1:200:17)

(g) acetic acid, butan-2-one, water (1:9:1, saturated with boric acid)

(h) butan-1-ol, ethanol, hydrochloric acid (0.1N) (1:10:5)[30]

(i) butan-1-ol, ethanol, phosphoric acid (0.1N) (1:10:5)[30].

Before using solvents (h) and (i), papers were dipped in an 0.3M-sodium dihydrogen phosphate solution and air-dried.

The following spray reagents were used:-

(1) Aniline oxalate. Reducing sugars were detected by spraying chromatograms with a saturated solution of aniline oxalate in ethanol, water [1:1 (v/v)], then heating at 150°C for ca. 5 min.

(2) Silver nitrate. Reducing sugars and sugar alcohols were detected by dipping chromatograms in silver nitrate reagent, prepared by adding saturated aqueous silver nitrate solution (1 ml) to acetone (100 ml), followed by sufficient water to redissolve the precipitate which formed. After drying, chromatograms were sprayed

with aqueous ethanolic N-sodium hydroxide solution [water, ethanol 1:9 (v/v)]. Chromatograms were preserved by treatment with 10% (w/v) sodium thiosulphate solution then washing with water.

(3) Periodate-permanganate. Reducing sugars and sugar alcohols were detected by spraying chromatograms with a mixture of 4 parts of 2% (w/v) sodium metaperiodate solution to 1 part (by volume) of 1% (w/v) potassium permanganate in 2% (w/v) sodium carbonate solution. After 10 mins, excess permanganate was removed by washing with water.

R_F values of sugars refer to distances moved relative to that of the solvent front. R_{gal} values of sugars refer to distances moved relative to that of D-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 (g.l.c.) of mixtures of O-methyl sugars [31,32] was carried out using a Pye Argon Chromatograph, at argon flow-rates of ca. 30 ml/min, on columns (120 × 0.5 cm) of:-

(i) 10% by weight of ethylene glycol adipate polyester on 45-60 mesh Gas-Chrom Z at 175°C

(ii) 10% by weight of butane-1,4-diol succinate polyester on 80-100 mesh Gas-Chrom P at 160°C.

Retention times (T) are quoted relative to methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside as standard.

Molecular-sieve chromatography (M-SC) was carried out on columns of:-

Bio-Gel P-10, Bio-Gel P-300, Bio-Gel A-5m (Bio-Rad Laboratories, Richmond, California)

Sephadex G-25M (Pharmacia Ltd., Uppsala, Sweden)

Sepharose 4B, Sepharose 2B (Pharmacia Ltd.)

Porasil Type A, Porasil Type B, Porasil Type C, Porasil Type E, (75-100 mesh; Waters Ltd., Stockport, Cheshire)

Bio-Glas-500, Bio-Glas-1500 (Bio-Rad Laboratories)

Corning CPG 10-1250 (120-200 mesh; Waters Associates, Framingham, Mass.).

Unless otherwise stated, M-sodium chloride solution, containing thymol (0.0005%) as a bacteriostatic agent, was used as eluant. The glass columns employed were pretreated with dichlorodimethylsilane (1% in benzene) at 60°C then oven-dried, to prevent deformation of elution patterns by "wall-effects". Care was taken to keep the "dead-space" at the column outlet to a minimum.

Gel bed materials were fully swollen by gentle stirring in the solution chosen as eluant, using several changes of solution. Except when using Bio-Gel P-10 or Sephadex G-25, the gel was deaerated. The gel slurry was added continuously to the column from a large filter funnel; the gel slurry in the funnel was stirred while excess eluant was allowed to percolate through the growing gel bed by regulating the flow of liquid from the tap at the bottom of the column to a rate of ca.0.5 ml/min. The top surfaces of columns of soft gel were stabilised by application

of a 1 cm layer of Sephadex G-25. Eluant was allowed to flow through the column for 2 days before use. Flow-rates of 0.5-1.0 ml/min were normally used for these columns.

Porous glass (silanised if necessary) and porous silica bed materials were added to the chosen eluant (ca. 400 ml); the suspension was deaerated then packed into a column as described for gel bed materials. Eluant flow-rates of 1.0-2.0 ml/min were used for these columns.

Best results were obtained by deaerating solvents before use, and using Mariotte flasks to ensure a constant pressure-head on columns.

Polysaccharides, dissolved in a solution (0.5-1.0 ml) slightly more concentrated than the eluant, were applied to the columns by careful layering on top of the bed material. Elution diagrams were obtained by the following methods:-

(i) Fractions (usually 2.1 ml) were collected by an automatic collector and screened by the phenol-sulphuric acid method[13]. The optical density was read on a Unicam SP1300 spectophotometer using filter 2. Collection of fractions was commenced as soon as the polysaccharide had been applied to the column.

(ii) Automatic recording of elution diagrams of dyed polysaccharides[33] is described in detail in Section V.

(iii) For detection of proteins or protein components of polysaccharides, the column effluent was automatically monitored at 254 nm using a Uvicord Type 4701A Optical Unit (L.K.B. Produkter A.B., Stockholm).

Zone electrophoresis of polysaccharides was carried out on strips (18 × 5 cm) of cellulose acetate film (Schleicher and Schüll, Dassel), using a Shandon Universal Electrophoresis Apparatus, Mark II, fed by a Vokam Power Unit, Model 2541, capable of providing constant voltage or constant current. 0.1M-ammonium carbonate buffer (pH 8.9) or 0.1M-acetate buffer (pH 4.7) was used as electrolyte. Electrophoresis was carried out at a field strength of 18.8 volts/cm for 2.5 hours. Polysaccharide bands were located by a modification of the periodate-rosaniline hydrochloride method [34] :-

(i) Strips were immersed in ethanol for 5 mins.

(ii) Strips were immersed in a solution of sodium metaperiodate [2% in water-ethanol, 1.5:10.0 (v/v)] for 10 mins.

(iii) Strips were immersed in a solution of reduced rosaniline hydrochloride (2%) [34], until staining was complete (ca. 15 mins). Iodine, from reduced periodate, normally appeared on the strips at this stage, but dissolved in the rosaniline hydrochloride solution.

(iv) Strips were washed four times in a solution of potassium metabisulphite (1 g) in N-hydrochloric acid (100 ml).

(v) Strips were washed in ethanol and dried between sheets of glass.

Polysaccharides were stained as a dark mauve band on a pale pink background.

Thin-layer electrophoresis of polysaccharides was carried out on "Phoroslides" (Millipore Ltd.) using 0.05M-ammonium

carbonate buffer (pH 8.9), 0.1M-ammonium carbonate buffer (pH 8.9), 0.1M-acetate buffer (pH 4.7) or 0.05M-borate buffer (pH 9.2) as electrolyte. The following procedure was adopted:-

(i) Slides (7.5 x 2.5 cm) were immersed in buffer, blotted to remove excess moisture and placed in a "Phoroslide" cell connected to a Vokam Power Unit.

(ii) Polysaccharide solutions (1% in buffer) were applied from a micropipette as thin bands 2 cm from the cathode end of the strip.

(iii) Electrophoresis was carried out at field strengths of 50-75 volts/cm along the strip for 0.5-10 mins.

(iv) Polysaccharide bands were located by the periodate-rosaniline hydrochloride technique[34] described for cellulose acetate electrophoresis; after electrophoresis of dyed polysaccharides, strips were dried in a current of air.

Amino acid analyses. Samples of polysaccharides, containing 1-2 mg protein (calculated from nitrogen content), were dissolved in constant-boiling hydrochloric acid (10 ml, redistilled and stored in the dark) in Pyrex tubes. Solutions were frozen in liquid nitrogen, and after evacuation to a pressure of < 0.1 mm Hg, the tubes were sealed then allowed to thaw. Hydrolyses were carried out at $105 \pm 0.5^{\circ}C$ for 24 hours, then the tubes were cooled, frozen and opened. The thawed solutions were filtered and concentrated to dryness on a rotary evaporator, and remaining hydrochloric acid was removed by repeated addition of deionised water followed by concentration to dryness; products were allowed to stand over sodium hydroxide and phosphorous pentoxide. Amino

acid analyses of hydrolysed samples were carried out using a Technicon Automatic Amino Acid Analyser, according to the method of Spackman et al[35] as modified by Benson and Patterson[36]. Mixtures of amino acids (ca. 0.2 mg in 0.5 ml buffer) were loaded on columns of:-

(1) Amberlite CG 120 resin (8 x 0.636 cm), using 0.35M-sodium citrate buffer, pH 5.22, as eluant at 55°C and L-2-amino-3-guanidopropionic acid (0.1 μM) as internal standard. This column was used to separate basic amino acids and ammonia.

(2) Technicon A resin (58 x 0.636 cm), using as eluant 0.2M-sodium citrate buffer, pH 3.28, with a buffer change after the elution of valine to 0.2M-sodium citrate, pH 4.25, at 52°C. Norleucine (0.1 μM) was used as internal standard. This column was used to separate neutral and acidic amino acids.

The peak area for each amino acid was calculated from the visible absorption trace on the chart, by hand measurement. Using the standard equivalent values for the amino acids and the known starting weight of sample, the quantity of each amino acid in the sample was calculated.

SECTION III

GUM EXUDATES FROM THE GENUS *LANNEA*

III A. A COMPARATIVE STUDY OF GUM EXUDATES FROM SOME SPECIES OF THE GENUS LANNEA A. RICH.

3A.1 INTRODUCTION

To date, the gum from only one species of the genus Lannea A. Rich. (Order, Sapindales; Family Anacardiaceae) has been studied. This species, properly described botanically as L. coromandelica (Houtt.) Merrill, has been studied under various synonyms, and extensive contradictions in the chemical literature have arisen as a result. The gum has been studied under the native vernacular names Modal,[37,38] Shemat,[37] Jeol[39,40] and also under the botanical synonyms Odina wodier,[40-45] L. grandis,[46-48] L. grandis Super,[49,50] L. grandis Engler[51-55] and L. coromandelica[56] . The publication[56] describing L. grandis and L. coromandelica as "closely related species" is not strictly correct. Other botanical synonyms exist for this species e.g. Dialium coromandelicum, Calesium grande, Haberlia grandis and Kalesiam[57] but have not appeared in the chemical literature. Problems of complex synonymy have already been encountered in other gum-forming genera[4,58] .

The chemical results quoted in several of these publications are mutually contradictory. The gum has been stated to be a neutral polysaccharide,[56] whereas all other investigators found it to be acidic. So far, the presence of only one aldobiouronic acid has been reported, but even this involves controversy, since the aldobiouronic acid has been stated to contain galacturonic acid,[45] the structure of the aldobiouronic acid being defined as

3-O-galacturonopyranosyl-D-galactose, and, in contrast, to contain 4-O-methylglucuronic acid,[37,38] the aldobiouronic acid being characterised rather more adequately, but still incompletely, as 4-methyl-1, 6-glucuronosido-galactose. Further disagreements involve other analytical parameters for the gum. Values for the specific rotation of the gum have been quoted as $+29^{\circ}$,[42] -44°[40] and $+45^{\circ}$[56]; the methoxyl content has been reported to be zero,[56] 2.38%[37] and 0.51%[40] - a value considered by the authors to be "very low and not of structural significance"; equivalent weight values varied from 1150[40] to 1245[37] and 1361.[47] A further unusual feature for a plant gum is the reported absence of nitrogen,[37,40] yet there are grounds for believing that the presence of nitrogen in a plant gum may be of greater fundamental significance than has been frequently assumed.[7,59,60] The ratio of galactose to arabinose has been found to be 1.3/1,[42] 3/1,[40] 4/1[56] and 5/1[37], and in complete disagreement, Dhar and Mukherjee[45] found the gum to contain more arabinose than galactose. Moreover, the molecular weight of the polysaccharide was reported to be 17.5×10^6,[46] but other investigators found the molecular weight of the methylated polysaccharide to be 1.68×10^5.[39]

Such a set of contradictions required investigation. Since all of the work cited above is of Indian origin, specimens of gum from L. coromandelica and from other Lannea species were obtained from different locations in an attempt to establish whether the genus Lannea is indeed unprecedented in its variability. Such a result would be surprising on botanical grounds. The Lannea genus occurs widely in Africa,[61-63] India[64] and eastward to

Indo-China and Hai-nan [57,65] but is less variable than many, and has relatively few, closely related species. [65] The gum exudates have been used medicinally, [64] as adhesives [66] and also in cloth printing. [64]

This section presents results obtained for a Ceylonese specimen of L. coromandelica gum, a Nigerian specimen of L. schimperi gum and two Sudanese specimens of L. humilis gum.

3A.2 ORIGIN OF SPECIMENS

The gum from L. coromandelica (Houtt.) Merrill was obtained in October, 1967 from the Research Officer of the Conservator of Forests, Colombo 2, Ceylon. Gum from L. schimperi (Hochst. ex A. Rich.) Engl. was collected at Shilca Research Station on 25th March, 1969 by Mr. G.O. Magaji for Professor D.M. Ramsay, Department of Plant Science, Ahmadu Bello University, Zaria, Nigeria. Gum from L. humilis (Oliv.) Engl. was obtained from the Gum Research Officer to the Republic of the Sudan; sample A was collected near El Obeid in April, 1969, and sample B from Layyuna Central Forest Reserve, Central Kordofan in May, 1969.

3A.3 RESULTS

Purification of Samples

Each of the four gum samples dissolved readily in cold distilled water after several hours. The solutions were filtered, then dialysed for several days; the polysaccharides were recovered as the freeze-dried products. Recoveries, on a dry weight basis, were in the range 75-80% for all four samples.

A portion of each of the four gum samples was dissolved in distilled water and exhaustively electrodialysed to convert the gum polysaccharides to the free acid form. The acidic polysaccharides were recovered by freeze-drying.

Analytical Comparison of the Purified Gums

The results of analyses of the purified, non-electrodialysed products are shown in table 3A.1, and those for the electrodialysed products in table 3A.2. Graphs of viscosity number against concentration for the four samples are shown in fig. 3A.1.

Periodate oxidations were carried out under conditions determined during a Smith-degradation study of L. humilis gum (see Section III B). Polysaccharide (40 mg) was dissolved in water (10 ml) then 0.5M-sodium metaperiodate solution (10 ml) was added. The mixture was left in darkness at room temperature for 72 hours. Aliquots (1 ml) were withdrawn, and the periodate reduced and formic acid released were determined.

Hydrolysis Studies

Each sample (25 mg) was hydrolysed with N-sulphuric acid for 7 hours on a boiling water bath. Paper chromatography of the hydrolysates in solvents (a), (b) and (c) showed the presence of galactose, arabinose, and a trace of rhamnose in each gum. Chromatograms run in solvent (c) also showed the presence of two components (R_{gal} 0.21, 0.61) which corresponded to aldobiouronic acids characterised for L. humilis gum (Section III B).

Each sample (25 mg) was hydrolysed with 2N-sulphuric acid for 7 hours on a boiling water bath. Paper chromatography of the hydrolysates in solvents (c) and (h) revealed the above

neutral sugars together with 4-O-methyl-glucuronic acid, galacturonic acid, and smaller amounts of glucuronic acid and glucurono-6,3-lactone from each sample.

Chromatograms for each of the four samples were similar, indicating no gross differences in constituent sugars of the polysaccharides.

Examination by Molecular-Sieve Chromatography (M-SC) and Ultracentrifugation

Each polysaccharide (50 mg) was dyed with Procion Red M-2B dye,[67] and examined by M-SC, using an automated method,[33] on the following columns:-

(a) Bio-Gel A-5m (40 × 1.5 cm)

(b) Porasil C (40 × 1.5 cm)

(c) Porasil B (40 × 1.5 cm)

(d) Porasil A (40 × 1.5 cm)

The eluant was M-sodium chloride solution. Elution diagrams are shown in fig. 3A.2, and further M-SC studies on Lannea gums are recorded in Section V.

In each case, two main peaks were found, one at the void volume of the column, and the other near the exclusion volume, except when Porasil A was employed; then a single, tailing peak at the void volume was recorded. The higher the molecular weight sieving-range of the column employed, the smaller was the void volume peak. Unpurified L. humilis gum sample B (50 mg) was dyed and examined on column (a); the elution diagram was the same as that obtained for the purified gum.

Solutions (0.5%) of the polysaccharides in 0.5M-sodium chloride solution were examined by ultracentrifugation at 44,770 r.p.m. Photographs revealed, in each case, a single smooth, broad peak.

Amino Acid Analyses

A portion of each sample (containing ca. 2 mg protein) was hydrolysed and examined for amino acids. Results are shown in table 3A.3.

The analyses are similar for all four Lannea samples, each having a very high proline content, and threonine, serine and leucine as the other major constituents. L. humilis gum differs from L. coromandelica and L. schimperi gums in possessing more proline than threonine or serine. The other amino acids are present in relatively small amounts.

In each analysis, a component was found which eluted more slowly than the amino acids on column (2). This component corresponded to glucosamine on this column, which was found to be capable of resolving a mixture of glucosamine and galactosamine.

In each case, the nitrogen recovery was exceedingly low, and each sample contained several components which eluted faster than the amino acids determined on column (2). These components are probably degradation products of amino acids and sugars, which can arise on hydrolysis.[68] A medium-sized and a large peak found between these unknown components and the first of the amino acid peaks were identified tentatively as methionine sulphoxide and hydroxyproline. The latter peak obscured that due to any aspartic acid present.

3A.4 DISCUSSION

Since the first study of a gum from the genus Lannea in 1948,[41] many conflicting results for the species concerned have been published. In an attempt to determine whether the chemical characteristics of Lannea gum exudates vary widely, samples of three Lannea species were obtained for study from widely differing locations, viz. Nigeria, Sudan and Ceylon. Each sample dissolved readily in distilled water, and the recoveries of purified polysaccharides were similar.

With such complex natural products as gum exudates, some inter- and intra-species variation in properties and composition would be expected, and recent studies have shown that variations in some genera e.g. Prunus[9] and Combretum[8], which, botanically, are best described as systems of complexes, are much larger than in others e.g. Acacia[4] and Araucaria.[30] Analyses of the four purified Lannea samples (tables 3A.1 and 3A.2) indicate that analytical parameters of Lannea species show only minor variations. The strong similarity found between the two samples of L. humilis gum suggests, moreover, that neither the inter- nor intra-species variation in the genus Lannea is likely to be great.

Molecular weights ($\bar{M}_w$) of the four polysaccharides were found to be in the range $(2.4 - 3.1) \times 10^6$. The value of $^{dn}/_{dc}$ found for L. coromandelica gum was used for all calculations, as it has been reported[69] that within one genus, values of $^{dn}/_{dc}$ of 23 species are almost identical. Limiting flow-time values of 8-14 ml g^{-1} were recorded for the Lannea samples, and these low viscosity values indicate a globular rather than rod-shaped

structure for the molecules. Furthermore, the graphs of viscosity number against concentration (fig. 3A.1) are of similar gradient, indicating that the molecular shapes are similar, with intermolecular attractions of the same order. Neutral sugar ratios were calculated on the assumption that the uronic acid residues were attached to galactose, since chromatography in solvent (c) showed the presence, in each hydrolysate, of components with the same mobility as the aldobiouronic acids characterised for L. humilis gum (Section III B) and no evidence for other aldobiouronic acids was obtained. As neutral O-methyl sugars were not found on chromatographic examination of hydrolysates, the methoxyl content of each sample can be assumed to arise entirely from 4-O-methylglucuronic acid, giving 4-O-methylglucuronic acid contents of 2.5% for both samples of L. humilis gum, 5.7% for L. schimperi gum and 10.1% for L. coromandelica gum. Only in this last case does 4-O-methylglucuronic acid occur as the major uronic acid. Characteristic features of this genus appear to be a high galactose/arabinose ratio and high values for the periodate reduced and formic acid released on periodate oxidation; indeed, the periodate oxidation results are similar to the highest values obtained for Araucaria gums,[30] and are appreciably higher than typical values for Acacia gums.[70,71] Hydrolysis studies on the Lannea gums revealed rhamnose in small amount, in addition to the neutral sugars (galactose and arabinose) found by earlier investigators of L. coromandelica gum, and, unlike previous reports[37,38,45] where a single uronic acid was found in this gum, the uronic acid system has been shown to be complex with galacturonic , glucuronic

and 4-O-methylglucuronic acids all present in each sample.

Comparison of these results with earlier investigations of L. coromandelica gum show that some previous values e.g. $[\alpha]_D$ + 29°(42) and neutralisation equivalent 1150(40) have been substantiated, but that the work of several investigators is clearly incorrect in several details. Perhaps the most misleading publication is that in which L. coromandelica gum is described as a neutral polysaccharide with no methoxyl content.(56) The presence of nitrogen and rhamnose was not established previously, and the ratio of galactose to arabinose found (ca. 6/1) is greater than in any of the earlier reports - clearly, the work in which more arabinose than galactose was reported(45) must be discounted. The quite arbitrary dismissal of a methoxyl content of 0.51%(40) as being of no structural importance was also a major error. Failure to appreciate the significance of this methoxyl content was perhaps partly responsible for the failure to identify 4-O-methylglucuronic acid in the gum. The value of -44° quoted for $[\alpha]_D$(40) must also be regarded as a major error. Light-scattering values obtained are in reasonable agreement with that of earlier workers,(39) allowing for some degradation during the methylation procedure, and contradict the unusually high value of 17.5×10^6 reported(46) for $\bar{M}_w$ of L. coromandelica gum. The value of $^{dn}/_{dc}$ employed(46) (0.1695) also appears to be unusually high for an acidic polysaccharide. It is interesting to observe that, allowing for the difference in $^{dn}/_{dc}$ value, $\bar{M}_w$ of L. coromandelica gum recorded above differs from that reported by Chaudhuri and Mukherjee(46) by a factor of almost exactly 10^2.

The most likely explanations of the confusion that has arisen in the analytical parameters of L. coromandelica gum are that the chemical analyses of previous workers have been faulty; or, perhaps more likely, that the studies were made on commercial mixtures, insufficient attention being given to the collection and correct botanical identification of the samples studied. The recommendations of a botanical authority in this respect have been recorded.[4,72] An implication of these analyses recorded above is that at least some, if not all, of the structural studies reported for L. coromandelica gum[39,40,56] cannot be correct.

Further studies on the four Lannea samples involved molecular-sieve chromatography (M-SC), ultracentrifugation and amino acid analyses.

M-SC of the dyed samples showed two peaks, the relative heights but not elution volumes of which depend on the dyeing procedure (see Section V). One peak appeared at the void volume of the column, and the other near the exclusion volume, except on the Porasil A column (fig. 3A.2). Porasil A appears to have such a low sieving-range that even the smallest gum molecules are eluted within the column sieving-range. This behavior is not consistent with each gum having two components. If a two-component system showed peaks at the void- and exclusion-volumes of one column, then the use of a column possessing a higher molecular weight sieving-range would leave the exclusion volume peak unaltered and sieve the void volume peak, at least partially. With Lannea gums, the void and exclusion volume peaks alter with each other. These results are consistent with the Lannea gums possessing a broad

molecular weight distribution. The void and exclusion volume peaks would be due to material not sieved by the column as its molecular weight range was higher and lower than the sieving limits of the column respectively. The use of a column of higher sieving-range would result in a smaller void volume peak and an enhanced exclusion volume peak, as found for the Lannea samples. In the elution diagrams on Bio-Gel A-5m, a small, broad peak between the two major peaks probably corresponds to the true molecular weight distribution in that molecular weight range. The other column materials are less efficient than Bio-Gel A-5m, so do not show such a feature in their elution diagrams. M-SC of dyed, crude L. humilis sample B gave an elution diagram identical to that obtained using purified gum, indicating that the gums had not been significantly altered on purification. Ultracentrifugation of the samples produced results confirming that Lannea gums have a broad molecular weight distribution.

The similarity in nitrogen recoveries of the amino acid analyses (table 3A.3) justifies a comparison, but the results must be regarded with caution. The analyses indicate that the Lannea samples contain proteinaceous material characterised by a large proline (and possibly also a high hydroxyproline) content, in which respect it is similar to certain animal, connective tissue proteins.[73,74] Tryptophan was not determined because of its modification and degradation during hydrolysis. The low nitrogen recoveries were probably due largely to the formation of degradation products on hydrolysis[68], and to the fact that aspartic acid was obscured and could not be estimated. Methionine sulphoxide and

hydroxyproline were identified tentatively in the hydrolysates, but were not estimated. The presence of a component corresponding to glucosamine in each sample is of interest, as it has been observed in all amino acid analyses of plant gums so far performed[7], and has been characterised from Azadirachta indica gum.[75] Nolan and Smith have observed[76] that hydrolysis for 12 hours with 6N-hydrochloric acid at 97-100°C in the presence of amino acids and monosaccharides resulted in 47% loss of glucosamine. About 95% destruction of glucosamine would therefore be expected to arise from hydrolysis conditions employed in these analyses. If this degradation is assumed, it can be calculated that glucosamine would be present in the original gums in an amount corresponding to ca. one residue per polysaccharide molecule, and a linkage of carbohydrate and protein moieties involving this amino sugar cannot be discounted.

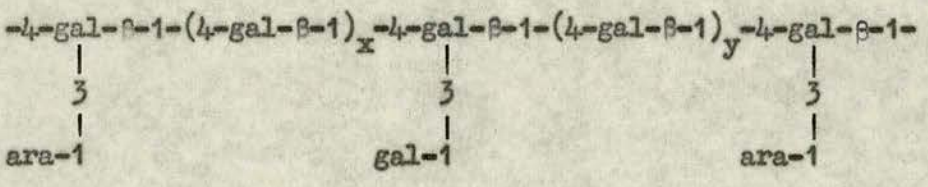
M-SC and amino acid analysis studies confirm that chemical and physical differences between the samples of the genus Lannea investigated in this section are small.

III B. SOME STRUCTURAL FEATURES OF THE GUM FROM LANNEA HUMILIS (OLIV.) ENGL.

3B.1 INTRODUCTION

From analytical results obtained in Section III A, there is no doubt that Lannea gum exudates are acidic polysaccharides that exist, as is customary, in the natural state as complex, nearly neutralised salts of the polysaccharide gum acid.

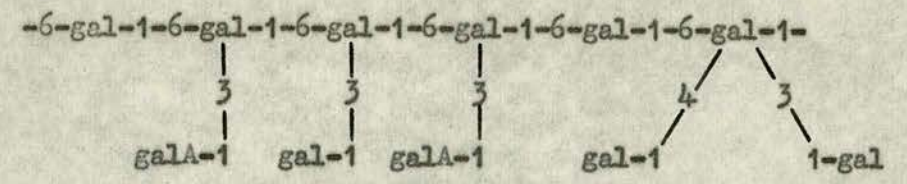
Two major attempts at establishing a structure for L. coromandelica gum have been made,[39,40,56] and have produced widely differing structures. Ramachandran and Joshi[56] suggest a structure based on a repeating unit of a backbone of β-1,4-linked galactose units, some of which are substituted at the 3-position by arabinose or galactose units, and may be represented by:-



(x + y = 4; gal, D-galactopyranose; ara, L-arabinofuranose)

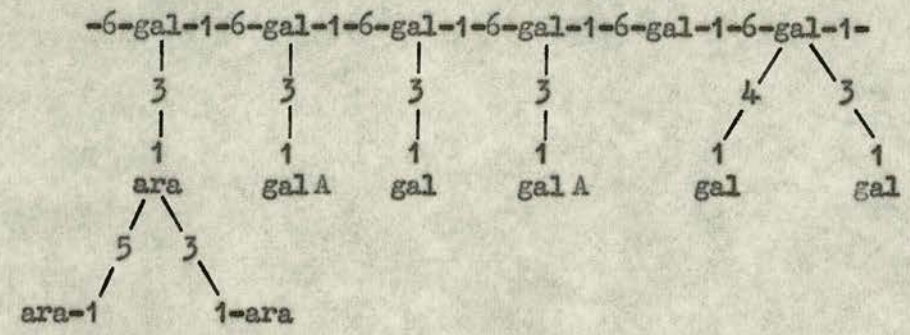
This structure is based on such erroneous results (e.g. that the gum is a neutral polysaccharide containing only galactose and arabinose), it must be largely discounted.

Bhattacharyya and Rao[40], on the other hand, propose a repeating structure for the degraded gum based on a main chain of 1,6-linked galactose units to some of which are attached galacturonic acid or galactose in the 3-position, or two galactose units in the 3- and 4-positions, and which may be represented by:-



(gal, galactopyranose; galA, galactopyranosyluronic acid)

This structure has been extended by Bhattacharyya and Mukherjee[39] to incorporate arabinose side-chains, producing a possible repeating structure for the whole gum, which may be represented by:-



(gal, D-galactopyranosyl; galA, D-galactopyranosyluronic acid; ara, L-arabinofuranosyl).

An attempt to correlate these structures with light-scattering measurements on the polysaccharide[46] has apparently been successful, but the structures are based on several doubts and inaccuracies. The arabinose content appears to be high, a strong negative optical rotation (-44°) was reported, rhamnose was not detected, nitrogen was not found, the methoxyl content was deliberately ignored and galacturonic acid was stated to be the only uronic acid involved.

Preliminary hydrolysis experiments on Lannea gums (Section III A) indicated that in all three species studies, rhamnose was present in small amount, and that the uronic acid system is complex,

with galacturonic, glucuronic and 4-O-methylglucuronic acids all present.

In this section, some structural features of a gum from the genus Lannea are elucidated. The gum from L. humilis (sample B) was chosen, as it was available in an amount sufficient to support a fairly complete study.

3B.2 RESULTS

Purification of Lannea humilis Gum

As described previously (Section III A), the crude gum was purified by extraction into distilled water, exhaustive dialysis and freeze-drying.

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), and on thin-layer electrophoresis in 0.1M-ammonium carbonate buffer (pH 8.9), 0.1M-acetate buffer (pH 4.7) and 0.05M-borate buffer (pH 9.2). Such conditions were shown able to resolve a mixture of gums from Acacia senegal (uronic acid, 14%) and A. pycnantha (uronic acid, 4%). L. humilis gum was chromatographed on a DEAE-cellulose column [29] (46 × 1.3 cm), buffered at pH 4.1 by 0.02M-acetate buffer. Gradient elution with sodium chloride (0.0 → 0.5M) in 0.02M-acetate buffer gave a single, symmetric peak. Examination of the gum by molecular-sieve chromatography and ultracentrifugation also revealed no sharp heterogeneity (Section III A).

Identification of Neutral Sugars

L. humilis gum (10g) was hydrolysed with N-sulphuric acid (500 ml) for 7 hours on a boiling water bath. The cooled solution was neutralised with barium carbonate, filtered, deionised with Amberlite IR-120(H) resin, and concentrated to a syrup, which was applied to a column (34 x 3.8 cm) of Duolite A-4 resin in the formate form. Elution with distilled water yielded the neutral sugars present in the hydrolysate. After concentration to a syrup, the neutral fraction was examined by paper chromatography in solvents (a), (b), (c), (h) and (i), which showed two components identical with authentic D-galactose and L-arabinose and a third, minor component identical with authentic L-rhamnose in these solvents. A portion of the neutral fraction was chromatographed on Whatman No. 3MM papers in solvent (a). The position of rhamnose was located by spraying papers on which L-rhamnose had been run as a marker, and the appropriate zone was eluted with water. This solution was concentrated to a syrup, and paper chromatography in the above solvents showed intense spots identical with authentic L-rhamnose.

An attempt to crystallise rhamnose from this syrup using aqueous ethanol produced a brown precipitate which did not melt within the normal range of monosaccharides. This precipitate was shown to give no colour with aniline oxalate spray after paper chromatography in solvents (a), (b) and (c), but was found to react in the phenol-sulphuric acid test [13].

Identification and Characterisation of Acidic Components

After elution of neutral sugars with distilled water from the N-sulphuric acid hydrolysate applied to the Duolite A-4 resin column, elution with 5% formic acid yielded the acidic components. This solution was concentrated to a syrup and the remaining formic acid was removed by repeated addition of water followed by reconcentration to a syrup. Paper chromatography of the syrup in solvent (c) indicated the presence of a trace of galactose and two acidic fractions having R_{gal} 0.21 and 0.61. After fractionation of the syrup on Whatman No. 3MM papers in solvent (c), these two acidic fractions were eluted and concentrated to syrups.

Fraction 1 (186 mg) had $[\alpha]_D + 48^\circ$ (c, 0.93). The syrup (10 mg) was hydrolysed (2N-sulphuric acid, 7 hours, boiling water bath), and paper chromatography of the hydrolysate in solvents (a), (c), (h) and (i) revealed galactose, galacturonic and glucuronic acids and glucurono-6, 3-lactone. These results suggest fraction 1 is a mixture of two aldobiouronic acids, one having an α-D-linkage, the other a β-D-linkage, one containing galacturonic acid and the other glucuronic acid. Paper chromatography of fraction 1 in solvent (c) still revealed only one spot having R_{gal} 0.21, but chromatography in solvent (d) revealed two merging spots having R_{gal} ca. 0.70, 0.73. Double development in solvents (c) and (d) produced no improvement in resolution. Zone electrophoresis of fraction 1 on Whatman No. 1 paper in 0.05M-borate buffer (pH 9.2) resulted in a single spot. Paper chromatography in solvent (a) for one week was found to produce a chromatogram in which two components (R_{gal} ca. 0.14, 0.19) were clearly visible,

and although merging somewhat, were sufficiently separated to be isolable in reasonably pure form. The remaining fraction 1 was fractionated on Whatman No. 3MM papers in solvent (a) for one week, and its two components were eluted and concentrated to syrups.

Fraction 1a (33 mg) had $[\alpha]_D$ + 107° (c, 1.09), suggesting the presence of an α-D-linkage. Paper chromatography in solvent (c) showed a single spot having R_{gal} 0.21, and in solvent (a) showed fraction 1a had R_{gal} ca. 0.14, and contained a trace of fraction 1b as impurity. Fraction 1a (7 mg) was hydrolysed (2N-sulphuric acid, 7 hours, boiling water bath), and paper chromatography of the hydrolysate in solvents (a), (c) and (h) revealed about equal amounts of galactose and galacturonic acid with a trace of glucurono-6, 3-lactone. Sodium borohydride (25 mg in 5 ml water) was added to fraction 1a (8 mg) and the mixture was left at room temperature for 18 hours. Excess borohydride was destroyed by the addition of Amberlite IR-120 (H) resin. Resin and solvent were removed, and borate was volatilised as methyl borate by repeated additions of methanol followed by concentration to dryness. The reduced product was hydrolysed (2N-sulphuric acid, 7 hours, boiling water bath) and paper chromatography of the hydrolysate in solvents (a), (c), (g) and (h) indicated about equal amounts of galactitol and galacturonic acid, with a trace of galactose still present. Fraction 1a (10 mg) was methylated by the Kuhn method. Methanolysis followed by g.l.c. examination of the mixture of methyl glycosides on column (i) indicated the presence of 2,3,4-tri-O-methyl-D-galacturonic acid (T 5.25) and 2,3,6-tri-O-methyl-D-galactose (T 2.30, 3.10, 3.58).

The methanolysate was then reduced with sodium borohydride. After removal of methyl glycosides by mild acid hydrolysis (N-sulphuric acid, 4 hours, boiling water bath), the products were examined by paper chromatography in solvents (e) and (f). This revealed 2,3,4-tri-O-methyl-D-galactose [R_G 0.74, solvent (e); 0.35, solvent (f)] and 2,3,6-tri-O-methyl-D-galactose [R_G 0.74 solvent (e); 0.50, solvent (f)]. These experiments led to the identification of fraction 1a as 4-O-(α-D-galactopyranosyluronic acid)-D-galactose.

Fraction 1b (48 mg) had $[\alpha]_D + 8^\circ$ (c, 1.06), suggesting the presence of a β-D-linkage, and was chromatographically identical to authentic 6-O-(β-D-glucopyranosyluronic acid)-D-galactose in solvents (a) (R_{gal} ca. 0.19), (c) (R_{gal} 0.21) and (d) (R_{gal} 0.72). Chromatography in solvent (a) also revealed a small amount of fraction 1a as impurity. Fraction 1b (10 mg) was hydrolysed (2N-sulphuric acid, 7 hours, boiling water bath), and paper chromatography of the hydrolysate in solvents (a), (c) and (h) revealed galactose together with glucuronic acid and glucurono-6, 3-lactone, in about equal amounts. A trace of galacturonic acid was also found. Fraction 1b (10 mg) was reduced with sodium borohydride as described for fraction 1a, and the reduced product was hydrolysed (2N-sulphuric acid, 7 hours, boiling water bath). Paper chromatography of the hydrolysate in solvents (a), (c), (g) and (h) indicated galactitol together with glucuronic acid and glucurono-6, 3-lactone, in about equal amounts. Fraction 1b (15 mg) was methylated by the Kuhn method. Methanolysis followed by g.l.c. examination of the mixture of methyl glycosides on column (i) indicated the presence of 2,3,4-tri-O-methyl-D-glucuronic acid

(T 2.14, 2.70), 2,3,4-tri-O-methyl-D-galactose (T 5.55) and 2,3,5-tri-O-methyl-D-galactose (T 3.69, 4.32). The methanolysate was then reduced with sodium borohydride. After removal of methyl glycosides by mild acid hydrolysis, the products were examined chromatographically in solvents (e) and (f). This revealed 2,3,4-tri-O-methyl-D-galactose [red spot: R_G 0.72, solvent (e); 0.36, solvent (f)] and a red-black spot [R_G 0.84, solvent (e); 0.72, solvent (f)] corresponding to 2,3,4-tri-O-methyl-D-glucose and 2,3,5-tri-O-methyl-D-galactose. Double development in solvent (f) 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 fraction 1b as 6-O-(β-D-glucopyranosyluronic acid)-D-galactose.

Fraction 2 (151 mg) had $[\alpha]_D + 10^\circ$ (c, 0.75), suggesting the presence of a β-D-linkage, and was chromatographically identical to 6-O-(4-O-methyl-β-D-glucopyranosyluronic acid)-D-galactose in solvents (c) (R_{gal} 0.61) and (d) (R_{gal} 0.92). These chromatograms also revealed a trace of galactose as impurity. Fraction 2 (8 mg) was hydrolysed (2N-sulphuric acid, 7 hours, boiling water bath) and paper chromatography of the hydrolysate in solvents (a), (c), (h) and (i) revealed about equal amounts of 4-O-methylglucuronic acid and galactose. Reduction of fraction 2 (15 mg) with sodium borohydride as described for fraction 1a was followed by hydrolysis of the reduced product (2N-sulphuric acid, 7 hours, boiling water bath). Paper chromatography of the hydrolysate in solvents (a), (c), (g) and (h) indicated about equal amounts of galactitol and 4-O-methylglucuronic acid, with a trace of unreduced galactose.

Fraction 2 (15 mg) was methylated by the Kuhn method. Methanolysis followed by g.l.c. examination of the mixture of methyl glycosides indicated the presence of the O-methyl sugars which were found for fraction 1b. Reduction of the methanolysate followed by removal of methyl glycosides then paper chromatography in solvents (e) and (f) again showed the presence of the O-methyl sugars which were found for fraction 1b. These experiments led to the identification of fraction 2 as 6-O-(4-O-methyl-β-D-glucopyranosyluronic acid)-D-galactose.

Preparation of Degraded Gum A

To determine conditions for preparation of degraded gum A, L. humilis gum (1g) was dissolved in 0.01N-sulphuric acid (40 ml) and heated on a boiling water bath for 120 hours. Aliquots (1 ml) were withdrawn at intervals, neutralised with barium carbonate, filtered, deionised with Amberlite IR-120(H) resin and concentrated to syrups, then examined by paper chromatography in solvent (c). It was found that arabinose appeared within 1 hour and the quantity released increased with time. Galactose first appeared in trace amount after 4 hours, then increased with time, but was present in much smaller quantity than arabinose. Traces of rhamnose were found on the chromatograms. Small spots corresponding to authentic 3-O-β-L-arabinofuranosyl-L-arabinose (R_{gal} 1.20), 3-O-β-L-arabinopyranosyl-L-arabinose (R_{gal} 0.76) and 3-O-α-D-galactopyranosyl-L-arabinose (R_{gal} 0.55) were also found. These components were maximal after 36-48 hours. Galactobioses and higher oligosaccharides appeared in trace amounts after 36 hours, but quantities were still extremely small after 100 hours.

Syrups in which the presence of disaccharides containing arabinose had been indicated were further chromatographed in solvents (a), (b) and (f). Results verified that these disaccharides were chromatographically identical with authentic 3-O-α-D-galactopyranosyl-L-arabinose [pink spot with aniline oxalate: R_{gal} 0.67, solvent (a); 0.73, solvent (b)], 3-O-β-L-arabinofuranosyl-L-arabinose [R_{gal} 1.40, solvent (f)] and 3-O-β-L-arabinopyranosyl-L-arabinose [R_{gal} 0.82, solvent (a); 0.91, solvent (b); 0.55, solvent (f)].

Degraded gum A was prepared by heating a solution of L. humilis gum (25g) in 0.01N-sulphuric acid (1l) for 100 hours on a boiling water bath. After cooling, the brown solution was filtered and exhaustively dialysed against running tap water. Degraded gum A was isolated as a freeze-dried, pale-brown product (18.9g; yield, 76%).

Examination of Degraded Gum A

An attempt to record the optical rotation of degraded gum A was unsuccessful due to the brown colour of its solution. $\bar{M}_n$ of the gum, determined by end-group analysis, was 4,320. Hydrolysis of the gum (50 mg) with N-sulphuric acid for 7 hours on a boiling water bath followed by paper chromatography of the resulting syrup in solvents (c) and (d) showed the presence of galactose, arabinose and the same aldobiouronic acids already characterised from the whole gum. Degraded gum A (322 mg) was methylated by the Haworth and Purdie procedures to give a product (219 mg) (Found: OMe, 39.5%). Methanolysis of a portion of this product, followed by g.l.c. examination of the mixture of methyl glycosides gave results

shown in table 3B.1. Hydrolysis of the mixture of methyl glycosides, and paper chromatographic examination of the resulting syrup in solvents (e) and (f) indicated the presence of 2-O-methyl-D-galactose in addition to those O-methyl sugars identified as their methyl glycosides by g.l.c.

Preparation of Degraded Gum B

Degraded gum A (12.7 g) was hydrolysed with 0.5N-sulphuric acid (1 l) for 1 hour on a boiling water bath. After removal of an aliquot (8 ml), the remaining brown solution was exhaustively dialysed against running tap water, and degraded gum B was isolated as a freeze-dried, pale-brown product (9.9 g; yield, 78%). The aliquot removed was neutralised with barium carbonate, filtered, deionised with Amberlite IR-120(H) resin and concentrated to a syrup. Paper chromatographic examination in solvents (a), (b) and (c) indicated galactose, a smaller amount of arabinose and three neutral disaccharides chromatographically identical with authentic 6-O-β-D-galactopyranosyl-D-galactose [R_{gal} 0.30, solvent (a); 0.38, solvent (b)], 3-O-β-D-galactopyranosyl-D-galactose [R_{gal} 0.49, solvent (a); 0.55, solvent (b)], (major components), and 3-O-α-D-galactopyranosyl-L-arabinose [R_{gal} 0.67, solvent (a); 0.73, solvent (b); 0.55, solvent (c)], (trace component, pink spot with aniline oxalate), together with higher oligosaccharides.

Examination of Degraded Gum B

Again, the optical rotation of degraded gum B could not be recorded due to the brown colour of its solution. $\bar{M}_n$ of the gum, determined by end-group analysis, was 3740. Hydrolysis of the gum

(50 mg) with 0.5N-sulphuric acid for 1 hour on a boiling water bath followed by paper chromatography of the resulting syrup in solvents (a) and (b) indicated the presence of galactose, a trace of arabinose, 3-O-β-D-galactopyranosyl-D-galactose, rather less 6-O-β-D-galactopyranosyl-D-galactose and higher oligosaccharides. Hydrolysis of the gum (50 mg) with N-sulphuric acid for 7 hours on a boiling water bath followed by paper chromatography of the resulting syrup in solvents (c) and (d) indicated the presence of the same aldobiouronic acids already characterised from the whole gum. Degraded gum B (342 mg) was methylated by the Haworth and Purdie procedures to give a product (294 mg) (Found: OMe, 39.9%). Methanolysis of a portion of this product followed by g.l.c. examination of the mixture of methyl glycosides gave results shown in table 3B.2. Hydrolysis of the mixture of methyl glycosides and paper chromatographic examination of the resulting syrup in solvents (e) and (f) indicated the presence of 2-O-methyl-D-galactose in addition to those O-methyl sugars already identified as their methyl glycosides by g.l.c.

Preparation of Degraded Gum C

Degraded gum A was subjected to a Smith degradation. Degraded gum A (2.04 g) was dissolved in water (50 ml) and 0.75M-sodium metaperiodate solution (50 ml) was added. After 96 hours in darkness at room temperature, 13.4 m moles periodate/g polysaccharide had been reduced, and 6.5 m moles formic acid/g polysaccharide had been formed. The reaction was stopped by addition of ethylene glycol (6 ml) and the solution was dialysed against running tap water for 3 days. Sodium borohydride (1 g) was added,

and after 30 hours at room temperature, the solution was dialysed for a further 3 days. The solution was made 1N with respect to sulphuric acid and the polyalcohol was hydrolysed for 48 hours at room temperature, then dialysed for 2 days. Degraded gum C was isolated as the freeze-dried product (0.514g; yield, 25%).

Examination of Degraded Gum C

$[\alpha]_D$ was found to be +25° (c, 0.92). $\bar{M}_n$ of degraded gum C, determined by end-group analysis, was 1950. Hydrolysis of the gum (50 mg) with 0.5N-sulphuric acid for 1 hour on a boiling water bath followed by paper chromatography of the resulting syrup in solvents (a) and (b) indicated the presence of galactose, 3-O-β-D-galactopyranosyl-D-galactose (major disaccharide), 6-O-β-D-galactopyranosyl-D-galactose (trace component), and higher oligosaccharides. Degraded gum C (268 mg) was methylated by the Haworth and Purdie procedures to give a product (177 mg) (Found: OMe, 39.1%). Methanolysis of a portion of this product, followed by g.l.c. examination of the mixture of methyl glycosides gave results shown in table 3B.3. Hydrolysis of the mixture of methyl glycosides and paper chromatographic examination of the resulting syrup in solvents (e) and (f) indicated the presence of 2-O-methyl-D-galactose in addition to those O-methyl sugars already identified as their methyl glycosides by g.l.c.

Methylation of L. humilis Gum

The gum (324 mg) was methylated by the Haworth and Purdie procedures to give a product (246 mg), $[\alpha]_D$ 0° (c, 1.15 in chloroform) (Found: OMe, 41.6%). The gum (372 mg) was also

methylated by the sodium hydride, methyl iodide, dimethylsulphoxide procedure[21] to give a product (319 mg), $[\alpha]_D$ -11° (c, 0.95 in chloroform) (Found: OMe, 40.5%). Methanolysis of a portion of each product followed by g.l.c. examination of the mixtures of methyl glycosides gave results shown in table 3B.4. The relative amounts of the O-methyl sugars could not be satisfactorily estimated due to incomplete resolution of several of the components, but in both methanolysates, 2,3,4,6-tetra-, 2,4,6- and 2,3,4-tri-O-methyl-D-galactose were present in ratios of approximately 8/5/1, and the ratio of 2,3,4- to 2,3,5-tri-O-methyl-L-arabinose was smaller for the sample employing the Haworth and Purdie methylation procedures. Large amounts of 2,4-di-O-methyl-D-galactose were found. Hydrolysis of the mixtures of methyl glycosides and paper chromatographic examination of the resulting syrups in solvents (e) and (f) indicated the presence in both of 2-O-methyl-D-galactose in addition to those O-methyl sugars already identified as their methyl glycosides by g.l.c. Only a trace of 2-O-methyl-D-galactose was found in the sample employing the sodium hydride, methyl iodide, dimethylsulphoxide methylation procedure, but a larger quantity was found in the sample methylated by the Haworth and Purdie procedures.

Preparation and Examination of Polysaccharide I

Preliminary, small-scale experiments established that an 0.25M-sodium metaperiodate solution and oxidation time of 72 hours were required for L. humilis gum.

L. humilis gum (44.4g) was dissolved in distilled water (1250 ml) and 0.5M-sodium metaperiodate solution (1250 ml) was

added. Oxidation was carried out in darkness at room temperature and was followed by measurement of release of formic acid with time (fig. 3B.1). After 72 hours, 10.0 m moles periodate/g polysaccharide had been reduced and 4.1 m moles formic acid/g polysaccharide released. The reaction was stopped by addition of ethylene glycol (26.5 ml), and the solution was dialysed against running tap water for 2 days. Sodium borohydride (13.3g) was added and the mixture kept at room temperature for 30 hours, then dialysed for 2 days. The solution was made 1N with respect to sulphuric acid, and the polyalcohol was hydrolysed for 48 hours at room temperature. A portion of this solution ($^1/_{10}$ of total volume) was neutralised with barium carbonate, filtered, dialysed against distilled water (4 l) for 24 hours, and against running tap water for 2 days, then mixed with the main portion of the solution, which had been dialysed against running tap water for 3 days. Polysaccharide I was isolated as the freeze-dried product (12.5g; yield, 28%), $[\alpha]_D + 27^\circ$ (c, 0.80) (Found: gal 95%, ara 5%; $\bar{M}_n$ by end-group analysis, 1960).

Polysaccharide I (30 mg) was hydrolysed with 2N-sulphuric acid for 7 hours on a boiling water bath. Paper chromatographic examination of the resulting syrup in solvents (a), (h) and (i) revealed galactose, arabinose and no uronic acids. The gum (20 mg) was hydrolysed with 0.5N-sulphuric acid for 1 hour on a boiling water bath. Paper chromatographic examination of the resulting syrup in solvents (a) and (b) indicated the presence of galactose, arabinose, two neutral disaccharides with the mobilities of 6-O-β-D-galactopyranosyl-D-galactose [minor component: R_{gal} 0.30,

solvent (a); 0.39, solvent (b)] and 3-O-β-D-galactopyranosyl-D-galactose [major component: R_{gal} 0.48, solvent (a); 0.55, solvent (b)] and higher neutral oligosaccharides. Polysaccharide I (259 mg) was methylated by the Haworth and Purdie procedures to give a product (202 mg), $[\alpha]_D$ -0.5° (c, 0.97 in chloroform) (Found: OMe, 40.4%). Polysaccharide I (294 mg) was also methylated by the sodium hydride, methyl iodide, dimethylsulphoxide procedure[21] to give a product (277 mg), $[\alpha]_D$ -36° (c, 0.99 in chloroform) (Found: OMe, 41.9%). A portion of each product was methanolysed and the mixtures of methyl glycosides were examined by g.l.c. A portion of each methanolysate was hydrolysed and the resulting syrups were examined by paper chromatography in solvents (e) and (f). Results are summarised in table 3B.5.

The dialysate obtained after the hydrolysis stage in the preparation of polysaccharide I was concentrated, deionised with Amberlite IR-120(H) resin and concentrated to a syrup. Paper chromatography in solvents (a) and (e) indicated the presence of traces of galactose [R_F 0.27, solvent (a); 0.15, solvent (e)] and arabinose [R_F 0.32, solvent (a); 0.21, solvent (e)] together with glycerol [major component: R_F 0.50, solvent (a); 0.40, solvent (e)], threitol [minor component: R_F 0.39, solvent (a); 0.30 solvent (e)] and small amounts of glycolaldehyde [R_F 0.66, solvent (a); 0.65, solvent (e)] and ethylene glycol [R_F 0.59, solvent (a); 0.55, solvent (e)].

Preparation and Examination of Polysaccharide II

Preliminary, small-scale experiments established that an 0.125M-sodium metaperiodate solution and oxidation time of 48 hours

were required for polysaccharide I. These conditions were also employed for subsequent degradations.

Borohydride reduction of periodate-oxidised polysaccharide I (11.5g polysaccharide) followed by controlled acid hydrolysis at room temperature for 48 hours, omitting the stage of dialysis against distilled water, gave polysaccharide II (6.08g; yield, 53%), $[\alpha]_D$ +21° (c, 0.80) (Found: galactose 98%, arabinose 2%; $\bar{M}_n$ by end-group analysis, 3290). Release of formic acid with time during periodate oxidation of polysaccharide I was followed (fig. 3B.1). After oxidation for 48 hours, 3.37 m moles periodate/g polysaccharide had been reduced and 1.20 m moles formic acid/g polysaccharide released.

Hydrolysis of polysaccharide II (40 mg) with 0.5N-sulphuric acid for 1 hour on a boiling water bath, followed by paper chromatography of the resulting syrup in solvents (a) and (b) indicated the presence of galactose, a small amount of arabinose, two neutral disaccharides with the mobilities of 3-O-β-D-galactopyranosyl-D-galactose (major component) and 6-O-β-D-galactopyranosyl-D-galactose (trace component), and higher neutral oligosaccharides. Polysaccharide II (262 mg) was methylated by the Haworth and Purdie procedures to give a product (181 mg), $[\alpha]_D$ -3.8° (c, 0.89 in chloroform) (Found: OMe, 40.0%). Methanolysis of a portion of this product was followed by g.l.c. examination of the mixture of methyl glycosides. A portion of the methanolysate was hydrolysed and the resulting syrup examined by paper chromatography in solvents (e) and (f). Results are summarised in table 3B.5.

Preparation and Examination of Polysaccharide III

Borohydride reduction of periodate-oxidised polysaccharide II (5.2g polysaccharide) followed by controlled acid hydrolysis at room temperature for 48 hours gave polysaccharide III (1.75g; yield, 34%), $[\alpha]_D$ +5° (c, 0.80) (Found: galactose 99%, arabinose 1%; $\bar{M}_n$ by end-group analysis, 2930). Release of formic acid with time during periodate oxidation of polysaccharide II was followed (fig. 3B.1). After oxidation for 48 hours, 2.20 m moles periodate/g polysaccharide had been reduced and 0.89 m moles formic acid/g polysaccharide released.

Hydrolysis of polysaccharide III (42 mg) with 0.5N-sulphuric acid for 1 hour on a boiling water bath, followed by paper chromatography of the resulting syrup in solvents (a) and (b) indicated the presence of galactose, a trace of arabinose, two neutral disaccharides with the mobilities of 3-O-β-D-galactopyranosyl-D-galactose (major component) and 6-O-β-D-galactopyranosyl-D-galactose (trace component), and higher neutral oligosaccharides. Polysaccharide III (273 mg) was methylated by the Haworth and Purdie procedures to give a produce (240 mg), $[\alpha]_D$ -13° (c, 0.97 in chloroform) (Found: OMe, 40.0%). Methanolysis of a portion of this product was followed by g.l.c. examination of the mixture of methyl glycosides. A portion of the methanolysate was hydrolysed and the resulting syrup examined by paper chromatography in solvents (e) and (f). Results are summarised in table 3B.5.

Preparation and Examination of Polysaccharide IV

Borohydride reduction of periodate-oxidised polysaccharide III (1.1g polysaccharide) followed by controlled acid hydrolysis at room temperature for 48 hours gave polysaccharide IV [0.40g; yield, 36% (uncorrected for polysaccharide removed during formic acid determinations)], $[\alpha]_D$ -19° (c, 0.70) (Found: galactose, 100%; $\bar{M}_n$ by end-group analysis, 2200). Release of formic acid with time during periodate oxidation of polysaccharide III was followed (fig. 3B.1). After oxidation for 48 hours, 2.20 m moles periodate/g polysaccharide had been reduced and 0.72 m moles formic acid/g polysaccharide released.

Hydrolysis of polysaccharide IV (34 mg) with 0.5N-sulphuric acid for 1 hour on a boiling water bath followed by paper chromatography of the resulting syrup in solvents (a) and (b) indicated the presence of galactose, two neutral disaccharides with the mobilities of 3-O-β-D-galactopyranosyl-D-galactose (major component) and 6-O-β-D-galactopyranosyl-D-galactose (trace component), and higher neutral oligosaccharides. Polysaccharide IV (234 mg) was methylated by the Haworth and Purdie procedures to give a product (157 mg), $[\alpha]_D$ -16° (c, 0.84 in chloroform) (Found: OMe, 39.0%). Methanolysis of a portion of this product was followed by g.l.c. examination of the mixture of methyl glycosides. A portion of the methanolysate was hydrolysed and the resulting syrup examined by paper chromatography in solvents (e) and (f). Results are summarised in table 3B.5.

Molecular-Sieve Chromatography (M-SC) of L. humilis and Some of its Degradation Products

Portions (50 mg) of L. humilis gum, degraded gums A and B and polysaccharides I and II were dyed with Procion Red M-2B dye[67]. M-SC[33] of degraded gums A and B was performed on a column (40 × 1.5 cm) of Bio-Gel A-5m; M-SC[33] of polysaccharides I and II was performed on a column (35 × 1.5 cm) of Bio-Gel P-10. M-sodium chloride solution at flow rates of 0.5 ml/min was used as eluant for both columns. Elution diagrams of the degraded gums are shown in fig 3B.2, together with elution diagrams of L. humilis for comparison. These show that degraded gums A and B are of lower molecular weight than L. humilis and do not appear to have such a broad molecular weight distribution. Polysaccharides I and II are also of low molecular weight, and appear to have retained a rather broad distribution.

3B.3 DISCUSSION

To evaluate structural evidence published for a gum from the genus Lannea,[39,40,56] the gum from Lannea humilis was studied in greater detail. The purified gum was examined by zone electrophoresis, thin-layer electrophoresis, ion-exchange chromatography,[29] molecular-sieve chromatography (M-SC)[33] and ultracentrifugation. As none of these experiments indicated any sharp discontinuity in the properties of the system of molecular species involved, it seems probable that L. humilis gum exhibits the same kind of hetero-polymolecularity as that postulated for Acacia gums,[2,70,77] but M-SC studies (Section IIIA) indicate that its molecular weight

range is greater than that of *Acacia* gums. The importance of assessing heterogeneity appears to have been overlooked in certain studied of *L. coromandelica* gum,[39,40] but other investigators of the same gum pronounced it homogeneous, on the evidence of hydrolysis products of fractions obtained by fractional precipitation and the results of acetylation and deacetylation.[56] These rather superficial studies would not have been thought sufficient to decide that a compound as complex as a plant gum is homogeneous.

L. humilis gum had $\bar{M}_w$ 2.57×10^5, and was found to contain D-galactose (75%), L-arabinose (11%) and L-rhamnose (2%). As rhamnose was present in small amount, it was concentrated by thick paper chromatography, producing a syrup giving strong spots corresponding to authentic L-rhamnose on chromatography. Attempts to crystallise rhamnose from this syrup precipitated a material found to react in the phenol-sulphuric acid test,[13] but which did not produce a colour with aniline oxalate spray after paper chromatography. These results suggest that the value quoted for rhamnose is high, due to the presence of an impurity with the mobility of rhamnose in that solvent used for separation of neutral sugars prior to their estimation by the phenol-sulphuric acid method.[13] The chromatographic identification of rhamnose is, however, unaffected by the presence of this impurity, the identity of which is uncertain. It probably originated from the bark present in the crude gum sample. A very low rhamnose content was confirmed by the presence of trace amounts of 2,3,4-tri-O-methyl-L-rhamnose in methylated *L. humilis* gum. The gum was also

found to contain uronic acid (12%) of which 2.5% was 4-O-methyl-D-glucuronic acid; the remaining uronic acid was present as D-galacturonic acid (major component) and D-glucuronic acid (minor component).

L. humilis gum was hydrolysed with N-sulphuric acid for 7 hours on a boiling water bath. Removal of neutral sugars followed by fractionation of the acidic component by thick paper chromatography gave two acidic fractions. The first of these was found to be a mixture of two aldobiouronic acids, which were separated by further thick paper chromatography and characterised as 4-O-(α-D-galactopyranosyluronic acid)-D-galactose and 6-O-(β-D-glucopyranosyluronic acid)-D-galactose. Due to shapes of the spots produced on paper chromatography, R_{gal} values of these compounds in solvent (a) were difficult to estimate. The second acidic fraction was characterised as 6-O-(4-O-methyl-β-D-glucopyranosyluronic acid)-D-galactose. Unlike 4-O-(α-D-galactopyranosyluronic acid)-D-galactose, the other two aldobiouronic acids are commonly found in Acacia gums.[4,78,79] Previous studies on L. coromandelica gum identified 6-O-(4-O-methyl-D-glucopyranosyluronic acid)-D-galactose,[37,38] but investigators who identified galacturonic acid[45] characterised the aldobiouronic acid as 3-O-(D-galactopyranosyluronic acid)-D-galactose.

Controlled hydrolysis of L. humilis gum, using 0.01N-sulphuric acid, gave degraded gum A in good yield. The hydrolysis was followed chromatographically, showing that rhamnose was liberated, arabinose was readily released in appreciable amount and galactose appeared within four hours (unlike the findings of Bhattacharyya and Rao[40] in a study of the autohydrolysis of L. coromandelica

gum, when galactose appeared after 25 hours). Oligosaccharides were released in trace amount after 36 hours. Also observed were fragments shown to have the same paper chromatographic mobilities as 3-O-α-D-galactopyranosyl-L-arabinose, 3-O-β-L-arabinopyranosyl-L-arabinose and 3-O-β-L-arabinofuranosyl-L-arabinose. These are well-known products of controlled acid hydrolysis of Acacia gums,[4,77] but only 3-O-arabinofuranosyl-L-arabinose has been reported in L. coromandelica gum.[39] All three disaccharides were released under relatively mild hydrolysis conditions, suggesting that in each case, the reducing arabinose residues were initially present in the furanose form. So the following are structural fragments of L. humilis gum:-

$$\cdots\cdot \underline{L}\text{-Ara}\underline{f} \xrightarrow{1\beta 3} \underline{L}\text{-Ara}\underline{f}^{\,1}\cdots\cdot \qquad \cdots\cdot \underline{L}\text{-Ara}\underline{p} \xrightarrow{1\beta 3} \underline{L}\text{-Ara}\underline{f}^{\,1}\cdots\cdot$$

$$\cdots\cdot \underline{D}\text{-Gal}\underline{p} \xrightarrow{1\alpha 3} \underline{L}\text{-Ara}\underline{f}^{\,1}\cdots\cdot$$

Degraded gum A gave a brown solution, and optical rotation of the gum could not be measured, a problem which has been encountered with L. coromandelica gum.[45] Estimated $\bar{M}_n$ of degraded gum A was 4320 (obtained by end-group analysis, suggesting that the reducing galactose residue is generally not substituted at C-6), showing that degradation on autohydrolysis was greater than could be expected by the simple removal of rhamnose and arabinose from the periphery of the molecule. The $\bar{M}_n$ value is in the range expected, if random hydrolysis of ca. 3% of the galactopyranosidic bonds had occurred[70]; this would also explain the liberation of traces of galactose and galactobioses, revealed by paper chromatography, during controlled acid hydrolysis. Methylated

degraded gum A, on methanolysis and hydrolysis, gave 2,3,5- and 2,3,4-tri- and 2,5-di-O-methyl-L-arabinose, 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 2,3,4-tri-O-methyl-D-galacturonic acid (table 3B.1). The presence of 2,6-di- and 2-O-methyl-D-galactose is probably due to undermethylation. This suggests that the following non-reducing end-groups are present in degraded gum A:-

L-Araf $^{1\cdots\cdot}$ L-Arap $^{1\cdots\cdot}$

D-Galp $^{1\cdots\cdot}$ D-GalpA $^{1\cdots\cdot}$

D-GpA $^{1\cdots}$ 4-O-Me-D-GpA $^{1\cdots\cdot}$

The presence of a large amount of 2,3,4,6-tetra-O-methyl-D-galactose indicates much terminal, non-reducing galactopyranosyl residues, a feature already noticed in methylated autohydrolysed L. coromandelica gum,[40] but not observed in any methylated degraded Acacia gum studied to date. With the identification of much 2,4-di-O-methyl-D-galactose, this is evidence for degraded gum A being highly branched.

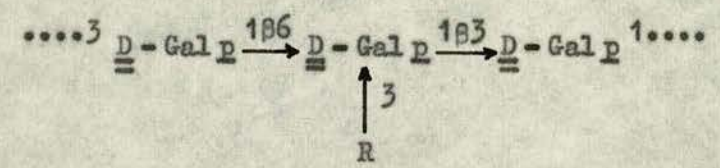
Hydrolysis of degraded gum A with 0.5N-sulphuric acid for 1 hour on a boiling water bath yielded degraded gum B. Paper chromatography of a portion of this hydrolysate revealed 3-O-β-D-galactopyranosyl-D-galactose and 6-O-β-D-galactopyranosyl-D-galactose, with residual traces of 3-O-α-D-galactopyranosyl-L-arabinose, indicating that the following are structural fragments of degraded gum A:-

$\cdots\cdot$D-Galp $\xrightarrow{1\beta3}$ D-Galp $^{1\cdots\cdot}$ $\cdots\cdot$D-Galp $\xrightarrow{1\beta6}$ D-Galp $^{1\cdots\cdot}$

Degraded gum B again formed a brown solution, preventing measurement of its optical rotation. Estimated $\bar{M}_n$ of degraded gum B was 3740 (obtained by end-group analysis, suggesting that the reducing galactose residue is generally not substituted at C-6), showing that drastic degradation of degraded gum A had not occurred during the hydrolysis reaction. Partial acid hydrolysis of degraded gum B again revealed the presence of the β-1,3- and β-1,6-galactobioses, and an examination of methylated degraded gum B revealed the same O-methyl sugars as were present in methylated degraded gum A, with the exception of 2,5-di-O-methyl-L-arabinose, not detected in methylated degraded gum B (table 3B.2). Again 2,3,4,6-tetra-O-methyl-D-galactose was the major product. The similarity of O-methyl galactose residues detected in methylated degraded gums A and B suggests their structures are similar.

To obtain information on the distribution of β-1,3- and β-1,6-linkages in the galactan core of the gum, degraded gum A was subjected to a Smith degradation, producing degraded gum C. Estimated $\bar{M}_n$ of degraded gum C was 1950 (obtained by end-group analysis, suggesting that the reducing galactose residue is generally not substituted at C-6). Partial acid hydrolysis of degraded gum C gave the β-1,3-galactobiose but only trace amounts of the β-1,6-galactobiose. Examination of methylated degraded gum C showed 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 (table 3B.3). The presence of 2,3,6-tri-, 2,6-di- and 2-O-methyl-D-galactose is probably due to undermethylation of the axial 4-position of galactopyranose. The proportions of O-methyl sugars detected is evidence that degraded gum C is a galactan composed predominantly of β-1,3-glycosidic

linkages with a few β-1,6-branch points. The identification of some 2,3,4-tri-O-methyl-D-galactose in methylated degraded gum C indicates a few β-1,6-glycosidic linkages exist in degraded gum C and that the following is a possible structural fragment of degraded gum A:-



where R = L-Araf$^{1\cdots\cdot}$, L-Arap$^{1\cdots\cdot}$, D-Galp$^{1\cdots\cdot}$,

$\cdots\cdot^{4}$ D-Galp$^{1\cdots\cdot}$ or $\cdots\cdot^{6}$ D-Galp$^{1\cdots\cdot}$

Methylated L. humilis gum was methanolysed and hydrolysed, giving 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-, 2,3,6-, 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 2,3,4-tri-O-methyl-D-galacturonic acid (table 3B.4). The presence of 2,6-di- and 2-O-methyl-D-galactose probably arises from undermethylation, and no 2-O-methyl-L-arabinose, found in large amount in methylated L. coromandelica gum by Bhattacharyya and Mukherjee,[39] was observed. The presence of 2,3,4-tri-O-methyl-L-rhamnose in trace amount indicates a few non-reducing, end-group L-rhamnose units in L. humilis gum and identification of 2,3,5- and 2,3,4-tri-O-methyl-L-arabinose indicates that arabinose-containing side chains are terminated by L-arabinofuranose or L-arabinopyranose residues. Identification of 2,5-di-O-methyl-L-arabinose is evidence for 3-O-substituted L-arabinofuranose residues in the arabinose-

containing side chains. The presence of an appreciable amount of 2,4-di-O-methyl-D-galactose indicates that an appreciable number of the D-galactose residues are 3,6-di-O-substituted, and identification of 2,3,6-, 2,4,6- and 2,3,4-tri-O-methyl-D-galactose is evidence for some 4-O-, 3-O- and 6-O- substituted D-galactose residues. The presence of a large amount of 2,3,4,6-tetra-O-methyl-D-galactose agrees with the results of Bhattacharyya and Mukherjee[39] for methylated L. coromandelica gum but contradicts the results of other investigators[56] of the same gum, and is evidence for the presence of many terminal, non-reducing D-galactopyranose residues in the gum. The identification of 2,3,4-tri-O-methyl-D-glucuronic acid and 2,3,4-tri-O-methyl-D-galacturonic acid suggests that D-glucuronic, 4-O-methyl-D-glucuronic and D-galacturonic acids occur as terminal non-reducing units in the whole gum. With the known structures of aldobiouronic acids present in the gum, this suggests that the following are structural fragments of L. humilis gum:-

D-GpA $\xrightarrow{1\beta6}$ D-Gal p $^{1\cdots\cdot}$ 4-O-Me-D-GpA $\xrightarrow{1\beta6}$ D-Gal p $^{1\cdots\cdot}$

D-Gal p A $\xrightarrow{1\alpha4}$ D-Gal p $^{1\cdots\cdot}$.

Further substitution of the galactose residues, to which uronic acids are attached, is not ruled out on the evidence obtained. Methylation evidence for L. humilis gum and degraded gums A and B, in which smaller amounts of arabinose were present, suggests that the arabinose-containing side chains are attached to the galactan framework at C-3 and C-6 positions of certain D-galactose residues.

L. humilis gum was subjected to four successive Smith degradations, giving polysaccharides I-IV. Percentage yields were low, especially from the first degradation in which a large quantity of periodate was reduced and much formic acid formed (fig. 3B.1). Dialysis after the acid hydrolysis stage of this degradation produced a syrup which, on chromatographic examination, revealed traces of ethylene glycol (expected from non-reducing L-arabinopyranose residues) and some threitol (expected from D-galactopyranose residues substituted in the 4-position, as in the α-1,4-linked aldobiouronic acid characterised for the gum).

$\bar{M}_n$ values for polysaccharides I-IV were obtained by end-group analysis, suggesting that in each case, the reducing galactose residue is generally not substituted at C-6. An extremely low value of $\bar{M}_n$ was recorded for polysaccharide I (1960), a value lower than that of polysaccharide II (3290). Values for polysaccharides III and IV (2930 and 2200 respectively) decrease in the expected manner. A possible explanation of these anomalous molecular weights is that in the first degradation stage much degradation occurs, shown by large values for periodate reduction and formic acid release. It is feasible that this drastic degradation would produce many fragments of which an appreciable proportion are just sufficiently large that they cannot be removed by dialysis. These would be equally estimated with larger molecules, as each has one reducing end-group, in a determination of $\bar{M}_n$, resulting in a low molecular weight. In a second degradation stage, further breakdown of these molecules would result in their removal on dialysis, leaving a smaller proportion of very low molecular

weight species. An estimation of $\bar{M}_n$ would then produce a value higher than in the first degradation; subsequent degradations would each lower the value of $\bar{M}_n$. This explanation agrees with results of M-SC studies on dyed polysaccharides I and II, which show the former to be of slightly higher molecular weight (fig. 3B.2). The desalting step of the dyeing procedure[67] would remove fragments of very low molecular weight, leaving the larger fragments present to be examined by M-SC. The value of $\bar{M}_n$ of degraded gum C agrees closely with that of polysaccharide I, suggesting that this feature must be a property of the galactan framework of L. humilis gum. The large degradation occurring on the first Smith degradation stage would be expected to remove the terminal, non-reducing D-galactopyranose residues shown by methylation studies of L. humilis gum and degraded gums A and B but must also have cleaved vulnerable β-1,6-linked D-galactose residues within the molecular framework to account for the drastic breakdown, implying that long chains of periodate-resistant β-1,3-linked D-galactose residues are not an important feature of this gum. The third and fourth degradation stages do not cause a large decrease in molecular weight, but yields are low, suggesting that in each stage, only low molecular weight peripheral units are being removed, or that polysaccharides II and III are structurally heterogeneous, some molecules being only slightly broken down whilst others are fragmented.

Optical rotation of the Smith-degraded products decreased to a negative value, a feature observed by Dea[71] during studies of Acacia drepanolobium and Acacia seyal gums. Partial acid hydrolysis

of polysaccharides I-IV gave, in each case, the β-1,3-galactobiose but the amount of β-1,6-galactobiose was small, decreasing to trace amounts in polysaccharide IV. That four successive Smith degradations were required to remove all the arabinose units from L. humilis gum indicates that the arabinose-containing side chains can be no more than four units long.

Examination of methylated polysaccharides I-IV gave the O-methyl sugars detailed in table 3B.5. The presence of 2,3,6-tri-, 2,6-di- and 2-O-methyl-D-galactose probably arises from under-methylation. The presence of only trace amounts of 2,5-di-O-methyl-L-arabinose in methylated polysaccharide I implies that few arabinose side chains are four units long, an observation confirmed by the presence of only trace amounts of 2,3,5-tri-O-methyl-L-arabinose in methylated polysaccharide III. Identification of 2,3,4-tri-O-methyl-L-arabinose in methylated polysaccharide I suggests the presence of some arabinopyranose residues but the proportion of this component depends on the methylation procedure employed. Arabinopyranose residues should have been removed during the first Smith degradation, and no evidence for chains of arabinopyranose residues has been obtained, so it was not thought that this component was of structural significance. The predominance of 2,4,6-tri-O-methyl-D-galactose in each methylated polysaccharide is evidence for most of the D-galactose residues being β-1,3-linked. Identification of 2,3,4-tri-O-methyl-D-galactose in each case suggests some D-galactose residues are β-1,6-linked, confirmed by identification of the β-1,6-galactobiose on partial acid hydrolysis. The continued presence of periodate-vulnerable β-1,6-linkages

indicates that they must be revealed at each stage by the progressive removal of arabinose-containing side chains or D-galactose residues from the C-3 position of 6-O-substituted D-galactose units. The presence of 2,3,4,6-tetra- and 2,4-di-O-methyl-D-galactose is evidence for branching occurring in the polysaccharides. Polysaccharides I-IV are therefore based on a branched galactan made up of β-1,3-linked D-galactose residues joined by β-1,6-linkages.

From the structural evidence obtained, some suggestions can be made for the structure of L. humilis gum. A Smith degradation study, and methylation analysis of degraded gum C indicate that long chains of β-1,3-linked D-galactose residues are unlikely to be an important feature of the gum. Detection of 2,3,4-tri-O-methyl-D-galactose in trace amounts in methylated polysaccharides I-IV and degraded gum C suggests the presence of a few β-1,6-linked D-galactose residues in each. The presence of 2,4-di- and 2,3,4,6-tetra-O-methyl-D-galactose in these products would indicate that if there is a "main chain" of β-1,3-linked D-galactose units in the galactan core of the gum, it must carry short side chains of β-1,3-linked D-galactose residues; the branch points for these would be through β-1,6-linkages [fig. 3B.3(A)]. Alternatively, there could be occasional β-1,6-linkages along a "main chain" of β-1,3-linked D-galactose units; the branch points would then be through β-1,3-linkages to the β-1,6-linked residues in the "main chain" [fig. 3B.3(B)]. A randomly branched structure of β-1,3-linked chains with β-1,6-branch points would also satisfy the available structural evidence [fig. 3B.3(C)]. The ratio of 2,4,6-tri- to 2,3,4,6-tetra- and

2,4-di-O-methyl-D-galactose, estimated from g.l.c. peak areas, did not vary greatly in polysaccharides I-IV, indicating that the same degree of branching was present in each. This implies that degradations II-IV merely removed peripheral residues, with degradation also of the few β-1,6-linkages revealed in the previous stage, and agrees with the observed small decreases in the values of $\bar{M}_n$. If certain molecules had β-1,6-linkages in positions such that their cleavage resulted in fragmentation of the molecule, the low yields obtained would be explained.

This branched galactan structure was produced essentially by a single Smith degradation, evidence for the presence of many periodate-vulnerable residues in L. humilis gum. Methylation analysis of L. humilis gum and degraded gums A and B revealed much 2,4-di- and 2,3,4,6-tetra-O-methyl-D-galactose, showing that many D-galactose residues are 3,6-di-O-substituted and many are terminal non-reducing units. This suggests a highly branched structure for the original gum, an observation in agreement with its low limiting flow-time number. Methylation and hydrolysis studies showed that the number of β-1,6-linkages decreased markedly on Smith degradation of L. humilis gum, indicating that many of these originally present were periodate-vulnerable; the remainder must have been substituted, either by galactose- or arabinose-containing side chains, at C-3, rendering them periodate-resistant. The branched galactan framework of L. humilis gum therefore appears to be composed of the small galactan structures described for the Smith degradation products and degraded gum C, linked by periodate-labile β-1,6-linkages (either single, or as a block, or as blocks interspersed with one

or more β-1,3-linkages). The number of these small galactan structures joined together must vary greatly as the molecular weight distribution of the gum appears to be extremely broad. The β-1,3-linked D-galactose residues must be heavily substituted at C-6 by single D-galactose residues or short galactose side chains, to account for the large number of terminal non-reducing D-galactose units.

Arabinose-containing side chains are short, only a few being four units long. As 2,5-di-O-methyl-L-arabinose was the only di-O-methyl-L-arabinose observed on examination of methylated L. humilis gum, it seems probable that these chains are 1,3-linked, a conclusion supported by the identification of 3-O-β-L-arabinofuranosyl-L-arabinose and 3-O-β-L-arabinopyranosyl-L-arabinose, released on controlled acid hydrolysis. These disaccharides indicate that the 1,3-linkages have the β-configuration. The arabinose side chains are terminated by non-reducing L-arabinopyranose or L-arabinofuranose residues, but the identification of 3-O-α-D-galactopyranosyl-L-arabinose suggests some may be terminated by D-galactopyranose residues. Uronic acids have been shown to be present as terminal, non-reducing units. The mode of attachment of L-rhamnose has not been established, but by analogy with Acacia gums,[7,80,81] may possibly be linked to the 4-position of glucuronic acid. On methylation analysis, the extremely small amount of resulting 2,3-di-O-methyl-D-glucuronic acid would not be detected.

The evidence obtained in this study suggests L. humilis gum molecules possess a branched galactan framework, based on short

chains of β-1,3-linked D-galactose residues joined together by β-1,3- and β-1,6-linkages. To this framework, at suitable C-3 and C-6 positions are attached very short D-galactose side chains or single D-galactose residues, short L-arabinose-containing side chains, and three different uronic acids. The attachment of L-rhamnose is uncertain. Many chains are terminated by D-galactopyranose; no unequivocal proof of a backbone or main chain was found.

Fig. 3B.4 shows a possible structural fragment of L. humilis gum; this is not intended to represent any repeating unit in the structure of the molecule, and is only one of many similar structures which, on the basis of evidence obtained, can be proposed for the gum.

This structure differs in several details from typical structures proposed for Acacia gums.[2,70,77,82] D-galacturonic acid has not been found in Acacia gums so far studied, and the number of terminal, non-reducing D-galactopyranose units present in L. humilis gum is greater than observed in any Acacia gum. The structure of L. humilis gum has also been shown to be more vulnerable to periodate. The galactan core produced by a series of Smith degradations was similar to that of certain Acacia gums.[70,77]

Analysis of gums from three species of the genus Lannea (Section IIIA) showed them to be similar, implying that variations in structure would be expected to be slight. However the structure reported by Ramachandran and Joshi[56] for L. coromandelica gum bears no relation to structural features of L. humilis gum elucidated above, and doubts must be raised as to whether the gum

they studied did in fact belong to the genus Lannea. Other studies on the structure of L. coromandelica gum [39] and its autohydrolysed product[40] reveal some features in common with L. humilis gum. The structural basis proposed for L. coromandelica gum was a highly-branched β-1,3- and β-1,6-linked galactan to which were attached short arabinose-containing side chains and galacturonic acid, but the importance (probably wrongly) attached to 2-O-methyl-L-arabinose and 2-O-methyl-D-galactose, which presumably arose due to incomplete methylation, meant that tri-O-substituted L-arabinose and D-galactose residues were prominent features of their proposed structure. The linkage of D-galacturonic acid did not accord with that described above for L. humilis gum and no glucuronic or 4-O-methylglucuronic acid was observed. Their structural studies were incomplete, leading to a proposed structure which would not reproduce the results obtained during a Smith degradation study of L. humilis gum, and expected in a similar study of L. coromandelica gum.

SECTION IV

THE GUM EXUDATE FROM *AZADIRACHTA INDICA* A. JUSS.

4.1 INTRODUCTION

Azadirachta indica (also known as Antelaea indica, Melia azadirachta, M. indica and widely recognised under the native vernacular names Nim, Neem and Margosa)[83,84] and Azadirachta integrifoliola are the only two known members of the genus Azadirachta A. Juss. (Order, Geraniales; Family, Meliaceae). A. integrifoliola is only found in the Phillipines and seems to be marginally different from A. indica.[85]

A. indica is common throughout India, Ceylon and Burma, being popular as an ornamental tree,[86] and is also widely cultivated for its medicinal value.[87] Its wood is like mahogany and its bark is bitter, yielding a gum which was said to be a stimulant[86] and which has been in pharmaceutical use in India for many centuries.[88] Widely introduced into the tropics from India,[89] A. indica trees have been found to improve the soil and it has been suggested that this tree could prove useful in Africa.[89]

The gum exudate from A. indica has already been the subject of chemical study. The electrochemistry of azrechtic acid, a polysaccharide prepared by electrodialysis of A. indica gum in the presence of mineral acid, has been studied,[90] and investigations into the colloidal properties of A. indica gum[91] have shown it to be a polyelectrolyte, behaving as a lyophilic colloid. It has been suggested[92] that differential thermogravimetric analysis of gums in the neutralised and free acid forms would provide a rapid means of identification and characterisation. Differential thermogravimetric records for A. indica gum have been obtained.[92] The gum has been found to have $[\alpha]_D$-71°,[93]

equivalent weight 1080[94] and to contain nearly 20% proteinaceous material.[75] Hydrolysis studies have shown[94] the presence of D-galactose and L-arabinose in the ratio 3/2, L-fucose, D-glucuronic acid and a trace of xylose; the gum contains the aldobiouronic acid 4-O-(D-glucopyranosyluronic acid)-D-galactopyranose. The presence of the amino sugar D-glucosamine in 2.9% amount has been demonstrated.[75]

A. indica gum therefore possess several unusual features: (a) the strongly negative optical rotation, (b) the presence of fucose, (c) the glucosamine content of 2.9%, and (d) the presence of about 20% proteinaceous material, (values of the protein content of purified plant gums usually range from 0.2 - 6%).

This section comprises experiments confirming the presence of fucose, xylose and appreciable amounts of an amino sugar, together with some studies on the proteinaceous component of A. indica gum.

4.2 RESULTS

Purification of Samples

A. indica gum, sample A, was obtained in October, 1967 from Mr L. Wijesinghe, Research Laboratory of the Conservator of Forests, 9, Kew Road, Colombo 2, Ceylon. A. indica gum, sample B, was obtained in May, 1968 from Dr. D.B. Deb, Regional Botanist, Botanical Survey of India, Southern Circle, Coimbatore.

The samples dissolved in cold water after several hours. The solutions were filtered then dialysed for several days; the purified gums were recovered as freeze-dried products. Recoveries,

on a dry weight basis, were 80% and 76% for samples A and B respectively.

Estimation of Hexosamine in A. indica Gum

Two procedures, based on the colorimetric method of Elson and Morgan,[95] were employed.

The first procedure was that of King[96] (which is essentially the method used by Belcher et al[97]), but was modified at the neutralisation stage. Portions (30 mg and 50 mg) of gum were hydrolysed with 2N-hydrochloric acid for 6 hours on a boiling water bath, conditions which should result in complete hydrolysis of hexosamine without its degradation.[75,76] Hydrolysis with 4N-hydrochloric acid for 6 hours on a boiling water bath gave a concordant result, but a duplicate experiment with 6N-hydrochloric acid gave a result that was slightly low, indicating that degradation effects were becoming important. The hydrolysate was decolourised with charcoal, made just alkaline to phenolphthalein, and its volume was adjusted to 25 ml. To aliquots (1, 2 and 3 ml) was added 1 ml of a solution of acetylacetone (1 ml) in N-sodium carbonate solution (50 ml), and volumes were adjusted to 4 ml with distilled water. The solutions were heated on a boiling water bath for 10 min, cooled, and volumes were adjusted to 9 ml with ethanol. After heating on a water bath at 75°C for 5 min, 1 ml of a solution of p-dimethylaminobenzaldehyde (0.8 g) in 10N-hydrochloric acid (60 ml) was added, and solutions were reheated at 75°C for 30 min with occasional shaking. After cooling, volumes were adjusted to 10 ml with ethanol and optical densities were read on a Unicam SP1300 spectrophotometer using filter 3,

against a blank prepared by treating a portion (2 ml) of the neutralised hydrolysate with distilled water (1 ml) instead of acetylacetone solution (1 ml) in the above procedure. The hexosamine content was read from a calibration curve prepared using D-glucosamine. This method gave:-

hexosamine (as glucosamine) in A. indica gum, sample A = 2.1%

The second procedure was that of Lakshmi and Pattabiraman,[75] based on the method described by Levvy and McAllan,[98] but modified at the hydrolysis stage, and at the colour development stage, where gentle heating was employed.[98] Portions (60 mg) of gum were hydrolysed, neutralised, and solution volumes were adjusted to 25 ml as described in the first procedure. To aliquots (0.5, 1.0 and 1.5 ml), made up to 1.5 ml with distilled water, were added 0.16M-sodium tetraborate solution (1.5 ml) and 5% acetic anhydride in acetone (0.15 ml). Solutions were mixed, heated on a boiling water bath for 5 min, cooled, and 1.5 ml p-dimethylamino-benzaldehyde solution [10%, in a mixture of glacial acetic acid (90 ml) and 10N-hydrochloric acid (10 ml)] was added. After heating on a water bath at 37°C for 20 min, volumes were made up to 10 ml with ethanol and optical densities were read on a Unicam SP1300 spectrophotometer using filter 3, against a blank prepared by treating the neutralised hydrolysate (1 ml) with acetone (0.15 ml) instead of acetic anhydride in acetone (0.15 ml) in the above procedure. The hexosamine content was obtained from a calibration curve for glucosamine. This method gave:-

hexosamine (as glucosamine) in A. indica gum samples A and B = 2.0 and 2.2% respectively.

Removal of Protein from A. indica Gum, Sample A

Attempts were made to deproteinise A. indica gum. Portions (1 g) of the crude gum were dissolved in water (20 ml), filtered through glass wool, then treated by the following methods:-

(a) The gum solution was adjusted to pH 5 with glacial acetic acid and 10 volumes of acetone were added with stirring. The precipitate was collected by vacuum filtration, washed with acetone, then ether, and dried over calcium chloride.(75) This whole gum served as a "blank". The same conditions of precipitation were also used as the final stage of each of the following procedures.

(b) To the gum solution (20 ml) was added ethanol, until a small precipitate had formed. The solution was centrifuged, the supernatant was dialysed for 2 days, then the gum was recovered by precipitation.

(c) The gum solution (20 ml) was shaken with chloroform (4 ml) and n-butanol (0.8 ml) for 30 min (Sevag technique), and the resulting emulsion was centrifuged. The aqueous layer was separated, the procedure was repeated, then the gum was recovered by precipitation.

(d) The gum solution (20 ml) was shaken with 1,2,2-trifluoro-1,1,2-trichloroethane (15 ml) for 30 min then centrifuged. The aqueous layer was separated from the gelatinous and non-aqueous layers. This procedure was repeated twice. The aqueous layer was dialysed for 2 days, then the gum was recovered by precipitation.

(e) To the gum solution (20 ml) was added trichloroacetic acid (1.6 g). After standing overnight, the solution was shaken for 90 min, gently heated and cooled, then filtered. The filtrate was dialysed for 2 days, then the gum was recovered by precipitation.

(f) To the gum solution (20 ml) was added 2N-trifluoroacetic acid (20 ml). After standing overnight, the solution was shaken for 90 min and centrifuged. The supernatant was dialysed for 2 days, then the gum was recovered by precipitation.

(g) The gum solution (20 ml) was treated with 10% sodium tungstate solution (6.7 ml) and 0.6N-sulphuric acid (6.7 ml) and allowed to stand for 10 min. The solution was heated to boiling, cooled and allowed to stand overnight, then filtered and dialysed for 2 days. The gum was recovered by precipitation.

(h) The gum solution (20 ml) was stirred with sodium borohydride (0.2 g) for 3 hours, then allowed to stand overnight. After filtration, the solution was dialysed for 2 days then the gum was recovered by precipitation.

(i) The pH of the gum solution (20 ml) was adjusted to 1.5 - 2.0 with N-hydrochloric acid, and pepsin (50 mg; twice crystallised from alcohol, L. Licht and Co. Ltd.) was added. The solution was incubated at 37°C for 48 hours, the pH being regularly readjusted to 1.5 - 2.0. The pH was then adjusted to 7.5 with 2N-sodium hydroxide solution, and trypsin (50 mg; twice crystallised, Sigma Chem. Co.) was added. The solution was again incubated at 37°C for 48 hours, the pH being maintained at 7.5. The reaction was stopped by addition of an equal volume of 20% trichloroacetic

acid solution, the mixture was centrifuged for 1 hour and the supernatant was dialysed for 3 days. To ensure that no enzyme remained, the solution was treated with 1,2,2-trifluoro-1,1,2-trichloroethane (25 ml) as described in method (d). After further dialysis for 3 days, the gum was recovered by precipitation.

(j) Purified A. indica gum (1 g) was dissolved in tris buffer, pH 8.0 (10 ml) then calcium chloride (8.1 mg) and pronase (6.6 mg; Sigma Chem. Co.) was added. This solution was heated under reflux at 60°C on a water bath for 24 hours, then reaction was stopped by addition of an equal volume of 20% trichloroacetic acid solution. After centrifugation, the solution was twice treated with 1,2,2-trifluoro-1,1,2-trichloroethane (20 ml) as described in method (d) to ensure no enzyme remained, then dialysed for 3 days. The gum was recovered by precipitation.

The nitrogen content of each of these products was determined by the Kjeldahl method, and results, together with alterations in protein content, are shown in table 4.1.

Molecular-Sieve Chromatographic Studies of the Protein Component of A. indica Gum, Sample A

In a study of Acacia campylacantha gum, it was proposed[7] that the use of a Bio-Gel A-5m molecular-sieve column with a sodium chloride/urea/dithiothreitol eluant led to "no significant reduction in the proportions of aggregated polysaccharide ..... but the protein aggregates were successively broken down". An absence of protein in the molecular-sieving range of the column using this eluant, was taken to imply that covalent linkages between the

carbohydrate and protein components of Acacia campylacantha gum do not exist. The use of such a column for the study of Azadirachta indica gum was investigated.

Azadirachta indica gum (50 mg) was dyed [67] with Procion Red M-2B dye. Molecular-sieve chromatography (M-SC) of dyed and undyed A. indica gum was performed on the following columns:-

(a) Bio-Gel A-5m (40 x 1.5 cm) using M-sodium chloride solution as eluant at flow-rate of 0.3 ml/min.

(b) Bio-Gel A-5m (36 x 1.5 cm) using M-sodium chloride/7M-urea/ 0.005% (w/v) dithiothreitol/0.05M-tris buffer (pH 9.0) as eluant at flow-rate of 0.1 ml/min.

Applications to column (a) were made in 2M-sodium chloride solution (1 ml). Applications to column (b) were made in 1 ml of a 2M-sodium chloride/7M-urea/0.1% (w/v) dithiothreitol/0.05M-tris buffer (pH 9.0) solution, after standing in this solvent for a stated time.

Portions (3 mg) of dyed A. indica gum were applied to column (a), and to column (b) after standing in solvent for 0 hours and 24 hours. Elution diagrams were obtained using an automated colorimetric method[33] (fig. 4.1). Portions (6 mg) of undyed gum were applied to column (a), and to column (b) after standing in solvent for 0, 24 and 114 hours. Elution diagrams were obtained by screening eluant for protein at 254 nm (fig. 4.1). Elution diagrams of dyed and undyed gums on column (a) were similar, but with enhancement of the void volume peak of the undyed sample. Elution volumes of peaks were smaller when column (b) was employed,

but the peak which appeared as a shoulder on column (a) was resolved on column (b). Allowing the dyed gum to stand for some time in the solvent before application to column (b) appeared to have little effect on its elution properties, but this treatment resulted in a diminution of the peak, appearing at elution volume 45 ml, in the elution diagram of the undyed sample (fig. 4.1). The large peak at elution volume 80 ml (fig. 4.1) was tentatively identified as dithiothreitol present in the applied sample solution. These chromatograms also indicate that, unlike Acacia campylacantha gum,[7] Azadirachta indica gum must stand in solvent for more than 24 hours for maximum breakdown to occur. The time of standing in solvent and dithiothreitol content of solvent were then varied, to determine whether further breakdown could be achieved.

Portions (10 mg) of A. indica gum were dissolved in solvent (1 ml) and applied to column (b) after 0, 24, 48, 72 and 100 hours. (These larger applications of gum were employed to enlarge the peaks present in the elution diagrams). Chromatograms obtained after standing in solvent for 0 and 24 hours were identical to figs. 4.1(5) and 4.1(6) respectively; traces obtained after periods of 48, 72 and 100 hours were all identical to fig. 4.1(7). Results indicate that the elution diagram undergoes no further alteration after 48 hours.

Portions (10 mg) of A. indica gum were dissolved in solvent (1 ml), the dithiothreitol concentration of which was varied, then applied to column (b) after 24 and 48 hours. Concentrations of dithiothreitol employed were 0.2%, 0.4% and 0.8% (w/v). Resulting chromatograms are shown in fig. 4.2. The peak eluted at 80 ml was

found to increase as dithiothreitol concentration in the solvent increased. An ultraviolet spectrum of solvent containing 0.1% dithiothreitol, using column eluant as blank, showed a strong absorption at 254 nm. As these solutions differed only in sodium chloride and dithiothreitol concentrations, the peak eluted at 80 ml must be due to dithiothreitol in the solvent. The proposal, by Munro,[7] that protein aggregates in Acacia campylacantha gum were broken down to small polypeptides, eluted near the exclusion volume, seems unlikely, this peak being largely (if not entirely) due to dithiothreitol. Chromatograms also showed that when the gum was allowed to stand for 48 hours in solvent, in which the dithiothreitol concentration was 0.4% (w/v), before application to column (b), no peak at elution volume 45 ml [fig. 4.2(4)] was found. There were no further changes in the elution diagram when the dithiothreitol concentration was increased to 0.8%. A further run using gum which had stood for 110 hours in solvent of dithiothreitol concentration 0.8% also produced an elution diagram identical to fig. 4.2(6).

Undyed A. indica gum (10 mg) was allowed to stand in solvent (1 ml, 0.4% (w/v) dithiothreitol) for 48 hours, then applied to column (b). Fractions were collected and screened by the phenol-sulphuric acid method,[13] producing the elution diagram shown in fig. 4.2(7). It is similar to the trace obtained by screening at 254 nm, but the main peak eluted at 36 ml is greatly enlarged, and dithiothreitol was not detected. Portions (5 mg) of dyed gum were dissolved in solvent (1 ml, 0.4% (w/v) dithiothreitol), applied to column (b) after 24 and 48 hours, and screened colorimetrically.[33]

Elution diagrams were identical to those obtained with solvent of dithiothreitol concentration 0.1% [figs. 4.1(3) and 4.1(4)]. When the dithiothreitol concentration was raised to 0.8% (w/v), the dye was degraded, producing a brown solution. Dyed polysaccharides are therefore unsuited to studies such as this.

Allowing A. indica gum to stand for 48 hours in a solvent containing 0.4% (w/v) dithiothreitol resulted in the disappearance of a peak appearing as a shoulder on column (a), and distinct on column (b) at lower dithiothreitol concentrations. To investigate the effect of this apparent breakdown, a quantity of gum was fractionated by M-SC using these same conditions. Two fractions were obtained, corresponding to material eluted before and after the elution volume of the trough in the elution diagram left by the disappearance of this peak (see fig. 4.2).

A column (31 × 3.7 cm) of Bio-Gel A-5m was prepared, using M-sodium chloride/7M-urea/0.005% (w/v) dithiothreitol/0.05M-tris buffer (pH 9.0) as eluant at a flow-rate of 0.5 ml/min. Applications were made in 8 ml of a 2M-sodium chloride/7M-urea/0.4% (w/v) dithiothreitol/0.05M-tris buffer (pH 9.0) solution, after standing in this solvent for 48 hours. A. indica gum (100 mg) was applied to this column and screened for protein at 254 nm. The resulting trace was identical to that obtained under the same conditions on column (b) [fig. 4.2(4)], but peak elution volumes were increased. A. indica gum (total 2 g) was fractionated in twenty consecutive portions each of 0.1 g, monitoring each fractionation at 254 nm. Fractions I and II were obtained by mixing the material eluted over the regions of the elution diagram

described above. Fractions I and II were exhaustively dialysed against running tap water, concentrated, centrifuged to remove a slight precipitate, then freeze-dried. The precipitate was shown to arise from a slight, continuous bleed from the column, by collection and examination of a portion of column eluant.

To obtain a reference sample, and to investigate further the action of the application solution on A. indica gum, a portion of gum (2 g) was allowed to stand in this solvent (160 ml) for 48 hours. The solution was exhaustively dialysed, filtered, and the product was recovered by freeze-drying (fraction III).

To ensure that urea did not remain in fractions I-III, each fraction was redissolved in distilled water (50 ml) and sodium chloride (2 g) was added, to weaken any hydrogen bonding present. Solutions were dialysed against several changes of distilled water, and fractions I-III were recovered as the freeze-dried products.

Analytical Examination of A. indica Gum Samples A and B and Fractions I-III

Yields and results of analyses of these products are shown in table 4.2.

Portions (20 mg) of samples A and B and fractions I-III were hydrolysed with N-sulphuric acid for 7 hours on a boiling water bath. Paper chromatography of the hydrolysates in solvents (a), (b) and (c) indicated the presence, in each, of galactose, arabinose, lesser amounts of mannose, small amounts of xylose and fucose, and trace amounts of rhamnose. Chromatography in solvent (c)

also indicated the presence in each sample of two acidic components with the mobilities of 4-O-(α-D-glucopyranosyluronic acid)-D-galactose (minor component, R_{gal} 0.28) and 4-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-galactose (major component, R_{gal} 0.66). Further portions (20 mg) of each product were hydrolysed with 2N-sulphuric acid for 7 hours on a boiling water bath. Paper chromatography of the hydrolysates in solvents (a), (c) and (h) indicated the presence, in each, of the above neutral sugars together with 4-O-methylglucuronic acid and small amounts of glucuronic acid and glucurono-6,3-lactone. On the chromatograms of each hydrolysate, the relative intensities of spots were similar, so the carbohydrate compositions of the samples are probably similar.

Portions (50 mg) of samples A and B and fractions I-III were dyed[67] with Procion Red M-2B dye. Dyed polysaccharides (3-4 mg) were dissolved in 2M-sodium chloride (0.5 ml) and examined by M-SC on a column (40 x 1.5 cm) of Bio-Gel A-5m using M-sodium chloride as eluant at a flow-rate of 0.5 ml/min. Elution diagrams, obtained by an automated colorimetric method[33] are shown in fig. 4.3. Samples A and B were similar but with sample B having a larger void volume peak, and a distinct peak rather than a shoulder at elution volume 69 ml. Fraction I was of higher molecular weight than fraction II (having a similar elution volume to samples A and B), and fractions I and II had traces of each other as impurity. Fraction III showed two peaks corresponding to those of fractions I and II, indicating that breakdown had occurred on standing with sodium chloride/urea/dithiothreitol/tris solvent, but complete reaggregation had not occurred on its removal.

Portions (containing ca. 2 mg protein) of samples A and B and fractions I-III were hydrolysed and examined for amino acids. Results are shown in table 4.3. Nitrogen recoveries were high, indicating satisfactory analyses. The major amino acid was, in each case, aspartic acid with the other common amino acids, except methionine, present in appreciable amount. A. indica gum samples A and B were similar, differing slightly in proline and methionine content, but sample A and fraction III were almost identical. Fractions I and II showed only slight differences from each other and from the other samples. A component whose elution corresponded to that of glucosamine was found in appreciable quantity in all five samples.

4.3 DISCUSSION

To investigate a series of results, unusual in a gum exudate, viz. high negative optical rotation,[93] the presence of fucose,[94] an appreciable amount of an amino sugar,[75] and about 20% of proteinaceous material,[75] found for Azadirachta indica gum, two samples of this gum were obtained for study. Preliminary investigations on the purified samples showed that the gum was readily soluble in distilled water, possessed a high negative optical rotation, though rather lower than that reported by Mukherjee and Srivastava,[93] and gave a nitrogen value which indicated a protein content almost twice that found (by a different method) by Lakshmi and Pattabiraman.[75] A more detailed investigation of the samples, together with fractions I-III obtained by molecular-sieve chromatography of A. indica gum, was

made, and results are discussed later.

Amino sugars have been widely studied, due to their occurrence in many of the natural products isolated from animal (including human) and microbial sources.[99,100] A glucosamine-containing oligosaccharide has been isolated from the heparin-protein linkage region,[101] protein preparations from skin tissue have been shown[102] to contain glucosamine and galactosamine and the hexosamine content of hen egg albumin has been investigated.[103] Other studies on amino sugars include their identification in the bacterial cell-wall,[104] and their distribution in soil.[105,106] Naturally, this widespread occurrence has led to much work on their identification,[107] and determination. Rapid, automated methods for hexosamine determination[108,109] and for the estimation of glucosamine and galactosamine in mixtures[110,111] using anion-exchange chromatography have been described. Methods have been adapted to micro-scale determinations,[112, 113] and analyses of amino sugars by gas-liquid chromatography[114] and mass spectrometry[115] have been suggested.

The distribution of amino sugars in plants, however, has so far appeared to be restricted to a few species[116] and, until 1967,[75] no study of their distribution in plant gum exudates had been made. A study of nine plant gums[75] produced evidence for the presence of an amino sugar, present in 2.9% amount and characterised as D-glucosamine, only in the case of A. indica gum. Analyses, described in this section, for amino sugars in A. indica gum samples A and B, confirm the presence of hexosamine, but in

amount (2.0 - 2.2%) rather lower than that claimed by Lakshmi and Pattabiraman.[75] The methods employed were similar to that of Lakshmi and Pattabiraman, but included modifications to take advantage of milder hydrolysis conditions, a more efficient neutralisation procedure than that of King,[96] and the improved colour development found on heating. Analyses for hexosamine in the gums from Khaya nyasica, K. senegalensis, Fagara macrophylla, F. xanthoxyloides and Boswellia papyrifera by the same methods were made, and produced negative results. These, together with the results of Lakshmi and Pattabiraman[75] would seem to indicate that few gum exudates possess hexosamine, but amino acid analyses of plant gums of the Lannea (section IIIA), Acacia[7] and Araucaria[7] genera all show small quantities of a component whose elution corresponds to that of glucosamine. It is possible that the colorimetric methods of hexosamine determination used above and used by Lakshmi and Pattabiraman[75] were subject to marked interference by the large quantity of carbohydrate and protein present in the gums, despite attempts to use suitable blank solutions, producing negative results for gums of low hexosamine content. This would imply that the value of 2-3% hexosamine in A. indica gum is probably low. Future work on this topic could possibly involve further investigation of the hydrolysis conditions resulting in maximum yield of amino sugars from plant gums, then careful separation of the amino sugars from other constituents by ion-exchange chromatography[110,111] before estimation by a suitable method. This would prevent the interference effects suggested by the above results.

Preliminary determination of nitrogen content of A. indica gum suggested a protein content of ca. 35%. Natural products in which polysaccharide and protein components are involved are very common, and may or may not involve covalent bonding between the components. It has been suggested that the presence of nitrogen in plant gums may be of fundamental significance[7,59,60] despite some reports[37,40] of a plant gum possessing no nitrogen (see Section III A). Attempts to remove most, if not all, of the protein from A. indica gum were largely unsuccessful, shown by nitrogen determinations on the products recovered (table 4.1). Preferential precipitation using ethanol was found to remove carbohydrate material, leaving a product enriched in protein. The reason for this is not clear; protein was obviously not preferentially precipitated, but a partial precipitation of the gum itself would be expected to remove the material of highest molecular weight, a fraction which has been shown[7,117] to contain more nitrogen than lower molecular weight fractions. This is confirmed by later studied on A. indica gum by M-SC [figs. 4.1(1) and 4.1(2)]. When using chloroform and butanol, or 1,2,2-trifluoro-1,1,2-trichloroethane, a small amount of material observed at the interface could not have been merely denatured protein, as a slight increase in protein content of the gum was observed in each case. Trichloroacetic and trifluoroacetic acids are in common use as protein precipitating agents, but were found to have little effect on the gum (even when solutions were shaken and heated to encourage precipitation), slightly increasing and decreasing the observed protein content respectively. The protein content was unaffected by sodium borohydride solution, which has been found to be the

best solvent for a water-insoluble gel present in Acacia drepanolobium gum,[118] possibly because it reduces protein disulphide bonds,[119] interfering with interactions causing aggregation. An appreciable drop in protein was found after treatment with tungstic acid solution, but the conditions employed (heating and standing at low pH) would be expected to adversely affect the polysaccharide. The use of enzymes, especially pronase, markedly decreased the protein content, but again the conditions employed (low pH with pepsin, heating at 60°C for 24 hours with pronase) could affect the polysaccharide component. It has been observed[120] that aggregation of protein molecules will only occur if the concentration is sufficiently high, but treatment of a 20% gum solution (5 ml) with 20% trichloroacetic acid solution (5 ml) still produced no precipitate, even after standing overnight. These results indicate that the protein and carbohydrate components of A. indica gum are strongly attached, perhaps covalently bonded, as the common agents for precipitation of protein from solution by alteration of protein bonds, both intra- and intermolecular, had little or no effect on the gum. Enzymes, on the other hand, did remove appreciable quantities of protein, but these act by cleavage of protein chains rather than by affecting the protein molecule as a whole, so their activity should be little influenced by the nature of any protein-polysaccharide bonding. The possibility, that the other methods did remove a large amount of protein, producing small polysaccharide fragments which did not precipitate with the remaining gum molecules, leaving the nitrogen content undisturbed, must be discounted, because at no stage in these methods was a

large amount of denatured protein observed, and because the product after treatment with pronase had lost more than 50% of its protein, yet still precipitated satisfactorily.

The effect, on A. indica gum, of M-SC on a column of Bio-Gel A-5m using a sodium chloride/urea/dithiothreitol/tris solution as solvent and eluant[7] [column (b)] was studied, and compared with results using a sodium chloride solution as solvent and eluant [column (a)]. Urea is a known protein denaturant[121] which acts by breaking hydrophobic bonds as well as by interacting with peptide bonds,[122] while dithiothreitol is known to reduce disulphide bonds in proteins.[119] The presence of sodium chloride should minimise electrostatic bonding. The solvent used with column (b) should therefore disrupt all but covalent bonds (if present) between carbohydrate and protein, and the use of a similar solution as eluant should prevent any recombination during M-SC. Such a solvent has been employed previously[123] to obtain consistent molecular weights of proteins, by breaking all but primary bonds.

Elution volumes on column (b) were found to be considerably lower than on column (a) (fig. 4.1), which is possibly due to the prevention of intramolecular hydrogen bonding by urea, resulting in expanded gum molecules; this expansion effect has been observed with serum albumin.[124] Column (b) was able to distinguish a peak observed only as a shoulder on column (a) (fig. 4.1), which perhaps implies slight differences in intramolecular bonding of the molecules of the two peaks. It was observed (fig. 4.1) that the quantity of dithiothreitol used by Munro[7] was insufficient to completely alter

the elution diagram of A. indica gum. Experiments to determine optimum time of standing in solvent before M-SC was performed, and dithiothreitol concentration required, showed (figs. 4.1 and 4.2), that standing in solvent of dithiothreitol concentration 0.4% for 48 hours resulted in breakdown of the peak which had appeared as a shoulder on column (a), and that the elution diagram underwent no further alteration when dithiothreitol concentration and/or time of standing was increased. These results might imply that the peak which had disappeared was due to molecules in which there was non-covalent bonding between the carbohydrate and protein components. The other main peaks were unaffected by this solvent, suggesting that they were perhaps due to molecules in which the carbohydrate and protein components were covalently bonded. Why a component involving non-covalent linkages, if present, was not removed during attempted deproteinisation is not clear. It is probable that this component would possess hydrogen, electrostatic, hydrophobic and disulphide linkages, but unlike the solvent employed in M-SC studies, which was designed to break all of these bonds, methods used in attempted deproteinisation must have been unable to break sufficient of these bonds to allow protein to precipitate. It was found that dyed polysaccharides were unsuited for studies involving dithiothreitol concentrations of greater than ca. 0.4%, but it is unlikely that results obtained using dyed gums would have been valid in any case; dye molecules bond to the constituent residues of the polysaccharide component and, to a lesser extent, of the protein component, probably altering the intramolecular bonds. Screening by the phenol-sulphuric acid method[13] after M-SC of undyed

A. indica gum on column (b), using the optimum conditions detailed above, produced an elution diagram [fig. 4.2(7)] similar to that obtained by screening for protein at 254 nm [fig. 4.2(4)], but the peak eluting at 37 ml was much enhanced, implying that the first (void volume) peak had a higher ratio of protein to carbohydrate than this second peak. This is consistent with elution diagrams of dyed gum (screened colorimetrically) and undyed gum (screened at 254 nm) on column (a) [figs. 4.1(1) and 4.1(2)], and agrees with previous investigations on the high molecular weight fraction of Acacia gums.(7,117)

A portion (2 g) of A. indica gum was fractionated on a large column of Bio-Gel A-5m using sodium chloride/urea/dithiothreitol/tris solution as solvent and eluant. Fractions I and II comprised material eluted before and after the position of the trough in the elution diagram due to disappearance of the peak, discussed above. Fraction I should correspond to the higher molecular weight protion of the original gum, and fraction II to material of lower molecular weight than the main carbohydrate peak of the gum (fig. 4.2). A third fraction was obtained by treatment of gum with solvent, then recovering the gum without having performed M-SC.

A. indica gum samples A and B were analysed, together with fractions I-III (table 4.2). Samples A and B were found to be similar, but sample B was of higher molecular weight, consistent with its higher limiting flow-time number. Otherwise the analytical parameters agree closely, and show that A. indica gum has a high negative optical rotation, and 35 - 37.5% proteinaceous material. The gum also has much uronic acid; the large methoxyl content and

hydrolysis studies show this to be largely 4-O-methylglucuronic acid. The values of $^{dn}/_{dc}$ found for samples A and B are appreciably higher than values for gums of the Lannea (see section III A) and Acacia[69] genera. Fraction III was found to be similar to sample A, showing that treatment of the gum with a sodium chloride/urea/dithiothreitol/tris solvent does not result in drastic breakdown. The limiting flow-time number and nitrogen content of fraction III are lower than those of sample A, suggesting perhaps some slight degradation and loss of a little loosely-bound protein. Fractions I and II do not differ greatly from the other samples, but fraction I has a low content of proteinaceous material. The high molecular weight of fraction I is consistent with it being the high molecular weight fraction of the gum, and the low limiting flow-time number of fraction II is expected, as it is the low molecular weight fraction. Fractions I and II also possess a high negative optical rotation.

M-SC examination (fig. 4.3) showed samples A and B to be similar, with minor differences in the peak appearing at the void volume (which depends on the dyeing procedure), and that appearing as a shoulder in sample A, but distinct in sample B. Fraction I showed a sharper peak than sample A, due to removal of low molecular weight material, with a trace of fraction II remaining. Fraction II contained a trace of fraction I and was found to be of low molecular weight. Fraction III showed peaks corresponding to fractions I and II which is consistent with its observed limiting flow-time number. Breakdown caused by the solvent appears, therefore, to be largely irreversible, with little reaggregation occurring on removal of

the sodium chloride/urea/dithiothreitol/tris solution. The effect of dithiothreitol by itself on Acacia campylacantha gum has been shown[7] to be reversible, reaggregation occurring on its removal.

Hydrolysis studies on samples A and B and fractions I-III verified the presence in A. indica gum of galactose, arabinose and small amounts of fucose and xylose, which were reported by earlier investigators,[94] but mannose in appreciable amount and rhamnose in trace amount were also detected. The uronic acids present in the gum were found to be glucuronic acid (minor component) and 4-O-methylglucuronic acid (major component), and evidence for the presence of the aldobiouronic acids 4-O-(α-D-glucopyranosyluronic acid)-D-galactose and 4-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-galactose was obtained. Mannose, rhamnose and 4-O-methylglucuronic acid were not identified in the earlier study.[94] Possibly the use of methylation analysis, by these investigators, to identify the aldobiouronic acid salt obtained, prevented distinction between glucuronic acid and 4-O-methylglucuronic acid. Chromatograms of the hydrolysates of samples A and B and fractions I-III were observed to be similar, indicating no gross differences in the carbohydrate constituents of each, suggesting that the solvent used for M-SC fractionation affected protein bonding, but had little or no effect on polysaccharide chains. This might imply that, at least in the molecules degraded by this solvent, the polysaccharide chains are relatively short, (dyed fraction II eluted near the exclusion volume of a Bio-Gel A-5m column), being compounded into larger aggregates by protein interactions.

Amino acid analyses of samples A and B and fractions I-III are shown in table 4.3, and reveal only minor variations between the samples. The large nitrogen recoveries would indicate that virtually all the nitrogen of A. indica gum is proteinaceous. The value for proteinaceous material in the gum reported by Lakshmi and Pattabiraman[75] would therefore seem to be low. The difference is not due to the different purification method employed, as a sample purified by their procedure was found to have a protein content of 34.4% (table 4.1), a value differing only slightly from that of gum purified in the usual way (table 4.2) (due probably to no correction for residual moisture in the former product being made). Almost all the common amino acids are present in appreciable amount in each sample, perhaps indicating that the proteins occurring in the gum are of fairly high molecular weight, rather than being present as many short peptide chains. Aspartic acid was found in largest amount. As expected, a component corresponding to glucosamine was found in samples A and B and also in each of the fractions. The possibility therefore exists of a linkage region between carbohydrate and protein components of the gum involving aspartic acid and glucosamine (or more probably N-acyl glucosamine in the original gum[75]). Such a linkage occurs in animal glycoproteins, and has been demonstrated in plants, in a glycopeptide from Soybean hemaglutinin[125] which contained mannose, N-acetyl glucosamine and aspartic acid. The aspartic acid was shown to be directly linked to N-acetyl glucosamine and the suggested linkage was 1-L-β-aspartamido-(2-acetamido)-1,2-dideoxy-β-D-glucose. Mannose has also been identified in A. indica gum. The quantities

of glucosamine, calculated from the amino acid analysis traces for each sample, were sample A 1.7%, sample B 1.2%, fraction I 1.0%, fraction II 2.7%, fraction III 1.6%. The degradation of glucosamine might be expected to be less than in the case of the Lanneas (Section III A) due to the presence of smaller amounts of carbohydrate, but would probably still be at least 80%.[7,76] Assuming this degradation, glucosamine contents of the original samples would be sample A 8.5%, sample B 6.0%, fraction I 5.0%, fraction II 13.5%, fraction III 8.0%. This would tend to confirm that earlier estimations of hexosamine gave rather low values. Future investigators of this topic might attempt to isolate glycopeptides from A. indica gum, probably by enzymic hydrolysis, for which details of any carbohydrate-protein covalent linkages could be elucidated.

SECTION V

AUTOMATED MOLECULAR-SIEVE CHROMATOGRAPHY OF POLYSACCHARIDES

5.1 INTRODUCTION

Molecular-sieve chromatography (M-SC), also known as gel filtration and gel permeation chromatography, is a rapid, high-resolution, analytical technique used in the measurement of molecular sizes and molecular weight distributions of heterogeneous systems, for estimation of molecular weight of macromolecules, and for studying equilibria and system interactions [126]. Since its development began about 1953, application of the technique has been extended to a wide range of compounds, especially those of a polymeric or biological nature. The mechanism of separation is unique among column fractionations, being primarily based on the differing rates with which molecules of various sizes in solution migrate through porous granules packed in a column [127], rather than depending on solubility or adsorption differences. When other such features occur, it is usually possible to turn their existence to additional advantage [128,129]. The method has been scaled up, making separation of gram-quantities possible [130], scaled down to separations on the micro scale [131,132], and speeded up to give separations in 0.3 - 30 min [131,133]. Thin layer M-SC is widely used [134,135].

A considerable range of porous gels, glass and silica materials is now available commercially for column packing, and this range will probably increase in the near future. Manufacturer's data for such products is usually obtained using characterised proteins, dextrans or polystyrene fractions; their performance with other types of polymer (e.g. acidic polysaccharides) frequently differs, and must be evaluated. Pore-size distribution [136] and particle

size of the packing material, eluant composition, flow rate and column shape[137] have significant influence on the degree of separation attainable. Except for polymer systems that have been rigorously characterised, the most suitable molecular-sieve and optimum chromatographic parameters are best found by experiment.

In earlier studies on acidic polysaccharides[82,117], elution patterns were obtained by collecting many small fractions, which were individually screened by the phenol-sulphuric acid method[13]. When many runs have to be performed, an automated method for continuous monitoring of effluent from columns is clearly required. Several methods are available, including differential refractometry[138], densitometry[139], spectroscopy[140], radioactive labelling[141]; flame-ionisation, conductivity[142,143] and differential vapour-pressure[144] detectors, and polarography[145]. Not all of these methods are suitable for column effluents containing polysaccharides; a suitable technique using available laboratory equipment was required.

Dudman and Bishop[67] recently described the reaction of "Procion" dyes (I.C.I. Ltd.) with polysaccharides. Coloured derivatives were studied by electrophoresis on cellulose acetate film, overcoming the difficulty of detection of undyed material studied by this technique. The authors found a pure polygalacturonan was completely resistant to the dyes tested, a polysaccharide containing ca. 30% uronic acid was dyed only to a slight extent, but they did not report results for acidic gum polysaccharides. Mention was made of the possibility of using dyed polysaccharides to monitor the behaviour of polysaccharides in gel filtration and other chromatographic systems.

In this study, the use of the dyeing technique for monitoring

M-SC is explored. Development work was shared with Mr. (now Dr.) A.C. Munro; unless stated otherwise, the results quoted here are from my own experiments.

5.2 EXPERIMENTAL

Polysaccharides were dyed according to the procedure used by Dudman and Bishop [67] ; polysaccharides (50 mg) in water (5 ml) were mixed with fresh solutions of Procion Brilliant Red M-2B dye (50 mg) in water (5 ml). After 5 min, sodium chloride (200 mg) was added, followed by sodium carbonate (10 mg) after a further 30 min. Solutions were allowed to stand overnight. M-SC on a column (35 × 2.5 cm) of Sephadex G-25 using distilled water as eluant separated the dyed polysaccharides, eluted at the void volume of the column, from residual dye and inorganic salts. The solutions of dyed polysaccharides were freeze-dried.

Dyed polysaccharide (2-10 mg) in 2M-sodium chloride solution (0.5 - 1.0 ml) was applied with care to the top of a column containing an appropriate molecular-sieve. Elution was carried out with M-sodium chloride solution at flow-rates of 0.5 - 2.0 ml/min, when automated analysis took ca. 2 hours.

The apparatus used to monitor the effluent from the column is shown in fig. 5.1(1). The effluent was passed into a stoppered glass cell (3 ml) in a Unicam SP1300 colorimeter set at filter 3. The cell-outlet tube was adjusted in length to give an internal cell volume of ca. 1 ml, and connected to a water-pump via a measuring cylinder (100 or 250 ml). The water-pump maintained constant cell

volume, and to ensure that no suction was applied to the column, a third tube was left open to the air. Polythene capillary tubing was used throughout for all connections. More recently, the apparatus was improved by disconnecting the filter-pump, blocking the air-inlet tube and ensuring the stopper fitted tightly on the cell [fig. 5.1(2)]. The column effluent then passed directly through the cell without suction being necessary. The colorimeter light beam was attenuated to below the solution level in the cell, and the photocell output from the colorimeter fed to a recorder (Kent Mark 3 Electronic) via an impedance matching device (Vibron Electrometer Model 33B). The input resistor of the recorder was increased from 1 to 2.5 Ohms, giving a recorder deflection of 10 inches at minimum output from the photocell. This particular combination of instruments is, of course, not unique; micro-flow colorimeters (Phoenix MFC 800) give an optical path length of 20 mm for a cell hold-up volume of only 0.035 ml if increased sensitivity is essential.

It was found that over the period of any run, column flow-rate was sufficiently constant for peak elution volumes to be obtained by interpolation, if the effluent volume in the measuring cylinder was read at the start and finish of each run. As the colorimeter output was logarithmic and recorder scale linear, peak heights and areas were estimated by calibrating the recorder chart for various readings on the colorimeter scale.

Some elution diagrams showed a small peak at the same elution volume as free dye. This peak was not always reproducible when polysaccharides were re-dyed and examined and could be eliminated by increasing the quantity of sodium carbonate used in the dyeing

procedure to 25 mg. Increased quantity (20-30 mg) of sodium carbonate was also required for satisfactory dyeing of polysaccharides of high uronic acid content, or containing sulphate groups.

The work described in this section was carried out using Procion Brilliant Red M-2B dye. With the appropriate filter in the colorimeter, Procion Blue M-3G dye was also found to give satisfactory results. A survey of a wide range of "Procion" dyes has shown [146] that they all readily dye acidic polysaccharides, but colorimeter response to the dye colours can vary, depending on efficiency of the filter available.

The method detailed above is unsuitable for compounds of molecular weight less than ca. 1500, as difficulty is encountered in freeing the dyed compound from unreacted dye and inorganic salts. In certain cases (see Section IV), conditions may have to be employed which destroy the dye, when again colorimetric monitoring is unsuitable.

5.3 RESULTS

Comparison of Molecular-Sieving Properties of Dyed and Undyed Polysaccharides.

Using M-sodium chloride solution as eluant at flow-rates of 1.0 - 2.0 ml/min, samples of dyed and undyed *Acacia cyanophylla* and *Acacia laeta* gum polysaccharides were run on a column (39 × 3 cm) of Sepharose 4B, and samples of dyed and undyed *Lannea humilis* gum were run on a system of columns (each 40 × 1.5 cm) of Porasil C, Porasil B

and Porasil A, connected in series. The following method was employed:-

(a) Undyed polysaccharide (3-6 mg) in 2M-sodium chloride solution (1 ml) was applied to the column. Fractions (2.1 ml) were collected and analysed for carbohydrate by the phenol-sulphuric acid method [13]. Optical density of the fractions was read on a Unicam SP1300 colorimeter (filter 2).

(b) Dyed polysaccharide (5-10 mg) in 2M-sodium chloride solution (1 ml) was applied to the column and fractions (2.1 ml) were again collected. Optical density of the fractions was read on a Unicam SP1300 colorimeter (filter 3). (This run was not performed with dyed Lannea humilis gum).

(c) The column was connected to the automatic monitoring system described above. Dyed polysaccharide (5-10 mg) in 2M-sodium chloride solution (1 ml) was applied to the column, and its elution diagram recorded.

Results are shown in fig. 5.2. In each case, good agreement in elution volume was found, although the peak appearing at the void volume of the column was enhanced after dyeing the polysaccharide.

Similar experiments with Acacia campylacantha gum on a column (39 × 3 cm) of Sepharose 4B also indicated no change in elution properties after dyeing [7].

Range of Acidic Polysaccharides to Which the Dyeing Technique May be Applied.

It was of interest to investigate whether the dyeing technique was widely applicable in the field of acidic gum polysaccharides.

Accordingly, samples of the gums from Acacia cyanophylla, Acacia campylacantha, Acacia laeta, Acacia pycnantha, Lannea coromandelica, Lannea schimperi, Lannea humilis, Smith-degraded and acid-degraded polysaccharides from Lannea humilis (see Section III B), Azadirachta indica, Combretum grandifolium, Prunus avium, Khaya senegalensis, Araucaria columnaris, Araucaria araucana, Albizia glaberrima, i-carrageenan (sulphate ester, 10%), and Dextran 10, Dextran 20, Dextran 80, Dextran 110 and Dextran 150 (Pharmacia Ltd.), were dyed. All but Khaya senegalensis and i-carrageenan were found to dye readily, and even these were sufficiently coloured for M-SC to be performed, when the quantity of sodium carbonate used in the dyeing procedure was increased from 10 mg to 20-30 mg.

M-SC of dyed samples was performed on columns of Bio-Gel A-5m (35 × 1.5 cm and 40 × 1.5 cm), Bio-Gel P-10 (35 × 1.5 cm), Sepharose 4B (35 × 1.5 cm) and a system of columns (each 40 × 1.5 cm) of Porasil C, Porasil B and Porasil A connected in series, using M-sodium chloride solution as eluant, at flow-rates of 0.5 - 2.0 ml/min. Results are shown in table 5.1, and some elution diagrams in fig. 5.3.

I would like to thank Mr. J.R.A. Millar for examining the behavior of Lannea humilis and dyed Khaya senegalensis and i-carragennan on the Porasil C, Porasil B, Porasil A system.

Evaluation of Some Commercially Available, M-SC Column Materials.

Until recently, M-SC has been performed on gel columns and much of the work on acidic gum polysaccharides restricted to Bio-Gel P-300 (71,147). The range of commercially available materials includes porous gels, glasses and silicas, and use was

made of the rapid, automated method described in this section, to evaluate a selection of these.

Using M-sodium chloride solution as eluant, at flow-rates of 0.5 - 2.0 ml/min, and employing the dyed Acacias, Lanneas and Dextrans detailed above, elution properties of columns of Bio-Gel A-5m (35 × 1.5 cm and 40 × 1.5 cm), Bio-Gel P-300 (40 × 1.5 cm), Bio-Gel P-10 (35 × 1.5 cm), Sepharose 2B (37 × 1.5 cm), Sepharose 4B (35 × 1.5 cm), Bio-Glas-500 (33 × 1.5 cm), Bio-Glas-1500 (43 × 1.5 cm), Corning CPG 10-1250 (43 × 1.5 cm), Porasil A (40 × 1.5 cm), Porasil B (40 × 1.5 cm), Porasil C (40 × 1.5 cm) and Porasil E (37 × 1.5 cm) were investigated. Elution properties were also investigated for a column system obtained by connecting the Porasil C, Porasil B and Porasil A columns in series; the minimum length of polythene capillary tubing was used for connections, and when system was in operation, it was arranged that bed material of the second and third columns was just covered by eluant, to minimise mixing. A comparison of column materials is given in table 5.2, and some typical elution diagrams are shown in fig. 5.4.

Because of its rigidity, porous glass or porous silica lends itself to work with higher flow-rates than can be attained using porous gels. Runs were performed on the column (40 × 1.5 cm) of Porasil C at flow-rates of ca. 10 ml/min, to determine whether resolution still was satisfactory; to apply a sample, eluant flow was stopped, polysaccharide (3-5 mg) in 2M-sodium chloride solution (0.5 ml) was carefully layered on the Porasil then eluant flow restarted. Flow-rates of ca. 10 ml/min were obtained by positioning the eluant reservoir about 95 cm above the top of the column. Under

these conditions, runs were complete in 9 min. Results are shown in fig. 5.5.

5.4 DISCUSSION

To obtain best possible results from M-SC, as well as other techniques, operational parameters must be optimised [148,149], e.g. the most useful column material, flow-rate and eluant must be chosen. This involves repeated trial runs, and when a commercial M-SC instrument is not available, an automated method of recording elution diagrams is essential, individual analysis of collected fractions being laborious and time-consuming. The automated, colorimetric technique described in this section is simple, inexpensive to set up, and has been shown to give satisfactory elution diagrams for the range of acidic polysaccharides studied. Caution should, however, be exercised when extending the method to other types of compounds. Elution diagrams of dyed and undyed material must be compared, to ensure that unexpected effects do not occur.

"Procion" dyes [150] are dichlorotriazinyl dyes which, by possessing sulphonic acid groups, are readily soluble in water. In neutral solution, physical adsorption and possibly some hydrogen bonding take place between dye and polysaccharide, but there is no formation of covalent bonds until alkali has been added. It has been determined that "Procion" dyes react with compounds containing primary hydroxyl groups approximately seven times faster than with compounds containing secondary hydroxyl groups [150]. So additional sodium carbonate must be added to overcome the acidity of a polysaccharide which has a high uronic acid content, although dyeing will

be less intense because uronic acid residues contain no primary hydroxyl group. Additional alkali must also be used when dyeing a carrageenan. The small, exclusion volume peak found occasionally in M-SC traces was probably dye which had been physically adsorbed, but not chemically bonded, then released on subsequent storage. This would account for its disappearance when more sodium carbonate was used in the dyeing procedure. Incomplete desalting by M-SC on the Sephadex G-25 column would, if present, enlarge this peak.

The automated M-SC method described involves application of dyed sample to a column and continuous monitoring of optical density of effluent. The progress of the sample through the column is visible (a return to true chromatography), enabling poor results due to inconsistencies in column flow to be immediately detected. The modification to the system, whereby the filter-pump may be removed, offers the advantage that liquid level in the colorimeter cell is steadier and precipitation of some salt from the eluant, at the top of the cell, is avoided. No difference in elution properties was noticed after removal of the filter-pump.

Elution volumes of dyed and undyed polysaccharides were shown to be equal, within experimental limits (fig. 5.2). This is surprising, as addition of dye to a molecule would be expected to increase its molecular weight, so decreasing its elution volume. From the increase in nitrogen content due to dyeing, quantity of dye incorporated into the polysaccharide could be calculated. This fell in the range 7% for Acacia cyanophylla (weakly dyed) to 16% for Acacia campylacantha (strongly dyed). These results are similar to values obtained by a different method by Dudman and Bishop[67], and correspond to a molecular weight increase of 8-18%. It is unlikely that this

relatively small increase would be resolved by the M-SC system used. The constancy of elution volumes is important for interpretation of subsequent M-SC elution diagrams.

The relative proportion of the peak eluted at the void volume of a column was usually enhanced after dyeing (fig. 5.2), and may be partially due to preferential reaction of dye with high molecular-weight material [7]. But after bonding with a hydroxyl group of a polysaccharide, the dye molecule still possesses a reactive chlorine atom, and can undergo hydrolysis, react with another hydroxyl group of the same polysaccharide, or react with a hydroxyl group of another polysaccharide. The occurrence of this last possibility would enhance the higher region of molecular weight distribution, and may contribute to an increased void volume peak without affecting elution volumes of other peaks. Similar effects have been noticed with molecular aggregates in a histochemical staining reaction for protein-bound carboxyl groups [151].

M-SC of a series of dextrans showed their elution to be in order of decreasing molecular weight (table 5.1), obeying generally accepted theories of M-SC [127]. Dextrans have been used to calibrate molecular-sieve columns for determination of molecular weights [71,147], so dyed dextrans would appear to be suitable standards for molecular weight determinations of dyed polysaccharides. Care must still be exercised when calibration is carried out with fractions of dissimilar structure to the polymer under investigation; methods of overcoming this difficulty have been described in the literature [152,153]. A wide range of acidic gum polysaccharides were found to dye readily, so molecular weights can be quickly and

easily estimated if care is taken over column calibration. An idea of molecular weight distribution may be readily obtained, if it is borne in mind that the distribution is probably weighted to the region of high molecular weight, compared to that found by screening fractions of undyed polysaccharide. The extent of application of this colorimetric method seems to cover gum exudates from many genera, which vary widely in structure and uronic acid content, and offers a quick, easy comparison of a series of samples.

Use was made of this rapid method to evaluate some of the commercially available M-SC column materials. It was found that porous gels give extremely good resolutions (figs. 5.2, 5.3, 5.4), with Bio-Gel A-5m and Sepharose 4B being the most generally useful for a range of gum exudates. The former has a slightly wider sieving-range, producing better resolution of peaks. Sepharose 2B is of use for material of high molecular weight (fig. 5.4), whilst Bio-Gel P-300 and Bio-Gel P-10 sieve in much lower molecular weight ranges (figs. 5.3, 5.4). Drawbacks to the use of softer gels are that packing a column takes some time, flow-rates are low, and tops of the gel beds require stabilisation (usually a 1 cm band of a firmer gel e.g. Bio-Gel P-4 or Sephadex G-15). All gels are prone to bacterial attack, although the use of sodium azide, thymol or other bacteriostatic agents delays its onset.

Results using porous glasses were poor. Bio-Glas-500 and Bio-Glas-1500 may have been incompletely silanised, and recently a more efficient silanisation method than that recommended by the manufacturer has been published [154]. Adsorption effects were noticed, but were saturated after the first few runs. Some

separation of peaks was obtained (fig. 5.4) but resolution was much poorer than that of porous gels. Corning CPG 10-1250 showed similar results. This material was not silanised before use (manufacturer's advice).

Although the sieving range of porous silicas was found to be rather less than that of porous gels, resolution still was satisfactory (figs. 5.4, 5.5). Porasil C and Porasil E were shown to be of suitable sieving range for a wide variety of acidic polysaccharides. Initial adsorption effects found with Porasil A and Porasil B were saturated in the first few runs, but the use of "deactivated Porasils" [155] would offer advantages. Ease of packing, stability and rigidity of column beds and resistance to bacterial attack, combined with satisfactory sieving properties mean that porous silica columns are extremely useful. In an attempt to extend sieving ranges, Porasil C, Porasil B and Porasil A were connected in series. A slight loss in resolution of high molecular weight material was noticed; Dextran 150 elutes just after the void volume on Porasil C, yet its elution volume on the three-column system was indistinguishable from the void volume. However, elution diagrams of dyed polysaccharides (figs. 5.2, 5.3) were satisfactory. The use of longer columns, taking advantage of the high flow-rates possible with porous glass and silica columns, would also overcome the problem of restricted sieving-ranges.

The stability of porous silica columns enables flow-rate to be increased, and elution times considerably shortened. A study of operational parameters of fast M-SC at flow rates up to 12.5 ml/min has shown [133] that sacrifices in terms of resolution and peak

width should be much smaller than predicted by theory. The use of small Sephadex columns [10.5 × (40-50) mm] at high pressure (up to 60 p.s.i.) enables runs to be completed in 20-100 sec, but the column must be renewed after each run [131]. At flow-rates of 10 ml/min on a column (40 × 1.5 cm) of Porasil C, elution was complete in 9 min (fig. 5.5). Fast M-SC with porous silica further enhanced the peak which appears at the void volume, and elution volumes were decreased somewhat (fig. 5.5), probably due to some compression of bed material, but were reproducible. M-SC at high flow-rates would be extremely useful when many runs had to be performed.

APPENDIX I

A COMPARISON OF TWO METHODS OF POLYSACCHARIDE METHYLATION

During a study of structural features of L. humilis gum, the opportunity was taken to compare two methods of methylation. The methods compared were (1) the combined Haworth and Purdie procedures [19,20] and (2) the sodium hydride, methyl iodide, dimethylsulphoxide procedure [21]; L. humilis gum and its Smith degradation product, polysaccharide I, were subjected to each of these methylation methods.

Gum Sample	Methylation Method	Yield (%)	Optical rotation	OMe (%)
L. humilis gum	(1)	76	0°	41.6
L. humilis gum	(2)	86	-11°	40.5
polysaccharide I	(1)	78	- 0.5°	40.4
polysaccharide I	(2)	94	-36°	41.9

Rather better yields were obtained using method (2), but its products had more negative optical rotations than those from method (1). An attempt to obtain a value of $\bar{M}_w$ of L. humilis gum methylated by each procedure failed, due to lack of material. Each product was methanolysed and examined by g.l.c. then the mixtures of methyl glycosides were hydrolysed and examined chromatographically in solvents (e) and (f) (tables 3B.4, 3B.5 and page 46). The ratio of major O-methyl-D-galactose sugars was found to be similar in each case, but some differences in the chromatograms were observed. The ratio of 2,3,4- to 2,3,5-tri-O-methyl-L-arabinose was higher after methylation by procedure (2), but this method resulted in only traces of 2,6-di- and 2-O-methyl-D-galactose from methylated L. humilis gum, and only traces of

2,3,6-tri-, 2,6-di- and 2-O-methyl-D-galactose from methylated polysaccharide I. The reason for a variation in the ratio of tri-O-methyl-L-arabinose residues is not obvious, but the absence of products of undermethylation suggests this procedure is more efficient.

Attempted remethylation, by further Purdie procedures, of the products obtained by procedure (1) produced a slight drop in methoxyl content, probably due to some silver impurity. Values of OMe $\geq$ 39% were therefore taken to imply complete methylation, but it would appear that the method employed for methoxyl determination is insufficiently sensitive to detect the minor variation in OMe value due to the small amounts of undermethylation products present in samples methylated by procedure (1). The assumption that 2,3,6-tri-, 2,6-di- and 2-O-methyl-D-galactose residues were produced by undermethylation, made during studies on degradation products of L. humilis gum, would appear to be justified from the results of methylation by procedure (2).

These experiments would indicate that the sodium hydride, methyl iodide, dimethylsulphoxide procedure results in a more efficient methylation than the Haworth and Purdie procedures. Investigation is, however, required into the reasons for variations in optical rotation and in ratios of 2,3,4- to 2,3,5-tri-O-methyl-L-arabinose residues. A previous study [156] advised caution in the interpretation of results obtained from gums having significant proportions of L-rhamnopyranose residues as end-groups, attached to position 4 of D-glucuronic acid, when methylated by the sodium hydride, methyl iodide, dimethylsulphoxide procedure, but such

residues do not occur to any great extent in L. humilis gum. It is suggested that both methods be compared over a wider range of polysaccharides to assess the importance of the variation of results observed.

APPENDIX II

THIN-LAYER ELECTROPHORESIS OF DYED POLYSACCHARIDES

Dudman and Bishop[67] prepared dyed polysaccharides for use in cellulose acetate electrophoresis. They reported cellulose acetate to be the only satisfactory support, agar and polyacrylamide gels, filter paper and glass-fibre paper giving broad bands with dyed polysaccharides and not resolving mixtures.

The use of thin-layer electrophoresis on Phoroslides (Millipore Ltd.), with 0.1M-ammonium carbonate buffer (pH 8.9), 0.1M-acetate buffer (pH 4.7) or 0.05M-borate buffer (pH 9.2) as electrolyte, for investigation of polysaccharides dyed with Procion Brilliant Red M-2B dye, was evaluated. Dyed polysaccharides were shown to migrate faster than undyed samples but useful separations, based primarily on uronic acid content of the polysaccharides, were observed (fig. A.1). Fig. A.2 shows separation of a mixture of dyed Dextran 110 (uronic acid = 0%), and dyed gums from Acacia campylacantha (uronic acid = 9%) and Acacia cyanophylla (uronic acid = 25%). Separation is clearly seen in as little as 1 min. The mobility of the dextran probably indicates the influence of dye on electrophoretic migration.

Thin-layer electrophoresis on Phoroslides appears to be a useful alternative to cellulose acetate electrophoresis for dyed polysaccharides, producing useful resolution of components of mixtures in a shorter time interval. As was found with M-SC (see Section V), the ability to observe migration of a dyed sample is extremely useful, and the use of dyed polysaccharides, by dispensing with the need for subsequent detection of bands after electrophoresis, offers advantages in terms of time and effort required, over corresponding work with undyed samples.

TABLES

TABLE 3A.1

ANALYSES OF PURIFIED LANNEA GUM SAMPLES

	Lannea coromandelica	Lannea humilis,A	Lannea humilis,B	Lannea schimperi
Moisture, %	11.8	10.6	12.9	7.2
Ash, %	3.5	2.5	2.6	4.2
Nitrogen, % (a)	0.22	0.28	0.29	0.27
Protein, % (N×6.25)	1.38	1.75	1.81	1.69
Methoxyl, % (b)	1.6	0.4	0.4	0.9
Uronic anhydride (decarboxylation), % (b)	17	13	14	17
$[\alpha]_D$ (b)	+27°	+36°	+43°	+30°
Limiting flow-time number, ml g^{-1} (a)	11.7	9.6	8.7	14.4
Molecular weight, $\bar{M}_w$ (a,c)	2.57×10^5	3.10×10^5	2.57×10^5	2.41×10^5
Sugar composition; (b,d)				
galactose, %	69.5	72.5	75	69.5
arabinose, %	11	13	11	10
rhamnose, %	2.5	3	2	3.5
uronic acid, % (e)	17	11.5	12	17
Formic acid released on periodate oxidation (mM/g polysaccharide) (b)	3.54	4.54	4.13	5.26
Periodate consumed (mM/g polysaccharide) (b)	9.26	10.13	10.00	10.43
Periodate consumed / Formic acid released	2.62	2.24	2.42	1.99

(a) corrected for moisture and ash contents.

(b) corrected for moisture, ash and protein contents.

(c) in M-sodium chloride at 27°C; the value $dn/dc = 0.154$ (found for the sample of L. coromandelica gum by Dr. I.C.M. Dea) was used for all samples.

(d) sugars calculated as anhydro forms.

(e) average of decarboxylation value and value calculated from equivalent weight.

TABLE 3A.2

ANALYSES OF PURIFIED ELECTRODIALYSED LANNEA GUM SAMPLES

	Lannea coromandelica	Lannea humilis,A	Lannea humilis,B	Lannea schimperi
Moisture, %	8.8	9.0	8.4	8.9
Ash, %	0.4	0.2	0.2	0.04
Equivalent weight, g (a)	1060	1595	1612	1059
Uronic anhydride, % (b)	17	11	11	17

(a) corrected for moisture and ash contents.

(b) calculated on the basis that all acidity arises from uronic acid groups.

TABLE 3A.3

AMINO ACID ANALYSES OF PURIFIED LANNEA GUM SAMPLES (a)

	Lannea coromandelica	Lannea humilis,A	Lannea humilis,B	Lannea schimperi
lysine	21	31	33	13
histidine	6	5	0	6
ammonia	(b)	(b)	(b)	(b)
arginine	18	21	25	15
threonine	161	134	135	210
serine	206	128	132	260
glutamic acid	79	73	78	34
proline	145	220	203	148
glycine	33	34	34	28
alanine	56	69	83	50
valine	39	54	45	30
isoleucine	39	42	45	17
leucine	138	110	115	130
tyrosine	37	42	40	37
phenylalanine	12	24	21	14
glucosamine	10	14	11	9
nitrogen recovery (c)	35%	26%	22%	22%

(a) values are expressed as μ moles amino acid per 1000 μ moles total.

(b) value included only in nitrogen recovery.

(c) expressed as % recovery of Kjeldahl nitrogen.

TABLE 3B.1

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED DEGRADED GUM A

O-methyl sugar identified **	Approximate relative molar proportions
2,3,5-tri-O-methyl-L-arabinose	0.1
2,3,4-tri-O-methyl-L-arabinose	trace
2,5-di-O-methyl-L-arabinose	trace
2,3,4,6-tetra-O-methyl-D-galactose	7
2,3,6-tri-O-methyl-D-galactose	1
2,4,6-tri-O-methyl-D-galactose	4
2,3,4-tri-O-methyl-D-galactose	0.5
2,6-di-O-methyl-D-galactose	0.5
2,4-di-O-methyl-D-galactose	5
2-O-methyl-D-galactose	1
2,3,4-tri-O-methyl-D-glucuronic acid*	2
2,3,4-tri-O-methyl-D-galacturonic acid*	1

* as methyl ester methyl glycoside

** see table 3B.4 for retention times and R_G values

TABLE 3B.2

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED DEGRADED GUM B

O-methyl sugar identified **	Approximate relative molar proportions
2,3,5-tri-O-methyl-L-arabinose	trace
2,3,4-tri-O-methyl-L-arabinose	trace
2,3,4,6-tetra-O-methyl-D-galactose	6
2,3,6-tri-O-methyl-D-galactose	0.5
2,4,6-tri-O-methyl-D-galactose	4
2,3,4-tri-O-methyl-D-galactose	0.2
2,6-di-O-methyl-D-galactose	0.2
2,4-di-O-methyl-D-galactose	4
2-O-methyl-D-galactose	1
2,3,4-tri-O-methyl-D-glucuronic acid*	1.5
2,3,4-tri-O-methyl-D-galacturonic acid*	0.5

* as methyl ester methyl glycoside

** see table 3B.4 for retention times and R_G values

TABLE 3B.3

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED DEGRADED GUM C

O-methyl sugar identified **	Approximate relative molar proportions
2,3,4,6-tetra-O-methyl-D-galactose	1
2,3,6-tri-O-methyl-D-galactose	0.1
2,4,6-tri-O-methyl-D-galactose	9
2,3,4-tri-O-methyl-D-galactose	trace
2,6-di-O-methyl-D-galactose	2
2,4-di-O-methyl-D-galactose	trace
2-O-methyl-D-galactose	1

** see table 3B.4 for retention times and R_G values

TABLE 3B.4

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED L. HUMILIS GUM

Relative retention time (T) of methyl glycosides.** column (i)	Relative retention time (T) of methyl glycosides.** column (ii)	R_G after hydrolysis. solvents (e)	R_G after hydrolysis. solvents (f)	O-methyl sugar identified.
0.54	0.49	1.02	1.03	2,3,4-tri-O-methyl-L-rhamnose
0.63,0.77	0.58,0.74	0.95	1.03	2,3,5-tri-O-methyl-L-arabinose
1.07	1.25	0.79	0.75	2,3,4-tri-O-methyl-L-arabinose
(1.42),(2.10)	(1.95),(2.35)	0.81	0.80	2,5-di-O-methyl-L-arabinose
1.62	1.78	0.88	0.80	2,3,4,6-tetra-O-methyl-D-galactose
(2.30),(3.10),(3.58)	(3.23),(4.00),(4.41)	0.74	0.48	2,3,6-tri-O-methyl-D-galactose
3.33,3.72	4.21,4.55	0.74	0.40	2,4,6-tri-O-methyl-D-galactose
(5.48)	(7.45)	0.74	0.35	2,3,4-tri-O-methyl-D-galactose
7.49	11.36	0.53	0.20	2,6-di-O-methyl-D-galactose
11.68,13.22	17.80,19.60	0.53	0.12	2,4-di-O-methyl-D-galactose
-	-	0.37	0.04	2-O-methyl-D-galactose
(2.10),(2.65)	(2.35),(3.21)	-	-	2,3,4-tri-O-methyl-D-glucuronic acid*
(5.48)	(7.45)	-	-	2,3,4-tri-O-methyl-D-galacturonic acid*

* as methyl ester methyl glycoside

** figures in parenthesis indicate T values of components which are not completely resolved.

TABLE 3B.5

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED POLYSACCHARIDES I - IV

O-methyl sugar identified **	Approximate relative molar proportions				
	Polysaccharide				
	I[1]	I[2]	II	III	IV
2,3,5-tri-O-methyl-L-arabinose	0.5	0.3	0.1	trace	nil
2,3,4-tri-O-methyl-L-arabinose	0.1	0.3	nil	nil	nil
2,5-di-O-methyl-L-arabinose	trace	trace	nil	nil	nil
2,3,4,6-tetra-O-methyl-D-galactose	1	1	1	1	1
2,3,6-tri-O-methyl-D-galactose	0.1	trace	0.1	0.2	0.1
2,4,6-tri-O-methyl-D-galactose	5	5	5	7	6
2,3,4-tri-O-methyl-D-galactose	0.1	0.1	trace	trace	trace
2,6-di-O-methyl-D-galactose	1.5	trace	1.5	2	1.5
2,4-di-O-methyl-D-galactose	trace	0.2	trace	trace	trace
2-O-methyl-D-galactose	0.5	trace	0.3	0.5	0.3

[1] methylated by the Haworth and Purdie procedures

[2] methylated by the NaH/MeI procedure

** see table 3B.4 for retention times and R_G values

TABLE 4.1

NITROGEN AND PROTEIN CONTENTS OF *AZADIRACHTA INDICA* PRODUCTS AFTER ATTEMPTED DEPROTEINISATION

	Deproteinisation Method	Nitrogen,%	Protein,% (N × 6.25)	Alteration in protein content,%
(a)	recovered gum	5.5	34.4	-
(b)	ethanol	7.5	46.9	+12.5
(c)	chloroform, butanol	5.6	35.0	+ 0.6
(d)	1,2,2-trifluoro-1,1,2-trichloroethane	5.7	35.6	+ 1.2
(e)	trichloroacetic acid	5.8	36.2	+ 1.8
(f)	trifluoroacetic acid	5.1	31.9	- 2.5
(g)	tungstic acid	4.4	27.5	- 6.9
(h)	sodium borohydride	5.5	34.4	nil
(i)	pepsin, trypsin	4.4	27.5	- 6.9
(j)	pronase	2.5	15.6	-18.8

TABLE 4.2

ANALYSES OF PURIFIED AZADIRACHTA INDICA

SAMPLES A AND B AND FRACTIONS I - III

	Gum Sample A	Gum Sample B	Fraction I	Fraction II	Fraction III
Yield, % (a,b)	80	76	59	26	81
Moisture, %	11.9	13.0	13.9	12.8	12.4
Nitrogen, % (b)	6.0	5.6	3.7	6.7	5.2
Protein, % (N×6.25)	37.5	35.0	23.2	41.9	32.5
$[\alpha]_D$ (b)	-62°	-58°	-54°	-45°	-59°
Methoxyl, % (b)	2.05	2.36	n.d.	n.d.	n.d.
Methoxyl, % (c)	3.3	3.6	n.d.	n.d.	n.d.
Equivalent weight, g (b)	990	957	n.d.	n.d.	835
Equivalent weight, g (c)	620	620	n.d.	n.d.	560
Uronic anhydride, % (d)	28.3	28.3	n.d.	n.d.	31.4
Limiting flow-time number, ml g^{-1} (b,e)	10.6	13.3	9.7	7.2	7.9
Refractive index, dn/dc (b,e)	0.166	0.163	n.d.	n.d.	n.d.
Molecular weight, $\bar{M}_w$ (b,e)	5.2×10^5	7.1×10^5	1.3×10^6	n.d.	5.0×10^5

n.d. - not determined.

(a) yields of samples A and B are recoveries from purification of the crude gums.
yields of fractions I and II are recoveries from fractionation of purified gum, sample A.
yield of fraction III is recovery from treatment of purified gum, sample A, (solvent used in M-SC studies employed).

(b) corrected for moisture content.

(c) corrected for moisture and protein contents.

(d) calculated from equivalent weight on the basis that all acidity arises from uronic acid groups.

(e) in M-sodium chloride at 27°C.

TABLE 4.3

AMINO ACID ANALYSES OF PURIFIED AZADIRACHTA INDICA SAMPLES A AND B AND FRACTIONS I - III (a)

	Gum Sample A	Gum Sample B	Fraction I	Fraction II	Fraction III
lysine	44	59	35	30	43
histidine	17	18	11	26	16
ammonia	(b)	(b)	(b)	(b)	(b)
arginine	27	28	35	25	28
aspartic acid	138	145	129	169	146
threonine	66	64	59	62	63
serine	75	79	66	88	76
glutamic acid	78	81	84	63	71
proline	73	57	83	91	68
glycine	73	71	79	70	70
half cystine	18	14	6	23	15
alanine	53	55	62	40	54
valine	75	76	71	80	76
methionine	3	0	0	6	1
isoleucine	51	51	64	41	59
leucine	84	80	87	59	83
tyrosine	30	31	22	28	24
phenylalanine	57	57	66	36	57
glucosamine	38	34	41	63	50
nitrogen recovery (c)	83%	74%	75%	77%	72%

(a) values are expressed as μ moles amino acid per 1000 μ moles total.

(b) value included only in nitrogen recovery.

(c) expressed as % recovery of Kjeldahl nitrogen.

TABLE 5.1

ELUTION VOLUMES (ml) OF PEAKS IN THE ELUTION DIAGRAMS OF DYED POLYSACCHARIDES

	Column (1)	Column (2)	Column (3)	Column (4)
Acacia cyanophylla	26, 42, 65 (i)	-	17, 45, 65(sh)	-
Acacia campylacantha	25, 43, 64(tr) (i)	-	18, 46	110, 178(tr)
Acacia laeta	24, 35, 62(tr) (i)	-	19, 41, 68(tr)	-
Acacia pycnantha	53, 64(tr) (i)	-	58	-
Lannea coromandelica	35, 43(tr), 71 (ii)	-	-	-
Lannea schimperi	35, 54(tr), 72 (ii)	-	-	-
Lannea humilis	34, 48(tr), 71 (ii)	25, 70(tr)	-	107, 179
degraded gum A*	66 (ii)	-	-	-
degraded gum B*	75 (ii)	-	-	-
polysaccharide I*	73 (ii)	24, 50	-	144(tr), 182
polysaccharide II*	-	28(sh), 50	-	183
Azadirachta indica	26(sh), 41, 61(sh) (i)	-	30(sh), 46, 60(sh)	-

tr - trace sh - shoulder

Column (1) Bio-Gel A-5m, 35 × 1.5 cm (i) or 40 × 1.5 cm (ii).

Column (2) Bio-Gel P-10, 35 × 1.5 cm.

Column (3) Sepharose 4B, 35 × 1.5 cm.

Column (4) System of linked columns of Porasils C, B and A, each 40 × 1.5 cm.

* from Lannea humilis gum (see Section III B).

(Table continued overleaf)

TABLE 5.1 (Continued)

ELUTION VOLUMES (ml) OF PEAKS IN THE ELUTION DIAGRAMS OF DYED POLYSACCHARIDES

	Column (1)	Column (2)	Column (3)	Column (4)
Combretum grandifolium	25, 63 (i)	-	31, 70	-
Prunus avium	22, 46, 62 (i)	-	17, 62, 75(tr)	-
Khaya senegalensis	-	-	-	97, 135, 175
Araucaria columnaris	27, 50, 66(tr) (i)	-	18, 53	-
Araucaria araucana	25, 42, 61 (i)	-	18, 48, 68(sh)	-
Albizia glaberrima	25(tr), 58 (i)	-	69	-
i-carrageenan	-	-	-	140(sh), 163
Dextran 10	68 (ii)	36	-	-
Dextran 20	52 (i) 60 (ii)	24	55	130, 177(tr)
Dextran 80	46, 62(tr) (i)	-	48	108
Dextran 110	44, 65(tr) (i)	-	42, 65(tr)	-
Dextran 150	-	-	-	105, 178(tr)

tr - trace sh - shoulder

Column (1) Bio-Gel A-5m, 35 × 1.5 cm (i) or 40 × 1.5 cm (ii).

Column (2) Bio-Gel P-10, 35 × 1.5 cm.

Column (3) Sepharose 4B, 35 × 1.5 cm.

Column (4) System of linked columns of Porasils C, B and A, each 40 × 1.5 cm.

TABLE 5.2

A COMPARISON OF SOME COLUMN MATERIALS FOR MOLECULAR-SIEVE CHROMATOGRAPHY

Material	Pretreatment	Ease of packing	Flow-rate	Sieving properties*	Application to study of gum exudates
Bio-Gel A-5m	swelling, deaeration	slow, top of column must be stabilised	slow	wide molecular weight range ($\bar{M}_n$ 5×10^3-10^6), resolution good	generally useful
Bio-Gel P-300	swelling, deaeration	slow, top of column must be stabilised	slow	low molecular weight range ($\bar{M}_n < 2\times10^5$), resolution good	useful for low molecular weight gums and degradation products
Bio-Gel P-10	swelling	moderate	moderate	low molecular weight range ($\bar{M}_n < 1.5\times10^4$), resolution good	restricted to low molecular weight products
Sepharose 2B	swelling, deaeration	slow, top of column must be stabilised	slow	high molecular weight range ($\bar{M}_n$ 10^5-5×10^6), resolution good	useful for studies of high molecular weight materials
Sepharose 4B	swelling, deaeration	slow, top of column must be stabilised	slow	wide molecular weight range ($\bar{M}_n$ 2×10^4-10^6), resolution good	generally useful
Bio-Glas -500	silanisation, deaeration	fast	fast	limited molecular weight range, resolution poor	limited by poor resolution
Bio-Glas-1500	silanisation, deaeration	fast	fast	limited (high) molecular weight range, resolution poor	limited by poor resolution and high molecular weight range.

*Molecular weight values quoted are approximations based on studies of typical gum exudates.

(Table continued overleaf)

TABLE 5.2 (Continued)

A COMPARISON OF SOME COLUMN MATERIALS FOR MOLECULAR-SIEVE CHROMATOGRAPHY

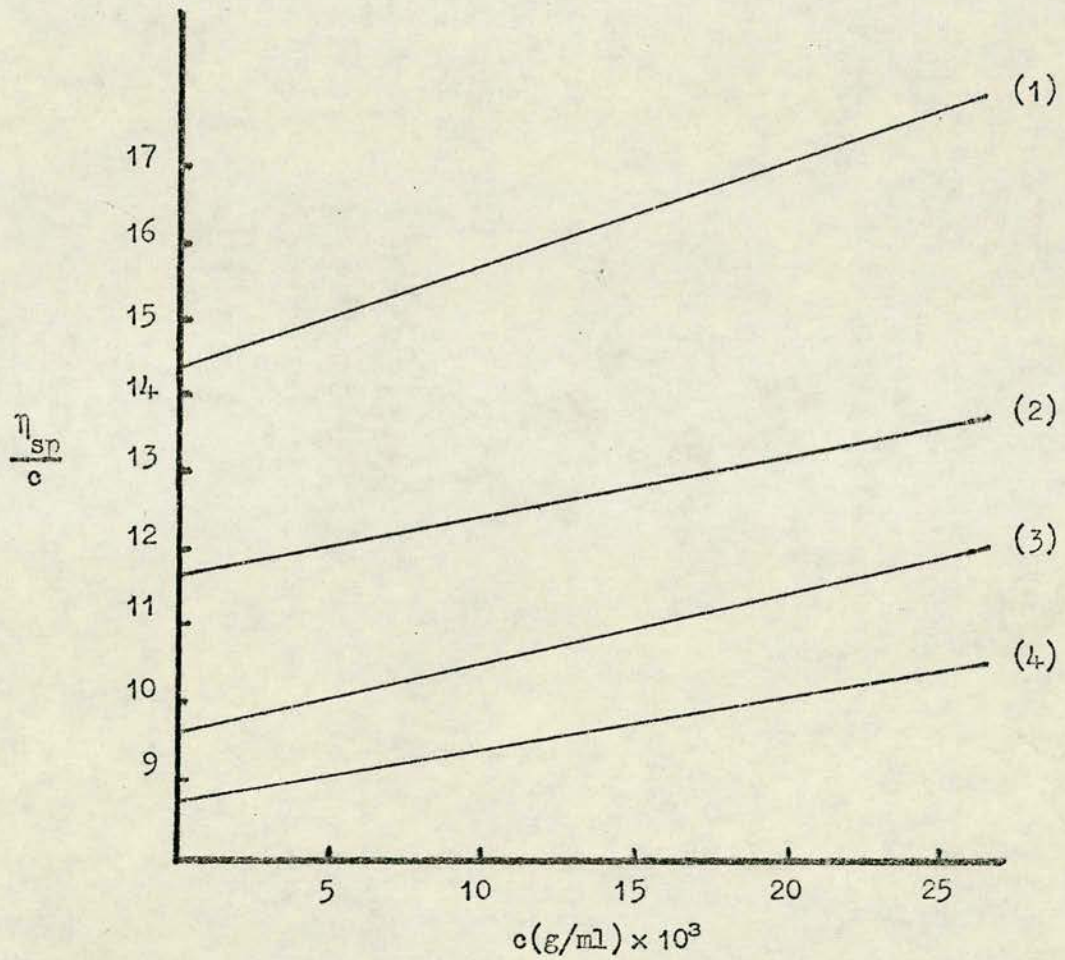
Material	Pretreatment	Ease of packing	Flow-rate	Sieving properties*	Application to study of gum exudates
Corning CPG 10-1250	deaeration	fast	fast	limited molecular weight range, resolution poor	limited by poor resolution
Porasil A	deaeration	fast	fast	low molecular weight range ($\bar{M}_n < 10^4$), resolution fairly good	restricted to low molecular weight products
Porasil B	deaeration	fast	fast	low molecular weight range ($\bar{M}_n < 5\times10^4$), resolution fairly good	useful for low molecular weight gums
Porasil C	deaeration	fast	fast	useful molecular weight range ($\bar{M}_n < 5\times10^5$), resolution fairly good	generally useful
Porasil E	deaeration	fast	fast	high molecular weight range ($\bar{M}_n < 2\times10^6$), resolution fairly good	useful for high molecular weight samples
System of Porasils C, B and A	as for the individual columns			wide molecular weight range ($\bar{M}_n < 2\times10^5$), resolution good	useful for low molecular weight gums and degradation products

*Molecular weight values quoted are approximations based on studies of typical gum exudates.

FIGURES

FIGURE 3A.1

GRAPHS OF VISCOSITY NUMBER ($\frac{\eta_{sp}}{c}$) AGAINST CONCENTRATION (c) FOR *LANNEA* POLYSACCHARIDES



(1) *L. schimperi*

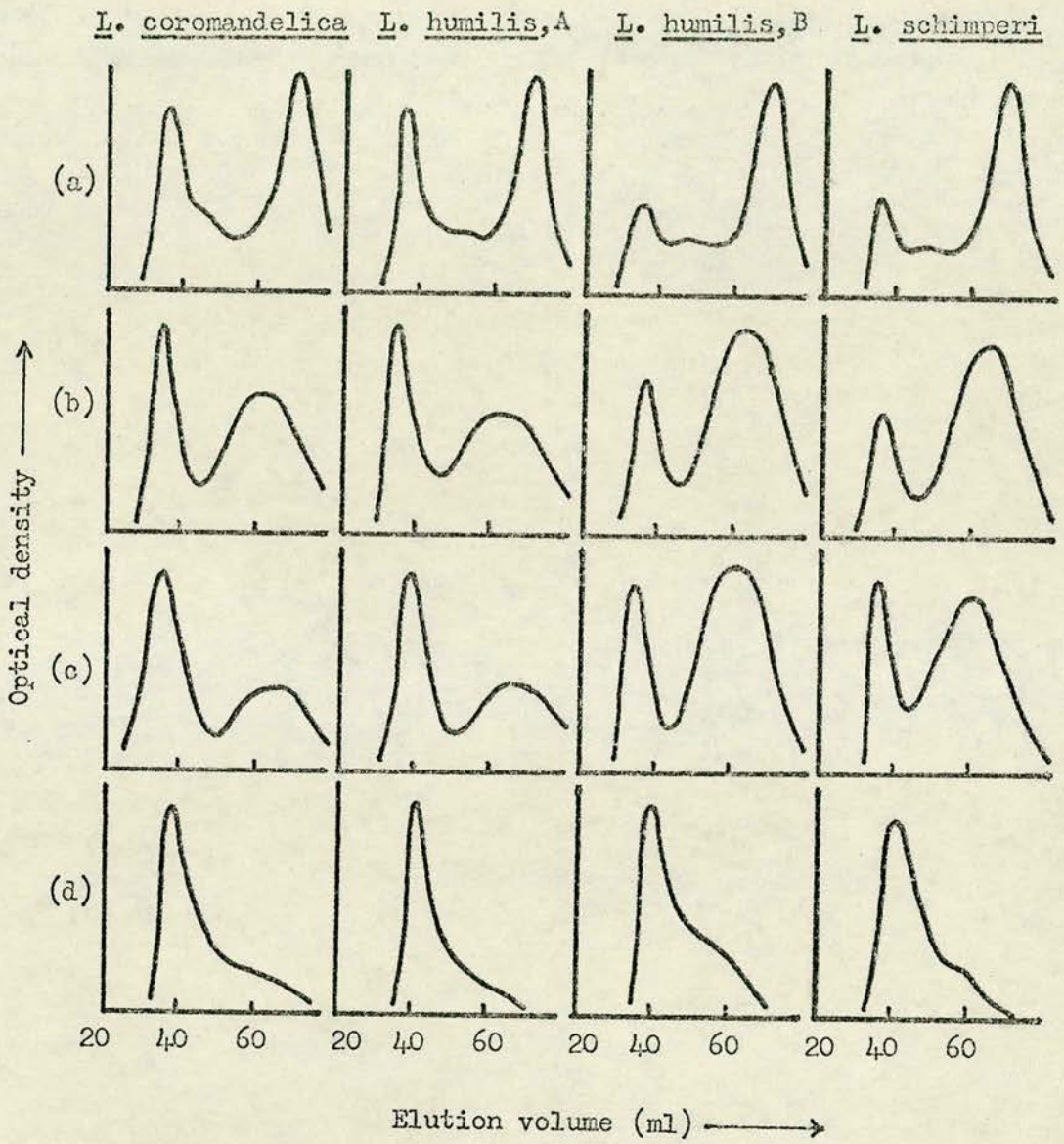
(2) *L. coromandelica*

(3) *L. humilis*, A

(4) *L. humilis*, B

FIGURE 3A.2

ELUTION DIAGRAMS OF *LANNEA* POLYSACCHARIDES



(a) Bio-Gel A-5m

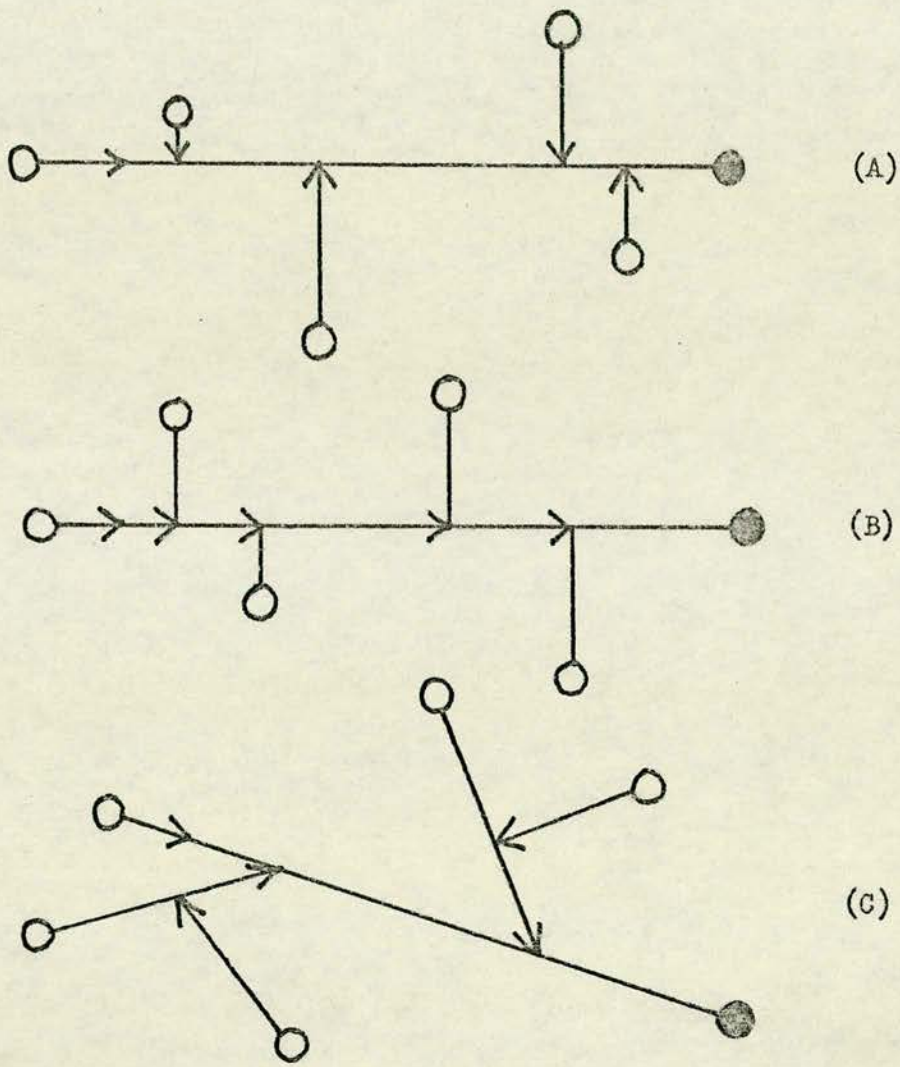
(b) Porasil C

(c) Porasil B

(d) Porasil A

FIGURE 3B.3

REPRESENTATION OF SOME OF THE POSSIBLE STRUCTURES FOR THE PERIODATE-RESISTANT GALACTAN FRAMEWORK OF LANNEA HUMILIS GUM



—— = chain of -1,3- linked D-galactose residues

⟶ = -1,6-linkage at branch point or in the chain

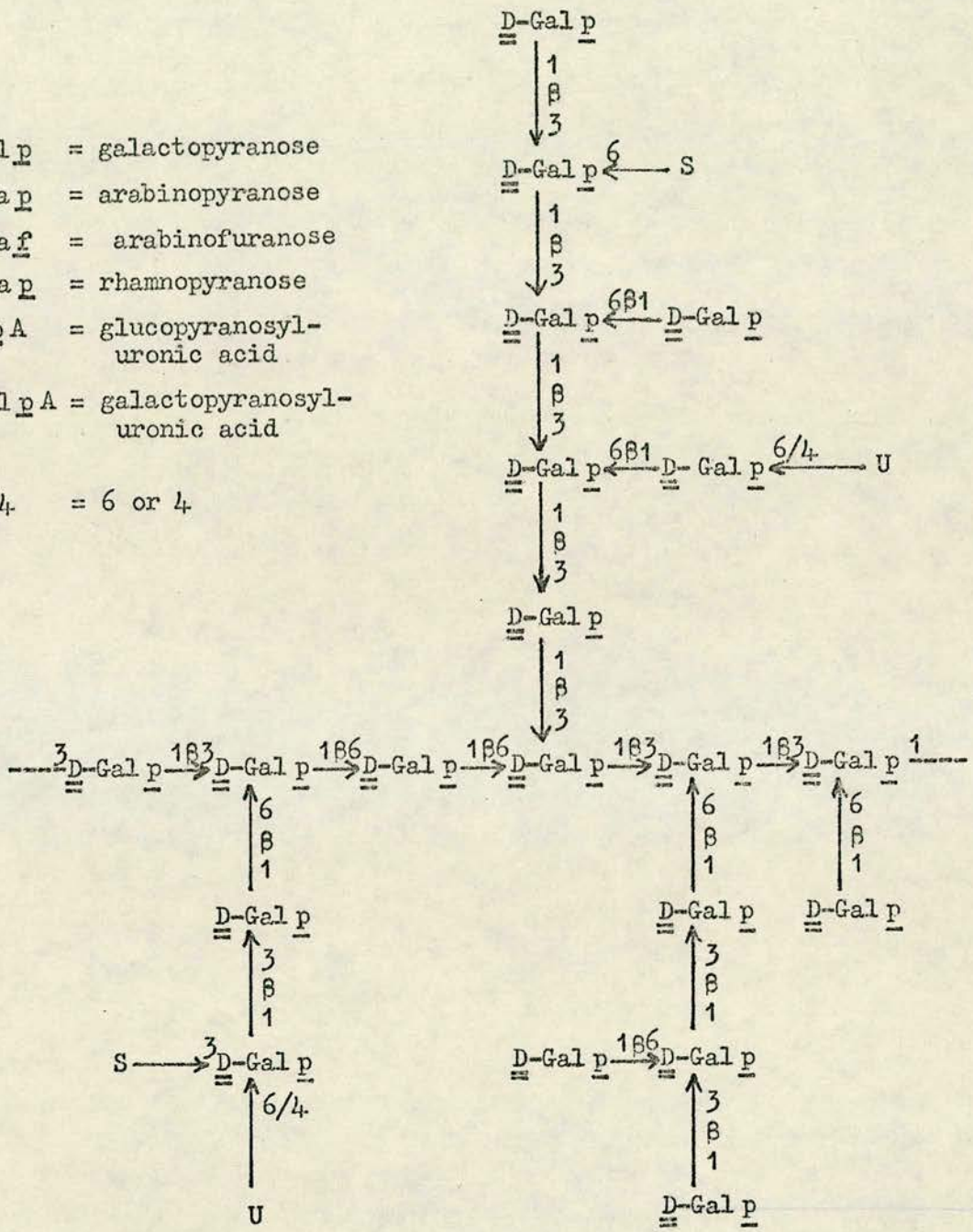
● = reducing end-group

○ = non-reducing end-group

FIGURE 3B.4

A POSSIBLE STRUCTURAL FRAGMENT OF LANNEA HUMILIS GUM

Gal p = galactopyranose
Ara p = arabinopyranose
Ara f = arabinofuranose
Rha p = rhamnopyranose
G p A = glucopyranosyl-uronic acid
Gal p A = galactopyranosyl-uronic acid
6/4 = 6 or 4

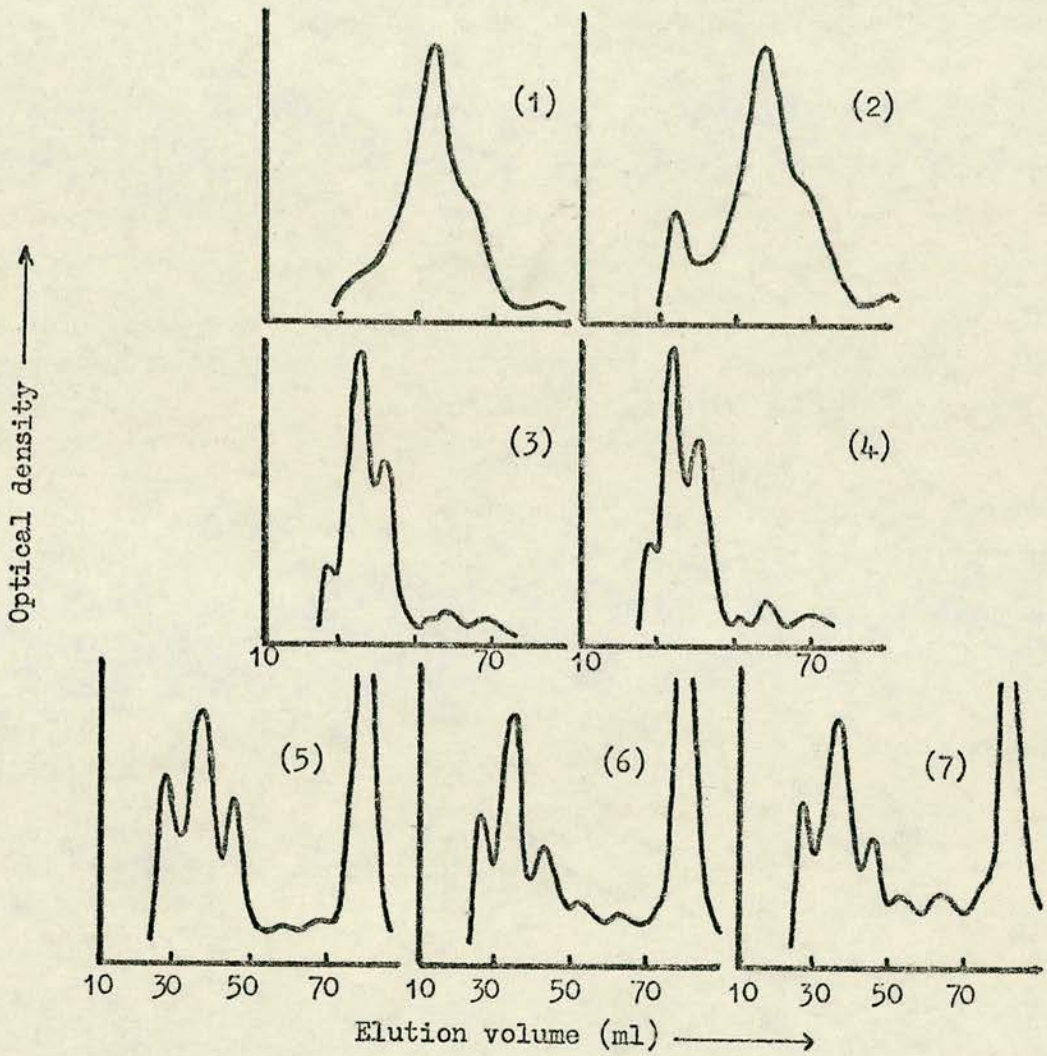


U represents uronic acid residues, which may be Gal p A > G p A ≃ 4-O-Me-G p A. A few G p A residues may be substituted by L-Rha p.

S represents D-Gal p -, L-Ara p -, and L-Ara f - containing side chains. These side chains may be up to 4 units long and contain ···3 L-Ara f 1··· residues. They may be terminated by L-Ara f 1···, L-Ara p 1···, or D-Gal p 1α3 L-Ara f 1··· residues.

FIGURE 4.1

ELUTION DIAGRAMS GIVEN BY AZADIRACHTA INDICA GUM ON BIO-GEL A-5m



(1) Dyed gum on column (a)
(2) Undyed gum on column (a)
(3) Dyed gum on column (b) (after 0 hours in solvent)
(4) Dyed gum on column (b) (after 24 hours in solvent)
(5) Undyed gum on column (b) (after 0 hours in solvent)
(6) Undyed gum on column (b) (after 24 hours in solvent)
(7) Undyed gum on column (b) (after 114 hours in solvent)

FIGURE 4.2

ELUTION DIAGRAMS GIVEN BY AZADIRACHTA INDICA GUM ON BIO-GEL A-5m AFTER TREATMENT WITH VARIOUS CONCENTRATIONS OF DITHIOTHREITOL (DTT)

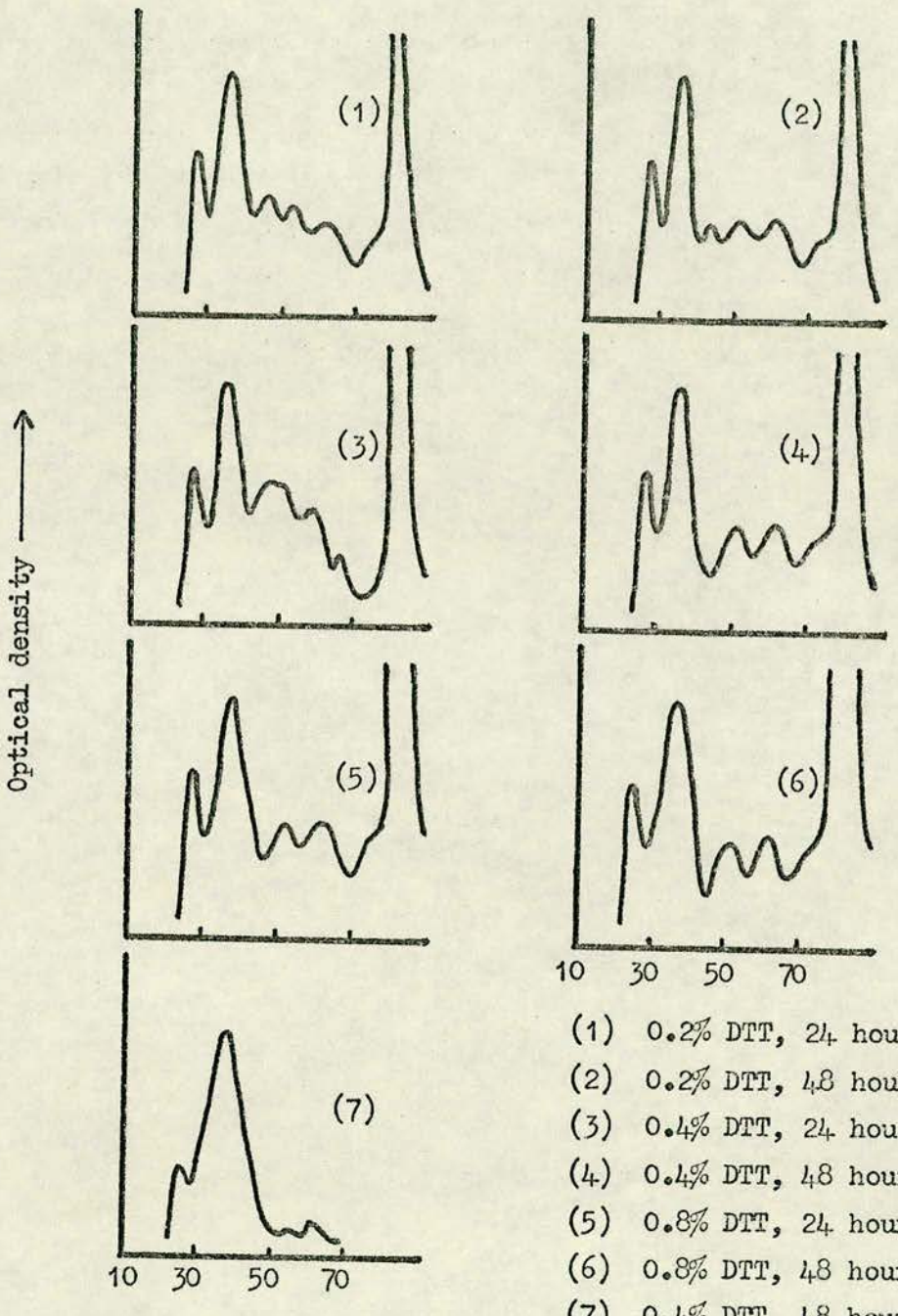
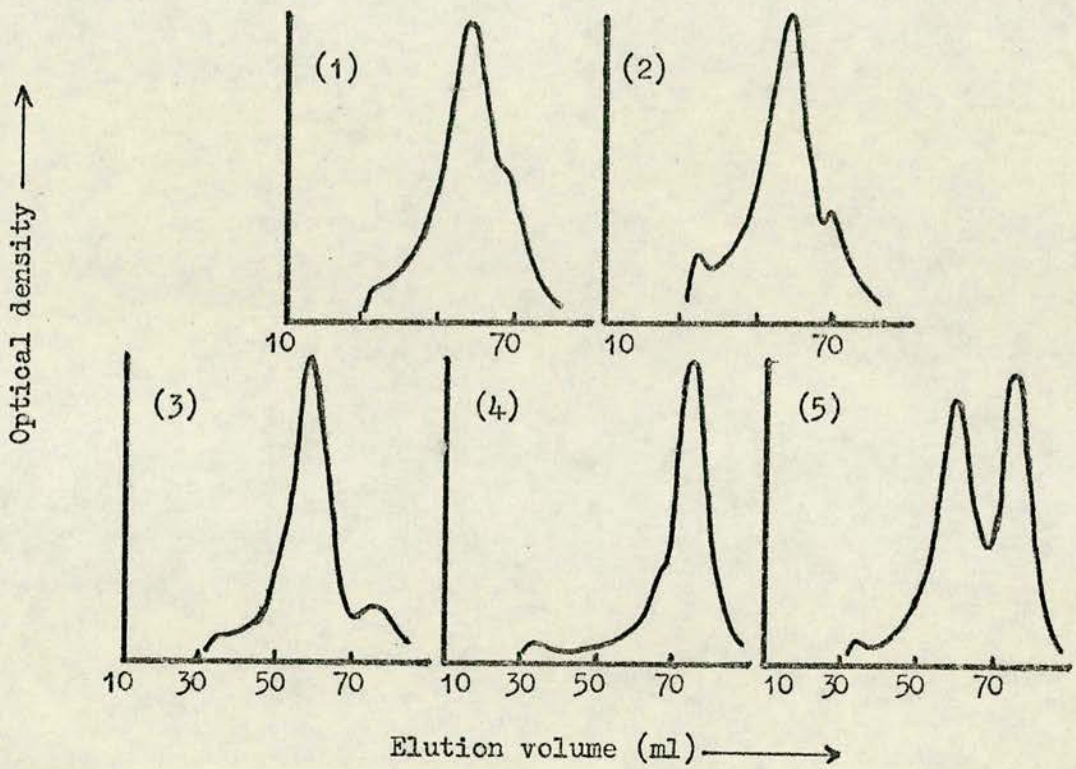


FIGURE 4.3

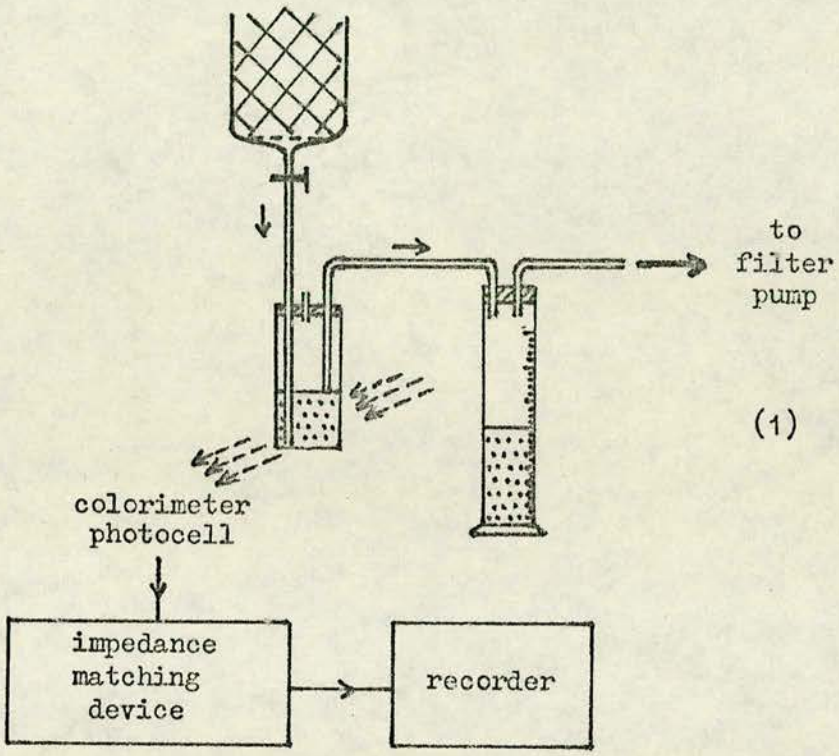
ELUTION DIAGRAMS GIVEN BY AZADIRACHTA INDICA SAMPLES A AND B AND FRACTIONS I - III ON BIO-GEL A-5m [M-SODIUM CHLORIDE ELUANT]



(1) Sample A
(2) Sample B
(3) Fraction I
(4) Fraction II
(5) Fraction III

FIGURE 5.1

APPARATUS FOR AUTOMATED COLORIMETRIC MOLECULAR-SIEVE CHROMATOGRAPHY



MODIFIED CELL ARRANGEMENT

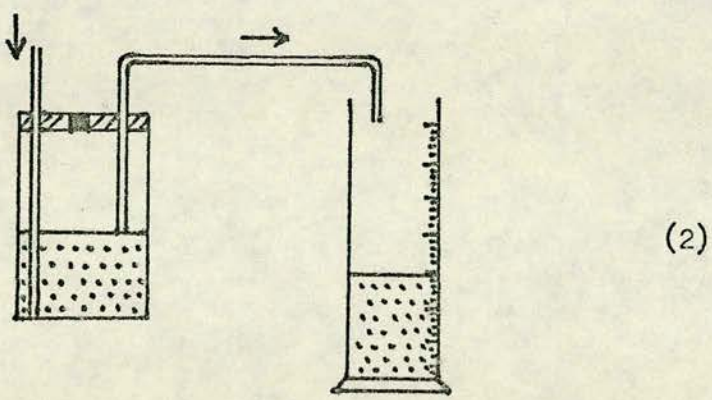
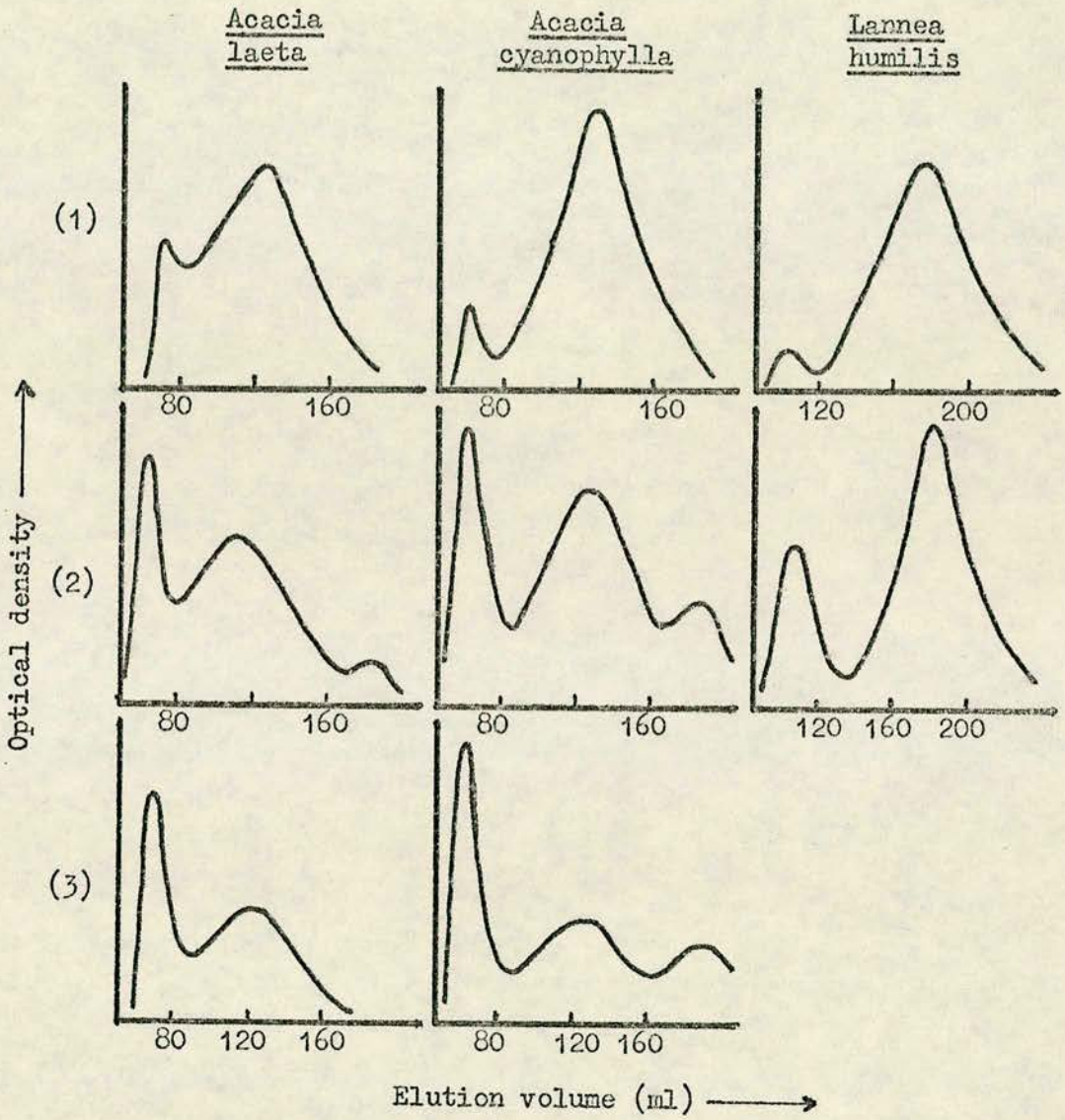


FIGURE 5.2

COMPARISON OF ELUTION DIAGRAMS OF DYED AND UNDYED POLYSACCHARIDES



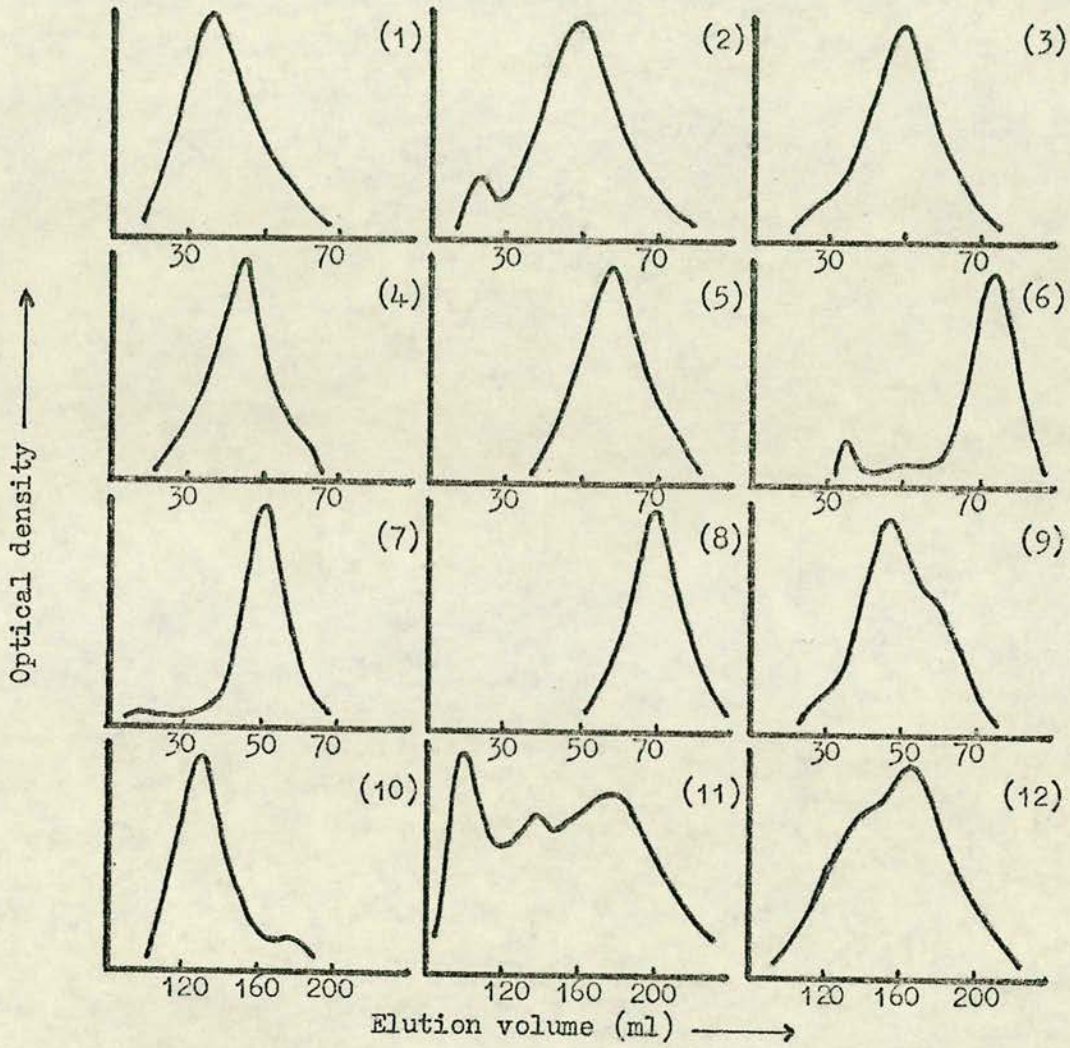
(1) Undyed gum - fractions screened by phenol-sulphuric acid method.

(2) Dyed gum - screened by automated colorimetric method.

(3) Dyed gum - optical density of fractions recorded.

FIGURE 5.3

ELUTION DIAGRAMS OF A RANGE OF DYED POLYSACCHARIDES



(1) Dextran 10
(2) Polysaccharide I from Lannea humilis } Bio-Gel P-10
(3) Polysaccharide II from Lannea humilis

(4) Dextran 80
(5) Acacia pycnantha } Bio-Gel A-5m
(6) Lannea schimperi

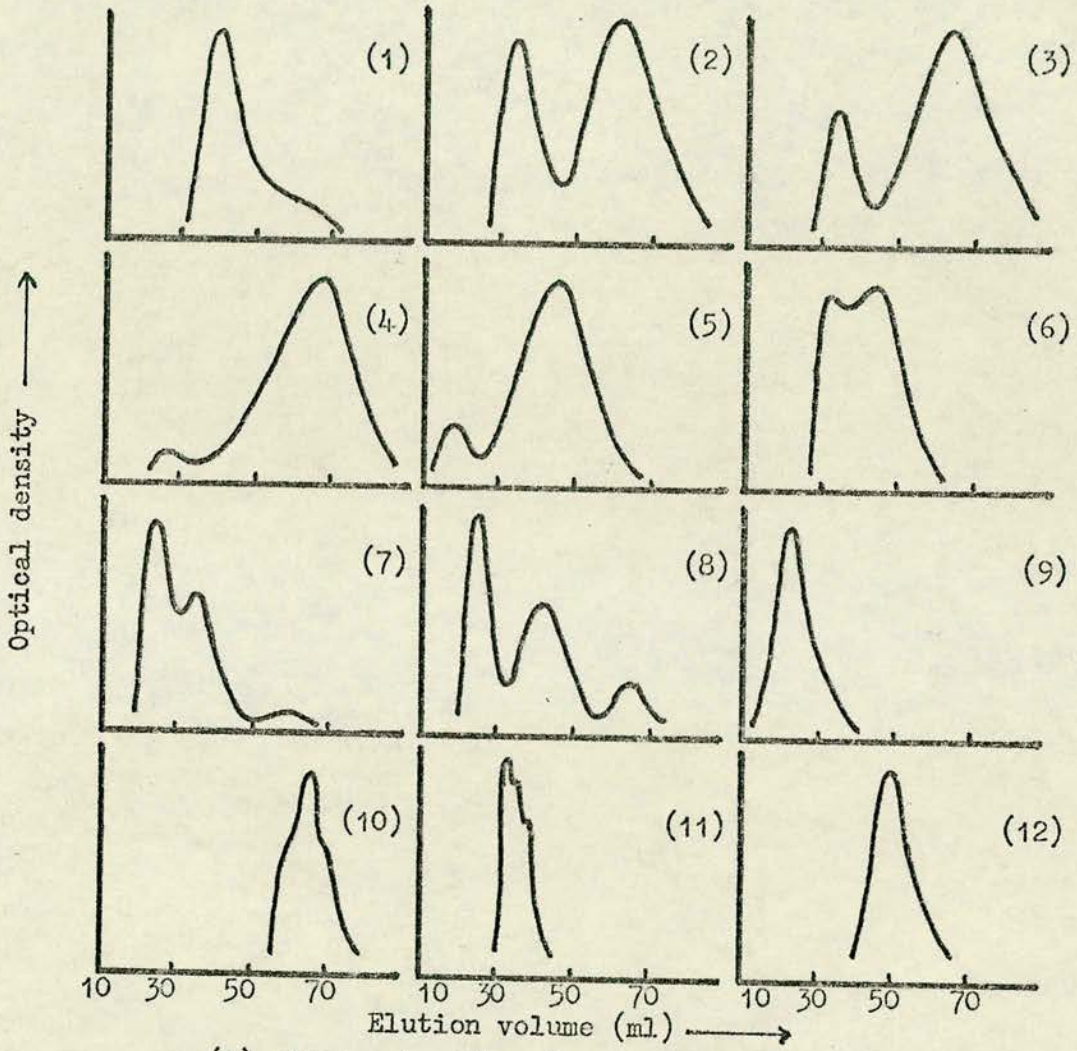
(7) Araucaria columnaris
(8) Albizia glaberrima } Sepharose 4B
(9) Azadirachta indica

(10) Dextran 20
(11) Khaya senegalensis } Porasil system
(12) i-carrageenan

FIGURE 5.4

ELUTION DIAGRAMS OF DYED POLYSACCHARIDES

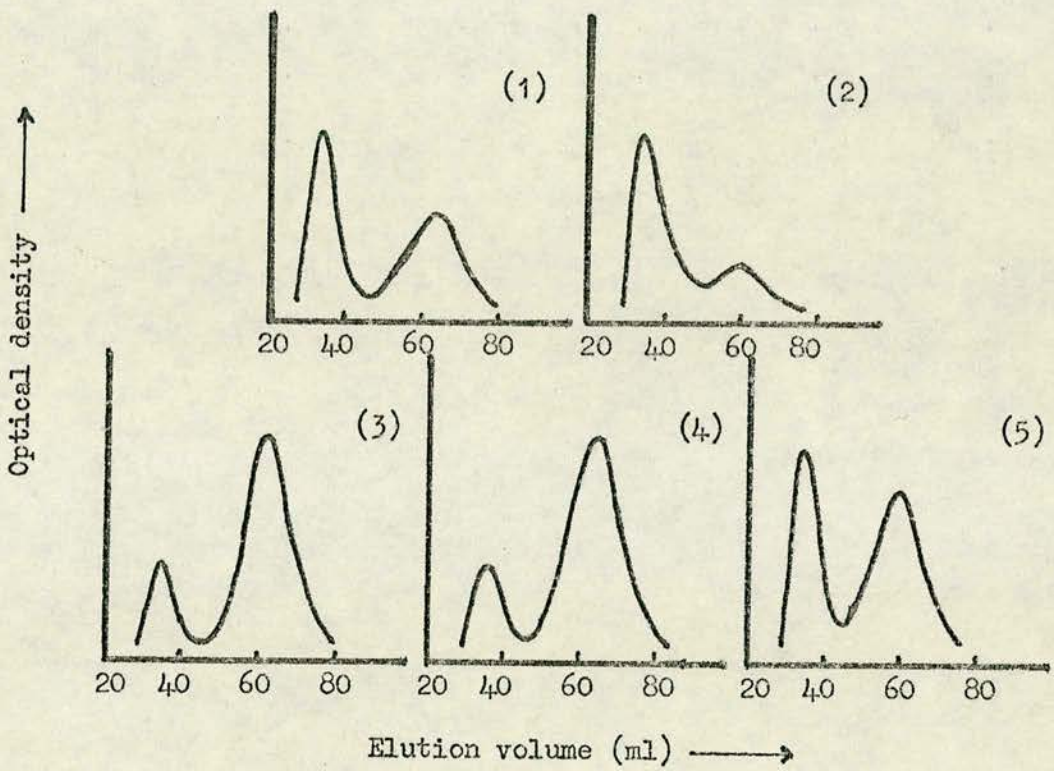
ON A RANGE OF COLUMNS



(1) Lannea humilis, Porasil A
(2) Lannea humilis, Porasil B
(3) Lannea humilis, Porasil C
(4) Acacia campylacantha, Sepharose 2B
(5) Acacia campylacantha, Sepharose 4B
(6) Acacia laeta, Porasil E
(7) Acacia laeta, Bio-Gel A-5m
(8) Acacia cyanophylla, Bio-Gel A-5m
(9) Acacia cyanophylla, Bio-Gel P-300
(10) Acacia cyanophylla, Corning CPG 10-1250
(11) Acacia cyanophylla, Bio-Glas-500
(12) Acacia cyanophylla, Bio-Glas-1500

FIGURE 5.5

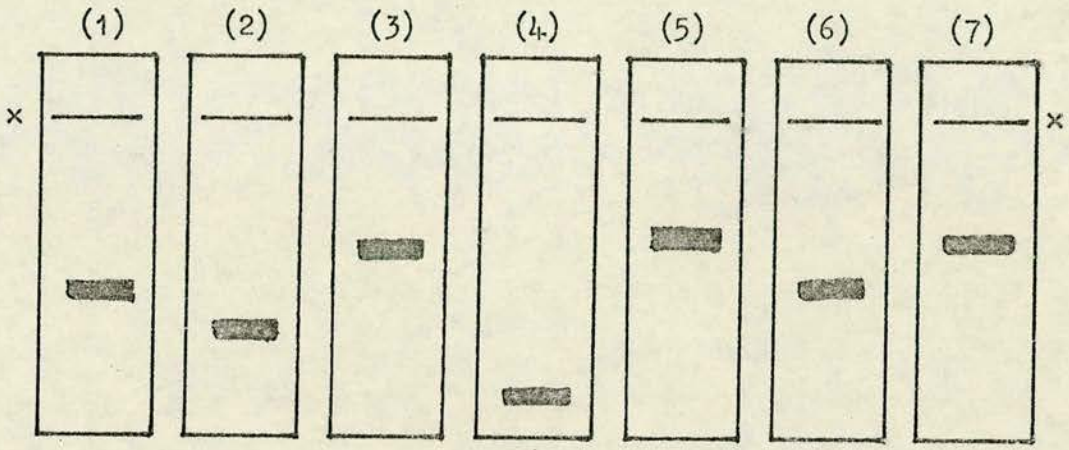
ELUTION DIAGRAMS OF DYED POLYSACCHARIDES ON PORASIL C AT DIFFERENT FLOW-RATES



(1) Lannea coromandelica, 2.5 ml/min
(2) Lannea coromandelica, 10 ml/min
(3) Lannea schimperi, 1 ml/min
(4) Lannea schimperi, 2.5 ml/min
(5) Lannea schimperi, 10 ml/min

FIGURE A.1

THIN-LAYER ELECTROPHORESIS OF DYED POLYSACCHARIDES

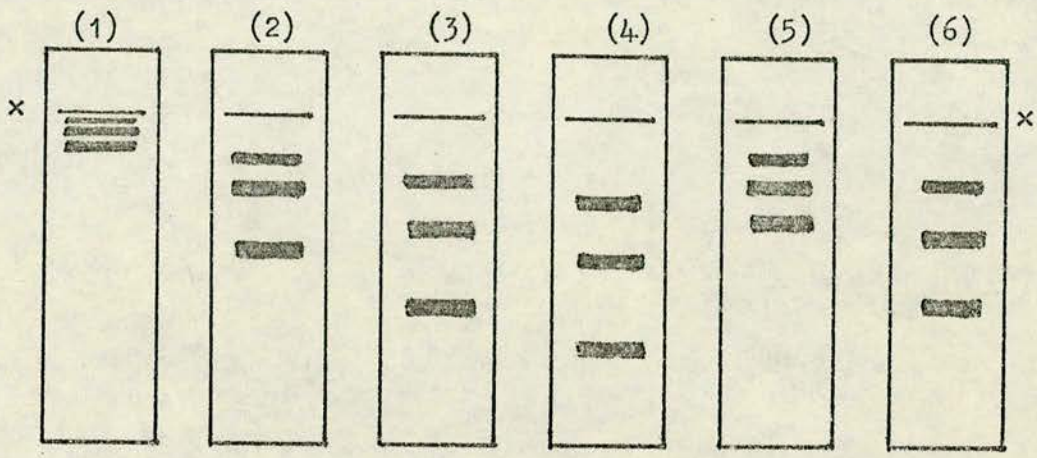


(1) Acacia campylacantha, (uronic acid 9%), ammonium carbonate buffer.
(2) Acacia campylacantha, acetate buffer.
(3) Acacia campylacantha, borate buffer.
(4) Acacia cyanophylla, (uronic acid 25%), ammonium carbonate buffer.
(5) Araucaria araucana, (uronic acid 10%), borate buffer.
(6) Lannea humilis (uronic acid 12%), borate buffer.
(7) Undyed Lannea humilis, borate buffer.

Each run was performed for 10 min at a field strength of 50 volts/cm.

FIGURE A.2

THIN-LAYER ELECTROPHORESIS OF A MIXTURE OF DYED POLYSACCHARIDES



× - × origin

(1) 1 min, field strength 50 volts/cm
(2) 3 min, field strength 50 volts/cm
(3) 5 min, field strength 50 volts/cm
(4) 8 min, field strength 50 volts/cm
(5) 1 min, field strength 75 volts/cm
(6) 2 min, field strength 75 volts/cm

Band of greatest mobility - Acacia cyanophylla gum
Band of intermediate mobility - Acacia campylacantha gum
Band of least mobility - Dextran 110

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Automated molecular-sieve chromatography of polysaccharides

Molecular-sieve chromatography (MSC) is a useful technique for investigating the polymeric properties of acidic polysaccharides[1,2]. In earlier studies[3,4], elution patterns were obtained by collecting many small fractions which were then screened tediously by a colorimetric method[5].

A considerable range of agarose gels and porous glass or silica beads is now available for studies of high molecular weight polymers. This range will probably increase in the future. Manufacturer's data for such products are usually obtained from tests with characterised proteins, dextrans, or polystyrene fractions; their performance with other types of polymer (*e.g.* acidic polysaccharides) frequently differs[6] and must therefore be evaluated.

The different basic types of agarose preparations (gelled beads, and granular, physically disintegrated gels) vary fundamentally in chromatographic characteristics. Similar considerations apply to porous glass beads, which appear to be offered as two distinct types—"Bio-Glas" (Bio-Rad Laboratories, California) and "Haller Glass" (Corning CPG glass). The choice of a molecular-sieve with a suitable pore-size distribution has a significant influence on the degree of separation attainable[7]. Although HEITZ AND KERN predicted[8] that separation efficiency will eventually be determined by molecular coil dimensions rather than by experimental refinement, it seems reasonable to suppose that, for complex branched molecules with associated steric and charge effects, the most suitable molecular-sieve and the optimum chromatographic parameters for a particular analysis can best be found by experiment. In addition to variables such as the eluant composition and flow-rate, particle size of the molecular-sieve etc., the column shape[9] can be important—in addition to the column dimensions—if optimum results are to be obtained for polymer systems that have not previously been characterised rigorously. There are no standard methods for the preparation, purification, and analysis of polysaccharides. Successful procedures are frequently established only by series of successive refinements, and conditions devised for one polysaccharide system should not be taken automatically as optimum for others, even if they are similar in type or origin.

In all chromatographic techniques there have always been two time-consuming tasks: evaluating new or modified materials[10], and establishing optimum conditions for their use with different classes of compounds. At the present time, the extent of these committments in MSC clearly calls for an automated method of monitoring the effluent from the columns continuously, *e.g.* by differential refractometry[11]; spectroscopy[12]; radioactive labelling[13]; flame-ionisation, conductivity[14,15], and differential vapour-pressure[16] detectors; polarography[17]; and, most interesting of all, a return to true chromatography[18].

Selective dyes such as Toluidine Blue[19,20], Alcian Blue[21], and Mucicarmine[22] have long been used in differential staining reactions for electrophoresis and chromatography, and the recent resurgence of attention to mucopolysaccharides (glycosaminoglycans) has led[23] to renewed interest in dyed complexes.

DUDMAN AND BISHOP[18] observed that the reactivity of "Procion" dyes with polysaccharides was proportional to the number of primary hydroxyl groups present;

a pure polygalacturonan was completely resistant to the dyes tested, and a polysaccharide containing *ca.* 30% uronic acid was dyed only to a slight extent. Tests on the behaviour of acidic gum polysaccharides were not reported. The uronic acid content of such materials frequently falls within the 5–25% range, but some botanical genera *(Khaya, Sterculia)* contain up to *ca.* 50%.

It was therefore of interest to evaluate the broad range of application of this colorimetric technique by studying the extent of the reactions of the dyes Procion Blue M3G and Procion Brilliant Red M2B (kindly provided by I.C.I. Ltd., Dyestuffs Division, Manchester) with a wide range of acidic gum polysaccharides from the *Acacia, Albizia, Araucaria, Azadirachta, Combretum, Khaya, Lannea* and *Sterculia* genera. Although the extent of reaction, as indicated by DUDMAN AND BISHOP[18], appears to be inversely proportional to the uronic acid content, those gums with the highest uronic contents available to us (*Brachychiton diversifolium*[24,25], 51%; *Khaya senegalensis*[26], 43%) nevertheless were dyed to an extent adequate for their colorimetric detection in the eluate from MSC columns containing the agarose gels "Bio-Gel A5" and "Sepharose 4B". For polysaccharides with low uronic acid content the amount of dyestuff used can be controlled, so that the molecular weight of the natural product is not significantly increased. Dextrans are also readily dyed; the characterised fractions available commercially can continue to be used as calibration standards for relative measurements[6] if more valid standards of closely similar chemical structure, characterised by fundamental methods, are not available.

Unfortunately, the use of Procion dyes is not of general applicability: a carrageenan did not react in the dyeing process.

The use of dyed polysaccharides (we prefer the use of Procion Brilliant Red M2B) leads to a simple method of monitoring the behaviour of the native polysaccharides during molecular-sieve chromatography, or for evaluating—with a selected, characterised polysaccharide—the performance of a molecular-sieve column. The dyed sample (1–3 mg) in 2 *M* sodium chloride (0.5 ml) is applied with care to the top of a silanised glass column (35 × 1.5 cm I.D.) containing an appropriate molecular sieve (porous glass or silica; agarose, dextran, or polyacrylamide gels). A suitable eluant is 1 *M* sodium chloride, at 0.5–1 ml/min; automated analyses then take about 2 h. The column effluent is passed directly via capillary tubing into the cell (modified to permit continuous flow) of a colorimeter (Unicam SP 1300). The photo-cell signal is fed via an impedance-matching device (Vibron 33B electrometer) into a recorder (Kent, 1 mV, chart speed 2 in./h). By increasing the recorder input resistor from 1 to 2.5 Ω the maximum signal from the photo-cell gives a recorder deflection of 10 in. This particular experimental combination of instruments is, of course, not unique. Micro-flow colorimeters (Phoenix MFC 800) give an optical path-length of 20 mm for a cell hold-up volume of only 0.035 mm, if increased sensitivity is essential.

Chemistry Department,
The University,
Edinburgh EH 9 3JJ (Great Britain)

D. M. W. ANDERSON
A. HENDRIE
A. C. MUNRO

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THE CHEMISTRY OF *LANNEA* GUM EXUDATES

D. M. W. Anderson and A. Hendrie
Chemistry Department, The University, Edinburgh, EH9 3JJ, Scotland

SUMMARY

The gum exudates from *Lannea coromandelica* (Houtt.) Merrill has been studied previously under its earlier synonyms *Odina wodier* and *Lannea grandis*, and also under the native vernacular names Jeol, Modal, and Shemat. Some of the results reported in these studies are mutually contradictory. An authenticated specimen of the gum has therefore been examined to ascertain which of the published data are incorrect.

As in other gum-forming genera (Anderson et al., 1966; Anderson and Dea, 1969) the genus *Lannea* (Order, Sapindales; Family, Anacardiaceae) contains examples of complex synonymy. The species *Lannea coromandelica* (Houtt.) Merrill has as synonyms the native vernacular names "Jeol", "Modal ", and "Shemat", and also the binomials *Dialium coromandelica* Houtt, *Calesium grande*, *Haberlia grandis*, *Odina wodier* Roxb., and *Lannea grandis* Engl. A recent publication (Ramachandran and Joshi, 1968), which describes *L. grandis* and *L. coromandelica* as "closely related species", is not strictly correct.

There have been many studies of this gum, e. g. under the names "Modal" and "Shemat" (Parikh et al., 1956); "Jeol" (Bhattacharyya and Mukherjee, 1964); *Odina wodier* (Mukherjee and Banerjee, 1948; Mukherjee and Chakravarti, 1948; Maitra, 1953; Dhar and Mukherjee, 1959; Bhattacharyya and Rao, 1964); *Lannea grandis* (Chaudhuri and Mukherjee, 1967; *Lannea grandis* Super (Mukherjee, 1948; Mukherjee and Rohatgi, 1948); and *Lannea*

grandis Engler (Mukherjee and Rohatgi, 1948; Mukherjee and Rai Choudhury, 1953; Mukherjee, 1953; Mukherjee and Sinha, 1953; Mukherjee et al., 1953).

Some of these publications are superficial or trivial in nature, and the results quoted in several are mutually contradictory. As a result, this *Lannea* gum is unique both in the extent to which it has been studied and the confusion that has arisen as a result. For example, the gum has been stated to be a *neutral* polysaccharide (Ramachandran and Joshi, 1968) and, alternatively, to be an *acidic* polysaccharide containing galacturonic acid—the only aldobiouronic acid detected being defined as 3-*O*-galacturono-pyranosyl-*D*-galactose (Dhar and Mukherjee, 1959). In direct contradiction, Parikh et al. (1956) found only one aldobiouronic acid, characterised rather more adequately, but still incompletely, as 4-methyl-1, 6-glucuronosido-galactose. Further disagreements involve other analytical parameters for the gum. For example, values reported for the specific rotation of the gum are +29° (Mukherjee and Banerjee, 1948), −44° (Bhattacharyya and Rao, 1964), and +45° (Ramachandran and Joshi, 1968): the methoxyl content has been reported to be zero (Ramachandran and Joshi, 1968), 2.38% (Parikh et al., 1956), and 0.51%—a value which Bhattacharyya and Rao (1964) considered to be "very low and not of structural significance". Parikh et al. (1956) reported that the gum does not contain any methylpentose; other investigators apparently did not trouble to carry out this analysis. A further unusual feature for a plant gum is the reported absence of nitrogen (Parikh et al., 1956; Bhattacharyya and Rao, 1964); we have yet to investigate a plant gum exudate which does not contain nitrogen, and there are grounds for believing that the presence of nitrogen in a plant gum may be of greater fundamental significance (Anderson and Herbich, 1963; Anderson and Rahman, 1967) than has been frequently assumed. Finally, the ratio reported for arabinose/galactose has varied extensively viz., 1/1.3 (Mukherjee and Banerjee,

1948), 1/3 (Bhattacharyya and Rao, 1964), 1/4 (Ramachandran and Joshi, 1968), 1/5 (Parikh et al., 1956), and, in complete disagreement, Dhar and Mukherjee (1959) found the gum to contain more arabinose than galactose.

To investigate this set of contradictions, unprecedented in gum chemistry, an authenticated sample of *Lannea coromandelica* gum, obtained in October 1967 from the Research Officer of the Conservator of Forests, Colombo 2, Ceylon, has been analysed by standard methods that have already been described (Anderson and Stoddart, 1966), with the exception that analyses involving infrared spectroscopy were carried out satisfactorily with the new, low-cost, Hilger and Watts H 1200 instrument.

The analytical data can be summarised as follows [*denotes value corrected for ash and moisture content; $^{+}$ denotes value corrected for moisture, ash, and protein (%N × 6.25) content]:

moisture, 11.8%; ash, 3.5%; nitrogen*, 0.22%; methoxyl^{+}, 1.6%; uronic anhydride^{+}, 17% (acidic decarboxylation), neutralisation equivalent,$^{+}$ 1060 (hence uronic anhydride = 17%); $[\alpha]_D$, +27° (C^{+} 1.0, water); $[\eta]^{+}$, 11.7 $ml^3{}_g{}^{-1}$; $\bar{M}_W$*, 0.33×10^6 (light scattering). (The values for $[\eta]$ and $\bar{M}_W$ were obtained in 1M sodium chloride). The acidic sugars present are 4-*O*-methylglucuronic acid, and smaller amounts of glucuronic and galacturonic acids: the neutral sugars are galactose, arabinose, and rhamnose. The carbohydrate composition of the gum can be expressed as galactose, 70%; arabinose, 11%; rhamnose, 2%; uronic anhydride, 17% (approx. 50% of this being present as 4-*O*-methylglucuronic acid).

Comparisons of these analytical results show that although some previous values e. g. $[\alpha]_D$+29° (Mukherjee and Banerjee, (1948), neutralisation equivalent 1150 (Bhattacharyya and Rao, 1964) have been substantiated, the work of earlier investigators is clearly incorrect in several details. The presence of nitrogen, rhamnose, and three uronic acids was not established previously, and the ratio of

arabinose to galactose (1:6) is less than that quoted in any of the earlier reports. Perhaps the most misleading of all the previous publications is the most recent (Ramachandran and Joshi, 1968) in which *L. coromandelica* gum is reported to be a neutral polysaccharide with no methoxyl content; to dismiss quite arbitrarily (Bhattacharyya and Rao, 1964) a methoxyl content of 0.51% as being of no structural importance was also a significant error. In addition, the value—44° quoted for $[\alpha]_D$ by Bhattacharyya and Rao (1964), and the ratio of arabinose to galactose (arabinose > galactose) reported by Dhar and Mukherjee (1959) must also be regarded as being major errors; alternatively, the identity of the materials studied would have to be questioned. In many early studies either commercial mixtures were used or insufficient attention was given to the collection and correct botanical identification of the sample studied; the recommendations of a botanical authority in this respect have been recorded [Anderson and Dea, 1969, 1969(a)].

An implication of the analyses reported here is that at least some, if not all, of the structural studies published for this gum cannot be correct. Analytical and structural studies of the gums from this and other *Lannea* species are therefore in progress, and will be reported elsewhere in due course.

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AN ANALYTICAL STUDY* OF GUM EXUDATES FROM SOME SPECIES OF THE GENUS *LANNEA* A. RICH.

D. M. W. ANDERSON and A. HENDRIE

Chemistry Department, The University, Edinburgh EH9 3JJ, Scotland

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Abstract—The composition and properties of the gum exudates from *Lannea coromandelica* (syn. *L. grandis*), *L. schimperi*, and two specimens of the gum from *L. humilis* have been studied. The analytical results suggest that inter- and intra-species differences in *Lannea* are unlikely to be large, and the evidence obtained indicates that a considerable amount of the work published on this genus earlier must be regarded as incomplete or inaccurate.

INTRODUCTION

TO DATE, the gum from only one species of the genus *Lannea* A. Rich. (Sapindales; Anacardiaceae) has been studied. This species, properly described botanically as *L. coromandelica* (Houtt.) Merrill, is, however, unique in gum chemistry because of the extent to which it has been studied under different synonyms, and also because of the extent of the contradictions in chemistry that have arisen as a result. The gum has been studied under the native vernacular names Modal,[2] Shemat,[2] Jeol,[3,4] and also under the botanical synonyms *Odina wodier*,[4–8] *L. grandis*,[9–11] *L. grandis* Super,[12,13] *L. grandis* Engler,[13–17] and *L. coromandelica*.[18] There are also other botanical synonyms for this species, e.g. *Dialium coromandelica*, *Calesium grande*, and *Haberlia grandis*, but mercifully chemistry appears to have been spared their use.

The chemical results quoted in several of these publications are mutually contradictory.

* This is Part 35 of the Series "Studies on Uronic Acid Materials"; for Part 34, see Ref. 1.

[1] D. M. W. ANDERSON and A. C. MUNRO, *Carbohyd. Res.* **12**, 9 (1970).
[2] V. M. PARIKH, T. R. INGLE and B. V. BHIDE, *J. Ind. Chem. Soc.* **33**, 119 (1956).
[3] A. K. BHATTACHARYYA and A. K. MUKHERJEE, *Bull. Chem. Soc. Japan* **37**, 1425 (1964).
[4] A. K. BHATTACHARYYA and C. V. N. RAO, *Can. J. Chem.* **42**, 107 (1964).
[5] S. N. MUKHERJEE and G. BANERJEE, *J. Ind. Chem. Soc.* **25**, 59 (1948).
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[16] S. N. MUKHERJEE and S. K. SINHA, *J. Ind. Chem. Soc.* **30**, 647 (1953).
[17] S. N. MUKHERJEE, *J. Ind. Chem. Soc.* **30**, 851 (1953).
[18] R. RAMACHANDRAN and B. C. JOSHI, *Phytochem.* **7**, 2057 (1968).

The gum has been most recently described[18] as a *neutral* polysaccharide; all previous investigators found it to be acidic. So far the presence of only one aldobiouronic acid has been reported, but even this involves controversy, since the aldobiouronic acid has been stated to contain galacturonic acid[8] and, in contrast, 4-*O*-methyl glucuronic acid.[2] The specific rotation of the gum has been quoted as +29°,[5] −44°,[4] and +45°;[18] the equivalent weight as 1245,[2] 1150,[4] and 1361;[10] the methoxyl content as 2·38%,[2] 0·51%,[4] and zero.[18] The ratio of galactose to arabinose has been found to be 1·3/1;[5] 3/1;[4] 4/1;[18] and 5/1:[2] the gum has also been reported[8] to contain more arabinose than galactose.

Clearly such an unparalleled set of contradictions required analytical investigation. Since all of the work cited above is of Indian origin, specimens of gum from *L. coromandelica* and from other *Lannea* spp. were sought from other locations in an effort to establish whether the genus *Lannea* is indeed unprecedented in its variability, a result which would be surprising on botanical grounds. This paper presents the results that have been obtained for a Ceylonese specimen of *L. coromandelica* gum, a Nigerian specimen of *L. schimperi* gum, and two Sudanese specimens of *L. humilis* gum.

DISCUSSION

Consideration of the analytical results shown in Tables 1 and 2 indicates that the gum exudates from these four *Lannea* spp. have very similar analytical parameters. A characteristic of the genus is the high ratio of galactose to arabinose. Indeed, our value of 6/1 for *L. coromandelica* gum is higher than any of the wide range of values published by earlier Indian investigators; a similarly high ratio also occurs in *L. humilis* and *L. schimperi*. Clearly, the work[8] in which more arabinose than galactose was reported must be discounted. Further, the strong similarity found between the two samples of *L. humilis* gum indicates that neither the inter- nor intra-species differences in *Lannea* are likely to be great, and this is in agreement with the known botanical characters of this genus. With such complex natural products as plant gums, both inter- and intra-species differences in properties and composition must be expected to occur; recent work has shown that for some genera, e.g. *Prunus*[19] and *Combretum*[20] (which botanically are best described as systems of complexes), the variations can be much larger than for others, e.g. *Acacia*,[21] *Araucaria*.[22] Nevertheless, the variations implied for *Lannea* spp. by the lack of agreement between the results presented in the earlier papers cited in this communication are barely credible botanically or chemically; the most likely explanation is that they have arisen either through faulty chemical analysis, faulty botanical identification of the species, or through working with mixtures or commercial gum samples.

There is no doubt whatsoever that *Lannea* gum exudates are acidic polysaccharides that exist—as is customary—in the natural state as complex, nearly neutralized salts of the polysaccharide gum acid. It is unfortunate that the recent work of Ramachandran and Joshi[18] suggests that the purified polysaccharide is neutral, with a structure containing only galactose and arabinose; this is so greatly in error that there is no alternative to discounting that work.

The other major attempt[4] to establish a structure for the gum polysaccharide must also be regarded with caution. It is based on several doubts and inaccuracies. The arabinose content appears to be high; a strongly negative optical rotation (−44°) was reported;

[19] I. C. M. DEA, forthcoming publication.
[20] C. E. SPEED, M.Sc. Thesis, Edinburgh University, 1969.
[21] For a review, see D. M. W. ANDERSON and I. C. M. DEA, *Phytochem.* **8**, 167 (1969).
[22] D. M. W. ANDERSON and A. C. MUNRO, *Carbohyd. Res.*, **11**, 43 (1969).

rhamnose was not detected; nitrogen was not found; the methoxyl content was deliberately ignored; and only galacturonic acid was stated to be involved. Our examination has indicated that the uronic acid system is complex, with galacturonic, glucuronic, and 4-*O*-methyl-glucuronic acids all present. Since Bhattacharyya and Rao's structure has been used[9–11] in attempted interpretations of physico-chemical measurements, it is important that a structural model that reflects all the known facts should be available. A structural study of the gums from *Lannea* spp. is therefore in progress.

TABLE 1. ANALYSES OF PURIFIED GUM SAMPLES

	L. coromandelica	*L. humilis* A	*L. humilis* B	*L. schimperi*
Moisture, %	11·8	10·6	12·9	7·2
Ash, %	3·5	2·5	2·6	4·2
Nitrogen, %*	0·22	0·28	0·29	0·27
Protein (%N × 6·25)	1·38	1·75	1·81	1·69
Methoxyl, %†	1·6	0·4	0·4	0·9
Uronic anhydride (decarboxyln.)†	17	13	14	17
$[\alpha]_D$†	+27°	+36°	+43°	+30°
Limiting flow-time number, $cm^3\ g^{-1}$*†	11·7	9·6	8·7	14·4
$\bar{M}_w$‡*	$2{\cdot}57 \times 10^5$	$3{\cdot}10 \times 10^5$	$2{\cdot}57 \times 10^5$	$2{\cdot}41 \times 10^5$
Sugar composition† §				
galactose, %	69·5	72·5	71	69·5
arabinose, %	11	13	12	10
rhamnose, %	2·5	3	5	3·5
uronic acid, % ‖	17	11·5	12	17

* Corrected for moisture and ash.
† Corrected for moisture, ash, and protein.
‡ In M-NaCl.
‖ Average of decarboxylation value and value calculated from equivalent wt.
§ Sugars calculated as anhydro forms.

TABLE 2. ANALYSES OF ELECTRODIALYSED SAMPLES

	L. coromandelica	*L. humilis* A	*L. humilis* B	*L. schimperi*
Moisture, %	8·8	9·0	8·4	8·9
Ash, %	0·4	0·2	0·2	0·04
Equivalent wt.*	1060	1753	1774	1059
Hence uronic anhydride, %†	17	10	10	17

* Corrected for moisture and ash content.
† If all acidity arises from uronic acid groups.

Prior to this study, only two values of $\bar{M}_w$, as determined by light-scattering, had been reported for a *Lannea* gum. Bhattacharyya and Mukherjee reported[3] that the fully methylated gum had $\bar{M}_w = 1{\cdot}68 \times 10^5$, but more recently it was reported[9] that "the original acid polysaccharide derived from the plant exudate, *L. grandis*" had $\bar{M}_w = 17{\cdot}5 \times 10^6$. This is an unusually high value of $\bar{M}_w$ for an acidic gum exudate. Molecular-sieve chromatography[23] has indicated that *Lannea* exudates are not of particularly high molecular weight, and our

[23] D. M. W. ANDERSON, A. HENDRIE and A. C. MUNRO, *J. Chromatog.*, **44**, 178 (1969).

light-scattering values are in reasonable agreement with that of the earlier workers.[3] The value of 0·1695 for *dn/dc* reported recently[9] also appears to be unusually high for an acidic polysaccharide; allowing for this difference from our value (0·154), the difference between our value for $\bar{M}_w$ and that of Chaudhuri and Mukherjee[9] appears to involve a factor of almost exactly 10^2.

EXPERIMENTAL AND RESULTS

Origin of Specimens

The gum from *Lannea coromandelica* (Houtt.) Merrill was obtained in October 1967 from the Research Officer of the Conservator of Forests, Colombo 2, Ceylon. Gum from *L. schimperi* (Hochst. ex A. Rich) Engl. was collected at Shilca Research Station on 25 March 1969 by Mr. G. O. Magaji for Professor D. M. Ramsay, Department of Plant Science, Ahmadu Bello University, Zaria, Nigeria. Gum from *L. humilis* (Oliv.) Engl. was obtained from the Gum Research Officer to the Republic of the Sudan; sample A was collected near El Obeid in April 1969, and sample B from Layyuna Central Forest Reserve, Central Kordofan, in May 1969.

Analytical Methods

The standard analytical methods have been described in detail,[24] with the exception that the Hilger and Watts H1200 i.r. spectrometer was used in analyses involving i.r. vapour-phase measurements.

Weight-average molecular weights, $\bar{M}_w$, were obtained from light-scattering measurements made with a Sofica Model 42000 Photogonio Diffusometer using unpolarized green light (546 nm); the values quoted in Table 1 are the average of two values obtained at concentrations of approx 0·2% and 0·1% respectively in M-NaCl. These solutions were clarified by passage through Millipore filters of pore-sizes 0·45 and 0·22 μm. In the evaluation of $\bar{M}_w$, the value $dn/dc = 0{\cdot}154$ (found for the sample of *L. coromandelica* gum by Dr. I. C. M. Dea) was used for all samples.

Optical rotations were determined with a Perkin–Elmer electronic polarimeter, Model 141.

Purification of Samples

Each of the four gum samples dissolved readily in cold distilled water after several hours. The solutions were filtered, then dialysed for several days; the polysaccharide was recovered as the freeze-dried product. The recoveries, on a dry wt. basis, were in the range of 75–80% for all four species. The results of analyses made on these products are shown in Table 1.

A portion of each of the four gum samples was dissolved in distilled water and exhaustively electrodialysed to convert the gum polysaccharides to the free acid form. The acidic polysaccharides were recovered by freeze-drying, then analysed as shown in Table 2.

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[24] D. M. W. ANDERSON and J. F. STODDART, *Carbohyd. Res.* **2**, 104 (1966).

ABSTRACT OF THESIS

Name of Candidate ANDREW HENDRIE

Address 4, LYNE STREET, EDINBURGH, EH7 5DW

Degree DOCTOR OF PHILOSOPHY *Date* APRIL, 1971

Title of Thesis ANALYTICAL AND STRUCTURAL STUDIES ON GUM EXUDATES FROM THE LANNEA AND AZADIRACHTA GENERA

An analytical study of gum samples from three species of the genus Lannea showed them to have similar chemical properties, suggesting that interspecies variation in this genus is unlikely to be large. Molecular-sieve chromatography indicated a broad molecular weight distribution for these gums.

A sample of Lannea humilis gum was subjected to hydrolysis, Smith degradation, and methylation studies, and these revealed its molecular structure to be based on a branched galactan framework of short chains of β-1,3-linked D-galactose residues joined by β-1,3- and β-1,6-linkages. To this framework, at suitable C-3 and C-6 positions, are attached short D-galactose and L-arabinose side chains, and three different uronic acids. The mode of attachment of L-rhamnose is uncertain. From an acid hydrolysate of the gum, the unusual aldobiouronic acid 4-O-(α-D-galactopyranosyluronic acid)-D-galactose, and the more common 6-O-(β-D-glucopyranosyluronic acid)-D-galactose and 6-O-(4-O-methyl-β-D-glucopyranosyluronic acid)-D-galactose, were isolated and characterised.

Two samples of Azadirachta indica gum, from different botanical sources, were found to contain an amino sugar (ca. 2%) and much proteinaceous material (ca. 35%). Attempts to remove protein, using common denaturing agents, were unsuccessful, but better results were achieved by enzymic hydrolysis. The amino acid composition of each sample was determined. Molecular-sieve chromatography of Azadirachta indica gum, using a denaturing solvent, revealed degradation of one of the peaks in its elution diagram; a portion of gum (2 g) was fractionated by molecular-sieve chromatography, and the two samples of Azadirachta indica gum and these fractionation products were compared.

An automated colorimetric method, of molecular-sieve chromatography was developed and evaluated. It was found to be inexpensive, rapid and to give reproducible results, although some difficulty over relative peak heights was encountered.

Preliminary studies were made on a comparison of two methylation methods and on the electrophoresis of dyed polysaccharides.

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