STUDIES ON THE STRUCTURE AND METABOLISM OF POLYSACCHARIDES

by

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PREFACE

The major part of this thesis consists of reprints of publications in which the results of biochemical investigations on polysaccharides completed in the period 1952-60 are reported. The chemical and enzymic studies were carried out under the supervision of the writer by his research students and colleagues, although in several publications (1, 3, 4, 5, 8, 9, 14, 16, 25, 26, 30)* the writer was responsible for a substantial part of the experimental work. The studies on laminarin and the lichen polysaccharides were directed by Professor E. L. Hirst and the writer, and the two investigations involving ultracentrifugal analysis were carried out in collaboration with Dr. C. T. Greenwood and his research students. A number of collaborators from other laboratories have provided samples of animal, algal and protozoal tissues and a number of glycogens, but in all cases the structural analyses were supervised by the writer.

The writer wishes to express his sincere thanks to Professor E. L. Hirst, C.B.E., F.R.S., for his valued advice and encouragement throughout this period, to his research students who have carried out the major part of the experimental work, and to his many colleagues for helpful discussions and the provision of polysaccharide material, and reference compounds.

* See pp. 2-6.

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2. Multiple-branching in Amylopectin. With E.L. Hirst. Chem. and Ind., 1954, 224.

3. The Alkali-stability and Molecular Size of Glycogens. With C.T. Greenwood. Proc.Chem.Soc., 1957, 26.

4. Observations on the Absorption Spectra of Polysaccharide-Iodine Complexes. With A.R. Archibald and A. Wright. Proceedings of the Biochemical Society, 25 March 1960. Biochem.J., 1960, 75, in the press.

5. α-1:4-Glucosans. Part II. The Molecular Structure of the Liver Glycogen from a Case of von Gierke's Disease. J.Chem.Soc., 1954, 3527.

6. α-1:4-Glucosans. Part III. The Molecular Structure of Brewer's Yeast Glycogen. With Khin Maung. J.Chem.Soc., 1955, 867.

7. α-1:4-Glucosans. Part IV. A re-examination of the Molecular Structure of Floridean Starch. With I.D. Fleming and E.L. Hirst. J.Chem.Soc., 1956, 2831.

8. α-1:4-Glucosans. Part V. End-group Assay of Glycogens by Periodate Oxidation, and the Oxidation of Maltose by Sodium Metaperiodate. With A.R. Archibald. J.Chem.Soc., 1957, 2205.

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STUDIES ON THE STRUCTURE AND METABOLISM OF POLYSACCHARIDES

The aim of the present investigations was to increase our knowledge of the molecular structure of several glucose-containing polysaccharides including starch, glycogen and laminarin; since these polysaccharides serve as carbohydrate reserves in various plant or animal tissues, an understanding of their metabolism is also of fundamental biochemical importance. The results of a series of chemical and enzymic studies on the polysaccharides, and of the properties of certain enzymes catalysing their synthesis and degradation are therefore recorded in this thesis.

The Structural Analysis of Polysaccharides

The main structural features of a polysaccharide are normally determined by the chemical methods of methylation and partial acid hydrolysis. The former procedure enables the nature and ring structure of the component monosaccharides, the repeating glycosidic linkages, the proportion of non-reducing terminal groups and possible types of inter-chain

linkages to be determined. The structures of isolichenin (19)[*], lichenin (19) and laminarin (21) have been examined by this means, and the methylation analysis of other polysaccharides has been reviewed (22, 23, 24). The more recent method of partial acid hydrolysis (linkage analysis) gives information on the configuration of repeating and inter-chain linkages, and in certain cases, on the sequence of linkages. A preliminary study of this type has enabled leucosin to be characterised as a β-1:3-glucosan (17). However, both methods suffer from the disadvantages of normally requiring gram quantities of material, and being unsuitable for the routine analysis of large numbers of polysaccharide samples of the same structural type. In the present essentially biochemical studies, two alternative methods of structural analysis have been employed, namely, periodate oxidation and enzymic degradation; with both of these methods, the analysis of decigram quantities of several polysaccharide samples can be readily carried out.

[*] Arabic numbers in parentheses refer to the author's publications.

Periodate Oxidation Studies

The periodate oxidation of polysaccharides was first described by Hirst and his co-workers (I)*, who showed that with sparingly soluble potassium metaperiodate as oxidant, the theoretical production of formic acid from various methyl glycosides of mono- and di-saccharides, and from starches and glycogens was obtained after 100-150 hr. at room temperature. This type of polysaccharide represents an ideal carbohydrate for periodate oxidation analysis as formic acid arises only from the non-reducing terminal groups (unlike the dextrans), and secondary reactions due to the oxidation of reducing groups are absent. Since the potassium periodate oxidation method is now established for the analysis of starch and glycogen-type polysaccharides (II), it has been applied to the end-group assay of glycogens from mammalian liver (1, 8), mammalian muscle (9, 13), invertebrate tissues (1, 8, 9) and to the amylopectin components of various starches (7, 12, 18). These analyses were carried out on 200-300 mg. polysaccharide; in one study (13), the scale of oxidation was reduced to 75 mg. glycogen.

* Roman numbers in parentheses refer to references in the bibliography (p.33).

The slow rate of oxidation by potassium metaperiodate has been criticised by Potter and Hassid (III) who proposed an alternative oxidation system using sodium metaperiodate in the presence of sodium chloride at 2°. Using maltose as a model compound, the theoretical yield of formic acid was stated to be produced after 25 hr. However, application of these conditions to various glycogens gave average chain length ($\overline{CL}$) values greater than those obtained by other methods (IV). A critical study (1, 8) of the conditions suggested by Potter and Hassid showed that after 25 hr., oxidation of both maltose and glycogen was incomplete. Moreover, the presence of sodium chloride caused the development of acidity which interfered with the estimation of formic acid. It was therefore concluded that reaction with aqueous sodium metaperiodate at 2° for 7-10 days provided a more reliable oxidation system (8), and this method has been successfully applied to various glycogens.

Determination of the periodate reduction and the production of formaldehyde may yield valuable information on polysaccharide structure (23, 24). Examples of the former are given in the analysis of isolichenin (19), lichenin (19) and leucosin (17), whilst the liberation of formaldehyde from laminarin (20, 21) and leucosin (17) enables the

proportion of reducing groups to be determined. The mannitol content of the insoluble and soluble forms of laminarin (20, 21, 23) may be similarly estimated.

Analysis of periodate-oxidized polysaccharides may also provide structural information (23, 24). For example, the presence of monosaccharides in acid hydrolysates indicates that in the original polysaccharide, such residues do not contain α-glycol groupings. Partial acid hydrolysis of the polyalcohol prepared by the reduction of periodate-oxidised lichenin gave glucose but not laminaribiose, suggesting that few, if any, sequences of adjacent 1:3-linked glucose residues were present (19). The proportion of 1:3-linkages in this polysaccharide as determined by periodate reduction, was confirmed by analysis of the polymers prepared from periodate-oxidised lichenin and isonicotinhydrazide and thiosemicarbazide (19).

It is clear that periodate oxidation methods have many useful applications in polysaccharide chemistry, provided that due regard is paid to the possible occurrence of 'under-oxidation' (e.g., 7), and 'over-oxidation' (e.g., 20, 21). Furthermore, for routine analysis, a knowledge of the structural type of polysaccharide is required before the significance of formic acid and formaldehyde production can be accurately assessed.

Enzymic Degradation Studies

The degradation of a polysaccharide by a purified hydrolytic enzyme (polysaccharase) provides a most useful method for selective partial hydrolysis. In contrast to acid hydrolysis, enzymic reactions have the great advantage of specificity. For example, the ability of β-glucosidase preparations to hydrolyse leucosin (17) is evidence for the presence of β-glucosidic linkages. Several examples of specific enzymic hydrolysis are given in a recent review (42).

With glycogen and starch-type polysaccharides present knowledge of the action patterns of various starch-metabolizing enzymes enables details of fine structure to be determined (for reviews, see 22, 23, 41 and 42). The enzymes concerned are α-amylase, β-amylase and muscle phosphorylase which degrade α-1:4-glucosidic linkages, and R-enzyme, amylo-1:6-glucosidase and isoamylase which are specific for the hydrolysis of α-1:6-inter-chain linkages.

Starches and polysaccharides containing adjacent α-1:4-glucosidic linkages are attacked by α-amylases, in a random manner, with the production of reducing sugars, particularly maltose. Many of the substrates of α-amylase give characteristic colorations with iodine (4), and with these, α-amylolysis is characterized by a rapid decrease in

iodine staining power. Hence, α-amylase and iodine staining have been used to characterise several polysaccharides as being of the starch and glycogen-type (6, 7, 9, 12, 16, 18). It must be noted that resistance of a polysaccharide to α-amylase does not imply the total absence of α-1:4-linkages, but that such linkages, if present, are not arranged adjacently, e.g., some dextrans contain α-1:4-inter-chain linkages, and nigeran consists of alternate α-1:3- and α-1:4-linked glucose residues; neither polysaccharide is attacked by α-amylase.

With branched α-1:4-glucosans, the inter-chain linkages and certain adjacent α-1:4-linkages are resistant to enzyme action (VI), and oligosaccharides with degree of polymerisation ($\overline{DP}$) 4-10 glucose residues are produced, in addition to maltose. The extent of degradation [expressed as apparent percentage conversion into maltose, P_M] should therefore be related to the degree of branching. An approximate relationship has been established experimentally, (9, 13, 15), and this method has enabled the degree of branching of a biopsy sample of human liver glycogen to be determined (see p. 20).

The extent of the stepwise degradation of a polysaccharide to maltose by β-amylase is of considerable

structural significance. With amyloses, it provides the only method for detecting the presence of a small number of anomalous linkages. Thus, amyloses prepared by thymol fractionation of potato starch have β-amylolysis limits of 70-80% and must contain some structural feature in addition to α-1:4-linked D-glucopyranose residues (10). The nature of this 'barrier' to β-amylase action has been considered (10, 36, 40). Since β-amylolysis of glycogen and amylopectin is confined to the outer chains, a knowledge of the $\overline{CL}$ and β-amylolysis limit enables the relative length of the exterior and interior chains to be determined (9, 12, 13, 15, 16 and 18).

The action of muscle phosphorylase on glycogen and amylopectin is also confined to the outer chains, the products being α-D-glucosyl phosphate (30-50%) and a limit dextrin (Ø-dextrin). The latter differs from the corresponding β-dextrin with respect to the length of the exterior chain 'stubs' (V). On this basis, a method for determining the degree of multiple branching has been proposed (11, 29).

The action of yeast isoamylase on various starch-type polysaccharides results in an increase in β-amylolysis limit. This enzyme has no action on α-1:3-, α-1:4- or

β-1:6-linkages (31, VII) and this observation characterises the outermost inter-chain linkages as being of the α-1:6-type. This method has been applied to various algal and protozoal polysaccharides (7, 12, 18).

The use of amylo-1:6-glucosidase and R-enzyme by other workers in investigating the fine structure of polysaccharides has been reviewed (41, 42). The specificity of the latter enzyme appears to be controlled by the relative length of the interior chains of the branched α-1:4-glucosan (34).

The Structure and Metabolism of Glycogen

The aim of the present studies was three-fold: first, to extend our knowledge of the structure of glycogens isolated from as wide a range of animal tissues as possible; secondly, to determine whether single- or multiple-branching was present; thirdly, to analyse the structure of mammalian glycogen isolated from certain physiological or pathological conditions which might yield information on the *in vivo* metabolism.

The basic structure of glycogen was established by methylation studies carried out by Haworth and Bell and their respective collaborators (for review, see 22) who showed the molecules to have a high molecular weight ($\sim 10^6$), and to be

composed of chains of α-1:4-linked D-glucose residues (average length ca 12 residues) interlinked to form a branched structure. The presence of α-1:6-glucosidic inter-chain linkages was shown by later chemical studies (22).

In the present investigations, some forty samples of glycogen have been examined (5, 6, 9, 13, 15, 16). The majority of glycogens, by potassium metaperiodate oxidation, had $\overline{CL}$ values of 10-15 glucose residues. Glycogen from Cardium, Mytilus edulis, and one particular rabbit liver had $\overline{CL}$ values of 8, 9 and 17 respectively (9). An unusual result of this comparative study was the finding that horse and ox muscle glycogens had $\overline{CL}$ values of 16-19 (13). On incubation with β-amylase, percentage conversions into maltose of 46 ± 7 were recorded with most samples; exceptions were Cardium glycogen (14%) and one rabbit liver glycogen (25%). From these results, the exterior and interior chain lengths of most glycogens were calculated to be 7-9 and 3-4 glucose residues respectively.

Inspection of the detailed results shows that small differences exist in degree and position of branching between glycogens from different biological sources, and in different samples from the same biological source, e.g., Mytilus edulis glycogens with $\overline{CL}$ values of 5, 9, 10, 12, 13, 14 and 17

have now been reported. Some variation in branching characteristics is not unexpected with a reserve carbohydrate of this type which is undergoing continual synthesis and breakdown in the animal cells. Indeed, in view of the complex enzyme system catalysing these processes, and the relationship of this system to the metabolic state of the animal (e.g., hormonal control, and factors affecting the synthesis of enzyme-proteins), it is perhaps surprising that glycogen from brewer's yeast (6), protozoa (8, 16), marine invertebrates (9) and mammalian liver (9) should have, in fact, essentially similar branched structures.

The molecular weights of many of the above glycogen samples have been measured by sedimentation-diffusion and/or light-scattering methods (3, 14). Several of the samples were polydisperse, so that the sedimentation constants of the major component had to be used in the evaluation of molecular weights; these were in the range 3-9 x 10^6. With four glycogens showing no polydispersity, there was good agreement between light-scattering and sedimentation-diffusion measurements. Glycogens are therefore amongst the largest known polysaccharides and with their low viscosities, represent ideal storage materials with little effect on the osmotic pressure and viscosity of tissue fluids.

The polydispersity may be characteristic of the 'native' polysaccharide, or caused by alkaline degradation during isolation. The finding (3, 14) that the molecular weight of isolated glycogen is not affected by hot alkali would support the former possibility.

The suggestion of Meyer that glycogen had a multiply branched structure, rather than a singly branched structure as proposed by Haworth, presented a difficult experimental problem, since chemical methods cannot differentiate between these structures. Enzymic methods have therefore been applied, and qualitative evidence in favour of multiple-branching was first obtained by Larner and his coworkers (VIII) who studied the stepwise degradation of glycogen by muscle phosphorylase and amylo-1:6-glucosidase. In an attempt to obtain quantitative data, the theoretical differences in $\overline{CL}$ values of β- and Ø-dextrins showing varying degrees of multiple-branching have been compared with experimental results (11, 29). The results support the concept of multiple-branching, and in addition, reveal the existence of small variations in degree of multiple-branching between different glycogen samples. Calculations on the experimental results of Larner and coworkers are in accord with these conclusions (22).

The inter-conversion of glycogen and glucose is catalysed by a multi-enzyme system including hexokinase, phosphoglucomutase, phosphorylase, branching enzyme, amylo-1:6-glucosidase and glucose 6-phosphatase. The relative activity of these enzymes may, in certain instances, be deduced from the structure of glycogen isolated from metabolically abnormal tissue.

During the development of rigor mortis in mammalian muscle, there is a marked decrease in glycogen content. However, analysis of glycogen isolated from horse l-dorsi, diaphragm and psoas, and from ox psoas muscle reveals little difference in branching properties between pre- and post-rigor samples (13). It is concluded that the relative activity of the phosphorylase-amylo-1:6-glucosidase system is unaltered during post-mortem glycogenolysis and glycolysis.

Glycogen-storage diseases constitute a group of pathological conditions in which glycogen is deposited in the liver and other tissues in unusually large amounts. For example, a liver may contain 15% of glycogen, compared with the normal content of ca. 5%. G.T. Cori has differentiated between four types of disease (IX); in type 1 (von Gierke's disease), glycogen of normal structure accumulates only in the liver and kidney, and in these

tissues, glucose 6-phosphatase activity is subnormal; in type 2, glycogen deposition is generalized and the structure is normal; in types 3 and 4, the structures are abnormal, resembling a Ø-dextrin and amylopectin respectively, indicating a deficiency of amylo-1:6-glucosidase and branching enzyme.

Three cases of glycogen-storage disease have been examined. In the first (5) the liver glycogen had a $\overline{CL}$ of only 6, and had a Ø-dextrin type structure; in the second (15), the liver and kidney glycogens had normal structures. The third case involved analysis of 34 mg. glycogen isolated from 233 mg. liver biopsy tissue; by α-amylolysis, a $\overline{CL}$ of ca. 8 was found, the β-amylolysis limit was only 27%, and the iodine staining power was low (X). The first and third cases thus belong to type 3, whilst the second is a true von Gierke's disease. This latter conclusion was confirmed by Dr. P.W. Kent (15) who reported an extremely low glucose 6-phosphatase activity of the liver tissue.

The Structure and Metabolism of Starch

The fine structure of the amylose and amylopectin components of starch from various plant sources, and the

properties of β-amylase, Z-enzyme and R-enzyme have been investigated.

Enzymic degradation studies (10) have shown that amylose is heterogeneous with respect to both $\overline{DP}$ and molecular structure. Thymol fractionation of potato starch gave amylose of $\overline{DP}$ 3,200 (determined viscometrically) and β-amylolysis limit 77% whilst aqueous leaching of the same starch gave a smaller (40%) yield of amylose of $\overline{DP}$ 1,800 which had a β-amylolysis limit of 99%, indicating a completely linear structure. It follows that the major part of the amylose has a $\overline{DP}$ of 5-6,000 and a β-amylolysis limit of 50-60%.

Starch-type polysaccharides have been isolated from the algae Dunaliella bioculata (12) and the protozoon Chilomonas paramecium (18) and characterised. The former contained only 13% of amylose which had a β-amylolysis limit of 73%, whereas the protozoal starch contained 45% of amylose of low $\overline{DP}$ (ca. 335) and high β-amylolysis limit (90%).

The mechanism of the β-amylolysis of amylose has been examined (33, 36). Measurement of the sedimentation constants and limiting viscosity numbers of amylose, and intermediate and β-limit dextrins showed no significant change in

molecular size. This is convincing evidence of a single-chain mechanism.

For the complete amylolysis of amylose, a second enzyme (Z-enzyme) is required. Previous investigators have suggested that Z-enzyme, which occurs with β-amylase in cereal preparations, removes the anomalous linkages in amylose by selective hydrolysis (for reviews, see 10, 23, 36, 42). However, a re-examination of the action of Z-enzyme on amylose has shown that slight random hydrolysis takes place, and that "Z-enzyme" is a minute trace of α-amylase impurity in the β-amylase preparation (40). The fact that Z-enzyme hydrolyses α-1:4-linkages considerably simplifies our views on the structure and metabolism of amylose.

The nature of the barriers to β-amylase in amylose has been discussed (10, 36, 40). From enzymic evidence, the presence of α-1:3- or α-1:6-glucosidic linkages is considered to be unlikely, but the existence of phosphate groups or oxidised glucose residues remains as a possibility.

The degree and position of branching of various amylopectin samples has been examined. The majority of these had $\overline{CL}$ values in the range 20-25 and β-amylolysis limits of 50-63% (11, 18, 23) corresponding to exterior and

interior chain lengths of ca. 15 and 7 respectively. Dunaliella amylopectin is an interesting exception (CL, 15-16; β-amylolysis limit 60%) (12). Pretreatment of algal and protozoal amylopectins with isoamylase results in an increase in β-amylolysis limit of 16-22% (12, 18); the nature of the outermost inter-chain linkages is therefore indicated (31, VII). The variation in branching characteristics in different amylopectins is not so marked as with glycogens, suggesting that the relative activity of P- and Q-enzymes (43) is more constant in the various plant species.

The average length of the interior chains in amylopectin is appreciably greater than that of glycogen. The significance of this structural difference has not hitherto been adequately recognised. However, this fact provides at least a partial explanation for (a) the greater susceptibility of amylopectin for α-amylase (9) and R-enzyme (34), (b) the marked difference between the iodine staining power of the two polysaccharides (4), (c) the failure of amylopectin β-dextrin to react with concanavalin-A (cf., 13, X), (d) the greater ease of oxidation of amylopectin by periodate, (e) the striking difference in hydrodynamic behaviour between amylopectin and glycogen (14, 35).

The occurrence of multiple-branching in amylopectin has been considered. Calculations (2) based on the experimental results of Peat and his coworkers (XI) suggested a lower degree of multiple-branching than in glycogen; subsequently, an error was noted in the above experimental results (XII), and with a correction for this, re-calculation indicated a fully developed 'tree'-type structure for amylopectin. This conclusion was substantiated by a study of the ∅- and β-dextrins of waxy maize and sorghum starch (11). It is now apparent that the differences in physico-chemical properties between glycogen and amylopectin cannot be ascribed to differences in degree of multiple-branching.

The structure of Floridean starch has also been examined; unlike normal starches, this algal polysaccharide does not contain an essentially linear amylose component. An earlier investigation of the periodate oxidation and β-amylolysis of Floridean starch had suggested the presence of 1:3-glucosidic linkages; these conclusions could not be confirmed in the present study (7). Determination of periodate reduction, and the extent of degradation by α-amylase, β-amylase and isoamylase showed that the polysaccharide was in fact, of the amylopectin-glycogen type.

It is concluded that the storage polysaccharides of

certain marine and fresh-water algae, and protozoa are closely related to the starches synthesized by photosynthetic terrestrial plants.

The Structure and Metabolism of β-Glucosans

Several aspects of the structure and catabolism of four β-glucosans have been examined.

As previously stated, lichenin, a reserve polysaccharide from Iceland moss, was investigated by methylation and periodate oxidation methods (19), and shown to be a linear molecule composed of 70% β-1:4- and 30% β-1:3-linked D-glucopyranose residues. The absence of β-1:6-linkages was indicated by the formaldehyde production (1 mol. per glucose residue) on periodate oxidation at pH 8 (cf. 20). Lichenin is hydrolysed in step-wise fashion to glucose by almond β-glucosidase (19) in contrast to barley enzyme preparations which yield glucose and oligosaccharides.

The insoluble and soluble forms of laminarin, from Laminaria cloustoni and L. digitata respectively, have been studied (20, 21). Physical and chemical analysis of methylated laminarin has provided clear evidence of a branched structure, since a fraction of $\overline{DP}$ 58 had a $\overline{CL}$ value of only 23. It is probable that β-1:6-inter-chain linkages

are present. Periodate oxidation analysis indicates that the two forms of laminarin differ chemically. A sample of insoluble laminarin contained 1.9% of mannitol, which was the terminal group of 46% of the molecules, and one free-reducing group per 45 glucose residues were present. In contrast, one sample of soluble laminarin had a mannitol content of 3.9%, and mannitol was the terminal group of 75% of the molecules, whilst the presence of one free-reducing group per 75 glucose residues was indicated.

The occurrence of a β-1:3-glucosan, named leucosin, in the flagellated protozoon Ochromonas malhamensis (17) is of biochemical interest, since certain algae and protozoa are morphologically related; indeed, there is considerable discussion on the precise classification of these organisms. Leucosin resembles laminarin with regard to periodate oxidation and behaviour on partial hydrolysis with acids and enzymes; it differs however in containing ca. 10% of a second sugar, tentatively identified as mannose. Mannitol has not been detected in hydrolysates of leucosin.

Leucosin and both forms of laminarin are hydrolysed by enzyme preparations from barley (17, 30), soya-beans and extracts of Cladophora rupestris and other marine algae (25, 26), with the formation of glucose and a series of

laminarisaccharides. This indicates random hydrolysis. In contrast, hydrolysis by almond emulsin yields only glucose. The *in vivo* significance of these hydrolytic enzymes is not yet known, but since these polysaccharides function as reserve carbohydrates, the various organisms must contain an efficient enzyme-system for the rapid mobilization of this storage material. The demonstration of trans-β-glucosylase activity by extracts of barley (37) and *Cladophora rupestris* (28) may indicate that transfer reactions (for review, see 44) also play an important part in the intermediary metabolism of these polysaccharides.

Carbohydrate-Metabolising Enzymes in Marine Algae

The polysaccharides of the marine algae are of special interest to the chemist, since many of them contain monosaccharide residues which are not common constituents of plant or animal polysaccharides e.g., L-fucose, L-galactose, 3:6-anhydro-D-galactose and 3:6-anhydro-L-galactose (24). In addition, D-mannuronic and L-guluronic acids may be present, together with monosaccharide residues esterified by sulphuric acid. Although structural investigations on the complex algal polysaccharides have been in progress for many years, virtually no attention has been paid to their

metabolism. Accordingly, a research programme on the enzyme systems in marine algae was initiated in 1953, and this thesis contains the results of some preliminary investigations (25-28).

Despite considerable difficulty in the extraction of protein material, evidence for the presence of a number of carbohydrases in extracts of Cladophora rupestris, L. digitata, Rhodymenia palmata and Ulva lactuca was obtained. These included maltase, cellobiase, amylase, xylanase, laminarinase, cellodextrinase, and carboxymethyl cellulase (25, 26). The maltase activity of C. rupestris resembled that of other maltase preparations in that oligosaccharides were synthesised from concentrated solutions of maltose (27), although specificity differences from other maltases were revealed. With cellobiose, β-linked glucose-oligosaccharides were synthesised, and with a cellobiose-xylose mixture 3-O-β-D-glucopyranosyl-D-xylopyranose was produced (28).

These studies indicate that the carbohydrate complements of the marine algae are generally similar to those of the cereals, and suggest that the pathways for the metabolism of the simpler polysaccharides such as starch, xylan and the β-glucosans are closely related. The more complex polysaccharides may be metabolised by relatively insoluble

enzyme-systems which are associated with the algal cell-wall, since the soluble extracts had no hydrolytic activity towards fucoidin, alginic acid, and Cladophoran and other heteropolysaccharides. It is significant to note that the latter polysaccharides have a structural function in the cells, whilst the simpler polysaccharides appear to act as carbohydrate reserves.

Carbohydrate-Metabolising Enzymes in Yeast

Although the metabolism of starch by plant enzymes, and of glycogen by mammalian liver and muscle enzymes have been extensively studied [for reviews of the fundamental studies by Peat and Cori, and their coworkers, see 41, 42 and 43] the enzyme-system catalysing the synthesis and degradation of the reserve polysaccharide of yeast has not been systematically investigated.

As a preliminary to such an investigation, the storage polysaccharide of brewer's yeast was examined both chemically and enzymically, and characterized as a glycogen (6). By analogy with the plant and animal systems, yeast should therefore contain *inter alia* a phosphorylase, a branching enzyme, and a debranching enzyme; these activities have been studied.

The presence of phosphorylase in yeast juice was first shown by Schaffner, Specht and Kiessling in the period 1938-9, but apart from a preliminary study by Meyer (XIII), the purification of this enzyme has not been reported. Extraction of air-dried brewer's yeast with sodium bicarbonate, followed by ammonium sulphate fractionation and freeze-drying in citrate-buffer gave protein preparations which have a phosphorylase activity of ca. 100 Green and Stumpf units per gm. (XIV), a value similar to that reported for bean and potato P-enzyme preparations (XV). Enzyme action was optimum at pH 5.8 and 35°, and was not activated by adenylic acid (unlike muscle phosphorylase). On incubation with glycogen or amylopectin, 40-50% conversion into α-D-glucosyl phosphate was observed. This preparation was contaminated with other enzymes, including maltase, a branching enzyme and a debranching enzyme.

Incubation of the phosphorylase with α-D-glucosyl phosphate resulted in polysaccharide formation, which had a branched rather than a linear structure (e.g., β-amylolysis limit 44%), indicating the presence of branching enzyme impurity. The latter was later (32, 39) prepared free from phosphorylase by ethanol-citrate fractionation of a yeast extract. The optimum pH and temperature of the yeast

branching enzyme and Q-enzyme are similar, but the former differs from its animal and plant counterparts in effecting the branching of both amylose and amylopectin. By this means, a novel transformation of a plant amylopectin into a polysaccharide with all the properties of an animal glycogen has been achieved (35).

The presence of a yeast debranching enzyme - isoamylase - acting on amylopectin has been reported by Maruo and Kobayashi (XVI). The action of a brewer's yeast protein fraction on glycogen was therefore studied, and with a large number of glycogen samples, an increase in both iodine staining power and β-amylolysis limit occurred (31, XIV). The specific action of this enzyme on α-1:6-glucosidic linkages has been used to characterize the inter-chain linkages in various polysaccharides (see p. 14 and 23).

Allied to these studies, the properties of yeast maltase have been examined with special reference to trans-α-glucosylase activity (44, VII). The yeast enzyme, like that from Cladophora rupestris (27) and Tetrahymena pyriformis (38), can transform maltose into a mixture of oligo-saccharides, some of which contain α-1:6-glucosidic linkages; however, the yeast enzyme has a relatively greater affinity for water than for carbohydrate as an acceptor substrate.

These investigations have established a general similarity in the type of enzyme system responsible for the metabolism of glycogen (and maltose) in brewer's yeast and in animal tissues, although some differences in the chemical properties of the individual enzyme proteins have been noted.

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Periodate Oxidation of Glycogens. By D. J. MANNERS. (*Department of Chemistry, University of Edinburgh*)

In continuation of studies on the structure of glycogens, end-group assays have been carried out on several additional samples of glycogen. By potassium periodate oxidation (Bell & Manners, 1952; cf. Halsall, Hirst & Jones, 1947) unit-chain lengths of 12 ± 1 glucose radicals were found for foetal-pig liver, rabbit liver III, cat liver IV, cat liver VI, and *Mytilus edulis* IV glycogens. *Helix pomatia* II, *Mytilus edulis* V and human liver (von Gierke disease) glycogens had chain lengths of 10, 9 and approx. 6 respectively. By methylation, rabbit liver glycogen had a chain length of 12 (Bell, 1935) and *H. pomatia* II one of 11–12 (Baldwin & Bell, 1940).

End-group assays have also been made by sodium periodate oxidation for 25 hr. at 2° C. (Potter & Hassid, 1948). Measurement of the formic acid liberated, and the periodate uptake, indicate that oxidation is not complete under these conditions. The end-group values so obtained are not in good agreement with those obtained by methylation or by potassium periodate oxidation of the same samples. Oxidation of maltose under similar conditions gave 2·4–2·6 moles formic acid per mole (Atherden and Manners, 1953; cf. Potter & Hassid, 1948; and Morrison, Kuyper & Orten, 1953, who obtained 3 and 2·5 moles respectively). This difference in the oxidation of the 'model' compound may account, in part, for the discrepancy between the end-group values of glycogens obtained by Cori & Larner (1951) from periodate oxidation (Potter & Hassid method) and enzymic assays.

Prolonged periodate oxidation of glycogens, followed by acid hydrolysis, and analysis of the hydrolysate for glucose, has been used to detect 1:2 or 1:3 glycosidic linkages (Hirst, Jones & Roudier, 1948; Gibbon & Boissonnas, 1950). No significant amounts of glucose could be detected either by chromatography or by glucose oxidase in experiments with five different glycogens, and two glycogen α-dextrins; it is concluded that these glycogens contain only 1:4 and 1:6 linkages.

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MULTIPLE-BRANCHING IN AMYLOPECTIN

By E. L. Hirst and D. J. Manners

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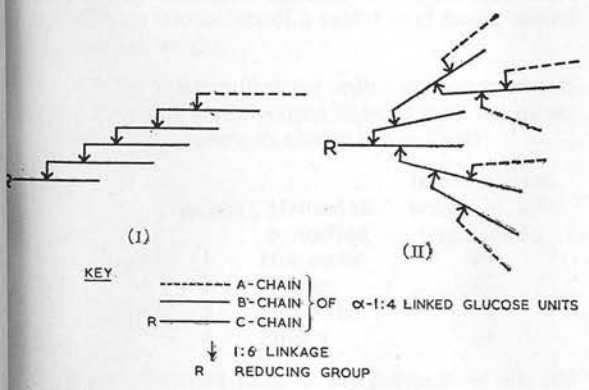
recent years, much progress has been made towards the dation of the molecular structure of amylopectin. Important nces have been made in the laboratories of C. F. Cori[1] S. Peat[2] where *qualitative* evidence of multiple-branching been obtained. The purpose of this communication is to ate how the evidence of S. Peat, *et al*[2] may be used to give *antitative* estimate of the degree of multiple-branching in opectin.

the various molecular structures postulated for amylo- n, a singly-branched (laminated) or a multiply-branched) structure appears to be most widely accepted. The er structure (I) was suggested by W. N. Haworth and E. L. [3] as the simplest structure compatible with the data available methylation studies; it was never intended to be a complete sentation of the amylopectin molecule[4]. The more complex " structure (II) was postulated by K. H. Meyer[5] as the t of chemical and enzymic studies. These structures are rated below:



at and co-workers[2] have defined three types of unit-chain; -type (side-chain) which is attached to the rest of the ule only by a linkage from its reducing group, (2) B-type -chain) to which are attached other chains, (3) C-type carries the single reducing group in the molecule. By tion, it can be seen that the ratio of A : B-chains in the inated " structure is 1 : $(n-2)$ where n is the number of hains in the molecule, while in the fully developed " tree " ure there are approximately equal numbers of A and B-.

successive action of β-amylase and *R*-enzyme has been to obtain qualitative evidence of multiple-branching in maize starch (W.M.S.)[2]. On β-amylolysis of an amylo- , A-chains are almost completely removed, leaving s " comprising two or three glucose units, while B-chains completely degraded since this enzyme cannot hydrolyse -pass the α-1 : 6 inter-chain linkages. These latter es can be hydrolysed by *R*-enzyme,[6] an A-chain giving rise ltose or maltotriose and B-chains yielding linear dextrins (degree of polymerisation) $\geqslant 6$. The amount of maltose riose liberated on treating a β-dextrin with *R*-enzyme is ore dependent on the number of A-chains in the molecule. W. M. S. has a chain length of 20 glucose units[7] and may be assumed to have a minimum D.P. of *ca.*6000[8] ; its β-amylolysis limit is 50% and the observed molar percentage of maltose and maltotriose, after treatment of the β-dextrin with *R*-enzyme, is 5·3%[2]. Since the molar percentage of maltose and maltotriose arising from a singly-branched β-dextrin of D.P. 3000 is 0·083%, S. Peat, *et al.* concluded that " multiple branching is an intrinsic part of the amylopectin structure."

The above data can be used to give a quantitative estimation of the ratio of A : B chains in the W.M.S. The observed yield of maltose and maltotriose is *ca.* 64 times that required by a singly-branched structure, indicating that *ca.* 64 A-chains are present in the β-dextrin, and hence, in the W.M.S. Crystalline β-amylase (free from α-amylase) was used in the preparation of the β-dextrin from W.M.S. and consequently both polysaccharides will contain the same number of unit-chains, namely, *ca.* 300. The ratio of A : B chains is therefore 64 : 236 or approximately 1 : 4. Further calculation shows that (*a*) for such high molecular weight polysaccharides, this ratio is independent of the actual D.P. of the W.M.S. and β-dextrin, (*b*) a fully developed " tree " structure would give *ca.* 12·5% maltose and maltotriose by the successive action of β-amylase and *R*-enzyme. It follows from these considerations that in the W.M.S. examined by S. Peat, *et al.*, only one unit-chain in every five contained more than one branch point. The molecular structure thus approximates rather more closely to a " laminated", and therefore elongated, molecule than to a compact " tree " structure. This suggestion is in accord with the observed approximately linear relationship between the viscosity and molecular weight of methylated[9] and native[10] amylopectins.

The evidence available from chemical and enzymic studies[1,11] indicates that glycogen has a multiply-branched structure similar to that proposed by K. H. Meyer for amylopectin, but differing in that the unit-chains normally comprise 10—14 and not 20—25 glucose units. Amylopectin therefore appears to differ from glycogen in the degree of multiple-branching. Physico-chemical studies, including those on the viscosity[9,10,12] and iodine-binding power[13] of amylopectin and glycogen, suggest that there is a fundamental difference in the arrangement of the constituent unit-chains. This difference may be due to different degrees of multiple-branching, and experiments are now in progress in this Laboratory to test this hypothesis.

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The Alkali-stability and Molecular Size of Glycogens

By C. T. Greenwood and D. J. Manners

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In view of present interest in the alkaline degradation of polysaccharides, and in particular, of starch[1] and its component amylose[2] and amylopectin,[3] we now report the effect of alkali on the molecular size of glycogen.

The classical Pflüger method for the preparation of glycogen involves digestion of the tissues with 20—60% aqueous potassium hydroxide at 100°, followed by precipitation of the glycogen with alcohol. The method has been criticised by Meyer and Jeanloz[4] who suggested that degradation of the glycogen occurred during the alkaline extraction. However, Bridgman[5] reported that glycogen extracted by the Pflüger method, or with trichloroacetic acid from two halves of a rabbit liver had a similar molecular weight.

We have determined the sedimentation constants of four samples of glycogen isolated from the halves of two rabbit livers, as shown in the Table:

	Sample	Method of extraction	Sedimentation const., $\times 10^{13}$ (c.g.s. units)
Liver A	1	Hot water	85
	2	Pflüger	86
Liver B	3	Hot water	76
	4	Pflüger	83

It is concluded that, in the presence of air, the extent of degradation of glycogen by 30% aqueous potassium hydroxide at 100° is no greater than that which might be caused by the action of boiling water. These results are in agreement with those of Staudinger[6] who showed that the molecular weights of guinea-pig liver and muscle glycogens were unaffected by digestion with 15—30% potassium hydroxide at 100° for 1 hour.

By contrast, hot dilute alkali appears to degrade glycogen. Digestion of another sample of rabbit-liver glycogen in 8% sodium hydroxide solution at 100° for 1·5 hours caused a reduction in the sedimentation constant from 86 to 57 $\times 10^{-13}$ c.g.s. units and an increase in polymolecularity[7] of the polysaccharide, as shown by a broadening of the peak of the schlieren pattern.

In continuation of physicochemical studies on starch-type polysaccharides, the sedimentation constants of 17 other samples of glycogen have been determined. The majority of the samples, from vertebrate and invertebrate tissues, were isolated by the Pflüger method, and the sedimentation constants were found to vary between 39 and 130 $\times 10^{-13}$ c.g.s. units. A mean value of 1·5 $\times 10^{-7}$ being assumed for the diffusion constant of glycogen,[8] these results correspond to molecular weights of 2—6 $\times 10^{6}$. All the samples were polymolecular. In addition, six of the glycogens were *polydisperse* and showed the presence of a second component, in some instances heavier and in others lighter than the bulk. These glycogens are unusual, although the polydispersity of samples of human-liver glycogen has previously been reported.[9]

It has been suggested[10] that purification of glycogen by precipitation with glacial acetic acid may render it unsuitable for ultracentrifugal analysis. The sedimentation constant and schlieren pattern of rabbit-liver glycogen (sample 4) is, however, unaltered after four precipitations of the glycogen with 80% acetic acid.

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Observations on the Absorption Spectra of Polysaccharide-Iodine Complexes

by A. R. Archibald, D. J. Manners and A. Wright,
(Department of Chemistry, University of Edinburgh)

In the course of structural studies on starch-type polysaccharides, we have measured the absorption spectra of the iodine complexes of more than fifty different samples of amylopectin and glycogen. Using conditions similar to those of Peat, Whelan, Hobson and Thomas (1954), in which the absorption spectra of 0.01% polysaccharide solution stained with a solution of 0.02% iodine in 0.2% potassium iodide is measured in a Unicam S.P.500 spectrophotometer, values for the wavelength of maximum absorption (λ_{max}) and the extinction (absorption value) at this wavelength (E_{max}) have been obtained.

Glycogens from various biological sources gave λ_{max} values in the range 420-490 mμ with E_{max} 0.1-0.4. The iodine staining power increased generally in the order glycogen β-limit dextrin, invertebrate glycogen, mammalian liver glycogen and mammalian muscle glycogen, but there was no correlation between λ_{max} or E_{max} values and the degree of branching, or the relative length of the exterior and interior chains.

With amylopectins, the corresponding λ_{max} and E_{max} values were 530-550 mμ and 0.8-1.2 respectively, and there was no apparent relationship between iodine staining power and branching characteristics. Floridean starches gave absorption spectra with λ_{max} ca 500 mμ and E_{max} ca 0.7.

Amylopectin and glycogen differ markedly in iodine staining properties in two additional respects. Firstly, during the β-amylolysis of glycogen, a decrease in both λ_{max} and E_{max} is observed whereas for amylopectin the λ_{max} of the intermediate and limit-dextrins is unaltered. The type of iodine binding in the two polysaccharides must therefore differ, and in the case of amylopectin, it is suggested that the length of the interior chains is a controlling factor. Secondly, the addition of ammonium sulphate (cf. Schlamowitz, 1951) to the polysaccharide-iodine-iodide solution causes an increase in iodine staining power. With amylopectins, the λ_{max} is unaltered but E_{max} is increased by 10-30%; with glycogens, λ_{max} is increased by ca 40 mμ, and E_{max} to the range 0.8-1.1. In these solutions, the dehydrating action of ammonium sulphate may facilitate the binding of iodine by a proportion of the exterior chains of glycogen.

We wish to thank Professor E. L. Hirst, F.R.S., for his interest in this work.

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α-1 : 4-*Glucosans. Part II.* The Molecular Structure of the Liver Glycogen from a Case of von Gierke's Disease.*

By D. J. MANNERS.

[Reprint Order No. 5375.]

The liver glycogen isolated from a case of von Gierke's disease has a chain length of 6 glucose residues, a molecular weight of $\sim 10^6$, and a β-amylolysis limit of 14%. Its $[\alpha]_D$ is +201° and it gives no colour with iodine. The glycogen is degraded by salivary α-amylase and is not attacked by muscle phosphorylase. The molecular structure resembles that of a phosphorylase limit-dextrin of a normal glycogen, but differs in having shorter exterior chains.

VON GIERKE'S or glycogen-storage disease is a metabolic disorder which is accompanied by the deposition of unusually large amounts of glycogen in the liver, skeletal muscle, and other tissues. Illingworth and Cori (*J. Biol. Chem.*, 1952, **199**, 653) isolated glycogen from the livers of ten cases of von Gierke's disease. Structural studies (by enzymic methods; cf. Cori and Larner, *ibid.*, 1951, **188**, 17; Larner, Illingworth, Cori, and Cori, *ibid.*, 1952, **199**, 641) showed that eight of these glycogens had structures similar to those of other mammalian glycogens, *viz.*, unit chains of 11—13 glucose residues, phosphorolysis limits of 33—42%, and absorption maxima of the iodine complexes at 470 mμ. In the ninth case, glycogen was obtained from both liver and muscle tissues which had shorter unit chains and was only slightly degraded by muscle phosphorylase (see Table 1). The tenth glycogen superficially resembled an amylopectin since the unit chains comprised 21 glucose residues, and were degraded 51% by phosphorylase, whilst the "glycogen"–iodine complex had an absorption maximum at 530 mμ. No estimate of the degree of multiple branching in these glycogens was recorded (cf. Hirst and Manners, *Chem. and Ind.*, 1954, 224). Polglase, Smith, and Tyler (*J. Biol. Chem.*, 1952, **199**, 97) examined glycogen obtained from two cases of von Gierke's desease: in one case the liver glycogen had a normal chain length; in the second the unit chains of the liver glycogen were *ca.* 25% shorter, whilst the muscle glycogen appeared to be normal. Sedimentation studies were also carried out on these glycogens (Polglase, Brown, and Smith, *ibid.*, p. 105; see Table 2). The present paper is concerned with the molecular structure of a von Gierke liver glycogen which was found to be different from those described above.

The liver, obtained at autopsy, was kindly placed at our disposal by Professor H. A. Krebs, F.R.S. Glycogen was isolated from the tissue by repeated extraction with hot water; the aqueous extracts were freed from protein by 4% trichloroacetic acid, and the glycogen was precipitated with ethanol and further purified by precipitation with 80% acetic acid (Bell and Young, *Biochem. J.*, 1934, **28**, 882). The purified glycogen dissolved in water ($[\alpha]_D$ +201°) to give an opalescent solution which, unlike that of other glycogens, was not stained brown with iodine. Quantitative measurements of the iodine-binding power by potentiometric titration (Anderson and Greenwood, *Chem. and Ind.*, 1953, 642) showed a much lower binding power than that of normal glycogens (Greenwood and Manners, unpublished work). Acid hydrolysis of the glycogen gave glucose and no other reducing sugar. Salivary amylolysis of the glycogen caused rapid breakdown to reducing sugars (maltose and higher oligosaccharides), showing the presence of α-1 : 4-glucosidic linkages. The absence of maltulose in the α-amylolytic digest indicates that fructose is absent in the original polysaccharide (cf. Peat, Roberts, and Whelan, *Biochem. J.*, 1952, **51**, xvii). End-group assay by potassium periodate (Bell and Manners, *J.*, 1952, 3641; Halsall, Hirst, and Jones, *J.*, 1947, 1399) showed that the unit chains contain, on the

* Part I, *J.*, 1954, 1891.

average, 6 glucose residues. This low value was not due to impurities of low molecular weight or to degradation products since the chain length was unaltered after prolonged dialysis of the glycogen. In an attempt to detect the presence of glucosidic linkages other than 1 : 4 or 1 : 6, a sample of periodate-oxidised glycogen was hydrolysed with acid, and the hydrolysate examined for glucose (cf. Hirst, Jones, and Roudier, *J.*, 1948, 1779; Part I, *loc. cit.*). No glucose could be detected (by paper chromatography) in the hydrolysate; 1 : 2- and 1 : 3-glucosidic linkages are therefore not present in the von Gierke glycogen. Treatment of the glycogen with a solution of crystalline sweet potato β-amylase resulted in a 14% conversion into maltose. The glycogen was not appreciably attacked by muscle phosphorylase in the presence of an excess of inorganic phosphate. Ultra-centrifugal examination of the glycogen, by Dr. C. T. Greenwood, showed that the glycogen was polydisperse and contained two components; the major component sedimented at 48 *S* * and the minor component at 200 *S* [equivalent to molecular weights of *ca.* 2 and 9 × 10^6 respectively, on the assumption that the diffusion constant is similar to that of rabbit-liver glycogen (see Bell, Gutfreund, Cecil, and Ogston, *Biochem. J.*, 1948, **42**, 405)]. The von Gierke glycogen is therefore a highly branched α-1 : 4-glucosan, composed of *ca.* 10^3 unit chains—each comprising, on the average, 6 glucose residues—which are interlinked only by 1 : 6-glucosidic linkages.

These results suggest that the von Gierke glycogen *qualitatively* resembles the phosphorylase limit-dextrin of a normal glycogen. Such a dextrin would have short unit chains and a low β-amylolysis limit [that of a normal glycogen is *ca.* 45% (Bell and Manners, *loc. cit.*)], and be resistant to attack by muscle phosphorylase. However, from a consideration of the action pattern of β-amylase and muscle phosphorylase, it will be shown that the von Gierke glycogen is not *identical* with a normal phosphorylase limit-dextrin.

Branched α-1 : 4-glucosans contain three types of unit chain; all three are linear chains of α-1 : 4-linked glucose residues, but they differ in the position of the linkage(s) to adjacent chains (Peat, Whelan, and Thomas, *J.*, 1952, 4546), *viz.*: A-chain, joined to the rest of the molecule only by a single glucosidic linkage from the reducing group; B-chain, joined to the molecule by its reducing group and one or more linkages involving $C_{(6)}$ of constituent glucose residues; C-chain, the sole chain in the molecule which is terminated by a free reducing group. An A-chain corresponds to a "side-chain," and a B-chain to a "main-chain" in the terminology of Cori and Larner (*loc. cit.*). Muscle phosphorylase has a different affinity for A- and B-chains; Cori and Larner (*loc. cit.*) have shown that in a phosphorylase limit-dextrin, the A-chain contains only one glucose residue whilst the exterior portion of the B-chain probably comprises 5 or 6 glucose residues (assuming that the dextrin contains equal numbers of A- and B-chains). This dextrin has a β-amylolysis limit of 24% (Hestrin, *J. Biol. Chem.*, 1949, **179**, 943), equivalent to the loss of one maltose residue per unit chain, enzyme action being limited to the B-chains from which two maltose residues are removed. The von Gierke glycogen thus differs from a phosphorylase limit-dextrin, since the β-amylolysis limit is only 14%. This figure represents the loss of *ca.* one glucose residue per chain, equivalent to the loss of one maltose residue from only half of the chains. The average exterior chain length of the von Gierke glycogen is therefore approximately half that of a normal phosphorylase limit-dextrin, *i.e.*, two glucose units. The interior chains of the von Gierke glycogen thus comprise three glucose residues. The low β-amylolysis limit obtained in the present study suggests either that the glycogen contains a relatively small proportion of B-chains (*i.e.*, a high degree of multiple branching) or that, *in vivo*, muscle phosphorylase has a higher affinity for the glucosidic linkages in B-chains than *in vitro*. The former possibility is now being investigated.

The occurrence of a glycogen with short unit chains of *ca.* 6 glucose residues is unusual, but not unique. Certain samples of glycogen from *Mytilus edulis* and *Helix pomatia* have chain lengths of *ca.* 5 and 7 glucose residues, respectively (Bell and Manners, *loc. cit.*), whilst Illingworth, Larner, and Cori (*J. Biol. Chem.*, 1952, **199**, 631) reported that a

* Sedimentation constants (S_{20}) are given in Svedberg units where $S = 1 \times 10^{-13}$ c.g.s. units.

specimen of tubercle bacillus glycogen had an apparent end-group value of 16% (equivalent to a chain length of *ca.* 6). The last glycogen was not attacked by phosphorylase, and appears to be a phosphorylase limit-dextrin, unlike the short-chain invertebrate glycogens which are partially degraded by phosphorylase and have β-amylolysis limits of 41 and 37% respectively. These invertebrate glycogens therefore differ from the von Gierke glycogen in having longer exterior chains and shorter interior chains, even though the constituent unit chains, on the average, are of approximately similar lengths.

The present sample of von Gierke glycogen differs from those examined by Illingworth and Cori and by Polglase *et al.* (*locc. cit.*) as shown in Tables 1 and 2.

TABLE 1. *Action of muscle phosphorylase on von Gierke glycogens.*

Sample	Source	Chain length	Conversion into glucose-1 phosphate (%)
Illingworth and Cori (*loc. cit.*)			
Case no. 1	Liver	12—13	36
Case no. 9	Liver	9	12
	Muscle	7—8	3
Case no. 10	Liver	21	51
Present study	Liver	6	1

When a glycogen is found to have a structure different from that in the " normal organism " one must postulate enzymic imbalance, *i.e.*, an abnormally greater or smaller activity in one or more of the several enzymes concerned in the equilibrium, glycogen $\rightleftharpoons$ glucose. The above data suggest that there are different types of glycogen storage disease. Cori and Cori (*ibid.*, p. 661) observed that, in two fatal cases of the disease,

TABLE 2. *Sedimentation constants of von Gierke glycogens.*

Sample	Source	Sedimentation constant (S_{20}) of major component	Sedimentation constant (S_{20}) of minor component
Polglase, Brown, and Smith (*loc. cit.*)	Liver (patient N.D.)	72—74	220
	Liver (patient P.N.)	75	—
	Skeletal muscle (N.D.)	59—67	29—36
	Cardiac muscle (N.D.)	75	38
Present study	Liver	48	200

glucose-6 phosphatase was " practically absent " from the liver tissue, whilst the formation of the amylopectin-like glycogen may have been due to a deficiency in branching enzyme. Although in the present study no biopsy samples were available for estimation of enzymic activity, it seems probable that the abnormal structure was caused, in part, by a deficiency in the debranching enzyme, amylo-1 : 6-glucosidase, thereby preventing complete phosphorolysis of the glycogen.

EXPERIMENTAL

Isolation of Glycogen.—Liver tissue (500 g.; from a 12-year-old female) which had been boiled in water (500 ml.) and stored under toluene (10 ml.) and octyl alcohol (3 ml.) was received from Professor H. A. Krebs. The solid material was strained off, ground with sand, and extracted four times with boiling water (600 ml.). To the combined, cooled, aqueous extracts and washings (3350 ml.), 40% trichloroacetic acid (380 ml.) was added and the mixture stored at 0° for 12 hr. The precipitated protein was removed by filtration through kieselguhr, and 2 vols. of ethanol were added to the filtrate. The precipitated glycogen was further purified by two precipitations from 80% acetic acid (Bell and Young, *loc. cit.*) and six precipitations from ethanol, and then dried *in vacuo* over phosphoric oxide for 4 hr. at 100°. The yield was 5·2 g.

Properties of the Glycogen.—The glycogen had $[\alpha]_D^{18}$ +201° (*c*, 0·68 in H_2O) (Found: N, 0·026%). Hydrolysis with 1·5N-sulphuric acid at 100° for 2 hr. gave glucose and no other sugar (paper chromatography).

Salivary α-Amylolysis of the Glycogen.—Glycogen (169·8 mg.) was incubated with 0·2M-phosphate buffer (pH 7·0; 10 ml.), 3% aqueous sodium chloride (2 ml.), distilled water (30 ml.), and salivary amylase solution (2 ml.) at 37°. Aliquot portions were removed at intervals for examination on a paper chromatogram and for determination of reducing power (as maltose)

by means of the Shaffer–Somogyi reagent (cf. Bell and Manners, *loc. cit.*). The course of hydrolysis was as follows :

Time of incubation (hr.)	0·08	0·33	0·67	2·50	4·00	6·00	24·00
Apparent conversion into maltose (%)	7·2	13·4	17·8	21·7	23·1	24·6	31·0

No further increase in reducing power was observed after 24 hr. The paper chromatograms showed the presence of maltose and higher maltosaccharides. Glucose and maltulose were absent.

Periodate Oxidation of the Glycogen.—Glycogen (235 mg.) was oxidised by a saturated aqueous solution of potassium periodate, as previously described (Bell and Manners, *loc. cit.*) :

Time (hr.)	168	265	432	576
Formic acid production (mg.)	8·2	9·8	10·6	10·9

The maximum formic acid titre is equivalent to a chain length of six glucose residues.

Glycogen (3·0 g.) was dialysed against successive changes of distilled water at room temperature for one week, and then isolated by freeze-drying. The dialysate was concentrated *in vacuo* at 45° ; no reducing carbohydrates were present (aniline oxalate spray on paper ; heating for 5 min. at 110°).

A sample of the dialysed glycogen (165 mg.) was oxidised with potassium periodate solution as above. The maximum yield of formic acid (obtained after *ca.* 400 hr.) was 7·8 mg., again equivalent to a chain length of six glucose residues.

The remaining solutions of periodate-oxidised glycogen from the assays were combined, dialysed, and freeze-dried. Periodate-oxidised glycogen (64 mg.) was hydrolysed with 2N-sulphuric acid (15 ml.) at 100° for 2 hr. The hydrolysate was cooled, neutralised to pH 5·0 (barium hydroxide) and, after removal of barium sulphate, was freeze-dried. This material was suspended in distilled water (1 ml.) and examined on a paper chromatogram No glucose was present, indicating the absence of 1 : 2- and 1 : 3-linkages in the original glycogen.

β-*Amylolysis of the Glycogen.*—Glycogen (14·0 mg.) was incubated with 0·2M-acetate buffer (pH 4·64 ; 5 ml.), distilled water (10 ml.), and crystalline sweet-potato β-amylase solution (1 ml.). The course of hydrolysis was :

Time of incubation (hr.)	2	24	48
Conversion into maltose (%)	7·7	14·1	14·3

Phosphorolysis of the Glycogen [with A. M. Liddle].—Glycogen (34·3 mg.) was incubated at 35° with 0·4M-phosphate buffer (pH 6·8 ; 2·5 ml.), adenylic acid (0·5 mg.), phosphorylase solution (0·25 ml.), and distilled water to a total volume of 10 ml. The phosphorylase was prepared in crystalline form from rabbit muscle by Green and Cori's method (*J. Biol. Chem.*, **1943**, **151**, 21). Samples (2 ml.) were withdrawn at intervals for estimation of glucose-1-phosphate, a slight modification of Allen's method being used (*Biochem. J.*, 1940, **34**, 858). Enzyme action gave *ca.* 1% conversion into glucose-1 phosphate after 45 hr. Under these conditions, 13-unit fœtal sheep-liver glycogen was degraded as follows :

Time (hr.)	1·3	4·0	25·5	49·5
Conversion into glucose-1 phosphate (%)	19	21	23	23

The author is indebted to Professor E. L. Hirst, F.R.S., and Dr. D. J. Bell for their interest in this work, and to Dr. C. T. Greenwood for the determination of the sedimentation constant of the glycogen.

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α-1 : 4-*Glucosans. Part III.* The Molecular Structure of Brewer's Yeast Glycogen.*

By D. J. Manners and Khin Maung.

[Reprint Order No. 5858.]

Brewer's yeast (*Saccharomyces cerevisiae*) contains a glucosan which is stained reddish-brown by iodine, is degraded by salivary α-amylase, and has a molecular weight of $\sim 2 \times 10^6$. The polysaccharide has an average chain length of 13, a β-amylolysis limit of 44%, and a phosphorolysis limit of 23%. The molecular structure therefore resembles that of a typical glycogen. The properties of the glycogens from baker's yeast and brewer's yeast are compared.

Although the glycogen from baker's yeast has been studied several times (*e.g.*, Northcote, *Biochem. J.*, 1953, **53**, 348; Jeanloz, *Helv. Chim. Acta*, 1944, **27**, 1501), little attention has been paid to the corresponding polysaccharide from brewer's yeast. A sample of the latter, isolated by Daoud and Ling (*J. Soc. Chem. Ind.*, 1931, **50**, 365T), had $[\alpha]_D$ +179° in water and was stained brownish-red by iodine; Yokoyama (*Beitr. Physiol.*, 1925, **3**, 95) found $[\alpha]_D$ +192° in water. No structural examination appears to have been published. The present communication deals with an investigation of the molecular structure of brewer's yeast glycogen; this is being used as a substrate in studies on the carbohydrate-metabolising enzymes in this organism.

The glycogen was extracted from yeast cell-wall material (obtained by disruption of the whole yeast with hot dilute sodium hydroxide) with 0·5N acetic acid (Bell and Northcote, *J.*, 1950, 1944; Northcote and Horne, *Biochem. J.*, 1952, **51**, 232), and was purified by several precipitations with 80% acetic acid (cf. Bell and Young, *Biochem. J.*, 1934, **28**, 882) and with ethanol. The purified glycogen gave an opalescent solution in water ($[\alpha]_D$ +198°), which was stained reddish-brown by iodine. The iodine binding power of the glycogen was quantitatively determined by Mr. D. M. W. Anderson, using the potentiometric titration method described by Anderson and Greenwood (*Chem. and Ind.*, 1953, 642); it was similar to that of mammalian glycogens. Acid hydrolysis of the glycogen gave glucose (96%) and no other reducing sugar. The glycogen was readily attacked by salivary α-amylase, as shown by the rapid loss of iodine staining power, and the production of glucose, maltose, and α-dextrins; the glucosidic linkages in the glycogen are therefore predominantly of the α-1 : 4 type. Since maltulose was absent from the α-amylolytic digest, fructose is not a constituent of the glycogen (cf. Peat, Roberts, and Whelan, *Biochem. J.*, 1952, **51**, xvii). Oxidation of the glycogen by potassium periodate (cf. Halsall, Hirst, and Jones, *J.*, 1947, 1399; Bell and Manners, *J.*, 1952, 3641) and determination of the maximum amount of formic acid produced indicated a unit-chain length of thirteen glucose residues. In an attempt to detect 1 : 2 or 1 : 3 linkages, periodate-oxidised glycogen was hydrolysed with acid, and the hydrolysate analysed for glucose (cf. Hirst, Jones, and Roudier, *J.*, 1948, 1779; Bell and Manners, *J.*, 1954, 1891). Paper chromatography showed that glucose was absent; the glycogen must therefore contain only α-1 : 4 and 1 : 6 glucosidic linkages. Treatment of the glycogen with β-amylase gave 44% conversion into maltose, indicating that the exterior chains comprise 8 glucose residues; the interior chains, on the average, thus contain 4 glucose residues. Treatment of the glycogen with muscle phosphorylase in the presence of excess of inorganic phosphate

* Part II, *J.*, 1954, 3527.

resulted in a 23% conversion into glucose 1-phosphate; 12-unit and 13-unit glycogens from various animal tissues have phosphorolysis limits of 20—25% (Liddle and Manners, unpublished). Examination of the glycogen in an ultracentrifuge, by Dr. C. T. Greenwood, showed it to be multimolecular, the sedimentation constant (S_{20}) being 52×10^{-13} c.g.s. units [equivalent to a molecular weight of *ca.* 2×10^6, the diffusion constant being assumed to be of the same order as that of other glycogens (cf. Bell, Gutfreund, Cecil, and Ogston, *Biochem. J.*, 1948, **42**, 405)]. An estimate of the shape of the molecule, and the degree of multiple branching in the molecule will be described in a later communication.

The available data indicate that brewer's yeast glycogen consists of *ca.* 10^3 unit-chains, each comprising, on the average, 13 α-1 : 4-linked glucose residues, and randomly interlinked by 1 : 6-glucosidic linkages. The molecular structure thus resembles that of the majority of the glycogens from mammalian, invertebrate, and protozoan tissues examined in our previous studies (Bell and Manners, *loc. cit.*; Manners and Ryley, *Biochem. J.*, 1952, **52**, 480); nevertheless, small but significant differences in degree and position of branching between the baker's yeast and the brewer's yeast glycogen are revealed (see Table).

A comparison of the properties of yeast glycogens with rabbit-liver glycogen.

Source :	Baker's yeast		Brewer's yeast	Rabbit-liver
	A	*B*		*C*
$[\alpha]_D$ in water	+184°	+187°	+198°	+196°
Unit-chain length	11—12	—	13	12—13
β-Amylolysis limit	50	46—48·5	44	43
Exterior chain length *	8	—	8	8
Interior chain length †	2—3	—	4	3—4

A, Data from Northcote (*loc. cit.*). *B*, Data from Jeanloz (*loc. cit.*). *C*, Data from Bell and Manners (*loc. cit.*).

* No. of glucose units removed on β-amylolysis plus 2·5. † Unit-chain length — Exterior chain length — 1.

In our previous papers (Bell and Manners, *loc. cit.*; Manners and Ryley, *loc. cit.*) exterior chain lengths were calculated on the assumption that the exterior " stubs " of β-limit dextrins contained 1·5 glucose residues (cf. Meyer, *Adv. Enzymology*, 1943, **3**, 109); Peat, Whelan, and Thomas (*J.*, 1952, 4546), however, have shown that a proportion of the " stubs " in a β-dextrin from waxy-maize starch contain two or three glucose residues. A mean of these latter figures has been used in the present calculation.

In view of the different conditions of growth and fermenting properties of baker's and brewer's yeast, and hence, enzymatic composition, it is not unexpected that the glycogens show small differences in molecular structure; the unit-chain length and the position of branching in the chains depend upon the " balance " between the activities of phosphorylase and the branching and debranching enzymes, *i.e.*, on the metabolic condition of the organism at the time of isolation of the glycogen.

Experimental

Analytical Methods.—(a) *Determination of reducing sugar.* Reducing sugars were determined by use of the Shaffer–Somogyi reagent 60 (*J. Biol. Chem.*, 1933, **100**, 695) as modified by Hanes and Cattle (*Proc. Roy. Soc.*, 1938, *B*, **125**, 387) or by the Somogyi reagent (*J. Biol. Chem.*, 1945, **160**, 61) which had been calibrated against glucose and maltose.

(b) *Paper chromatography.* Descending chromatograms were carried out at room temperature with Whatman No. 1 paper and benzene–pyridine–butanol–water (1 : 3 : 5 : 3) as solvent. Development was by spraying with aniline oxalate or with a silver nitrate–sodium hydroxide reagent (Trevelyan, Procter, and Harrison, *Nature*, 1950, **166**, 444).

(c) *Iodine stain.* Polysaccharide solution (2 ml.) was added to iodine solution (1 ml. containing 1 mg. of iodine and 10 mg. of potassium iodide per ml.) and water (2 ml.), and the absorption value of the polysaccharide–iodine complex measured on a Spekker Photoelectric Absorptiometer (1 cm. cells), an Ilford filter No. 603 being used, against an iodine blank.

(d) *Determination of glucose 1-phosphate.* Glucose 1-phosphate was determined by a slight modification of Allen's method (*Biochem. J.*, 1940, **34**, 858).

Preparation of Glycogen.—A dispersion of washed brewer's yeast (1·5 kg.) in 3% sodium hydroxide (1 l.) was heated at 95° for 6 hr. (cf. Northcote and Horne, *loc. cit.*). After the mixture had cooled, the cell-wall material was collected on the centrifuge and treated again with hot 3% sodium hydroxide. (The sodium hydroxide extracts did not contain any appreciable

amount of glycogen.) The cell-wall material was extracted by three successive treatments with 0·5N-acetic acid (each 500 ml.) at 75° for 2 hr. The combined acetic acid extracts were concentrated under reduced pressure to about 500 ml., and ethanol (6 vols.) was added. The crude precipitate of glycogen was purified by three precipitations from 80% acetic acid (Bell and Young, *loc. cit.*) and finally from ethanol. The yield was 17·7 g.

Properties of the Glycogen.—The glycogen had $[\alpha]_D^{18}$ +198° (*c*, 0·25 in H_2O); +175° (*ca.* 0·50 in N-NaOH) (Found: N, 0·05%; P, nil; Ash, 0·10%). An aqueous solution was opalescent, and was stained red-brown with iodine. Hydrolysis by 1·5N-sulphuric acid at 100° for 2 hr. gave glucose and no other sugar (paper chromatography). The glycogen had a glucose content of 96%, determined by quantitative acid hydrolysis (Pirt and Whelan, *J. Sci. Food Agric.*, 1951, **2**, 224).

Salivary α-Amylolysis of the Glycogen.—Salivary α-amylase solution was prepared by dissolving freeze-dried human saliva in distilled water, and removing insoluble material by centrifugation. The amylase solution showed no maltase activity, but was contaminated with maltotriase. During the digestion of waxy-maize starch, maltose, maltotriose, and α-dextrins were the initial products of the reaction, and glucose, maltose, and α-dextrins the end-products (cf. Whelan and Roberts, *Nature*, 1952, **170**, 748; *J.*, 1953, 1298).

An enzymic digest was set up containing glycogen (50·0 mg.), phosphate–citrate buffer (0·16M with respect to phosphate) of pH 7·0 (20 ml.), sodium chloride (25·0 mg.), salivary amylase solution (1 ml.), and water (29 ml.). Aliquot portions (2 ml.) were analysed at intervals (Shaffer–Somogyi reagent 60), after incubation at 35°.

The decrease in iodine-staining power was as follows:

Time of incubation (min.)	0	4	10	20
Absorption value	0·305	0·035	0·020	0·010

The apparent percentage conversion into maltose after 1, 2, and 48 hours' incubation was 62, 68, and 95, respectively.

Paper chromatography showed the presence of glucose ($R_G = 1$), maltose ($R_G = 0·55$), and a series of sugars of higher molecular weight ($R_G < 0·09$) in the digest. Maltulose was absent.

Potassium Periodate Oxidation of the Glycogen.—Glycogen (549·0 mg.) was dissolved in 5% potassium chloride solution (100 ml.); 10 ml. were withdrawn for a control determination. 8% (w/v) sodium metaperiodate (20 ml.) was added to the bulk, from which 10-ml. portions were withdrawn at intervals for determination of formic acid by titration in a carbon dioxide-free atmosphere against 0·01N-sodium hydroxide, methyl-red being used as indicator (cf. Halsall, Hirst, and Jones, *loc. cit.*; Bell and Manners, *loc. cit.*). The following results were obtained.

Time (hr.)	96	168	266	386
Total formic acid produced (mg.)	8·9	10·2	10·6	10·5
Apparent chain length * (glucose residues) ...	15·6	13·6	13·2	13·2

* Calculated from the weight of glycogen oxidised.

A 12-unit or 14-unit glycogen would yield 11·6 or 10·0 mg. of formic acid, respectively.

The remaining solution of periodate-oxidised glycogen was neutralised with ethylene glycol (5 ml.) and dialysed against running tap water for 36 hr., and the non-diffusible material collected by freeze-drying. 50 mg. of this were hydrolysed by 2N-sulphuric acid (2 ml.) at 100° for 3 hr. No glucose could be detected in the hydrolysate by paper chromatography; brewer's yeast glycogen does not therefore contain 1 : 2- or 1 : 3-glucosidic linkages.

β-Amylolysis of the Glycogen.—β-Amylase was prepared from soya beans by Bourne, Macey, and Peat's method (*J.*, 1945, 882). A solution of β-amylase was prepared by dissolving soya bean β-amylase (50 mg.) in 0·2M-acetate buffer (pH 4·6; 20 ml.), and removing insoluble material by centrifugation; the supernatant liquid had an activity of 125 units/ml., estimated by Hobson, Whelan, and Peat's method (*J.*, 1950, 3566). Control experiments with maltose and starch showed it to be free from maltase and α-amylase. Glycogen (48·4 mg.) was incubated with 0·2M-acetate buffer (pH 4·6; 6 ml.), water (21 ml.), and β-amylase solution (3 ml.; 375 units) at 37°. Samples (3 ml.) were withdrawn at intervals and analysed for maltose. The course of degradation was as follows:

Time of incubation (hr.)	1	2	20	44
% Conversion into maltose	30·7	38·4	43·5	44·0

In a duplicate experiment with 50·4 mg. of glycogen, the β-amylolysis limit was 43·8%.

Phosphorolysis of the Glycogen [with A. MARGARET LIDDLE].—Glycogen (52·4 mg.) was incubated at 35° with 0·5M-phosphate buffer (pH 6·8; 2·0 ml.), adenylic acid (1 mg.), muscle

phosphorylase solution (0·20 ml.), and water to a total volume of 10 ml. Crystalline rabb muscle phosphorylase was prepared by Green and Cori's method (*J. Biol. Chem.*, 1943, **151**, 2

Time of incubation (hr.)	5	24	48
% Conversion into glucose 1-phosphate	20·0	23·2	23·2

In a duplicate experiment with 53·4 mg. of glycogen, the phosphorolysis limit was 22·8%

The authors are indebted to Professor E. L. Hirst, F.R.S., for his interest and advice, to D C. T. Greenwood for determination of the sedimentation constant of the glycogen, and to M D. M. W. Anderson for the potentiometric titration. They also thank the Government of t Union of Burma for the award of a State Scholarship (to K. M.) and Messrs. Wm. Younger a Company Ltd. for supplies of yeast.

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553. α-1 : 4-*Glucosans. Part IV.* A Re-examination of the Molecular Structure of Floridean Starch.*

By I. D. Fleming, E. L. Hirst, and D. J. Manners.

The structure of Floridean starch from *Dilsea edulis* has been re-investigated. The available evidence, from periodate oxidation and enzymic degradation studies, shows that it resembles the amylopectin–glycogen class of polysaccharides. It is hydrolysed by β-amylase and does not contain 1 : 3-glucosidic linkages (cf. Barry, Halsall, Hirst, and Jones, *J.*, 1949, 1468).

The molecular structure of Floridean starch, an iodophilic glucose polysaccharide present in various red algæ, has been the subject of several investigations. Early studies by Colin *et al.*[1] showed that the glucosan was stained violet with iodine, and was dextrorotatory, and Kylin [2] found that it was degraded by a dialysed malt extract to maltose. Barry, Halsall, Hirst, and Jones [3] examined a specimen of Floridean starch (referred to below as sample I) isolated from *Dilsea edulis*; it was a glucosan, $[\alpha]_D$ +156° in H_2O, which resisted attack by crystalline sweet-potato β-amylase. On oxidation with potassium metaperiodate, one mol. of formic acid was liberated per 18 glucose residues; the observed uptake was 0·6 mol. of periodate per glucose residue, suggesting that 40% of 1 : 3-glucosidic linkages were present. A second sample of Floridean starch, also from *Dilsea*, was investigated by O'Colla.[4] This material, which was contaminated with galactan (18%), had $[\alpha]_D$ +166° in H_2O, gave 50% of maltose on treatment with wheat β-amylase, and on periodate oxidation an uptake of 0·77 mol. per glucose residue was observed: one mol. of formic acid was released per 12 glucose residues. The material was partially methylated (OMe 28·2%) and, on hydrolysis, 2 : 3 : 4 : 6-tetra- (3·3%) and 2 : 3 : 6-tri-*O*-methylglucose (42%) were obtained. A third sample of Floridean starch, free from galactan, was subjected to periodate oxidation by Barry, McCormick, and Mitchell; [5] the observed periodate uptake was 0·97 mol. per glucose residue, and this figure was confirmed by analysis of the thiosemicarbazide and isoniazid derivatives of the periodate-oxidised Floridean starch. Further, an acid hydrolysate of the latter did not contain glucose (cf. Hirst, Jones, and Roudier [6]). This sample could not, therefore, contain an appreciable proportion of 1 : 3-glucosidic linkages.

The present communication is concerned with a re-investigation of sample I, and the results of a preliminary examination of two further samples of Floridean starch, which has been carried out in an attempt to resolve the discrepancies in the above investigations.

Paper chromatography of an acid hydrolysate of sample I showed the presence of glucose and a barely detectable trace of galactose. The latter is not significant since the traces of contaminating galactan can be removed on further purification. The polysaccharide failed to react with the acid resorcinol (ketose) reagent, or with the naphtharesorcinol (uronic acid) reagent. By cuprimetric titration, the reducing sugar content was 93%; the remaining constituents are inorganic material and protein, which have no structural significance. In the following section, analytical figures have been corrected for the presence of 7% of non-carbohydrate material in the sample; the small destruction of glucose (*ca.* 1—2%) during acid hydrolysis has been neglected.

An aqueous solution of sample I had $[\alpha]_D$ +163° (corr. as pure glucosan, +176°), and was stained reddish-brown with iodine; the iodine complex gave an absorption spectrum with a maximum at 500 mμ. On incubation with salivary α-amylase, a rapid decrease in iodine-staining power was observed. The apparent percentage conversion into maltose (R_m) after enzyme action had ceased was 65, the main end-product being a reducing sugar with the same paper chromatographic mobility as maltose. Under identical conditions, the R_m values for waxy maize starch and rabbit-liver glycogen were 88 and 70 respectively.

* Part III, *J.*, 1955, 867.

A solution of crystalline sweet-potato β-amylase rapidly degraded sample I, giving 46% conversion into maltose, whilst an amorphous preparation of β-amylase gave an R_m value of 44—45%. The latter β-amylase, from soya beans, contained the Z-factor [7] (a mixture of a group-specific β-glucosidase, an endo-β-1 : 4-glucosanase, and an endo-β-1 : 3-glucosanase [8]). The barriers to β-amylase in sample I are hydrolysed by yeast *iso*amylase [9] (which hydrolyses the α-1 : 6-glucosidic interchain linkages in glycogen and amylopectin), since after incubation with *iso*amylase, a 9% increase in β-amylolysis limit was observed. Sample I is also degraded by potato phosphorylase; in the presence of inorganic phosphate, 35% of the polysaccharide was degraded, yielding glucose 1-phosphate. Oxidation of sample I by sodium metaperiodate at 1° resulted in the consumption of 1·05 mol. of periodate per glucose residue; under similar conditions, waxy maize starch consumed the normal amount of periodate (1·04 mol. per glucose residue). Determination of the maximum amount of formic acid liberated by potassium metaperiodate indicated an average chain length of nine glucose residues.

The above properties suggest, in general, that sample I belongs to the amylopectin-glycogen class of polysaccharides (see Table). Thus, enzymic degradation shows that the main repeating linkage is of the α-1 : 4-glucosidic type, and that the molecule is branched; further, the branch points are not adjacent to the non-reducing terminal groups. Since the iodine complex showed little absorption at 620 mμ, the presence of a linear amylose-type component in the sample is excluded. The extent of periodate oxidation indicates the absence of any appreciable proportion of 1 : 3-glucosidic linkages; it now seems probable that the earlier [3,4] periodate uptake values of 0·6 and 0·77 mol. per anhydroglucose unit resulted from incomplete oxidation of the Floridean starch. In the earlier experiments, an oxidation period (with potassium metaperiodate) of only 8 days was used, whereas it has been found that at least 12 days are required for the complete oxidation of normal glycogens and starches.[10,11] Under such conditions, the production of formic acid follows the periodate uptake.[11] The increased yield of formic acid, and hence the decrease in average chain length reported in the present study, is in accord with this view.

The specific rotation (+176°) of sample I, which is slightly lower than that of glycogens [12] (184° to 201°), is an unusual property, for which no explanation is yet available. The presence of β-glucosidic linkages has been considered; however, since β-glucosidases have no effect on the β-amylolysis limit, the barriers to β-amylase action cannot be β-glucosidic linkages. The possibility that sample I is contaminated with a small proportion of a β-glucosan, or contains a small number of β-glucosidic linkages in the interior parts of the molecule, is being investigated.

A preliminary investigation of two additional samples of Floridean starch has been carried out. Sample II was kindly provided by Dr. V. C. Barry, and sample III by Dr. A. G. Ross. Both contained small amounts of a contaminating galactan, and the analytical data are therefore recorded only in terms of the weights of material analysed. The presence of this galactan does not affect the mode of action of the several enzymes used in this study and, hence, the general conclusions drawn from their use.

Floridean starches II and III, which did not react with the resorcinol reagent, formed iodine complexes with absorption spectra showing a single maximum at 525 mμ. The samples were rapidly attacked by the following enzymes: (*a*) salivary α-amylase (R_m *ca.* 57); (*b*) crystalline sweet-potato β-amylase, yielding *ca.* 33% of maltose; (*c*) amorphous soya-bean β-amylase, giving *ca.* 37% of maltose; and (*d*) potato phosphorylase, with a 27% conversion into glucose 1-phosphate. These observations, which represent mean values for samples II and III, show the presence of branched chains of α-1 : 4-linked glucose residues in the molecules. The barriers to β-amylase in sample II appear to be similar to those in glycogen; after incubation with yeast *iso*amylase, the β-amylolysis limit was increased to 57%. There is no evidence for the presence of β-glucosidic linkages in the outer chains * of the Floridean starches; emulsin has no action on these samples. After oxidation of the samples with sodium metaperiodate, the observed uptakes were 0·96 and 0·86 mol. of periodate per anhydrohexose unit, whilst the formic acid liberation during potassium metaperiodate oxidation corresponded to average chain lengths of 12 and

* *I.e.*, those parts of a chain between the branch point and the non-reducing terminal group.

13 glucose residues respectively. If a correction is applied for the contaminating galactan, the chain length values would be even smaller.

The three samples of Floridean starch appear to have similar molecular structures; they are branched α-1 : 4-glucosans, are partially hydrolysed by β-amylase, and do not contain appreciable proportions of 1 : 3-glucosidic linkages. In these properties, they resemble the glucosans which have been isolated [13] from other algæ, *e.g.*, Odonthalia and *Ulva expansa*.

Comparison of the properties of glycogen, Floridean starch, and amylopectin.

Property	Glycogen [12]	Floridean starch	Amylopectin [12]
$[\alpha]_D$ in H_2O	+196°	+176°	+212°
Iodine coloration	Reddish-brown	Deep reddish-brown	Purple
$\lambda_{max.}$ (mμ) of absorption spectrum of iodine complex	460	500	540
β-Amylolysis limit [a]	45	46	54
Potato-phosphorolysis limit [b]	—	35	41
α-Amylolysis limit [a]	70	65	88
Periodate uptake (mols. per anhydroglucose unit)	1·08	1·05	1·04
Average chain length (glucose residues)	12	9	20

[a] Percentage conversion into maltose. [b] Percentage conversion into glucose 1-phosphate.

Experimental

Analytical Methods.—(a) *Reducing-sugar determination.* Somogyi's improved method [14] was used.

(b) *Nitrogen.* Total nitrogen was determined by Johnson's method,[15] the reagents being calibrated by use of a mixture of soluble starch and lysozyme.

(c) *Iodine absorption spectra.* Polysaccharide (2·5 mg.) and iodine solution (0·2% of iodine in 2% aqueous potassium iodide; 2·5 ml.) in a total volume of 25 ml. were mixed. The absorption spectrum in the range 400—700 mμ was then determined using a Unicam SP. 600 Spectrophotometer (1 cm. cells; iodine–water blank).

The other methods used were those described in Part III of this series.

Enzymic Reactions.—Except as stated, the enzyme preparations have been described in Part III and earlier papers.[10,12] Enzymic reactions were carried out at 35° (with the exception of the digests containing *iso*amylase), in the presence of toluene. Acetate buffer pH 4·6 and phosphate–citrate buffer pH 7·0 were used for β- and α-amylolysis, respectively.

Properties of the Floridean Starches.—(a) *Sample I.* Hydrolysis with 1·5N-sulphuric acid at 100° for 2 hr. gave glucose and a trace of galactose (paper chromatography). By quantitative acid hydrolysis, sample I had a reducing sugar content of 92·7%. No reaction was observed with the acid resorcinol or naphtharesorcinol reagent; ketoses and uronic acids were therefore absent. Sample I had $[\alpha]_D^{15}$ +163° (*c* 1·30 in H_2O) (Found : N, 0·92%, equiv. to 5·7% of protein). Corrected for the presence of this protein and 0·8% of ash, the glucosan has $[\alpha]_D$ +176°. Sample I had a very slight reducing power towards the Somogyi reagent, equivalent to D.P. 67; no evidence of molecular size can be deduced from this figure. An aqueous solution of sample I showed no appreciable opalescence; the molecular weight is therefore much lower than that of glycogen (~10^6).

(b) *Samples II and III.* Qualitative acid hydrolysis showed the presence of glucose and a small proportion of galactose. The samples did not react with the acid resorcinol reagent, and the reducing-sugar contents by cuprimetric titration, as glucose, were 87·9 and 84·1% respectively. Aqueous solutions of the starches were unsuitable for polarimetric observation. Sample II had an apparent D.P. of 86 (Somogyi reagent) (Found : N, 0·34%). Sample III was also analysed (Found : N, 0·53%).

Degradation by Salivary α-Amylase.—Enzymic digests were set up containing Floridean starch (*ca.* 20 mg.), buffer (1 ml.), water (24 ml.), and maltase-free salivary α-amylase solution (5 ml.). Aliquot portions (3 ml.) were analysed, at intervals, for reducing sugar (as maltose). After 48 hours' incubation, the apparent percentage conversions into maltose (R_m) were: sample I, 65; II, 56; III, 58. In similar experiments with rabbit-liver glycogen (20·7 mg.) and waxy maize starch (25·6 mg.) the R_m values were 70 and 88 respectively.

Qualitatively, salivary α-amylase–Floridean starch digests become achroic within a few minutes. Paper chromatography of these digests showed the presence of maltose (intense

spot) together with smaller amounts of glucose (R_G 1·0) and a series of higher saccharides of low chromatographic mobility ($R_G \leqslant 0{\cdot}17$).

Action of Crystalline Sweet-potato β-Amylase on Floridean Starches.—Sample I (15·5 mg., ≡14·4 mg. of glucosan) was incubated with buffer (4 ml.), water (20 ml.), and β-amylase solution (1 ml.). Samples were analysed, at intervals, for maltose. After 22 and 48 hr., 46% conversion into maltose was obtained.

Sample II (45·0 mg.) was added to buffer (10 ml.), water (38 ml.), and β-amylase solution (2 ml.). The percentage conversion into maltose was 38 and 37 respectively, after 24 and 48 hr. A portion (7 ml.) of the digest was added to a solution of sample II (16·7 mg.; 3 ml.) and incubated for a further 48 hr. At this time, 37% of the newly added sample II had been hydrolysed to maltose. A second portion (7 ml.) of the main digest was added to a solution of sample III (23·1 mg.; 3 ml.). After incubation for a further 48 hr., 28% conversion into maltose was observed.

It follows, therefore, that all three samples of Floridean starch are susceptible to hydrolysis by crystalline sweet-potato β-amylase.

Action of Amorphous Preparation of β-Amylase on Floridean Starches.—A solution of β-amylase was prepared by dissolving soya-bean β-amylase (50 mg.) in 0·2M-acetate buffer (pH 4·6; 20 ml.), and removing insoluble material by centrifugation; the supernatant liquid had an activity [16] of 196 units/ml. Enzyme digests containing Floridean starch (*ca.* 20 mg.), β-amylase (980 units; 5 ml.), and water (20 ml.) were set up. Samples (3 ml.) were analysed, at intervals, for maltose, with the following results:

	β-Amylolysis limit		
Time of incubation (hr.)	4	24	48
Sample I (*a*)	—	43	44
(*b*)	—	44	45
Sample II	36	37	—
Sample III	35	37	—

Paper chromatography of β-amylase–Floridean starch digests showed that maltose was the sole reducing sugar present. Control experiments showed that the β-amylase had no maltase activity, and slowly hydrolysed salicin and laminarin.

The Floridean starches were also hydrolysed by an amorphous preparation of β-amylase from barley, yielding 30—40% of maltose.

Action of iso*Amylase and β-Amylase on Floridean Starches.*—*iso*Amylase [9] was isolated from brewer's yeast by Manners and Khin Maung. Sample I (10·0 mg.), 0·2M-acetate buffer (pH 5·89; 1 ml.), water (4·5 ml.), and *iso*amylase (40 mg. per ml.; 0·5 ml.) were incubated at 18° for 24 hr. The enzyme was then inactivated by heating the digest to 100° for 2—3 min. Barley β-amylase (500 units; 1 ml.) was added. After 24 hours' incubation at 35°, a 3 ml. sample was withdrawn, and after deproteinisation (zinc sulphate–barium hydroxide) analysed for maltose. The β-amylolysis limit was 54%. In a similar experiment with sample II, the β-amylolysis limit of the *iso*amylase-treated starch was 54% after 24 hours' incubation with β-amylase, and 57% after 48 hours'.

Phosphorolysis of Floridean Starches [with A. MARGARET LIDDLE].—Digests were prepared containing Floridean starch I, II, III (*ca.* 25 mg.), potato phosphorylase (100 mg., 6 Green and Stumpf [17] units), 0·5M-phosphate buffer (pH 6·8; 2 ml.), and water to 10 ml. Aliquot portions (2 ml.) were analysed at intervals for glucose 1-phosphate, as described previously:

	Conversion (%) into glucose 1-phosphate.		
Time of incubation (hr.)	10	24	40
Sample I	—	33	35
Sample II	23	24	—
Sample III	—	27	28

In a control experiment with waxy maize starch, a phosphorolysis limit of 41% was obtained. Similar limits have been obtained by other workers.[7]

Action of Emulsin on the Floridean Starches.—Digests were set up containing samples II or III (49·8 or 46·8 mg.), 0·2M-acetate buffer (pH 5·0; 3·5 ml.), 0·01M-mercuric chloride (0·5 ml.), distilled water (18·0 ml.), and emulsin solution (40 mg./ml.; 3·0 ml.). Aliquot portions (3 ml.) were analysed, after deproteinisation (zinc sulphate–barium hydroxide), for reducing sugar. No significant increase in reducing power occurred within 48 hr. In control experiments, the emulsin rapidly attacked laminarin and salicin, and had no action on soluble starch.

Sodium Metaperiodate Oxidation of Floridean Starches.—Sample I (96·8 mg., ≡90·0 mg. of glucosan) was oxidised with sodium metaperiodate (3·2% w/v; 10 ml.) at 1°. Samples (2 ml.) were removed at intervals, and the periodate uptake determined by Fleury and Lange's method.[18] After 72 and 168 hr., 0·93 and 1·05 mol. respectively of periodate were consumed per anhydroglucose unit.

Sample II [(*a*) 152·7 and (*b*) 152·0 mg.] were oxidised under similar conditions. The periodate consumption, after 96 and 192 hr., was (*a*) 0·90 and 0·98 and (*b*) 0·95 and 0·94 mol. per anhydrohexose unit.

Sample III [(*a*) 147·4 and (*b*) 151·0 mg.] were oxidised as above. After 90 and 216 hr., the periodate uptakes were (*a*) 0·82 and 0·87 and (*b*) 0·83 and 0·85 mol. per anhydrohexose unit.

If reducing-sugar contents of 88 and 84% are assumed for samples II and III, respectively, these figures are equivalent to periodate uptakes of 1·03 and 1·02 mol. per anhydrohexose unit.

In a control experiment waxy maize starch (211·5 mg.) was oxidised at 1° with sodium metaperiodate (4·0% w/v; 20 ml.). After 50 and 120 hr., 1·03 and 1·04 mol. of periodate were consumed per anhydroglucose unit.

Control titrations of arsenite in the presence of Floridean starches against iodine showed that the impurities present (galactan and protein) did not interfere with the titration, and hence with measurement of the periodate uptake.

Potassium Metaperiodate Oxidation of Floridean Starches.—Floridean starch (55—100 mg.) was dissolved in 3% potassium chloride solution (40 ml.) and the pH adjusted to 5·8 (glass electrode) by the addition of sodium hydroxide. 4% w/v Sodium metaperiodate solution (10 ml.) was added; 10 ml. portions were withdrawn at intervals for determination of formic acid.[10] A mixture of potassium chloride and sodium metaperiodate was analysed similarly. The following results were obtained :

Sample	I	II	III
Weight oxidised (mg.)	90·9	98·8	55·8
Total formic acid produced (mg.)	2·80	2·43	1·22
Apparent chain length (glucose residues)	9·2	11·6	13·0

The formic acid release was complete after 210 hours' oxidation at room temperature (15—17°); similar titres were obtained after 260 hr.

The oxidised sample I was isolated by freeze-drying (after decomposition of excess of periodate with ethylene glycol, and dialysis), and hydrolysed with acid. Paper chromatography of the neutralised and de-ionised hydrolysate showed the presence of galactose (moderate spot) and glucose (faint spot). The amount of glucose is less than that of the insignificant quantity of galactose present in the original acid hydrolysate (p.) and represents less than 1% of the original molecule.

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423. α-1 : 4-*Glucosans. Part V.* End-group Assay of Glycogens by Periodate Oxidation, and the Oxidation of Maltose by Sodium Metaperiodate.*

By D. J. Manners and (in part) A. R. Archibald.

Fifteen samples of glycogen have been assayed by potassium metaperiodate oxidation, and with only two exceptions, average chain lengths of 10—14 glucose residues were obtained.

Oxidation of glycogen and of maltose, dissolved in sodium chloride, by sodium metaperiodate for 25 hr. at 2° (Potter and Hassid [1]) has been studied. Under these conditions, oxidation is incomplete.

Periodate oxidations have been widely used in structural investigations of polysaccharides, in particular, of starches, glycogens, and dextrans. By estimating the formic acid produced during oxidation, the proportion of triol groups in the polysaccharide can be assessed and, in starches and glycogens, the ratio of non-terminal to non-reducing terminal glucose residues (*i.e.*, average chain length, $\overline{CL}$) determined.[2] Further, examination of an acid hydrolysate of a periodate-oxidised glucosan enables 1 : 2- or 1 : 3-glucosidic linkages to be detected.[3] In the present investigation, the average chain lengths of several samples of glycogen have been determined by (*a*) potassium metaperiodate oxidation at room temperature, and (*b*) sodium metaperiodate oxidation at 2°. A preliminary account of part of this work has been published.[4]

Table 1. *End-group assay of glycogens by oxidation with potassium periodate.*

Source of glycogen [a]	Method of isoln.[b]	Method of purifn.[c]	$[\alpha]_D$ (in H_2O)	$\overline{CL}$	Source of glycogen [a]	Method of isoln.[b]	Method of purifn.[c]	$[\alpha]_D$ (in H_2O)	$\overline{CL}$
Cat liver IV	P	A	—	13	Rabbit liver III...	W	A	+196°	13
Cat liver VI	P	A	—	12	Rabbit liver IV	W	A	—	13
Fœtal pig liver ...	W	A	+191°	11	Rabbit liver V ...	W	A	+196	12
Helix pomatia I ...	P	A	+192	10	Rabbit liver X ...	C	E	+193	12
Helix pomatia II	P	A	+182	7	*Tetrahymena pyriformis* II	P	A	+195	14
Mytilus edulis IV	W	PA	+196	12	*Trichomonas gallinae* II	P	A	+200	13
Mytilus edulis V...	W	A	—	9					
Mytilus edulis VI	C	A	+195	13					
Rabbit liver II ...	P	A	+198	12					

[a] Roman numerals refer to different samples from the same biological source. [b] P = Pflüger method; W = hot-water extraction; C = commercial preparations. [c] A = acetic acid precipitation (Bell and Young, *Biochem. J.*, 1934, **28**, 282); PA = deproteinisation with picric acid; E = electrodialysis.

Assay of glycogen by oxidation with potassium periodate was first made by Halsall, Hirst, and Jones,[5] $\overline{CL}$ values being calculated from the production of formic acid after 150 hours' oxidation. Later, Bell and Manners [6] found that samples of mammalian-muscle glycogen ($\overline{CL}$ 12, by methylation) had apparent chain lengths of 15—16 after 150 hours' oxidation. However, when oxidation was continued to maximum production of formic acid (after 300—400 hours' oxidation), $\overline{CL}$ values of 12 ± 1 were obtained. This apparent discrepancy was ascribed to variation in the oxidation rate with room temperature.[7] A similar effect was also noted by Carlquist,[8] who found that in 144 hr. at 15° or 21° a glycogen sample gave 8·4 or 9·5 moles of formic acid per 100 glucose residues. Accordingly, end-group assays have been carried out on 15 different samples of glycogen, and $\overline{CL}$ values, evaluated from the final constant concentrations of formic acid, are recorded in Table 1, together with the specific rotations and methods of preparation of the glycogens.

* Part IV, *J.*, 1956, 2831.

During isolation of glycogen by the Pflüger method (digestion of the tissues with hot 30% potassium hydroxide), there is no appreciable alkaline-degradation of the polysaccharide.[9] By methylation, the glycogens from rabbit liver III and X and *Helix pomatia* I had chain lengths of 12.[10-12] The latter type of glycogen therefore differs significantly in degree of branching from *Helix pomatia* II glycogen isolated in 1949; the presence of 7-unit chains in this has been confirmed. Fœtal pig liver glycogen, which has not previously been studied, resembles the majority of mammalian glycogens in degree of branching.

An alternative periodate method used by Potter and Hassid [1] involves oxidation at 2° of a solution of the polysaccharide in 1·5% sodium chloride with 1·5 mols. of sodium metaperiodate and determination of the formic acid produced after 25 hr. Chain lengths of 22—27 were reported for various amylopectins, although these results do not appear to have been confirmed by methylation assay of the *same* samples. Other end-group assays of glycogens and amylopectins by this method have been reported; [13-15] the $\overline{CL}$ values were greater than those obtained by other methods of assay of the same samples. In particular, the results differed from enzymic assays (using phosphorylase and amylo-1 : 6-glucosidase), as shown in Table 2.

TABLE 2. *Chain lengths of glycogens and amylopectins determined by periodate oxidation and enzymic methods.*

Sample	Sodium periodate oxidn.[a]	Enzymic assay	Ref.	Sample	Sodium periodate oxidn.[a]	Enzymic assay	Ref.
Rabbit liver glycogen	18	14·7	13	Potato amylopectin	27	21·8	13
Rabbit liver glycogen	22	15·9	16	Sago amylopectin ...	22	17	13
Rabbit liver glycogen	23	17·2	16	Wheat amylopectin...	23	18·5	13
Corn amylopectin ...	26	21·2	13				

[a] Procedure of Potter and Hassid.[1]

It appears that with Potter and Hassid's procedure periodate oxidation and, hence, production of formic acid, are incomplete after 25 hr.; it has therefore been applied to glycogens already assayed by potassium periodate. Six samples of glycogen (and one of amylopectin), in sodium chloride solution, were oxidised with sodium metaperiodate at 2°, and the concentration of formic acid was determined after 25 hr. (Table 3). Since the

TABLE 3. *End-group assays of glycogens by periodate oxidation.*

Sample	Potassium periodate oxidn.*	Sodium periodate oxidn.†		Sample	Potassium periodate oxidn.*	Sodium periodate oxidn.†	
		A	*B*			*A*	*B*
Ascaris lumbricoides	12	15—16	11	*Mytilus edulis* VI...	13	—	14
Cat liver VI	12	—	12	Rabbit liver I	13	—	13
Commercial ‡	—	—	12	Rabbit liver II ...	12	14	—
Helix pomatia II ...	7	9	—	Rabbit liver V ...	12	—	12
Human liver	6	8	—	Rabbit liver X.....	12	—	12
Human muscle ...	12	—	11	*Trichomonas fœtus*	15	18	15
Mytilus edulis V ...	9	13	—				

* See Table 1 and ref. 6. † *A*, Potter and Hassid's procedure; *B*, modified procedure.

‡ Purchased from British Drug Houses Ltd. [waxy maize starch had $\overline{CL}$ values of 18 and 22 by potassium and sodium periodate oxidation (method *A*) respectively].

$\overline{CL}$ values from the titres after 25 hr. were greater than those from potassium periodate assay, production of formic acid was incomplete. Six further quantitative experiments showed that only 80—90% of the theoretical periodate was reduced after 25 hr. Schlamowitz's report [14] that formic acid was completely liberated within 20—25 hr. could not be confirmed (see p.).

The period of 25 hr. for oxidation was chosen by Potter and Hassid [1] on the grounds that their " model " saccharide, maltose, yielded the theoretical 3 mols. of formic acid in this time. Their method is therefore based on the unproved assumption that oxidation of a disaccharide occurs at the same rate as that of a polysaccharide of molecular weight

$\sim 10^7$. We have noted, however, that different samples of glycogen are oxidised, under identical conditions, at slightly differing rates. We have re-examined the periodate oxidation of maltose in sodium chloride. In one experiment, after 25 hr. 2·3 mols. of formic acid were produced, and 4·7 mols. of periodate reduced. In additional experiments, maltose, in water or in 3% sodium chloride, was oxidised with varying amounts of sodium metaperiodate. After 25 hr. 1·7—2·6 mols. of formic acid were present, and after *ca.* 120 hr. 2·4—3·1 mols. Although strictly reproducible results could not be obtained in the presence of sodium chloride (see p.), release of formic acid and uptake of periodate were never theoretical after 25 hours' oxidation at 2°.

The formation and subsequent hydrolysis of a formyl ester were incidentally indicated, since production of the third mol. of formic acid continues in the *absence* of periodate. A mixture of maltose in sodium chloride and sodium metaperiodate was divided after 48 hr. at 2° when it contained 2·6 mols. of formic acid. One half was stored at 2° for 24 hr. : the formic acid content increased to 2·9 mols. To the other half ethylene glycol was added, to reduce the remaining periodate, and the formic acid determined immediately and at intervals thereafter. During 24 hr. the formic acid content increased slowly from 2·6 to 2·9 mols. In a similar oxidation by aqueous sodium metaperiodate, we found 2·4 mols. of formic acid released after 48 hours' oxidation, 2·7 mols. after neutralisation of periodate and storage at 2° for 24 hr., and 2·6 mols. after 72 hours' total oxidation. Aliphatic formyl esters are also slowly hydrolysed in presence of sodium metaperiodate at 2° without consumption of periodate. It is apparent that the periodate oxidation of maltose resembles that of lactose [17] and cellobiose,[18] and involves (*a*) an initial oxidation in which 4 mols. of periodate are reduced and 2 mols. of formic acid produced, and (*b*) a slower stage involving the release of a third mol. of formic acid by hydrolysis of a formyl ester.

The above results on the rate of production of formic acid have been confirmed by others. Morrison and his co-workers [19] found that maltose oxidised by Potter and Hassid's procedure gave 2·5 mols. of formic acid, whilst Potter and his collaborators [20] observed that at 3° the expected 3 mols. of formic acid were not produced after 9 days.

For the end-group assay of glycogens, Potter and Hassid's method has therefore been modified : oxidation in *aqueous* solution is continued for 7—10 days, and the maximum concentration of formic acid determined. With " model " compounds consisting of glycogens already assayed by potassium periodate, the oxidation (determined by periodate-uptake and formic acid production) was normally complete within 7 days. Typical results are reported in Table 3. We noted, however, that with certain mammalian liver glycogens formic acid is produced unexpectedly slowly; [21] the reasons for this are being investigated.

The present study provides further evidence that the *average* length of the chains in glycogens is normally *ca.* 12 glucose residues. We have now assayed some 30 samples of glycogen and, of these, 23 had $\overline{CL}$ values of 10—14. These results agree with those of Abdel-Akher and Smith [22] who for another 37 individual glycogens found average chain lengths of 10—14. This suggests that in most animal tissues the *relative* activity of phosphorylase and branching enzyme during glycogen synthesis is very similar. However, the activity of this enzyme system in different specimens of *Mytilus edulis* appears to vary since glycogens with $\overline{CL}$ values of *ca.* 5, 9, 12, 13, and 17 have been isolated (see Table 1 and ref. 6).

Experimental

Glycogen Samples.—We are indebted to Dr. J. S. D. Bacon for *Mytilus edulis* V glycogen, to Dr. G. D. Greville for the cat liver glycogens, to Dr. E. E. Percival for rabbit liver X glycogen and to Dr. J. F. Ryley for the protozoal glycogens (cf. ref. 23). *Helix pomatia* I glycogen was prepared by deacetylation of the corresponding acetate which was kindly provided by Dr. D. J. Bell. *Mytilus edulis* VI glycogen was purchased from L. Light and Co. Ltd. The remaining

samples were isolated and purified as indicated in Table 1. The rotations of 0·2—0·4% glycogen solutions were measured in 2 dm. polarimeter tubes.

Potassium Periodate Oxidation of Glycogens.—The method previously described [6] was used.

Sodium metaperiodate oxidations.

Analytical Methods.—Formic acid was determined, after neutralisation of periodate with ethylene glycol, by titration with 0·01N-sodium hydroxide in a stream of carbon dioxide-free air with (*a*) methyl-red as indicator, when repeating Potter and Hassid's experiments,[1] or (*b*) a glass electrode and pH meter to an end-point at pH 5·8. Periodate uptake was estimated by the methods of Barnebey [24] or Fleury and Lange.[25] Results are expressed as moles per mole of maltose or, for glycogens, moles per mole of anhydroglucose residue.

Oxidations in Presence of Sodium Chloride.—During oxidations of glycogen or maltose in 1·5% sodium chloride solution with sodium metaperiodate, reagent blanks were also analysed. On storage at 2°, these became acid (pH *ca.* 3), and periodate was precipitated. Production of formic acid is therefore calculated from sodium hydroxide titres after correction for the *initial* acidity of the reagent blanks. Further, the results are approximate since the sodium hydroxide titrations include not only release of formic acid by periodate oxidation, but also, and to an unknown extent, acidity due to the interaction of sodium chloride and metaperiodate. The precipitation of periodate, which occurred within 24—96 hr., prevented accurate measurement of periodate reduction.

(a) *Glycogens.* Glycogen (30—280 mg.) in 3% sodium chloride (5 ml.) was oxidised with 0·27—0·40M-sodium metaperiodate (5 ml.) at 2° for 25 hr. Ethylene glycol (neutral; 3 ml.) was added, and the mixture kept for 1 hr. at room temperature in the dark, before titration. A control of sodium chloride and metaperiodate was similarly analysed. The results are given in Table 3.

The rate of formic acid production was studied by treating rabbit liver IV glycogen (189·8 mg.) and *Mytilus edulis* I glycogen (110·0 mg.) in 3% sodium chloride (10 ml.) with 0·37M-sodium metaperiodate (10 ml.) at 2° :

Time of oxidn.	Apparent chain length (glucose residues)	
(hr.)	Rabbit liver IV glycogen	*Mytilus edulis* I glycogen
51	16·2	16·2
100	15·0	15·2
166	14·6	14·2
291	12·6	13·2

For measurement of periodate uptake, 50 mg. of glycogen were oxidised for 25 hr. The results were glycogen from *Ascaris lumbricoides*, 0·98 mole per anhydroglucose residue; cat liver IV, 0·89; cat liver VI, 0·94; horse muscle, 0·92; fœtal sheep liver, 0·95, and rabbit liver IV, 0·94. On complete oxidation, these glycogens reduce 1·08—1·09 mols. of periodate.

(b) *Maltose.* Maltose hydrate (223·4 mg.), dissolved in 3% sodium chloride (10 ml.), was oxidised with 0·37M-sodium metaperiodate (10 ml.; 6 mols.) at 2°. The results were :

Time of oxidn. (hr.)	2	4	25	48	72
Formic acid prodn. (mols.)	1·5	1·7	2·3	2·7	3·1

In a second experiment, anhydrous maltose (162·8 mg.) in 3% sodium chloride was treated with 0·27M-sodium metaperiodate (10 ml.; 6 mols.) at 2° :

Time of oxidn. (hr.)	0·5	1	2	22	25	96	145
Periodate uptake (mols.)	3·2	3·9	4·0	4·6	4·7	—	—
Formic acid prodn. (mols.)	1·1	1·2	1·4	2·3	2·3	2·7	3·3

Maltose was also oxidised with varying amounts of sodium metaperiodate (4·4—34·0 mols.); after 25 hr. 1·7—2·5 mols. of formic acid were released, and after 95 hr. 2·5—2·9 mols.

In the above experiments, free iodine was present after *ca.* 90 hr. showing that over-oxidation had occurred.

Oxidations in Absence of Sodium Chloride.—In these oxidations, the reagent control of aqueous sodium metaperiodate was stable on storage at 2°.

(a) *Maltose.* Maltose hydrate (220·4 mg.) in water (10 ml.) was oxidised with 0·37M-sodium metaperiodate (10 ml.; 6 mols.) at 2° with results as follows :

Time of oxidn. (hr.)	2	4	25	48	72
Periodate uptake (mols.)	3·6	—	4·2	4·2	4·4
Formic acid prodn. (mols.)	1·4	1·6	2·2	2·5	2·8

On repetition, 2·1 mols. of formic acid were produced, and 4·2 mols. of periodate consumed after 25 hr. Oxidation of maltose with 4·0 or 11·6 mols. of periodate gave the following results :

Time of oxidn. (hr.)	2	4	25	48	72
Formic acid prodn. (mols.) :					
(*a*) 4·0 mols. of oxidant	1·3	1·4	2·0	2·1	2·3
(*b*) 11·6 mols. of oxidant	1·8	1·9	2·6	2·9	3·0

In the above experiments, the " formic acid " production is slightly lower (*ca.* 0·2 mol. of apparent formic acid) than in those in presence of sodium chloride. The difference is attributed to the acidity developed during the interaction of sodium chloride and metaperiodate.

(b) *Glycogens.* *Trichomonas fœtus* glycogen [23] (106·4 mg.) in water (25 ml.) was oxidised with 0·37M-sodium metaperiodate (5 ml.) at 2°. Portions (5 ml.) were removed at intervals, ethylene glycol (1 ml.) was added, and the formic acid titrated, with the following results :

Time of oxidn. (days)	2	4	6	10
Formic acid prodn. (mg.)	1·66	1·81	1·93	2·00
Apparent chain length (glucose residues)	18·0	16·4	15·4	15·0

Under similar conditions, *Ascaris lumbricoides* glycogen (100 mg.) gave 2·26 mg. of formic acid after 3 days, and 2·35 mg. after 5 days; 0·99 and 1·05 mols. of periodate were reduced within 2 and 3 days, respectively.

For end-group assays, glycogen (*ca.* 100 mg.) in water (23 ml.) was oxidised with 0·4M-sodium metaperiodate (2 ml.) at 2°. Portions (5 ml.) were removed at intervals for determination of formic acid. $\overline{CL}$ values, calculated from the final formic acid concentration, are given in Table 3; 1·06—1·09 mols. of periodate were reduced during these oxidations.

Ascaris lumbricoides glycogen (102·5 mg.) and *Mytilus edulis* VI glycogen (95·8 mg.) were also oxidised at room temperature (15—17°). The final production of formic acid (after 8 days) was 2·54 and 2·13 mg. respectively, corresponding to average chain lengths of 11 and 13 glucose residues. During the oxidation of glycogens with a *limited* excess of periodate (*ca.* 30%), appreciable " over-oxidation " does not therefore occur.

Hydrolysis of Formyl Esters.—Mixtures of maltose (*a*) in sodium chloride and (*b*) in water with sodium metaperiodate were divided after 48 hours' oxidation at 2°. One-half of each solution was stored at 2° for a further 24 hr. Ethylene glycol was added to the remaining solutions, which were then titrated with sodium hydroxide (methyl-red). The neutralised solutions were stored at 2° for 24 hr., alkali being added at intervals to maintain the pH. Results were :

Time after redn. of periodate (hr.)	0	2	3	24
Formic acid prodn. (mols.) (*a*)	2·6	2·7	2·8	2·9
(*b*)	2·4	2·5	2·6	2·7

The normal oxidations resulted in the production of (*a*) 2·9 and (*b*) 2·6 mols. of formic acid after 24 hr.

At 2°, and in presence of 3% sodium chloride and 0·37M-sodium metaperiodate, ethyl and *n*-propyl formate were slowly hydrolysed. No periodate was consumed.

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682. α-1 : 4-*Glucosans. Part VI.* Further Studies on the Molecular Structures of Glycogens.*

By A. Margaret Liddle and D. J. Manners.

Twenty-five samples of glycogen, with average chain lengths ranging from 8 to 18 glucose residues, have been degraded by barley β-amylase. The percentage conversions into maltose were 46 ± 7 in all but two instances. *Cardium* glycogen and one sample of rabbit liver glycogen had unusually low β-amylolysis limits of 14 and 25% respectively. These results illustrate the variation in molecular structure which can exist in glycogens.

The highly branched nature of the interior of several glycogens has been confirmed by α-amylolysis.

The use of acetic acid in the purification of glycogen is discussed.

Our earlier studies [1,2] indicated differences in the degree and position of branching among (*a*) glycogens from different biological sources, and (*b*) different glycogen samples from the same source. These observations are extended in the present paper, which reports structural analyses of several additional glycogens by periodate oxidation, and by the use of β- and α-amylase. A preliminary account of part of this work has been published.[3]

Twenty-five samples of glycogen have been analysed. A number of these were kindly provided by Drs. D. J. Bell and G. R. Tristram and the determination of certain chain lengths ($\overline{CL}$) has already been reported.[4] The remainder were isolated by extraction of the tissues with hot water or 30% potassium hydroxide (Pflüger method) and purified by precipitation first from 80% acetic acid and then from ethanol.[5] The polysaccharides had glucose contents of 94—97%, specific rotations (in water) of 191° to 201°, and the glycogen–iodine complexes showed maximum absorption at 420—470 mμ. Average chain lengths were determined by measurement of the maximum amount of formic acid liberated by potassium metaperiodate oxidation at room temperature. This method of analysis gives reproducible results which are in agreement with enzymic or methylation analyses of the *same* samples.[2,4]

The glycogens were incubated with barley β-amylase, at pH 4·6 and 35°, and the percentage conversions into maltose determined (see Table 1). The enzyme preparation was free from maltase and α-amylase, but contained Z-enzyme [6] and showed slight hydrolytic activity towards maltotriose. The hydrolytic activities due to Z-enzyme and that towards maltotriose are not significant, since Z-enzyme does not affect the β-amylolysis of glycogens, and maltotriose is not a product of enzyme action.

Peat, Whelan, and Thomas [7] have shown that the side chains in an amylopectin β-limit dextrin contain 2 or 3 glucose residues, whilst Summer and French,[8] from a study of the action of β-amylase on model substrates, concluded that the exterior chain "stubs" in a glycogen or amylopectin β-limit dextrin "would never be any shorter than 2·5 glucose units." The exterior and interior chain lengths reported in Table 1 have therefore been calculated on the assumption that β-amylase action on glycogen ceased at the second or third glucose residue from the outermost branch points.

It has been suggested [10] that purification of glycogens by precipitation with acetic acid renders them less susceptible to degradation by the enzymes muscle phosphorylase and amylo-1 : 6-glucosidase. After treatment with hot alkali, the glycogens could then be completely degraded by these enzymes. Fœtal sheep liver glycogen, purified by the acetic acid method, has therefore been digested with 0·1N-sodium hydroxide at 100° for 30 min. This treatment did not increase the β-amylolysis limit. Further, four precipitations of rabbit liver or yeast glycogen from 80% acetic acid, followed by an ethanol

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TABLE 1. *The action of barley β-amylase on some glycogens.*

Sample no.	Source of glycogen	$\overline{CL}$ [a]	β-Amylolysis limit (%)	Exterior [b] chain length	Interior [c] chain length
1	*Arenicola*	11	43	7—8	2—3
2	*Cardium*	8	14	3—4	3—4
3	Cat liver IV	13[4]	53 [d]	9—10	2—3
4	,, VI	12[4]	52 [d]	8—9	2—3
5	Cock liver	13	39	7—8	4—5
6	Fœtal pig liver	11[4]	49	8	2
7	Fœtal sheep liver (alkali-treated) [e]	13[2]	49	9	3
8	Human muscle II	11	40	7	3
9	*Mytilus edulis* IV	12[4]	51	8—9	2—3
10	,, V	9[4]	40	6	2
11	,, VI	13[4]	46	8—9	3—4
12	,, VII	13	46	8—9	3—4
13	,, VIII	13	45	8—9	3—4
14	,, IX	10	51	7—8	1—2
15	,, X	14	45	8—9	4—5
16	Rabbit liver I	13	25	5—6	6—7
17	,, III	13[4]	51	9	3
18	,, IV	13[4]	45	8—9	3—4
19	,, V	14[4]	51	9—10	3—4
20	,, VI	18[9]	52	12	5
21	,, X	12[4]	49	8—9	2—3
22	,, XII	17	43	9—10	6—7
23	,, XIII	15	46	9—10	4—5
24	Rabbit muscle II	11	39	6—7	3—4
25	Skate liver	13	45	8—9	3—4

[a] Superscript numbers refer to chain-length determination reported in refs. 2, 4, or 9. [b] No. of glucose residues removed by β-amylase + 2·5. [c] $\overline{CL}$ − exterior chain length − 1. [d] Incubation with crystalline sweet-potato β-amylase gave β-amylolysis limits of 54 and 52% respectively. [e] Before alkali treatment, this glycogen had a β-amylolysis limit of 49%.[2]

precipitation, did not decrease the β-amylolysis limits. We conclude that the purification of glycogen with acetic acid does not affect the extent of degradation by β-amylase.

The above results clearly demonstrate the variation in branching characteristics which exist in glycogens, and in particular, the structural differences in samples from the same biological source. Thus, $\overline{CL}$ values for rabbit liver glycogen range from 12 to 18, whilst glycogens (samples 16, 17, 18) with the same chain length differ in position of branching in the constituent chains. Rabbit liver I glycogen is unusual in that both the exterior and interior chains contain *ca.* 6 glucose residues; normally, the exterior chains are roughly twice the length of the interior chains. The presence of relatively short chains in rabbit liver I glycogen has been confirmed, since the extent of degradation by muscle and potato phosphorylase was abnormally low.[11]

Glycogens from *Arenicola* (lug worm), cock liver, and *Cardium* (cockle) do not appear to have been examined previously. The first two samples have normal structures, but the *Cardium* glycogen has unusually short exterior chains (3—4 glucose residues) although the interior chains are of normal length.

The action of purified salivary α-amylase on a number of glycogen-type polysaccharides has also been investigated. Although α-amylases catalyse random hydrolysis of α-1 : 4-glucosidic linkages in these polysaccharides, the extent of degradation should be related to the proportion of α-1 : 6-glucosidic linkages present, since these linkages and certain adjacent α-1 : 4-glucosidic linkages resist enzymic action.[12] Several polysaccharides were therefore incubated (at pH 7·0 and 35°) with salivary α-amylase, and the apparent percentage conversion into maltose (P_M) determined at intervals (see Table 2). Paper chromatography showed that extensive random hydrolysis had occurred, the products being glucose, maltose, maltotriose (trace), and oligosaccharides with R_G 0·10, 0·05, and lower chromatographic mobilities.

Since the relative proportion of enzyme and substrate was constant, the variation in P_M values must reflect differences in molecular structure. In general, the extent of α-amylolysis appears to be inversely proportional to the degree of branching in the sub-

TABLE 2. *The α-amylolysis of some branched α-1 : 4-glucosans.*

Source of polysaccharide	Percentage of α-1 : 6-glucosidic linkages	Apparent % conversion into maltose (P_M) * 2 hr.	6 hr.	24 hr.
Glycogens :				
Arenicola	9	56	71	76
Cardium	13	39	49	54
Cock liver	8	57	72	76
Helix pomatia	14	51	64	67
Mytilus edulis VII.....	8	60	73	80
,, VIII.....	8	61	76	80
,, IX.....	10	62	78	83
,, X.....	7	61	75	80
Skate liver	8	59	75	80
Amylopectin :				
Waxy maize starch	5	78	93	95
β-Limit dextrins : †				
Helix pomatia glycogen	22	21	26	29
Waxy maize starch	10	48	59	66

* All digests contained 4·6 units of α-amylase per mg. polysaccharide (see p.).

† Prepared by the prolonged action of β-amylase on the polysaccharide, removal of maltose by dialysis, and isolation by freeze-drying.

strate. The low P_M values for *Helix pomatia* [2] and *Cardium* glycogens are in accord with the earlier evidence for compact and highly branched structures. Conversely, waxy maize starch and its β-limit dextrin have relatively open interior structures, since *ca.* 47 and 33% of the glucosidic linkages in the molecules can be hydrolysed by α-amylase.

Peat, Roberts, and Whelan [13, 12] reported that the products of α-amylolysis of a sample of rabbit liver glycogen included *ca.* 5% of maltulose and small quantities of fructose-containing α-dextrins, suggesting that fructose was a minor component of the glycogen. Examination of our α-amylase digests by paper chromatography with the orcinol spray reagent [14] or the acid resorcinol reagent [15] failed to show the presence of ketoses. It is concluded that fructose is not a constituent of the above glycogens.

EXPERIMENTAL

Preparation of Glycogens.—We are indebted to Dr. D. J. Bell for glycogen samples 7, 8, 16, and 17 and the cock liver tissue, and to Dr. G. R. Tristram who provided samples 14 and 15 and many of the animal tissues. The isolation and certain properties of these glycogens are recorded in Table 3. The yield varied from 0·3 to 1·6 g. per 100 g. of wet tissue, and the polysaccharides

TABLE 3. *The preparation and properties of some glycogens.*

Source of glycogen	Method of isoln.[a]	Method of purifn.[b]	$[\alpha]_D$, H_2O	$\lambda_{max.}$ of iodine complex (mμ) [c]
Arenicola	P	A	+200°	420
Cardium	W	PA	+201	420
Cock liver	W	PA	+191	440
Human muscle	P	A	+195	—
Mytilus edulis VII.....	W	PA	+200	420
,, VIII.....	W	PA	+194	420
,, IX.....	W	PA	+196	—
,, X.....	W	PA	+199	—
Rabbit liver XII.....	W	A	+193	470
,, XIII.....	W	A	+199	470
Rabbit muscle II.....	P	A	+194	—
Skate liver	P	A	+196	420

[a] P = Pflüger method; W = hot-water extraction. [b] A = precipitated with acetic acid and ethanol; PA = deproteinised with picric acid, then as A. [c] Determined as by Peat, Whelan, Hobson, and Thomas (*J.*, 1954, 4440) and corrected for light scattering.

had glucose contents of 94—97% (paper chromatography, and Shaffer–Somogyi estimation [16] after hydrolysis with 1·5N-sulphuric acid for 2 hr. at 100°).

Potassium Metaperiodate Oxidation of Glycogens.—The method was similar to that previously described,[2, 4] the formic acid being titrated potentiometrically to pH 5·7 with carbonate-free [17] 0·01N-sodium hydroxide.

β-Amylolysis of Glycogens.—Barley β-amylase, from the Wallerstein Laboratories, New York, had an activity of 118 units/mg. by Hobson, Whelan, and Peat's method.[18] The enzyme preparation had a negligible reducing power and showed no maltase activity. It was free from α-amylase since it did not reduce the iodine-staining power of amylopectin β-limit dextrin, or the molecular weight of glycogen β-limit dextrin.[19] On incubation with potato amylose at pH 3·6 and 4·6, 72% and 95% conversion respectively into maltose was observed. This indicates the presence of Z-enzyme in the barley preparation (compare ref. 6). Maltotriose was very slowly hydrolysed by high concentrations of the enzyme (86 units/mg. of maltotriose).

Enzyme digests contained 0·1M-acetate buffer of pH 4·6, and toluene, and were incubated at 35°. Preliminary experiments in which rabbit liver V glycogen was incubated with 40, 60, 80, and 100 units of β-amylase per mg. of glycogen showed that in every case enzyme action was virtually complete within 4 hr. and that on incubation for a further 44 hr. the conversion into maltose did not increase by more than 1%. β-Amylolysis limits were therefore determined, in duplicate, by incubating glycogen (1 mg./ml.) with β-amylase (40—50 units/mg. of glycogen) at pH 4·6 in a total volume of 50 ml. Samples (3 ml.) were analysed at intervals for maltose,[16] and the mean results obtained after 24 and 48 hr. are recorded in Table 1. Duplicate analyses were identical or differed by no more than 1%.

After 48 hours' incubation under the above conditions, the digests contained active β-amylase. Addition of 1 drop of digest to 1% starch solution (2 ml.) caused immediate liberation of reducing sugar. Further, the addition of fresh β-amylase after 48 hr. did not increase the maltose content of the digest.

Portions of the digests were examined by paper chromatography. Ethanol (2 vol.) was added to precipitate protein and polysaccharide, and the supernatant solutions were deionised with IR-4B(OH) and IR-120(H) ion exchange resins, and concentrated. The chromatograms, developed with a silver nitrate–sodium hydroxide reagent,[20] showed the present of maltose and no other sugar.

α-Amylolysis of Glycogens.—α-Amylase was prepared from human saliva by the method of Fischer and Stein [21] except that the final crystallisation stage was omitted, and the enzyme isolated by freeze-drying in 0·2M-citrate buffer (pH 7·0). The enzyme preparation, with an activity of 42 units/mg. by a modification of Fischer and Stein's method,[21] had a negligible reducing power, slowly hydrolysed maltotriose (27 units/mg. of substrate), and contained a trace of maltase activity (37 units/mg. of substrate).

Standard digests containing polysaccharide (*ca.* 50 mg.), 0·5% sodium chloride solution (10 ml.), α-amylase solution (*ca.* 230 units, 1 ml.), and distilled water to 100 ml. were incubated at 35°. Portions (5 ml.) were analysed at intervals, for reducing sugar (see Table 2). The small (*ca.* 2%) increase in reducing power of the digest with waxy maize starch between 6 and 24 hours' incubation showed the virtual absence of maltase activity under the above conditions (4·6 units α-amylase/mg. of substrate.)

After 48 hr. a 10 ml. sample was removed from each digest, boiled, concentrated, and examined by paper chromatography. The silver nitrate–sodium hydroxide reagent showed the presence of glucose, maltose (R_G 0·49), maltotriose (traces only), and higher oligosaccharides including sugars with R_G 0·10 and 0·05. When duplicate chromatograms were sprayed with an orcinol reagent,[14] as used by Roberts *et al.*,[12, 13, 22] neither maltulose nor ketose-containing oligosaccharides could be detected. An authentic sample of maltulose had R_G 0·48, and a mixture of glucose and fructose (97 : 3) gave a positive ketose reaction under similar conditions.

Portions (4 ml.) of the digests were also heated with resorcinol (0·45% in water; 2 ml.) and hydrochloric acid–glycerol reagent (5 ml.) at 100° for 15 min. and the solutions examined colorimetrically.[15] If the fructose content of the glycogens was only *ca.* 3% then each aliquot would contain *ca.* 60 μg. of ketose. Under the above conditions, 30 μg. of fructose could be detected, and the intensity of colour with this was greater than that with any of the samples from the α-amylase digests.

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893. α-1 : 4-*Glucosans. Part VII.* The Enzymic Degradation and Molecular Structure of Amylose.*

By J. M. G. Cowie, I. D. Fleming, C. T. Greenwood, and D. J. Manners.

Amylose, prepared in the absence of oxygen, is only partially degraded (70—80%) by purified β-amylase, but is completely degraded by the concurrent action of β-amylase and Z-enzyme. Treatment of amylose with oxygen, either during fractionation or in presence of hot alkali, does not introduce barriers to the action of these enzymes.

β-Amylolysis of potato amylose and a subfraction obtained by aqueous leaching indicates that amylose is heterogeneous.

The specificity of phosphorylase, β-amylase, and barley Z-enzyme is discussed; the last enzyme, which hydrolyses anomalous linkages in amylose, has no action on α-1 : 3-, α-1 : 4-, or α-1 : 6-glucosidic linkages, or on β-linked disaccharides.

Although the main features of the degradation of starch-type polysaccharides by β-amylase have been known for many years, the extent of hydrolysis of amylose and the mode of action (*i.e.*, single- or multi-chain attack) remain uncertain.[1] Recent investigations by Peat,[2] Hopkins,[3] Hassid,[4] and their collaborators have shown that, in contrast to earlier reports, *pure* β-amylase converts only *ca.* 70% of amylose into maltose. This incomplete degradation is not due to retrogradation of the substrate or to inactivation of the enzyme.[2] Since the action of β-amylase ceases when glucosidic linkages other than α-1 : 4 are encountered in the substrate, the amylose samples appear to contain a small number of anomalous linkages. Peat and his co-workers [2] believe that these linkages are structural features of the native amylose; they can be hydrolysed by a specific enzyme (Z-enzyme [5]) which occurs, together with β-amylase, in soya-beans. Unpurified β-amylase preparations from this source, therefore, degrade amylose completely. Although the nature of the anomalous linkages is not yet known, Baum, Gilbert, and Scott [6] have suggested that they are introduced into the amylose, by oxidation, during the fractionation of the starch. The specificity of Z-enzyme has not yet been determined, and has been the subject of some controversy.[2-5, 7]

The present communication describes a study of the action of β-amylase, in the presence and absence of Z-enzyme, on highly purified amyloses of high molecular weight. These amyloses, which have a degree of polymerisation ($\overline{D.P.}$) of several thousand glucose residues, were prepared by the fractionation of starch in the presence and in the absence of oxygen. The effect of oxygen and hot alkali on the β-amylolysis limit of certain amyloses has also been examined. In addition, the specificity of barley Z-enzyme has been investigated.

The β-Amylolysis Limit of Amylose.—Purified soya-bean β-amylase was caused to act on several samples of amylose, which had been prepared under neutral conditions in the presence and in the absence of oxygen. The reaction was followed by determination of the liberated maltose rather than by measurement of changes in the iodine-staining power: the latter method is less precise, and gives results which are *ca.* 5% higher than those from reducing-power measurements (see p. and ref. 2); in addition, the maltose production is independent of the mode of enzyme action (cf. ref. 8). The enzyme preparation, which was free from Z-enzyme [5] and α-amylase, gave 69—86% conversion into maltose with eight amylose samples (see Table 1). Amylose IV had a β-amylolysis limit of 99%. The low β-amylolysis limits were not due to inactivation of the enzyme, since they were unaffected by changes in initial enzyme : substrate ratio, or by addition of fresh enzyme to the digests after 24 hours' incubation.

* Part VI, *J.*, 1957, 3432.

TABLE 1. *β-Amylolysis limits of amyloses.*

Sample	Conditions of prepn.[a]	D.P.	β-Amylolysis limit † A	B	C	D	E
I	N_2	3,200	77	92	—	99	98
II	O_2	2,800	75	—	—	98	—
III	O_2	1,600	76	—	—	99	—
IV	N_2	1,800	99	—	—	—	—
V	N_2	2,700	86	—	—	—	—
VI	Air	1,850	73 *	86	72	95	94
VII	Air	*ca.* 1,500	69	83	75	94	94
VIII	N_2	*ca.* 1,500	77	87	74	92	93
IX	N_2	*ca.* 600	77	85	79	99	—

[a] Atmosphere during fractionation (see p.).

* Crystalline sweet-potato β-amylase (free from Z-enzyme) also gave 73% conversion into maltose.

† Soya-bean β-amylase at pH 4·6 on (A) untreated amylose, and (B) amylose treated with oxygen in alkali. Barley β-amylase at pH 3·6 on (C) untreated amylose, and at pH 4·6 on (D) untreated amylose, and (E) amylose treated with oxygen in alkali.

By contrast, barley β-amylase caused 92—99% degradation of the same amylose samples, when incubated at pH 4·6. Although this enzyme preparation was free from α-amylase and maltase, previous studies [9] have indicated that amorphous barley β-amylase shows Z-enzyme activity. The complete degradation of the amylose is therefore due to the combined action of barley Z-enzyme and β-amylase. Since soya-bean Z-enzyme is inactivated at pH 3·6, our barley β-amylase preparation was incubated at this pH with four amylose samples. Only 72—79% conversion into maltose was observed, the detailed results being similar to those obtained with soya-bean β-amylase. Control experiments showed that despite the decreased stability of β-amylase itself at pH 3·6, the digests contained sufficient β-amylase to allow the limit to be reached within 6 hr., and that this limit was unchanged when the initial enzyme concentration was increased five-fold.

It appears, therefore, that barley β-amylase alone is unable to degrade amylose completely, and that barley Z-enzyme, like soya-bean Z-enzyme, is acid-labile.

Further evidence for the presence of Z-enzyme in barley was obtained by dividing a digest of amylose I and soya-bean β-amylase and adding equivalent amounts of barley and soya-bean β-amylase severally to the two portions. The addition of soya-bean enzyme had no effect; the barley preparation caused a rapid increase in the maltose content, from 77 to 98%.

The possibility that the Z-linkages may arise from changes in the physical state of the substrate, *i.e.*, " ageing," [10] has also been considered. Incubation of amylose in acetate buffer (pH 4·6) for 48 hr. at room temperature *before* the addition of barley β-amylase resulted in only a small decrease (*ca.* 8%) in β-amylolysis limit. Ageing is not therefore responsible for the resistance of some 20—30% of the amylose to pure β-amylase. Further, when digests containing soya-bean and barley β-amylase were prepared under *identical* conditions, enzyme action was complete within 2—6 hr., *i.e.*, before any appreciable ageing is likely to have taken place. (This result is substantiated by unpublished sedimentation measurements on amylose during β-amylolysis.)

The various amylose samples used in these experiments were prepared either in the presence or in the absence of oxygen, and with the exception of amylose IV (see Table 1), 14—31% of the samples was resistant to pure β-amylase. Probably, therefore, the barriers to β-amylolysis are a structural feature of the native amylose and are not introduced, by oxidation, during the *fractionation* of the starch. However, the possibility of inadvertent modification of the starch during *isolation* cannot be entirely disregarded.

The β-Amylolysis Limit of Amylose treated with Oxygen in Alkali.—Evidence suggesting that oxidation might be the source of the anomalous linkages in amylose was obtained by Gilbert and his co-workers.[6] Amylose, after treatment with oxygen at 95° in both neutral and alkaline solution, was incubated with potato phosphorylase. Only 67—81% degradation occurred whereas, under similar conditions, 90% of the original amylose was degraded (as measured by decrease in iodine-staining power). (It must be noted, however, that

phosphorolysis is more accurately assayed by measurement of the production of α-D-glucosyl phosphate.) It was concluded [6] that barriers to phosphorylase action, and by analogy, to β-amylolysis, can be introduced into amylose by molecular oxygen.

We have studied the degradation, by barley β-amylase, of four amylose samples which had been heated at 98° for 20 min. in 0·5M-sodium hydroxide in a stream of oxygen. The results (Table 1) show that this treatment does not alter the percentage conversion into maltose. It follows that, under the conditions employed by Gilbert, molecular oxygen does not introduce barriers to β-amylase or Z-enzyme action.

The above experiments were repeated with pure soya-bean β-amylase, and in all instances, a small *increase* (*ca.* 10%) in β-amylolysis limit was observed. If anomalous linkages had been introduced by oxygen, then the β-amylolysis limit would *decrease*. The increase is attributed to alkaline degradation of the amylose, involving the rupture of non-terminal α-1 : 4-glucosidic linkages and the liberation of new non-reducing end-chains which are susceptible to β-amylase. The alkaline degradation, which is not detected by the combined action of β-amylase and Z-enzyme, may be followed viscometrically. In one experiment a 60% decrease in specific viscosity was observed, indicating that 1—2 α-1 : 4-glucosidic linkages per molecule had been broken. It is clear, however, that the majority of the Z-linkages in the oxygen-treated amylose are intact since the β-amylolysis limit is only 83—92%, and the residual polysaccharide is still stained blue by iodine.

Neufeld and Hassid [4] found that after dissolution of amylose β-limit dextrin in hot alkali (3 min. in 0·19N-potassium hydroxide at 100° *in air*), the polysaccharide had a β-amylolysis limit of *ca.* 50%. Dissolution of the same dextrin in hot water resulted in an increase of only 5% in β-amylolysis limit. In view of our results, it is probable that alkaline degradation of α-1 : 4-glucosidic linkages rather than Z-linkages is responsible for the increased susceptibility of the limit dextrin to β-amylase.

Comparison of our results with those of Gilbert [6] suggests that β-amylase and phosphorylase differ slightly in specificity. Phosphorolysis of an amylose chain, in which successive α-1 : 4-glucosidic linkages are broken, appears to be arrested when an " oxidised " glucose residue is encountered. In contrast, β-amylase action, involving hydrolysis of alternate glucosidic linkages, is not limited by such residues. Although the nature of the oxidised residue is not known (oxidation at $C_{(3)}$ or $C_{(6)}$ is most likely [1b]), it must differ only slightly from a normal α-1 : 4-linked glucopyranose residue since β-amylase itself has a relatively high degree of specificity.

It will be noted that amylose from two varieties of potato starch, and from wrinkled pea starch, behave in the same way towards β-amylase, Z-enzyme, and oxygen in hot alkali. The above results may, therefore, be typical of the amyloses of plant starches. It is of interest that sago, tapioca, maize, and potato amyloses are similarly degraded by β-amylase and soya-bean Z-enzyme,[2] whilst Neufeld and Hassid [4] have reported that amyloses from seven different sources are incompletely hydrolysed by pure β-amylase.

The Heterogeneity of Potato Amylose.—Several workers have reported that amylose is heterogeneous.[1b] Neufeld and Hassid [4] showed that subfractions of potato and maize amylose had different β-amylolysis limits. Meyer *et al.*[10] have reported $\overline{\text{D.P.}}$'s of 200 and 700 for two fractions obtained by aqueous leaching of potato starch, and Hopkins and Bird [3] found that similar fractions had β-amylolysis limits of 80 and 64%.

We found (see Table 2) that aqueous leaching at 70° extracts *ca.* 40% of the amylose from the granule. This fraction (amylose IV) has a $\overline{\text{D.P.}}$ of 1800, and it must be linear since it is completely degraded by purified β-amylase. In contrast, amylose I, which represents the whole amylose in the potato starch, has a $\overline{\text{D.P.}}$ of 3200 and a β-amylolysis limit of only 77%. It follows that the residual amylose remaining after aqueous leaching has a $\overline{\text{D.P.}}$ of 5000—6000 and a β-amylolysis limit of 50—60%. Aqueous extraction of the granules at 100° gives a polysaccharide (amylose V) with properties intermediate between those of amylose I and IV. The presence of the small proportion of anomalous

linkages is therefore confined to the amylose fractions of higher molecular weight. Moreover, the anomalous linkages are situated, on the average, near the centre of these molecules, and not near the non-reducing end.

The Specificity of Z-enzyme and the Nature of the Anomalous Linkages.—Peat, Thomas, and Whelan [5] originally isolated Z-enzyme from a soya-bean β-amylase preparation as a stable powder which was free from glycerophosphatase and amylase activity, and with the general properties of a group-specific β-glucosidase. The fact that almond emulsin (a mixture of β-glucosidases) also showed Z-enzyme activity supported this suggestion. However, a later investigation by Neufeld and Hassid [4] showed that the laminarinase, cellobiase, and Z-enzyme activities of almond emulsin were due to distinct enzymes.

In an attempt to determine the specificity of barley Z-enzyme, the action of β-amylase (soya-bean at pH 4·6 or barley at pH 3·6) and that of β-amylase plus Z-enzyme (barley at pH 4·6) on various carbohydrates have been compared. This method of investigation is, however, limited by the availability of suitable substrates, since the action of a carbohydrase is dependent on (*a*) the specificity for the glycosidic linkage, (*b*) the nature of adjacent glycosidic linkages, and (*c*) steric factors arising from the size and shape of the substrate. For example, R-enzyme,[11] which is specific for α-1 : 6-glucosidic linkages, cannot hydrolyse isomaltose, panose, or the α-1 : 6-inter-chain linkages in glycogen and in the interior of amylopectin. The above requirements are satisfied only by the inter-chain linkages in the outer regions of an amylopectin molecule. In the present study, the fact that Z-enzyme does not hydrolyse a disaccharide containing a particular linkage is not, therefore, considered to be conclusive proof of non-identity with the anomalous linkage.

The possibility that Z-enzyme action could be due to traces of α-amylase has been emphasised by Hopkins and Bird,[3] although Peat *et al.*[5, 7] have reported enzymic evidence against this view. However, the constancy of the molecular weight of amylose during barley β-amylolysis [12] shows clearly that random α-amylolytic action is not occurring, and that barley α-amylase and Z-enzyme must be distinct enzymes.

If oxidation of anhydroglucose residues occurred during the isolation of a linear amylose molecule (cf. ref. 6), then the barriers to β-amylase could be " oxidised " residues situated at the non-reducing end or at some position in the chain itself. Such residues could only be removed by Z-enzyme, by the hydrolysis of one of more α-1 : 4-glucosidic linkages, *i.e.*, by an amylase-type action. Since Z-enzyme is not an amylase, it follows that the anomalous linkages cannot involve oxidised glucose residues situated as above.

By similar reasoning, the tentative suggestion [13] that the anomalous linkages are glucose residues attached by a β-linkage to the non-reducing end of a linear α-1 : 4-glucosan is considered to be unlikely.

Recent studies of partial acid hydrolysis [14] have indicated that amylopectin contains a small proportion of α-1 : 3-glucosidic linkages. These linkages may arise through the action of an amylo-1 : 4 ⟶ 1 : 3-transglucosidase acting either with phosphorylase during the synthesis of linear chains, or with Q-enzyme during the amylose ⟶ amylopectin conversion. The presence of an α-1 : 3-glucosidase in Nature has also been reported.[15]

The experiments described below indicate that Z-enzyme is not an α-1 : 3-glucosidase, and that amylose samples I—IX do not contain α-1 : 3-glucosidic linkages. A comparison has been made of the action of β-amylase, in the presence and absence of Z-enzyme, on (*a*) α-dextrins produced by the salivary α-amylolysis of isolichenin [16] and (*b*) a partial acid hydrolysate of isolichenin. This polysaccharide is a linear polymer of D-glucose containing both α-1 : 3- and α-1 : 4-linkages, and should closely satisfy the structural and steric requirements of a substrate for Z-enzyme. Measurements of reducing power showed that no hydrolysis occurred within 48 hr. It is concluded that Z-enzyme has no action on either the non-terminal or the terminal α-1 : 3-glucosidic linkages, which are liberated by the enzymic or chemical degradation of isolichenin.

The barriers to β-amylase action are unlikely to be α-1 : 6-glucosidic linkages, for the following reasons: (*a*) potato amylopectin, on incubation with barley and soya-bean β-amylase, gave similar conversions into maltose (54—56%); (*b*) treatment of amylose IX with isoamylase [17] (which hydrolyses α-1 : 6-inter-chain linkages in glycogen and amylopectin) before addition of β-amylase did not cause a significant increase in β-amylolysis; and (*c*) isomaltose and panose were not hydrolysed by either β-amylase preparation.

The suggestion [5] that amylose contains a small number of β-glucosidic linkages, which are hydrolysed by Z-enzyme, has been considered, although our studies have been limited by the fact that carbohydrates containing *both* α-1 : 4- and β-glucosidic linkages are not available as model substrates. Barley Z-enzyme appears to be distinct from laminarinase and salicinase; whereas Z-enzyme is inactivated at pH 3·6, the laminarinase and salicinase activities are only partly reduced. In addition, the barley preparation had no action on cellobiose or gentiobiose, at pH 4·6, and therefore differs from the soya-bean Z-enzyme preparation.[5]

From the above studies, it is concluded that barley Z-enzyme is not an amylase, and does not hydrolyse α-1 : 3- or α-1 : 6-glucosidic linkages; these linkages are therefore unlikely to be the barriers to β-amylase action. In addition, although barley Z-enzyme has no action on simple β-glucosides, the possibility that amylose contains a small proportion of β-glucosidic linkages remains.

Experimental

Analytical Methods.—The general methods used were as described in previous papers of this series, except that paper chromatograms were developed with ethyl acetate–pyridine–water (10 : 4 : 3) as solvent.[18] The iodine binding-power of amyloses was measured by the potentiometric-titration method described by Anderson and Greenwood.[19] Limiting viscosity numbers $[\eta]$ were determined in M-potassium hydroxide and number-average $\overline{D.P.}$ values calculated [20] from $\overline{D.P.} = 7{\cdot}4[\eta]$.

Preparation of Amylose.—The methods of fractionating the potato and wrinkled pea starches are shown in Table 2.

Enzyme Preparations.—β-Amylase was isolated from soya-beans and purified by Peat, Pirt, and Whelan's method [2] in which Z-enzyme is inactivated by a short heat treatment (30 min. at 60° at pH 4·8). The purified solution had an activity of *ca.* 20,000 units/ml. when

Table 2. *Preparation of amyloses.*

Sample	Source	Fractionation procedure: Method	Ref.	Iodine affinity *
I †	Potato var. Redskin	Thymol and BuOH	20	19·5
II	,, ,,	,, ,,	21	19·5
III	,, ,,	,, ,,	21	19·5
IV ‡	,, ,,	Aq. leaching at 70°	20	19·5
V	,, ,,	Aq. leaching at 98°	20	19·5
VI	,, King Edward	Pyridine	22	19·1
VII	,, ,,	$Al(OH)_3$ and thymol	23	15·8
VIII	,, ,,	,, ,,	23	17·0
IX	Wrinkled pea var. Laxton's Progress	Thymol and BuOH	20	18·5

* Expressed as mg. of iodine bound per 100 mg. of starch.[19]
† Yield approx. 17 g. per 100 g. of starch.
‡ Yield approx. 7 g. per 100 g. of starch.
Samples I, II, IV, and V were recrystallised from butan-1-ol under nitrogen; amylose III was recrystallised in the presence of oxygen.

tested under the conditions suggested by Hobson, Whelan, and Peat.[24] It did not reduce the iodine-staining power of amylopectin β-dextrin and contained only an insignificant trace of maltase. The properties of the barley β-amylase have been described by Liddle and Manners.[25] The stability of this enzyme was investigated by determining the activity after varying periods of incubation at pH 4·6 and 3·6:

Time of incubation (hr.)	0	5	24
Activity units,[24] at pH 4·6	138	123	85
Activity units,[24] at pH 3·6	18	9	0

Isoamylase was isolated from brewer's yeast, as described by Manners and Khin Maung.[17]

Determination of β-Amylolysis Limits.—Digests were normally prepared by incubating, at 35° in the presence of toluene, amylose (20—50 mg.), 0·2M-acetate buffer pH 3·6 or 4·6, β-amylase (*ca.* 50—100 units/mg. of amylose), and distilled water (to a final volume of 50 ml.). Dried amylose samples were moistened with ethanol, dissolved in 0·2N-potassium hydroxide, with shaking (and warming, if necessary), and then neutralised with N-hydrochloric acid (to phenolphthalein). Amylose–butanol complexes were dissolved in distilled water, as required. The amylose concentration was determined, in triplicate, by acid hydrolysis of a portion (1 ml.). of the digest, and estimation of the liberated glucose, as described by Pirt and Whelan.[26] Portions of the digest (1—3 ml.) were removed at intervals for determination of maltose or A.V. (absorption value of the iodine complex at 680 mμ) (see Table 3). The iodine-staining method gave slightly higher β-amylolysis limits. The β-amylolysis limit of potato amylopectin was remaindetermined similarly.

TABLE 3.

Digest	Time of incubation (hr.)	1	2	4	7	24	48
Amylose VII, pH 4·6	Conversion into maltose (%)	77	85	89	—	94	94
	Decrease in A.V. (680 mμ) (%)	73	78	91	—	98	—
Amylose VII, pH 3·6	Conversion into maltose (%)	66	69	—	73	75	75
	Decrease in A.V. (680 mμ) (%)	65	74	—	79	80	80

Action of Varying Amounts of Barley and Soya-bean β-Amylase on Amylose.—Amylose I (21·8 mg.) was incubated with barley β-amylase (100 units/mg. of substrate) at pH 4·6; after 24 hours' incubation, a further 100 units of β-amylase/mg. of substrate were added to the remainder of the digest. Results were:

Time of incubation (hr.)	1	2	6	24	48
β-Amylolysis limit (%)	89	95	98	98	98

A similar experiment with amylose I (46·2 mg.) and soya-bean β-amylase (50 units/mg. of substrate) at pH 4·6 gave 77% conversion into maltose after 24 hr. The digest was divided; to portion A, fresh soya-bean β-amylase (50 units/mg. of substrate) was added, and to portion B a similar amount of barley β-amylase. After 24 hours' incubation, the β-amylolysis limits were: A, 77; B, 98%. The residue of portion A gave an intense blue colour with iodine ($\lambda_{max.}$ 660 mμ) whilst that of digest B was achroic.

In a similar experiment with amylose VIII the following results were obtained; β-amylolysis limit (24 hours' incubation with soya-bean enzyme) 77%; after addition of fresh soya-bean β-amylase, 77%; after addition of a similar amount of barley β-amylase, 92%.

The action of barley β-amylase on amylose VIII at pH 3·6 was studied; two digests were prepared containing (*a*) 150 mg. of enzyme (initial activity *ca.* 12,000 units) and (*b*) 30 mg. of enzyme (initial activity *ca.* 2400 units). Portions from digest (*a*) were deproteinised (zinc sulphate–barium hydroxide) before analysis. The β-amylolysis limits were (*a*) 73, (*b*) 75% after 24 and 48 hours' incubation.

Effect of Oxygen on the β-Amylolysis Limit.—Amylose (samples I, VI, VII, VIII, IX) (20—30 mg.), dissolved in 0·5N-sodium hydroxide (10 ml.), was heated in a stream of oxygen at 95° for 20 min. The solution was cooled, neutralised, and incorporated into digests with soya-bean or barley β-amylase. The β-amylolysis limits, determined after 24 hours' incubation, are shown in Table 1, columns B and E. No significant increase was observed when incubation was continued for a further 24 hr. The specific viscosity of amylose VIII fell from 0·40 to 0·16 during the oxygen treatment.

Effect of "Ageing" on the β-Amylolysis Limit.—Amylose I (treated with oxygen and alkali as above) (20·4 mg.) was incubated with barley β-amylase at pH 4·6; after 24 and 48 hr. 97 and 98% conversion into maltose was observed. A similar digest in which 24·5 mg. of amylose and buffer were mixed, at room temperature (17—19°), for 48 hr. before addition of the enzyme was also examined. After a further 24 hr., 92% conversion into maltose had occurred.

The experiment was repeated with amylose VII which has a β-amylolysis limit of 94%. When digests containing 19·6 mg. of amylose dried *in vacuo* at 70°, or 20·7 mg. of air-dried amylose were incubated with buffer for 48 hr. at 18°, the β-amylolysis limits were 86 and 85%. Although no retrogradation of the amyloses was visible, it is probable that, under these extreme conditions, some ageing had occurred, with a small (5—9%) lowering of the β-amylolysis limit.

Action of β-Amylases on Isolichenin Dextrins.—Isolichenin (150 mg.) was incubated with freeze-dried salivary α-amylase [25] (15 mg.) in a 50 ml. digest at 35° for 24 hr. 20 ml. portions

were then incubated in 0·2M-acetate buffer (pH 4·6) with soya-bean β-amylase solution (0·1 ml.) or barley β-amylase (25 mg.) in a total volume of 25 ml. In all three digests, the apparent percentage conversion into glucose was 10. In a second experiment, in 0·1M-citrate buffer pH 6·5 (near the pH optimum for soya-bean Z-enzyme [5]), the conversions into glucose were 10, 11, and 9% respectively.

A similar quantity of isolichenin was hydrolysed with 0·5N-sulphuric acid (10 ml.) at 98° for 45 min., to give 32% apparent conversion into glucose. Portions of the neutralised hydrolysate (3 ml.) were incorporated into 10 ml. digests containing 0·2M-acetate buffer (pH 4·6) and β-amylase. After 24 hours' incubation, 32% conversion into glucose was observed with both soya-bean and barley β-amylase.

Action of Isoamylase on Wrinkled-pea Amylose.—Amylose IX (6·9 mg.), 0·2M-acetate buffer pH 5·89 (1 ml.), water (4·5 ml.), and isoamylase (40 mg./ml.; 0·5 ml.) were incubated at 18° for 24 hr. The enzyme was inactivated by heat, the digest cooled, and β-amylase in 0·2M-acetate buffer (pH 4·6) (250 units; 2 ml.) added. The β-amylolysis limit was 77%. In a control experiment without isoamylase, the β-amylolysis limit was 75%.

Action of Barley β-Amylase on Simple Saccharides.—Digests containing gentiobiose, isomaltose, panose, and cellobiose (*ca.* 10 mg.) in buffer pH 4·6 (1 ml.) containing barley β-amylase (10 mg.) were incubated at 35° for 24 hr. Glucose could not be detected (paper chromatography).

Action of Barley β-Amylase Preparation on Laminarin and Salicin.—The laminarinase activity of the enzyme at pH 4·6 and 3·6 was determined by a method due to Anderson and Manners; [27] the activities were 13·5 and 11·1 units respectively. In similar experiments with salicin, the activities at pH 4·6 and 3·6 were 10·1 and 8·8 units respectively.

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950. α-1 : 4-*Glucosans. Part VIII.* Multiple-branching in Glycogen and Amylopectin.*

By A. Margaret Liddle and D. J. Manners.

The degree of multiple-branching in a glycogen or amylopectin can be evaluated from the chain lengths of the corresponding muscle-phosphorylase and β-amylase limit dextrins.

Fifteen samples of glycogen, from various biological sources, show small but significant differences in degree of multiple-branching. Amylopectins show a similar range of values. Accordingly, the marked physicochemical differences between glycogen and amylopectin cannot be related to differences in degree of multiple-branching.

A recent development in the chemistry of glycogen and amylopectin has been the recognition that multiple-branching is a characteristic structural feature. Multiple-branching was first postulated by Meyer,[1] and has been confirmed experimentally by Peat [2] and Cori [3] and their respective co-workers. We now describe a method for the quantitative estimation of the degree of multiple-branching in a branched α-1 : 4-glucosan. A preliminary account of part of this work has been published.[4]

The degree of multiple-branching may be conveniently expressed as the ratio ($\overline{A/B}$) of A-chains to B-chains. An A-chain (side-chain) is linked to the molecule only by the reducing group, whilst B-chains (main-chains) which are similarly linked, also have other chains attached to them.[2] In a glycogen-type molecule containing x chains, $\overline{A/B} = 1 : (x - 1)$ for a singly-branched "laminated" structure of the type originally suggested by Haworth, Hirst, and Isherwood,[5] whereas a multiply-branched "tree" structure as postulated by Meyer [1] contains approximately equal numbers of A- and B-chains.

For the proposed method of estimation of $\overline{A/B}$, a knowledge of the average chain length ($\overline{CL}$), muscle-phosphorolysis limit and β-amylolysis limit is required. From these, the difference (Δ) in $\overline{CL}$ values of the phosphorylase limit dextrin (φ-dextrin) and the β-amylase limit dextrin (β-dextrin) can be calculated. Normally, muscle phosphorylase removes 30—40% of glycogen as α-D-glucosyl phosphate, and β-amylase 40—50% as maltose, this degradation being limited to the exterior chains of the polysaccharide. The observed value Δ can be related to $\overline{A/B}$ as follows: in a β-dextrin, A-chain "stubs" contain 2 or 3 glucose residues,[2] whilst B-chain "stubs," which are probably of a similar length, are considered to contain n glucose residues. In a φ-dextrin, the A-chain "stubs" contain a single glucose residue, whereas the B-chain "stubs" contain 4 glucose residues more than those of the corresponding β-dextrin, *i.e.* $(4 + n)$.[6] In a branched α-1 : 4-glucosan with $\overline{A/B} = 1 : 1$, the average length of the exterior chains in the φ-dextrin is $[1 + (4 + n)]/2$, and in the β-dextrin is $(n + 2{\cdot}5)/2$, *i.e.* $\Delta = 1{\cdot}25$ glucose residues. [It will be noted that this calculation is independent of the length of the B-chain "stub" of a β-dextrin.] Similarly, when $\overline{A/B} = 1 : 2$, the exterior chain lengths of the φ- and β-dextrins are $[1 + 2(4 + n)]/3$ and $(2{\cdot}5 + 2n)/3$ so that $\Delta = 2{\cdot}17$. The values of Δ in the range $\overline{A/B} = 2 : 1 \longrightarrow 1 : 8$ have been calculated, and a graph of Δ against $\overline{A/B}$ prepared. The degree of multiple-branching can therefore be evaluated from experimental determinations of Δ.

In the present study, 15 samples of glycogen and 2 of amylopectin have been examined. Values of $\overline{CL}$ were determined by oxidation with potassium periodate,[7] and the

* Part VII, *J.*, 1957, 4430.

β-amylolysis experiments are described in Part VI of this Series.[8] The polysaccharides were then incubated with rabbit-muscle phosphorylase [9] in presence of 0·1M-phosphate (pH 6·8) and 0·001M-adenylic acid (activator), and the percentage conversion into α-D-glucosyl phosphate determined. Control experiments showed that the enzyme was free from α-amylase and amylo-1 : 6-glucosidase (the "debranching" enzyme of rabbit muscle [6]), so that enzyme action must be confined to the outer chains of the polysaccharide. Although phosphorolysis was continued for 24 hr., enzyme action, with 90 ± 15 units * of phosphorylase per mg. of substrate, was complete within 1·5 hr.; moreover, the phosphorolysis limits were not altered by a four-fold increase in initial enzyme concentration, or by the addition of fresh enzyme after 4 hr. Since the enzyme was dissolved in a 0·03M-cysteine–1% glycerophosphate buffer, the amount of cysteine (traces of which are required for maximum solubilisation and activity of the enzyme [9]) could not be a limiting factor. Under these conditions, 14—36% of various glycogens and 40—41% of the amylopectin samples were converted into α-D-glucosyl phosphate. It will be noted that *Helix pomatia* II glycogen and rabbit liver I glycogen, both of which have low β-amylolysis limits,[8] have phosphorolysis limits of only 22 and 14%, respectively.

Multiple-branching in glycogen and amylopectin.

Polysaccharide	$\overline{CL}$	φ-limit (%)	$\overline{CL}$ of φ-dextrin	β-limit (%)	$\overline{CL}$ of β-dextrin	Δ	$\overline{A/B}$
Glycogens							
Rabbit liver I	13	14	11·2	25	9·8	1·4	1 : 1·1
,, III	13	31	9·0	51	6·4	2·6	1 : 2·9
,, V	14	32	9·5	51	6·9	2·6	1 : 2·9
,, XIII	15	30	10·5	46	8·1	2·4	1 : 2·4
Cat liver IV	13	36	8·3	53	6·1	2·2	1 : 2·0
,, VI	12	34	7·9	52	5·8	2·1	1 : 1·9
Fœtal sheep liver	13	29	9·2	49	6·6	2·6	1 : 2·9
Rabbit muscle I	13	25	9·8	45	7·2	2·6	1 : 2·9
Human muscle II	11	22	8·6	40	6·6	2·0	1 : 1·8
Mytilus edulis V	9	21	7·1	40	5·4	1·7	1 : 1·4
,, VI	13	28	9·4	46	7·0	2·4	1 : 2·4
Ascaris lumbricoides	12	31	8·3	49	6·1	2·2	1 : 2·0
Helix pomatia II	7	22	5·5	37	4·4	1·1	1 : 0·9
Tetrahymena pyriformis I	13	31	9·0	44	7·3	1·7	1 : 1·4
Brewer's yeast	13	30	9·1	44	7·3	1·8	1 : 1·5
Amylopectins							
Waxy maize starch	20	41	11·8	50	10·0	1·8	1 : 1·5
Waxy sorghum starch	22	40	13·2	52	10·6	2·6	1 : 2·9

Comparable calculations based on phosphorolysis and β-amylolysis results published by G. T. Cori and her co-workers [3] show that rabbit liver glycogen, wheat amylopectin, and corn amylopectin have Δ values of 1·8, 2·6, and 2·7, respectively, equivalent to $\overline{A/B} = 1 : 1{\cdot}5$, 1 : 2·9, and 1 : 3·2.

It is concluded that different glycogens show small but significant differences in multiple-branching. The variation in $\overline{A/B}$ values is greater than that caused by a small experimental error in the analytical procedures. For example, if the phosphorolysis and β-amylolysis limits of rabbit liver I glycogen were 15 and 24%, respectively, then Δ would be 1·1, equivalent to $\overline{A/B} = 1 : 0{\cdot}9$; alternatively, if the limits were 13 and 26%, respectively, then the calculated $\overline{A/B}$ value is 1 : 1·4. Both of these $\overline{A/B}$ values are significantly different from those of the other rabbit liver glycogens.

Our previous studies [7, 8] have already shown that glycogens differ in degree and position of branching, and it now seems clear that variations in degree of multiple-branching also exist. These properties do not appear to be related to the biological source of the glycogen. Furthermore, amylopectins show small variations in $\overline{A/B}$, over a similar range of values.

* For definition, see ref. 9.

The above results are in agreement with other studies on multiple-branching. Calculations based on the yield of (*a*) maltose and maltotriose liberated by the action of R-enzyme on amylopectin β-dextrin [2] and (*b*) glucose liberated from a glycogen or amylopectin ϕ-dextrin by amylo-1 : 6-glucosidase [10] indicate that all these polysaccharides contain a high proportion of A-chains, $\overline{A/B}$ ranging from *ca.* 1 : 1 to 1 : 3. The marked physicochemical differences [10] between glycogen and amylopectin (*e.g.* molecular shape, interaction with iodine and with concanavalin-A) are therefore not directly due to different degrees of multiple-branching, but arise from different conformations of A- and B-chains. This latter probably reflect differences in the site and mode of biosynthesis of the polysaccharides. In the animal cell, glycogen synthesis appears to be a three-dimensional polymerisation, whilst that of amylopectin must be sterically limited during the formation of mixed layers of amylose and amylopectin in the starch granule.

Experimental

Analytical Methods.—Inorganic phosphate was estimated by Allen's colorimetric method,[11] except that a 10% solution of ammonium molybdate was used.

For the estimation of α-D-glucosyl phosphate in presence of 0·1M-inorganic phosphate a modification of Hanes's method [12] has been used. Aliquot parts of the enzyme digests (2 ml.) were diluted with distilled water (2 ml.), and magnesia solution [0·86% (w/v) magnesium chloride hexahydrate, 1·35% (w/v) ammonium chloride, 3·5% (v/v) aqueous ammonia; 10 ml.] was added, the mixture being warmed to *ca.* 50°. After about 45 min., the mixture was diluted to 25 ml. and the magnesium ammonium phosphate precipitate removed. An aliquot part of the filtrate (5 ml.) was hydrolysed with 1N-hydrochloric acid (0·65 ml.) for 7 min. at 98°, the solution cooled and diluted to 25 ml., and the inorganic phosphate content measured. A portion of the original filtrate, without acid hydrolysis, was also examined. Control experiments showed that glucose 6-phosphate was not hydrolysed under these conditions, and that adsorption of α-D-glucosyl phosphate on the magnesium ammonium phosphate precipitate did not occur. By contrast, if the inorganic phosphate is precipitated without dilution at room temperature, coprecipitation of the α-D-glucosyl phosphate occurs.

Phosphorylase.—Rabbit muscle phosphorylase was prepared by methods similar to those of Green and Cori, and Illingworth and Cori.[9] Several preparations were made; they varied in degree of crystallinity, and in the relative proportion of *a* and *b* forms. The phosphorylase was stored at 0° in 0·03M-cysteine hydrochloride–1% sodium β-glycerophosphate buffer.

We are indebted to Professor C. H. Waddington, F.R.S., and Dr. R. A. Beatty for the supply of rabbits, and to Mrs. R. M. Clayton who anæsthetised the rabbits; in some preparations, magnesium sulphate [13] was used in place of Nembutal.

Phosphorolysis of Polysaccharides.—Digests were prepared containing brewer's yeast glycogen [14] (40—50 mg.), 0·5M-phosphate buffer (pH 6·8; 4 ml.), 0·01M-adenylic acid (2 ml.), and phosphorylase solution (7500 units/ml.; 0·5 or 2·0 ml.) in a total volume of 20 ml. After incubation at 35°, portions (2 ml.) were removed for determination of α-D-glucosyl phosphate.

Time of incubation (hr.)	0·02	1·0	5·0	24·0
Phosphorolysis (%)				
(*a*) 80 units/mg. of glycogen	5	30	30	30
(*b*) 320 units/mg. of glycogen	19	31	31	29

In a subsequent experiment, glycogen (from *Helix pomatia*) on incubation with 110 units of phosphorylase/mg. gave 20% conversion into α-D-glucosyl phosphate within 1 hr. After 4 hr. the enzyme concentration was increased to 250 units/mg.; no further degradation occurred in the following 20 hr.

The phosphorolysis limits of the polysaccharides were determined, in duplicate, 75—105 units of phosphorylase/mg. of substrate being used. The α-D-glucosyl phosphate contents of samples of the digests examined after incubation for 1·5, 3, and 24 hr. were identical, or differed by only 1%.

Under the above conditions, normal mammalian glycogens had phosphorolysis limits of 29—36% and isolated ϕ-dextrins were resistant to further enzyme action. With less active enzyme preparations, the percentage conversions into α-D-glucosyl phosphate were 20—25%.[14]

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575. α-1 : 4-*Glucosans. Part IX.* The Molecular Structure of a Starch-type Polysaccharide from* Dunaliella bioculata.

By B. P. Eddy, I. D. Fleming, and D. J. Manners.

A glucose-containing polysaccharide, isolated from the unicellular salt-water alga *Dunaliella bioculata*, has been examined by chemical and enzymic methods.

It resembles plant starches in many respects, but contains only 13% of amylose, and the amylopectin component has an average chain length of 15—16 glucose residues. Both components are incompletely degraded by β-amylase, the barriers to this enzyme being hydrolysed by Z-enzyme and isoamylase, respectively.

Although the polysaccharides isolated from red and brown seaweeds (Rhodophyceae and Phaeophyceae) have been extensively studied,[1,2] present knowledge of similar materials from green and blue-green algae (Chlorophyceae and Myxophyceae) is limited. The last two are, on the whole, less readily available. It is generally accepted [3] that the reserve carbohydrate of *Chlorella* is a starch, but precise structural details have not been reported. The green alga *Ulva expansa* contains an iodophilic polysaccharide which, unlike normal starches, shows no appreciable birefringence and gives an abnormal *X*-ray powder diagram.[4] Polysaccharides from *Oscillatoria* have been characterised as amylopectin [5] or glycogen [6] in type, and *Nitella* [5] contains a cellulose-type material. In contrast, *Nostoc* [5] yields a complex mucilage composed of uronic acids, rhamnose, xylose, galactose, and glucose, whilst green algae of the *Chlamydomonas* species synthesise soluble extracellular polysaccharides with galactose and arabinose as the main constituents.[7] This paper describes an examination of the storage polysaccharide from the halophytic unicellular alga *Dunaliella bioculata* (class, Chlorophyceae; order, Volvocales; family, Polyblepharidaceae). The food value of this organism has been reported elsewhere.[8]

A preliminary study of the dried algal cells showed the presence of glucosan which was extracted with water at 70° or 100°, or with potassium hydroxide solution (5% or 24%) at 20°. Since this material was stained blue-black with iodine, and was degraded by α-amylase, the presence of a starch-type polysaccharide was indicated. A sample of polysaccharide material extracted with hot water contained glucose (69%) and protein (23%). Attempts to separate these by extraction with chloral hydrate [9] or trichloroacetic acid and toluene were not successful. Although the protein content decreased to 6% and 3% respectively, some degradation of the polysaccharide (content 78 and 82% respectively) occurred. As an alternative method of extraction, the procedure of MacWilliam and his co-workers [10] was examined.

The dried cells were extracted with 30% aqueous perchloric acid at room temperature, and the resulting polysaccharide was then purified *via* the iodine complex. The product was amorphous, contained 99% of glucose and *ca.* 0·1% of protein, and an aqueous solution, which had $[\alpha]_D$ +169°, was stained blue with iodine, exhibiting maximum absorption at 600 mμ. On incubation with salivary α-amylase, an apparent percentage conversion into maltose (P_M) of 85 was observed; under similar conditions, potato starch had a P_M value of 88. The polysaccharide was rapidly degraded by soya-bean β-amylase, giving 62% conversion into maltose; this degradation was increased to 83% by prior incubation with yeast isoamylase. The latter enzyme hydrolyses α-1 : 6-glucosidic inter-chain linkages in amylopectin and glycogen.[11]

The polysaccharide was oxidised at room temperature with potassium metaperiodate; [12]

* Part VIII, Liddle and Manners, *J.*, 1957, 4708.

the production of formic acid corresponded to an average chain length ($\overline{CL}$) of 18 glucose residues, and the absence of glucose in an acid hydrolysate of the periodate-oxidised polysaccharide indicated that 1 : 2- or 1 : 3-glucosidic linkages were absent.[13]

The presence of an amylose-type component was shown by potentiometric titration[14] of the iodine complex, and the iodine binding power, kindly determined by Mr. J. M. G. Cowie, indicated an amylose content of 12—14%. The $\overline{CL}$ value of the amylopectin component was therefore 15—16 glucose residues. The small amount of formic acid which is liberated from the end-groups of amylose can, to a first approximation, be neglected. The polysaccharide was fractionated by the thymol method,[15] in the absence of oxygen, and the resulting fractions treated with β-amylase. The amylose component, with pure β-amylase, had a β-amylolysis limit of 73%, and on addition of barley β-amylase (which contains Z-enzyme[16]) the limit increased to 93%. The amylopectin fraction had β-amylolysis limits of 60% before, and 76% after, pre-treatment with isoamylase. The polymeric chains are therefore mainly composed of α-1 : 4-glucosidic linkages, and the outermost inter-chain linkages in the amylopectin are of the α-1 : 6-type.

The above evidence shows that the polysaccharide isolated from *Dunaliella bioculata* resembles normal plant starches in many respects. The interaction with iodine, the degradation by α- and β-amylase, and the separation into two components by thymol are typical properties. It is of interest that the algal amylose, like that from the potato and the wrinkled pea,[16] contains a small number of anomalous linkages which are not attacked by β-amylase.

The amylose content of the algal starch is unusually low (plant starches normally contain 20—30% of amylose[17]) and is similar to that of the starch synthesised by the flagellated protozoan *Polytomella coeca*.[18] Further, the amylopectin component has a relatively high degree of branching ($\overline{CL}$ values of 20—25 are usual[19]), which is similar to that of the polysaccharide from *Oscillatoria princeps*.[6] Both these organisms show high Q-enzyme (*i.e.*, amylo-1 : 4 ⟶ 1 : 6-transglucosidase) activity, and the present study suggests that *D. bioculata*, which can be readily cultivated under laboratory conditions, may also be a convenient source of this enzyme.

Experimental

The analytical methods and enzyme preparations used have been described in Parts IV,[20] VI,[21] and VII[16] of this series.

Isolation of Polysaccharide.—The algal cells, grown in artificial double-strength sea-water, were harvested by centrifugation, dried, and stored *in vacuo* over P_2O_5. Before extraction, the dried cells were disintegrated in a ball-mill and sieved (200 mesh).

In a preliminary experiment, ethanol was added to a hot-water extract of the powdered cells, to give an impure polysaccharide material [Found: reducing sugar (as glucose), 69·4%; N, 3·7%, equiv. to 23% of protein]. A portion was extracted with aqueous 33% chloral hydrate to give sample I [Found: glucose (by paper chromatography and cuprimetric titration), 77·7; N, 0·9%]. A second portion was largely freed from protein by shaking it with 10% aqueous trichloroacetic acid and toluene, to yield sample II (Found: glucose, 81·7; N, 0·46%). Analysis by periodate oxidation and potentiometric iodine titration indicated that both samples had been degraded during purification.

A pure sample of undegraded polysaccharide was finally prepared by purification of a perchloric acid extract of the dried cells.[10] The latter (5 g.) were extracted three times with water (80 ml.) and 72% perchloric acid (60 ml.) by shaking for 30 min. at room temperature. Cell debris was removed by centrifugation, and the crude polysaccharide was precipitated and washed with acetone. An aqueous solution was then dialysed for 48 hr. against running tap-water. The volume was adjusted to 100 ml. and, to this, 20% sodium chloride solution (25 ml.) and iodine solution (3% in 3% potassium iodide; 10 ml.) were added with stirring. After several hours, the polysaccharide–iodine complex was collected, washed with alcoholic sodium chloride (2% in 75% ethanol), and decomposed by treatment with alcoholic sodium hydroxide (25 ml. of 5N-aqueous solution in 125 ml. of ethanol). The polysaccharide was washed again with alcoholic sodium chloride, dissolved in water, dialysed for 48 hr., and precipitated with

acetone. This gave sample III (1 g.) (Found: glucose, 99·5; N, 0·02%). Sample III was a white amorphous powder which dissolved in warm water. The aqueous solution had $[\alpha]_D$ +169° (*c* 0·54) and was stained blue with iodine, exhibiting maximum absorption at 600 mμ.

The iodine-binding power of sample III was determined by Mr. J. M. G. Cowie using the method of Anderson and Greenwood.[14] It gave a typical starch titration curve, and extrapolation indicated the presence of 12—14% of amylose.

Enzymic Degradation.—(a) *Salivary α-amylase.* Polysaccharide (27·8 mg.), 0·1M-citrate–phosphate buffer (pH 7·0; 5 ml.), sodium chloride (5 mg.), and freeze-dried salivary α-amylase [21] (15 mg.) in a total volume of 50 ml. were incubated at 35° for 48 hr. The P_M value was 85. In a control experiment, potato starch (31·4 mg.) gave a P_M value of 88.

(b) *Soya-bean β-amylase.* Sample III (15·1 mg.) was incubated with 0·2M-acetate buffer (pH 4·6; 3 ml.), containing soya-bean β-amylase solution (0·05 ml.; 1000 units), in a total volume of 25 ml. After 24 and 48 hr., the β-amylolysis limit was 62%.

(c) *Isoamylase and β-amylase.* Sample III (29·1 mg.) in acetate buffer (pH 5·9; 5 ml.) and water (8 ml.) was treated with isoamylase solution (80 mg. in 2 ml.) at room temperature for 24 hr. (The isoamylase was extracted from brewer's yeast by Miss Zeenat H. Gunja.) After inactivation of the isoamylase by heat, soya-bean β-amylase solution (0·05 ml.) and water (to 50 ml.) were added. The β-amylolysis limit, after 24 hours' incubation at 35°, was 83%.

Potassium Metaperiodate Oxidation.—Sample III (174·5 mg.), dissolved in 3% potassium chloride solution (50 ml.), was oxidised with 8% sodium metaperiodate solution (8 ml.) at room temperature. Portions (10 ml.) were analysed at intervals:

Time of oxidn. (hr.)	50	100	200	300
Total formic acid prodn. (mg.)	1·99	2·50	2·76	2·80
Apparent $\overline{CL}$ (glucose residues)	24·9	19·8	18·0	17·7

Since the starch contains 13% of amylose, the amylopectin component has a $\overline{CL}$ value of 15—16 glucose residues. The remaining solution of periodate-oxidised starch was treated with ethylene glycol (5 ml.), dialysed for 48 hr., and freeze-dried. After acid hydrolysis with 2N-sulphuric acid for 2 hr. the neutralised hydrolysate was examined by paper chromatography. Glucose could not be detected.

Fractionation of the Starch.—Polysaccharide (*ca.* 200 mg.) was dissolved in boiling water (150 ml.) in an atmosphere of nitrogen. The solution was allowed to cool to 70° and powdered thymol (100 mg.) added. The temperature was maintained at 70° for 30 min. with continuous stirring, then the solution was set aside at room temperature for 2 days. The thymol–amylose complex was collected and dissolved in water (30 ml.). The supernatant solution was treated with acetone, and the precipitated polysaccharide collected, washed with acetone and dried (yield, *ca.* 170 mg.). This material gave a solution which was stained with iodine, showing maximum absorption at 560 mμ.

Enzymic Degradation of Starch Components.—(a) *Amylose.* The above solution of amylose (0·62 mg./ml. determined by acid hydrolysis) was incubated with acetate buffer (pH 4·6; 5 ml.) and soya-bean β-amylase solution (0·1 ml.) in a 50 ml. digest. After 24 hr. the β-amylolysis limit was 73%. Barley β-amylase (which contains Z-enzyme; 1500 units; 15 mg.) was then added. After a further 24 hr. the β-amylolysis limit was 93%.

(b) *Amylopectin.* The above precipitate (48·1 mg.) was incubated at pH 4·6 with barley β-amylase (3000 units; 30 mg.) in a final volume of 50 ml. The β-amylolysis limit was 60%.

[With Zeenat H. Gunja] Amylopectin (30·1 mg.) was incubated with isoamylase (50 mg.) at pH 5·9 and 20° in a 15 ml. digest for 18 hr. The isoamylase was inactivated by heat, β-amylase solution (3 ml.) added, and the maltose content determined. After 24 hr., the β-amylolysis limit was 76%.

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α-1:4-Glucosans

10. GLYCOGEN STRUCTURE AND *RIGOR MORTIS* IN MAMMALIAN MUSCLES*

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Numerous chemical studies have demonstrated that glycogens from various sources may differ in such characteristics as molecular weight, average chain length and in the relative number of D-glucose residues in external and internal chains (Manners, 1957). That constitutional differences in the glycogen molecule have metabolic significance has been indicated by Stetten, Katzen & Stetten (1956), who showed that in the rat and rabbit the larger molecules of muscle glycogen are more reactive, and by Stetten & Stetten (1955), who found that D-glucose residues located peripherally in the chains have a higher rate of turnover than those more centrally situated. Another aspect of the apparent physiological inhomogeneity of mammalian-muscle glycogen has arisen during investigations on post-mortem glycolysis, and on the concomitant onset of *rigor mortis*. In general, provided that pre-slaughter stress has not seriously depleted glycogen reserves, post-mortem glycolysis will continue until pH 5·4–5·5 is reached in the muscles, at which the enzyme systems producing lactic acid are inactivated and some residual glycogen may be found. At a final pH of 5·9, 0·6–1 % of glycogen frequently remains in the psoas and diaphragm muscles of the horse (Lawrie, 1955), and 0·6 % at a final pH of 6·1 in the sternocephalicus muscle of the ox (Howard & Lawrie, 1957). This could signify that the glycogens remaining in such muscles after the completion of *rigor mortis* were either inaccessible or insusceptible to attack during post-mortem glycolysis, or that they might differ in

* Part 9, Eddy, Fleming & Manners (1958).

constitution from the glycogens which predominate in the pre-rigor state. The analytical methods employed in previous papers of this series have therefore been applied to glycogens prepared from some mammalian muscles during the pre- and post-rigor phases.

MATERIALS AND METHODS

Muscles. Samples (0·5–1 kg.) from 7–8-year-old draught horses and 3–4-year-old steers were excised 15–20 min. after death. The following locations were studied: heart (left ventricular wall); diaphragm (junction of pars sternalis with pars costalis); psoas (portion of psoas major at level of sacral vertebrae); longissimus dorsi (l. dorsi; portion posterior to first, second and third lumbar vertebrae); sternocephalicus (entire muscle).

Definition of pre-rigor and post-rigor samples. General features of the time course of *rigor mortis*, and of its associated biochemical changes, in the horse (Lawrie, 1953) and the ox (Howard & Lawrie, 1956, 1957), indicate that muscles from these species, with the exception of horse heart, if sampled when at a pH greater than 6·8 (horse muscles, ox sternocephalicus) or than 6·4 (ox psoas), will then be in the pre-rigor condition provided that they eventually attain a pH of 6·1 (horse muscles, ox sternocephalicus) or 5·7 (ox psoas). Samples of glycogen characteristic of the pre-rigor condition were accordingly prepared from these muscles at a standard time of 1 hr. *post mortem*, when the pH was above the prescribed limit.

Horse heart, however, goes into *rigor mortis* within an hour of death and thus, for practical purposes, yields only post-rigor glycogen, unless special techniques are used.

Samples of glycogen characteristic of the post-rigor phase were prepared from muscle samples held in moist nitrogen at 37° for 5 hr. and thereafter stored for 17 hr. at 0°. The high temperature ensures that conversion of glycogen into lactic acid will occur with the minimum accumulation of hexose phosphate intermediates (Bendall & Davey, 1957); the elapse of 24 hr. from the time of death ensures that the ultimate pH has been attained when the muscle is processed.

pH was determined by glass electrode on samples of muscle, 0·5–1·0 g. being homogenized in 10 ml. of 5 mM-sodium iodoacetate (Bate-Smith & Bendall, 1947).

Preparations of glycogen for analysis. Small pieces cut from the intact muscle were quickly added to hot 30% (w/v) NaOH held in a boiling-water bath (1 g. of muscle to 9 ml. of alkali) and heated until homogeneous. On cooling, ethanol was added to give a final concentration of approximately 50% (v/v). The impure glycogen was centrifuged down after settling overnight and washed with a mixture of 1 vol. of ethanol with 2 vol. of 20% (w/v) NaOH. From this point the procedure followed that described by Somogyi (1957) for the preparation of glycogen free from nitrogen and phosphorus. Each glycogen sample used for structural analysis represented the bulked yields from the muscles of several animals.

Electrophoresis of glycogen. This was kindly examined by Dr D. H. Northcote, using the method of Fuller & Northcote (1956).

Analytical methods

Glucose content. Glycogen was hydrolysed in 2N-sulphuric acid (0·1%) at 100° for 2 hr. and the glucose content of the hydrolysate determined by the Somogyi (1952) reagent. Hydrolysis of glycogen under the conditions devised by Pirt & Whelan (1951) for the analysis of starch (with 1·5N-sulphuric acid) gave values 1–2% lower than the above. The slightly stronger acid is necessitated by the greater proportion of α-1:6-glucosidic linkages. A control experiment showed that no destruction of glucose occurred (reducing-power measurements).

Estimation of glycogen. The procedure of Good, Kramer & Somogyi (1933) was employed.

Enzymic degradation and iodine-staining. These analyses were carried out as described by Liddle & Manners (1957), except that, in the α-amylolysis digests, 3·7 units of enzyme/mg. of polysaccharide was used. P_M denotes apparent percentage conversion into maltose. $E_{max.}$ represents the extinction (absorption value) of the iodine-stained polysaccharide at the $\lambda_{max.}$.

Glycogen value. The interaction of glycogen and concanavalin-A prepared from jack-bean meal was examined under the conditions of Cifonelli, Montgomery & Smith (1956), except that extinctions were measured on a Unicam SP. 500 spectrophotometer. The extinction given under these conditions by 1 mg. of glycogen was compared with that of a standard sample of rabbit-liver glycogen (glycogen value 1·00) kindly provided by Professor F. Smith. Amylopectins did not react under these conditions (see Calderbank, Kent, Lorber, Manners & Wright, 1960).

Periodate oxidation. The horse l. dorsi (post-rigor) muscle glycogen (about 250 mg.) was oxidized with a mixture of 20 ml. of 8% (w/v) sodium metaperiodate solution and 80 ml. of 5% (w/v) potassium chloride solution at room temperature, as described by Bell & Manners (1952). A second sample (about 100 mg.) was oxidized with sodium metaperiodate at 2° under the conditions of Manners & Archibald (1957).

The remaining glycogens (about 75 mg.) were oxidized with a suspension of potassium metaperiodate (5 ml. of sodium metaperiodate and 20 ml. of potassium chloride solutions) at room temperature. The oxidation was completed after 9–10 days, and 10 ml. samples were removed for analysis of formic acid after 10 and 12 days. A reagent control was also prepared and analysed.

RESULTS

Glycolytic criteria of horse and ox muscles

Characteristic pre- and post-rigor values for the pH and glycogen concentration in the muscles studied are given in Table 1. There is a considerable concentration of residual (or post-rigor) glycogen in all the muscles except horse heart. In horse l. dorsi and ox psoas, where the final pH is about 5·5, inactivation of glycolytic enzymes presumably accounts for the unattacked glycogen. This reason cannot automatically be adduced for horse psoas and diaphragm and ox sternocephalicus muscles, since the final pH is 5·8–6·0.

Table 1. *Characteristic* pH *values and glycogen contents of muscles investigated*

Values in parenthesis indicate the number of specimens examined. Initial pH and initial glycogen values were obtained 1 hr. *post mortem*.

Muscle	Initial pH	Final pH	Initial glycogen (pre-rigor) (mg./100 g.)	Residual glycogen (post-rigor) (mg./100 g.)
Horse heart	6·14±0·07 (4)	5·86±0·06 (4)	723± 64 (4)	79± 45 (4)
Horse psoas*	6·77±0·04 (6)	5·85±0·06 (6)	1229±190 (6)	606±143 (6)
Horse diaphragm	6·92±0·03 (6)	5·86±0·04 (6)	1883±127 (6)	1184± 43 (6)
Horse l. dorsi	6·87±0·05 (6)	5·45±0·08 (6)	2216±125 (6)	1179±101 (6)
Ox psoas†	6·41±0·07 (12)	5·53±0·07 (12)	772±125 (12)	205± 43 (9)
Ox sternocephalicus	7·11±0·04 (3)	6·01±0·15 (3)	1333±350 (3)	492±110 (3)

* From Lawrie (1955). † From Howard & Lawrie (1956).

Table 2. *Properties of horse- and ox-muscle glycogens*

$E_{max.}$ represents the extinction at $\lambda_{max.}$ after iodine treatment. ECL represents the average length of the exterior chains (no. of glucose residues removed by β-amylase + 2·5).

Sample	Chain length (average)	Glycogen value	$\lambda_{max.}$ (mμ)	$E_{max.}$	P_M	β-Amylolysis limit (%)	ECL
Horse, l. dorsi pre-rigor	17	0·87	480	0·34	83	53	11–12
Horse, l. dorsi post-rigor	17	0·82	490	0·36	78	47	10
Horse, diaphragm pre-rigor	17	0·81	475	0·31	80	53	11–12
Horse, diaphragm post-rigor	17	0·85	480	0·32	80	51	11
Horse, psoas pre-rigor	16–17	—	490	0·32	79	48	10–11
Horse, psoas post-rigor	17	0·76	490	0·35	76	46	10
Horse, heart, post-rigor	16–17	0·83	470	0·29	78	48	10–11
Ox, psoas pre-rigor	16–17	0·86	490	0·34	82	50	11
Ox, psoas post-rigor	15	—	480	0·30	79	50	10
Ox, sternocephalicus pre-rigor	19	0·78	475	0·27	83	51	12
Ox, sternocephalicus post-rigor	15	0·89	485	0·21	79	44	9

Characterization of purified glycogens

Glucose contents. The glycogen samples had glucose contents in the range 96–100 %, and the following analyses are based on these figures.

Iodine staining. The glycogens stained deep red–brown with iodine, and the spectra showed maximum absorption in the range 470–490 mμ. The $\lambda_{max.}$ values in Table 2 represent the mid-point of a wide absorption peak covering 20–30 mμ. Mammalian-liver glycogens under similar conditions show maximum absorption over the range 450–470 mμ ($E_{max.}$ 0·2–0·3), whereas amylopectins have $\lambda_{max.}$ values of 530–540 mμ and $E_{max.} > 0·6$.

Glycogen values. The glycogen values of the muscle glycogens varied between 0·76 and 0·89, indicating a slightly lower degree of branching than usual. There was no appreciable difference between pre- and post-rigor samples.

Average chain length. Potassium metaperiodate oxidation of horse l. dorsi (post-rigor) muscle glycogen (252 mg.) gave formic acid corresponding to chain lengths of 16·9, 16·6 and 16·6 glucose residues after 9, 10 and 11 days. In a duplicate analysis, the production of formic acid from 251 mg. of this glycogen corresponded to an average chain length of 16·6 glucose residues.

Sodium metaperiodate oxidation of the same glycogen (105 mg.) at 2° for 20 days indicated an average chain length of 16·8 glucose residues.

The remaining glycogens were analysed after 10–12 days' oxidation; the sodium hydroxide titres after 10 and 12 days were identical. The production of formic acid indicated average chain length values as follows: Pre-rigor samples: horse l. dorsi, 16·8; horse diaphragm, 16·8; horse psoas, 16·5; ox sternocephalicus, 18·6; ox psoas, 16·5. Post-rigor samples: horse diaphragm, 16·6; horse psoas, 17·1; horse heart, 16·5; ox sternocephalicus, 14·7 and 15·0 in duplicate analyses; ox psoas, 16·5. These values are considered to be accurate to within ±0·5 glucose residue. These results, to the nearest whole number, are summarized in Table 2.

β-Amylolysis limits. Incubation of the glycogens at pH 4·6 and 35° with barley β-amylase resulted in 44–53 % conversion into maltose.

α-Amylolysis limits. On incubation with purified salivary α-amylase at pH 7·0 and 35° for 24 hr., P_M values in the range 76–83 were obtained. The majority of these figures are significantly higher than those observed (approx. 75 %) with mammalian-liver glycogens (average chain length 13–14), and indicate a slightly lower degree of branching.

Electrophoresis. On zone electrophoresis (glass-fibre paper) in 0·1 M-sodium borate buffer, pH 9·3, at 2000 V and 140 mA for 90 min., the glycogens moved 6–8 cm. as one component. The apparent homogeneity of the muscle glycogens may be contrasted with the studies of Lewis & Smith (1957), who found that a variety of glycogens gave two distinct components on electrophoresis in 2 N-sodium hydroxide solution. There were small differences between the mobilities of the pre- and post-rigor samples examined, but these were not in a consistent direction.

The properties of the mammalian-muscle glycogens are summarized in Table 2.

DISCUSSION

Extraction and isolation of glycogen from animal tissues

The conventional solvents for the extraction of glycogen from animal tissues are hot water, cold dilute aqueous (5–10 %) trichloroacetic acid and hot concentrated aqueous (30 %) potassium or sodium hydroxide (Pflüger method). The last-named method has been criticized by many workers (e.g. Meyer & Jeanloz, 1943; Stetten, Katzen & Stetten, 1958; Bryce, Greenwood & Jones, 1958) as causing considerable degradation of the glycogen molecules. There is ample evidence to show that glycogen is degraded by hot dilute alkali (e.g. Greenwood & Manners, 1957; Stetten *et al.* 1958), but the available data indicate that hot concentrated alkali does not cause a progressive reduction in molecular weight. Thus Staudinger (1948) found that treatment of guinea-pig liver and muscle glycogen with 15 or 30 % potassium hydroxide solution at 100° for 1 hr. did not alter the molecular weight (light-scattering measurements). Bryce *et al.* (1958) showed that the sedimentation constant of rabbit-liver glycogen was not progressively reduced after treatment for 3 hr. with hot 30 % potassium hydroxide. Furthermore, Cori & Cori (1958) reported that the distribution of sedimentation constants of glycogen was unaffected after treatment with hot concentrated alkali. This marked difference in the degradative action of dilute and concentrated alkali may be related to the low solubility of oxygen in the latter.

The Pflüger method has therefore been used throughout the present study since it gives a high yield of protein-free glycogen (extraction of muscle tissue with hot water or trichloroacetic acid is extremely inefficient), and does not appear to cause appreciable degradation of the constituent glucopyranosidic linkages.

Previous studies on muscle glycogen

Although the molecular structures of mammalian liver and invertebrate glycogens have been extensively studied (Abdel-Akher & Smith, 1951; Manners, 1957), considerably less attention has been paid to mammalian-muscle glycogens. This may be attributed to the relative difficulty in obtaining adequate samples of tissue, and their low glycogen contents (usually < 1 %). The presence of glycogen in muscle tissue was first noted by Sanson (1857), but some 80 years elapsed before the first chemical investigations were reported (Young, 1937; Bell, 1937). These indicated that rabbit-muscle and liver glycogens had similar properties, and that a sample of horse-muscle glycogen (pooled tissues) had an average chain length of 11–12 by methylation. A summary of the results of other end-group assays of muscle glycogens is given in Table 3. These differ markedly from those reported in Table 2. Furthermore, the β-amylolysis limits (42 ± 4 %) are significantly lower than those of our present analyses.

Molecular structure of pre- and post-rigor muscle glycogens

Although glycogens have been isolated with average chain length values ranging from 6 to 18, the majority of samples have values of 12 ± 2 (Manners, 1957). The most noticeable feature of the mammalian-muscle samples as a whole (and, in particular, of the sternocephalicus pre-rigor glycogen) is

Table 3. *Properties of some mammalian-muscle glycogens*

Methods of assay are indicated by: (m) methylation; (p) periodate oxidation; (e) enzymic.

Sample	Chain length (average)	β-Amylolysis limit (%)	References
Horse	11–12 (m, p)	42	Bell (1937); Bell & Manners (1952)
Human	11 (p)	—	Halsall, Hirst & Jones (1947)
	12 (p)	41	Bell & Manners (1952)
	11 (p)	40	Liddle & Manners (1957)
Rabbit	13 (p)	—	Halsall *et al.* (1947)
	11–13 (m, p)	45	Bell (1948*a*); Bell & Manners (1952)
	11 (p)	39	Liddle & Manners (1957)
	15 (e)	—	Illingworth, Larner & Cori (1952)
	13 (p)	46	Manners & Wright (unpublished work)

therefore the significantly lower degree of branching, as determined by potassium periodate oxidation. This method is well established in carbohydrate chemistry for the analysis of non-reducing polysaccharides, and the possibility of any inherent error is reduced by the close agreement reported by other workers between methylation, enzymic and periodate-oxidation analyses of the same samples.

The potassium periodate-oxidation results in Table 2 are supported by the glycogen and P_M values.

The interaction of concanavalin-A and glycogen was first examined in detail by Cifonelli *et al.* (1956), who noted that the glycogen values of various glycogens were increased by β-amylolysis. They concluded that the reaction mainly involved the interior chains. However, it seems probable that other factors are also significant since measurement of the glycogen values of various human glycogens (from cases of glycogen-storage disease) have indicated an approximate relationship with the degree of branching (Calderbank *et al.* 1960). The glycogen values of the horse-muscle glycogens are significantly lower than those of 12–14 unit glycogens and are therefore consistent with the lower degree of branching and the relatively longer exterior chains.

The extent of degradation by α-amylase may, to a first approximation, be related to the degree of branching in the polysaccharide, since the interchain linkages and certain adjacent α-1:4-glucosidic linkages are resistant to enzyme action (Whelan & Bines, 1955). The observed trend of P_M values for the muscle glycogens is fully in accord with the significantly lower degree of branching, as the values are intermediate between those of a normal glycogen (average chain length 14, P_M 75) and an amylopectin (average chain length 22, P_M 89).

The iodine-staining power of mammalian-muscle glycogen is appreciably greater than that of liver or invertebrate glycogens (Manners, 1957). However, this feature of muscle glycogens, which has been noted by other workers (Young, 1937; Bell, 1948*b*), is not apparently directly related to the molecular structure. The iodine-staining data in Table 2 serve to characterize the muscle polysaccharides as 'glycogens' rather than 'amylopectins'. For example, the amylopectin component of malted barley starch has an average chain length of only 18 (Aspinall, Hirst & McArthur, 1955) but has $\lambda_{max.}$ 540 mμ and $E_{max.}$ 0·77 (unpublished data).

The results recorded in Tables 1 and 2 show that the glycogens remaining after the development of *rigor mortis* in horse muscles, and in ox psoas, do not differ significantly in molecular structure from the initial pre-rigor polysaccharides. On the other hand, in ox sternocephalicus muscle there is a significant shortening of the outer chains, from 12 to 9 glucose residues, during post-mortem glycolysis.

Biochemical and physiological implications

The biochemically significant quantity of glycogen which remains unattacked by the glycolysis system at a final pH of 6·0 in ox-sternocephalicus muscle appears to represent a molecular species having a shorter average external chain length than that initially present. In this instance, the presence of residual glycogen at a high final pH may be rationalized at the chemical level. Although in horse psoas and diaphragm residual glycogen at a high final pH is found, this does not apparently differ from the pre-rigor glycogens in these muscles. The pre- and post-rigor glycogens in these muscles need not be physiologically equivalent, however, since fractionation of a given glycogen sample can yield species of different molecular weight but similar branching characteristics (Stetten & Stetten, 1958). This may have physiological importance as glycogen of high molecular weight appears to be preferentially degraded *in vivo* and *in vitro* by muscle phosphorylase (Stetten *et al.* 1958; Larner, Ray & Crandall, 1956) and to exchange its glucose units more vigorously with the environment than that of low molecular weight (Stetten *et al.* 1956). In general, post-rigor glycogens would thus be expected to have a low molecular weight and a more compact molecular-weight distribution. If this is so, there would seem to be no relationship between the degree of branching and molecular size in the horse-muscle glycogens examined.

Dr W. A. J. Bryce has kindly determined the sedimentation constants of the l. dorsi muscle glycogens; the polysaccharides were polymolecular, values of 96 and 80×10^{-13} c.g.s. unit being obtained for the major components of the pre- and post-rigor samples, corresponding to molecular weights of 4·4 and $3{\cdot}7 \times 10^6$ [assuming a diffusion constant of $1{\cdot}50 \times 10^{-7}$ (Bell, Gutfreund, Cecil & Ogston, 1948)]. These results are qualitatively in agreement with the above suggestion.

In view of the similar average chain length values of the pre- and post-rigor glycogens from horse muscle and ox psoas, and assuming that the major pathway of glycogen breakdown involves phosphorylase, it is clear that the relative activity of phosphorylase and amylo-1:6-glucosidase is largely unaltered during post-mortem glycolysis. This may be contrasted with other metabolic conditions, e.g. glycogenesis, in which an alteration in activity is manifested by the presence of glycogen with relatively short exterior chains and a higher degree of branching (Manners, 1957). On the other hand, with ox-sternocephalicus muscle, the significant shortening of the outer chains from 12 to 9 glucose residues may indicate either a partial inactivation

of the debranching enzyme, or *in vivo* heterogeneity with respect to branching characteristics.

The concentration of glycogen in muscle has hitherto been recognized as an aspect of functional specialization, since it tends to be high in so-called white muscles, which derive energy for short bursts of activity by anaerobic glycolysis, and low in so-called red muscles, which are equipped for sustained energy production by respiration. That this relatively simple concept might be inadequate was indicated by the work of Bloom, Lewis, Schumpert & Shen (1950) and Kits van Heyningen & Kemp (1955). It now appears that distinct molecular species of glycogen, exhibiting different susceptibilities to glycogen breakdown, may occur in a given muscle. This presumably reflects some further aspect of functional specialization.

SUMMARY

1. The molecular structure of glycogen isolated by the Pflüger method from some mammalian muscles during the pre- and post-phases of *rigor mortis* has been examined by chemical and enzymic methods.

2. Glycogen from pre- and post-rigor horse-muscle tissues has an average chain length of approximately 17 glucose residues. This indicates a significantly lower degree of branching than is present in other samples of mammalian and invertebrate glycogen.

3. The glycogen isolated from ox-sternocephalicus muscle after the onset of *rigor mortis* has significantly shorter exterior chains than that isolated from the pre-rigor muscle. This could indicate either partial inactivation of the enzyme system catalysing the glycogen $\rightleftharpoons$ glucose 1-phosphate interconversion or a true heterogeneity of the glycogen in this muscle *in vivo*.

4. The chemical and physiological significance of these observations is discussed.

5. The possibility of the degradation of glycogen during alkaline extraction is considered.

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138. *Physicochemical Studies on Starches. Part XII.* The Molecular Weight of Glycogens in Aqueous Solution.*

By W. A. J. Bryce, C. T. Greenwood, I. G. Jones, and D. J. Manners.

Molecular weights are presented for 23 samples of glycogens isolated from various biological sources. Ultracentrifugal analysis showed that most of the samples were polydisperse. The molecular weights of the main components lie in the range (3—9) × 10^6. The polydisperse nature of the glycogens has been confirmed by light-scattering measurements. The effects of varying the isolation procedure, and of alkali, on the molecular weight have also been studied.

Glycogen and amylopectin are both highly branched, essentially α-1 : 4-linked glucosans. However, their hydrodynamic properties are completely different. This must be related to fundamental differences in fine structure and molecular shape.[1,2] In this paper, we describe the solution properties and hydrodynamic behaviour of glycogens isolated from a variety of biological sources. Estimations of molecular weight and its distribution have been obtained, and the effects of variations in the method of isolation, and of alkali, on the molecular weight have been studied. A preliminary account of some of this work has already appeared.[3]

Experimental

Sedimentation Measurements.—The methods described in Part XI [4] were employed. M- and 0·1M-sodium chloride and 0·2M-potassium hydroxide were used as solvents.

The sedimentation constant (S_{20}) was virtually independent of the solvent, and the majority of the measurements were carried out in either M- or 0·1M-sodium chloride. Results were corrected to water at 20°.

The apparent amount of each component in a resolvable polydisperse system was estimated by direct measurement of the areas under the refractive-index gradient curves. An enlarged image (3×) of the photographic plates was projected on smooth paper and the upper outline traced. An image of the base line (from a comparative run with solvent alone in the cell) was then superimposed by alignment of the reference lines, and traced on. The refractive-index gradient curves were carefully divided, in the usual manner, on the assumption that each component had a symmetrical distribution, and the appropriate areas between the peaks and the base-line were measured with a planimeter. Values were expressed to the nearest 5%.

Estimations of the polymolecularity of the major component of some of the glycogen samples were obtained by using Gralen's function,[5] dB/dX, where B is an estimate of the "width" of the sedimentation gradient curve and is equal to H/A (A = area of the Schlieren diagram; H = the height of the maximum ordinate), and X = the distance of the peak from the axis of rotation. In all instances, B varied linearly with X. Although this function should be extrapolated to infinite dilution, the value at c = 1 g. per 100 ml. was taken as a standard for comparison of the polymolecularity of different samples.

Diffusion Measurements.—The method is outlined in Part X.[6] The solvent was 0·1M-sodium chloride, and values of the diffusion constant (D_m) were calculated by the moment method.

Partial Specific Volume.—The partial specific volume ($\bar{V}$) of glycogen was taken as 0·62, the value calculated from density measurements on aqueous solutions of one sample.

Light-scattering Measurements.—The apparatus and the methods used to clarify and dilute the glycogen solutions were similar to those previously described for the *Zea mays* polysaccharides,[6] 0·1M-sodium chloride being the solvent. Although 15% aqueous magnesium chloride has been recommended,[7,8] we found this solvent to have no advantages. The value of the molecular weight of a given sample was the same in both the above solvents. Glycogen

* Part XI, preceding paper.

solutions were clarified by careful filtration (cf. ref. 8) through sintered glass (G4). Repeated filtration caused some small loss in turbidity, whilst little improvement occurred in the apparent dissymmetry. (For example, a sample after one filtration had $M = 8{\cdot}4 \times 10^6$, dissymmetry = 1·41; after five filtrations, $M = 7{\cdot}7 \times 10^6$, dissymmetry = 1·34, the concentration being assumed to be unchanged by filtration.) Solutions were therefore filtered once, before dilution. This procedure gave reproducible results. Hc/τ was independent of c for all samples. The particle scattering factor ($P_{90°}$) was calculated from the dissymmetry, the molecules being assumed to be spherical.[6] The refractive-index increment (dn/dc) for glycogen was found to be 0·146 (c = g./ml.) in 0·1M-sodium chloride at 546 mμ.

Isolation of Glycogens.—Unless otherwise stated, samples of glycogen had been isolated from the tissue by the classical Pflüger method involving digestion with 30% aqueous potassium hydroxide at 100°, followed by precipitation of the glycogen with ethanol and with acetic acid.[9] Commercial samples of glycogen from British Drug Houses Ltd. (I), and Nutritional Biochemicals Corporation, Ohio, U.S.A. (II), were also examined. Methylated horse-muscle glycogen was kindly provided by Dr. D. J. Bell.

Results and Discussion

Sedimentation Coefficients.—Typical sedimentation data are shown in Table 1. It was apparent that for all the glycogens studied in detail, the sedimentation constant (S_{20}) was dependent on the concentration (c), and varied by about 10% for a 1% change in concentration. This is in general agreement with Larner, Ray, and Crandall's results,[10] but, whilst these authors suggested that S_{20} was a function of c^2, our values were best

Table 1. *Typical sedimentation results.*

		$10^{13}S_{20}$ at c (g./100 ml.)							
Glycogen sample	Solvent	1·0	0·75	0·50	0·25	0·16	0·125	0·08	0 (extrapol.)
Ascaris lumbricoides ...	0·1M-NaCl	47	47	47	48	—	—	—	48
Brewer's yeast	0·1M-NaCl	56	—	60	61	—	62	—	64
,, ...	1M-NaCl	54	—	—	—	—	—	—	—
,, ...	0·2M-NaOH	56	58	60	61	62	—	63	64
Commercial, II	0·1M-NaCl	65	67	69	71	—	—	—	73

represented by a linear function. The relations was expressed by $S_{20} = (S_{20})_0(1 - kc)$, where $(S_{20})_0$ is the value of S_{20} at infinite dilution, and c was expressed in g./100 ml. With the exception of the *Ascaris lumbricoides* glycogen (which was relatively concentration-independent; see Table 1), the average value of k was $0{\cdot}10 \pm 0{\cdot}02$. Values of $(S_{20})_0$ for glycogens examined at only one concentration were therefore calculated from this value, and are shown in parentheses in the second and third columns of Table 2.

Molecular Weight and its Distribution.—Table 2 shows the results of the sedimentation measurements for the 23 samples examined. Typical sedimentation diagrams are shown in the Figure. Most samples proved to be polydisperse on ultracentrifugation. Diagrams *a* and *b* (for oyster and *Helix pomatia* glycogen) illustrate the type of Schlieren diagram observed for the most obviously polydisperse samples. This feature is unusual, although Polglase, Brown, and Smith [11] reported similar results for samples of human-liver glycogen. The amounts of main components quoted in the Table are only approximate as no attempt was made to correct for boundary anomaly effects.[12] For many samples, an extremely wide molecular-weight distribution was indicated; the leading sedimentation boundary was extremely asymmetric and reached nearly to the bottom of the cell after a short time of centrifugation. It was difficult to prove whether or not this leading boundary was a second component, and hence no attempt was made to estimate either its amount or its approximate sedimentation constant. Samples which showed this probable fast component are indicated by the symbol S_{20}(F) in the Table. In some other samples, a corresponding asymmetric lower molecular weight distribution was apparent. Again, no analysis of this was attempted and this is indicated by the symbol S_{20}(S) in Table 2.

Diffusion measurements showed that for methylated horse muscle the diffusion coefficient (D_m) = $1{\cdot}0 \times 10^{-7}$; for brewer's yeast glycogen, $D_m = 1{\cdot}1 \times 10^{-7}$; for com-

mercial glycogen I, $D_m = 2{\cdot}0 \times 10^{-7}$; and for commercial glycogen II, $D_m = 1{\cdot}1 \times 10^{-7}$ The molecular weights shown in Table 2 for the main components in the other samples are calculated by assuming a value of $1{\cdot}1 \times 10^{-7}$ for D_m in agreement with other workers.[2] All the molecular weights are in the range (3—9) $\times 10^6$, and, together with the values of the frictional ratio (f/f_0), are of the same order as those previously reported from sedimentation and diffusion measurements.[2,13] It should be noted, however, that the values for

Typical sedimentation diagrams. For all samples, c = 1 g./100 ml.; solvent, 1·0M-sodium chloride; speed = 20,000 r.p.m. Movement of the peaks is from right to left. The figures in parentheses after the times indicate the angle of the Schlieren bar.

Oyster glycogen: 8 (55°), 15 (45°), 18 (35°), 30 (30°), *and* 47 (25°) *min. after reaching full speed.*

Helix pomatia *glycogen*: 6 (65°), 14 (45°), 25 (45°) 37 (45°), *and* 50 (35°) *min. after reaching full speed.*

Brewer's yeast glycogen: 9 (65°), 17 (50°), 26 (45°), 37 (40°), *and* 52 (40°) *min. after reaching full speed.*

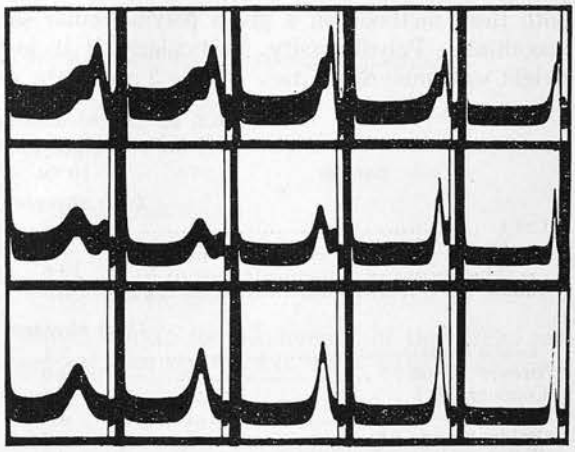

TABLE 2. *Sedimentation results.*

Glycogen sample	$10^{13}S_{20}$ of components [a] major	minor	Major component (%)	dB/dx [b]	$10^{-6}M$ [c]	f/f_0 [d]
	(a) *Mammalian livers*					
Cat I	75	F, S	—	0·8	4·4	—
,, IV	84	F, S	—	—	4·9	—
,, VI	(102)	F, S	—	1·0	5·9	—
Human (glycogen-storage disease)	(53)	(220)	70	1·8	3·1	—
Fœtal sheep	110	F	—	1·1	6·4	—
Fœtal pig	(49)	(11)	70	0·8	2·9	—
Rabbit II	94	—	95+	—	5·5	1·7
,, (fructose-infused *)	(80)	F	—	1·1	4·7	—
,, (galactose-infused *)	(153)	S	—	—	9·0	—
,, (normal *)	(145)	F	—	1·8	8·4	—
	(b) *Mammalian muscles*					
Horse (methylated)	23	—	95+	—	2·8	1·4
Human	(85)	(20)	85	0·8	4·9	—
Rabbit I	79	—	95+	0·7	4·6	1·9
	(c) *Other glycogens*					
Ascaris lumbricoides	48	F	—	—	2·8	—
Brewer's yeast	64	—	95+	—	3·7	2·0
Commercial, I	24	—	95+	—	0·7	1·9
,, II	73	—	95+	—	4·0	1·7
Helix pomatia II	(63)	(300, 7)	80	0·9	3·6	—
Mytilus edulis I	(93)	F	—	0·9	5·4	—
Oyster *	(45)	(90, F)	—	—	2·6	—
Tetrahymena pyriformis I	(69)	S	—	—	4·0	—
Trichomonas foetus I	(70)	S	—	—	4·0	—
Trichomonas gallinae I	(84)	S	—	—	4·9	—

[a] For values in parentheses and meaning of F and S, see text. [b] Value for main component at *c* (total) = 1·00 g./100 ml. [c] Molecular weight calculated from $M = \boldsymbol{R}T(S_{20})_0/(1 - \bar{V}\rho)D_m$. [d] Frictional ratio calculated from $f/f_0 = 10^{-8}[(1 - \bar{V}\rho)/D_m{}^2(S_{20})_0\bar{V}]^{1/3}$.

* Samples kindly supplied by Dr. M. Schlamowitz.

rabbit liver and muscle are considerably lower than those recently reported by Stetten, Katzen, and Stetten [8] (see below).

The values of (dB/dx) confirm qualitatively the extremely polymolecular nature of glycogen (cf. ref. 5) in agreement with the distributions evaluated by Larner and his co-workers.[10] Further, in agreement with these authors, mammalian-muscle glycogens appear to be less polymolecular than liver glycogens.

The polydisperse nature of most of the samples studied was confirmed by turbidimetric measurements. Although the molecular weight from these measurements is a true weight-average whilst that from sedimentation and diffusion is less well-defined,[14] the results from both these methods on a given polymolecular sample should be of the same order of magnitude. Polydispersity, particularly if it involves components of high molecular weight will cause disparities. Table 3 shows the results of light-scattering measurements

TABLE 3. *Light-scattering results.*

Sample	Uncorr. 10^{-6}M	Dissymmetry	$1/P_{90°}$	Corr. 10^{-6}M
Liver glycogens				
Cat I	10·5	1·48	1·30	13·6
,, IV	8·8	1·85	1·52	13·4
,, VI	12·8	1·67	1·40	17·9
Rabbit II *	6·9	1·20	1·13	7·8
Other glycogens				
Ascaris lumbricoides	7·1	1·40	1·26	8·9
Brewer's yeast *	4·0	1·15	1·10	4·4
Commercial, I	1·7	1·19	1·12	1·9
,, II *	4·9	1·15	1·10	5·4
Rabbit muscle I *	3·7	1·17	1·11	4·1
Tetrahymena pyriformis I	6·1	2·50	1·85	11·3

* Samples exhibiting no polydispersity.

on the ten samples which appeared to be the least obviously polydisperse on ultracentrifugation. For four of these, the agreement is reasonably good, indicating that they were only polymolecular, whilst the presence of S_{20}(F) in the other samples is convincingly illustrated by the higher turbidimetric molecular weight. It is therefore suggested that a given glycogen sample should be examined by both the sedimentation and the light-scattering method in order to prove unambiguously whether or not it is polydisperse. Without further investigations, it is not possible to decide whether polydispersity occurs in native glycogen in the tissue or is an artefact resulting from degradation during isolation. Polglase and his co-workers [11] consider that such variations occur naturally.

Effect of Isolation Procedure on Molecular Weight.—The classical Pflüger method involving digestion of tissue with 30% potassium hydroxide has often been criticised [15] on the assumption that alkaline degradation occurs. Table 4 shows the results for the determination of S_{20} for glycogen samples isolated from the halves of two rabbit livers severally with boiling water and 30% aqueous potassium hydroxide. Within experimental error, S_{20} is the same for all samples. It is concluded that, in the presence of air, the extent of degradation of glycogen by 30% potassium hydroxide solution at 100° is no greater than that which might be caused by boiling water. Similar results have been obtained by Staudinger,[16] and Bridgman [17] reported that glycogen extracted with cold trichloroacetic acid and hot alkali from two halves of a rabbit liver had a similar molecular weight. However, recent light-scattering work by Stetten, Katzen, and Stetten [8] has shown that if extraction with trichloroacetic acid is for a limited time at 0° the glycogen from rabbit liver has an average molecular weight of $(11—80) \times 10^6$ rather than the $(2—6) \times 10^6$ as in hot potassium hydroxide extractions. This suggests that it is difficult to avoid degradation during extraction, and that the molecular weights reported here and previously [2] may not be representative of " native " glycogen.

Effect of Dilute Alkali and Acetic Acid.—In contrast to the behaviour of hot 30% alkali,

hot *dilute* alkali appears to degrade glycogen rapidly. Digestion of rabbit-liver glycogen in 8% aqueous sodium hydroxide at 100° for 1·5 hr. reduced S_{20} from 86 to 57 $\times$ 10^{-13} c.g.s. units (see Table 4), and increased the polymolecularity (as shown by a broadening of the peak of the Schlieren pattern).

It has been suggested [18] that purification of glycogen by precipitation with glacial acetic acid may render it unsuitable for ultracentrifugal analysis. However, when rabbit-liver and brewer's yeast glycogens were reprecipitated with 80% acetic acid there was no change in the value of S_{20} (see Table 4). Precipitation of glycogen by acetic acid does not, therefore, alter the hydrodynamic properties or cause degradation of glucosidic linkages to any appreciable extent.

TABLE 4. *Effect of isolation procedure on the sedimentation constant.*

Sample	Method of isolation	$10^{13}S_{20}$ at c = 1 g./100 ml.
Rabbit liver XII	Hot water	85
	30% KOH at 100°	86
Rabbit liver XIII	Hot water	76
	30% KOH	83
	30% KOH + repptn. with AcOH	83
Rabbit liver IV	30% KOH	86
	30% KOH + 8% NaOH at 100° for 1½ hr.	57
Brewer's yeast	30% KOH	64
	30% KOH + repptn. with AcOH	63

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15.

Biochemical Investigation of a Case of Glycogen-Storage Disease (von Gierke's Disease)

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Glycogen-storage disease is characterized by the deposition of unusually large quantities of glycogen, particularly in the liver and kidneys. In a survey of a number of cases, Cori (1952–53) has recognized the existence of four types of the disease. In type 1 (also known as von Gierke's disease), the physical properties and the chemical structure of the glycogen appear to be normal and the biochemical defect lies in a striking deficiency of glucose 6-phosphatase both in the liver and kidney. Type 2 is shown by the generalized deposition of glycogen in many tissues, notably in heart and skeletal muscle. The glycogen has a normal structure, glucose 6-phosphatase is present and the biochemical abnormality has not so far been defined. In types 3 and 4, of which few cases are known, the glycogen accumulated in the liver and kidney has an unusual structure; in type 3 it has very short outer chains and in type 4 the glycogen resembles amylopectin in having relatively long inner and outer branches.

The present studies are concerned with the investigation of a fatal case of the disease in which the molecular structure of the liver and kidney glycogen and the enzymic activity of the liver have been examined. The patient (A.K.) was a sibling of one (S.K.) investigated earlier (Manners, 1954), which corresponded to type 3 of Cori's classification. This

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liver glycogen (S.K.) had an average chain length of only six glucose residues (i.e. a degree of branching twice that of a normal glycogen), the molecular structure resembling that of a phosphorylase-limit dextrin. In this case, a deficiency in amylo-1:6-glucosidase was probable. Some additional properties of the S.K. glycogen are also reported.

METHODS AND MATERIALS

Normal liver. This was from a 2-year-old child who died after a short illness. The liver was removed 21 hr. after death and stored at −18°.

Diseased liver. Tissues were removed from the refrigerated body of a 4-year-old boy (A.K.) 50 hr. after death and were stored at −18°. A portion of the tissue was dropped into boiling water and heated for 10 min. Glycogen was later extracted from this boiled tissue (see Results section).

Dipotassium D-*glucose* 6-*phosphate.* This was prepared from the barium salt (104 mg.; C. F. Boehringer und Söhne, Mannheim, Germany) in water (1 ml.) by addition of solid K_2SO_4 (38 mg.). The solution was centrifuged and the supernatant fluid was made up to 2 ml. with 0·2 M-potassium citrate buffer, pH 6·5.

Dipotassium α-D-*glucose* 1-*phosphate.* This was obtained by phosphorolysis of starch. A solution (0·02 M) made in 6 mM-$MgSO_4$ was used.

Triphosphopyridine nucleotide. This was kindly given by Dr H. L. Kornberg.

Analytical methods

Glucose content. The glycogens, 0·1% concentration, were hydrolysed in 2 N-H_2SO_4 at 100° for 2 hr. and the reducing sugar was determined with the Somogyi (1952) reagent.

Paper chromatography. Descending chromatograms were carried out at room temperature on Whatman no. 1 paper with ethyl acetate–pyridine–water (10:4:3, by vol.) as solvent, and aniline oxalate as development reagent.

Absorption spectra of iodine complexes. The conditions of Peat, Whelan, Hobson & Thomas (1954) were used.

Periodate oxidation. The glycogens were oxidized at room temperature with potassium metaperiodate (Bell & Manners, 1952). The liver glycogen was also oxidized with a limited excess of sodium metaperiodate under the conditions of Manners & Archibald (1957).

Glycogen value. A solution of concanavalin A was prepared from a 0·9% NaCl extract of jack-bean meal (Cifonelli & Smith, 1955). For the determination of glycogen values, 9 ml. of the above solution was added to 1 ml. of aqueous glycogen solution containing 0·1–1·0 mg. of polysaccharide, and the extinction measured after 10 min. at 420 mμ with a Unicam SP. 500 spectrophotometer. The extinction given by 1 mg. of glycogen under these conditions was compared with that of a standard sample of rabbit-liver glycogen (glycogen value 1·00) kindly provided by Professor F. Smith (see Cifonelli, Montgomery & Smith, 1956).

Viscosity measurements. The viscosity of glycogen solutions of various concentrations was measured at 25° in a modified Ubbelohde viscometer. The limiting viscosity number $[\eta]$ was obtained by linear extrapolation of η sp./C to zero concentration (C as g./ml.).

Phosphate estimations. These were performed by the method of Berenblum & Chain (1938).

Enzymic degradation: α- and β-amylolysis. The enzyme preparations and conditions of hydrolysis were those described by Liddle & Manners (1957). α-Amylolysis results are given as P_M values, i.e. apparent percentage conversion into maltose.

Isoamylase. This enzyme (cf. Manners & Khin Maung, 1955) was extracted from brewer's yeast by Miss Z. H. Gunja.

Enzymic assays

Glucose 6-*phosphatase estimation.* Homogenates were prepared by grinding 1 g. of abnormal liver in a chilled mortar. The homogenate was stirred with ice-cold glass-distilled water, filtered through muslin and the filtrate made up to 3·5 ml. A homogenate of normal liver (800 mg.) was prepared similarly except that the final volume of the supernatant fluid was 1·8 ml. Incubations were carried out at 30° for 15, 30 or 60 min., when the reaction was terminated by the addition of 1 ml. of trichloroacetic acid (10%, w/v). Water (1 ml.) was then added and the mixture was centrifuged (445 *g* for 5 min.). A sample (1 ml.) of the supernatant liquid was withdrawn for determination of phosphate.

Phosphoglucomutase estimation. Abnormal liver (1 g.) was homogenized with glass-distilled water (3 ml.) in a Potter glass homogenizer. The product was centrifuged (30 min. at 10 000 *g*) and the supernatant liquid was used for enzyme assay immediately, and after standing for 24 hr. at 2° and being diluted 10-fold, 100-fold and 500-fold with the cysteine buffer. Glucose 1-phosphate was incubated at 30° with the enzyme preparation (0·1 ml.), in the presence of 0·05 M-cysteine brought to pH 7·5 by addition of 0·1 N-NaOH, for 5 or 10 min. The reaction was terminated by the addition of 5 N-H_2SO_4 (1 ml.) and the solution was diluted with water to a final volume of 5 ml. The resulting solution was heated on a boiling-water bath for 3 min., cooled, centrifuged (445 *g*, 3 min.) and 1·5 ml. of the supernatant was withdrawn for determination of phosphate.

Glucose 6-*phosphate dehydrogenase estimation.* Abnormal liver (1 g.) was homogenized with water (4·5 ml.) in an all-glass apparatus at 0° and centrifuged (3500 rev./min.) for 20 min. The supernatant liquid was used for enzyme assay. In parallel experiments, normal liver (850 mg.) was homogenized in water (2·6 ml.) in the same way. Incubation mixtures were prepared containing 0·25 M-glycylglycine (pH 7·6, 3·5 ml.); 0·1 M-$MgCl_2$ (3·5 ml.); water (5·6 ml.); enzyme preparation (0·7 ml.); triphosphopyridine nucleotide (TPN; 3·5 ml. containing 1·4 mg.). For the 'zero time' reading (i.e. immediately after addition of the enzyme), 2·4 ml. of the mixture was mixed with ethanol (2·4 ml.) and 10% (w/v) Na_2SO_4 (0·1 ml.). After 15 min. the solution was centrifuged (2000 rev./min.) and the extinction of the supernatant was measured at 340 mμ. The reaction commenced when 0·05 M-dipotassium glucose 6-phosphate (0·6 ml.) was added to the remaining incubation mixture at 20°. Samples (2·4 ml.) were withdrawn and investigated at various intervals in the manner described. Spectral measurements were made in comparison with an 'enzyme blank' identical with 'zero time' incubation mixture save that the TPN was substituted by an equivalent volume of water.

RESULTS

Isolation and purification of A.K. glycogen

The boiled liver tissue (243 g.) was ground with sand and extracted three times with boiling water in an atmosphere of nitrogen. The combined extracts were cooled to 0° and deproteinized by the addition of 0·1 vol. of 40 % (w/v) trichloroacetic acid. After removal of protein, the glycogen was precipitated with ethanol, purified by five further precipitations and dried with ether. Yield, 18·9 g. (equivalent to 7·8 % yield from the tissue).

Glycogen was similarly extracted from boiled kidney tissue (78 g.). Yield 3·4 g. (i.e. 4·4 % yield from the tissue). Extraction of the kidney residue with 30 % (w/v) KOH at 100° did not yield any additional glycogen.

Boiled brain and muscle tissue were extracted successively with water and 30 % KOH at 100°. No glycogen could be isolated.

Characterization of purified A.K. glycogens

The liver glycogen had $[\alpha]_D + 196°$ (*c*, 0·2 in water), and on acid hydrolysis gave glucose (96 %) and no other sugar. The aqueous solution stained deep red–brown with iodine ($\lambda_{max.}$ 460–465 mμ; $E_{max.}$ 0·21).

The kidney glycogen had the following properties: $[\alpha]_D + 200°$ (*c*, 0·2 in water); glucose content 98 %; iodine complex, $\lambda_{max.}$ 445–450 mμ, $E_{max.}$ 0·19.

Periodate oxidation. Liver glycogen (262·2 mg.) gave 5·06, 5·11 and 5·11 mg. of formic acid after potassium metaperiodate oxidation for 9, 14 and 16 days. The final figure corresponds to an average chain length of 14·6 glucose residues. In a duplicate experiment, 261·0 mg. of liver glycogen gave 5·17 mg. of formic acid on complete oxidation, equivalent to average chain length 14·5.

Kidney glycogen (253·7 mg.) was similarly oxidized; the final production of formic acid, after 17 days, was 5·06 mg., equivalent to average chain length 14·3. In a repeat oxidation, 253·7 mg. of glycogen gave 5·07 mg. of formic acid, i.e. average chain length 14·1.

Liver glycogen (100·3 mg.) in water (22 ml.) was then oxidized at 2° with 3 ml. of 8 % (w/v) sodium metaperiodate. The final production of formic acid, after 20 days' oxidation, was 2·0 mg., corresponding to an average chain length 14·4 glucose residues.

Glycogen values. The measurement of the extinction of 1 mg. of A.K. glycogen in the presence of concanavalin-A gave glycogen values: liver glycogen, 0·97; kidney glycogen, 1·01. Samples of amylopectin isolated from potato starch (var. Kerr's Pink) and the starch from the freshwater alga *Dunaliella bioculata* (Eddy, Fleming & Manners, 1958) did not react with concanavalin-A.

Limiting viscosity number. In 0·1 M-NaCl solution, the liver and kidney A.K. glycogens had $[\eta]$ 8·3 and 5·3 respectively. These figures are about one-twentieth of those for amylopectin.

Enzymic degradation

α-Amylolysis. The results of a comparison of the action at 37° of purified salivary α-amylase on the human glycogens, and on control samples of rabbit-liver glycogen (average chain length 13·8) and potato amylopectin (average chain length 22·0) are shown in Table 1.

β-Amylolysis. On treatment with barley β-amylase at pH 4·6 and 37°, the liver and kidney glycogens both had a β-amylolysis limit of 46 %. Under similar conditions, the rabbit-liver glycogen and potato amylopectin gave 53 and 61 % conversion into maltose.

β-Amylolysis after pretreatment with isoamylase. The glycogens (26–28 mg.) were incubated with isoamylase (50 mg.) at pH 5·9 for 24 hr. at 20° in a total volume of 10 ml. The enzyme was inactivated by heating; 5 ml. of 0·2 M-acetate buffer, pH 4·6, β-amylase (2600 units) and water to 25 ml. were then added. After 24 hr. at 37° the β-amylolysis limits were: liver glycogen 60 %, kidney glycogen 59 %. Potato amylopectin on similar treatment had a β-amylolysis limit of 77 %. The outermost branch points in these polysaccharides are therefore of the α-1:6-glucosidic type.

Further investigation of S.K. glycogen

This glycogen, the structure of which has already been discussed by Manners (1954), gave a pale-yellow–brown stain with iodine and the solution did not show an absorption peak in the region 440–480 mμ; at 430 mμ the extinction was only 0·05. The glycogen had glycogen value 1·61, $[\eta]$ 10·6 in 0·1 M-NaCl and, on α-amylolysis (see Table 1), the P_M values were 24, 27 and 33 respectively. After treatment with isoamylase, the β-amylolysis limit was 20 %.

Table 1. *Action of salivary α-amylase on polysaccharides*

The polysaccharides (0·5 mg./ml.) were incubated at 37° with freeze-dried α-amylase (2·3 units/ml.) in the presence of 0·05 % NaCl in a total volume of 100 ml. Samples (5 ml.) were analysed, at intervals, for reducing sugar (as maltose).

	P_M		
Time of incubation (hr.) ...	2·5	5	24
Liver glycogen (A.K.)	62	66	74
Kidney glycogen (A.K.)	63	67	76
Rabbit-liver glycogen	65	69	75
Potato amylopectin	74	79	89

Determination of liver-enzyme activities

A survey of the activity of four enzymes, namely glucose 6-phosphatase, phosphoglucomutase, glucose 6-phosphate dehydrogenase and phosphohexoisomerase, was carried out on homogenates of the diseased (A.K.) liver. Results of six experiments showed that with incubation times between 15 and 30 min. the tissue hydrolysed glucose 6-phosphate at a rate of 1·81–2·72 μmoles/hr./g. of frozen tissue. Under identical conditions, enzyme preparations of normal liver hydrolysed the substrate much more quickly, at a rate of 129·8–130·0 μmoles/hr./g. of frozen tissue.

Table 2. *A comparison of the properties of glycogens from glycogen-storage disease*

Property	Kidney glycogen (A.K.)	Liver glycogen (A.K.)	Liver glycogen (S.K.)
$[\alpha]_D$ (water)	+200°	+196°	+201°
Iodine complex $\lambda_{max.}$ (mμ)	445–450	460–465	—
Glycogen value	1·01	0·97	1·61
Average chain length (glucose residues)	14·2	14·5	6·1
α-Amylolysis limit (%)	76	74	33
β-Amylolysis limit (%) (*b*)*	46	46	14
β-Amylolysis limit (%) (*a*)*	59	60	20
Molecular weight (see Addendum)	7×10^6	7×10^6	2×10^6
Limiting viscosity number	5	8	11

* (*b*) Before and (*a*) after treatment with isoamylase.

Table 3. *Glucose 6-phosphatase activity of abnormal- and normal-liver homogenate*

In these experiments the substrate was 0·1 M-dipotassium glucose 6-phosphate (0·1 ml.) in 0·1 M-potassium citrate buffer (pH 6·5). The enzyme preparations are as described in the Methods and Materials section. Final volume, 0·5 ml. Results of duplicate experiments are shown in parentheses.

Additions	Duration of incubation (min.)	Phosphate found (μg.)	Phosphate liberated/hr./g. of frozen tissue (μmoles)
Abnormal liver (A.K.)			
Buffer (0·2 ml.); enzyme (0·2); water (0·1 ml.)	60	32·5	—
Buffer (0·2 ml.); substrate; water (0·2 ml.)	60	3·75	—
Buffer (0·2 ml.); substrate; enzyme (0·2 ml.)	0*	37·2	—
Buffer (0·2 ml.); substrate; enzyme (0·2 ml.)	15	38·0 (38·4)	1·81 (2·72)
Buffer (0·2 ml.); substrate; enzyme (0·2 ml.)	30	39·5 (39·5)	2·60 (2·60)
Buffer (0·2 ml.); substrate; enzyme (0·2 ml.)	60	42·0 (42·0)	2·72 (2·72)
Normal liver			
Buffer (0·3 ml.); enzyme (0·1 ml.); water (0·1 ml.)	15	40·0	—
Buffer (0·3 ml.); substrate; water (0·1 ml.)	15	12·25	—
Buffer (0·3 ml.); substrate; enzyme (0·1 ml.)	15	97·0	129·8
Buffer (0·3 ml.); enzyme (0·1 ml.); water (0·1 ml.)	30	38·9	—
Buffer (0·3 ml.); substrate; water (0·1 ml.)	30	13·7	—
Buffer (0·3 ml.); substrate; enzyme (0·1 ml.)	30	148·8	130·0

* Zero-time control; trichloroacetic acid was added before the enzyme.

Table 4. *Phosphoglucomutase activity of abnormal liver*

The reaction mixtures were made with 0·02 M-dipotassium α-glucose 1-phosphate (0·1 ml.) as substrate. The buffer mixture contained 0·05 M-cysteine (0·2 ml., pH 7·5) and 6 mM-$MgSO_4$ (0·1 ml.). The enzyme preparation is as described in the Methods and Materials section. Final volume, 0·4 ml.; incubated at 30°. Results of duplicate experiments are shown in parentheses.

Additions	Duration of incubation (min.)	Phosphate liberated (μg.)	Hydrolysable phosphate used (μmoles)
Enzyme; buffer	10	26·65	—
Enzyme; buffer; substrate*	10	93·0	—
Substrate; buffer; water (0·1 ml.)	10	67·7	—
Enzyme; substrate; buffer	5	31·3 (29·3)	2·04 (2·10)
Enzyme; substrate; buffer	10	31·3 (30·0)	2·04 (2·08)

* Zero-time control; sulphuric acid was added before the enzyme.

Table 5. *Phosphoglucomutase activity of aged and diluted enzyme preparations of abnormal liver*

Tubes contained 0·05 M-cysteine (0·2 ml.) and enzyme (0·1 ml., appropriate preparations are described in the Methods and Materials section). In some cases, 6 mM-$MgSO_4$ (0·1 ml.) was also added. Final volume, 0·4 ml.; incubated at 30° for 5 min.

Additions	Hydrolysable P remaining (μg.)	Glucose 1-phosphate used (μmoles/hr./g. of frozen tissue)
Enzyme (1:10); $MgSO_4$	7·37	—
Enzyme (1:100); $MgSO_4$	4·37	—
Glucose 1-phosphate; water (0·1 ml.)	68·2	—
Glucose 1-phosphate; enzyme (1:10)	40·7	5088
Glucose 1-phosphate; enzyme (1:100)	63·0	1536
Glucose 1-phosphate; enzyme (1:500)	66·0	1704

Table 6. *Glucose 6-phosphate dehydrogenase activity of abnormal and normal liver*

The enzyme preparation and incubation mixture is described in the Methods and Materials section; 0·24 μmole of TPN was present.

Time (min.)	TPN reduced (μmoles/g. of frozen tissue): Abnormal liver	Normal liver
0	—	—
2	2·32	0·49
5	3·06	2·78
10	5·36	6·30
15	7·37	6·42
20	8·85	7·73

On the other hand, the diseased tissue was found to possess a high level of phosphoglucomutase activity. The enzyme activity was measured by the method of Najjar (1948), in which an enzyme preparation is incubated with dipotassium α-glucose 1-phosphate and the residual glucose 1-phosphate is estimated by acidic hydrolysis and determination of the liberated inorganic phosphate.

Table 4 shows the rapid action on glucose 1-phosphate in 10 min. at 30°. A further experiment (Table 5), in which the enzyme preparation was diluted (1:10), showed that the substrate is used at a rate of 5088 μmoles/hr./g. of frozen tissue. At higher dilutions (1:100; 1:500), the rate fell to 1536 and 1704 μmoles/hr./g. of frozen tissue respectively [Weber & Cantero (1957) give values for 37°].

Because of the turbidity of homogenates of the diseased liver, a modification of the method of Glock & McLean (1953, 1956) was used for determination of the glucose 6-phosphate dehydrogenase activity of the tissue. In this modification, the enzymic reaction was terminated by the addition of ethanol, and addition of Na_2SO_4 facilitated flocculation of the opalescent substance. The rate of reduction of TPN by the diseased liver was 3·06 μmoles/g. of frozen tissue in 5 min., in contrast with a value of 2·78 μmoles/g. of frozen normal tissue.

A qualitative investigation of the phosphohexoisomerase activities of normal- and diseased-liver homogenates by the method of Glock & McLean (1956) showed that both tissues were highly active.

Glycogen content of liver and other tissues

The content of glycogen in the abnormal muscle and liver was determined by the method of Kemp & Kits van Heijningen (1954) (performed by Dr A. J. M. van Beusekom-Kits van Heijningen). No fixed glycogen was to be found in the muscle, and, in the liver, the amount of glycogen extracted by cold trichloroacetic acid varied between 9·91 and 10·50 %, and a further 0·53–0·67 % of non-extractable polysaccharide remained. The amount of free sugars (including glucose 1-phosphate) was 0·06–0·09 % (Table 7).

The nitrogen content (Kjeldahl) of homogenates of the diseased liver was 21–23 mg. of nitrogen/g. of frozen tissue, whereas for the normal liver the values were 28·6–29·2 mg. of nitrogen/g. of tissue.

DISCUSSION

The present case of glycogen-storage disease is characterized by the accumulation of liver and kidney glycogen which appears to have a 'normal' structure. Glycogen is not deposited in the skeletal muscles, i.e. glycogenesis is not generalized, as in the type 2 disease. Although samples of human liver and kidney glycogen from comparable normal tissues were not available for study, the branching properties of the A.K. glycogens are similar to those of the majority of mammalian glycogens hitherto examined (for a review of glycogen structure, see Manners, 1957).

The interaction of the A.K. glycogens with iodine and concanavalin-A, the average chain length determined by periodate oxidation, the α-amylolysis limit and the low limiting viscosity number differentiate them from amylopectins, and hence eliminate the possibility of a deficiency in amylo-1:4 → 1:6-transglucosidase (branching

Table 7. *Glycogen content of diseased liver (A.K.)*

The pairs of figures represent the results of duplicate experiments.

	Free glycogen* (%)	Fixed glycogen (%)	Total (%)	Free sugars† (%)	
				Anthrone	H_2SO_4 reaction
Immediately deproteinized	10·21, 10·50	0·53, 0·63	10·74, 11·03	0·09, 0·06	0·06, 0·06
Stored for 9 hr. at room temp.	9·91, 10·38	0·60, 0·67	10·58, 10·95	0·09, 0·09	0·09, 0·09

* Values for cold trichloroacetic acid extract corrected for free sugar values.
† 80% (v/v) methanol extracts.

enzyme) as the biochemical lesion (type 4 disease). The extent of β-amylolysis shows that the outer chains contain, on the average, about nine glucose residues and are clearly much longer than those in phosphorylase-limit dextrins. The above reactions are consistent with the view that the present case corresponds to type 1 disease, i.e. a true von Gierke's disease. It follows that the probable enzymic deficiency is of glucose 6-phosphatase and experimental investigations have shown that this is in fact the case.

The previous conclusions (Manners, 1954) on the unusually high degree of branching in the S.K. glycogen have been confirmed. The significantly greater reaction with concanavalin-A, and lower P_M value on α-amylolysis are to be expected; the extremely weak iodine-binding power from measurements of absorption spectra is also of interest. The S.K. and A.K. liver glycogens thus show very marked differences in molecular structure (see Table 2). It is unlikely that these glycogens were markedly changed during their isolation, particular precautions (exclusion of oxygen, etc.) being taken to minimize degradation.

The 'normal' structure of the A.K. glycogen suggests that the biochemical lesion does not reside in a deficiency of amylo-1:6-glucosidase, as has been found by Illingworth & Cori (1952) and Illingworth, Cori & Cori (1956) for some forms of glycogen-storage disease characterized by glycogen accumulation in the heart and kidneys as well as in the liver.

A survey of the activities of glucose 6-phosphatase, phosphoglucomutase, glucose 6-phosphate dehydrogenase and phosphohexoisomerase in the diseased liver revealed that the first of these was present in much smaller amounts than in normal human liver from a child of similar age. The other enzymes were highly active. An enzyme preparation from the diseased liver (A.K.) resulted in the liberation of 8·3 μg. of inorganic phosphate/hr./100 mg. of liver from glucose 6-phosphate, compared with 406 μg. with normal liver. Despite unavoidable differences in the age of the diseased and normal tissue, these figures clearly indicate a marked difference in the levels of glucose 6-phosphatase activity. In two cases of the disease reported by Cori & Schulman (1954), values were 20 and 17 μg. of inorganic phosphate liberated/hr. as compared with 362 μg. for normal liver.

The present case thus corresponds to type 1 of Cori's classification, i.e. a case of von Gierke's disease in which the biochemical lesion is a much reduced level of glucose 6-phosphatase. In these circumstances glucose 6-phosphate may be expected to be retained in the liver, not being available to other tissues without removal of the phosphate group. Its conversion into glucose 1-phosphate and hence into glycogen is thus favoured either by the action of the usual phosphorylase mechanisms or by the alternative route suggested by the results of Leloir & Cardini (1957).

The high concentration of free glycogen (10·21; 10·50%) in the frozen diseased liver is characteristic of the disease. The slight decrease in the values (9·91 and 10·38%) after the liver has been kept at room temperature for 9 hr. suggest that little glycogenolysis has occurred during storage of the liver. It has to be said, however, that the diseased liver was not obtained until 50 hr. after the death of the patient and, although the body was refrigerated, the possibility of some changes in glycogen content during this period cannot be excluded. Furthermore, the diseased liver was stored at −18° for 3 months before it was examined. It has not been possible to obtain 'normal tissue' of comparable age and origin.

It is of interest that the present case (A.K.; boy, 4 years old) of type 1 glycogen-storage disease is a sibling (brother) of the patient (S.K.; girl, 12 years old) who suffered from the type 3 disease (Manners, 1954).

Two other cases of siblings having glycogen-storage disease have been reported (Illingworth & Cori, 1952; Cori & Cori, 1952). The first was a girl (M.A.S.) aged 15 months having type 1 von Gierke's disease; a sibling died with similar symptoms though biochemical details were not given. The second was a case of male twins (Daniel B. and Dennis B., aged 3½ years). In both, the structures of the liver glycogens were apparently 'normal', and examination of the liver of Dennis B. showed a decreased level of glucose 6-phosphatase activity. These twins may be presumed to have suffered from

the type 1 form of the disease. Another such family has also been reported by di Sant'Agnese, Andersen, Mason & Bauman (1950).

SUMMARY

1. Liver glycogen ($[\alpha]_D + 196°$ in water) isolated in a case of glycogen-storage disease has a normal structure (average chain length 14·5) and resembles rabbit-liver glycogen in respect of α- and β-amylolysis and the action of isoamylase.

2. The diseased liver has high phosphoglucomutase, glucose 6-phosphate dehydrogenase and phosphohexoisomerase activities.

3. Glucose 6-phosphatase is present in the diseased liver in much reduced quantity, indicating that the disease corresponds to type 1 of Cori's classification, i.e. von Gierke's disease.

4. The present case (A.K.) is a sibling of one (S.K.) investigated earlier and found to be suffering from type 3 of the disease, i.e. a deficiency of amylo-1:6-glucosidase. It is notable that these siblings suffered from different forms of glycogen-storage disease.

5. The kidney glycogen (A.K.) was also examined and was found to have a normal structure.

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Studies on the Metabolism of the Protozoa

6. THE GLYCOGENS OF THE PARASITIC FLAGELLATES *TRICHOMONAS FOETUS* AND *TRICHOMONAS GALLINAE**

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The molecular structures of the intracellular carbohydrates synthesized by a number of Protozoa have been the subject of several recent investigations. Bourne, Stacey & Wilkinson (1950) have examined the polysaccharide synthesized by the flagellate *Polytomella coeca*; it was found to resemble starch, and could be fractionated into linear and branched components (amylose and amylopectin). The polysaccharides produced by the ciliate *Cycloposthium* and by a holotrich ciliate present in the rumen of the sheep differ in that they are homogeneous, and have branched structures similar to that of an amylopectin (Forsyth, Hirst & Oxford, 1953; Forsyth & Hirst, 1953). In contrast, the ciliate *Tetrahymena pyriformis* synthesizes a homogeneous polysaccharide which has the properties of a typical animal glycogen (Manners & Ryley, 1952). During a study of the immunologically specific substances of *Trichomonas foetus*, Feinberg & Morgan (1953) isolated a glycogen-like polysaccharide from dried cells in a yield of 5–10 %. The present paper is concerned with the intracellular polysaccharides synthesized by *Trichomonas foetus* and *T. gallinae* (hereafter designated TFP and TGP respectively) which have been purified and subjected to a detailed structural analysis. The available data indicates that they are branched α-1:4-glucosans which resemble glycogen rather than amylopectin in branching characteristics.

MATERIALS AND METHODS

Materials

Flagellate preparations. Trichomonas gallinae and the Belfast strain of *T. foetus* were cultivated in a peptone–lemco medium containing 2% (w/v) glucose as described by Ryley (1955). Cells of *T. gallinae* were harvested after 24 hr. and those of *T. foetus* after 48 hr. incubation at 37° on the centrifuge, and were washed in 0·9% (w/v) NaCl.

Maltulose. Maltulose was prepared from maltose as described by Peat, Roberts & Whelan (1952).

Methods

Before analysis, the polysaccharides were dried *in vacuo* at 100°, over P_2O_5 for several hours.

Blue value (B.V.) was determined by the method of Bourne, Haworth, Macey & Peat (1948).

Paper chromatography. Descending chromatograms were carried out at room temperature using Whatman no. 1 paper and a mixture of 10 ml. glacial acetic acid and 90 ml. of 75% (v/v) aqueous *iso*propanol as solvent (Bird & Hopkins, 1954). Development was by spraying with aniline oxalate (Partridge, 1949), urea oxalate (McGilvray, 1949) and ammoniacal silver nitrate (Trevelyan, Procter & Harrison, 1950). The rate of movement (R_G values) of sugars was determined by dividing the distance moved by the sugars from the starting line by the distance moved by D-glucose ($R_G = 1$) under identical conditions.

α-Amylolysis. α-Amylolysis was investigated by adding salivary amylase solution (5 ml.) to the glycogen (*ca.* 40 mg.) dissolved in 0·2M phosphate buffer (pH 7·0; 4 ml.), aqueous sodium chloride (3%, w/v; 1 ml.) and distilled water (40 ml.). Samples (2 or 3 ml.) were withdrawn at intervals for determination of reducing power, as maltose. The salivary amylase solution was prepared by dissolving freeze-dried saliva in distilled water, and removing insoluble material with the centrifuge; the solution contained traces of maltotriase but showed no maltase activity.

Sedimentation constant. The rates of sedimentation of the glycogens (1%, w/v, solution in M-NaCl) were determined using an electrically driven 'Spinco' ultracentrifuge (Specialized Instruments Corporation, Belmont, California), as described by Greenwood & Manners (1955). We are indebted to Dr C. T. Greenwood for these determinations.

* Part 5, Ryley (1955).

Other methods. End-group assay, β-amylolysis and other methods were the same as those described previously (Manners & Ryley, 1952), except that reducing sugars were determined using the Shaffer & Somogyi (1933) reagent, as modified by Hanes & Cattle (1938).

RESULTS

Glycogen content of the cells

Trichomonas foetus during growth synthesizes glycogen; the cell glycogen content (dry weight basis) usually lies between 10 and 30 %, although on one occasion a value of 55 % was observed. Growth of the flagellate will take place in the lemco–peptone medium without the addition of glucose; in one experiment such cells had a glycogen content of 8·1 %. In the presence of 1 % glucose, 6 times the yield of organisms was obtained, and these had a glycogen content of 17·4 %. Increasing the glucose content of the medium to 2–6 % reduced growth by half, and gave cells with a glycogen content of about 14 %. Similar values for intracellular glycogen were obtained with *T. gallinae*.

Isolation and purification of glycogen

Cells from 6 to 12 l. batches of culture were treated with 2 vol. 30 % (w/v) KOH at 100° for 30 min., cooled, centrifuged to remove insoluble material, and the crude glycogen was precipitated with 1·1 vol. ethanol. The precipitate was removed on the centrifuge, dissolved in a little water, any insoluble residue centrifuged off, and the glycogen reprecipitated with 1·1 vol. ethanol. The precipitate was washed twice with ethanol, and then with ether, and was air-dried.

The combined preparations of crude glycogen were purified by precipitation with 80 % (v/v) acetic acid (Bell & Young, 1934). Crude glycogen (13 g. TFP; 1·58 g. TGP) was dissolved in water to give a 7 % (w/v) solution, centrifuged to remove any insoluble material, and 4 vol. glacial acetic acid were then added. The precipitated glycogen was recovered by centrifuging and subjected to five more acetic acid precipitations. The glycogen was then 'deacetylated' by treating with 100 ml. 15 % (w/v) NaOH at 100° for 1 hr. (Illingworth, Larner & Cori, 1952). Glycogen was precipitated from the alkaline solution with ethanol, washed in ethanol and ether and air-dried. Yields: TFP 10·13 g., TGP 0·94 g.

Characterization of purified glycogens

The purified glycogens were white powders: TFP, N = 0·04 %, P = nil, ash = 0·2 %; TGP, N = nil, P (inorganic) = 2·30 %, P (organic) = nil, ash = 12·08 %. Weights of TGP are corrected for 12·08 % ash in the material which has no structural significance. The glycogens were freely soluble in water, giving opalescent solutions which stained yellow-brown with dilute iodine (B.V. = 0·01). TFP had $[\alpha]_D^{18} + 199°$ in water (2 dm. tube, *c*, 0·4 or 0·5). TGP had $[\alpha]_D^{18} + 197°$ in water (2 dm. tube, *c*, 0·4 or 0·6).

Glucose content. Hydrolysis of the glycogens by 1·6 N-sulphuric acid at 100° gave the following percentage conversions into glucose: TFP, 96 % in 2 hr., 98 % in 3 hr.; TGP, 82 % in 2 hr., 85 % in 3 hr. Paper chromatography showed the presence of glucose in the hydrolysates; no other reducing sugar was present.

α-Amylolysis. Treatment of the polysaccharides with salivary α-amylase at pH 7·0 and 35° caused a rapid decrease in turbidity and iodine-staining power; the glycogens were achroic within 5 min. Paper chromatography showed the presence of glucose, maltose ($R_G = 0·62$) and α-dextrins ($R_G \leqslant 0·11$) in the α-amylolytic digests. Maltulose ($R_G = 0·66$) was absent. The extent of α-amylolysis (see Table 1) and the presence of α-dextrins in the digest indicates that the glycogens are branched α-1:4-glucosans (cf. Manners, 1954). The slow increase in reducing power after 49 hr. was due to traces of maltotriase in the α-amylase (cf. Manners, 1954). The absence of maltulose is evidence that fructose is not a constituent of the glycogens (cf. Peat *et al.* (1952), who isolated *ca.* 5 % of maltulose from an α-amylolytic digest of pregnant doe-liver glycogen).

End-group assay. Potassium periodate oxidation at room temperature. On complete oxidation, 421 mg. TFP gave 8·11 mg. formic acid, corresponding to a unit-chain length of fifteen glucose residues. 144 mg. TGP under identical conditions gave 4·66 mg. formic acid, corresponding to a unit-chain length of nine glucose residues.

β-Amylolysis. TFP (43·2 mg.) on treatment with crystalline sweet-potato β-amylase (Balls, Thompson & Walden, 1946; Balls, Walden & Thompson, 1948), at 35° and pH 4·54, gave 27·2 mg. maltose, equivalent to a β-amylolysis limit of 60 %. TGP (19·5 mg.), under similar conditions, gave 10·5 mg. maltose, equivalent to a β-amylolysis limit of 51 %.

Sedimentation constants. The ultracentrifugal examination showed that TFP was polydisperse, and contained two components. The major component had $S_{20} = 63 \times 10^{-13}$, and the minor component $S_{20} = 7 \times 10^{-13}$. TGP, although polydisperse, contained only one component which had

Table 1. *Action of salivary α-amylase on glycogens*

The glycogens (0·1 %, w/v) were incubated with salivary α-amylase at 35° in the presence of 0·02 M phosphate buffer and aqueous NaCl (0·075 %, w/v). Samples were analysed at intervals for reducing sugar (as maltose).

Time of incubation (hr.)	Apparent % conversion into maltose	
	TFP	TGP
0·75	30	31
20	58	61
49	61	69
112	63	73

Table 2. *A comparison of the properties of certain protozoal polysaccharides with rabbit liver glycogen and an amylopectin (waxy maize starch)*

Property	Waxy maize* starch	*Cyclo-posthium*†	Holotrich† ciliate	*Tricho-monas foetus*	*Tetra-hymena*‡ *pyriformis*	Rabbit liver§ glycogen	*Tricho-monas gallinae*
$[\alpha]_D$ (water)	+212°	—	—	+199°	+195°	+198°	+197°
$[\alpha]_D$ (N-NaOH)	+153°	+154°	+171°	—	—	+169°	—
Iodine coloration	Red-purple	Purple	Reddish purple	Yellow-brown	Yellow-brown	Reddish brown	Yellow-brown
Unit-chain length							
(*a*) KIO_4 method	—	23–24	22	15	13	13	9
(*b*) Methylation method	22	22	21	—	—	13	—
β-Amylolysis limit	54	—	—	60	44	45	51
Mean exterior chain length‖	14–15	—	—	11–12	8–9	8–9	6–7
Mean interior chain length‖	6–7	—	—	2–3	3–4	3–4	1–2
Approx. mol.wt.							
(*a*) Sedimentation–diffusion	—	—	—	3×10^6	3×10^6	4×10^6	$3–4 \times 10^6$
(*b*) Viscosity of methyl ether	8×10^5	2×10^5	2×10^5	—	—	—	—

Sedimentation–diffusion data taken from Greenwood & Manners (1955).

* Data from Haworth, Hirst & Woolgar (1935), and Halsall, Hirst, Hough & Jones (1949).
† Data from Forsyth *et al.* (1953), and Forsyth & Hirst (1953).
‡ Data from Manners & Ryley (1952).
§ Data from Bell & Manners (1952) and Manners (1952).
‖ Mean exterior chain length = no. of glucose residues removed on β-amylolysis plus 2·5. Mean interior chain length = unit-chain length – (exterior chain length plus 1). Cf. Manners (1954).

$S_{20} = 76 \times 10^{-13}$. Assuming that the diffusion constants were similar to those of other glycogens (see Bell, Gutfreund, Cecil & Ogston, 1948), these sedimentation constants are equivalent to molecular weights of 2·9, 0·3 and $3·5 \times 10^6$ respectively.

DISCUSSION

The present investigation has shown that TFP and TGP superficially resemble other known protozoal polysaccharides in being branched α-1:4-glucosans; they differ, however, from these, and from each other, in degree of branching and in the mean position of branching in the unit-chains, as shown in Table 2. The major proportion of the molecules of TFP are composed of *ca.* 1200 unit-chains, each comprising an average of 15 α-1:4-linked glucose residues, whereas those of TGP contain *ca.* 2000 unit-chains, consisting of only 9 α-1:4-linked glucose residues. TGP thus has a higher degree of branching than that of any other protozoal polysaccharide hitherto described. These differences in degree of branching presumably reflect differences in the enzyme system catalysing the synthesis of glycogen; in *T. gallinae* the ratio of the activity of branching enzyme:phosphorylase must be greater than in *T. foetus* and in *Tetrahymena pyriformis*. The β-amylolysis limits of TFP and TGP are *ca.* 10% higher than those of animal glycogens of similar chain length (cf. Bell & Manners, 1952); hence the ratio of the mean exterior chain length to that of the mean interior chain length is proportionally larger (see Table 2).

Further work is now in progress, with a view to determining the degree of multiple-branching in TFP. It seems probable that 'amylopectins' and 'glycogens' are more correctly differentiated by this property, rather than by chain length (cf. Hirst & Manners, 1954); a comparison of the degrees of multiple-branching in TFP, *T. pyriformis* glycogen and the sheep protozoal polysaccharide will therefore be of considerable interest.

SUMMARY

1. Glucose-containing polysaccharides have been isolated from the parasitic flagellates *Trichomonas foetus* and *T. gallinae*.

2. The purified polysaccharides resemble, but are not identical with, animal glycogens; they have $[\alpha]_D^{18}$ 197–199°, stain yellow-brown with dilute iodine, are degraded by salivary α-amylase and have molecular weights of *ca.* 3×10^6.

3. The 'glycogens' from *T. foetus* and *T. gallinae* have unit-chain lengths of 15 and 9 glucose residues respectively, and β-amylolysis limits of 60 and 51%.

4. The relationship between these 'glycogens' and other polysaccharides, including those from other Protozoa, are discussed.

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STRUCTURE OF A RESERVE POLYSACCHARIDE (LEUCOSIN) FROM *OCHROMONAS MALHAMENSIS*

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In view of present interest in the molecular structure and metabolism of cellulose, laminarin and related polysaccharides,[1] we report the isolation of a polysaccharide containing a high proportion of β-1 : 3-glucosidic linkages from the flagellated protozoon *Ochromonas malhamensis*.

Hot-water extraction of the protozoal cells gave a mixture of polysaccharides composed of glucose, galactose and mannose. Fractionation with Cetavlon[2] and acetone gave a polysaccharide containing glucose and a small quantity (*ca* 10%) of a second component sugar, tentatively identified as mannose. The polysaccharide was soluble in cold water ($[\alpha]_D$ $+10°$), was non-dialysable, and had a low reducing power. The corresponding acetate, which appeared to be homogeneous, had $[\alpha]_D$ $-59°$ in chloroform (*cf* $[\alpha]_D$ $-60°$ for laminarin acetate[3]).

Partial hydrolysis with dilute sulphuric acid or with an *endo*-β-glucosidase preparation gave a series of oligosaccharides with the R_G values of glucose, laminari-biose, -triose, -tetraose and -pentaose. The polysaccharide was slowly degraded by an *exo* β-glucosidase.* In one experiment, 9% conversion into glucose was observed; under similar conditions, 10% degradation of laminarin occurred.

The presence of 1:3-linkages was shown by oxidation with sodium metaperiodate; only 0·17 mol. periodate per hexose residue was consumed. The corresponding production of formaldehyde and formic acid indicated that the polysaccharide had an essentially linear structure with a degree of polymerisation of 36-40. On periodate oxidation in presence of phosphate buffer (pH 8)[4], the polysaccharide was rapidly over-oxidised with the formation of 0·5 mol. formaldehyde per hexose residue (*cf* laminarin which also gives 0·5 mol.).[4,5] The presence of a small proportion of linkages other than β-1 : 3-glucosidic is therefore established.

The occurrence of iodophobic glucose-containing polysaccharides in various species of algae and flagellates has been noted by several workers,[6] although detailed chemical studies on these materials, termed leucosin and paramylon (paramylum), appear to be limited. The present demonstration of a structural similarity between laminarin, the characteristic reserve carbohydrate of the Phaeophyceae, and the reserve polysaccharide of *Ochromonas malhamensis* is, therefore, of considerable biochemical interest.

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* *Exo*-β-glucosidases catalyse a stepwise hydrolysis of successive linkages, in contrast to *endo*-β-glucosidases, which cause random hydrolysis.

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115. *Studies on the Metabolism of the Protozoa. Part VIII.* The Molecular Structure of a Starch-type Polysaccharide from* Chilomonas paramecium.

By A. R. Archibald, E. L. Hirst, D. J. Manners, and J. F. Ryley.

Chilomonas paramecium when grown in an acetate-containing medium synthesizes a starch-type polysaccharide. This contains *ca.* 45% of an essentially linear amylose, which has a lower degree of polymerization and lower iodine-binding power than potato amylose. The amylopectin component has an average chain length of 22 glucose residues, and is similar in many properties to a typical plant amylopectin.

Although the formation of starch is normally considered to be characteristic of the higher terrestrial plants, the presence of water-insoluble iodophilic granules has been noted in many other organisms. These include bacteria, *e.g.*, *Streptococcus pyogenes* [1] and *Corynebacterium diphtheriae*,[2] flagellated protozoa, *e.g.*, *Polytomella coeca*,[3] and fresh-water algæ, *e.g.*, *Dunaliella bioculata*.[4] We now describe a chemical and enzymic investigation of the starch-type polysaccharide synthesized by the flagellate *Chilomonas paramecium* (class, Cryptophyceae; order, Cryptomonadina).

Chilomonas paramecium is a free-living, motile organism (length 18—30 μ, breadth 9—12 μ, with two anterior flagellæ 10 μ in length); when it is grown on a synthetic medium, starch is slowly synthesized (rate 22 μg. per 10^6 cells per hr. at 25°).[5] Hutchens and his co-workers [5] showed that hot water extracted material giving a deep blue stain with iodine ($\lambda_{max.}$ 625 mμ), whilst a suspension of the insoluble residue gave a reddish-purple colour. These appear to be amylose and amylopectin-type polysaccharides respectively. Extraction of the cells with 0·5N-sodium hydroxide at 100° destroyed the cell structure; the resulting solution gave a purple-blue colour with iodine ($\lambda_{max.}$ 550 mμ). No fractionation of the starch was attempted.

A pure culture of the flagellate was grown on an acetate-containing medium (120 l.), and starch (yield, 2·3 g.) was extracted from the harvested cells by the chloral hydrate method.[6] The starch, originally present as iodophilic polygonal granules (diameter 2—5 μ), was obtained as a white amorphous powder, insoluble in water, but soluble in dilute sodium hydroxide solution ($[\alpha]_D$ +157° in N-NaOH). A dilute neutralized solution gave a blue stain with iodine [blue value [7] (B.V.) 0·54; $\lambda_{max.}$ 590 mμ]. An acid hydrolysate contained glucose as the sole carbohydrate (paper chromatography and treatment with glucose oxidase) and failed to react with an acid-resorcinol [8] (ketose) reagent. By cuprimetric titration, the glucose content was 78%; † the starch sample also contained inorganic material (10%) and protein (6%) which in view of the conditions of growth of the organism, and extraction of the polysaccharide, were considered to have no structural significance. Potentiometric titration of the iodine complex,[9] kindly performed by Dr. A. W. Arbuckle, indicated an iodine affinity of 5·0%; on the assumption that the amylose component has an iodine affinity of 10·6% (p.), this corresponds to an amylose content of 47%. Incubation with barley β-amylase (which also contains Z-enzyme [10]) gave 75% conversion into maltose. The above evidence indicates the presence of a two-component starch, since the corresponding β-amylolysis limits of normal amylose and amylopectin are *ca.* 100 and 60% respectively.

The starch was then treated with 0·1N-sodium hydroxide at 100° for 10 min., in an atmosphere of nitrogen. After centrifugation, the supernatant solution was neutralized and treated with acetone to give alkali-soluble polysaccharide (fraction I, yield 56%).

* Part VII, Ryley, *Biochem. J.*, 1956, **62**, 215.

† All analytical figures of starch fractions are based on the observed glucose contents.

TABLE 1. *Properties of* Chilomonas paramecium *starch fractions.*

Fraction	Original starch	I	Ia	Ib	II
Glucose content (%)	78	88	97	82	95
Iodine affinity (%)	5·0	6·1	10·6	0·09	1·60
B.V.	0·54	0·65	0·98	0·13	0·20
Iodine complex, $\lambda_{max.}$ (mμ)	590	620	645	540	520
β-Amylolysis limit (%)	75	81	95	60	64
Amylose content calc. from					
(*a*) iodine affinity	47	58	—	—	15
(*b*) B.V.	48	62	—	—	8
(*c*) β-amylolysis limit	43	60	—	—	11

Acetone was also added to a suspension of alkali-insoluble material, causing coagulation and giving a precipitate (fraction II, yield 29%). Analysis of the two fractions (see Table 1) indicated partial separation into components showing marked differences in iodine-binding power (cf. ref. 11).

β-Amylolysis of fraction II resulted in 64% conversion into maltose. On prolonged oxidation with sodium metaperiodate at 2°,[12] 1·07 moles of periodate were reduced per glucose residue, showing the apparent absence of 1,2- or 1,3-glucosidic linkages. The production of formic acid corresponded to an average chain length ($\overline{CL}$) of 25 glucose residues, a value similar to that of many amylopectins.

Since fraction I had many of the properties of a plant starch of high amylose content (cf. refs. 13 and 14), a portion was fractionated by the thymol method, and the resulting amylose complex purified by three precipitations with butanol.

The amylose (Fraction Ia) had a glucose content of 97%, B.V. 0·98, $\lambda_{max.}$ 645 mμ, and iodine affinity 10·6%. Incubation with purified soya-bean β-amylase gave 90% conversion into maltose; in presence of Z-enzyme, the β-amylolysis limit was 95%. The protozoal amylose thus contains few, if any, anomalous linkages. An estimate of the degree of polymerisation ($\overline{DP}$) was made viscometrically, the observed limiting viscosity number indicating $\overline{DP}$ 300—350. Fraction Ia therefore consists of essentially unbranched chains of α-1,4-linked D-glucose residues, but differs markedly in iodine-binding power and $\overline{DP}$ from highly purified potato amylose (*e.g.*, iodine affinity 19·5%, $\overline{DP}$ 3200 [10]). However, the $\overline{DP}$ is of the same order of magnitude as that reported for amylose preparations from apple,[15] maize,[16] malted barley,[17] and wheat starches.[18]

Since the amylopectin (fraction Ib; yield 80 mg.) had a glucose content of only 82% (it was isolated by freeze-drying, and contained inorganic material), analysis was confined to the interaction with iodine and enzymic degradation. Fraction Ib had an iodine affinity of 0·09%, showing the absence of amylose-type material, and B.V. 0·13. The β-amylolysis limit was 60 and 82%, before and after treatment with yeast isoamylase. The outermost inter-chain linkages are therefore of the α-1,6-type.

The present study has shown that *Chilomonas paramecium* synthesizes a starch of small granular size which can be fractionated into essentially linear (amylose) and branched (amylopectin) components. Chloral hydrate extraction of the cells yields a starch-type polysaccharide which, however, differs from potato starch by virtue of its relative insolubility in hot water (compare *P. coeca* starch [3]), of its significantly higher iodine binding power and β-amylolysis limit, and of the properties of the amylose component (the corresponding figures for potato starch are B.V. 0·4, iodine affinity 4·0%, and β-amylolysis limit 60—70%) (see Table 2). On the assumption that fraction Ia represents the pure amylose, the amylose content of the original starch is 45 ± 5%, *i.e.*, higher than that of most plant starches (20—30%) * and markedly different from *P. coeca* starch (13—16%).[3] The low iodine-binding power of the amylose may be correlated with the

* However, the amylose content of the starch from certain varieties of pea [13] and maize [14] is much higher, *e.g.*, wrinkled pea (var. Laxton's progress) starch has an amylose content of 43% (unpublished work).

TABLE 2. *Properties of protozoal and potato starches.*

Property	*Chilomonas paramecium*	*Polytomella coeca* [3]	*Holotrich ciliates* [22]	Potato starch [24]
Whole starch				
$[\alpha]_D$ in N-NaOH	+157°	+160°	+171°	+159° [a]
Amylose content (%)	45	13—16	0	20
B.V.	0·54	0·36	0·05	0·4
Amylose component				
B.V.	0·98	1·13	—	1·4—1·5
β-Amylolysis limit (%)				
(*a*) β-amylase alone	90	—	—	77 [10]
(*b*) β-amylase + Z-enzyme	95	89	—	99 [10]
Amylopectin component				
$\overline{CL}$	22	—	22	22
Iodine complex, $\lambda_{max.}$ (mμ)	540	—	540 [b]	545
B.V.	0·13	0·11	0·05	0·06—0·13
β-Amylolysis limit (%)				
(*a*) β-amylase alone	60	48	63 [c]	53, 61 [c]
(*b*) after pretreatment with isoamylase	82	—	80 [c]	80, 77 [c]

[a] Determined by Mr. A. Wright. [b] Determined by Dr. A. M. Liddle.
[c] Data from Gunja, Manners, and Khin Maung, *Biochem. J.*, in preparation.

low $\overline{DP}$; thus, there is evidence that the $\lambda_{max.}$ of amylose-type chains is directly related to the $\overline{DP}$,[19] provided that $\overline{DP}$ <500. In view of recent reports [20] on the lability of amylose to oxygen and alkali, the question of degradation during isolation must be considered. In our experiments, degradation during anaerobic fractionation is unlikely; nevertheless, the possibility of inadvertent degradation during the original extraction remains, and it is being examined. It must be noted, however, that the chloral hydrate method has been successfully used for the extraction of other protozoal starches.[3,21,22]

The *Chilomonas* amylopectin appears to be similar to most plant amylopectins. Fraction II contains *ca.* 11% of amylose; with allowance for this, the production of formic acid on periodate oxidation corresponds to a $\overline{CL}$ of 22. This is identical with that for potato amylopectin.[23] Further, the β-amylolysis limits of fraction Ib are similar to those of two samples of potato amylopectin (Table 2). The average lengths of the exterior and interior chains (15—16 and 5—6 respectively) agree closely with those found for other amylopectins.[24]

The present results provide further information on the biochemical relations between the various groups of protozoa. Ciliates, *e.g.*, *Cycloposthium*,[21] holotrichs,[22] and *Tetrahymena pyriformis*,[25] and certain parasitic flagellates (the so-called animal-like group), *e.g.*, *Trichomonas foetus* [26] *and Trichomonas gallinae*,[26] synthesize amylopectin or glycogen-type polysaccharides. In contrast, the plant-like flagellates store either laminarin-type polysaccharides, *e.g.*, *Ochromonas malhamensis* [27] and *Euglena* [28] or starches, *e. g.*, *Polytoma* [29] and *P. coeca*.[3] *Chilomonas paramecium* can now be added to the latter group.

EXPERIMENTAL

The analytical methods employed have been described in Parts II [25] and VI [26] of this Series; the enzyme preparations were those reported in refs. 4 and 10.

Flagellate Preparation.—A pure culture of *Chilomonas paramecium* was maintained at 24°, with sub-inoculations at intervals of one week, in a sterile medium adjusted to pH 6·0—6·5, containing 0·1% (w/v) of sodium acetate and 0·1% (w/v) of " Oxoid " brand Lab-lemco. In large-scale cultures (20 two-litre flasks each containing 1·5 l. of medium) the flagellate was grown at 28° for 5 days, and the cells, which contained polygonal iodophilic granules (2—5 μ diam.), were harvested by gentle centrifugation.

Isolation and Properties of the Starch.—The cells were extracted twice with 33% chloral hydrate solution (100 ml.) at 80° for 1 hr.[6] Acetone (2 vol.) was added to the cooled centrifuged extract, giving an impure starch contaminated with cell debris. The pooled yield from four 30 l. preparations was 2·74 g. This product was purified by a further extraction with chloral

hydrate, precipitated, washed with acetone, and air-dried at 37° (yield 2·25 g.). Before further examination, contaminating chloral hydrate was removed by extraction (Soxhlet) with methanol.

The starch was a white amorphous powder, insoluble in water but soluble in dilute sodium hydroxide solution. An acid hydrolysate contained glucose (paper chromatography and glucose oxidase treatment) and on treatment with acid-resorcinol [8] gave a reaction of 0·011 unit per mg. Under identical conditions, fructose, maltose, and " AnalaR " soluble starch gave values of 3·00, 0·016, and 0·014 unit per mg. respectively. The following properties of the starch were noted: $[\alpha]_D$ +157° (*c* 0·54 in N-NaOH); Found: glucose, 78·4%; ash 9·7%; protein-N, 0·94% (determined by the colorimetric biuret method of Robinson and Hogden [30]). A dilute solution was stained deep blue with iodine (B.V. 0·54), showing maximum absorption at 590 mμ. On potentiometric titration, the iodine complex gave a typical starch curve, extrapolation indicating an iodine affinity of 5·0%. Incubation with barley β-amylase [10] at pH 4·6 and 35° (with 54 units of β-amylose per mg. of starch) gave 75% conversion into maltose after 24 hr. The reducing power of the digest did not increase on further incubation.

Extraction of the Starch with Alkali.—The starch (1·5 g.) was stirred in 0·1N-sodium hydroxide (100 ml.) at 100° for 10 min. in an atmosphere of nitrogen. After cooling and neutralization by dilute sulphuric acid (phenolphthalein), an insoluble gelatinous residue was collected by centrifugation. Acetone precipitation of the solution gave fraction I (840 mg.) (Found: glucose, 88·0%; ash, 5·9%; B.V., 0·65; $\lambda_{max.}$ of iodine complex, 620 mμ; iodine affinity, 6·1%; β-amylolysis limit, 81%). Treatment of the residue with acetone gave fraction II (440 mg.) (Found: glucose, 94·5%; ash, 2·7%; B.V., 0·196; $\lambda_{max.}$ of iodine complex, 520 mμ; iodine affinity, 1·60%; β-amylolysis limit, 64%).

Periodate Oxidation of Fraction II.—Fraction II (160 mg.) was moistened with alcohol and shaken overnight with 2N-sodium hydroxide (10 ml.). The solution was then neutralized with dilute sulphuric acid (Methyl Red) and diluted with water to 25 ml. 20 ml. of this solution (equivalent to 121·2 mg. of glucosan) were oxidized in the dark at 2° with 8% sodium meta-periodate solution (3 ml.) and water (to 25 ml.). A solution of periodate in water was also prepared. Samples (3 ml. or 2 ml.) were analysed at intervals for the production of formic acid and the reduction of periodate:

Time of oxidation (days)	7	10	12
Total formic acid prodn. (mg.)	1·17	1·40	1·37
Reduction of periodate (mole/glucose residue)	1·04	1·07	1·07

The production of formic acid indicates a $\overline{CL}$ value of 25 glucose residues; the theoretical periodate reduction is 1·04 mol. if only 1,4- and 1,6-glucosidic linkages are assumed to be present.

Thymol Fractionation of Fraction I.—Fraction I (0·5 g.) was suspended in water (20 ml.) and added to vigorously stirred boiling water (65 ml.), in an atmosphere of nitrogen. The solution was heated at 98° for 20 min., then allowed to cool, and a small insoluble residue was removed by centrifugation. The clear solution was heated to 60°, powdered thymol (1·5 g.) added, and the mixture stirred at 60° for 30 min. and then kept at room temperature for 3 days. The thymol–amylose complex was collected by centrifugation and directly dispersed in boiling water (50 ml.) under nitrogen, and redistilled butanol (5 ml.) was added. The mixture was stirred at 95° for 30 min., then allowed to cool slowly to room temperature, and the butanol–amylose complex removed by centrifugation. The amylose was purified by two further precipitations with butanol, and isolated by treatment of the butanol complex with butanol. This gave fraction Ia (129 mg.) [Found: glucose, 97·0%; B.V., 0·98; $\lambda_{max.}$ of iodine complex, 645 mμ; iodine affinity, 10·6%; limiting viscosity number (in 0·2M-potassium hydroxide), 45·4]. By the approximate relation $-\overline{DP} = 7{\cdot}4[\eta]$,[31] this corresponds to $\overline{DP}$ 335.

The supernatant solution from the thymol precipitation was freeze-dried, to give fraction Ib. This was extracted (Soxhlet) with methanol to remove thymol, redissolved in water, and freeze-dried (yield 80 mg.) (Found: glucose, 82%; B.V., 0·125; iodine affinity, 0·09%).

Enzymic Degradation of Fractions Ia *and* Ib.—Fraction Ia (18·5 mg.) was dissolved in 0·2M-acetate buffer (pH 4·6; 5 ml.), and purified soya-bean β-amylase solution [10] (0·1 ml.; 1000 units) and water (to 25 ml.) were added. After incubation at 35° for 24 hr., the β-amylolysis limit was 90%. On being treated with barley β-amylase containing Z-enzyme [10] (50 β-amylase units/mg.), fraction Ia (10·4 mg.) in a final volume of 25 ml. gave 95% conversion into maltose.

Fraction Ib (9·8 mg.) was dissolved in 0·2N-sodium hydroxide (2 ml.), then neutralized,

and 0·2M-acetate buffer (pH 5·8; 5 ml.) added, followed by aqueous yeast isoamylase (5 mg./ml.; 2 ml.). After incubation at 20° for 18 hr., the isoamylase was inactivated by heat, and β-amylase solution (500 units; 1 ml.) and water (to 25 ml.) were added. The maltose content after 36 hours' incubation at 35° indicated a β-amylolysis limit of 82%. In a control experiment without isoamylase, the β-amylolysis limit was 60%.

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375. *A Comparison of* iso*Lichenin and Lichenin from Iceland Moss* (Cetraria islandica).

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Purified *iso*lichenin and lichenin, from Iceland moss, have been characterised by methylation and periodate oxidation.

*iso*Lichenin has been found to consist solely of D-glucose residues united by α-1 : 3- and α-1 : 4-glucosidic linkages which are present in the relative proportion of 3 : 2. The molecule appears to be linear, with an average chain length of 42—44 glucose residues. The structure of lichenin as a linear polymer of β-D-glucose containing both 1 : 3- and 1 : 4-linkages in the proportion of 3 : 7 has been confirmed.

Several structural investigations of the polysaccharides of lichens, which are symbiotic organisms in the phylum *Thallophyta*, have been reported recently. The major polysaccharide in Iceland moss (*Cetraria islandica*) is lichenin, which was shown by Meyer and Gürtler [1] to contain D-glucose residues united by 1 : 3- and 1 : 4-linkages. In addition, Iceland moss contains *iso*lichenin and hemicelluloses. The latter are composed of D-galactose, D-mannose, and uronic acid residues.[2] Lichenin and *iso*lichenin, from the Indian lichens *Usnea longissima* and *Roccella montagnei* respectively, are both polymers of D-glucose, although they differ in physical and chemical properties.[3] Pustulan, from *Umbilicaria pustulata*, is a linear glucan in which the component residues are united by β-1 : 6-linkages.[4] In contrast, the alkali-soluble polysaccharides of reindeer moss (*Cladonia alpestris*) are highly branched molecules composed of D-galactose, D-glucose, and D-mannose residues.[5] In this communication, a structural investigation of *iso*lichenin from Iceland moss is described, and previous observations on the structure of lichenin have been confirmed and extended.

iso*Lichenin*.—The presence of *iso*lichenin in Iceland moss has been known for many years. This polysaccharide differs markedly from lichenin in being freely soluble in water, giving a dextrorotatory solution, which is stained blue by iodine. Pringsheim [6] concluded that *iso*lichenin was a glucan related to amylose, but Karrer [7] and Meyer [8] and their co-workers considered it to be a mixture of polysaccharides derived from glucose, galactose, and mannose. Attempts to purify *iso*lichenin were, in general, unsuccessful. In the present study it has been found that *iso*lichenin is, in fact, composed solely of glucose residues.

Iceland moss, freed from fatty materials and lichen acids, was extracted with boiling water. The impure lichenin which came down on cooling was removed and smaller amounts of this polysaccharide were then separated by alternate freezing and thawing of the solution. Other contaminating polysaccharides were removed as insoluble alkaline-copper complexes, and further purification was effected by fractional precipitation with acetone of the water-soluble copper complex of *iso*lichenin. After dialysis and treatment with ion-exchange resins, the purified *iso*lichenin was precipitated by alcohol. From 6 kg. of Iceland moss, 2·7 g. of pure *iso*lichenin were finally obtained.

On acid hydrolysis, *iso*lichenin was converted almost quantitatively into glucose. The presence of other sugars and uronic acids could not be demonstrated. In water *iso*lichenin had an unusually high positive rotation ($[\alpha]_D$ +255°) which may be attributed to α-1 : 3-linked glucopyranose residues (compare nigeran,[9] $[\alpha]_D$ +270° to +283° in N-sodium hydroxide). The *iso*lichenin showed slight reducing action towards alkaline hypoiodite (apparent D.P. 34). The aqueous solution gave a greenish-blue colour with very dilute iodine solution (absorption $\lambda_{max.}$ at 600 mμ), but the " blue value " [10] was too low to permit accurate measurement (B.V. *ca.* 0·01; cf. *ca.* 1·30 for amylose and *ca.* 0·10 for amylopectin).

The *iso*lichenin reduced 0·4 mole of sodium metaperiodate per glucose residue, indicating that some 60% of the glucose residues were unattacked by periodate. The formic acid produced by potassium metaperiodate oxidation corresponded to 1 mole per 14 glucose residues. If (see below) *iso*lichenin is a linear glucan, and the non-reducing and reducing end-groups give rise to one and two molecules of formic acid respectively, this would be equivalent to an average chain length of 42 glucose residues.

Hydrolysis of the methylated polysaccharide (OMe, 44·4%) gave 2 : 4 : 6- and 2 : 3 : 6-tri-*O*-methyl-D-glucose together with small amounts of 2 : 3 : 4 : 6-tetra- and mixed di-*O*-methyl-D-glucoses. The latter (*ca.* 2%) most probably arose from undermethylation of the polysaccharide (a tri-*O*-methyl glucan has OMe, 45·6%) and from hydrolytic demethylation. The proportion of tetra-*O*-methyl-D-glucose in the products of hydrolysis indicated the presence of 1 non-reducing terminal group per 44 glucose residues. Analysis of the tri-*O*-methylglucose fraction showed that 2 : 4 : 6-tri-*O*-methyl-D-glucose was the major component. From this evidence, and that previously cited, it is concluded that the molecule of *iso*lichenin is unbranched, and contains *ca.* 60% of α-1 : 3- and 40% of α-1 : 4-glucosidic linkages.

Measurements of the viscosity of solutions of *iso*lichenin acetate in chloroform indicated a low molecular weight, the D.P. being of the order of 40—50 glucose residues.

*iso*Lichenin was not attacked by barley or soya-bean β-amylase and it follows that α-1 : 3-glucosidic linkages must be situated near the non-reducing end of the molecule. This evidence makes it clear also that the *iso*lichenin investigated could not be a mixture of an amylose and an α-1 : 3-glucan.

The demonstration of α-1 : 3-glucosidic linkages in *iso*lichenin reveals an unusual structural feature in this glucan, and differentiates it from amylose (compare ref. 6). *iso*-Lichenin thus resembles nigeran, the intracellular polysaccharide synthesised by a strain of *Aspergillus niger*, which contains approximately equal numbers of α-1 : 3- and α-1 : 4-glucosidic linkages.[9] The analogy, however, is not complete in that the average chain length of nigeran is 300—350 glucose residues, and the detailed structures must differ since the proportion of 1 : 3- and 1 : 4-linked residues in *iso*lichenin does not permit an alternating sequence of these linkages such as is present in nigeran.

Lichenin.—Earlier investigations had established a structural similarity between lichenin and cellulose. For example, on acetolysis, cellobiose octa-acetate is formed,[11] and on methanolysis of the methylated polysaccharides, methyl 2 : 3 : 6-tri-*O*-methyl-D-glucoside is obtained,[12] although in both cases the yields from lichenin were smaller than those from cellulose. Nevertheless, lichenin differs from cellulose in being soluble in hot water,[13] and more susceptible to enzymic degradation. In 1947, Meyer and Gürtler [1] provided the first clear evidence of a chemical difference between these polysaccharides. Hydrolysis of methylated lichenin gave tetra-*O*-methyl-D-glucose (0·6%, equivalent to an average chain length of *ca.* 170 glucose residues), and a mixture of 2 : 3 : 6- and 2 : 4 : 6-tri-*O*-methyl-D-glucose, thereby indicating the presence of both 1 : 4- and 1 : 3-linkages. Periodate oxidation showed that *ca.* 27% of 1 : 3-linkages were present. The relative proportions of 1 : 3- and 1 : 4-linkages in lichenin were confirmed by Boissonnas.[14]

In the present investigation two samples of lichenin have been studied. They were composed of D-glucose residues (94—96%) united by β-linkages, as shown by the specific rotation ($[\alpha]_D$ +8° to +10° in N-sodium hydroxide) and by hydrolysis to glucose by exo-β-glucosidase preparations * from *Helix aspersa* and almond emulsin. Partial hydrolysis, with dilute acid or with endo-β-glucosidase preparations from malted barley and *Cladophora rupestris*,[15] gave a series of sugars including glucose, cellobiose, laminaribiose, and higher oligosaccharides (paper chromatography). On periodate oxidation, 0·7 mole of periodate per anhydroglucose unit was consumed.

* Exo-β-glucosidases cause a stepwise hydrolysis of successive linkages in a β-glucan, in contrast to endo-β-glucosidases, which catalyse random hydrolysis.

Chromatographic analysis of a hydrolysate of a methylated lichenin showed the presence of (*a*) tetra-*O*-methyl-D-glucose equivalent to an average chain length of 62 glucose residues, (*b*) 2 : 4 : 6-tri- (*ca.* 30%) and 2 : 3 : 6-tri-*O*-methyl-D-glucose (*ca.* 70%), thus confirming the presence of 1 : 3-linkages, and (*c*) no more than a trace of di-*O*-methyl-D-glucoses, showing the virtual absence of branch points. This sample of lichenin has, therefore, a shorter chain length than that examined by Meyer and Gürtler [1] and this difference probably arises from the more extensive fractionation of the lichen polysaccharides in the present study.

Lichenin does not appear to be a mixture of a β-1 : 3- and a β-1 : 4-glucan. A lichenin acetate was prepared, fractionated, and deacetylated. The regenerated polysaccharide consumed 0·7 mol. of periodate per anhydroglucose residue, indicating that no preferential removal of material containing a larger proportion of 1 : 3-linked glucose residues had occurred. Furthermore, treatment of periodate-oxidised lichenin with *iso*nicotinhydrazide or thiosemicarbazide gave the corresponding polymers,[16] and analysis of these (for N) gave values for the α-glycol content of lichenin similar to those previously obtained. If lichenin was chemically heterogeneous, then a complex of a periodate-oxidised β-1 : 4-glucan would be formed, with a correspondingly higher N content (cf. Barry and his co-workers [16]).

The 1 : 3-linkages appear to be randomly situated in the lichenin molecule. Periodate-oxidised lichenin was reduced to the corresponding polyalcohol with potassium borohydride, and partially hydrolysed with acid. Paper chromatography of the hydrolysate showed the presence of glucose but not of laminaribiose. It is concluded from this evidence that few, if any, sequences of two or more adjacent 1 : 3-linkages were present in the periodate-oxidised lichenin.

The lichenin from Iceland moss is therefore similar to the β-glucans isolated recently from barley [17] and oats,[18] although the relative proportions of 1 : 3- and 1 : 4-linkages and average chain lengths are not identical. Barley β-glucan (D.P. *ca.* 100) contains a higher proportion of 1 : 3-linkages (*ca.* 50%) than oat β-glucan (D.P. *ca.* 180; 33%) and lichenin.

Experimental

Analytical Methods.—The methods used were those described in earlier papers.[19] The following solvents were used for the paper chromatography of (*a*) unsubstituted sugars, and (*b*) methylated sugars: butan-1-ol–benzene–pyridine–water [5 : 1 : 3 : 3, v/v; (*a*)], butan-1-ol–ethanol–water [4 : 1 : 5, v/v; (*a*) and (*b*)], and ethyl acetate–pyridine–water [10 : 4 : 3, v/v; (*a*)].

iso*Lichenin*

Isolation of iso*Lichenin.*—Iceland moss (500 g.), after extraction with benzene and methanol (Soxhlet) to remove waxy materials, was treated with cold 2% sodium carbonate solution (1·5 l.) to remove lichen acids. The moss residue was washed free from alkali, and extracted with boiling water (3 l.) for 2—3 hr. After filtering, the solution was allowed to cool slowly. Lichenin was precipitated, and was separated by centrifugation. The solution was concentrated and centrifuged, and residual lichenin removed by repeated freezing and thawing of the solution, the lichenin being partially precipitated during the thawing. Crude *iso*lichenin was isolated by precipitation with acetone, dissolution in cold 1% hydrochloric acid, and reprecipitation with acetone. The product, *iso*lichenin A, had $[\alpha]_D^{17}$ +165° (*c* 0·62 in H_2O) and contained glucose together with small amounts of galactose, mannose, and a pentose. From a total of 4 kg. of moss, 5·2 g. of *iso*lichenin A were obtained. The *iso*lichenin was treated with Fehling's solution, and the copper complex shaken with water (only two-thirds of the complex dissolved). The insoluble material, after regeneration to the polysaccharide with acetic acid, gave, on acid hydrolysis, mannose and galactose and a small amount of glucose. The polysaccharide regenerated from the soluble fraction [Fraction A; yield 2·8 g.] contained glucose and only traces of mannose and galactose. Fraction A had $[\alpha]_D^{17}$ +212° (*c* 0·40 in H_2O). An alkaline copper-complex of Fraction A was then fractionally precipitated with acetone. A gelatinous precipitate was rejected, and a flocculent precipitate collected, dissolved in water, acidified (acetic acid), and reprecipitated with acetone. The product, *iso*lichenin B, on acid hydrolysis

gave glucose and no other sugar. An aqueous solution of *iso*lichenin B was dialysed for 2—3 days, passed through basic and acidic ion-exchange resins, and finally precipitated with alcohol (yield 1·9 g.).

In a second series of extractions, 0·8 g. of *iso*lichenin was obtained from 2 kg. of moss.

Examination of iso*Lichenin.*—The *iso*lichenin precipitate was a white fibrous solid. Hydrolysis with 0·5N-hydrochloric acid at 100° for 3—4 hr. gave glucose (96%) and no other sugar (quantitative paper chromatography [20]). No uronic acid could be detected in the polysaccharide (naphtharesorcinol test) or on paper chromatography of the acid hydrolysate. *iso*Lichenin had $[\alpha]_D^{15}$ +255° (*c* 1·0 in H_2O), contained 0·32% of ash and had a low reducing power towards hypoiodite with an apparent D.P. of 34. An aqueous solution was stained greenish-blue with iodine, the absorption spectrum having a maximum at 600 mμ, and a blue-value [10] of 0·01.

Periodate Oxidation of iso*Lichenin.*—The polysaccharide (99·6 mg.) was oxidised with potassium metaperiodate at room temperature, under the conditions described previously.[19,21] The formic acid production was as follows :

Time of oxidation (hr.)	24	48	96	144	168	192
Moles formic acid per $C_6H_{10}O_5$ residue, × 10^2	3·8	4·7	5·7	6·1	6·7	6·9

On the assumption that each molecule of *iso*lichenin gives rise to 3 moles of formic acid, the observed yield of formic acid corresponds to a chain length of about 42 units.

In a second experiment, *iso*lichenin (115·4 mg.) was oxidised with sodium metaperiodate (0·25M ; 50 ml.). The periodate uptake was determined, at intervals, by Fleury and Lange's method : [22]

Time of oxidation (hr.)	24	48	72	96
Moles of periodate consumed per anhydroglucose unit	0·40	0·41	0·41	0·42

*iso*Lichenin therefore contains about 60% of periodate-resistant glucose residues.

Acetylation of iso*Lichenin.*—*iso*Lichenin (1·8 g.) was acetylated by Pacsu and Mullen's method [23] giving a chloroform- and acetone-soluble acetate (3·0 g.) [Found : CH_3·CO, 43·8. Calc. for $(C_{12}H_{16}O_8)_n$: CH_3·CO, 44·8%] with $[\alpha]_D^{16}$ +160° (*c* 1·04 in $CHCl_3$) and η_{sp}^{20}/c 0·02254 (*c* 1·035 in $CHCl_3$) corresponding to an apparent D.P. of *ca.* 43, on the assumption that $Km = 5{\cdot}3 \times 10^{-4}$.[24]

Methylation of iso*Lichenin.*—*iso*Lichenin *O*-acetate (2·7 g.) in acetone solution was methylated four times with dimethyl sulphate and sodium hydroxide solution, and three times with methyl iodide and silver oxide. The partly methylated polysaccharide (OMe, 43·3%) was isolated and then fractionated by the solution method, with successive mixtures of chloroform and light petroleum (b. p. 65—70°), as follows :

Fraction	Petroleum–chloroform	Yield (g.)	OMe (%)
1	95 : 5	—	—
2	90 : 10	0·056	—
3	85 : 15	1·398	43·6
4	80 : 20	0·661	42·2

Fractions 3 and 4 were combined, and methylated by Freudenberg and Boppel's liquid-ammonia method.[25] At the end of the reaction, the neutralised mixture was extracted with chloroform, and the extract filtered, dried, and concentrated. Methylated *iso*lichenin was obtained as a white flocculent precipitate on pouring the concentrated solution into light petroleum (b. p. 40—60°) (Found : Ash, 0·5; OMe, 44·3. Calc. for tri-*O*-methylglucan, OMe, 45·6%). The methylated polysaccharide was then fractionated by the solution method, with chloroform–light petroleum (b. p. 70—80°) as follows :

Fraction	Petroleum–chloroform	Yield (g.)	η_{sp}/c (in $CHCl_3$)	$[\alpha]_D^{14}$ (in $CHCl_3$)	OMe (%)
1	90 : 10	0·036	—	—	—
2	85 : 15	1·42	0·0137	+218°	44·5
3	80 : 20	0·34	0·0184	+216	44·1

Fractions 2 and 3 were combined (Found : OMe, 44·4%).

Hydrolysis of Methylated iso*Lichenin and Separation of Methylated Sugars.*—An acid hydrolysate of a sample (10 mg.) was examined by paper chromatography. 2 : 3 : 4 : 6-Tetra- (R_G 1·00), 2 : 4 : 6-tri- (R_G 0·76), 2 : 3 : 6-tri- (R_G 0·83), and di-*O*-methylglucose were present.

Methylated *iso*lichenin (1·40 g.) was hydrolysed with (1%) methanolic hydrogen chloride

(100 ml.) under reflux for 13 hr. (constant rotation). After neutralisation (diazomethane), the hydrolysate was concentrated, and further hydrolysed with boiling 0·5N-hydrochloric acid (100 ml.) for 10 hr. The neutralised and concentrated hydrolysate was extracted with chloroform, and this extract concentrated. The syrup (1·50 g.) was fractionated on a cellulose column [26] (70 × 2 cm.), light petroleum (b. p. 100—120°)–butan-1-ol (6 : 4) saturated with water being used as solvent. Three fractions were isolated, which contained 1·40 g. of sugars (93% recovery). Elution of the column with water gave a fourth fraction (13 mg.) containing traces of mono-*O*-methylglucose and glucose, which was not examined further.

Fraction 1. The syrup (0·161 g.) contained tetra-*O*-methylglucose (18·0%, by hypoiodite oxidation) and methyl glucosides of tri-*O*-methylglucoses. It was rehydrolysed with 0·5N-hydrochloric acid (10 ml.), neutralised, and chromatographed on a second cellulose column, giving fraction 1*a* (19 mg.) and 1*b* (118 mg.). Fraction 1*a* was identified as 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose. After three recrystallisations from light petroleum (b. p. 40—60°), it had m. p. 83—85°, $[\alpha]_D^{17}$ +84° (*c* 0·53 in H_2O). Fraction 1*b* was a mixture of sugars with the same R_G values as 2 : 3 : 6- and 2 : 4 : 6-tri-*O*-methylglucose.

Fraction 2. This fraction (1·205 g.) was a mixture of 2 : 4 : 6- (major component) and 2 : 3 : 6-tri-*O*-methylglucose (paper chromatography). It was 90·3% pure, by hypoiodite oxidation. 2 : 3 : 4- and 3 : 4 : 6-Tri-*O*-methylglucose were absent; the sugars gave a negative Weerman reaction. Part of the fraction (300 mg.) was rechromatographed three times on a cellulose column, giving fraction 2*a* (150 mg.) and 2*b* (50 mg.). Fraction 2*a* was pure 2 : 4 : 6-tri-*O*-methyl-D-glucose. After recrystallisation from ether, it had m. p. 120—122°, $[\alpha]_D^{18}$ +75° (equil.) (*c* 1·1 in H_2O) (Found: OMe, 41·1. Calc. for $C_9H_{18}O_6$: OMe, 41·9%). The corresponding 2 : 4 : 6-tri-*O*-methyl-*N*-phenyl-D-glucosylamine had m. p. 143—145°. Fraction 2*b* was identified as 2 : 3 : 6-tri-*O*-methyl-D-glucose. On recrystallisation from ether, it had m. p. 114—116°, $[\alpha]_D^{17}$ +67° (equil.) (*c* 0·6 in H_2O). The phenylhydrazide of the derived 2 : 3 : 6-tri-*O*-methyl-D-gluconic acid had m. p. 145° (Found: OMe, 28·9. Calc. for $C_{15}H_{24}O_6N_2$: OMe, 28·4%).

Fraction 3. This material (40 mg.; 70% pure by hypoiodite oxidation) appeared to contain 2 : 3-di-*O*-methylglucose (21%) and other di-*O*-methylglucoses (79%) as indicated by quantitative paper chromatography.

The weights and mol. proportions of sugars were: tetra-*O*-methyl- (0·031 g.; 1 mol.), tri-*O*-methyl- (1·220 g.; 42 mol.) and di-*O*-methyl-glucoses (0·028 g.; 1 mol.). *iso*Lichenin is therefore unbranched, and contains one non-reducing terminal group per 44 glucose residues.

Action of β-*Amylase on* iso*Lichenin.*—β-Amylase was prepared from barley by the method of Preece and Shadaksharaswamy.[27] The following digest was prepared: *iso*lichenin (50 mg.), 0·04M-acetate buffer (pH 4·6; 2 ml.), β-amylase solution (0·1%; 0·5 ml.), and water (5 ml.). After 48 hours' incubation at 38°, chromatography failed to show the presence of reducing sugars. The iodine-staining power of the polysaccharide was unchanged. In similar digests containing soluble starch or a mixture of starch and *iso*lichenin, maltose was detected after only 15 minutes' incubation.

A sample of purified soya-bean β-amylase kindly supplied by Professor S. Peat, F.R.S., likewise failed to attack *iso*lichenin.

Lichenin

Isolation of Lichenin.—The material which was precipitated from the hot-water extract of Iceland moss (p.) was freed from *iso*lichenin by repeated precipitations from hot water. Addition of Fehling's solution to an alkaline solution of the lichenin gave an insoluble copper complex; after regeneration (acetic acid), the lichenin was precipitated with acetone and dried (yield 3 g. from 500 g. of moss). The polysaccharide was finally shaken with water for 5—6 hr. and reprecipitated with acetone.

Examination of Lichenin.—The lichenin preparations were white powders, insoluble in cold water, and soluble in hot water, or alkali; they had $[\alpha]_D$ +8° to +10° (*c* 1·0 in N-sodium hydroxide). Paper chromatography of acid hydrolysates gave glucose and no other sugar. Sample I had a glucose content of 94% (by polarimetric determination and quantitative paper chromatography [20]), ash content 0·88%, and a slight reducing action towards alkaline hypoiodite (apparent D.P. 80—86). Sample II had a glucose content of 96% (by cuprimetric titration) and an ash content of 0·23%.

Periodate Oxidation of Lichenin.—(a) *Sodium metaperiodate.* Lichenin (60—70 mg.) was

shaken, in the dark, with 0·22M-sodium metaperiodate solution (15 ml.) for periods up to 72 hr. The periodate-uptake was determined as before. After 24, 48, and 72 hours' oxidation, both sample I and II had reduced 0·7 mole of periodate per anhydroglucose residue. Approximately 30% of the glucose residues in lichenin are therefore resistant to periodate oxidation.

(b) *Preparation of* iso*nicotinhydrazide and thiosemicarbazide polymers.* Lichenin (sample II) was treated with sodium metaperiodate for 72 hr. at room temperature, and the periodate-oxidised lichenin then isolated, washed, and dried. 50 Mg. portions, dissolved in hot water (2·5 ml.), were cooled and mixed with *iso*nicotinhydrazide (85 mg.) or thiosemicarbazide (56 mg.) in water (5 ml.). The precipitated polymers were collected, washed and dried: *iso*nicotinhydrazide polymer (Found: N, 11·6, equiv. to 66% of α-glycol groups); thiosemicarbazide polymer (Found: N, 12·7, equiv. to 67% of α-glycol groups).

(c) *Partial hydrolysis of the polyalcohol.* Periodate-oxidised lichenin (Sample II; 40 mg.) was treated with potassium borohydride (25 mg.) in water (2 ml.) for 5 hr. The polyalcohol was precipitated with alcohol, then hydrolysed with 0·5N-sulphuric acid (4 ml.) at 100° for 1·5 hr., and the neutralised concentrated hydrolysate examined by paper chromatography. Glucose was the only sugar present. Under similar conditions, a partial hydrolysate of lichenin contained glucose, laminaribiose, cellobiose and higher oligosaccharides.

Acetylation of Lichenin.—Lichenin (Sample I; 2·5 g.) on acetylation (Pacsu and Mullen's method [23]) gave lichenin acetate (3·4 g.) (Found: ash content 0·5; CH_3·CO, 44·0. Calc. for $(C_{12}H_{16}O_8)_n$: CH_3·CO, 44·8%). The acetate (3·0 g.) was fractionated from chloroform solution with light petroleum (b. p. 40—60°), as follows:

Fraction	Yield (g.)	Acetyl (%)	$[\alpha]_D^{16}$ (*c* 1·0 in $CHCl_3$)
1	0·4	43·8	−30°
2	2·2	44·2	−34

Lichenin acetate (Fraction 2) was deacetylated (sodium methoxide), and the regenerated polysaccharide treated with sodium metaperiodate. 0·7 Mole of periodate per anhydroglucose unit was consumed.

Methylation of Lichenin.—Lichenin (Sample I, 6·0 g.) on methylation (seven treatments with dimethyl sulphate and sodium hydroxide) gave a product with OMe 40·8%. Fractionation, by the solution method, with dry benzene–light petroleum (b. p. 75—80°), gave the following fractions:

Fraction	Petroleum–benzene	Yield (g.)	OMe (%)	$[\alpha]_D^{17}$ (in $CHCl_3$)
1	95 : 5	0·08	41·5	−8·2°
2	90 : 10	2·13	43·9	−8·1
3	85 : 15	1·34	43·1	−7·9
4	80 : 20	0·10	39·8	—

Fractions 2 and 3 were combined, and treated twice with methyl iodide and silver oxide. The product had $[\alpha]_D^{17}$ −8·2° (*c* 0·74 in $CHCl_3$) (Found: ash content 0·81; OMe, 44·4%).

Hydrolysis of Methylated Lichenin and Separation of Methylated Sugars.—Paper chromatography of an acid hydrolysate of 10 mg. methylated lichenin showed the presence of 2 : 3 : 4 : 6-tetra- (R_G 1·00), 2 : 3 : 6-tri- (R_G 0·83), 2 : 4 : 6-tri- (R_G 0·76) and di-*O*-methylglucoses.

Methylated lichenin (3·0 g.) was hydrolysed successively with methanolic and aqueous hydrogen chloride, and the resulting syrup (3·2 g.) fractionated on a cellulose column [26] (90 × 2·5 cm.) as described previously.

Fraction	Yield (g.)	Components	Purity (%, by hypoiodite oxidation)
1	0·455	Tetra-*O*-methylglucose and methyl tri-*O*-methylglucosides	10·3
2	2·469	Tri-*O*-methylglucoses	91·5
3	0·039	Di-*O*-methylglucoses	85·1

A fourth fraction (15 mg.) was eluted from the column with water.

Fraction 1. Rehydrolysis and chromatography gave fraction 1*a* (32 mg.) and 1*b* (410 mg.). Fraction 1*a* crystallised, and was identified as 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose; after recrystallisation from light petroleum (b. p. 40—60°) it had m. p. 83—86°, $[\alpha]_D^{15}$ +83° (*c* 0·67 in H_2O). Fraction 1*b* was a mixture of tri-*O*-methylglucoses.

Fraction 2. Paper chromatography showed that 2 : 3 : 4-tri-*O*-methylglucose (R_G 0·85) was absent. The material gave a negative Weerman reaction, indicating the absence of the

3 : 4 : 6-isomer. Part of fraction 2 (500 mg.) was rechromatographed on cellulose columns; fractions 2*a* (333 mg.), 2*b* (129 mg.), and 2*c* (18 mg.) were obtained. Fraction 2*a* was pure 2 : 3 : 6-tri-*O*-methyl-D-glucose. Recrystallisation from ether gave crystals, m. p. 120—122°, $[\alpha]_D^{16}$ +67° (equil.) (*c* 0·53 on H_2O) (Found : OMe, 40·9. Calc. for $C_9H_{18}O_6$: OMe, 41·9%). The phenylhydrazide of the derived aldonic acid had m. p. 145° (Found : N, 8·3; OMe, 27·7. Calc. for $C_{15}H_{24}O_6N_2$: N, 8·6; OMe, 28·4%). Fraction 2*b* was identified as 2 : 4 : 6-tri-*O*-methyl-D-glucose. After recrystallisation (dry ether), it had m. p. 119—123°, $[\alpha]_D^{17}$ +74° (equil.) (*c* 0·82 in H_2O) (Found : OMe, 40·5. Calc. for $C_9H_{18}O_6$: OMe, 41·9%). Treatment with aniline gave 2 : 4 : 6-tri-*O*-methyl-*N*-phenyl-D-glucosylamine with m. p. 143—144°. Fraction 2*c* was a mixture of tri-*O*-methylglucoses.

The rotation of the tri-*O*-methyl fraction in cold methanolic 2% hydrogen chloride was $[\alpha]_D$ +36° ⟶ −15° constant in 24 hr. (*c* 1·04). An authentic mixture of 2 : 3 : 6- (68%) and 2 : 4 : 6-tri-*O*-methyl-D-glucose (32%) showed $[\alpha]_D$ +57° ⟶ −13° constant in 24 hr. (*c* 1·08 in methanolic 2% hydrogen chloride).

Fraction 3. The syrup contained 2 : 3-di-*O*-methylglucose (53%) together with other di-*O*-methylglucoses (47%) by quantitative paper chromatography. In qualitative experiments, treatment of pure 2 : 4 : 6-tri- and 2 : 3 : 6-tri-*O*-methylglucose with N-hydrochloric acid at 100° for 3 hr. gave about 1% of mixed di-*O*-methylglucoses and a trace of mono-*O*-methylglucose. Fraction 3 is therefore considered to be due to (*a*) hydrolytic demethylation and (*b*) under-methylation.

Since 2·724 g. of methylated sugars contained 0·047 g. of pure tetra-*O*-methylglucose, lichenin has an average chain length of *ca.* 62 glucose residues.

Action of β-Glucosidase Preparations on Lichenin.—A fine suspension of lichenin was prepared either by dissolution in 0·2N-sodium hydroxide followed by neutralisation with hydrochloric acid (phenolphthalein) or by warming an aqueous suspension of lichenin to 50°. β-Glucosidase action was studied qualitatively by incubating a lichenin suspension (1% ; 2 ml.), 0·2M-acetate buffer (pH 4·6 or 5·0; 0·5 ml.), and β-glucosidase solution (0·5 or 1·0 ml.) at 37°. Samples were analysed at intervals, by paper chromatography, using authentic specimens of glucose, laminaribiose, and cellobiose as reference compounds.

Type of β-glucosidase	Source of enzyme	Method of prep. ref.	Product(s)
Exo-	Almond emulsin	28	Glucose
Exo-	*Helix aspersa*	29	Glucose
Endo-	Malted barley	30	Glucose, cellobiose, laminaribiose and higher oligosaccharides
Endo-	*Cladophora rupestris*	15	Glucose, cellobiose, laminaribiose and higher oligosaccharides.

In a further experiment, lichenin suspension (3 mg./ml. ; 5 ml.), 0·2M-acetate buffer (pH 5·0; 2 ml.), and almond β-glucosidase [28] (10 mg. in water ; 1 ml.) were incubated at 35°. After 3 days, 12% conversion into glucose was observed (Shaffer–Somogyi estimation). In a control experiment, laminarin {$[\alpha]_D$ +9° (*c* 2·7 in N-NaOH)} gave 15% conversion into glucose.

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PERIODATE OXIDATION OF LAMINARIN

By F. B. Anderson, E. L. Hirst and D. J. Manners
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The proportion of free reducing groups in a glucosan may be assayed by measurement of the formaldehyde liberated on periodate oxidation of the polysaccharide and the corresponding alcohol. In laminarin the reducing groups are attached to the rest of the molecule at C(3), and the yield of formaldehyde before and after conversion into laminaritol is therefore one and two mol. per reducing group. In addition, the above method of analysis enables the proportion of molecules which are terminated by a non-reducing mannitol group[1] to be determined. The formaldehyde production from the mannitol-terminated chains (which amounts to 1 mol. per chain, since the mannitol is linked through a primary alcohol group[1]) is not altered by the reduction of laminarin. By this means, Unrau and Smith[2] concluded that 30% of the molecules in a sample of laminarin (type unspecified) were terminated by a mannitol residue.

In an independent study, we have examined both the " soluble " and " insoluble " forms of laminarin,[3,4] formaldehyde production being determined with a phenylhydrazine-ferricyanide reagent[5] in preference to the chromotropic acid reagent (cf. ref. [2]).

The soluble laminarin gave 0·053 mol. formaldehyde per anhydrohexose residue, while the corresponding laminaritol yielded 0·066 mol. formaldehyde. It follows that *ca.* 75% of the laminarin molecules are terminated by a mannitol residue, and the average chain length is *ca.* 19. The formic acid production was also equivalent to a chain of *ca.* 19 residues.

This sample of soluble laminarin therefore contains one mannitol residue per 24 glucose residues. This result is supported by estimations of the glucose and mannitol contents of an acid hydrolysate of the polysaccharide.

A sample of insoluble laminarin has also been examined. Some 46% of the molecules were terminated by a mannitol residue while the production of formaldehyde and formic acid, and the nitrogen content of the *iso*niazid derivative[6] indicated an average chain length of *ca.* 24 residues.

The soluble and insoluble forms of laminarin thus differ significantly in mannitol content, and slightly in average chain length. The former conclusion is supported by the much lower reducing power of the soluble form.[3,7]

The " over-oxidation " of insoluble laminarin by periodate[8] has been studied. Molecules terminated by mannitol are converted into substituted acetaldehyde derivatives of the type R—O—CH_2—CHO, which are stable. By contrast, *linear* molecules, which carry a normal reducing group, are completely degraded, giving one mol. formaldehyde per glucose residue.

On periodate oxidation, in phosphate buffer pH 8,[8] the insoluble laminarin gave 0·52 mol. formaldehyde per glucose residue. This value would be expected if *ca.* 48% of the molecules were terminated by a mannitol residue. In view of the agreement between this figure and that previously determined, and since periodate " over-oxidation " ceases when 1 : 6-linkages are encountered, it is suggested that the presence of β-1 : 6-glucosidic linkages[1] in laminarin is confined to those molecules which contain a mannitol end-group.

It is hoped to publish full details of these experiments, and the results of complementary methylation studies elsewhere.

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663. *The Constitution of Laminarin. Part III.* The Fine Structure of Insoluble Laminarin.*

By F. B. Anderson, E. L. Hirst, D. J. Manners, and A. G. Ross.

Insoluble laminarin, a mixture of glucans isolated from *Laminaria cloustoni*, has been investigated by periodate oxidation and methylation. The presence of mannitol (2%) has been confirmed, and approximately half the molecules are terminated by a mannitol residue. Some of the molecules have a low degree of branching, and the constituent chains appear to be interlinked by β-1 : 6-glucosidic linkages. Many of the branched molecules contain mannitol.

The polysaccharide material isolated after the reduction of laminarin with potassium borohydride, and after treatment with lime-water, has also been examined; their structures support the above conclusions on the fine structure of laminarin.

The molecular structure of laminarin, a mixture of glucans which form a reserve carbohydrate in various species of brown marine algae (Phaeophyceae), has been the subject of several recent investigations. The material exists in two forms which differ in solubility in cold water. A "soluble" form is present mainly in *Laminaria digitata*, whilst "insoluble" laminarin has been isolated from the fronds of a number of species of Laminariaceae. The present paper is concerned only with the laminarin from *L. cloustoni*.

Methylation studies carried out by Barry [1] showed that insoluble laminarin was composed of β-1 : 3-linked D-glucopyranose residues, whilst successive oxidation with periodate and bromine indicated [2] an apparent chain length of sixteen glucose residues. However, a later investigation (Part I [3]) suggested that laminarin was composed of about twenty β-1 : 3-linked glucose residues, although the reducing power was unexpectedly low. It was concluded [3] that a proportion of the reducing glucose groups were in some way modified. A similar investigation of soluble laminarin from *L. digitata* was described in Part II [4] and will be considered in detail in a later communication.

An important development in laminarin chemistry was the discovery that mannitol (*ca.* 2%) was a constituent residue.[5] After partial acid hydrolysis of laminarin, Peat, Whelan, and Lawley separated from the mixed saccharides small quantities of mannitol, 1-*O*-β-D-glucosylmannitol and 1-*O*-laminaribiosylmannitol in addition to much larger amounts of glucose, laminaribiose, and higher laminarisaccharides. This finding, together with other evidence, showed that a *proportion* of the molecules were terminated by a mannitol residue linked through one of the two primary alcohol groups. A further discovery, which suggested that laminarin contained a few β-1 : 6-glucosidic linkages, was the isolation of a small quantity (0·26%) of gentiobiose, together with two isomeric trisaccharides, 6-*O*-β-laminaribiosylglucose and 3-*O*-β-gentiobiosylglucose. Since Peat and his co-workers considered that positive evidence for branching was not available (a significant quantity of 3 : 6-di-*O*-β-glucosylglucose could not be isolated), they suggested that one of the possible structures for laminarin was "a linear molecule of β-glucose residues in which repeating sequences of 1 : 3-links are occasionally interrupted by a 1 : 6-linkage." We are indebted to Professor Peat and Dr. Whelan who kindly allowed us to see two papers [5] before publication.

The present communication describes further methylation and periodate oxidation studies of insoluble laminarin. Degradation of laminarin by lime-water has also been examined. A preliminary account of part of this work has appeared elsewhere.[6] The following abbreviations are here used: average chain length ($\overline{CL}$) denotes the average number of residues per non-reducing glucose end-group; degree of polymerisation ($\overline{DP}$) refers to the average number of residues per molecule. Chains terminated at the reducing end by glucose or mannitol are described as G-chains or M-chains, respectively.

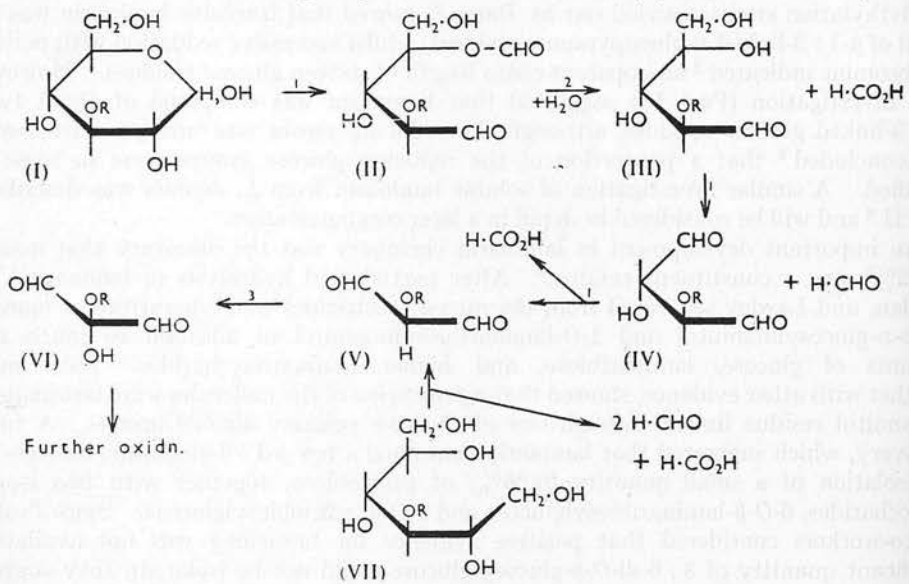
Isolation and Properties of Laminarin.—The laminarin was one sample, prepared by

* Part I, *J.*, 1950, 3494; Part II, *J.*, 1951, 720.

Dr. W. A. P. Black. It was extracted from the seaweed at 55° with dilute hydrochloric acid (pH 3·4) in 1 hr. and when purified had a glucose content of 94%, $[\alpha]_D$ −9° in H_2O, +9° in N-sodium hydroxide, and contained one reducing group per 47 residues (hypoiodite oxidation). (The insoluble laminarin examined in Parts I [3] and II [4] had reducing powers corresponding to one reducing group per 40 and 45 residues respectively.)

A partial acid hydrolysate was shown by paper chromatography to contain glucose, laminaribiose, laminaritriose, and unidentified sugars with $R_{Glucose}$ values of 0·45 (non-reducing), 0·34, 0·24, and 0·16. A complete acid hydrolysate contained mannitol. Since D-glucose and mannitol have similar R_G values in the usual paper chromatographic solvents, two different experiments were carried out. In the first, the glucose was converted into the less mobile D-gluconic acid by addition of D-glucose oxidase [7] to the neutralised hydrolysate. A non-reducing carbohydrate with the R_G value of mannitol was then revealed. The presence of mannitol (and a non-reducing disaccharide) has also been shown by using a special solvent (ethyl methyl ketone–acetic acid–water, saturated with boric acid) kindly communicated by Dr. W. R. Rees.

Periodate Oxidation Studies.—Periodate oxidation [8] of a 3-*O*-substituted glucose (I) may involve at least three reactions: (1) normal Malapradian oxidation of α-glycol groups, (2) slow hydrolysis of a formyl ester (II), followed by further Malapradian oxidation, and (3) oxidation of an activated hydrogen atom in a structure (V) of malondialdehyde type.



In contrast, periodate oxidation of 3- or 6-*O*-substituted hexitols (VII and VIII) requires only normal Malapradian-type reactions to yield respectively the malondialdehyde derivative (V) or the substituted acetaldehyde derivative (IX):

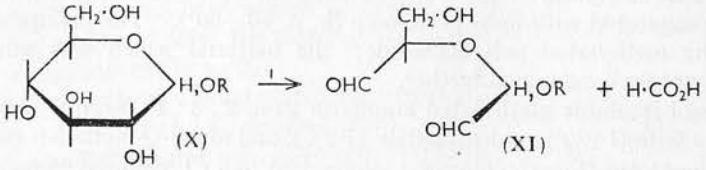
$$\underset{\text{(VIII)}}{RO{\cdot}CH_2{\cdot}[CH(OH)]_4{\cdot}CH_2{\cdot}OH} \xrightarrow{I} \underset{\text{(IX)}}{RO{\cdot}CH_2{\cdot}CHO} + 3H{\cdot}CO_2H + H{\cdot}CHO$$

Partial acid hydrolysis [5] and methylation [1,3] indicate that in laminarin the chains are terminated at the potential reducing end by either a reducing glucose residue linked at $C_{(3)}$ or a mannitol group, as represented in formulæ (I) and (VIII) respectively (where R is a chain of β-1 : 3-linked glucose residues).

Oxidation of non-reducing end-groups (X) also proceeds without a formyl-ester intermediate, to give the stable dialdehyde (XI).

The relative rates of these reactions have been controlled by using three oxidation

conditions: (*a*) Initial primary oxidation (I ⟶ II, VII ⟶ V, VIII ⟶ IX, and X ⟶ XI) by sodium metaperiodate at 2°, whereby the rate of hydrolysis of formyl esters is greatly reduced,[9] (*b*) total primary oxidation (I ⟶ V, and normal oxidation of VII, VIII, and X) with potassium metaperiodate [10] or a limited excess of sodium metaperiodate [11] at room temperature, and (*c*) sodium metaperiodate buffered at pH 8 at room temperature, enabling reactions (1), (2), and (3) to take place.[12]



Control experiments have shown that the initial primary oxidation of laminaribiose (giving structures II and XI) required some 14 hr., and that formaldehyde production from this sugar, or from 3-*O*-methyl-D-glucose, was not observed until after 3 days' oxidation. In contrast, the oxidation of laminarin under similar conditions gave formaldehyde within 1 hr. This observation is not only consistent with the presence of terminal-mannitol residues, but also provides a method of estimation. The initial yield * of formaldehyde (0·02 mol.) is equivalent to the presence of 2% of mannitol in the laminarin.

Measurements of the formaldehyde liberated during the total primary oxidation of laminarin and the corresponding alcohol (laminaritol) have been used to determine the number average $\overline{DP}$ and the proportion of M-chains. This oxidation (with a limited excess of sodium metaperiodate at room temperature) gave 0·041 mol. of formaldehyde from laminarin. Since the oxidation of either G- or M-chains gives rise to one mol. of formaldehyde per chain, this value corresponds to a $\overline{DP}$ of 24. After reduction of laminarin with potassium borohydride (p.) the laminaritol so produced yielded 0·063 mol. of formaldehyde on similar oxidation. It follows that 0·022 mol. of formaldehyde arises from the G-chains in laminarin, and that the remaining 0·019 mol. is liberated from M-chains. Calculations from these data show that the laminarin sample of $\overline{DP}$ 24 contains (*a*) 46% of M-chains, (*b*) 1·9% of mannitol, and (*c*) one reducing group per 45 glucose residues.

During the oxidation of laminarin at pH 8, only the residues (VIII) and (X) of the M-chains will be attacked, whilst linear G-chains will be degraded in stepwise fashion with the production of one mol. of formaldehyde per glucose residue. This oxidation process is inhibited by 1 : 6-linkages since these, like the mannitol residue, give rise to stable acetaldehyde derivatives (IX).[12] Our laminarin sample gave 0·52 mol. of formaldehyde, a value similar to that reported by Hough and Perry.[12] This result is in good agreement with that expected for a laminarin containing approximately equal numbers of M- and G-chains, many of the latter being linear.

The $\overline{CL}$ of laminarin can be deduced from the amount of formic acid produced during the initial primary oxidation. The observed figure, after 6 hours' oxidation with potassium metaperiodate at room temperature, or 24 hours' oxidation with sodium metaperiodate at 2° (see p.), is 0·10 mol., *i.e.*, 10 mol. of formic acid per 100 hexose residues. Since each mannitol residue gives rise to 3 mol. of formic acid, 5·7 mol. will originate from these, and the remainder (4·3 mol.) must arise from non-reducing end-groups. The proportion of these is therefore one per 23 glucose residues. In view of later evidence of heterogeneity, this numerical agreement between the $\overline{DP}$ and $\overline{CL}$ values is considered to be fortuitous, and not an indication of a linear structure.

The proportion of free reducing groups in the polysaccharide may be estimated from

* The products liberated on periodate oxidation are normally reported as mol. per anhydrohexose residue.

the rate of production of formic acid. As stated above, the initial primary oxidation yields 0·10 mol. whereas the total oxidation [after the hydrolysis of formyl ester groups (II) which requires 8—10 days] gives 0·15 mol. Since 0·05 mol. of formic acid is liberated from reducing glucose end-groups, the proportion of these is one per 40 glucose residues (cf. one per 45—47 by other methods).

Methylation Studies.—Laminarin was methylated with dimethyl sulphate and sodium hydroxide, and a chloroform solution of the methylated polysaccharide (OMe, 44·0%) was fractionally precipitated with light petroleum (b. p. 40—60°). The precipitate amounted to 78% of the methylated polysaccharide; the material which was soluble in light petroleum has not been examined further.

Hydrolysis of insoluble methylated laminarin gave 2 : 3 : 4 : 6-tetra- (4·4%), 2 : 4 : 6-tri- (84·6%), 4 : 6-di- (1·9%), unidentified di- (7·2%), and mono-*O*-methyl-D-glucose (1·9%). The proportion of tetra-*O*-methylglucose is equivalent to a $\overline{CL}$ value of 23 (not 20 as stated in ref. 13). No tri-*O*-methylglucose other than the 2 : 4 : 6-isomer could be detected.

The molecular weights of the whole and of insoluble methylated laminarin, as determined by isothermal distillation,[13] were respectively 1900 and 12,000, equivalent to $\overline{DP}$ values of 9 and 58. It follows that the material (22%) soluble in light petroleum had a molecular weight of *ca.* 500 ($\overline{DP}$ 2—3).

Although the measurement of molecular weights in the range 2000—20,000 is extremely difficult, the isothermal distillation measurements (which in this range are accurate to within 10%) show that the $\overline{DP}$ of methylated laminarin is much larger than the $\overline{CL}$, and a number of the molecules must therefore contain a small number of branch points. On the average, two branch points per molecule appear to be present. Evidence on the nature of the interchain linkage has not been obtained from the methylation analysis since undermethylation of the polysaccharide and hydrolytic demethylation of 2 : 4 : 6-tri-*O*-methylglucose [3,4] both give rise to di-*O*-methylglucose. However, the presence of oligosaccharides containing β-1 : 6-glucosidic linkages in partial hydrolysates of laminarin [5] suggests that these are, in fact, present as interchain linkages. These would number only 2—3% of the total glucosidic linkages, in agreement with the small observed yield of gentiobiose. The failure to isolate 3 : 6-di-*O*-β-glucosylglucose from a partial hydrolysate is not inconsistent with this view. In analogous experiments with other branched polysaccharides, hydrolysis of glycosidic linkages adjacent to interchain linkages does not appear to be random. Thus, the major trisaccharide containing an interchain linkage in a partial hydrolysate of glycogen or amylopectin is panose;[14] significant quantities of 4 : 6-di-*O*-α-glucosylglucose have not been isolated.

Degradation of Laminarin with Lime-water.—The susceptibility of reducing glucosaccharides and, in particular, laminarin to degradation by aqueous alkali is well known (see, for example, Part II [4]). Recent studies by Corbett and Kenner [15] showed that insoluble laminarin, on prolonged treatment with oxygen-free lime-water at 25°, is degraded in stepwise fashion from the reducing group to give D-glucometasaccharinic acid in 40—50% yield. Termination of the reaction was ascribed " to some inhibitive variation in chain structure " and Corbett and Kenner pointed out that degradation by lime-water would cease when 1 : 6-linkages were encountered in the molecule. It is now clear, however, that the degradative action of lime-water must be confined to the G-chains, and in laminarin samples containing an appreciable number of M-chains incomplete conversion into D-glucometasaccharinic acid is to be expected.

We carried out similar experiments, but at 60°, and subjected the residual polysaccharide to methylation analysis. Laminarin was treated with oxygen-free lime-water for 23 hr. at 60°. Unattacked polysaccharide which separated was collected (46% recovery). Re-treatment of this material with lime-water did not cause any appreciable degradation, so that more than 50% of the laminarin had been transformed into soluble products. The lime-treated laminarin had $[\alpha]_D^{20}$ −7° (in H_2O), only a very slight reducing

power, and contained mannitol. On partial hydrolysis with oxalic acid, a series of oligosaccharides similar to those in the laminarin hydrolysate (p.) were obtained.

Methylated lime-treated laminarin was prepared (OMe, 44·1%) and fractionated with light petroleum. It proved to be more homogeneous than the methyl ether of laminarin as 88% of the material was insoluble in light petroleum. This fraction, which had a molecular weight of 13,600 by isothermal distillation [13] (equivalent to $\overline{DP}$ 65), will be referred to as " methylated lime-treated laminarin." Since the whole methylated sample had a molecular weight of 2500, the material (12%) soluble in light petroleum consisted of methylated saccharides of $\overline{DP}$ *ca.* 2.

An acid hydrolysate of " methylated lime-treated laminarin " (see above) contained the following sugars: 2 : 3 : 4 : 6-tetra- (5·2%), 2 : 4 : 6-tri- (84·4%), 4 : 6-di- (1·5%), unidentified * di- (7·2%) and mono-*O*-methyl-D-glucose (1·8%). These proportions are generally similar to those present in the hydrolysate of methylated laminarin (p.). The " methylated lime-treated laminarin," like methylated laminarin, thus contains molecules with a low degree of branching, namely, an average of two branch points per molecule.

The sedimentation of lime-treated laminarin in the ultracentrifuge has been compared, by Broatch and Greenwood,[13] with that of the original polysaccharide. The insoluble laminarin was extremely heterogeneous and had a sedimentation constant of $0{\cdot}5 \times 10^{-13}$ c.g.s. units, whereas the lime-treated material was more homogeneous and had a much larger sedimentation constant ($1{\cdot}0 \times 10^{-13}$ c.g.s. units). These results indicate that the lime-water treatment has preferentially degraded the polysaccharide material of low molecular weight. This degradation is confined to G-chains, and many of the molecules of higher molecular weight (branched) must therefore be terminated by mannitol residues.

Preparation and Methylation of Laminaritol.—Free reducing groups in a polysaccharide can be reduced to the corresponding alcohol residue. The conversion of laminarin into laminaritol (with G-chains terminated as in VII) was first reported by Abdel-Akher, Hamilton, and Smith [16] who used sodium borohydride as reducing agent. With potassium borohydride, we have prepared laminaritol which was virtually non-reducing, with $[\alpha]_D$ −8° (in H_2O) and a glucose content of 94%.

If our previous deductions on the structure of laminarin and its periodate oxidation are correct, then laminaritol will consist of a mixture of M-chains and sorbitol-terminated chains (S-chains). The laminaritol should therefore be resistant to lime-water, should be overoxidised to virtually the same extent as laminarin, and on periodate oxidation the rate of release of formaldehyde and formic acid should be increased.

The above conclusions have been verified experimentally. Lime-water at 60° had no appreciable action on laminaritol. On overoxidation with periodate, 0·55 mol. of formaldehyde was produced. On oxidation at pH 5, the formaldehyde production from laminaritol before and after lime-water treatment was unaltered. The maximum constant yield after only 6 hours' oxidation was 0·063 mol., equivalent to a $\overline{DP}$ of 24. Further, the amount of formic acid (*ca.* 0·13 mol.) released on total primary oxidation was, as expected, less than from laminarin, and the rate of production was increased.

Methylation of laminaritol gave a polysaccharide methyl ether with OMe 43·3%, and only 6% of this failed to be precipitated when light petroleum was added to a chloroform solution. The precipitated methylated laminaritol had a number-average molecular weight of 3800 ($\overline{DP}$ 19) from isothermal-distillation measurements.[17] This fractionation differed from that of methylated laminarin in that material of higher molecular weight ($\overline{DP}$ *ca.* 60) was not selectively precipitated.

* *Added May 10th,* 1958: This di-*O*-methyl sugar has now been identified as the 2 : 4-isomer since it could be differentiated from 2 : 6- and 4 : 6-di-*O*-methylglucose by paper electrophoresis, and on periodate oxidation formaldehyde was liberated. This finding is in accord with, but not proof of, a branched structure.

The Molecular Structure of Laminarin.—From the evidence cited above we conclude that " laminarin " is heterogeneous with respect both to molecular weight and to chemical structure. The conditions used for its extraction (pH 3·4 and 55°) do not cause marked degradation (see p.) and it seems probable that " native " laminarin is also heterogeneous. In agreement with Peat and his co-workers, we find that insoluble laminarin contains *ca.* 2% of mannitol, and that approximately one-half of the molecules are terminated by mannitol. Unrau and Smith [18] have concluded, independently, that 30% of M-chains were present in an unspecified sample of laminarin. The heterogeneous nature of the latter was also shown [18] by electrophoresis on glass-fibre paper. In addition, although the average $\overline{DP}$ of our laminarin is *ca.* 24, some of the molecules (78% *by weight*) have a $\overline{DP}$ of *ca.* 60 and are slightly branched with β-1 : 6-inter-chain linkages. The alternative suggestion [5] that the β-1 : 6-glucosidic linkages are located in non-terminal positions in a linear chain of β-1 : 3-linked glucose residues seems less likely since this structure, on methylation analysis, would yield a small quantity of 2 : 3 : 4-tri-*O*-methyl-glucose. This sugar has not been detected. Further, the number of triol groups in laminarin is the same by both methylation and periodate oxidation analysis, in agreement with a branched structure. In a linear-type structure, the number of triol groups detected by periodate would exceed that found from methylation.

The methylation analysis of laminarin is complicated, in part, by the presence of alkali-sensitive reducing groups and, in contrast to laminaritol, laminarin is partly degraded on methylation, the number-average molecular weight decreasing from *ca.* 4000 to *ca.* 1900. Similar observations have been made by Friedlaender, Cook, and Martin [19] who found partly methylated laminarin with OMe 2·7 and 6·9% had molecular weights (weight-average values from sedimentation-diffusion measurements) of 3700 and 2900 respectively. This degradation is presumably limited to G-chains, which are partly converted into methylated oligosaccharides with $\overline{DP}$ 2—3. (In our previous study,[3] these oligosaccharides would be separated from the methylated laminarin during the purification of the latter by dialysis.) However, the residual laminarin (78%) has an average chain length of 23, and a proportion of these molecules are slightly branched. It is of interest that many of these branched molecules are resistant to lime-water at 60° and are therefore terminated by mannitol residues.

Experimental

Analytical Methods.—(a) *Paper chromatography.* The following solvents were used for the separation of glucose and mannitol: (*a*) phenol–water (72 : 28) with a silver nitrate identification spray; (*b*) ethyl methyl ketone–acetic acid–water, saturated with boric acid (9 : 1 : 1, v/v) [20] with a periodate–benzidine spray reagent.[21] The solvents used for the chromatography of oligosaccharides and methylated sugars have been described previously.[22]

(b) *Reducing power.* Somogyi's alkaline copper reagent [23] or Hagedorn and Jensen's potassium ferricyanide method [24] was used, with glucose and laminaribiose as standard sugars. Reducing power (R.P.) values are expressed as 100/No. of glucose residues per apparent reducing group (against laminaribiose standard).

(c) *Periodate oxidation.* The consumption of periodate and the production of formic acid were determined as described by Manners and Archibald.[11] Formaldehyde was estimated qualitatively with a chromotropic acid reagent [25] and quantitatively by the method of Hough, Powell, and Woods.[26]

Preparation and Properties of Insoluble Laminarin.—The laminarin was prepared by Dr. W. A. P. Black, Institute of Seaweed Research, Inveresk, as follows: Fresh *L. cloustoni* fronds, collected at Campbeltown (November, 1954), were minced (¼" mesh) and extracted for 1 hr. at 55—60° with ten parts (by wt.) of dilute hydrochloric acid solution (pH 3·4). After centrifugation, the weed residue was washed with water, and the combined extract and washings were set aside for 48 hr. The polysaccharide deposited was collected and washed with alcohol and ether. The yield represented 43% of the laminarin present in the fronds. The laminarin, purified by recrystallisation from hot water, had $[\alpha]_D$ −9° (*c* 1·5 in H_2O), +9° (*c* 2·7 in N-NaOH) [adsorbed alcohol,[27] 0·20; ash content, 0·45; glucose content (by cuprimetric

titration), 94%; R.P. values, 2·5 (Somogyi), 5·9 (potassium ferricyanide)]. Hypoiodite oxidation indicated the presence of one reducing group per 47 glucose residues.

Stability of Laminarin at pH 3·4 *and* 55°.—Laminarin (1 g.) was heated at 55° with dilute hydrochloric acid solution (pH 3·4; 100 ml.). Samples were removed after 0, 30, and 60 min., cooled, and neutralised, and the reducing powers of 5 ml. portions were determined. No increase in reducing power was observed. The residual solution was cooled, neutralised, and evaporated to dryness. Oligosaccharides could not then be detected by paper chromatography.

Partial Acid Hydrolysis of Laminarin.—Laminarin (1 g.) was heated at 100° with 0·1N-oxalic acid (40 ml.) for 5·5 hr., cooled, and neutralised with calcium carbonate, and the filtrate, after concentration, was examined by paper chromatography. The hydrolysate contained glucose, laminaribiose, laminaritriose (by comparison of $R_{Glucose}$ values with those of authentic samples), R_G 0·45 (non-reducing), R_G 0·34, 0·24, and 0·16 (reducing). Laminaritetraose would have an R_G value of 0·24 under these conditions. In addition, smaller amounts of non-reducing oligosaccharides were present.

Complete Acid Hydrolysis of Laminarin.—Laminarin (1 g.) was hydrolysed with N-sulphuric acid (100 ml.) for 7·5 hr. at 100°, and the solution cooled, neutralised with barium carbonate, filtered, and evaporated to a syrup. The presence of glucose was shown by paper chromatography, with both aniline oxalate and silver nitrate sprays. Part of the syrup (0·1 g.) was incubated with glucose oxidase solution (5 ml.) at pH 7·0 for 40 hr. The residual sugar with R_G *ca.* 1 reacted with silver nitrate but not with aniline oxalate. Chromatography in phenol–water revealed a non-reducing carbohydrate with the R_G value of mannitol.

In later experiments, neutralised hydrolysates were chromatographed in the solvent containing boric acid. Authentic samples of mannitol and sorbitol had R_G 2·8 and 3·3 respectively. The hydrolysate contained non-reducing carbohydrates with R_G 2·8 and 1·6.

Methylation Analysis of Laminarin.—The polysaccharide (15 g.) was methylated four times at room temperature under nitrogen, with dimethyl sulphate and sodium hydroxide solution. The methylated polysaccharide was extracted with chloroform, and the extract washed with water, dried, and concentrated (yield, 18·5 g., 98%) (Found: ash, 1·0; OMe, 44·0. Calc. for tri-*O*-methyl-laminarin: OMe, 45·6%).

Methylated laminarin (16·9 g.) was dissolved in chloroform (100 ml.), and light petroleum (b. p. 40—60°) added slowly until precipitation occurred. The material which was precipitated between 91 and 92·5% v/v light petroleum content was collected, washed, and dried (12·8 g., 78%; $[\alpha]_D$ −5·7° at *c* 1·1 in $CHCl_3$). No further polysaccharide was precipitated when the concentration of light petroleum was increased to 95%.

By isothermal distillation,[13] the original and the precipitated sample of methylated laminarin had molecular weights of 1900 and 12,000 respectively. An acid hydrolysate of the original methylated laminarin contained tetra-, tri-, and di-*O*-methylglucose (paper chromatography).

Precipitated methylated laminarin (5 g.) was hydrolysed with methanolic 4% hydrogen chloride (160 ml.) in a sealed tube at 100° for 6·5 hr. After neutralisation with silver carbonate, the filtrate was concentrated and hydrolysed with boiling aqueous N-hydrochloric acid (300 ml.) for 14 hr. The neutralised, concentrated hydrolysate was extracted with acetone and on evaporation 5·3 g. of methylated glucose derivatives were obtained.

The mixed sugars were chromatographed in butan-1-ol (15 ml.) on cellulose [28] (50 × 3 cm.), with light petroleum (b. p. 100—120°)–butan-1-ol saturated with water (7 : 3) as eluant. Three fractions were collected, comprising 4·85 g. of methylated sugars. Elution of the column with light petroleum–butan-1-ol (1 : 1) and then water yielded two further fractions (0·59 g.).

Fraction 1 (6 mg.) was not examined.

Fraction 2 (0·246 g.) contained 2 : 3 : 4 : 6-tetra-*O*-methylglucose (94%, by hypoiodite oxidation) and was homogeneous on paper chromatography; recrystallised from light petroleum (b. p. 40—60°) it had m. p. 85—86°; the derived anilide had m. p. 133—135°.

Fraction 3 (4·593 g.) was crystalline. It contained 2 : 4 : 6-tri-*O*-methylglucose (97%, by hypoiodite oxidation) {$[\alpha]_D$ +91° ⟶ +72° (*c* 0·92 in H_2O, after 24 hr.); m. p. and mixed m. p. 122—124°}. The corresponding anilide had m. p. and mixed m. p. 160—161°. The fraction was homogeneous on paper chromatography, even after prolonged development. The R_g values (tetra-*O*-methylglucose standard) of authentic 2 : 3 : 4- and 2 : 4 : 6-tri-*O*-methylglucose were 0·85 and 0·80 respectively.

Fraction 4, a syrup (0·491 g.), contained two methylglucoses [paper chromatography, R_g

0·59 (brown spot), R_g 0·62 (pink spot)], and gradually some crystals were formed. The fraction was dissolved in ethyl acetate and, on cooling, 4 : 6-di-*O*-methylglucose (R_g 0·59), m. p. 155—157°, mixed m. p. 154—156° (0·097 g.), was deposited. Evaporation of the ethyl acetate solution yielded a syrup (0·379 g.) which contained a sugar with R_g 0·62.

Since the mixed sugars contained 0·231 g. of tetra-*O*-methylglucose, the proportion of non-reducing end-groups is 4·4% or one per 23 glucose residues.

Degradation of Laminarin by Lime-water.—In extensive small-scale experiments laminarin was treated with lime-water for varying periods, at 25° or 60° in the presence or absence of oxygen. In a large-scale experiment laminarin (12 g.) was treated with saturated oxygen-free lime-water (1500 ml.) at 60° under nitrogen. Samples (5 ml.) were removed at intervals, and the consumption of lime-water determined by titration with 0·01N-hydrochloric acid. Reaction was complete after 23 hr. The mixture was neutralised with dilute nitric acid and concentrated to *ca.* 50 ml. The polysaccharide which was deposited on storage was collected by centrifugation, dissolved in water, and freeze-dried (yield, 5·5 g., 46% recovery). To the remaining solution, ethanol (80% v/v) was added and the resulting precipitate also dissolved in water and freeze-dried (yield, 4·5 g.). This material contained carbohydrates of low molecular weight which gave glucose on acid hydrolysis, saccharinic acid (48%), and non-lactonisable acid (19%) (estimated by the method of Bamford, Bamford, and Collins [29]), and had an ash content of 15%.

The lime-treated laminarin had $[\alpha]_D$ −7° (*c* 5·0 in H_2O), ash, 0·5%, and R.P. 0·3 (Somogyi), 1·7 (potassium ferricyanide); an acid hydrolysate contained mannitol (paper chromatography). Re-treatment with lime-water caused little further degradation, *ca.* 90% of polysaccharide being recovered.

On partial hydrolysis with oxalic acid, the hydrolysate of lime-treated laminarin contained the same series of sugars as that from the original laminarin. In a similar experiment with another sample of lime-treated laminarin, the presence of a reducing sugar with the R_G value of gentiobiose was noted.

Methylation Analysis of Lime-treated Laminarin.—This polysaccharide (8·5 g.) was methylated eight times with dimethyl sulphate and sodium hydroxide, as described for laminarin (p.). The methylated product (10·3 g., 96%) had ash 1·0, OMe, 44·1%. On addition of 94% v/v light petroleum (b. p. 40—60°) to a solution of methylated polysaccharide (9·2 g.) in chloroform (50 ml.), a precipitate (7·5 g., 88%) was obtained, having $[\alpha]_D$ −7·1° (*c* 1·3 in $CHCl_3$). The addition of further light petroleum (1 l.) to the supernatant solution did not give a precipitate.

The original and the precipitated sample of methylated lime-treated laminarin had molecular weights of 2500 and 13,600 respectively.[13]

The sample (4 g.) of high molecular weight was hydrolysed successively with methanolic 4% hydrogen chloride (130 ml.) and N-hydrochloric acid (300 ml.) as described previously, to give 4·4 g. of methylated glucose derivatives. Partition chromatography on a cellulose column gave five fractions:

Fraction 1, a syrup (0·294 g.) contained 2 : 3 : 4 : 6-tetra-*O*-methylglucose and methyl tri-*O*-methylglucoside. The major portion (0·240 g.) was re-hydrolysed with N-sulphuric acid (25 ml.) for 6 hr., neutralised, and concentrated. The resulting syrup was partitioned on a second cellulose column to give two fractions.

Fraction 1a (0·174 g.) contained crystalline 2 : 3 : 4 : 6-tetra-*O*-methylglucose, which after recrystallisation had m. p. 84—86°; the derived aniline derivative had m. p. and mixed m. p. 134—135°. Fraction 1b, a syrup (0·081 g.), contained 2 : 4 : 6-tri-*O*-methylglucose.

Fraction 2 (0·036 g.) contained tetra- (7 mg.) and 2 : 4 : 6-tri-*O*-methylglucose (29 mg.) (paper chromatography).

Fraction 3 (3·676 g.) crystallised. It was chromatographically homogeneous and contained 2 : 4 : 6-tri-*O*-methylglucose (94%, by hypoiodite oxidation). After recrystallisation from ether, the crystals had m. p. 124—126°, mixed m. p. 123—124°, $[\alpha]_D$ +94° ⟶ +73° (*c* 1·68 in H_2O, after 24 hr.). Its aniline derivative had m. p. and mixed m. p. 160—162°.

Fraction 4 (0·375 g.) was partly crystalline, and contained two sugars. One of these, after crystallisation from ethyl acetate, was identified as 4 : 6-di-*O*-methylglucose, m. p. and mixed m. p. 156—159°. The remaining sugar gave a pink colour with aniline oxalate.

Fraction 5 (0·074 g.) was a white amorphous solid which contained mono-*O*-methylglucose and a trace of glucose. It was not further investigated.

The percentage composition of the hydrolysate was: tetra- 5·2, tri- 84·4, 4 : 6-di- 1·5, unidentified di- 7·2, mono-*O*-methylglucose 1·8, indicating the presence of one non-reducing end-group per 20 glucose residues.

Preparation and Properties of Laminaritol.—Potassium borohydride (5 g.) was added to an aqueous solution of laminarin (10 g. in 500 ml.). After 48 hr., the mixture was neutralised (pH 7; acetic acid), and the polysaccharide precipitated with alcohol. The laminaritol was then dissolved in warm water, reprecipitated, redissolved, and finally freeze-dried. The product (9·6 g.) had glucose content 94%, ash content 1·2%, and R.P. 0·1 (Somogyi). A neutralised acid hydrolysate was examined by paper chromatography; comparison with authentic specimens showed the presence of glucose, mannitol, and sorbitol. Laminaritol (5·2 g.) was then treated with saturated lime-water (650 ml.) in an atmosphere of nitrogen at 60° for 23 hr. The solution was cooled, neutralised (pH 7; dilute nitric acid), and concentrated. Polysaccharide was slowly precipitated and after recovery by centrifugation was washed with water and freeze-dried (yield, 4·8 g.) (Found: ash content, 2·7%). Addition of alcohol to the lime-water solution gave a small precipitate (0·58 g.).

Methylation of Laminaritol.—Laminaritol (5 g.) was methylated six times with dimethyl sulphate and sodium hydroxide; the product was isolated by chloroform extraction (yield, 6·2 g.) (Found: OMe, 43·3%). It was dissolved in chloroform (35 ml.), and light petroleum (b. p. 40—60°) added until precipitation ceased (500 ml.). The recovery was 94%. The material (0·4 g.) which was soluble in light petroleum was recovered.

By isothermal distillation,[17] the insoluble material had a molecular weight of 3800.

Periodate Oxidation of Laminarin and Related Compounds.—(a) *Sodium metaperiodate at 2°.* Laminaribiose (150 mg.) and 3-*O*-methylglucose (50 mg.) were oxidised with 0·3M-sodium metaperiodate (10 ml.) in water (final vol. 200 ml.). Formaldehyde could not be detected during 3 days with the chromotropic acid reagent. The production of formaldehyde from mannitol, under similar conditions, was complete within 1 hr. and the concentration of formaldehyde (1·8 mol.; theory 2·0) remained constant for 44 hr.

Laminarin (90·0 mg.) and laminaritol (74·9 mg.) were oxidised with 0·3M-sodium metaperiodate (2 ml.) in water (23 ml.). The liberation of formaldehyde from laminarin was as follows:

Time of oxidn. (hr.)	1	3	5	12	48
Formaldehyde prodn. (mol.)	0·022	0·023	0·023	0·023	0·025

This is equivalent to an initial release of 0·021 mol.

The results with laminaritol were:

Time of oxidn. (hr.)	22	44	88
Formaldehyde prodn. (mol.)	0·057	0·058	0·060

The production of formic acid from the control experiments was as follows:

Time (hr.)	2	5	24	72	4—6 days	8 days
Formic acid (mol.)						
Laminaribiose	0·50	0·77	1·18	1·51	1·72	2·08
3-*O*-Methylglucose	—	0·28	0·33	0·58	0·68	—

Under these conditions, 1 mol. of formic acid is produced from laminaribiose after *ca.* 14 hr.

The following results were obtained when laminarin (1 g.) was oxidised:

Time (hr.)	5	24	48	96	168
Periodate uptake (mol.)	0·16	0·19	0·19	0·20	0·21
Formic acid (mol.)	0·09	0·10	0·10	0·11	0·11

It follows that 0·10 mol. of acid is released during the initial primary oxidation.

The production of formic acid from laminaritol was 0·12 mol. after 22 and 44 hr., and 0·13 mol. after 66 and 94 hr., representing a total primary oxidation yield of 0·12 mol.

(b) *Sodium metaperiodate at room temperature.* Laminarin and laminaritol (*ca.* 50 mg.), dissolved in water, were treated with 0·3M-sodium metaperiodate (2 ml.) in a total volume of 25 ml. Samples (2 ml.) were analysed at intervals for formaldehyde:

Time of oxidn. (hr.)	6	23	48
Formaldehyde prodn. (mol.)			
Laminarin	0·032	0·040	0·042
Laminaritol	0·064	0·064	0·064

A duplicate analysis gave 0·040 and 0·061 mol. of formaldehyde, respectively, after 16 and 36 hours' oxidation. Lime-treated laminaritol gave 0·061 mol. under similar conditions.

(c) *Sodium metaperiodate at* pH 8 *and room temperature.* The polysaccharides (*ca.* 8 mg.; concentration determined by acid hydrolysis) were dissolved in water, phosphate buffer (0·1M, pH 8: 12·5 ml.), and 0·3M-sodium metaperiodate (2 ml.) in a total volume of 25 ml. The production of formaldehyde was constant after oxidation for 18 hr. and amounted to 0·52 mol. from laminarin, and 0·55 mol. from laminaritol. Under these conditions, the residual polysaccharide was stable, even after oxidation for 9 days. In a further experiment, lime-treated laminaritol gave 0·57 mol. of formaldehyde.

(d) *Potassium metaperiodate at room temperature.* Laminaribiose (35·5 mg.) and mannitol (17·8 mg.) were dissolved in 3% potassium chloride solution (25 ml.), and 0·25M-sodium metaperiodate (25 ml.) was added. The production of formic acid was determined at intervals, and a reagent control was also analysed. Liberation of acid from mannitol ceased after 4 hr. and corresponded to 3·7 mol. Laminaribiose gave the following results:

Time (hr.)	2	4	6	24	48	96	144	168
Formic acid (mol.)	0·38	0·65	0·96	1·38	1·81	2·60	3·08	3·13

Under these conditions (pH 5; cf. Part I), the initial and the total primary oxidation require *ca.* 6 and 130 hr. respectively, and over-oxidation after this time is slight.

Laminarin (235·9 mg.) was oxidised as above:

Time (hr.)	3	6	18	42	168	240
Formic acid (mol.)	0·088	0·096	0·114	0·126	0·156	0·163

The initial and total primary oxidations therefore yield *ca.* 0·10 and *ca.* 0·15 mol. of formic acid, respectively.

Laminaritol (244·9 mg.) was similarly oxidised:

Time (hr.)	2	13	20	38	144	240
Formic acid (mol.)	0·081	0·113	0·122	0·131	0·153	0·168

The total primary oxidation corresponded to *ca.* 0·13 mol., and the rate of production of formic acid was significantly greater than from laminarin. It is apparent that slow overoxidation of laminarin and laminaritol occurs after potassium metaperiodate oxidation for approximately 150 and 100 hr. respectively.

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[22] Chanda, Hirst, and Manners, *J.*, 1957, 1951.
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THE MOLECULAR STRUCTURE OF GLYCOGENS

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I. Introduction

1. *Historical Introduction*

A review on the molecular structure of glycogens is particularly appropriate at the present time, as 100 years have now elapsed since Claude Bernard[1] announced, in March 1857, the isolation of glycogen from dog liver. Following his discovery, in 1850, of the glycogenic function of the liver, Bernard observed that liver contains a substance which slowly changes into sugar. He isolated this substance, "matière glycogène animale," and found it to be a white amorphous powder, soluble in water to give an opalescent solution. It was precipitated by both alcohol and acetic acid. Bernard observed that, although glycogen is resistant to hot alkali, it is readily broken down by hot acids and by diastatic enzymes to give fermentable sugars; furthermore, aqueous solutions give a characteristic red-brown coloration with iodine.

The presence of glycogen was then reported in skeletal muscle,[2] placental tissue,[3] surface epithelial cells,[4] and cells of the intestinal mucosa[4]; none could be detected in bone or in glandular or nervous tissue.[4] In 1861, glycogen (isolated from human liver) was found[5] to have the empirical formula $C_6H_{10}O_5$ (recalculated on the basis of modern atomic weights). During investigations on yeast, Errera[6] noted the presence of a substance which gave a brown coloration with iodine, and nine years later, Cremer[7] isolated glycogen from yeast as a white powder, soluble in water ($[\alpha]_D$ +198.9°) and having the properties of the animal glycogen described by Bernard. Improved methods for the preparation of yeast glycogen, free from yeast-gum (mannan), have been described by several workers, including Harden and Young.[8] Cremer's observations on yeast glycogen were confirmed by Clautriau,[9] who also found that the properties of glycogen from rabbit liver and from two species of fungi were identical with those of yeast glycogen.

Although it had been assumed that glycogen was a polymer of glucose, detailed proof that glucose was produced by the complete acid hydrolysis of glycogen was not published until 1881. Külz and Bornträger[10] compared

(1) C. Bernard, *Compt. rend.*, **44**, 578 (1857).
(2) A. Sanson, *Compt. rend.*, **44**, 1159, 1323 (1857).
(3) C. Bernard, *Journal de la physiologie de l'homme et des animaux*, **2**, 30 (1859).
(4) C. Bernard, *Journal de la physiologie de l'homme et des animaux*, **2**, 326 (1859).
(5) E. von Gorup-Besanez, *Ann.*, **118**, 229 (1861).
(6) L. Errera, *Compt. rend.*, **101**, 253 (1885).
(7) M. Cremer, *Münch. med. Wochschr.*, **41**, 525 (1894).
(8) A. Harden and W. J. Young, *J. Chem. Soc.*, **81**, 1224 (1902); **101**, 1928 (1912).
(9) G. Clautriau, cited in ref. 8.
(10) E. Külz and A. Bornträger, *Pflügers Arch. ges. Physiol.*, **24**, 28 (1881).

the analysis, optical activity, reducing power, microscopic appearance, and compound formation with sodium chloride, of glucose and the sugar isolated from an acid hydrolyzate of horse-liver glycogen; they were identical.

The enzymic degradation of glycogen received considerable attention in this period. In 1879, Seegen[11] showed that diastase, saliva, and pancreatic juice rapidly bring about a 60–70 % degradation of dog-liver glycogen. The products were a sugar, which differed from glucose in having a lower reducing power and a higher specific rotation, and a mixture of "dextrins"; a proportion of the "dextrins" was unfermentable. Two years later, Külz[12] identified the sugar from the enzymic degradation of glycogen as maltose. The structural significance of this finding did not, of course, become apparent until the constitution of maltose had been determined[13] many years later.

During the period 1880–1920, interest in glycogen was focused mainly upon improving methods of preparation and estimation, and upon its physiological role as a carbohydrate reserve; progress in purely chemical studies (see p. 269) was not possible until the ring structure of D-glucose had been established. In addition, the superficial properties (specific rotation, opalescence, iodine coloration, D-glucose content, and analysis for carbon, hydrogen, oxygen, nitrogen, and phosphorus) of "glycogens" from a variety of biological sources were compared.

2. *General Properties*

In this article, the term "glycogen" is used to describe a group of highly branched polysaccharides, isolated from animals or microorganisms, which conform to the following criteria.

(*1*) *Empirical formula* $C_6H_{10}O_5$ (*inorganic material, nitrogen, phosphorus and sulfur being absent*).—The majority of workers now agree that the empirical formula of glycogen is $C_6H_{10}O_5$, although in the period 1875–1900 a number of analyses[14] had suggested 6 $C_6H_{10}O_5 \cdot H_2O$. Harden and Young[8] found that oyster glycogen, when dried at 100° in air, had the analysis required for 6 $C_6H_{10}O_5 \cdot H_2O$, whereas a sample dried over phosphoric oxide at 100° under diminished pressure, gave analytical results corresponding to $C_6H_{10}O_5$.

Carefully purified preparations of glycogen are free from significant amounts of inorganic material, nitrogen, phosphorus, and sulfur, despite

(11) J. Seegen, *Pflügers Arch. ges. Physiol.*, **19**, 106 (1879).

(12) E. Külz, *Pflügers Arch. ges. Physiol.*, **24**, 81 (1881).

(13) W. N. Haworth and S. Peat, *J. Chem. Soc.*, 3094 (1926); J. C. Irvine and I. M. A. Black, *ibid.*, 862 (1926).

(14) For example, E. Külz and A. Bornträger, *Pflügers Arch. ges. Physiol.*, **24**, 19 (1881).

many reports to the contrary in the literature. For example, Pantlitschko and Matula[15] have claimed that glycogen contains four phosphate ester groups (and one uronic acid group) per 500 anhydro-D-glucose residues, whilst Wajzer[16] believed that glycogen from the livers of a rabbit or guinea-pig contained 0.2 % of organically bound phosphorus (mainly as α-D-gluco-pyranose 6-phosphate).

(*2*) *The aqueous solution has a high dextrorotation of about +196°.*—Figures given in the literature range from +179 to +233°, the majority being between 191–199°. In a typical comparative study, Harden and Young[8] found glycogen from oysters, rabbit muscle, and yeast to have $[\alpha]_D$ +191, +191, and +198°, respectively.

(*3*) *The aqueous solution gives a red-brown coloration with iodine.*—Although the staining power of glycogen with iodine is a characteristic property, the tint and intensity of coloration vary with the source of glycogen. For example, the iodine coloration given by rabbit-muscle glycogen is reddish-violet[17] whereas liver glycogens, under similar conditions, stain reddish-brown; in general, the iodine colorations of glycogens from invertebrates are much paler than those from tissues of vertebrates, although a number of exceptions to this generalization have been reported.[8] The staining power of glycogen with iodine must therefore be regarded as a superficial property, having uncertain structural significance. The iodine coloration of glycogen has been widely used for the detection of this polysaccharide in various tissues; in some instances, it is regrettable that no alternative method of identification has been applied, since other substances (for example, certain varieties of rice starch) are also stained red with iodine.

(*4*) *The aqueous solution has a negligible reducing power.*—The apparent reducing power of a glycogen solution varies with the reagent used. The alkaline dinitrosalicylic acid, ferricyanide, or copper reagents give reducing powers of the order of 0.1 % of D-glucose, whilst with the (less specific) alkaline hypoiodite reagent, values of the order of 1 % may be obtained. The accurate determination of the apparent reducing power of glycogen has formed the basis of a method for the determination of the molecular weight (see pp. 274–5).

(*5*) *The aqueous solution is opalescent.*—Aqueous solutions of glycogen show a variable but pronounced bluish-white opalescence; this opalescence may be so great that 1 % solutions are unsuitable for polarimetric observations. Quantitative measurements of the turbidity of glycogen solutions have been used for estimation of the glycogen content of a solution (see pp. 268–9), and for determination of the molecular weight (see Section II).

(15) M. Pantlitschko and J. Matula, *Monatsh.*, **81**, 179 (1950); *Chem. Abstracts*, **44**, 8969 (1950).

(16) J. Wajzer, *Compt. rend.*, **144**, 808 (1950).

(17) F. G. Young, *Biochem. J.* (London), **31**, 711 (1937).

(*6*) *Hydrolysis by dilute mineral acid at 100° gives an almost quantitative yield of* D-*glucose.*—Complete hydrolysis of glycogen may be effected by dilute mineral acid (0.5–1.0 *N* hydrochloric acid or 1.0–2.0 *N* sulfuric acid) at 100° within 2–5 hours, about 97 % conversion to D-glucose being obtained. A small amount of D-glucose is destroyed by the acid, and a further amount is lost by acid-reversion yielding isomaltose and gentiobiose.[18, 19] Significant amounts of monosaccharides other than D-glucose have not been detected in acid hydrolyzates of glycogen.

(*7*) *Chemical analysis indicates that the* D-*glucose residues are united by* α-(1 $\rightarrow$ 4)-*linkages, and that the ratio of non-terminal to terminal residues is normally 11 to 1.*—Structural investigations (p. 269) established the relationship between glycogen and starch, in that both polysaccharides contain α-D-(1 $\rightarrow$ 4)-glucosidic linkages. In amylopectin, the branched component of starch, the average chain length ($\overline{C.\ L.}$) is[20] 18–27.

(*8*) *The molecular weight is of the order of* 10^6.—Molecular-weight determinations have shown glycogens to be amongst the largest of natural polymers. The majority of samples have mean molecular weights of (1–10) $\times$ 10^6, and are polymolecular[21]; in addition, some samples are polydisperse and contain molecules with molecular weights averaging around two or three different values; for example, a human-liver glycogen (glycogen-storage disease) contains[22] two "components" with molecular weights of about 9 $\times$ 10^6 and 2 $\times$ 10^6. Accordingly, molecular-weight determinations on glycogens must be regarded as giving the order of magnitude of the mean molecular size, rather than absolute values (see Section II).

(*9*) *Hydrolysis by* β-*amylase normally results in* 45 $\pm$ 5 % *conversion to maltose.*—This criterion[23] serves to differentiate between glycogens, amylopectins, and α-amylodextrin (the β-limit dextrin of amylopectin), since amylopectins have β-amylolysis limits of 55 $\pm$ 5 %, whilst the latter, in which $\overline{C.\ L.}$ = 10–12, is not attacked by β-amylase.[24]

(*10*) *Glycogen shows the characteristic infrared absorption spectrum of starch-type polysaccharides.*—The infrared spectrum of glycogen, in the frequency range 730–960 cm.$^{-1}$, has three absorption peaks, at 928 $\pm$ 3, 838 $\pm$ 3, and 760 $\pm$ 2 cm.$^{-1}$; the absorption peak at 838 cm.$^{-1}$ is displayed by all carbohydrates containing α-D-glucopyranose units, whilst the peaks

(18) E. Elizabeth Bacon and J. S. D. Bacon, *Biochem. J.* (London), **58**, 396 (1954).

(19) A. Thompson, Kimiko Anno, M. L. Wolfrom and M. Inatome, *J. Am. Chem. Soc.*, **76**, 1309 (1954).

(20) D. J. Bell, *Ann. Repts. on Progr. Chem.* (Chem. Soc. London), **44**, 223 (1947).

(21) For reviews, see C. T. Greenwood, *Advances in Carbohydrate Chem.*, **7**, 289 (1952); **11**, 387 (1956).

(22) D. J. Manners, *J. Chem. Soc.*, 3527 (1954).

(23) D. J. Bell and D. J. Manners, *J. Chem. Soc.*, 3641 (1952).

(24) For a review see K. Myrbäck, *Advances in Carbohydrate Chem.*, **3**, 251 (1948).

at 928 and 760 cm.$^{-1}$ are shown only by (1 → 4)-linked glucans.[25] Hence, glycogen may be readily distinguished by infrared spectrophotometry from all other polysaccharides, except related α-D-(1 → 4)-glucans. Infrared spectrophotometry over the frequency range 900–1,700 cm.$^{-1}$ has been used for the preliminary identification and estimation of glycogen in cultures of enteric bacteria.[26]

(*11*) *X-ray analysis shows glycogen to be amorphous.*—Glycogens give rise to a diffuse x-ray pattern,[27] in contrast to starches, in which certain regions of the granules exist in crystalline form, thereby producing definite x-ray diffraction patterns.[28]

3. *Occurrence*

Glycogen has been isolated from livers, brains, and skeletal and cardiac muscles of many mammals, and has been detected in most animal cells, including those of adipose tissue. Human liver may contain 1–10% (by wet weight) of glycogen, which is also present in the tissues of invertebrates (for example, *Ascaris lumbricoides*, *Helix pomatia*, and *Mytilus edulis*), bacteria (for example, *Aerobacter aerogenes*, *Bacillus megatherium*, and *Neisseria perflava*) and protozoa (for example, *Tetrahymena pyriformis* and *Trichomonas gallinae*). In all these organisms, glycogen is important as the storage form of carbohydrate, and hence, as a source of energy.

Polysaccharides which are stained red-brown with iodine and have chain lengths of about 12 have been isolated from certain plants (for example, *Zea mays*)[29]; these have been termed "phytoglycogens." Dvonch and Whistler[30] consider that such polysaccharides should be regarded as highly branched amylopectins.

4. *Isolation and Purification*

Glycogen may be isolated from tissues by extraction with concentrated alkali at 100°, with chloral hydrate at 80°, or with cold aqueous trichloroacetic acid.

The most widely used method, developed by Bernard and Pflüger, involves digestion of the tissue with concentrated potassium hydroxide solution (20–60%) at 100°. Cellular constituents other than glycogen (for

(25) S. A. Barker, E. J. Bourne, M. Stacey and D. H. Whiffen, *J. Chem. Soc.*, 171 (1954).

(26) S. Levine, H. J. R. Stevenson, E. C. Tabor, R. H. Bordner and L. A. Chambers, *J. Bacteriol.*, **66**, 664 (1953).

(27) R. S. Bear and C. F. Cori, *J. Biol. Chem.*, **140**, 111 (1941).

(28) R. S. Bear and D. French, *J. Am. Chem. Soc.*, **63**, 2298 (1941).

(29) S. Peat, W. J. Whelan and J. R. Turvey, *J. Chem. Soc.*, 2317 (1956); see also K. H. Meyer and Maria Fuld, *Helv. Chim. Acta*, **32**, 757 (1949).

(30) W. Dvonch and R. L. Whistler, *J. Biol. Chem.*, **181**, 889 (1949).

example, proteins and nucleic acids) are destroyed, and glycogen is precipitated by the addition of alcohol. Further purification may be effected by several reprecipitations from aqueous solution with alcohol.

For many tissues (for example, mammalian liver), repeated extraction with boiling water has proved satisfactory; the combined extracts are then deproteinized with 4% trichloroacetic acid, and glycogen is precipitated with alcohol.[31] Alternatively, the aqueous extracts may be deproteinized by using excess, concentrated, aqueous picric acid solution.[32] Highly purified rabbit-liver glycogen has been prepared[33] by thoroughly grinding the tissue under 3% trichloroacetic acid solution. Addition of alcohol to the supernatant liquor from the centrifuged extract yielded crude glycogen which, after further purification, contained only 0.20% of ash and 0.03% of phosphorus. Extraction of glycogen, by the use of hot water or cold trichloroacetic acid solution should, however, be limited to liver tissue from animals in good nutritional condition, since these solvents are inadequate for the extraction of skeletal muscles, or livers of low glycogen content.[17, 34] For the latter tissues, the Pflüger technique is used.

Many glycogen samples prepared by the above methods, and particularly by the Pflüger technique, contain small amounts of inorganic material, including silica; this can be removed by electrodialysis of an aqueous solution of the glycogen. By this means, the ash and phosphorus content of *Mytilus edulis* glycogen were reduced from 0.25 and 0.12% to 0.08 and 0.03%, respectively.[35] Alternatively, repeated precipitation of glycogen with 80% acetic acid has been found to be a simple method of obtaining virtually ash-free preparations.[31]

The methods of isolation of glycogen outlined above have been subjected to a number of criticisms. It has been suggested that degradation of glycogen occurs during digestion of the tissues by the Pflüger technique, and, as an alternative, the use of chloral hydrate for the extraction of glycogen has been recommended.[36] Evidence on the alkali-stability of glycogen is conflicting. Bridgman[37] reported that glycogen extracted from one half of a rabbit liver by 3% trichloroacetic acid had a molecular weight of 5.2×10^6 (sedimentation–diffusion measurements), whilst glycogen isolated by the Pflüger technique from the remaining half had a molecular weight of 4.6×10^6; the difference was stated to be not significant. In a similar study,

(31) D. J. Bell and F. G. Young, *Biochem. J.* (London), **28**, 882 (1934).
(32) L. G. Petree and C. L. Alsberg, *J. Biol. Chem.*, **82**, 385 (1929).
(33) M. Sahyum and C. L. Alsberg, *J. Biol. Chem.*, **89**, 33 (1930).
(34) W. L. Bloom, G. T. Lewis, Mary Z. Schumpert and T. Shen, *J. Biol. Chem.*, **188**, 631 (1950).
(35) Margaret McDowell, *Proc. Soc. Exptl. Biol. Med.*, **25**, 85 (1927).
(36) K. H. Meyer and R. W. Jeanloz, *Helv. Chim. Acta*, **26**, 1784 (1943).
(37) W. B. Bridgman, *J. Am. Chem. Soc.*, **64**, 2349 (1942).

Greenwood and Manners[38] found that glycogen isolated by the Pflüger method from half of the livers of two rabbits had sedimentation constants (S_{20}) of 83 and 86 S; glycogen extracted by means of hot water from the remaining liver tissue had S_{20} = 76 and 85 S, respectively. Further evidence of the apparent stability of glycogen to alkali was obtained by Staudinger,[39] who showed, by a light-scattering technique, that the molecular weights of samples of guinea-pig liver glycogen and muscle glycogen were unaltered after treatment with 15–30 % potassium hydroxide at 100° for one hour.

In contrast, digestion of rabbit-liver glycogen with 8 % sodium hydroxide at 100° for 1.5 hours reduced[38] the sedimentation constant from 86 to 57 S. Moreover, Schlamowitz[40] found that the molecular weight of rabbit-liver glycogen decreased to about one third of the original value on treatment with 30 % potassium hydroxide for 2 hours at 100°. In view of the susceptibility of amylose and starch to alkali in the presence of oxygen,[41] digestion should preferably be carried out under anaerobic conditions.

In the tissue cells, glycogen is closely associated with protein; indeed it has been suggested[42] that two forms of glycogen exist—an insoluble form (desmoglycogen) which is bound to protein by "residual valencies," and a free, soluble form (lyoglycogen) which is readily extracted. Thus, only 27 % of the glycogen content of sclerotia of *Phymatotrichum omnivorum* could be extracted with hot water; digestion of the residue with 35 % potassium hydroxide solution yielded the remainder of the glycogen.[43] More recently, Bloom and coworkers[34] found that only 55 and 85 % of the glycogen in rat muscle and liver tissues, respectively, was extracted by cold 10 % trichloroacetic acid solution. The experiments of Meyer and Jeanloz[36] would suggest that the association between protein and glycogen is of a physical nature, involving entrapping of protein by the glycogen chains, and not a true chemical combination.

5. *Estimation*

A number of methods are now available[44] for the estimation of glycogen in tissues, either involving acid hydrolysis of the glycogen and determina-

(38) C. T. Greenwood and D. J. Manners, *Proc. Chem. Soc.*, 26 (1957); sedimentation constants (S_{20}) are given in Svedberg units, where $S = 1 \times 10^{-13}$ in c.g.s. units.

(39) H. Staudinger, *Makromol. Chem.*, **2**, 88 (1948).

(40) M. Schlamowitz, *J. Biol. Chem.*, **190**, 523 (1951).

(41) For example, R. T. Bottle, G. A. Gilbert, C. T. Greenwood and K. N. Saad, *Chemistry & Industry*, 541 (1953); H. Baum and G. A. Gilbert, *ibid.*, 489 (1954).

(42) R. Willstätter and Margarete Rohdewald, *Hoppe-Seyler's Z. physiol. Chem.*, **225**, 103 (1934); see also E. M. Mystkowski, *Biochem. Z.*, **278**, 240 (1935).

(43) D. R. Ergle, *J. Am. Chem. Soc.*, **69**, 2061 (1947).

(44) For reviews, see J. van der Vies, *Biochem. J.* (London), **57**, 410 (1954), and

tion of the D-glucose produced, or measurement of the iodine coloration[44] or turbidity[45] of tissue extracts. For certain biochemical and clinical investigations, special methods for the estimation of microgram quantities of glycogen have been developed.[46]

6. *Basic Structure*

Chemical studies reported by Karrer,[47] in 1921, indicated that glycogen and starch have closely related structures. Acidic or enzymic hydrolysis gave similar products from both polysaccharides, and, on methylation with methyl sulfate and barium or sodium hydroxide, methyl ethers of similar composition were isolated. Furthermore, both glycogen and starch degraded by acetyl bromide gave "acetobromomaltose" (in about 60 % yield).

Details of the chemical structure of glycogen remained unknown, however, until the polysaccharide attracted the attention of Haworth, Hirst, and Bell, and their respective collaborators.

In their first investigation on this subject, Haworth, Hirst and Webb[48] examined the acetylation and methylation of glycogen. Treatment with acetic anhydride in pyridine, or with sulfur dioxide and chlorine catalysts gave a tri-*O*-acetyl derivative in almost quantitative yield; on deacetylation with alcoholic potassium hydroxide, a polysaccharide with properties (specific rotation, staining power with iodine) similar to the original glycogen was obtained. Further proof that D-glucose is the sole component monosaccharide was afforded by methanolysis of glycogen triacetate, which gave a 98 % yield of methyl α- and β-D-glucopyranosides.

Earlier attempts by Karrer[47] and by Macbeth and Mackay[49] to prepare tri-*O*-methylglycogen (OMe, 45.6 %) by direct methylation of the polysaccharide had not been successful, but gave partially methylated products of OMe 32–37 %. Haworth and coworkers[48] found, however, that by simultaneous deacetylation and methylation of glycogen triacetate, followed by five or six further methylations, a trimethyl ether (OMe, 43.7) could be isolated in 90 % yield. The preliminary acetylation, during which degrada-

T. R. Niederland, J. Gvozdják and M. Triznová, *Chem. Zvesti*, **10**, 242 (1956); see also A. Kemp and Adrienne J. M. Kits van Heijningen, *Biochem. J.* (London), **56**, 646 (1954).

(45) R. G. Hansen, W. J. Rutter and E. M. Craine, *J. Biol. Chem.*, **195**, 127 (1952); L. Gyermek and G. Fekete, *Nature*, **175**, 386 (1955); *Acta Physiol. Acad. Sci. Hung.*, **8**, 259 (1955).

(46) N. G. Heatley, *Biochem. J.* (London), **29**, 2568 (1935); O. Walaas and Eva Walaas, *J. Biol. Chem.*, **187**, 769 (1950); Jean Fong, F. L. Schaffer and P. L. Kirk, *Arch. Biochem. and Biophys.*, **45**, 319 (1953).

(47) P. Karrer and C. Nägeli, *Helv. Chim. Acta*, **4**, 263 (1921); P. Karrer, *ibid.*, **4**, 994 (1921).

(48) W. N. Haworth, E. L. Hirst and J. I. Webb, *J. Chem. Soc.*, 2479 (1929).

(49) A. K. Macbeth and J. Mackay, *J. Chem. Soc.*, **125**, 1513 (1924).

tion of the molecule did not occur (see p. 276), thus facilitated etherification. Tri-*O*-methylglycogen was then hydrolyzed, giving 2,3,6-tri-*O*-methyl-D-glucopyranose in 76% yield, thereby providing evidence that the D-glucose residues are united by (1 → 4) linkages.

Further evidence for the presence of continuous chains of α-D-glucopyranose units in glycogen (and in starch) was obtained by Haworth and Percival,[50] who degraded tri-*O*-methylglycogen (or tri-*O*-methylstarch) with acetyl bromide, and isolated from the resulting mixture a disaccharide which, on oxidation and methylation, gave methyl octa-*O*-methylmaltobionate. Control experiments showed that under similar conditions 2,3,6-tri-*O*-methyl-D-glucose does not undergo resynthesis to a disaccharide. The same authors[51] then examined the methanolysis of tri-*O*-methylglycogen, and from the resulting mixture of methyl D-glucosides isolated, by fractional distillation, 8.9% of methyl tetra-*O*-methyl-D-glucopyranoside. From all the above evidence, it was suggested that glycogen consists of a linear chain of 12 α-(1 → 4)-linked D-glucose residues.

The proposed structure did not, however, explain the observed lack of reducing power of glycogen; Haworth and Percival[51] suggested that, during extraction of glycogen from animal tissues with concentrated alkali, modification of the reducing group occurs.

The results of Haworth and Percival were fully substantiated by Bell and associates. In 1935, the preparation and properties of *O*-acetyl and *O*-benzoyl derivatives of rabbit- and fish-liver glycogens were reported.[52] Glycogen regenerated from these acyl derivatives had the same optical rotation, iodine coloration, reducing power, and low phosphorus content as the original polysaccharides. Methylation of the above glycogens,[53] by simultaneous deacetylation and methylation of their triacetates, yielded trimethyl ethers (OMe, 45.5%); after acid hydrolysis, 9% of tetra-*O*-methyl-D-glucopyranose could be isolated, together with 2,3,6-tri-*O*-methyl-D-glucose and about 15% of di-*O*-methyl-D-glucose. From these findings, it was deduced that both fish- and rabbit-liver glycogen are built up of unbranched chains of 12 D-glucose residues. In the following year, a methylation end-group assay of rabbit-liver glycogen (formed after the ingestion of D-galactose) was performed[54]; hydrolysis of the trimethyl ether gave 6% of 2,3,4,6-tetra-*O*-methyl-D-glucose, corresponding to a chain length of 18 D-glucose residues. Glycogen prepared by the Pflüger technique from the whole tissues of *Mytilus edulis* was also shown[55] to be

(50) W. N. Haworth and E. G. V. Percival, *J. Chem. Soc.*, 1342 (1931).
(51) W. N. Haworth and E. G. V. Percival, *J. Chem. Soc.*, 2277 (1932).
(52) D. J. Bell and H. Kosterlitz, *Biochem. J.* (London), **29**, 2027 (1935).
(53) D. J. Bell, *Biochem. J.* (London), **29**, 2031 (1935).
(54) D. J. Bell, *Biochem. J.* (London), **30**, 1612 (1936).
(55) D. J. Bell, *Biochem. J.* (London), **30**, 2144 (1936).

composed of 18-unit chains. It was therefore believed that glycogen could exist in two forms, with either 12-unit or 18-unit unbranched chains of α-(1 → 4)-linked D-glucose residues. It will be noted that no structural significance was attributed to the presence of di-*O*-methyl-D-glucose in acid hydrolyzates of tri-*O*-methylglycogen. Furthermore, attempts to detect glycosidic methyl groups in methylated glycogen were unsuccessful.[56]

In 1937, two different molecular structures of glycogen were postulated by Haworth and Hirst,[57] and by Staudinger,[58] respectively. The former structure arose from a consideration of methylation data and molecular-weight determinations on glycogen. Measurements of the osmotic pressure exerted by certain glycogen derivatives led Carter and Record[59] to suggest that glycogens have a molecular weight of the order of 10^6, equivalent to a degree of polymerization (D. P.) of 3,400–17,000. Glycogen is therefore a highly branched molecule containing several hundred chains of some 12 to 18 D-glucose residues. The di-*O*-methyl-D-glucose previously mentioned may have arisen from the branch points; since a proportion of it was shown to be 2,3-di-*O*-methyl-D-glucose,[57] the inter-chain linkages were believed to involve C6 of the branching residue. Glycogen was accordingly formulated as a singly-branched molecule as shown in Fig. 1(a). This "laminated" structure was the simplest molecular structure compatible with the data then available from chemical studies; methylation assay would thus permit determination of the mean length of the chains.

An alternative structure, proposed by Staudinger,[58] originated with the belief that the glycogen molecule is almost spherical. The experimental evidence for this hypothesis, namely that from viscosity determinations, is discussed on p. 276. It was suggested that glycogen is composed of a central chain of up to 100 D-glucose units linked through C1 and C4, to which is attached (at C2, C3, and C6 of each unit) a side chain consisting of 12 or 18 α-(1 → 4)-linked D-glucose residues (Fig. 1(b)). The application of methylation end-group assay to such a polysaccharide would, in effect, allow determination of the length of the side chains.

The Staudinger formulation of the glycogen molecule is no longer accepted; more recent physicochemical studies indicate that the molecule is not spherical (see pp. 276–7). Furthermore, methylation and hydrolysis of such a polysaccharide would give D-glucose from the central chain, whilst the origin of the di-*O*-methyl-D-glucose in the hydrolyzate is not explained.[56]

A third molecular structure for glycogen was postulated by Meyer[60] in 1941; it was based on the methylation assay of glycogen and of the limit-

(56) D. J. Bell, *Biochem. J.* (London), **31**, 1683 (1937).
(57) W. N. Haworth, E. L. Hirst and F. A. Isherwood, *J. Chem. Soc.*, 577 (1937).
(58) H. Staudinger and E. Husemann, *Ann.*, **530**, 1 (1937).
(59) S. R. Carter and B. R. Record, *J. Soc. Chem. Ind.* (London), **55**, 218 (1936).
(60) K. H. Meyer and Maria Fuld, *Helv. Chim. Acta*, **24**, 375 (1941).

dextrin remaining after β-amylolysis. β-Amylase catalyzes a stepwise hydrolysis of alternate linkages in a chain of α-(1 → 4)-linked D-glucose residues, thereby liberating maltose.[24, 61] Enzyme action commences at the nonreducing end of the chain and ceases when glucosidic linkages other

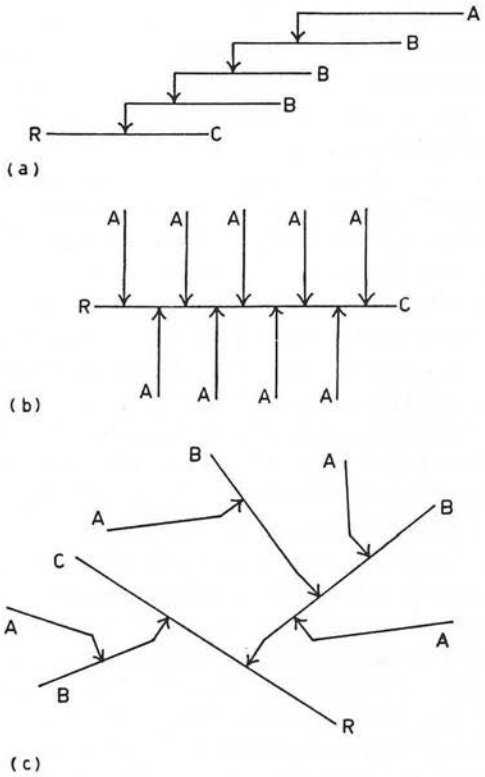

FIG. 1.—Molecular Structures for Glycogen: (a) Haworth "laminated" form, (b) Staudinger "comb" form, and (c) Meyer "tree" form. Key:—Linear chain of α-(1 → 4)-linked D-glucose residues; ↓ Inter-chain linkage [1→6-glucosidic in structures (a) and (c); 1 → 2, 1 → 3, and 1 → 6-glucosidic in (b)]; A, B, and C are types of chain (see p. 285) and R = free reducing group.

than α-D-(1 → 4) are encountered. The action of β-amylase on glycogen is therefore incomplete and is confined to the exterior portions of the chains, the products being maltose and a high molecular-weight limit-dextrin (β-dextrin) containing all the inter-chain linkages.

A sample of mussel glycogen (C. L. = 11) was treated with wheat

(61) C. S. Hanes, *New Phytologist*, **36**, 101, 189 (1937).

β-amylase, yielding 47 % of maltose and a β-dextrin. Methylation and hydrolysis of this dextrin gave 18 % of tetra-*O*-methyl-D-glucose, corresponding to a chain length of 5.5 D-glucose residues. Since the exterior "stubs" of β-dextrins were believed to contain one or two D-glucose residues,[62] it was concluded that the exterior chains contain 6–7 D-glucose residues, and hence, the interior chains consist of 3 D-glucose units. This finding was interpreted as indicating that glycogen has a compact, multiply-branched "tree" structure as shown in Fig. 1(c).

Although it is now generally accepted that glycogen has a multiply-branched "tree" structure, the above evidence does not, in fact, constitute proof of multiple branching, and the β-amylolysis data are equally in accord with a "laminated" formulation. β-Amylolysis eventuates only in a shortening of exterior chains, the number of nonreducing terminal groups in the molecule remaining constant. Hence, if 50 % of the molecule, whether of a laminated, tree, or comb-type structure, is removed by β-amylase, the *relative* proportion of end groups in the residual β-limit dextrin must be doubled.

In parallel studies on amylopectin, evidence for multiple branching was deduced from the results of a stepwise degradation.[62] Treatment of amylopectin with β-amylase gave 55 % of maltose and 45 % of limit dextrin (Dextrin I). On incubation of Dextrin I with yeast "maltase" preparation, slow degradation to D-glucose (17 %) and Dextrin II occurred; the latter was now susceptible to further attack by β-amylase. Dextrin II thus yielded maltose and a second β-dextrin (Dextrin III) which gave a red-brown coloration with iodine. These findings were stated to be explicable only by a multiply-branched structure. If amylopectin had a "comb"-type structure it was claimed that Dextrin II would consist of a linear chain of D-glucose residues which would give a blue coloration with iodine, and be completely saccharified by β-amylase.

Two years later, it was stated[63] that a proportion of the inter-chain linkages of glycogen β-dextrin are degraded by a yeast-enzyme preparation. The close structural relationship between glycogen and amylopectin was thereby confirmed, although conclusive proof of multiple branching in glycogen had not been obtained. It must be noted that the yeast preparation was heterogeneous, and that "debranching" was believed to be due to a phosphorylase. This view is now known to be incorrect; yeast extracts contain an enzyme (isoamylase) which can hydrolyze α-D-(1 → 6)-glucosidic linkages.[64]

(62) K. H. Meyer and P. Bernfeld, *Helv. Chim. Acta*, **23**, 875 (1940).
(63) K. H. Meyer and P. Bernfeld, *Helv. Chim. Acta*, **25**, 399 (1942).
(64) D. J. Manners and Khin Maung, *Chemistry & Industry*, 950 (1955).

Recent developments in the structural chemistry of glycogens are described in later Sections of this article, which is intended to supplement those published in 1943 by Meyer,[65] and in 1948 by Bell.[66]

II. Physicochemical Properties

1. *Molecular Weight*

Although estimates of the molecular weight of several glycogens have been published, only a brief discussion will be presented here, since many of the results have already been reviewed.[21]

The physicochemical methods employed include measurements of osmotic pressure, giving number-average values, and of the viscosity, sedimentation, and diffusion of glycogen and derivatives in various solvents. The latter methods provide weight-average values, which, for a polymolecular system, are larger than number-average molecular weights.[21] The homogeneity and particle size of glycogen have also been studied by electrophoresis[67] and ultramicroscopy,[68] respectively. More recently, two forms of light-scattering technique have been used; in the first,[39, 69] molecular-weight values (M) are calculated from a reduced form of the Rayleigh equation in which

$$M = \tau_{sp}/K$$

where τ_{sp} is the specific turbidity (that is, the absolute turbidity of a solution containing 1 g. of glycogen per liter) and K is a constant, dependent on the wave-length of the incident light. The value of K is determined by using either glycogen[39] or amandin[69] of known molecular weight. Alternatively, light-scattering may be considered as a problem in fluctuation theory, and results are then evaluated from the equation[70]

$$HC/\tau = (1/M) + 2BC$$

where H and B are constants, and C is the concentration of glycogen. By this method, six glycogens had molecular weights[71] in the range (3–15) $\times 10^6$.

Chemical methods of molecular-weight determination are based upon

(65) K. H. Meyer, *Advances in Enzymol.*, **3**, 109 (1943).

(66) D. J. Bell, *Biol. Revs. Cambridge Phil. Soc.*, **23**, 256 (1948).

(67) D. H. Northcote, *Biochem. J.* (London), **58**, 353 (1954).

(68) E. Husemann and H. Ruska, *Naturwissenschaften*, **28**, 534 (1940); *J. prakt. Chem.*, **156**, 1 (1940).

(69) P. Putzeys and L. Verhoeven, *Proc. Intern. Colloq. Macromolecules, Amsterdam*, 267 (1949).

(70) P. Debye, *J. Appl. Phys.*, **15**, 338 (1944); *J. Phys. & Colloid Chem.*, **51**, 18 (1947).

(71) B. S. Harrap and D. J. Manners, *Nature*, **170**, 419 (1952).

measurements of the reducing power of glycogens (which are assumed to contain one reducing group per molecule) with alkaline 3,5-dinitrosalicylic acid[72] or with ferricyanide[73] reagent. The results obtained are lower than those from physicochemical methods; protozoal and yeast glycogen (M = (2–3) $\times$ 10^6 from sedimentation data) gave M = (1–2) $\times$ 10^5 with the dinitrosalicylic acid reagent.[74] These discrepancies are due to alkaline-oxidative degradation of the molecules.[75]

Evidence that glycogen has a high molecular weight was published in 1923. Samec and Isajevic found that a sample of dog-liver glycogen had a molecular weight of 1.1 $\times$ 10^5, from osmotic-pressure measurements.[76]

TABLE I

Molecular Weight Determinations on Glycogens

Sample	*Derivative*	*Method[a] of measurement*	*Mean molecular weights* ($\times 10^{-6}$)	*References*
Rabbit liver	unsubstituted	O. P.	1.2–2.3	77
Fish liver	acetate	O. P.	1.3–3.5	78
Dog liver	acetate	O. P.	1.5	73
Rabbit liver	unsubstituted	S. D.	3.9–13.9	37
Rabbit muscle	unsubstituted	S. D.	2.6	79
Guinea-pig liver	unsubstituted	L. S.	3.7–7.6	39
Cat liver	unsubstituted	L. S.	10.0	71
Commercial	*p*-iodobenzoate	U. M.	3–6	68

[a] O. P. = osmotic pressure; S. D. = sedimentation and diffusion; L. S. = light scattering; U. M. = ultramicroscopy.

This observation appears to have been overlooked by many workers. A few results reported since 1936 are summarized in Table I. Essentially similar results have been obtained[38] by ultracentrifuge measurements on 20 samples of glycogen, all of which were polymolecular; the molecular weights varied between (2–6) $\times$ 10^6, six of the preparations being polydisperse. Polglase and coworkers[80] likewise found samples of human glycogen to be

(72) K. H. Meyer, G. Noelting and P. Bernfeld, *Helv. Chim. Acta*, **31**, 103 (1948).

(73) R. W. Kerr, F. C. Cleveland and W. J. Katzbeck, *J. Am. Chem. Soc.*, **73**, 111 (1951).

(74) D. J. Manners, A. R. Archibald, I. D. Fleming, I. G. Jones, A. Margaret Liddle and Khin Maung, unpublished observations.

(75) R. T. Bottle and G. A. Gilbert, *Chemistry & Industry*, 1201 (1954).

(76) M. Samec and V. Isajevic, *Compt. rend.*, **176**, 1419 (1923).

(77) H. B. Oakley and F. G. Young, *Biochem. J.* (London), **30**, 868 (1936).

(78) S. R. Carter and B. R. Record, *J. Chem. Soc.*, 664 (1939).

(79) D. J. Bell, H. Gutfreund, R. Cecil and A. G. Ogston, *Biochem. J.* (London), **42**, 405 (1948).

(80) W. J. Polglase, D. M. Brown and E. L. Smith, *J. Biol. Chem.*, **199**, 105 (1952).

polydisperse; both liver glycogen and muscle glycogen contained two components.

Examination of sedimentation diagrams has shown[38, 79] that glycogens are extremely polymolecular, and glycogen preparations can be fractionated. Guinea-pig liver glycogen (mean molecular weight, 3.7×10^6), on fractional precipitation with methanol, gave[39] fractions with molecular weights of 19.6, 6.8, 2.2, 1.7, 1.1, and 0.9×10^6, whilst *Ascaris lumbricoides* glycogen ($M = 8.8 \times 10^6$) contains a fraction, 3 % by weight, with[71] $M = 22.5 \times 10^6$.

Glycogen can be acetylated (by means of acetic anhydride and pyridine) without appreciable degradation; this is important, since acetylation is usually a preliminary to methylation. Glycogen samples ($\overline{\text{D. P.}}$, 410, 1,750, and 5,090, by osmometry) gave acetates with $\overline{\text{D. P.}}$ 390, 1,680, and 5,300, respectively; on deacetylation, the regenerated glycogens had[58] $\overline{\text{D. P.}}$ 410, 1,730, and 5,350.

To summarize, physicochemical measurements show that glycogens have molecular weights of $\sim 10^7$, and are therefore amongst the largest of natural polymers.

2. *Molecular Shape*

The majority of measurements of the molecular shape of glycogens indicate that the molecules are asymmetric, although Staudinger and Husemann[58] had suggested that glycogen molecules were spherical. They found that the specific viscosity of three glycogens ($\overline{\text{D. P.}}$ 410, 1,750, and 5,000) in various solvents was the same; the specific viscosity of a series of spherical polymers is independent of molecular weight. The glycogens used (commercial preparations) had relatively low molecular weights ($\sim 10^5$); similar viscosity–molecular-weight relationships do not hold[36] for glycogens with molecular weights of $(2\text{–}6) \times 10^6$.

Later viscometric observations have shown that glycogen molecules deviate from spherical form, and also vary in shape. *Ascaris lumbricoides* glycogen ($M = 7 \times 10^5$) has a higher specific viscosity than have mammalian glycogens of molecular weight[79] $(2\text{–}4) \times 10^6$. Baker's-yeast glycogen[81] has a greater viscosity than rabbit-liver glycogen; measurements on the yeast glycogen indicate an axial ratio of 1:8 or 1:13 for a prolate or oblate ellipsoid, respectively. The axial ratio of tubercle-bacillus glycogen is about 1:10 (from viscosity and diffusion measurements), or 1:11 (from sedimentation studies[82]).

Further evidence that glycogen molecules are elongated has been ob-

(81) D. H. Northcote, *Biochem. J.* (London), **53**, 348 (1953).
(82) E. Chargaff and D. H. Moore, *J. Biol. Chem.*, **155**, 493 (1944).

tained from ultracentrifuge measurements. The frictional ratios of various samples of rabbit-liver[37] and methylated, fish-liver[83] glycogens were 1.8–2.8 and 1.7–2.1, respectively. According to Bridgman,[37] a frictional ratio of 1.9 "corresponds to an ellipsoid of revolution having an axis ratio of 1 to 18 for a prolate ellipsoid or a ratio of 1 to 25 for an oblate ellipsoid." It must be noted, however, that the observed frictional ratios are due to the combined effects of molecular asymmetry and hydration.

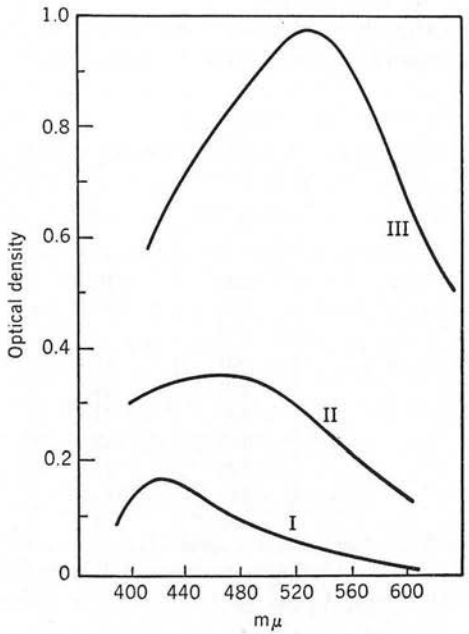

FIG. 2.—Absorption Spectra of Polysaccharide–Iodine Complexes. (I) *Mytilus edulis* glycogen, (II) rabbit liver glycogen, (III) waxy-maize starch (amylopectin). [Solutions contained 0.01% of polysaccharide and 0.02% of iodine in 0.2% of potassium iodide, and were read against an iodine—potassium iodide reference solution.]

Thus, glycogens from different sources differ not only in molecular weight, but also in degree of molecular asymmetry.

3. *Interaction with Iodine*

a. Absorption Spectrum.—The absorption spectra of the iodine complexes of several samples of glycogen have been measured[74]; in Fig. 2, the spectra of iodine complexes of rabbit-liver glycogen, *Mytilus edulis* glycogen, and amylopectin are compared.

(83) B. R. Record, *J. Chem. Soc.*, 1567 (1948).

The absorption maximum of the iodine complex of short, linear α-D-(1 → 4)-glucans appears to be related to the chain length[84]; for branched α-D-(1 → 4)-glucans, there are indications of a similar relationship, although neither the average nor exterior chain length is the sole factor (see Table II). Thus, rabbit-liver glycogen and muscle glycogen ($\overline{C.\ L.}$ = 12–13) form iodine complexes having different absorption spectra. The optical density at the wavelength of maximum absorption also increases with average chain-length.

In contrast with the above spectra (measured in aqueous iodine–potassium iodide), Schlamowitz[40] found that, in the presence of half-saturated

TABLE II

Wavelength of Maximum Absorption of Glycogen–Iodine Complexes

Sample	λ_{max} (mμ)	$\overline{C.\ L.}$	*Approximate exterior chain length*[b]	*References*
Mytilus edulis	420	13	8–9	74
Human liver[a]	430	6	3	74
Helix pomatia	430	7	4–5	74
Tetrahymena pyriformis	440	13	8	74
Rabbit liver	460	13	8	74
Human liver[a]	470	14	8–9	85
Rabbit muscle	490	13	8–9	74
Rabbit liver	490	18	12	74
Bacillus megatherium	520	10–11	7–8	86
Human liver[a]	530	21	15	85

[a] Glycogen-storage disease samples; data on normal, human-liver glycogen is not available. [b] Calculated from $\overline{C.\ L.}$ and the β-amylolysis or phosphorolysis limit (see Section IV).

ammonium sulfate, rabbit-liver glycogens of different chain-lengths and molecular weights had a similar absorption maximum at 490–500 mμ. At this maximum, the optical density increased with apparent chain-length.

In general, little information on the absorption spectra of iodine complexes of glycogens of known molecular structure is available, and attempts to relate spectra with details of fine structure appear to be premature.

b. Iodine-binding Power.—Measurements of the iodine-binding power of glycogen by potentiometric titration have shown that iodine has a much

(84) Marjorie A. Swanson, *J. Biol. Chem.*, **172**, 825 (1948); W. J. Whelan and J. M. Bailey, *Biochem. J.* (London), **58**, 560 (1954).

(85) Barbara Illingworth and Gerty T. Cori, *J. Biol. Chem.*, **199**, 653 (1952).

(86) C. Barry, R. Gavard, G. Milhaud and J. P. Aubert, *Ann. inst. Pasteur*, **84**, 605 (1953).

lower affinity for glycogen than for starch components.[87] For accurate measurements, a differential, potentiometric titration method must be used, and by this technique[88] the uptake of iodine by glycogen has been studied. The slopes of potentiometric titration curves show that the iodine-binding power of glycogen ($\overline{C.\ L.}$ = 12–13) is one quarter that of 18-unit glycogen, and about one tenth that of amylopectins ($\overline{C.\ L.}$ = 20–23).

The interaction of iodine and amylose involves formation of inclusion complexes in which iodine molecules are arranged, endwise and axially, inside a series of helices of α-(1 → 4)-linked D-glucose residues; each helix of 6 D-glucose residues contains one iodine molecule.[89] With branched α-D-(1 → 4)-glucans, similar complex formation, limited to the exterior chains, probably occurs. Glycogen ($\overline{C.\ L.}$ = 12) has[23] an average, exterior-chain length of 8; only a proportion of the exterior chains will contain a helix of six D-glucose residues and form an inclusion complex with iodine. Amylopectins (average, exterior chain-length, 13–18) have a relatively greater iodine-binding power since *every* exterior chain comprises 2–3 helices which will form complexes with iodine. Under the above conditions, with very low iodine concentrations, adsorption effects would be negligible.[88]

There appears to be an approximate relationship between the iodine binding power of branched α-D-(1 → 4)-glucans and the exterior chain length[89a]; evidence in support of the suggestion[88] that it is related to the degree of multiple branching is not available.

4. *Interaction with Proteins*

In solution, glycogens interact with certain proteins (for example, serum albumin and globulin, and myosin) to form complexes which may be examined by such physicochemical methods as ultracentrifugal analysis,[90] nephelometry,[91] ultraviolet spectrophotometry,[92] and electrophoresis.[93] Since glycogens have a higher affinity for myosin than φ-dextrin (muscle

(87) F. L. Bates, D. French and R. E. Rundle, *J. Am. Chem. Soc.*, **65**, 142 (1943).

(88) D. M. W. Anderson and C. T. Greenwood, *J. Chem. Soc.*, 3016 (1955).

(89) R. E. Rundle and D. French, *J. Am. Chem. Soc.*, **65**, 1707 (1943); R. R. Baldwin, R. S. Bear and R. E. Rundle, *ibid.*, **66**, 111 (1944).

(89a) However, certain *Zea mays* polysaccharides ($\overline{C.\ L.}$ = 12–13, exterior chain-length, 8–9) bind about three times more iodine than animal glycogens of similar branching characteristics.[88]

(90) E. M. Mystkowski, *Biochem. J.* (London), **31**, 716 (1937).

(91) S. J. von Przylecki, H. Andrzejewski and E. M. Mystkowski, *Kolloid-Z.*, **71**, 325 (1935).

(92) E. L. Rozenfel'd and E. G. Plyshevskaya, *Biokhimiya*, **19**, 161 (1954); *Chem. Abstracts*, **48**, 9423 (1954).

(93) T. T. Bolotina and E. L. Rozenfel'd, *Doklady Akad. Nauk S. S. S. R.*, **87**, 643 (1952); *Chem. Abstracts*, **47**, 6461 (1953).

phosphorylase limit-dextrin) or β-dextrin,[92] complex formation mainly involves combination of protein with exterior chains of glycogen; the nature of the linkages has not been clearly established.

An unusual glycogen–protein interaction has been investigated recently.[94] Addition of concanavalin-A, a globulin from jack-bean meal, to a solution of glycogen results in the formation of an insoluble complex. This interaction is most marked with short-chain glycogens and glycogen β-limit dextrins; amylopectin gives no reaction. Accordingly, concanavalin-A has been used for the identification and estimation of "glycogen" from various biological sources.

III. Structural Analysis by Chemical Methods

1. *End-group Assay*

Chemical determinations of nonreducing end-groups are based on methylation or periodate oxidation studies.[95]

a. Methylation Studies.—In these, acid hydrolyzates of gram quantities of methylated glycogen are analyzed for tetra-*O*-methyl-D-glucopyranose, which originates only from nonreducing terminal groups. Analysis of mixed methyl ethers of D-glucose was formerly done through fractional distillation[96] of the methyl glucosides[51, 57] or by chloroform–water extraction of tetra-*O*-methyl-D-glucopyranose from the remaining sugars.[53-56] More recently, chromatographic methods have been used.

b. Periodate Oxidation Studies.—To a large extent, methylation end-group assay of amylosaccharides can be replaced by simpler decigram-scale methods involving periodate oxidation. In these procedures, the formic acid which arises only from the nonreducing terminal groups of the amylosaccharide is determined, for example, by titration with sodium hydroxide or barium hydroxide. Originally,[97] sparingly soluble potassium metaperiodate at 15° was used as oxidant (see also ref. 23); modifications using sodium metaperiodate at temperatures ranging from 2–20° for 1–7 days have since been devised.[98-102]

(94) J. A. Cifonelli, R. Montgomery and F. Smith, *J. Am. Chem. Soc.*, **78,** 2485 (1956).

(95) Results are usually expressed as average chain lengths ($\overline{C.L.}$), which represent the number of D-glucose residues per end-group. Individual chains in glycogen molecules vary considerably in length, probably from 6–20 D-glucose residues.

(96) For experimental details see (a) W. N. Haworth and H. Machemer, *J. Chem. Soc.*, 2270 (1932); (b) E. L. Hirst and G. T. Young, *ibid.*, 1247 (1938); (c) I. Levi, W. L. Hawkins and H. Hibbert, *J. Am. Chem. Soc.*, **64,** 1957 (1942); (d) J. S. D. Bacon, E. Baldwin and D. J. Bell, *Biochem. J.* (London), **38,** 198 (1944).

(97) T. G. Halsall, E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.*, 1399 (1947).

(98) Under the conditions suggested by A. L. Potter and W. Z. Hassid, *J. Am. Chem. Soc.*, **70,** 3488 (1948), oxidation is incomplete, giving high $\overline{C.L.}$ values values (see ref. 101); chain lengths of 18–23 thus assessed [M. Schlamowitz, *J. Biol. Chem.*, **188,** 145 (1951)] in fact, represent 15–17-unit glycogens.

More than 80 different samples of glycogen have now been assayed by periodate oxidation,[108] and of these, 70 had $\overline{C.\ L.}$ values of 10–14.[23, 97, 99, 100, 101] Many results agree with those from methylation assays of the *same* samples.

TABLE III

End-group Assay of Glycogens by Methylation

Source of glycogen	*Method of separation of methylated sugars*[a]	$\overline{C.\ L.}$	*References*
Ascaris lumbricoides	S. G.	15	103
Dog liver	P.	12	104
Helix pomatia	P.	11–12	105
Horse liver	D.	18	106
Horse muscle	S. G.	12	103
Rabbit liver (D-fructose fed)	D.	11–12	96(d)
Rabbit liver (sucrose fed)	D.	18–19	96(d)
Rabbit liver	A.	18–19	97
Rabbit liver	Q. P.	11–12	107
Rabbit muscle	S. G.	11	108

[a] A. Light petroleum–water partition of methyl glucosides. D. Fractional distillation of methyl glucosides. P. Chloroform–water partition of methylated sugars. Q. P. Quantitative paper chromatography of methylated sugars. S. G. Partition chromatography of methylated sugars on silica gel.

2. *Characterization of Inter-chain Linkages*

a. Methylation Studies.—On hydrolysis, a fully methylated, branched α-D-(1 → 4)-glucan should give a mixture of 2,3,4,6-tetra-, 2,3,6-tri-, and one or more di-*O*-methyl-D-glucoses. Since the last fraction, theoretically equimolar with the tetra-*O*-methyl-D-glucopyranose, arises from the branch points, its characterization would identify the inter-chain linkage.

(99) K. H. Meyer and P. Rathgeb, *Helv. Chim. Acta*, **31**, 1540, 1545 (1948); M. Morrison, A. C. Kuyper and J. M. Orten, *J. Am. Chem. Soc.*, **75**, 1502 (1953).

(100) M. Abdel-Akher and F. Smith, *J. Am. Chem. Soc.*, **73**, 994 (1951).

(101) D. J. Manners and A. R. Archibald, *J. Chem. Soc.*, 2205 (1957).

(102) J. M. Bobbitt, *Advances in Carbohydrate Chem.*, **11**, 1 (1956).

(102a) By an alternative, periodate oxidation assay [M. Abdel-Akher, J. K. Hamilton, R. Montgomery and F. Smith, *J. Am. Chem. Soc.*, **74**, 4970 (1952)], a glycogen ($\overline{C.\ L.}$ = 12, by methylation) had a chain length of 11.

(103) D. J. Bell, *J. Chem. Soc.*, 473 (1944).

(104) W. Z. Hassid and I. L. Chaikoff, *J. Biol. Chem.*, **123**, 755 (1938).

(105) E. Baldwin and D. J. Bell, *Biochem. J.* (London), **34**, 139 (1940).

(106) F. A. Isherwood, Ph.D. Thesis, Birmingham, Engl., 1936.

(107) E. L. Hirst, L. Hough and J. K. N. Jones, *J. Chem. Soc.*, 928 (1949); see also J. K. Bartlett, L. Hough and J. K. N. Jones, *Chemistry & Industry*, 76 (1951).

(108) D. J. Bell, *J. Chem. Soc.*, 992 (1948).

The hydrolyzate of methylated, 18-unit, rabbit-liver glycogen contained a proportion of 2,3-di-*O*-methyl-D-glucose[57]; Haworth and coworkers therefore concluded that the inter-chain linkage was probably (1 → 6). Several years later,[108] the di-*O*-methyl sugars from methylated rabbit-liver and muscle glycogens were analyzed by a periodate oxidation procedure. 2,6-Di-*O*-methyl-D-glucose was the main component, providing tentative evidence for (1 → 3) inter-chain linkages; the 2,3- and 2,6-isomers were also present.

Experimentally, it seems to be impossible to methylate glycogen completely, despite repeated treatment with various methylating reagents.[108] Consequently, hydrolysis of methylated, 12-unit glycogens yields tetra-, tri-, and di-*O*-methyl-D-glucose in the molecular ratio of approximately 1:9:2, instead of 1:10:1 as expected.[53, 56] In addition, during acid hydrolysis, appreciable demethylation of tri-*O*-methyl-D-glucose can occur, yielding di-*O*-methyl-D-glucoses.[108] Undermethylation and hydrolytic demethylation therefore give rise to di-*O*-methyl-D-glucose which cannot be differentiated from that arising from the branch point. Thus, paper-chromatographic analysis[107] of hydrolyzed, methylated, rabbit-liver glycogen gave 8.7 % of tetra-, 69.0 % of tri-, 8.9 % of 2,3-di, 10.8 % of 3,6-di-, and 2.4 % of mono-*O*-methyl-D-glucose. Despite these difficulties, methylation was, until 1948, the only method available for characterization of inter-chain linkages.

b. Acid Hydrolysis Studies.—Partial hydrolysis of glycogen yields a mixture of sugars; apart from D-glucose, maltose and maltotriose will arise from linear portions of the chains. Sugars other than these maltosaccharides will contain unhydrolyzed branch points, and analysis of such sugars will identify the inter-chain linkages.

After partial (75 %), acid hydrolysis of 5 g. of rabbit-liver glycogen, chromatography of the acetylated sugars yielded 92 mg. of β-isomaltose octaacetate[109]; none was formed by "acid reversion" during a similar treatment of amylose. The isolation of a derivative of isomaltose provides evidence for α-D-(1 → 6)-glucosidic linkages in the polysaccharide.

Isomaltose (164 mg.) has been obtained[18] by carbon–Celite chromatography of a (neutralized) partial, acid hydrolyzate of 4 g. of rabbit-liver glycogen. In control experiments, D-glucose and maltose gave much less isomaltose by "acid reversion." It was concluded "that the isomaltose structure is an integral part of the glycogen molecule."

Peat and coworkers[110] have similarly characterized the inter-chain linkages in baker's-yeast glycogen. D-Glucose, maltose, isomaltose, and

(109) M. L. Wolfrom, E. N. Lassettre and A. N. O'Neill, *J. Am. Chem. Soc.*, **73**, 595 (1951).

(110) S. Peat, W. J. Whelan and T. E. Edwards, *J. Chem. Soc.*, 355 (1955).

panose (4-*O*-α-isomaltosyl-D-glucose) were isolated from a partial hydrolyzate, thus showing the presence of α-D-(1 → 6) inter-chain linkages.

In addition to isomaltose, isomaltotriose (about 0.7 %) and nigerose (about 0.001 %) have been isolated from partial, acid hydrolyzates of beef-liver glycogen.[110a] A small proportion of the branch points are therefore directly joined to an adjacent branch point by an α-D-(1 → 6) glucosidic linkage, and a very small proportion of α-D-(1 → 3) linkages may also occur.

Partial, acid hydrolyses therefore show that glycogens contain both α-D-(1 → 4) and α-D-(1 → 6) as the principal glucosidic linkages; however, small numbers of other linkages may also be present.

c. Periodate Oxidation Studies.—A method for the detection of (1 → 2)- and (1 → 3)-linkages in amylosaccharides has been developed by Hirst and coworkers.[111, 112] In a chain of aldopyranose residues, every residue except those substituted at C2 or C3 will be oxidized by periodate. Assuming complete oxidation, the finding of D-glucose in an acid hydrolyzate of the oxidized polysaccharide indicates that it originally contained (1 → 2) or (1 → 3) linkages.

In an application of this method to a glycogen (source unspecified), 6.0 mg. of periodate-oxidized glycogen yielded 0.016 mg. of D-glucose, showing that, at the most, only 2–3 % of the inter-chain linkages could involve C2 or C3.[113] This D-glucose might have arisen from incomplete oxidation of the glycogen.

In similar studies, glycogens (of cat liver, *Helix pomatia*, *Mytilus edulis*, and *Tetrahymena pyriformis*) after prolonged periodate oxidation, were hydrolyzed with acid.[114] The hydrolyzate of *Helix* glycogen yielded a trace of D-glucose, equivalent to <1 % of (1 → 2) or (1 → 3) linkages; the other hydrolyzates did not contain D-glucose. Acid hydrolyzates of periodate-oxidized glycogens (brewer's yeast,[115] human liver[22]) and oxidized α-dextrins from fetal-sheep liver,[114] rabbit liver,[74] and *Ascaris lumbricoides* glycogens,[114] likewise gave no evidence of (1 → 2) or (1 → 3) inter-chain linkages.

The acid hydrolyzate of a periodate-oxidized glycogen of unspecified origin, and of the derived polyalcohol, contained 1 % of D-glucose.[102a] Although it was suggested that this arose from (1 → 2) or (1 → 3) linkages, the

(110a) M. L. Wolfrom and A. Thompson, *J. Am. Chem. Soc.*, **78**, 4182 (1956); **79**, 4214 (1957).

(111) T. G. Halsall, E. L. Hirst, J. K. N. Jones and A. Roudier, *Nature*, **160**, 899 (1947).

(112) E. L. Hirst, J. K. N. Jones and A. Roudier, *J. Chem. Soc.*, 1779 (1948).

(113) G. C. Gibbons and R. A. Boissonnas, *Helv. Chim. Acta*, **33**, 1477 (1950).

(114) D. J. Bell and D. J. Manners, *J. Chem. Soc.*, 1891 (1954).

(115) D. J. Manners and Khin Maung, *J. Chem. Soc.*, 867 (1955).

possibility of incomplete oxidation remains, and, without additional information, the significance of this finding is uncertain.

Periodate-oxidation studies have thus shown that, in several glycogens, over 99 % of the inter-chain linkages are (1 → 6), and experiments employing partial hydrolysis by acid indicate that these linkages have an α-D-configuration.

IV. Structural Analysis by Enzymic Methods

Glycogens are attacked by three known groups of enzymes; amylases and phosphorylases degrade α-D-(1 → 4)-linkages, whilst α-D-(1 → 6) inter-chain linkages are hydrolyzed by "debranching" enzymes.[24, 116, 117]

In contrast with β-amylases (see p. 272), α-amylases catalyze random hydrolysis of α-D-(1 → 4) linkages in both exterior and interior chains of glycogens, giving maltose as the main end-product. Action of salivary α-amylase also yields maltotriose and α-dextrins (D. P. usually 5–8, containing one or more α-D-(1 → 6) linkages) as end products. Other α-amylases, from malt and *Aspergillus oryzae*, hydrolyze maltotriose so that glycogen breakdown finally gives maltose, D-glucose, and α-dextrins. The rate of α-amylolysis of glycogen is lower than that of amylose or amylopectin since the enzymes have a much lower affinity for glycogen than for the starch components.[118]

Phosphorylases,[117] in the presence of inorganic phosphate, remove D-glucose residues from the exterior chains of glycogen, according to the equation

$$[G]_n + m\ HO\cdot\mathbf{P} \rightarrow [G]_{n-m} + m\ G\cdot 1\text{-}\mathbf{P}$$

where $[G]_n$ or $[G]_{n-m}$ represents a chain of n or $(n - m)$ residues, and HO·P and G·1-P represent inorganic phosphate and α-D-glucosyl phosphate.

Phosphorylases cannot bypass inter-chain linkages. The affinity of phosphorylase for glycogen depends upon the enzyme source. Yeast[119] and muscle[120] phosphorylases readily attack glycogens (and amylopectins) yielding 30–50 % of D-glucosyl phosphate. By contrast, potato phosphorylase, under similar conditions, gives about 10 and 40 % of D-glucosyl phosphate from glycogen and amylopectin, respectively.[121] Muscle phosphorylase does not

(116) W. J. Whelan, *Biochem. Soc. Symposia* (Cambridge, Engl.), **11**, 17 (1953).

(117) D. J. Manners, *Ann. Repts. on Progr. Chem.* (Chem. Soc. London), **50**, 288 (1954); *Quart. Revs.* (London), **9**, 73 (1955).

(118) S. Schwimmer, *J. Biol. Chem.*, **186**, 181 (1950); Virginia M. Hanrahan and Mary L. Caldwell, *J. Am. Chem. Soc.*, **75**, 2191 (1953).

(119) Khin Maung, Ph.D. Thesis, Edinburgh, Scotland, 1956.

(120) Gerty T. Cori and J. Larner, *J. Biol. Chem.*, **188**, 17 (1951); Barbara Illingworth, J. Larner and Gerty T. Cori, *ibid.*, **199**, 631 (1952).

(121) A. Margaret Liddle, Ph.D. Thesis, Edinburgh, Scotland, 1956; A. Margaret Liddle and D. J. Manners, *J. Chem. Soc.*, in press (1957).

degrade all exterior chains to the same extent.[120] In the singly-branched "laminated" and multiply-branched "tree" structures for glycogen (and amylopectin) (see Fig. 1), three different types of chain may be distinguished[122]: *A-chain* (side chain), attached by a single (1 → 6) linkage from the reducing group; *B-chain* (main chain) to which one or more A-chains are linked and itself attached by the reducing group to an adjacent chain; and *C-chain*, to which other chains are linked, and which is probably terminated by a free reducing group. [In a molecule consisting of n chains, the the ratio of A-:B-chains (A. B.) for a "laminated" structure is $1:(n-2)$; a "tree" structure contains equal numbers of A- and B-chains.] In a glycogen (or amylopectin) muscle phosphorylase limit-dextrin (ϕ-dextrin), the A- and B-chain stubs contain one and 6–7 D-glucose residues, respectively.[120]

Several "debranching" enzymes have now been discovered. R-enzyme (from higher plants) hydrolyzes (1 → 6)-linkages in glycogen α-dextrins, although it has no action on glycogen.[116] Amylo-(1 → 6)-glucosidase (from rabbit muscle) also does not attack glycogen, but will remove the A-chain stubs of a ϕ-dextrin as D-glucose.[120] In contrast, yeast isoamylase hydrolyzes (1 → 6) linkages in glycogen, α-dextrin, and ϕ-dextrin.[64]

The action patterns of the above enzymes were determined by using the starch components as substrates; with certain reservations, these enzymes may be used for studying the fine structure of glycogen.

1. *End-group Assay*

Glycogen-type polymers contain, in effect, equal numbers of nonreducing end-groups and (1 → 6) inter-chain linkages[123]; determination of the proportion of either will enable the average chain-length to be calculated. In contrast to chemical methods, the enzymic methods of end-group assay measure the proportion of (1 → 6) linkages; two enzymes are required, one specific for α-D-(1 → 4)-linkages, and a debranching enzyme to hydrolyze the small proportion (5–10 %) of α-D-(1 → 6)-glucosidic linkages.

In the method of Cori and Larner,[120] glycogen (or amylopectin) is completely digested by the *concurrent* action of muscle phosphorylase and amylo-(1 → 6)-glucosidase. D-Glucose (which arises only from residues attached to C6 of an adjacent residue) and D-glucosyl phosphate (which is obtained from all other residues) are determined, and the branch-point content is calculated from the proportion of D-glucose.[124] Only 10–15 mg. of glycogen is required for each assay; the method has been applied to more

(122) S. Peat, W. J. Whelan and Gwen J. Thomas, *J. Chem. Soc.*, 4546 (1952).

(123) A molecule with n inter-chain linkages has $(n+1)$ end-groups; for glycogen, n may be 2,000.

(124) In a glycogen containing 8.0% of branch-points (that is, end-groups), the average chain length (C. L.) is 100/8.0 = 12.5.

than 50 glycogens, 36 of which had chain lengths[85, 120, 125] of 10–14. Many of the results are in good agreement with those from assays of the *same* samples by methylation or by potassium periodate oxidation.

Whelan and Roberts have devised an alternative method involving the *successive* action of salivary α-amylase and R-enzyme on glycogen.[116, 126] By determining the number of reducing groups produced by action of R-enzyme on the α-dextrins, the proportion of $(1 \rightarrow 6)$ linkages can be calculated. Rabbit-liver glycogen (C. L., 13.6, by periodate oxidation) had a chain length of 12.5 by this method.

2. *Determination of Exterior and Interior Chain Lengths*

β-Amylolysis of glycogen produces maltose and a high-molecular-weight dextrin (β-dextrin) with exterior chains consisting of perhaps two or three D-glucose residues.[23, 24, 61, 117] The exterior-chain length can therefore be calculated from the average chain-length and the β-amylolysis limit. Using crystalline, sweet-potato β-amylase, glycogens (C. L. 12 ± 1) had[23] β-amylolysis limits of $45 \pm 4\%$. The average, exterior and interior chain-lengths are therefore about 8 and 3 D-glucose residues, respectively, assuming that the exterior chain stubs average 2.5 residues.[127]

Several results are given in Table IV; in general, the length of the exterior chains is roughly twice that of interior chains. Exterior chain lengths cannot be measured by purely chemical methods.

3. *Evidence of Random Branching*

Enzymic studies have shown that the interior structure of glycogens is randomly branched. For example, on β-amylolysis, the exterior chains of rabbit-muscle glycogen are shortened by 5.4 residues, although maltose is the only sugar produced by enzyme action.[23] This result must represent a statistical average of the loss of even numbers of D-glucose residues from individual chains of different, exterior lengths. Furthermore, although the mean, interior, chain length of a 12-unit glycogen is 3–4, there is evidence that a proportion of the interior chains contain 7–8 residues. α-Amylases hydrolyze interior chains in glycogen and its β-dextrin, glucosidic linkages in the middle of these chains being most readily hydrolyzed. Some of the α-dextrins so produced contain exterior chains of about 4 D-glucose residues, as they are partially degraded by β-amylase[23] and show priming ac-

(125) Barbara Illingworth, Gerty T. Cori and C. F. Cori, *J. Biol. Chem.*, **218**, 123 (1956).

(126) W. J. Whelan and P. J. P. Roberts, *Nature*, **170**, 748 (1952).

(127) In ref. 23, exterior-chain lengths were calculated on the assumption that the stubs contained 1.5 residues (see refs. 60, 62, and 65); more recent evidence suggests that the stubs contain two or three residues.

tivity toward potato phosphorylase.[128] Hence, the original interior chains must contain about 8 residues. A study of the absorption spectrum of the iodine complex of the β-dextrin from glycogen also led Swanson[84] to suggest that glycogen contains some interior chains of 8 D-glucose residues.

From the mean, interior chain length of 3–4, and the above evidence, it follows that a number of interior chains must comprise only 1–2 residues. Evidence in favor of this hypothesis has been obtained, during studies of R-enzyme action on glycogen α-dextrins,[126] which shows that a few α-dextrins contain two branch points which are separated by only one D-glucose residue.

TABLE IV

Determination of Exterior and Interior Chain Lengths of Glycogens

Sample	$\overline{C.\ L.}$	*β-Amylolysis limit, %*	*Exterior chain length*[a]	*Interior chain length*[b]	*References*
Cat liver IV	13	54	9–10	2–3	121
Cat liver VI	12	52	8–9	2–3	121
Human muscle II	11	40	7	3	121
Mytilus edulis I	12	43	7–8	3–4	23
Mytilus edulis V	9	40	6	2	121
Mytilus edulis VI	13	46	8–9	3–4	121
Rabbit liver IV	13	45	8–9	3–4	121
Rabbit liver V	14	51	9–10	3–4	121
Rabbit liver VI	18	53	12	5	129
Rabbit muscle II	11	39	6–7	3–4	121
Yeast (baker's)	12	50	8–9	2–3	81
Yeast (brewer's)	13	44	8	4	115

[a] Number of D-glucose residues removed by β-amylase, + 2.5. [b] Average chain length ($\overline{C.\ L.}$) − exterior chain length − 1.

It is therefore very probable that the interior structure of glycogens is irregular.

4. *Determination of Multiple Branching*

A recent development in glycogen chemistry is the recognition that multiple branching is an essential structural feature. Enzymic experiments provide the only means of differentiating between singly- and multiply-branched structures, and methods for the qualitative and quantitative assessment of $\overline{A.\ B.}$ (degree of multiple branching) have been devised.

(128) Marjorie A. Swanson and C. F. Cori, *J. Biol. Chem.*, **172**, 815 (1948). The minimum substrate requirement for both these enzymes is a linear chain of 4 D-glucose residues.

(129) T. G. Halsall, E. L. Hirst, L. Hough and J. K. N. Jones, *J. Chem. Soc.*, 3200 (1949).

One method involves the *successive* action of muscle phosphorylase and amylo-(1 → 6)-glucosidase on glycogen.[130] Treatment of a muscle-phosphorylase limit-dextrin (ϕ-limit dextrin; L.D. 1) with amylo-(1 → 6)-glucosidase yielded D-glucose (about 5 %) and a polysaccharide which, on incubation with muscle phosphorylase, gave a second ϕ-limit dextrin (L.D. 2). Repetition of the digestion with amylo-(1 → 6)-glucosidase and muscle phosphorylase gave L.D. 3. For rabbit-liver glycogen, L.D. 1, L.D. 2, and L.D. 3 represent 64, 38, and 23 % of the original polysaccharide; 30 and 20 % of the original branch-points are removed in the successive digestions with amylo-(1 → 6)-glucosidase. These findings support the "tree" type of

TABLE V

Calculation of $\overline{A.\ B.}$ *in Glycogens*[a]

Sample	*Chain length of ϕ-dextrin*	*Moles of (A + B) chains*[b]	*Degradation of ϕ-dextrin by amylo-(1→6)-glucosidase, %*	*Moles of A chains*[c]	$\overline{A.\ B.}$
Human liver (normal)[d]	8.7	0.071	4.3	0.024	1:2.0
Human liver (glycogen-storage disease)[d]	7.6 8.7	0.081 0.071	5.2 4.7	0.029 0.026	1:1.8 1:1.7
Human liver (glycogen-storage disease)[d]	10.8	0.057	4.6	0.025	1:1.3
Rabbit liver[e]	9.4	0.065	2.7	0.015	1:3.3
Rabbit liver[f]	8.5	0.072	3.2	0.018	1:3.0
Rabbit muscle[f]	8.9	0.070	5.4	0.030	1:1.3

[a] The sole C-chain in the molecule has been neglected in these calculations. [b] Expressed per 100 g. of ϕ-dextrin, and equal to 100/(162 × chain length of ϕ-dextrin). [c] Expressed per 100 g. of ϕ-dextrin and equal to (per cent degradation of ϕ-dextrin by amylo-(1 → 6)-glucosidase)/180. [d] See ref. 85 for experimental figures. [e] See ref. 120 for experimental figures. [f] See ref. 130 for experimental figures.

structure for glycogen, since a "laminated" structure would lose only one branch point with each treatment.

Since the yield of D-glucose released from a ϕ-dextrin by amylo-(1 → 6)-glucosidase is dependent on the proportion of A-chains, the above method can be used to determine $\overline{A.\ B.}$; recorded in Table V are typical results which have been calculated from the experimental data of Gerty T. Cori and coworkers[85, 120, 130] on the assumption that debranching of the ϕ-limit dextrin was complete.

Similar calculations have been made by Beckmann,[131] using the data in reference 130.

(130) J. Larner, Barbara Illingworth, Gerty T. Cori and C. F. Cori, *J. Biol. Chem.*, **199**, 641 (1952).

(131) C. O. Beckmann, *Ann. N. Y. Acad. Sci.*, **57**, 384 (1953).

In a similar study,[119] an abnormal-liver glycogen ($\overline{C.\ L.} = 6$), which approximated in structure to a ϕ-dextrin,[22] was treated with isoamylase.[64] Some 7.3% of D-glucose was liberated; if $\overline{A.\ B.}$ is 1:1, 8.3% of D-glucose would be released.

Further evidence that glycogens may vary in degree of multiple branching has been obtained from examination of their β- and ϕ-limit dextrins,[121, 132] which differ only in exterior-chain length. If all glycogens contained equal numbers of A- and B-chains, the chain-length difference between the ϕ- and β-dextrin should be constant. A-Chain stubs of β-[122] and ϕ-dextrins[120] contain, on the average, 2.5 and 1 residue, respectively, whilst the B-chain stubs of a β-dextrin contain n D-glucose residues (the most probable value of n is 2.5) and those of a ϕ-dextrin comprise $(n + 4)$ residues.[120] The *average* length of the exterior chains of a ϕ-dextrin is therefore $[1 + (n + 4)]/2$ and of a β-dextrin is $(2.5 + n)/2$. The difference in chain length should therefore be 1.25 D-glucose residues. Experimentally, the chain length difference for fifteen glycogens varied from 1.2 to 2.7, equivalent to $\overline{A.\ B.}$ values of 1:1.0 to 1:2.9.

There can be little doubt that glycogens are multiply-branched molecules, as originally suggested by K. H. Meyer, although variations in the degree of multiple branching exist. However, in view of unavoidable limitations in the experimental and analytical procedures employed during the enzymic degradation of glycogen (each molecule may contain some 2,000 exterior chains), the numerical results for $\overline{A.\ B.}$ quoted in this Section represent approximate rather than absolute values.

V. Molecular Structure of Glycogens

Glycogens are highly branched macromolecules composed of several thousand chains; on the average, each chain contains 10–14 α-(1 → 4)-linked D-glucose residues and is joined to an adjacent chain by a (1 → 6)-glucosidic linkage. Individual chains vary considerably in length. Glycogen of molecular weight 5×10^6 contains about 31,000 D-glucose residues and about 2,500 α-D-(1 → 6) inter-chain linkages. The interior of such a molecule is very compact, some 10,000 of the D-glucose residues being arranged in chains so that the branch points are separated by an average distance of only 3–4 D-glucose residues. The over-all structure is multiply-branched, being tree- or bush-like in form; glycogens do not appear to consist of ordered arrangements of chains of similar lengths. Although average chain-lengths of 10–14 residues are usual, values ranging from 6–18 are occasionally found.

Glycogen preparations from a single biological source are polymolecular, and, sometimes, polydisperse. The molecules range in molecular weight

(132) A. Margaret Liddle and D. J. Manners, *Biochem. J.* (London), **61**, xii (1955); *J. Chem. Soc.*, in press (1957).

from 10^5–10^7, but are believed to have similar structures. However, there is tentative evidence of structural inhomogeneity in mussel glycogen, since two fractions, separated by electrodialysis, differed in solubility and viscosity, and had β-amylolysis limits of 30 and 43 %, respectively.[36]

1. *Glycogens of Vertebrates*

a. Mammalian Glycogens.—The properties of several mammalian glycogens are recorded in Table VI.

TABLE VI

Properties of Some Mammalian Glycogens

Sample	$[\alpha]_D$, *degrees* (H_2O)	*Molecular weight*[a] $\times 10^{-6}$	$\overline{C.L.}$[b]	*β-Amylolysis limit, %*	*References*
Cat liver	+193	10.0(l)	13(p)	48	23, 71, 74
Dog liver	+193	—	12(p)	—	100
Fetal-pig liver	+191	2.0(s)[c]	11(p)	49	38, 74
Fetal-sheep liver	+196	14.8(l)	13(p)	49	23, 71, 74
Guinea-pig liver	+190	—	10(p)	—	100
Horse liver	+190	—	11(p)	—	100
Horse muscle	+198	2.9(s)	11–12(m, p)	42	23, 56, 79
Human liver	+195	—	11(p)	—	100
Human muscle	+195	2.4(s)	12(p)	41	23, 74, 79
Ox liver	+192	—	12–13(p)	—	100
Rabbit liver	+198	1.1(o)	12(m)	51	52, 53, 77, 121
Rabbit liver	+188	1.9(s)	18(m)	52	57, 83, 121
Rabbit liver	+198	6.8(l)	12–13(m, p)	43	23, 71
Rabbit liver	+200	—	18(m)	—	54
Rabbit muscle	+196	2.6–2.8(s, l)	11–13(m, p)	45	23, 71, 79, 108
Rat liver	+193	—	11(p)	—	100

[a] Methods of measurement: (l) = light-scattering; (s) = sedimentation-diffusion; (o) = osmotic pressure of the methylated glycogen. [b] Methods of assay: (m) = methylation; (p) = periodate oxidation. [c] This sample was polydisperse; a minor component had a molecular weight of 0.5×10^6.

Apart from differences in molecular weight, 14 of the 16 samples show little variation in branching characteristics; the constituent chains contain 10–14 D-glucose residues per end-group, and 41–51 % of these are removed, as maltose, by β-amylase. The average interior and exterior chain-lengths are, therefore, approximately 3 and 8 D-glucose residues, respectively.

It is of interest that liver glycogens of the fetal pig and sheep, formed only during the later stages of embryonic development,[133] have structures similar to adult, mammalian-liver glycogen.

(133) Compare A. M. Nemeth, W. Insull, and L. B. Flexner, *J. Biol. Chem.*, **208**, 765 (1954).

The assumption that muscle and liver glycogens are chemically identical is partly confirmed by the data in Table VI, which show them to have similar branching properties. However, they differ in iodine coloration,[17] and muscle glycogens have a greater affinity for myosin than have liver glycogens (see p. 279). The reasons for these differences are not yet known.

b. Fish Glycogens.—The properties of several fish-liver glycogens are summarized in Table VII.

In general, fish glycogens show little variation in degree of branching

TABLE VII

Properties of Some Fish-liver Glycogens

Sample	$[\alpha]_D$, *degrees* (H_2O)	*Molecular weight*[a] $\times 10^{-6}$	$\overline{C.L.}$[b]	*References*
Bass (*Micropterus dolamieu*)	+191	—	14(p)	100
Bullhead (*Ameiurus melas*)	+190	—	12(p)	100
Carp (*Cyprinus carpio*)	+198	—	12(p)	100
Crappie (*Pomoxis annularis*)	+194	—	12(p)	100
Dogfish[c]	+195	3.6	12(m)	134
Gadidae[d]	+196	—	12(m)	31, 53
Garfish (*Lepisosteus osseus*)	+195	—	12(p)	100
Haddock (*Gadus aeglefinus*)	+180	1.0	12(m)	134
Hake (*Merluccius vulgaris*)	+190	1.1	12(m)	134
Northern pike (*Esox estor*)	+194	—	12–13(p)	100
Skate	+196	—	13(p)	121
Walleyed pike (*Stizostedion vitreum*)	+197	—	13(p)	100

[a] Determined by sedimentation–diffusion of the methylated glycogen (ref. 83). [b] Methods of assay: (m) = methylation; (p) = periodate oxidation. [c] For dogfish-muscle glycogen, the figures are +190–195; 2.5 and 12(m), respectively (refs. 83 and 134). [d] Pooled livers of cod (*Gadus morrhua*), haddock (*Gadus aeglefinus*), and whiting (*Gadus merlangus*).

and resemble most mammalian glycogens. Apart from skate-[121] and Northern pike-liver[94] glycogens which have β-amylolysis limits of 45 and 34%, respectively, the enzymic degradation of fish glycogens has not been studied.

c. Glycogen-storage Disease.—This disease, of which there are several distinct types, is characterized by the accumulation of glycogen in the liver and in other tissues. In some cases, this glycogen has an abnormal structure. These conditions result, in part, from a deficiency of one of the enzymes concerned in the interconversion of D-glucose and glycogen; glycogen breakdown is usually affected. The liver-enzyme system is shown in Fig. 3.

(134) W. N. Haworth, E. L. Hirst and F. Smith, *J. Chem. Soc.*, 1914 (1939).

Present knowledge of the biochemistry of this disease is due largely to Gerty T. Cori and coworkers[85, 125, 135] and, in Table VIII, the characteristics of four types of the disease and the properties of several glycogens are recorded.

The glycogen has a normal structure in types 1 and 2; in the former, there is deposition of fat in the liver and kidney, and a decreased activity of glucose-6-phosphatase. The enzyme deficiencies in type 2 are unknown. In types 3 and 4, which are less common, the glycogens have abnormal

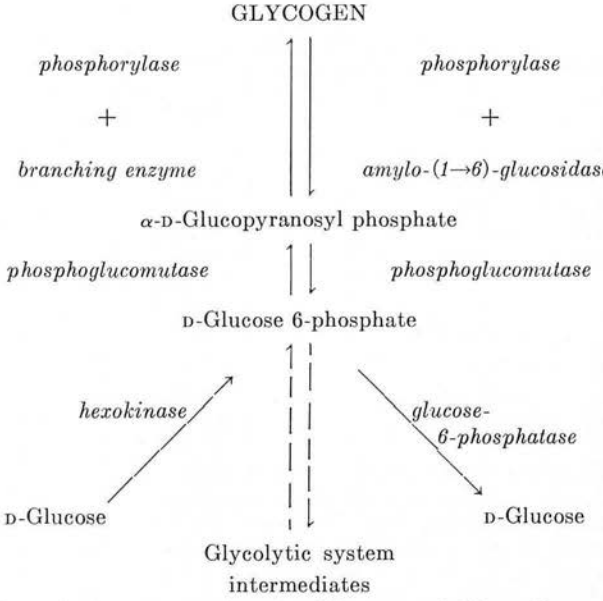

FIG. 3.—Enzymic Interconversion of D-Glucose and Liver Glycogen. [a] In this reaction, 5–10% of D-glucose is produced, in addition to α-D-glucopyranosyl phosphate.[120]

structures. In type 3, the short, exterior chains indicate absence of amylo-(1 → 6)-glucosidase[22, 85]; this has been confirmed experimentally in two cases of the disease.[125] Glycogen in type 4 resembles amylopectin in degree of branching, solubility, and x-ray diffracting properties.[85]

2. *Glycogens of Invertebrates*

Table IX summarizes the properties of several invertebrate glycogens, including protozoan glycogens.

(135) For a review, see Gerty T. Cori, *Harvey Lectures*, **48**, 145 (1953).

The variation in degree of branching of these glycogens probably reflects differences in the *relative* activity of phosphorylase and branching enzyme during glycogen synthesis.

In contrast to the above-mentioned protozoa which synthesize glycogen-type polysaccharides, certain ciliates (for example, *Cycloposthium*) contain an amylopectin-type of polysaccharide, whilst the flagellate *Polytomella coeca* stores a typical two-component starch.[136]

TABLE VIII

Types of Glycogen-storage Disease,[125, 135] *and Properties of Glycogens*

Type of disease	*Affected organ*	*Enzyme deficiency*	*Sample of glycogen*	$\overline{C.L.}$	*Phosphorolysis limit, %*[a]	*References*
1 (von Gierke's disease)	liver and kidney	glucose-6-phosphatase	liver	13	36	85
			muscle	12	38	85
			liver	11	36	85
			kidney	11	33	85
2	generalized	?	liver	14	35	85
			liver	11	33	85
			heart	13	42	125
			liver	12	40	125
3	generalized	amylo-(1 → 6)-glucosidase	liver	9	12	85
			muscle	8	3	85
			liver[b]	6	1	22
			heart	8	6	125
			psoas	8	5	125
			diaphragm	8	3	125
4	probably generalized	branching enzyme	liver	21	51	85

[a] Percent conversion to α-D-glucopyranosyl phosphate by muscle phosphorylase; this is a measure of the exterior chain length. [b] This glycogen was incorrectly described as coming from a case of von Gierke's disease.[22]

3. *Bacterial and Yeast Glycogens*

Polysaccharides which resemble animal glycogens in chemical and physical properties have been isolated from bacterial cells, including avian[82] and human[137] strains of *Mycobacterium tuberculosis* and surface cultures of enteric bacteria[26] (for example, *Escherichia coli*[138] and *Salmonella montevideo*), and also from yeasts.[6, 81, 110, 115, 139]

(136) E. J. Bourne, M. Stacey and I. A. Wilkinson, *J. Chem. Soc.*, 2694 (1950); the properties of several protozoal polysaccharides are compared in ref. 140.

(137) P. W. Kent and M. Stacey, *Biochim. et Biophys. Acta*, **3**, 641 (1949).

(138) H. Palmstierna, *Acta Chem. Scand.*, **10**, 567 (1956).

(139) R. W. Jeanloz, *Helv. Chim. Acta*, **27**, 1501 (1944).

4. *Comparison of Glycogens with Amylopectins*

Glycogens and amylopectins are structurally similar in that both contain chains of α-(1 $\rightarrow$ 4)-linked D-glucose residues which are mutually

TABLE IX

Properties of Some Glycogens of Invertebrates

Phylum	*Species*	$[\alpha]_D$, degrees H_2O	*Molecular weight,*[a] $\times 10^{-6}$	$\overline{C.L.}$[b]	*β-Amylolysis limit, %*	*References*
Protozoa	*Trichomonas foetus*	+199	2.9(s)[c]	15(p)	60	140
	Trichomonas gallinae	+197	3.5(s)	9(p)	51	140
	Tetrahymena pyriformis	+195	9.8(l)	13(p)	44	141
Platyhelminthes	*Moniezi expansa* (sheep tapeworms)	+194	—	12(p)	—	100
Nematoda	*Ascaris lumbricoides* (pig roundworms)	+196	0.7(s)	12–15(m, p)	49	23, 79, 142
Annelida	*Arenicola* sp. (lugworm)	+200	—	11(p)	43	121
Mollusca[d]	*Anodonta*[e]					
	Fraction I	+192	6.1(o)	—	43	36
	Fraction II	—	2.1(o)	—	34	36
	Fraction III	—	3.0(o)	—	30	36
	Mytilus edulis I	+192	—	12(p)	43	23, 74
	Mytilus edulis II	+195	—	16–18(m, p)	47	23, 55
	Mytilus edulis IV	+196	3.8(s)	12(p)	52	38, 121
	Mytilus edulis IX	+196	—	10(p)	51	121
	Cardium sp.	+201	—	8(p)	14	121
	Helix pomatia I	+192	—	11–12(m)	—	105
	Helix pomatia II	+182	2.6(s)[f]	7(p)	37	23, 38, 74

[a] Methods of measurement: (o) = osmotic pressure of the acetylated glycogen; (s) = sedimentation-diffusion, (l) = light-scattering. [b] Methods of assay: (m) = methylation; (p) = periodate oxidation. [c] This specimen was polydisperse, a minor component having a molecular weight of 0.3×10^6. [d] These invertebrates are commonly known as fresh-water mussel, common mussel, cockle, and edible snail, respectively. [e] Fractionation by electrodialysis. [f] This specimen was polydisperse; minor components had molecular weights of 12.9×10^6 and 0.3×10^6.

interlinked by α-D-(1 $\rightarrow$ 6)-glucosidic linkages; the chains are multiply-branched to a similar degree. Approximately equal numbers of A- and B-chains are present in waxy-maize starch[121, 143] and in wheat and corn

(140) D. J. Manners and J. F. Ryley, *Biochem. J.* (London), **59**, 369 (1955).
(141) D. J. Manners and J. F. Ryley, *Biochem. J.* (London), **52**, 480 (1952).
(142) E. Baldwin and H. K. King, *Biochem. J.* (London), **36**, 37 (1942).
(143) S. Peat, W. J. Whelan and Gwen J. Thomas, *J. Chem. Soc.*, 3025 (1956).

(maize) amylopectins (as shown by calculations similar to those in Table V), as well as in glycogens.

Differences between glycogen and amylopectin include the proportion of (1 → 6) linkages (average chain length), affinity for iodine, molecular shape, and interaction with concanavalin-A. The average chain-length in amylopectin is approximately twice that of glycogen[20]; most amylopectins contain 18–27 D-glucose residues per end-group, although samples with $\overline{C.\ L.}$ values of[29, 30] 11–13 or[144] 36 have been reported. The *relative* position of branching appears to be similar, and the length of the exterior chains is

TABLE X

Properties of Some Bacterial and Yeast Glycogens

Sample	$[\alpha]_D$, *degrees* (H_2O)	*Molecular weight,*[a] $\times 10^{-6}$	$\overline{C.\ L.}$[b]	*β-Amylolysis limit, %*	*References*
Aerobacter aerogenes	+200	9.2(s)	—	—	26
Bacillus megatherium	+192	0.2(d)	10–11(m, p)	46	86
Neisseria perflava	—[c]	—	—	57–59	145
Neisseria perflava	—[d]	—	11–12(m)	55–59	146
Neisseria perflava	+196	—	11(p)	—	100
Yeast (baker's)[e]	+187	—	—	49	139
Yeast (baker's)	+184–188	—	12(m, p)	50	81
Yeast (brewer's)	+198	2.4(s)	13(p)	44	115

[a] Methods of measurement: (s) = sedimentation-diffusion; (d) = reducing-power estimation, using a dinitrosalicylic acid reagent. [b] Methods of assay: (m) = methylation; (p) = periodate oxidation. [c] $[\alpha]_D$ + 175° in 0.5 *N* NaOH. [d] $[\alpha]_D$ + 178° in 0.5 *N* NaOH. [e] The glycogen was fractionated by electrodialysis giving a "soluble" fraction (27%) with the properties recorded; the "insoluble" fraction (73%) had a β-amylolysis limit of 46%.

normally twice that of the interior chains, as calculated from the β-amylolysis limit (50–60 %) and the average chain-length. It follows that the interior of amylopectin, although randomly branched, is less compact than that of glycogen; it is therefore more susceptible to enzymic attack. For example, α-amylases degrade interior chains (of 6–9 residues) in amylopectin more readily than those of glycogen (3–4 residues),[118] whilst R-enzyme, which has no appreciable action on glycogen, hydrolyzes many of the α-D-(1 → 6) linkages in amylopectin.[146a] It is probable that the inter-chain

(144) A. L. Potter, V. Silveira, R. M. McCready and H. S. Owens, *J. Am. Chem. Soc.*, **75**, 1335 (1953).

(145) E. J. Hehre, *J. Biol. Chem.*, **177**, 267 (1949).

(146) S. A. Barker, E. J. Bourne and M. Stacey, *J. Chem. Soc.*, 2884 (1950).

(146a) S. Peat, W. J. Whelan, P. N. Hobson and Gwen J. Thomas, *J. Chem. Soc.*, 4440 (1954).

Variations in the structure of glycogen may be due, in part, to differences in *relative* activity of phosphorylase and branching enzyme. With a deficiency in branching enzyme, a glycogen with a low degree of branching would be expected. The amount of active enzyme in a tissue depends on many factors; phosphorylase activity is related to the rates of enzymic synthesis and breakdown of the phosphorylase–enzyme protein and nucleotide (adenosine 5-phosphate) activator,[155] the number of primer molecules available, and the inorganic phosphate content of the cells. In higher animals, the enzyme systems are under hormonal control.[156] The rate and type of glycogen formation will therefore depend on the metabolic condition of the whole animal.

VII. Summary and Conclusions

Glycogens, from vertebrate, invertebrate, bacterial, and yeast cells, are multiply-branched molecules (molecular weight $\sim 10^7$) consisting of chains of α-(1 $\rightarrow$ 4)-linked D-glucose residues. The chains, which are arranged in a tree- or bush-like form, normally contain an average of about 12 D-glucose residues; 40–50 % of these may be removed by β-amylase. The exterior portions of the chains are therefore longer than those in the interior of the molecules, where adjacent branch points are separated by only 3–4 D-glucose residues. Small variations in molecular structure are shown by glycogens from different biological sources.

It seems probable that, with the improved chemical and enzymic semimicro-analytical methods now available, the structure of glycogen from hitherto-unexamined biological sources will be characterized and that structural studies will also be directed toward the quantitative determination of multiple branching and investigation of the compact interior of the molecules. A small proportion of glycogens may be expected to have abnormal structures. In this connection, the isolation of maltulose (some 5 %) from an α-amylolytic digest of glycogen[157] of pregnant-rabbit liver would suggest that D-fructose may be an extremely rare but minor component of certain glycogens.

(155) The enzymic synthesis and inactivation of mammalian phosphorylases is discussed in "Enzymes and Metabolism," Elsevier, Amsterdam, 1956, pp. 69, 150; *Biochim. et Biophys. Acta*, **20**, No. 1 (1956).

(156) For example, the conversion of D-glucose into liver glycogen is stimulated by insulin, and inhibited by adrenaline and glucagon [J. Berthet, P. Jacques, H. G. Hers and C. de Duve, *Biochim. et Biophys. Acta*, **20**, 190 (1956)].

(157) S. Peat, P. J. P. Roberts and W. J. Whelan, *Biochem. J.* (London), **51**, xvii (1952).

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LECTURES, MONOGRAPHS AND REPORTS, 1959

No. 2

STRUCTURAL ANALYSIS OF POLYSACCHARIDES

BY

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This monograph is based on the Meldola Medal Lecture, delivered by Dr Manners at the Royal Institution, London, on 17 *November*, 1958—*Professor H. V. A. Briscoe, Vice-President, in the Chair*

RECENT ADVANCES IN THE STRUCTURAL ANALYSIS OF POLYSACCHARIDES

By D. J. MANNERS, M.A., PH.D., F.R.I.C.

The past few years have seen a remarkable increase in our knowledge of the molecular structures of naturally occurring high polymers. This is especially true of the polysaccharides, and the purpose of this review is first, to describe some of the techniques and methods which have made this progress possible, and second, to illustrate (on pp. 15–35) their use, with an account of studies on certain glucose-containing polysaccharides.

The polysaccharides are high-molecular-weight polymers of anhydromonosaccharide units which are glycosidically linked to form non-cyclic polymeric chains. The degree of polymerization ($\overline{DP}$) may vary from *ca* 30 to $\sim 10^5$, and on complete hydrolysis one or more monosaccharides may be liberated. Homopolysaccharides may be defined as being composed of essentially one monosaccharide, whereas the more complex heteropolysaccharides may contain 2–6 different component sugars. In addition, small amounts of non-carbohydrate constituents may be present, *e.g.* ester groups derived from phosphate, sulphate, malonate or pyruvate. The constituent sugars may be arranged to give linear or branched structures (Fig. 1).

Progress in this field may be divided into a number of phases. The first, covering the nineteenth century, was concerned with the isolation, purification and composition of polysaccharides. Detailed chemical studies were limited, since the ring structure of the various monosaccharides had not been adequately characterized. The second phase (1900–1940) saw the establishment of the *basic* structures of the commonly occurring plant and animal polysaccharides by the classical method of methylation (p. 4) and by a limited number of physico-chemical methods (*e.g.* measurement of the viscosity and osmotic pressure of certain polysaccharide derivatives). The work of Sir Norman Haworth and his many collaborators, at Birmingham, was predominant during the latter part of this period. The third and fourth phases, which are developing concurrently, are concerned with the determination of the *fine* structure of polysaccharides by specialized techniques, and with the biological synthesis and degradation of these polymers.[1] The major part of this review will be devoted to certain aspects of these last two phases.

The first and often the most difficult stage in the structural examination of a polysaccharide is its isolation in an unmodified and undegraded state. The conventional methods of selective extraction with water, dilute salt, acid or alkali, followed by fractional precipitation with organic solvents, are adequate in only a few instances. Other methods involve the selective precipitation of certain types of polysaccharide as copper or

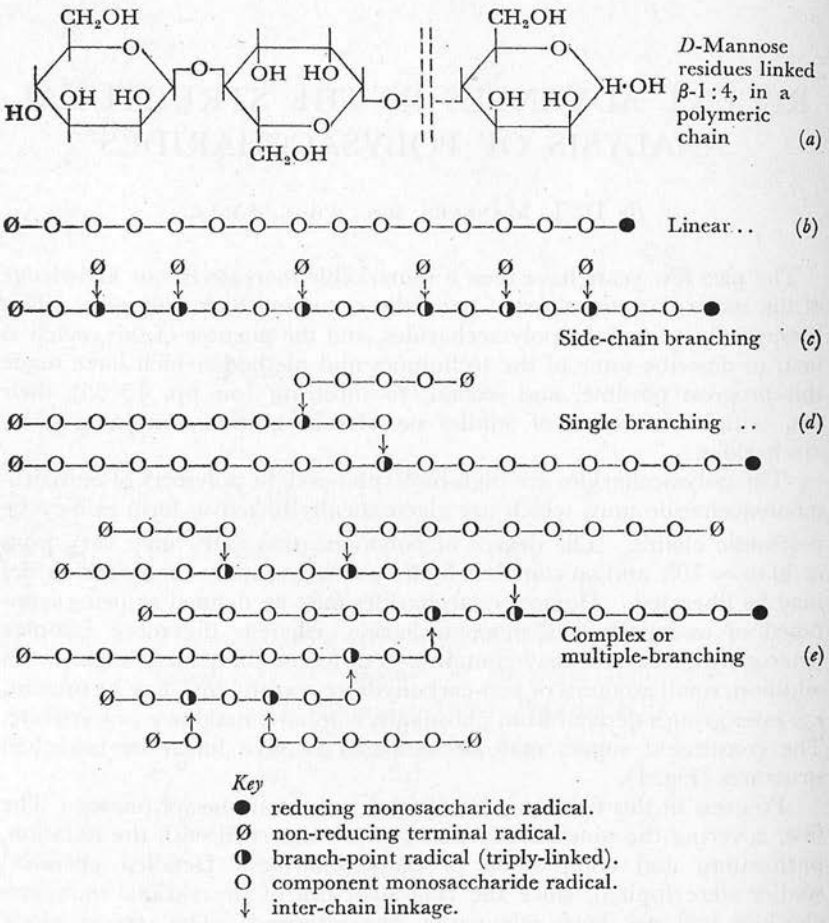


FIG. 1. Types of polysaccharide structure

iodine complexes or by the use of Cetavlon or barium hydroxide. Ionophoretic methods are becoming increasingly important, and a small-scale preparative column has now been developed[2] from an earlier analytical method. However, most carbohydrate chemists would agree that the development of new methods for the isolation of polysaccharide material free from lignin and protein, and the separation of individual polysaccharides, merits a high priority, particularly in view of recent findings on the degradative action of dilute alkali and oxygen on many polysaccharides.

For the structural analysis of a purified polysaccharide, a number of different techniques are required. Firstly, the component sugars are identified by suitable examination of an acid hydrolysate. Secondly, application of the methylation procedure may provide, in many instances, information on the ring structure of the constituent sugars, the C atoms

involved in the repeating glycosidic linkage(s), and the proportion of non-reducing terminal groups. By analysis of a partial acid hydrolysate, the anomeric configuration of the repeating linkage(s), the nature of inter-chain linkages and, in some cases, the order of monosaccharide residues in the polymeric chains can be determined. Finally, estimates of the molecular size and shape may be obtained by certain physico-chemical methods.

Polysaccharides containing uronic acid residues or sulphate ester groups require special methods, since they tend to resist hydrolytic attack and methylation. These difficulties may be partly overcome by reduction of carboxylic acid groups to primary alcohol groups with lithium aluminium hydride and by desulphation (using methanolic hydrogen chloride) to yield modified polysaccharides composed of unsubstituted monosaccharide residues.

The outstanding advance in the field of structural polysaccharide chemistry has been the application of chromatographic methods. It is now possible to separate by column chromatography a mixture of five or six different monosaccharides or their methyl ethers in an acid hydrolysate of a polysaccharide or the corresponding methylated derivative. Further, the quantitative separation of milligram quantities of sugars can be effected by paper chromatography. In contrast, the earlier separations of monosaccharides, by selective precipitation of derivatives or of methylated sugars by fractional distillation, required gram quantities of material and were seldom quantitative. The application of chromatographic methods to carbohydrate chemistry has been described in detail by Hirst, Hough and Jones.[3]

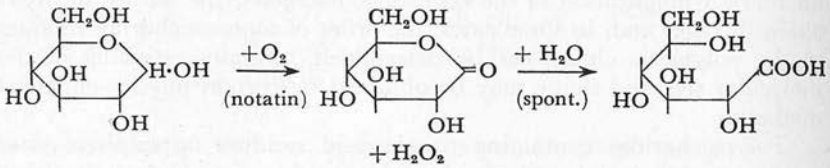
A second technique of increasing importance is zone electrophoresis (ionophoresis).[4] In many instances it provides a method for separating mono- and oligo-saccharides and their derivatives with similar chromatographic mobilities. By a combination of chromatographic and zone-electrophoretic methods, many of the more complex mixtures of sugars have been separated.

THE COMPOSITION OF POLYSACCHARIDES

In this analysis, the polysaccharide is hydrolysed with hot mineral acid under conditions whereby loss of monosaccharides by decomposition or by 'acid-reversion' is minimal. The conditions for hydrolysis depend upon the nature of the polysaccharide; for example, fructosans and glucosans require heating in 0·05N- and 1·5N-sulphuric acid for 10 and 90 min. respectively. The neutralized hydrolysate is examined qualitatively by paper chromatography, and the component sugars then separated quantitatively by chromatography on a column of cellulose, starch, charcoal–Celite or ion-exchange resin, and characterized by the measurement of physical constants and the preparation of crystalline derivatives.

Hydrolysates containing D-glucose and certain other monosaccharides may be analysed by enzymic methods. The oxidation of D-glucose to

D-gluconic acid is catalysed by notatin (glucose oxidase)[5] and the reaction may be followed by (*a*) manometric determination of the oxygen



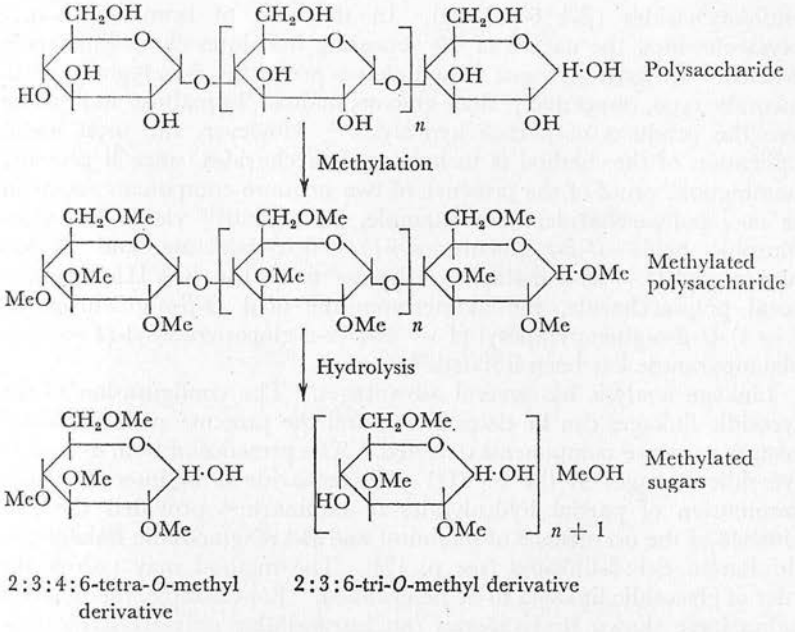
uptake,[5] (*b*) titration of the D-gluconic acid with alkali,[6] and (*c*) measurement of the change in reducing power.[7] Using method (*a*), the glucose content of hydrolysates of various fructosans has been determined,[8] and method (*c*) has been applied to *Brachychiton diversifolium* gum[9] and to protozoal polysaccharides.[7]

ANALYSIS BY METHYLATION

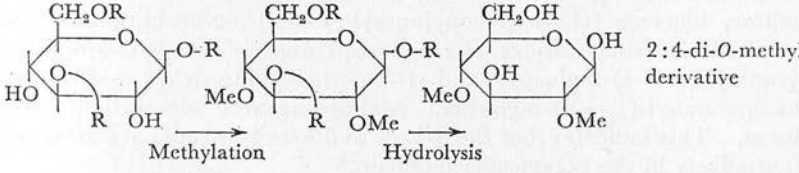
The methylation procedure, which was devised by Purdie and Irvine, and developed by Haworth and Hirst, is the most important analytical method in polysaccharide chemistry. Experimentally,[10] the method (Fig. 2) involves complete methylation of the polysaccharide (dimethyl sulphate and sodium hydroxide are normally used), hydrolysis with acid and quantitative separation and estimation of the resulting mixed methylated sugars. As mentioned previously, the application of chromatographic methods has greatly improved and facilitated the separation and has also markedly reduced the amount of methylated polysaccharide required for investigation.

As illustrated in Fig. 2 (*a*), the repeating linkage of the component sugars can be determined, the nature and proportion of non-reducing terminal groups examined (*i.e.* end-group assay), and, since each polysaccharide chain contains only one end-group, the average chain length ($\overline{CL}$) can then be measured. A comparison of this with the $\overline{DP}$ reveals the linear or branched nature of the polysaccharide. If the polysaccharide is branched, then the nature of the inter-chain linkages may be determined, as indicated in Fig. 2 (*b*).

The method has some limitations. The configuration of the glycosidic linkages, the order of monosaccharides in the polymeric chains of a heteropolysaccharide or the types of branching (side-chain, single or multiple) are not revealed. In practice, complete methylation of highly branched polysaccharides (*e.g.* glycogen, plant gums and mucilages) is difficult, and the hydrolysate may contain mono- or di-methyl sugars arising from undermethylation of non-terminal residues; a small amount (*ca* 2–3 per cent) of demethylation may also occur during the hydrolysis of the methylated polysaccharide. Both processes may therefore prevent the unambiguous identification of inter-chain linkages. Despite these limitations, methylation remains the fundamental method of structural analysis, and many examples of its application are given by E. L. Hirst in his recent reviews on the chemistry of fructosans,[11a] hemicelluloses,[11b] seaweed polysaccharides[11c] and plant gums.[11d]



(*a*) Methylation of a linear polysaccharide (repeating linkage 1:4-glucosidic)



(*b*) Methylation of a branched polysaccharide (R = chain of monosaccharide residues)

FIG. 2. Methylation Analysis

ANALYSIS BY PARTIAL ACID HYDROLYSIS

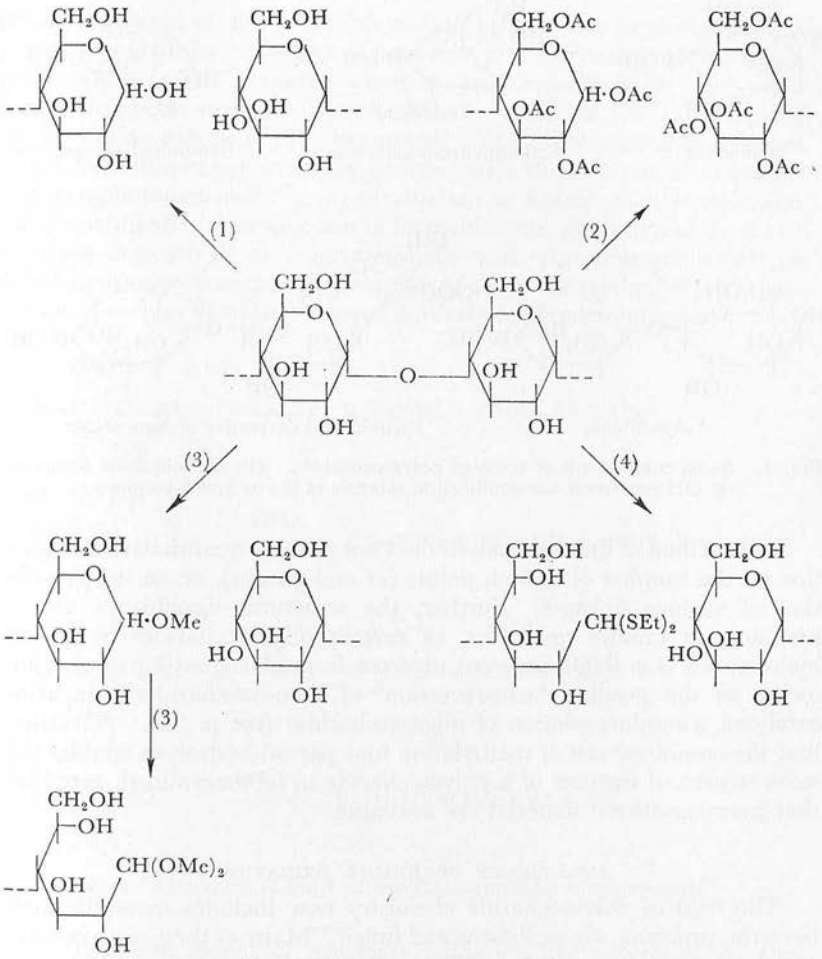
A methylation study of a polysaccharide may be supplemented by an examination of the products of partial acid hydrolysis, involving chromatographic separation and chemical identification of the mixed oligosaccharides. The success of this method, termed 'linkage analysis' by Peat and his co-workers[12] is largely dependent on the development of charcoal–Celite column chromatography[13,14,15] whereby most mixtures of mono- and oligo-saccharides can be readily separated. The resolving power of such columns may be improved by the presence of borate[16] or molybdate.[17]

An essentially linear homopolysaccharide yields, on linkage analysis, the constituent monosaccharide and a homologous series of oligosaccharides of increasing $\overline{DP}$, each containing the repeating linkage. Thus, amylose[14] gives glucose and maltosaccharides (α-1:4-linkage) and pustulan[18] (from the lichen *Umbilicaria pustulata*) yielded glucose and

gentiosaccharides (β-1 : 6-linkage). In the case of branched homopolysaccharides, the nature of the repeating and inter-chain linkages is revealed. In yeast glycogen these linkages are of the α-1 : 4- and α-1 : 6-glucosidic type, respectively, since glucose, maltose, isomaltose and panose were the products of partial hydrolysis.[12] However, the most useful application of the method is to heteropolysaccharides, since it provides unambiguous proof of the presence of two or more component sugars in the *same* polysaccharide. For example, gum ghatti[19] yields two aldobiouronic acids—*O*-β-D-glucuronosyl-(1 → 6)-D-galactose and *O*-β-D-glucuronosyl-(1 → 2)-D-mannose—whereas from the S. VIII pneumococcal polysaccharide, the aldotetraouronic acid *O*-β-D-glucuronosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 4)-D-galactopyranose has been isolated.[20]

Linkage analysis has several advantages. The configuration of the glycosidic linkages can be determined, and the presence of 'anomalous' linkages or minor components detected. The presence of both α- and β-glycosidic linkages in the S. VIII polysaccharide is of interest, whilst examination of partial hydrolysates of laminarin[21] provided the first evidence of the occurrence of mannitol and β-1 : 6-glucosidic linkages, in addition to β-1 : 3-linkages (see p. 17). The method may permit the order of glycosidic linkages to be determined. For example, methylation studies have shown that nigeran (an intracellular polysaccharide synthesized by *Aspergillus niger*) is linear and contains equal numbers of α-1 : 3- and α-1 : 4-glucosidic linkages. A partial hydrolysate contained maltose, nigerose (*O*-α-D-glucopyranosyl-(1 → 3)-D-glucopyranose) and the isomeric trisaccharides *O*-α-D-glucopyranosyl-(1 → 3)-*O*-α-D-glucopyranosyl-(1 → 4)-D-glucose and *O*-α-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 3)-D-glucose. Maltotriose and 'nigerotriose' were absent. This indicates that the α-1 : 3- and α-1 : 4-linkages are arranged alternatively in the polysaccharide chain.[22]

Some modifications of the method of partial acid hydrolysis have been introduced (Fig. 3). Certain insoluble polysaccharides may be more conveniently degraded by means of a mixture of acetic anhydride, acetic acid and sulphuric acid (partial acetolysis). The free sugars are then obtained by deacetylation of the corresponding acetates. This procedure has provided evidence for the presence of a small number of α-1 : 4-linked D-mannose residues in ivory-nut mannan, in addition to β-1 : 4-linked sugars.[23] Mercaptolysis and methanolysis are alternative methods in which the polysaccharides are depolymerized by mixtures of hydrochloric acid and ethyl mercaptan or methyl alcohol. The resulting diethyl mercaptals or methyl glycosides (or dimethyl acetals) can be readily isolated and crystallized. These last two techniques are particularly useful in studies on the complex seaweed polysaccharides that contain labile anhydro-sugars. For example, mercaptolysis of a polysaccharide from *Chondrus crispus* yielded[24] the diethyl mercaptal of the rare sugar 3 : 6-anhydro-D-galactose (Fig. 4). Araki and his co-workers have examined the products of the mercaptolysis of agar in some detail[25]; these included the diethyl mercaptals of D-galactose, DL-galactose, 3 : 6-anhydro-L-galactose and a disaccharide—agarobiose. The constitution

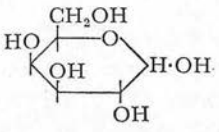


(1) Acidic or enzymic hydrolysis
(2) Acetolysis
(3) Methanolysis
(4) Mercaptolysis

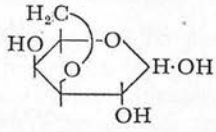
For convenience, the degradation of *glucosidic* linkages by methanolysis and mercaptolysis is shown; in practice, these procedures are normally applied to polysaccharides containing anhydro-sugars.

FIG. 3. Methods of degradation of glycosidic linkages

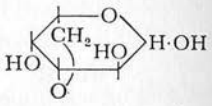
of the latter (*O*-β-D-galactosyl-(1 → 4)-3 : 6-anhydro-L-galactose) characterizes one of the repeating linkages in agar. Methanolysis studies have been equally important in this field. An interesting example is the isolation from agar of the dimethyl acetal of a disaccharide linked in ketal form to pyruvic acid.[26] This has been characterized as 4,6-*O*-1′-carboxyethylidene-β-D-galactopyranosyl-(1 → 4)-3 : 6-anhydro-L-galactose (Fig. 4).



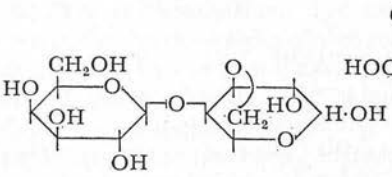
D-galactose



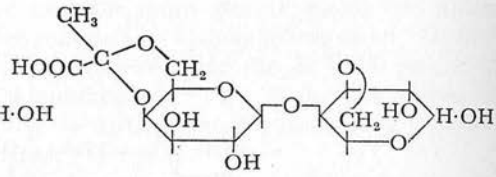
3:6-anhydro-D-galactose



3:6-anhydro-L-galactose



Agarobiose



Pyruvic acid derivative of Agarobiose

FIG. 4. Some components of seaweed polysaccharides. (In carbohydrate formulae >H·OH represents the equilibrium mixture of the α- and β-anomers.)

The method of linkage analysis does not provide quantitative information on the number of branch points (or end-groups), or on the proportion of various linkages. Further, the structural significance of the presence, in minute quantities, of certain oligosaccharides in partial hydrolysates (*e.g.* 0·001 per cent nigerose from glycogen[27]) is uncertain, owing to the possible 'acid-reversion' of monosaccharides[28] or acid-catalysed transglucosylation of oligosaccharides (see p. 34). Nevertheless, the combined use of methylation and partial hydrolysis enables the main structural features of a polysaccharide to be determined, provided that *gram quantities* of material are available.

ANALYSIS BY PERIODATE OXIDATION

The field of polysaccharide chemistry now includes materials from bacteria, protozoa, algae, lichens and fungi. Many of these are available only in very small quantities, and there has been a natural trend towards the development of semi-micro analytical methods. One such method, namely, periodate oxidation, has been widely used, and its application to the analysis of α- and β-glucosans by the writer and his collaborators forms part of this review.

A study of the periodate oxidation of a polysaccharide may, in many instances, provide quantitative information on the nature of the repeating and inter-chain linkages and enable the $\overline{CL}$ and, or, the $\overline{DP}$ to be determined. The reagent was introduced by Malaprade during studies on polyhydric alcohols and was first applied to carbohydrates in general by C. S. Hudson. During the past 13 years, the method[29] has been successfully applied to structural investigations of polysaccharides[30] by Hirst, Jones, Bell, Smith and many other workers.

In aqueous solution, periodate will oxidize compounds containing α-β diol- or α-β-γ triol-groups with the formation of aldehydes, iodate and, in certain cases, formic acid, formaldehyde and carbon dioxide (Fig. 5).

The arrangement of diol or triol (α-glycol) groups may be deduced from measurements of the reduction of periodate and the nature of the oxidation products. The required analyses are semi-micro in scale, and usually simple in operation, so that small quantities (10–1,000 mg) of polysaccharide can be readily examined. The experimental procedures are (*a*) reduction of periodate, by titration with thiosulphate or arsenite[29] or spectrophotometrically[31]; (*b*) production of formic acid, by titration with dilute alkali; (*c*) production of formaldehyde, gravimetrically as the dimedone derivative[29] or colorimetrically with chromotropic acid[32] or phenyl hydrazine–ferricyanide reagents (Schryver's method)[33]; (*d*) production of carbon dioxide, by use of a Warburg-type manometer.[34]

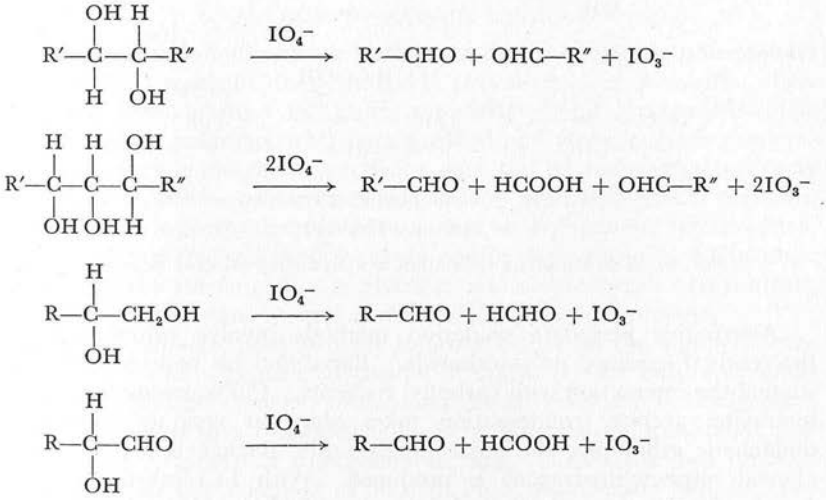


FIG. 5. Oxidation of Polyhydroxy Compounds by Metaperiodate

The rate and extent of periodate oxidation are controlled by the reaction conditions, and the equations in Fig. 5 represent ideal Malapradian-type oxidations. If the oxidation is carried out in presence of a large excess of oxidant, at an elevated temperature (>20°), in alkaline solution or in sunlight,[35] then 'over-oxidation' will occur, *i.e.* the oxidation proceeds beyond the simple Malapradian reaction with the reduction of further quantities of periodate, and the normal products of oxidation (formic acid, formaldehyde) will themselves be oxidized. A careful control of oxidation conditions is therefore essential.

The periodate oxidation of reducing groups in oligo- and polysaccharides may involve (*a*) normal Malapradian oxidation of diol or triol groups, with the production of a formyl ester, (*b*) slow hydrolysis of this ester followed by further oxidation, or (*c*) oxidation of activated H-atoms in malondialdehyde-type structures (Fig. 6). (The mechanism of the latter oxidation is uncertain: free iodine may appear, and iodate rather than periodate may be reduced.) Hough and Perry[36] have shown that all three reactions occur readily at pH 8·0, and that 1 : 3- or

1 : 4-linked aldohexose polymers are completely oxidized, by a step-wise process, with the production of 1 mol. formaldehyde per hexose residue. Oxidation under these conditions thus makes it possible to detect the presence of 1 : 6-linkages in a 1 : 3- or 1 : 4-linked polymer.

Recent studies on starches,[37] glycogens,[38] dextrans,[39] lichenin (p. 15) and laminarin (p. 17) provide examples of the above type of periodate oxidation analysis.

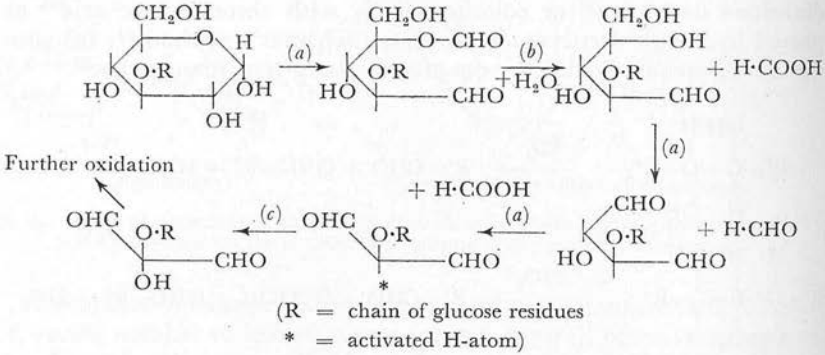


FIG. 6. The Periodate Oxidation of a Reducing Glucose Residue

Alternative periodate oxidation methods involve examination of the residual oxidized polysaccharide. Barry and his co-workers[40] have studied the interaction with carbonyl reagents. On warming in phenylhydrazine acetate, condensation takes place at residues containing dialdehyde groupings, the adjacent glycosidic linkage is ruptured and glyoxal bisphenylhydrazone is produced. With 1 : 3-linked glucosans (*e.g.* laminarin, yeast glucan), the reaction yields a polysaccharide from which the non-reducing terminal group has been removed. Repetition of the oxidation and phenylhydrazine acetate treatment results in the step-wise degradation of the polymeric chain. By contrast, a polysaccharide containing adjacent 1 : 3- and 1 : 4-linkages is fragmented, the former linked residue appearing as the monosaccharide osazone whilst the latter gives glyoxal bisphenylhydrazone. This Barry degradation has given new structural information on several polysaccharides, *e.g.* snail galactogen[41] and arabic acid.[42]

The Barry degradation method has been extended recently to polysaccharides containing either 1 : 5-linked pentofuranose or 1 : 6-linked hexopyranose residues.[43] In these, the glycosidic linkage from a non-oxidized residue to an adjacent oxidized pentose or hexose residue was found to be resistant to cleavage by phenylhydrazine. The products therefore included the osazones of di- or tri-saccharides rather than glyoxal bisphenylhydrazone and residual polysaccharide. The sequence of glycosidic linkages and the arrangement of inter-chain linkages in beet araban and yeast mannan have been examined by this method.

Alternatively,[44] isonicotinhydrazide or thiosemicarbazide can be condensed with the periodate-oxidized polysaccharide, and analysis (for S

and/or N) of the resulting polymer yields data on the proportion of α-glycol groups (*see* p. 15).

Acid hydrolysis of a periodate-oxidized polysaccharide is a useful procedure for the identification of monosaccharide residues devoid of α-glycol groups.[45] These are liberated as the free monosaccharide. However, the oxidized polysaccharides are readily *decomposed* by acid, and Smith and his co-workers[30] recommend reduction to the corresponding polyalcohol prior to acid hydrolysis, which is then quantitative. This method has been used to detect 1 : 3-linkages in starches and dextrans, and in plant gums and mucilages.

ANALYSIS BY ENZYMIC METHODS

The application of enzymic methods to the structural analysis of polysaccharides is a recent development in polysaccharide chemistry. Progress has been greatest in studies on starches and glycogens.[46] The hydrolysis of a polysaccharide by a purified and tested enzyme preparation has the advantage of specificity, and the degradative action may follow paths which cannot be traced out by present chemical methods. For example, α-glucosidases have no action on β-glucosans, whilst hydrolysis by β-amylase (β-amylolysis) is confined to the α-1 : 4-glucosidic linkages in the *exterior* chains of glycogen and amylopectin. In contrast, hydrolysis of glucosans by acid is an essentially random process.

Enzymic analysis may provide information on (*a*) the anomeric configuration of the repeating glycosidic linkages, (*b*) the nature of interchain linkages and sequence of monosaccharide residues, (*c*) the location of branch points in a branched polysaccharide, and (*d*) qualitative and quantitative evidence of multiple branching. The latter two types of analysis are mainly confined to branched α-1 : 4-glucosans, and are considered in detail on pp. 26–34.

Examples of (*a*) and (*b*) may be cited from recent studies on hemicelluloses. Whistler and Smith[47] examined the degradation of guaran by an enzyme preparation from guar seeds. The products included the trisaccharide mannotriose (7·5 per cent) and the disaccharide *O*-α-D-galactopyranosyl-(1 → 6)-D-mannopyranose. These findings confirmed previous conclusions based on chemical methods that guaran is a linear β-1 : 4-mannan, to alternate residues of which are attached, at C_6, an α-galactopyranose residue. This polysaccharide structure is therefore of the type shown in Fig. 1 (*c*).

Proof that L-arabofuranose residues are true components of wheat-straw xylan was provided by Bishop and Whitaker[48] who isolated a trisaccharide containing two D-xylopyranose and one L-arabofuranose residues from the products of enzymic hydrolysis. Partial hydrolysis with acid was quite unsuitable in this case, since arabofuranosidic linkages are extremely acid-labile, especially when compared with xylopyranosidic linkages.

A further example of the specificity of enzymic hydrolysis was reported recently by Araki and Arai.[49] The main polysaccharide fraction of agar, named agarose, was treated with an agar-digesting bacterium. The

products included a disaccharide (neoagarobiose) and a tetrasaccharide (neoagarotetraose) which contain 3:6-anhydro-L-galactose residues α-linked to C_3 of D-galactose. This result supports the linear structure of agarose as an alternating polymer of 1:3-linked β-D-galactopyranose and 1:4-linked 3:6-anhydro-α-L-galactopyranose residues. In contrast to enzymic hydrolysis, acids selectively hydrolyse the α-glycosidic linkages, giving agarobiose as the main product (Fig. 7).

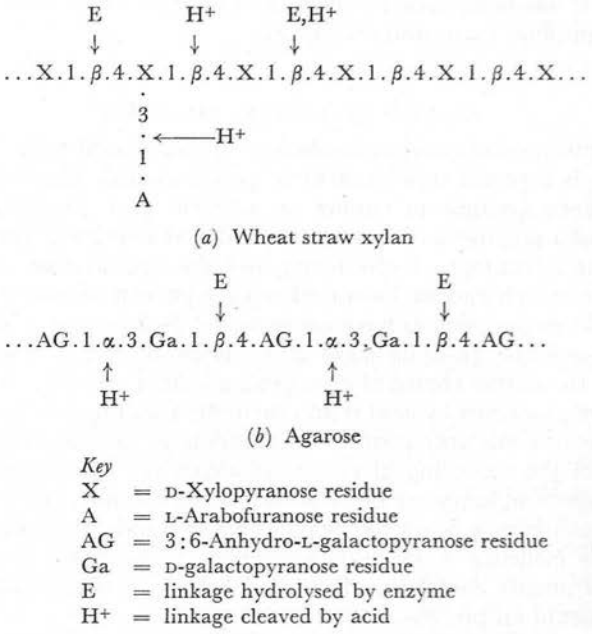


FIG. 7. The Partial Hydrolysis of Polysaccharides

In the field of fructosan chemistry, enzymic methods[50] have enabled the fructosan synthesized by *Acetobacter acetigenum* to be characterized as a levan, *i.e.* β-2:6-fructosan; a related polysaccharide from the fungus *Aspergillus sydowi* was shown to be of the inulin type, *i.e.* β-2:1-fructosan. The specific fructosanase preparations were culture filtrates from various micro-organisms grown on either inulin or the Acetobacter fructosan. The former had no action on *Bacillus subtilis* levan and the bacterial fructosan, whereas the latter did not hydrolyse inulin or the *Aspergillus* fructosan.

Other examples of partial enzymic depolymerization are the isolation of isomaltose and isomaltotriose from dextran–dextranase systems,[51] of di- and tri-galacturonic acid from the degradation of pectic acid,[52] and of xylose, xylobiose (*O*-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose), rhodymenabiose (*O*-β-D-xylopyranosyl-(1 → 3)-D-xylopyranose) and higher xylosaccharides from the action of rumen bacteria on *Rhodymenia palmata* xylan.[53]

It is clear from this short survey, and the examples reported elsewhere,[1] that enzymic methods provide a valuable means for the controlled depolymerization of polysaccharides, with the proviso that experimental conditions do not allow the synthesis of oligosaccharides by transglycosylation reactions to occur. An illustration of this possible artefact is provided[54] by the synthesis of laminaribiose, gentiobiose and higher β-glucosaccharides during the action of a barley β-glucosidase preparation on cellobiose.

ANALYSIS BY PHYSICO-CHEMICAL METHODS

Marked improvements have been made recently in the experimental techniques for the measurement of the size and shape of high polymers. In the earlier studies, viscometry and osmometry were among the principal methods for the investigation of polysaccharides (usually as their acetylated or methylated derivatives).[55] These methods have been improved; new types of viscometer and osmometer have been designed,[56] and membranes with known permeability characteristics prepared,[57] thereby extending the range of molecular-weight determinations.

New techniques have been introduced into this field, a number of which can be applied to unsubstituted polysaccharides. This is an important advantage, since the possibility of inadvertent degradation of a polysaccharide during the preparation of the corresponding acetate or methylated derivative is considerable. The development of the ultracentrifuge by Svedberg and his collaborators[58] has permitted the sedimentation properties of polysaccharides to be determined. From these, estimates of the polydispersity can be obtained and, with a knowledge of the diffusion constant, the weight-average molecular weight and molecular-weight distribution calculated. The application of light-scattering[59] is particularly useful for the higher-molecular-weight polymers, in the range 10^5–10^7. The availability of commercial instruments, *e.g.* the electrically-driven ultracentrifuge, and several designs of light-scattering photometer, has greatly facilitated the application of these procedures, both of which are now in routine use for the determination of the size and shape of polysaccharides and of the effect on these properties of certain chemical reagents and enzymes.

The use of the ultracentrifuge and light-scattering photometer may be illustrated by recent studies on glycogen.[60] The sedimentation constants of 23 different samples were measured; the majority of these were polydisperse, and the main components had molecular weights in the range $3–9 \times 10^6$. Glycogen isolated by hot water extraction or by the classical Pflüger method (using 30 per cent potassium hydroxide solution) had similar molecular weights; however, hot dilute alkali rapidly degraded the polysaccharide. The polydisperse nature of many of the glycogens was confirmed by light-scattering which gave much higher molecular-weight values. In contrast, the results with samples showing no polydispersity were in good agreement with the sedimentation data (Table I). It was therefore suggested that unambiguous proof of polydispersity

could be obtained by a comparison of sedimentation and light-scattering measurements.

TABLE I

A COMPARISON OF MOLECULAR WEIGHT VALUES OF SOME GLYCOGENS

Sample	Sedimentation-diffusion		Light-scattering
	10^{-6}M	Other components	10^{-6}M
Ascaris lumbricoides	2·8	F	8·9
Brewer's yeast	3·7	—	4·4
Cat liver I	4·4	F,S	13·6
„ „ IV	4·9	F,S	13·4
„ „ VI	5·9	F,S	17·9
Commercial I	0·7	—	1·9
„ II	4·0	—	5·4
Rabbit liver II	5·5	—	7·8
Rabbit muscle I	4·6	—	4·1

F = Fast component. S = Slow component.

The method of isothermal distillation[61] is proving to be particularly valuable in studies on polysaccharides in the molecular-weight range 3,000–20,000 which are not amenable to investigation by conventional osmometry or sedimentation methods. The numerous polysaccharides of the hemicellulose group fall into this category, and much information is now being collected.[62]

Although the measurement of the size and shape of polysaccharides has been adequately reviewed,[63] the use of other physico-chemical techniques for the determination of the type and anomeric configuration of the repeating glycosidic linkages has not often been described. These techniques include infra-red spectroscopy,[64] the comparison of the optical rotations of certain polysaccharides in aqueous and cuprammonium solutions,[65] and of the corresponding tricarbanilates in pyridine and morpholine,[66] and, for starch-type polysaccharides, the measurement of iodine binding-power by differential potentiometric titration.[67] In Table II are summarized some data for a number of glucosans. Thus, polysaccharides containing β-glucosidic linkages, *e.g.* bacterial cellulose and luteose, give an infra-red absorption band at 891 $\pm$ 7 cm^{-1} of moderate or strong intensity (designed type 2*b* by Barker and his co-workers[64]), do not show 'type 2*a*' absorption (at 844 $\pm$ 8 cm^{-1} as shown by polymers of α-D-glucose) and have only very weak 'type 3' absorption, at *ca* 770 cm^{-1}. The correlation between the nature of the glycosidic linkages and the infra-red absorption spectra provides a speedy micro-method for the *preliminary* examination of a polysaccharide, despite certain experimental difficulties, *e.g.* adequate drying of the material without structural modification. The complexing reaction between cuprammonium and carbohydrates results in a change of optical rotation, which is characteristic of the type of glycosidic linkage. Studies of this rotation change have provided the first evidence of a small proportion of 1 : 2-glucosidic linkages in certain dextrans.[68]

TABLE II

PROPERTIES OF SOME GLUCOSANS[64,65,66]

Polysaccharide	Main glucosidic linkage	Infra-red absorption peaks, frequencies (cm^{-1})			[α] 436 mμ of Polysaccharide		[α]D of Carbanilate	
		Type 1	Type 2*a* or 2*b*	Type 3	H_2O or NaOH	Cupram-monium	Pyridine	Morpholine
Crown-gall	β-1 : 2	919	888,880	—	−23	+960	+50	−11
Laminarin	β-1 : 3	917	890	—	−29	+34	−63	−63
Cellulose	β-1 : 4	914,933	894	(766)	−20	−1,200	−152	−85
Amylose	α-1 : 4	938	857,838	756	—	—	−83	−7
Amylopectin ..	α-1 : 4†	931	840	756	+375*	−715*	−62	−4
Glycogen	α-1 : 4‡	928	840	760	+366	−597	−32	+4
Dextran	α-1 : 6§	919	840	768	+297	−128	Insol.	+343

*Data for soluble starch.
† Contains 4% α-1 : 6-glucosidic linkages.
‡ Contains 8% α-1 : 6-glucosidic linkages.
§ May contain a small proportion of 1 : 3- and 1 : 4-glucosidic linkages.

The biological chemist may require a knowledge of the three-dimensional organization of the polysaccharides as laid down in plant-cell walls. This type of information has been obtained by use of the electron microscope and by X-ray analysis. Recent advances in this specialized field have been reviewed by Preston [69] and Northcote.[70]

FINE STRUCTURE OF GLUCOSE-CONTAINING POLYSACCHARIDES

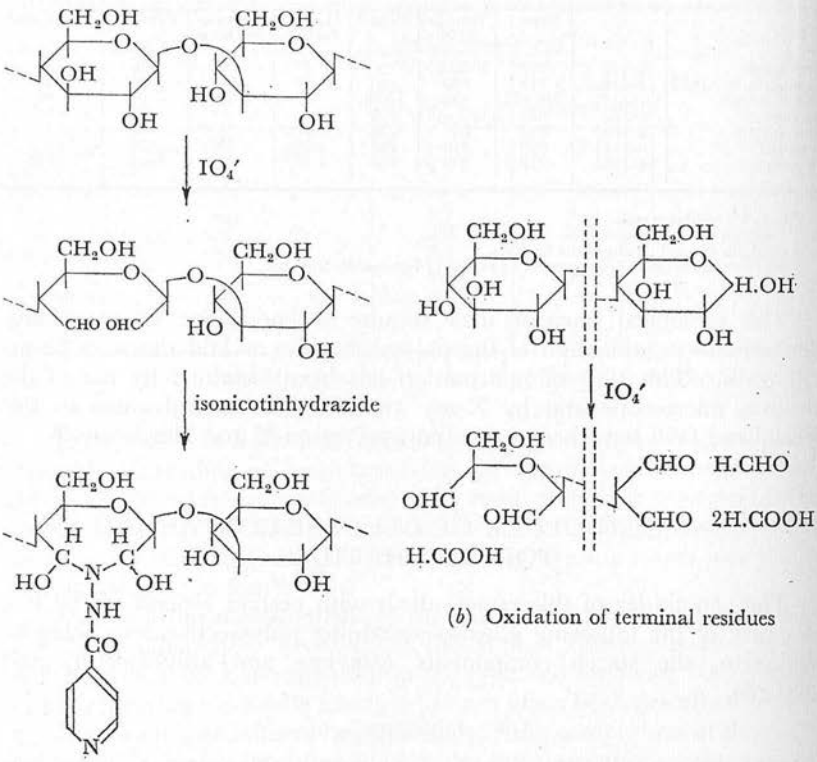
The remainder of this review deals with certain aspects of the fine structure of the following glucose-containing polysaccharides: lichenin, laminarin, the starch components (amylose and amylopectin) and glycogen.

LICHENIN

Lichenin, a glucose-containing polysaccharide, occurs in various lichens, including Iceland moss (*Cetraria islandica*). Early methylation studies[71] indicated that the molecule was essentially linear and contained both β-1 : 3- and β-1 : 4-glucosidic linkages. These conclusions have been confirmed and extended by periodate oxidation.[72]

On oxidation with sodium metaperiodate, 0·73 mol. per anhydroglucose residue was reduced. Since 1 : 3-linked residues do not contain α-β diol groups, this suggests that *ca* 30 per cent of these residues are present. Treatment of periodate-oxidized lichenin with isonicotinhydrazide[44] gave an insoluble product containing 11·6 per cent N. Under these conditions, 1 mol. of isonicotinhydrazide reacts with each oxidized glucose residue; this reaction, together with one possible structure for the product, is shown in Fig. 8. The observed N-content indicates the presence of *ca* 66 per cent diol groups, *i.e.* 1 : 4-linked glucose residues, and hence, *ca* 34 per cent of 1 : 3-linked units. The agreement between this figure and that from the reduction of periodate shows that the lichenin molecules contain *both* types of linkage, and are not a *mixture* of two distinct polysaccharides, one a β-1 : 3-glucosan, and the other, of the β-1 : 4-cellulose-type.[44] If lichenin was such a mixture, the

isonicotinhydrazide would react only with the β-1 : 4-component and the resulting derivative would have a correspondingly higher N content (14·1 per cent) equivalent to the presence of 100 per cent diol groups.



(*a*) Oxidation of non-terminal residues

(*b*) Oxidation of terminal residues

FIG. 8. The periodate oxidation of lichenin

Evidence that the 1 : 3-linkages are randomly situated in the molecule was then obtained. Periodate-oxidized lichenin was reduced to the corresponding polyalcohol and partially hydrolysed with acid. Paper chromatography showed the presence of glucose; laminaribiose was absent. It follows that few, if any, sequences of 2 or more adjacent 1 : 3-linkages were present in the original polysaccharide, since only 1 : 3-linkages would resist oxidation. Peat and his co-workers[73] have independently reached a similar conclusion, by the method of linkage analysis.

Further periodate oxidation studies showed the molecule to be entirely linear and to have an average chain length ($\overline{CL}$) of 63 glucose residues. The overoxidation of lichenin, at pH 8,[36] was investigated; 1·0 mol. formaldehyde per glucose residue was produced. Since this step-wise oxidation process is inhibited by 1 : 6-linkages, these cannot be

present in the lichenin molecule. The $\overline{CL}$ was determined from the amount of formic acid liberated on oxidation under controlled conditions, with potassium metaperiodate, in the absence of sunlight, at room temperature. After 10–14 days' oxidation, 0·0475 mol. formic acid per glucose residue was liberated. The non-reducing and reducing end-groups in a 1 : 3- or 1 : 4-linked glucosan yield one and two mol. formic acid respectively (Fig. 8*b*), and the experimental figure therefore corresponds to a $\overline{CL}$ value of 63. This result, obtained on 0·3 g polysaccharide, may be compared with a $\overline{CL}$ of 62 by methylation analysis of 6·0 g material. It is noteworthy that the whole periodate oxidation analysis was carried out on only *ca* 1 g purified polysaccharide.

LAMINARIN

Many species of brown marine algae, particularly the *Laminaria*, contain a reserve polysaccharide, laminarin, which is essentially a polymer of β-1 : 3-linked D-glucopyranose residues.[74,75] However, recent partial acid hydrolysis studies by Peat, Whelan and Lawley[21] of laminarin from *L. cloustoni* have indicated the presence of small proportions of other types of glucosidic linkages (Table III). The hydrolysate contained small quantities of mannitol, 1-*O*-β-glucosylmannitol and 1-*O*-β-laminaribiosylmannitol, showing that some of the molecules were terminated by a mannitol residue (Fig. 9). Generally similar results were obtained with the so-called 'soluble' laminarin from *L. digitata* except that a rather higher mannitol content was indicated. Other products of partial hydrolysis, in addition to β-1 : 3-glucosaccharides, were gentiobiose (0·26 per cent), 3-*O*-β-gentiobiosylglucose and 6-*O*-β-laminaribiosylglucose. The isolation of these provides the first evidence for β-1 : 6-glucosidic linkages in laminarin.

TABLE III

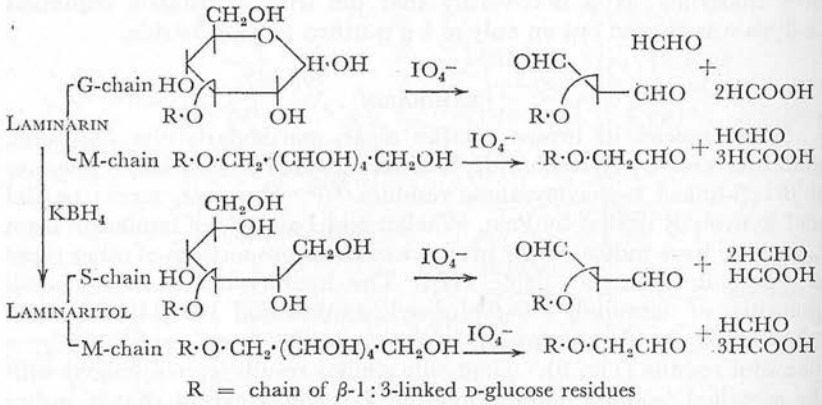
THE PRODUCTS OF PARTIAL ACID HYDROLYSIS OF LAMINARIN[21]

Yield* (g)		Type of linkage
100	Glucose Laminaribiose Laminaritriose	—3.G.1.β.3.G.1.β.3.G.1—
4·0	Mannitol 1-*O*-β-Glucosylmannitol 1-*O*-β-Laminaribiosylmannitol	—3.G.1.β.Mannitol
1·2	Gentiobiose 6-*O*-β-Laminaribiosylglucose 3-*O*-β-Gentiobiosylglucose	—3.G.1.β.6.G.1.β.3.G—

* From 137 g insoluble laminarin.
G signifies anhydroglucose residue throughout.

Our recent investigations[76] have been concerned with the mannitol and β-1 : 6-linkages.

The relative proportion of mannitol-terminated and glucose-terminated chains (designated M- and G-chains respectively) was determined by measurement of the amount of formaldehyde liberated on periodate oxidation of laminarin and the corresponding alcohol (laminaritol). F. Smith and his co-workers[77] have shown that free reducing groups in a polysaccharide may be reduced to the corresponding hexitol residue (Fig. 9). We have used potassium borohydride rather than sodium borohydride as the reducing agent, to convert G-chains into S-chains (sorbitol-terminated chains).



R = chain of β-1 : 3-linked D-glucose residues

FIG. 9. The Periodate Oxidation of Laminarin and Laminaritol

With a *limited* excess of sodium metaperiodate at room temperature as oxidant, laminarin and laminaritol gave 0·041 and 0·063 mol. formaldehyde per anhydrohexose residue. Since both G- and M-chains yield one mol. formaldehyde per chain, the laminarin sample has a $\overline{DP}$ of *ca* 24. Further, since the production of formaldehyde from G-chains is doubled during the conversion to laminaritol (Fig. 9), it follows that the observed yield of 0·041 mol. from laminarin consists of 0·022 mol. from the G-chains, and hence 0·019 mol. from the M-chains. The polysaccharide sample therefore contains 45 per cent M-chains, 1·9 per cent of mannitol and 1 reducing group per 45 residues. By hypoiodite oxidation, the value of 1 reducing group per 47 residues was obtained.

Two different samples of 'soluble' laminarin, which has a much lower reducing power towards alkaline hypoiodite and copper reagents than the insoluble form, were also examined (Table IV). The results confirm the general conclusion from linkage analysis that the mannitol content of 'soluble' laminarin is greater than that of other forms. Further, the significantly higher proportion of M-chains is in accord with the low reducing power, and the relatively lower production of saccharinic acid during degradation by aqueous alkali; the insoluble and soluble forms yield *ca* 40 and 19 per cent D-glucometasaccharinic acid respectively.[78] A complete chemical explanation of the marked difference in solubility is not yet available.

TABLE IV

THE PERIODATE OXIDATION OF LAMINARIN

Sample	Insoluble laminarin	Soluble laminarin 'A'	Soluble laminarin 'B'
Formaldehyde production (mols):			
(*a*) laminarin	0·041	0·050	0·053
(*b*) laminaritol	0·063	0·068	0·066
$\overline{DP}$ (hexose residues)	24	20	19
M-chains (%)	46	64	75
No. of hexose residues per reducing group	45	55	76
Mannitol content (%)	1·9	3·2	3·9

The analysis of methylated laminarin has provided evidence of a slightly branched structure. The methylated polysaccharide, which had a molecular weight of 1900 by isothermal distillation,[79] was fractionally precipitated from chloroform solution with light petroleum (b.p. 40–60°). The insoluble material, 78 per cent by weight, had[79] a molecular weight of 12,000 (*i.e.* $\overline{DP}$ 59); it follows that the soluble fraction must have a molecular weight of only *ca* 500. An acid hydrolysate of the *insoluble* methylated laminarin contained 4·4 per cent tetra-*O*-methyl glucose, corresponding to a $\overline{CL}$ of 23. It is therefore clear that a low degree of branching is present, and, on the average, these molecules contain two branch points. Although Peat and his co-workers[21] did not obtain positive evidence for branching, the above results would suggest that the β-1 : 6-linkages are present as inter-chain linkages, and the small number required is in agreement with the observed low yield of gentiobiose and related sugars.

The Barry degradation of laminarin has also been investigated,[80] and the results are in accord with the methylation evidence for a branched structure.

The overall structure of laminarin is therefore that of a mixture of β-1 : 3-glucosans, a proportion of which (*a*) are terminated at the potential reducing-end by a mannitol residue, and (*b*) are slightly branched and contain a small number of β-1 : 6 inter-chain linkages.

AMYLOSE

The heterogeneous nature of starch, the major reserve polysaccharide of photosynthetic plants, is now generally accepted.[81] The majority of starches contain two structurally different components, amylose and amylopectin. The latter has a highly branched structure and accounts for 70–80 per cent of most starches. [The possible existence of a third and minor component differing in branching properties from amylopectin cannot yet be ignored.]

The amylose component of starch, which can be separated from amylopectin as an insoluble complex by the addition of an alcohol of low solubility (*e.g.* butanol or thymol), is an essentially linear molecule composed of α-1 : 4-linked glucose residues. Methylation and periodate

oxidation studies have shown the presence of 1 : 4-linked D-glucopyranose residues, and the α-configuration is indicated by the high dextrorotation shown by solutions of amylose and its derivatives, the isolation of maltose and maltosaccharides from partial acid hydrolysates and the formation of maltose during amylolytic degradation. Estimates of the molecular size by various chemical and physical methods have given a wide range of results, with $\overline{DP}$ values ranging from *ca* 100 to 6,000. This variation is partly the result of inadvertent degradation and sub-fractionation during isolation.

An examination of the action of β-amylase[46] on amylose has provided new structural information. This enzyme, which occurs only in higher plants, catalyses a step-wise hydrolysis of alternate α-1 : 4-linkages with the liberation of maltose. β-Amylolysis ceases when phosphate ester groups or glucosidic linkages other than α-1 : 4 are encountered in the substrate. Enzyme action is therefore confined to the *exterior* chains of branched α-1 : 4-glucosans.

Early β-amylolysis studies indicated that amylose was a linear polysaccharide, since *ca* 100 per cent conversion into maltose was observed. However, Peat and his co-workers[82] discovered that whereas the action of amorphous preparations of soya-bean β-amylase gave this type of result, the action of crystalline sweet-potato β-amylase (and of a highly purified soya-bean β-amylase) was incomplete; only *ca* 70 per cent conversion into maltose resulted. This indicated that amylose contained a small number of linkages or residues which arrested the step-wise degradation; these will be referred to as 'anomalous linkages.' The amorphous soya-bean preparation on fractionation gave β-amylase and a second factor, named Z-enzyme, which could specifically hydrolyse the anomalous linkages. Thus, the concurrent action of crystalline sweet-potato β-amylase and soya-bean Z-enzyme gave complete amylolysis of amylose. Since the Z-enzyme preparation showed β-glucosidase activity, and almond emulsin* could also hydrolyse the anomalous linkages in amylose, it was suggested that the 'barriers' to β-amylase were a small number of β-glucosidic linkages. The general observation that *purified* β-amylase causes only *ca* 70 per cent degradation of amylose has been confirmed by many workers,[83,84,85] although Meyer[86] has concluded that the low conversion limits are the result of impurities in the substrate, or of retrogradation or 'ageing' (in which the amylose molecules aggregate and then precipitate) during enzyme action.

In 1956, Gilbert and his co-workers[87] postulated that oxidation was the source of the anomalous linkages. The degradation of amylose by potato phosphorylase to α-D-glucosyl phosphate in the presence of inorganic phosphate (phosphorolysis) was virtually complete when the substrate was prepared under anaerobic conditions. However, treatment of the substrate with oxygen at 95° in neutral or alkaline solution lowered the phosphorolysis limit to 67–81 per cent. It was therefore concluded that barriers to phosphorylase (and by analogy, to β-amylase) could be introduced by oxidation.

* A mixture of carbohydrases, including β-glucosidases.

A critical study of the β-amylolysis of amylose has been carried out by the writer and his colleagues.[85] The action on various amyloses of purified soya-bean β-amylase and an amorphous preparation from barley that contained Z-enzyme, was examined. The conversion limits into maltose were 69–77 per cent and 92–99 per cent respectively; the amyloses had been prepared by fractionation of starch in the presence of either nitrogen, oxygen or air (Table V).

TABLE V

β-AMYLOLYSIS LIMITS OF AMYLOSE

Amylose source	Atmosphere during fractionation	$\overline{DP}$*	β-Amylolysis limit (%)	
			β-Amylase	β-Amylase + Z-enzyme
Potato I	Nitrogen	3,200	77	99
„ II	Oxygen	2,800	75	98
„ III	Oxygen	1,600	76	99
„ VI	Air	1,850	73	95
„ VII	Air	*ca* 1,500	69	94
„ VIII	Nitrogen	*ca* 1,500	77	92
Wrinkled-pea	Nitrogen	*ca* 600	77	99
Dunaliella bioculata† ..	Nitrogen	—	73	93

* Number-average value calculated from $\overline{DP} = 7{\cdot}4$ (limiting viscosity number).
† A halophytic unicellular alga.

The results indicate that the barriers to β-amylolysis are not introduced by oxidation during the fractionation of starch, and therefore represent a structural feature of the native amylose. Ageing of the substrate was not responsible for the lower limits; under extreme conditions, namely the incubation of amylose at pH 4·6 for 48 hours, the lowering of the β-amylolysis limit was only 5–9 per cent.

The effect of oxygen on the β-amylolysis limit was then investigated. This treatment could modify amylose in at least two ways—the oxidation of non-terminal glucose residues (probably at C_3 or C_6) or fragmentation of the amylose molecule, and the formation of oxidized non-reducing terminal groups[88]; either of these modifications might arrest the action of the highly specific β-amylase.

Four amylose samples were heated at 98° for 20 min., in 0·5M-sodium hydroxide solution in a stream of oxygen, and the β-amylolysis limit determined with the barley preparation. The conversion into maltose was 93–98 per cent. It is clear that under the conditions employed by Gilbert,[87] molecular oxygen does not introduce barriers to the action of β-amylase or Z-enzyme. This suggests that phosphorylase and β-amylase show a slight and hitherto unexpected difference in specificity, since the oxygen-treatment does lower the phosphorolysis limit.

The action of purified soya-bean β-amylase on the four oxygen-treated amylose samples resulted in a small *increase* in β-limit, from 69–77 per cent to 83–92 per cent. This is attributed to the rupture of a small number of α-1 : 4-linkages (confirmed experimentally by viscosity

measurements), with the liberation of new non-reducing end-groups that are susceptible to β-amylase. The new end-groups cannot therefore differ appreciably in structure from normal α-D-glucopyranose residues (*cf.* ref. 88). Further, the anomalous linkages in amylose are stable to oxygen and hot sodium hydroxide solution.

The molecular structure of potato amylose has been examined in some detail (Table VI). Samples were prepared by aqueous leaching at 70° and at 98°, in addition to thymol–butanol fractionation. The former was completely degraded by purified β-amylase and can therefore contain only α-1 : 4-glucosidic linkages. Aqueous leaching at 98° gives an amylose preparation intermediate in properties between the above linear amylose and a normal sample.

TABLE VI

PROPERTIES OF POTATO AMYLOSES

Sample	Method of preparation*	Approx. yield†	$\overline{DP}$	Iodine‡ affinity	β-Amylolysis limit (%)
Potato I ..	Thymol and butanol	17	3,200	19·5	77
„ IV	Aq. leaching at 70°	7	1,800	19·5	99
„ V ..	Aq. leaching at 98°	13	2,700	19·5	86

* In an atmosphere of nitrogen.
† As g per 100 g starch.
‡ Expressed as mg of iodine bound per 100 mg; these values indicate the absence of amylopectin impurity (*cf.* ref. 86).

Since amylose IV represents *ca* 40 per cent (by weight) of the total amylose (*i.e.* sample I) in the starch granule, the residual amylose must have a $\overline{DP}$ of *ca* 6,000 and a β-amylolysis limit of 50–60 per cent. Potato amylose is therefore heterogeneous as regards both molecular size and structure. A large proportion of the molecules are completely linear and have a $\overline{DP}$ of *ca* 2,000, the remainder, with $\overline{DP}$ 6,000, containing a small number of anomalous linkages.

The nature of these anomalous linkages is at present unknown, despite investigations by many workers. The proportion of these linkages is very low, perhaps 0·1 per cent, and the chemical methods of methylation, periodate oxidation and linkage analysis are not sufficiently sensitive; enzymic methods have therefore been employed.

The anomalous linkage may either join a single group, or a chain of α-1 : 4-linked glucose residues, to a main chain (*i.e.* side-chain or single branching), or may be situated in the main chain. This latter would imply that potato phosphorylase may not be completely specific for the synthesis and degradation of α-1 : 4-glucosidic linkages.

Ester-phosphate groups and single glucose units have been considered[82] as side-chains. However, bone phosphatase (which dephosphorylates starch) does not eliminate the anomalous linkages,[82] and Hopkins and Bird could not detect the presence of side-chains of single glucose residues in amylose β-limit dextrin.[83]

Several workers have noted a discrepancy between the $\overline{CL}$ and $\overline{DP}$ values (*i.e.* the number of non-reducing end-groups and reducing groups) of various amylose preparations.[81] This would indicate some type of branching. The specificity of Z-enzyme, which might function as a 'de-branching' enzyme, has therefore been examined. As previously noted (p. 20) soya-bean Z-enzyme preparations show β-glucosidase activity[82] but the significance of this was reduced by the observation of Neufeld and Hassid[84] that the Z-enzyme activity of almond emulsin could be separated from the β-glucosidase.

The specificity of barley Z-enzyme was examined[85] by determining the degradation of enzymic and partial acid hydrolysates of isolichenin (a polysaccharide containing α-1 : 3- and α-1 : 4-linkages[72]) and of amylopectin (containing 5 per cent α-1 : 6- and 95 per cent α-1 : 4-linkages) by (*a*) purified soya-bean β-amylase, and (*b*) a barley preparation containing β-amylase and Z-enzyme. The two enzyme preparations gave identical results, and it follows that barley Z-enzyme has no action on α-1 : 3- or α-1 : 6-glucosidic linkages which are situated adjacent to α-1 : 4-linked glucose residues. It must also be noted that Z-enzyme appears to have no action on α-1 : 4-glucosidic linkages.[85,89]

The presence of anomalous linkages in the amylose component of potato starch implies that similar linkages may be present in amylopectin, and that the enzymic synthesis of starch from α-D-glucosyl phosphate may involve catalysts other than phosphorylase, D-enzyme and Q-enzyme.[81] Furthermore, the potato enzyme system may differ from that in other tissues. For example, the amylose components of starches isolated from malted barley[99] and the flagellated protozoon *Chilomonas paramecium*[7] do not appear to contain anomalous linkages; these polysaccharides are completely hydrolysed by purified β-amylase preparations.

The mechanism of the β-amylolysis of amylose has been the subject of several investigations.[46] Two types of reaction have been proposed involving either 'single-chain' action, in which the enzyme completely degrades one amylose molecule to maltose before attacking a second molecule, or 'multi-chain' action, by which all the substrate molecules are simultaneously attacked and progressively shortened. In the former, only maltose and unattacked amylose with the same $\overline{DP}$ as the original will be present at intermediate stages of hydrolysis, whereas with multi-chain action the $\overline{DP}$ of the residual amylose will decrease in proportion to the conversion into maltose.

The β-amylolysis of amylose of high molecular weight ($\overline{DP}$ 2,000 and 3,200) at pH 4·6 and 35° is now known to proceed by an essentially 'single-chain' mechanism.[85] The molecular properties (sedimentation constant, limiting viscosity number and iodine affinity) of amylose isolated at intermediate stages were identical with those of the original substrates, and there was no evidence for the presence of sugars other than maltose. These observations are independent of the presence of Z-labile linkages in amylose; the mechanism of β-amylase action is not influenced by Z-enzyme.

In contrast to the above findings, short-chain amyloses ($\overline{DP} < 50$)

are degraded by 'multi-chain' action, the exact nature of which is dependent on reaction conditions (pH, temperature and $\overline{DP}$ of substrate).

AMYLOPECTIN AND GLYCOGEN

It is convenient to consider together the molecular structures of amylopectin[81] and glycogen,[91,92] since these polysaccharides have many features in common (Table VII) and are synthesized and degraded by closely related enzyme systems.[1] Amylopectin and glycogen are high-molecular-weight (*ca* 10^7) polymers containing chains of α-1 : 4-linked D-glucopyranose residues which are inter-linked to form a highly branched structure. The polysaccharides are readily degraded by α-amylase, β-amylase and phosphorylase, and their solutions give characteristic colours with iodine. They differ in degree of branching, the average chain lengths ($\overline{CL}$) being *ca* 24 and 12; in the interior of the molecules, the branch points are separated by *ca* 6 and 3 glucose residues respectively. These polysaccharides also differ in hydrodynamic properties, *e.g.* the limiting viscosity number of glycogen is only *ca* 5–10 per cent that of amylopectin.

TABLE VII

SOME PROPERTIES OF AMYLOPECTIN AND GLYCOGEN*

Property	Amylopectin	Glycogen
$[\alpha]_D$, H_2O	+212°	+198°
$[\alpha]_D$, NaOH	+153°	+169°
Iodine coloration	Purple-brown	Red-brown
λ_{max} of absorption spectra (mμ)	530–540	450–460
Average chain length (glucose residues)	20–25	10–14
β-Amylolysis limit (%)	55 ± 5	45 ± 5
Exterior chain length (glucose residues)	14–19	5–8
Interior chain length (glucose residues)	5–9	3–5
β-Amylolysis limit (%) :		
(*a*) after incubation with R-enzyme	70	47
(*b*) after incubation with iso-amylase	70	57
Limiting viscosity number	155	5–8
Molecular weight	*ca* 10^7	*ca* 10^7

* Data collected from ref. 91, 92, 105.

In the past decade, interest has focused upon the nature of the inter-chain linkage, the relative position of these linkages in the polymeric chains, the type of branching (single or multiple) and the presence of anomalous residues and linkages.

The inter-chain linkages. Ideally, a hydrolysate of methylated amylopectin or glycogen should contain a mixture of tetra-, tri- and di-*O*-methyl glucose (the first and last in equimolar amounts), and characterization of the latter should identify the type(s) of inter-chain linkage (Fig. 2); the presence[93] of 2 : 3-di-*O*-methyl glucose in such hydrolysates would indicate some 1 : 6-glucosidic linkages. In practice, however, other methyl glucoses are also present; for example, hydrolysates of methylated waxy maize starch contained[94] 2 : 3-di-*O*-methyl glucose

(4·0 per cent); 3:6-di-*O*-methyl glucose (10·4 per cent) and mono-*O*-methyl glucose (1·3 per cent). For methylated rabbit-liver glycogen the corresponding figures[94] were 8·9, 10·8 and 2·4 per cent. In both instances, the combined yield of di-*O*-methyl glucose should not have exceeded that of tetra-*O*-methyl glucose, namely 4·2 and 8·7 per cent respectively. Thus the methylation method has not yielded conclusive results, although some of the di-*O*-methyl sugars were believed to be artefacts arising from demethylation of 2:3:6-tri-*O*-methyl glucose during hydrolysis of the methylated polysaccharide, and from under-methylation of non-terminal residues.

An alternative method of analysis[45] involves examination of an acid hydrolysate of the periodate-oxidized polysaccharide. If the inter-chain linkages are solely of the 1:6-type, then all the glucose residues in the polysaccharide contain an α-β diol group at C_2 and C_3 and will be oxidized by periodate. However, if 1:2- or 1:3-inter-chain linkages are present in the original polysaccharide, the branch-point residue will appear as D-glucose in the hydrolysate of the oxidized polysaccharide. In practice, the complete oxidation of a polysaccharide is difficult, and the hydrolysates of many periodate-oxidized amylopectins contain *ca* 1 per cent glucose. Nevertheless this result indicates that the majority ($>$ 75 per cent) of the inter-chain linkages are of the 1:6-type, and most workers have concluded that the glucose arises from under-oxidation of the polysaccharide, and not from 1:3-linkages. In the case of glycogen, this method has shown that 97–99 per cent of the inter-chain linkages are 1:6.[95]

It must be noted that the above methylation and periodate oxidation studies do not reveal the anomeric configuration of the inter-chain linkages. This has been identified as α by analysis of partial acid and enzymic hydrolysates.

The partial acid hydrolysis of amylopectin has been examined in detail by Wolfrom and his collaborators. The products include isomaltose (*ca* 1 per cent),[96] maltotriose,[96] and panose (*ca* 0·1 per cent)[97]. The yield of isomaltose is 200 times that formed by 'acid-reversion' from glucose under similar conditions, and therefore provides conclusive evidence for the presence of α-1:6-glucosidic linkages in amylopectin.[98] Isomaltose has also been isolated from the products of α-amylolysis (*Aspergillus oryzae* enzyme) of waxy corn starch.[99] This enzyme preparation did not synthesize isomaltose from amylose, or from maltose or glucose. More recently Whelan and Bines[100] have obtained the pentasaccharide 6^3-α-maltosylmaltotriose* by the action of salivary α-amylase on waxy maize starch; this finding confirms previous evidence on the inter-chain linkage.

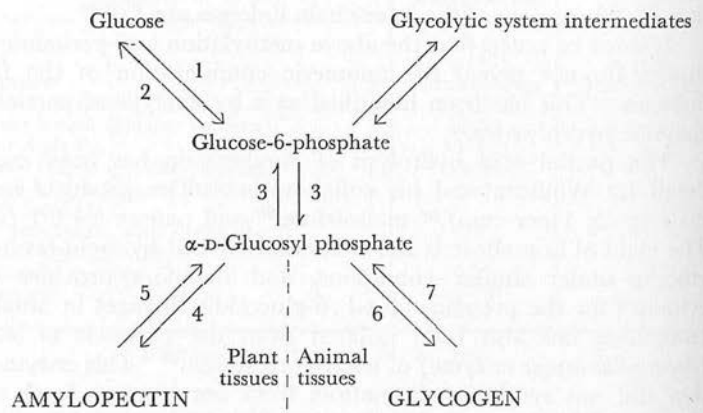
The presence of isomaltose in partial acid hydrolysates of rabbit-liver[101,102] and baker's-yeast glycogen[12] has been reported. Control experiments have shown that the sugar did not arise from acid-reversion. Panose[12,27] and isomaltotriose[27] have also been isolated from glycogen hydrolysates; the latter indicates that a small number of branch points are directly linked to an adjacent branch point.

* Systematic name *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-D-glucopyranose.

The presence of α-1 : 6-glucosidic inter-chain linkages in amylopectin and glycogen is therefore definitely established.

Determination of exterior and interior chain-lengths. The products of β-amylase action on amylopectin or glycogen are maltose (*ca* 50 per cent) and a high-molecular-weight limit dextrin (β-dextrin) containing short exterior chains of two or three glucose residues.[46] As shown in Table VIII, the average length of the exterior and interior chains can be calculated from the $\overline{CL}$ and β-amylolysis limit. In amylopectin, the interior chain length is roughly twice that of glycogen. This type of information, which cannot be obtained by purely chemical methods, may enable the metabolism of these polysaccharides to be examined.

The enzymic synthesis and degradation of amylopectin–glycogen type polysaccharides requires a number of distinct enzymes.[1] Phosphorylases catalyse the synthesis (and degradation) of linear chains of α-1 : 4-linked glucose residues, and these are converted into branched molecules containing *ca* 4 and 8 per cent α-1 : 6-glucosidic linkages by Q-enzyme and branching-enzyme respectively. For complete degradation, phosphorylase and a 'de-branching' enzyme (named R-enzyme[103] and amylo-1 : 6-glucosidase respectively) are required (Fig. 10). It follows that the structure of the reserve polysaccharide is dependent on the relative activity of several enzymes and is therefore related to the metabolic state of the organism.



Key

1. hexokinase + ATP
2. glucose-6-phosphatase
3. phosphoglucomutase
4. plant phosphorylase (P-enzyme) + Q-enzyme
5. P-enzyme + R-enzyme
6. muscle phosphorylase + branching enzyme
7. muscle phosphorylase + amylo-1 : 6-glucosidase.

FIG. 10. The enzymic interconversion of glucose and amylopectin or glycogen

The glycogen from two cases of glycogen-storage disease[104,105] has been examined recently (Table VIII). This metabolic dysfunction is characterized by the deposition of unusually large amounts of glycogen

in the liver, kidney and other tissues. One sample of liver glycogen (S.K.) had a $\overline{CL}$ of only 6 glucose residues, representing a degree of branching twice that of normal glycogens.[104] The unusually low β-amylolysis limit (14 per cent) indicated very short exterior chains of perhaps 2 or three glucose residues; the length of the interior chains was almost normal. The structure approximates to that of a muscle-phosphorylase limit dextrin (*see* p. 30) and it seems probable that the biochemical deficiency was a lack or inactivation of the debranching enzyme amylo-1 : 6-glucosidase. A second liver glycogen (A.K.) had a normal structure, and in this case the enzymic imbalance was not connected with the system catalysing the glycogen $\rightleftharpoons$ α-D-glucosyl phosphate interconversion.[105] The kidney tissue from the same source also contained a glycogen of normal structure.[105] The two glycogens would appear to correspond to disease types 3 and 1 respectively, in the classification of G. T. Cori.[106]

TABLE VIII

DETERMINATION OF EXTERIOR AND INTERIOR CHAIN LENGTHS OF α-1 : 4-GLUCOSANS

Sample	$\overline{CL}$	β-Limit	$\overline{ECL}$	$\overline{ICL}$
Amylopectins :				
Potato	23	61	16–17	5–6
Protozoal	22	60	15–16	5–6
Waxy maize I	20	58	14	5
„ „ II	19	59	13–14	4–5
Waxy sorghum I	22	56	14–15	6–7
„ „ II	25	58	17	7
Glycogens :				
Horse diaphragm muscle				
(*a*) pre-rigor	17	53	11–12	4–5
(*b*) post-rigor	17	51	11	5
Human liver (S.K.)	6	12	2–3	2–3
„ „ (A.K.)	14	46	9	4
Human kidney (A.K.)	14	46	9	4
Rabbit liver VII	14	53	10	3
„ „ VIII	13	48	9	3
„ „ X	12	49	8–9	3–4
Tetrahymena pyriformis II	14	48	9	4
Trichomonas gallinae II	13	47	8–9	3–4

$\overline{ECL}$ = Exterior chain length (no. of glucose residues removed by β-amylase + 2·5).

$\overline{ICL}$ = Interior chain length ($\overline{CL}$–$\overline{ECL}$–1).

In a second study on glycogen structure and metabolism, Lawrie, Manners and Wright[107] have examined glycogen isolated from mammalian muscles before and after the development of *rigor mortis*. The marked physiological changes, which are described elsewhere,[108] are accompanied by a decrease in glycogen content. The results shown in Table VIII indicate little or no change in overall glycogen structure; there is, therefore, no significant alteration in the *relative* activity of phosphorylase and amylo-1 : 6-glucosidase in this metabolic condition.

Multiple-branching in amylopectin and glycogen. The mode of arrangement of the constituent chains in an amylopectin or glycogen molecule has been the subject of repeated investigation during the past twenty or so years.

In 1937 Haworth, Hirst and Isherwood[109] proposed a singly branched 'laminated' structure for glycogen (Fig. 11*a*). This was the simplest molecular structure compatible with available experimental data on the $\overline{CL}$ (12–18 glucose residues) and the $\overline{DP}$ (*ca* 10^3); this structure is also in accord with the results of β-amylolysis, provided that the branch points are situated, on the average, near the mid-point of the individual chains. A similar type of laminated structure was later proposed for amylopectin, with the difference that the chains contained 20–25 glucose residues.

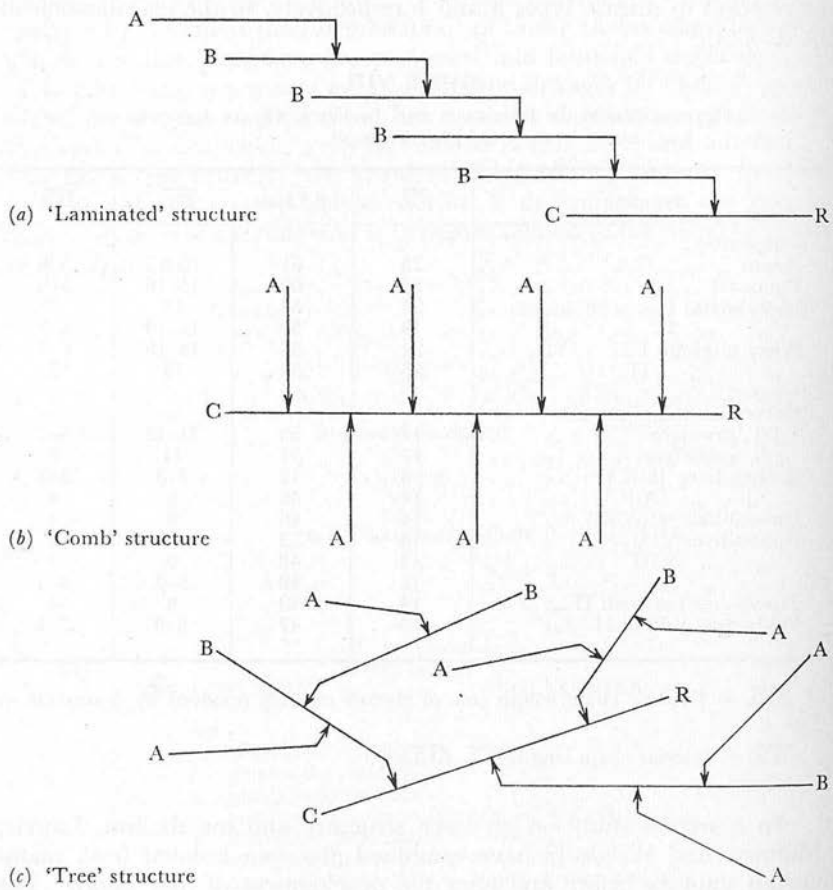


FIG. 11. Molecular Structures for Amylopectin and Glycogen

In the same year, Staudinger and Husemann suggested 'comb' or 'herring-bone' type structures for these polysaccharides (Fig. 11*b*).[110] The amylopectin molecule was regarded as containing a central linear chain of α-1 : 4-linked glucose residues, *alternate* residues of which served as branch points. The side-chains, each of *ca* 20 glucose residues, were linked to either C_3 or C_6 of the central chain residues. This structure was considered to account for the presence of both 2 : 6- and 2 : 3-di-*O*-methyl glucose (in addition to the tri- and tetra-methyl derivatives) in hydrolysates of the methylated polysaccharide (*cf.* Fig. 2) and for the observed colloidal properties and viscosity. The Staudinger formulation for glycogen, which originated with the belief that the molecule was spherical, was generally similar, except that side-chains of 12–18 glucose residues were attached to either C_2, C_3 or C_6 of *every* glucose residue in the central chain. However, in view of more recent chemical, enzymic and physico-chemical studies, these comb-type structures are no longer accepted.[81,86,91]

A third type of structure, put forward by K. H. Meyer,[111] postulated random multiple-branching, giving a 'tree' or 'bush' type molecule (Fig. 11*c*). This structure was initially based upon the results of methylation analysis of the polysaccharides and their β-limit dextrins.

Peat, Whelan and Thomas[112] have pointed out that the various structures contain linear chains of α-1 : 4-linked glucose residues which may be linked to the remainder of the molecule in three different ways: A-chain (side-chain) attached only by a single $1 \rightarrow 6$ linkage from the reducing group; B-chain (main chain), to which one or more A-chains are linked, and itself attached by the reducing group to an adjacent chain; C-chain, to which other chains are attached, and terminated by the sole free reducing group in the molecule. In a molecule of n chains, the ratio of A-chains : B-chains ($\overline{A/B}$) is $1 : (n-2)$ for a 'laminated' structure and approximately 1 : 1 for a fully developed 'tree' structure.[113] Although A-chains and B-chains are chemically indistinguishable, a final choice between these structures has been made by enzymic methods.

Qualitative evidence of multiple-branching in amylopectin and glycogen has been obtained by several workers. Larner and his co-workers[114] examined the *successive* action of muscle phosphorylase and amylo-1 : 6-glucosidase on amylopectin (wheat and maize) and glycogen (rabbit muscle and liver). Phosphorylase catalyses an end-wise degradation of the exterior chains of branched α-1 : 4-glucosans, giving α-D-glucosyl phosphate (*ca* 40 per cent) and a high-molecular-weight limit dextrin (LD. 1). This enzyme can differentiate between A- and B-chains, the length of the residual exterior chains in LD. 1 being one and *ca* 6 glucose residues respectively (Fig. 12). On treatment with amylo-1 : 6-glucosidase, the A-chain stubs are removed, as glucose (3–5 per cent).[115] The polysaccharide is now susceptible to a further but limited phosphorolysis; a second dextrin (LD. 2) can be isolated, retreated with amylo-1 : 6-glucosidase, and then phosphorylase, to give LD. 3. This dextrin amounts to 12–23 per cent of the original polysaccharide and its isolation gives evidence of multiple-branching. A 'comb' structure at

the LD. 1 stage would be completely degraded by the action of amylo-1 : 6-glucosidase, and then phosphorylase, whilst over 95 per cent of a 'laminated' structure would remain unattacked. This analysis, which requires only *ca* 0·2 g polysaccharide, has been applied to glycogen isolated from glycogen-storage diseased tissues,[116] and to a 'synthetic' amylopectin,* with similar results.

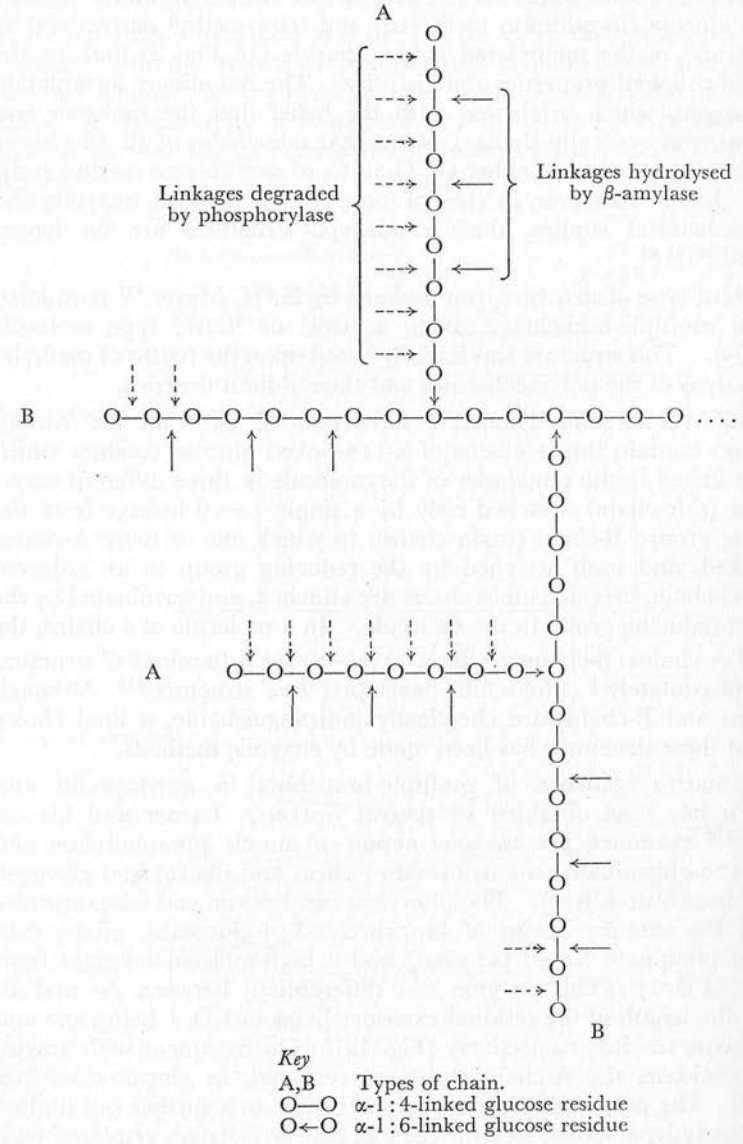


FIG. 12. The degradation of exterior chains of branched α-1 : 4-glucosans by β-amylase (⟶) and muscle phosphorylase (⇠---).

* Synthesized by the action of potato Q-enzyme on amylose.[117]

The analytical data may give a semi-quantitative indication of the degree of multiple-branching, *i.e.* $\overline{A/B}$, since the amount of glucose released from LD. 1 by amylo-1 : 6-glucosidase is, assuming complete enzymic hydrolysis, dependent on the proportion of A-chains. Calculations on this basis (Table IX) indicate that *both* amylopectins and glycogens have $\overline{A/B}$ values in the range *ca* $1:1 \rightarrow 1:3$.

TABLE IX

CALCULATION OF $\overline{A/B}$ VALUES IN AMYLOPECTIN AND GLYCOGEN

Polysaccharide	End-groups in LD.I (%)*†	Moles of (A + B) chains	Glucose from LD.I (%)*	Moles of A chains	$\overline{A/B}$
Amylopectin :					
Corn	10·2	0·063	5·3	0·030	1 : 1·1
Synthetic	7·2	0·044	3·7	0·021	1 : 1·1
Wheat	10·9	0·067	7·3	0·040	1 : 0·7
Glycogen :					
Human liver (normal) ..	11·5	0·071	4·3	0·024	1 : 2·0
(glycogen storage disease)	12·3	0·076	5·0	0·028	1 : 1·7
(glycogen storage disease)	9·3	0·057	4·6	0·025	1 : 1·3
Rabbit liver	11·7	0·072	3·2	0·018	1 : 3·0
Rabbit muscle	11·3	0·070	5·4	0·030	1 : 1·3

* The experimental figures are given in ref. 114, 116, 117.

† The $\overline{CL}$ of a polysaccharide containing 10·9 per cent of end-groups is $100/10{\cdot}9 = 9{\cdot}2$.

Studies on the action of salivary α-amylase on amylopectin and glycogen have also provided qualitative evidence of multiple-branching.[118] The products included maltose, maltotriose, a pentasaccharide and a mixture of higher oligosaccharides. Analysis of the latter indicated that a proportion of the α-dextrins contained two branch points. The interior chains in parts of the amylopectin and glycogen molecules therefore appear to comprise none, one or perhaps two glucose residues.

An alternative method[112] involves the *successive* treatment of amylopectin with β-amylase and R-enzyme (which hydrolyses the outermost α-1 : 6-inter-chain linkages). During β-amylolysis, A-chains are degraded to two or three residues and these are released as maltose or maltotriose during the action of R-enzyme on amylopectin β-limit dextrin. Waxy maize starch ($\overline{CL}$ 24) under these conditions gave 12·8 per cent maltose and maltotriose. If the polysaccharide contained equal numbers of A- and B-chains, the calculated yield would be 10·4 per cent, whereas singly branched or Staudinger-type structures would give $< 0{\cdot}1$ per cent or 20·8 per cent maltosaccharides, respectively. This method, which requires the preparation of maltose-free β-dextrin and the complete 'debranching' of the outermost chains, is limited to amylopectins, since R-enzyme has no appreciable action on glycogens.[119]

During a comparison of the degradation of the *exterior* chains of branched α-1 : 4-glucosans by muscle phosphorylase and β-amylase, it was noted that the difference (Δ) between the average chain lengths of

the residual phosphorylase limit dextrin (ϕ-dextrin or L.D.1) and β-amylase limit dextrin (β-dextrin) was not constant.[120] This variation has been interpreted as indicating small differences in degree of multiple branching ($\overline{A/B}$). The observed value Δ and $\overline{A/B}$ may be correlated (*see* Fig. 12) as follows: in a β-dextrin the A-chain 'stubs' contain either 2 or 3 glucose residues (depending upon whether the original A-chain comprised an even or odd number of glucose residues), *i.e.* an average value of 2·5 residues.[112] The B-chain 'stubs' are probably of a similar length, but for the present, are considered to contain n residues. By contrast, the A-chain in a ϕ-dextrin consists of a single glucose residue, whereas the B-chain 'stubs' contain 4 glucose residues more than those o the corresponding β-dextrin, *i.e.* $(4 + n)$ residues.[115] In a polysaccharide containing equal numbers of A- and B-chains, the *difference* in the *average* exterior chain lengths of the ϕ- and β-dextrins is therefore $[1 + (4 + n)]/2 - [2{\cdot}5 + n]/2$, or 1·25 glucose units. If $\overline{A/B} = 1:2$, then the corresponding figures are $[1 + (8 + 2n)]/3 - [2{\cdot}5 + 2n]/3 = 2{\cdot}17$ glucose residues. It must be noted that this calculation is independent of the length (n) of the B-chain 'stubs' in the β-dextrin. The values of Δ for theoretical $\overline{A/B}$ values in the range $2:1 \rightarrow 1:8$ have been similarly calculated, and from this, $\overline{A/B}$ values obtained from experimental determinations of Δ. Typical results are shown in Table X. This method can be applied equally to amylopectins and glycogens.

TABLE X

MULTIPLE-BRANCHING IN AMYLOPECTIN AND GLYCOGEN[120]

Polysaccharide	$\overline{CL}$*	$\overline{CL}$ of ϕ-dextrin†	$\overline{CL}$ of β-dextrin‡	Δ	$\overline{A/B}$
Amylopectin:					
Waxy maize starch	20	11·8	10·0	1·8	1:1·5
Waxy sorghum starch	22	13·2	10·6	2·6	1:2·9
Glycogen:					
Ascaris lumbricoides	12	8·3	6·1	2·2	1:2·0
Cat liver	13	8·3	6·1	2·2	1:2·0
Foetal sheep liver	13	9·2	6·6	2·6	1:2·9
Helix pomatia	7	5·5	4·4	1·1	1:0·9
Human muscle	11	8·6	6·6	2·0	1:1·8
Mytilus edulis	13	9·4	7·0	2·4	1:2·4
Tetrahymena pyriformis	13	9·0	7·3	1·7	1:1·4
Yeast (Brewer's)	13	9·1	7·3	1·8	1:1·5

* Determined by potassium periodate oxidation.

† $\overline{CL} \times (100 - \phi\text{-limit})$.

‡ $\overline{CL} \times (100 - \beta\text{-limit})$.

We have therefore concluded that glycogens from different biological sources show small differences in multiple branching but that the two amylopectin samples show a similar range of values. These studies, and the data in Table IX, clearly show that amylopectin and glycogen do not differ significantly in degree of multiple branching.

Although this structural feature of amylopectin and glycogen is now well established, it is important to note that this does not in any way

define or limit the overall molecular shape. These polysaccharides show marked differences in hydrodynamic properties,[60] *e.g.* the limiting viscosity number of glycogen is very much lower than that of amylopectin of similar molecular size, and the relationships between sedimentation constant and concentration are quite different. These differences reflect variations in molecular shape, which arise from differences in the site and mode of biosynthesis of the polysaccharides, and not from differences in degree of multiple-branching. Fig. 13 illustrates two molecular structures of different molecular shape but with similar branching properties.

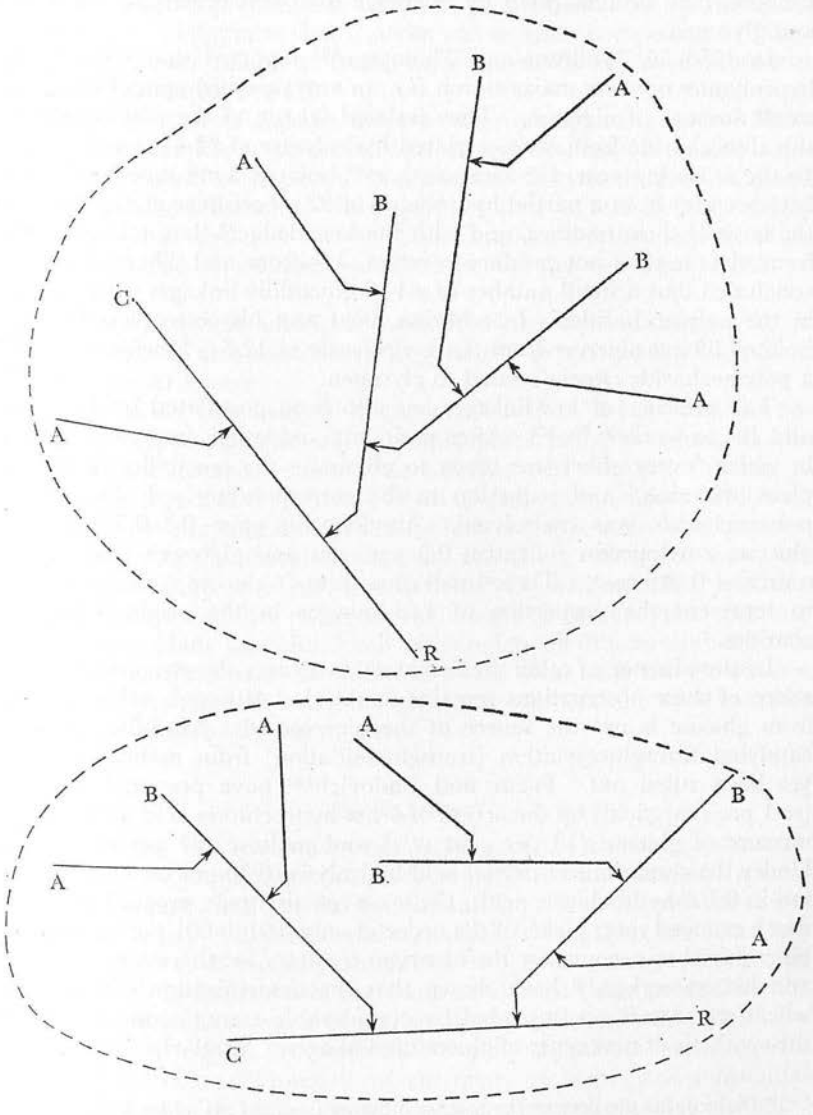


FIG. 13.

Many of the physico-chemical differences between amylopectin and glycogen can be understood, by extrapolation of this concept to molecules of some 10^3 chains, and with the realization that glycogen *must*, by virtue of the average interior chain-length of only *ca* 3–5 glucose residues, be a relatively compact molecule.

*Anomalous linkages.** With the advent of chromatography and other improved methods for the detection and isolation of carbohydrates, a new type of problem has arisen in polysaccharide chemistry, namely, the assessment of the structural significance of minor constituents. This problem may be illustrated by reference to recent researches on starch and glycogen.

In 1955–56, Wolfrom and Thompson[121] reported that partial acid hydrolysates of waxy maize starch (*i.e.* an amylopectin) contained a very small amount of nigerose. They isolated 50 mg of the octa-acetate of this disaccharide from the acetylated hydrolysate of 32·4 g amylopectin. In the following year, the same authors[27] isolated 2 mg nigerose (as the octa-acetate) from a partial hydrolysate of 92 g beef-liver glycogen. On the basis of these findings, and with the knowledge[98] that acid-reversion from glucose does not produce nigerose, Wolfrom and Thompson have concluded that a small number of α-1 : 3-glucosidic linkages were present in the polysaccharides. In addition, Peat and his co-workers[121a] have isolated 35 mg nigerose from the hydrolysate of 12·5 g Floridean starch, a polysaccharide closely related to glycogen.

The presence of 1 : 3-linkages has also been postulated by F. Smith and his co-workers.[30, 122] After periodate oxidation, under conditions in which 'every effort was made to eliminate the possibility of incomplete oxidation,' and reduction to the corresponding polyalcohol, the polysaccharide was hydrolysed. Amylopectin gave 0·2–0·5 per cent glucose, amylopectin β-dextrin 0·3 per cent and glycogen (unspecified source) 1·0 per cent. These small quantities of glucose were considered to represent the proportion of 1 : 3-linkages in the original polysaccharides.

In the absence of other confirmatory evidence, the structural significance of these observations remains doubtful. Although acid-reversion from glucose is not the source of the nigerose, the possibility of acid-catalysed transglucosylation (transglucosidation) from maltose has not yet been ruled out. Pazur and Budovich[123] have prepared nigerose (*ca* 1 per cent yield) by the action of 0·1 N-hydrochloric acid at 100° on a mixture of glucose (13 per cent w/v) and maltose (27 per cent w/v). Under the conditions of partial acid hydrolysis (0·36 per cent w/v glucosan in 0·1 N-hydrochloric acid), the same reaction may proceed at a very much reduced rate; yields of the order of only 0·01–0·001 per cent would be required to account for the observed results. Furthermore, F. Smith and his co-workers[124] have shown that the dextrinization of corn and wheat starches is accompanied by considerable transglucosidation with the synthesis of new types of glucosidic linkages. Similarly, the presence

* Defined, for the present purpose, as differing from α-1 : 4- and α-1 : 6-glucosidic linkages.

of periodate-resistant glucose residues may be due to steric hindrance effects near the branch points, or to the protection of a limited number of diol groups by traces of impurities.

Two further types of anomalous linkages may be mentioned. In 1952, Peat and his co-workers[125] noted the presence of maltulose (*O*-α-D-glucopyranosyl-(1 → 4)-D-fructopyranose; *ca* 5 per cent yield) in α-amylolytic hydrolysates of rabbit-liver glycogen. Fructose-containing α-dextrins were also detected. Similar results were reported[118] for a sample of waxy maize starch, although full details are not yet available. Since the enzyme preparation had no action on maltose or maltosaccharides, it would appear that fructose was a minor component. Other workers have been unable to detect ketose sugars in different samples of glycogen and amylopectin.[92]

A further type of linkage involves ester phosphate groups. Phosphorus is a variable trace constituent of starches, and is usually associated with the amylopectin component. Posternak has obtained evidence for the presence of glucose-6-phosphate in acid hydrolysates of various starches and has isolated phosphomalto-hexaose and -tetraose (containing glucose residues esterified at C_6) by the α-amylolysis of potato starch.[126]

The structural relationship between the above types of linkage, the Z-labile linkages of amylose and the reported[127] oxygen-sensitive bonds in starch is not yet known.

The possible existence of linkages other than α-1 : 4- and α-1 : 6-glucosidic in amylopectin and glycogen poses many problems to the polysaccharide chemist, since their solution will demand the use of modern techniques at the extreme limits of their accuracy and sensitivity. The mode of biosynthesis of these polysaccharides must also be considered : whether the phosphorylases and branching enzymes show a dual specificity, or undergo 'synergic' reactions[46] to yield modified products, whether these anomalies represent the results of non-enzymic reactions in the living plant or animal cell or whether additional and hitherto undetected enzymes exist in the cells. It is clear that the elucidation of the fine structure of starch and glycogen will continue to attract the attention of chemists for many years.

CONCLUSIONS

This review has covered certain aspects of the chemistry of homopolysaccharides containing two, three or perhaps four different types of glycosidic linkage; and yet the determination of their fine structure has required a variety of experimental techniques. It is therefore clear that investigations on heteropolysaccharides are even more difficult; for example, acid hydrolysates of the water-soluble polysaccharide from the green algae *Cladophora rupestris*, and its methylated derivative, yield five and seventeen sugars, respectively.[128] Polysaccharides showing this degree of molecular complexity present many problems, and substantial progress can only be made by the *combined* efforts of organic, biological and physical chemists.

The next decade should see marked progress in this, the third phase of polysaccharide chemistry, and in related problems of the mode of synthesis and degradation, and metabolic function in living tissues, of these complex high polymers. In the writer's opinion, this aspect of the subject promises to be the most interesting and, indeed, exciting of them all.

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Chemical Section

The Composition and Structure of Polysaccharides,

Structural Analysis of Polysaccharides by Methylation,

Structural Analysis of Polysaccharides by Periodate Oxidation.

Abstract *

The Composition and Structure of Polysaccharides

The article surveys the methods available for determination of the composition and structure of polysaccharides, and in tabulated form, summarizes data on the following:-

(a) Simple polysaccharides (glucosans, galactans, mannans, fructosans, pentosans) with respect to type of branched structure, nature of structural linkages, degree of polymerization and average chain length.

(b) Complex polysaccharides (algal polysaccharides, animal polysaccharides, microbial polysaccharides and plant hemicelluloses) with reference to type of branched structure, and nature of component sugars and main structural linkages.

(c) The composition of some plant gums and mucilages. A bibliography of 49 references is included.

Structural Analysis of Polysaccharides by Methylation and Periodate Oxidation

In these two articles, a critical survey of the application of these two techniques to polysaccharide chemistry is made. The respective advantages and limitations of each method is discussed, and references are given to the latest improvements in experimental methods.

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Biochem. J., 1954, Vol. 57, Part 3, p. xviii.]

Observations on the Carbohydrase Activity of certain Seaweed Extracts. By W. A. M. DUNCAN, D. J. MANNERS and A. G. ROSS. (*Department of Chemistry, University of Edinburgh*)

By extraction of minced seaweed (from *Cladophora rupestris*, *Laminaria digitata* and *Rhodymenia palmata*) under weakly alkaline conditions, dilute solutions of proteins showing hydrolytic activity towards several carbohydrates were obtained. These carbohydrases have been concentrated by precipitation with ammonium sulphate and freeze-drying. Whole extracts from these seaweeds will hydrolyse certain α-glucosides (maltose, sucrose, αα-trehalose), several β-glucosides [cellobiose, 'cellodextrin' (an acid-degraded cellulose), laminaribiose, laminarin, barley β-glucosan, methyl β-glycoside, phenyl β-glucoside, amygdalin and salicin], lactose, xylotriose and the xylans from Esparto grass and *R. palmata*. No significant activity could be detected towards methyl α-glucoside, methyl α-galactoside, methyl α-mannoside, soluble starch, fucoidin, and inulin. Partial separation of these activities has been achieved by ammonium sulphate fractionation.

The 'maltase' present in the seaweeds shows both hydrolytic and synthetic activity; during enzyme action on maltose, glucose and small amounts of maltotriose and maltotetraose are formed. The maltase activity is unaffected by inorganic phosphate and is optimum at about pH 5·5.

During the hydrolysis of laminarin by a seaweed extract, glucose and a series of oligosaccharides were produced; the latter, on prolonged incubation, were hydrolysed to glucose. 'Cellodextrin' on incubation with seaweed extract yielded glucose and cellobiose during the initial stages of hydrolysis; the cellobiose was eventually hydrolysed to glucose. These results may indicate that the extracts contain a group specific β-glucosidase which can only hydrolyse terminal β-linked glucose units, and a second β-glucosidase capable of hydrolysing non-terminal β-glucosidic linkages. The seaweed β-glucosidases, unlike emulsin, do not show synthetic activity (cf. Peat, Whelan & Hinson, 1952); the 'laminarinase' activity is maximal at about pH 5·1.

The seaweed extracts show marked xylanase activity, the course of which is unaffected by inorganic phosphate. From the xylans from Esparto grass or *R. palmata*, xylose, xylobiose, xylotriose and higher saccharides are produced. It seems probable that the 1:3 linkages in the algal xylan are more easily hydrolysed than the 1:4 linkages (cf. Percival & Chanda, 1950).

We wish to thank Prof. E. L. Hirst, F.R.S., for his interest in this work which forms part of a research programme sponsored by the Institute of Seaweed Research.

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Enzyme Systems in Marine Algae. The Carbohydrase Activities of Unfractionated Extracts of *Cladophora rupestris, Laminaria digitata, Rhodymenia palmata* and *Ulva lactuca*

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(*Received 9 June* 1955)

Present knowledge of the metabolism of terrestrial plants and freshwater algae is considerable (cf. Bonner, 1950; Fogg, 1953) and a large number of enzymes and enzyme systems have been detected in plant tissues and extracts; subsequently, many of these have been isolated and purified. In addition, certain freshwater algae (e.g. *Chlorella* and *Scenedesmus*) have been widely used in studies of photosynthesis and intermediary carbohydrate metabolism. By contrast, similar information on marine algae is lacking, and few investigations of the enzyme systems of marine algae have been reported, although the chemical structure of many of the end-products of anabolism has been investigated (cf. Black, 1953).

In co-operation with the Institute of Seaweed Research, a survey of the enzyme systems of marine algae has been commenced, and, in view of our previous interest in the enzymic hydrolysis of glucosides and glucosans (Manners, 1952, 1955), our preliminary experiments have been directed towards the detection of carbohydrases in extracts of marine algae. In the present paper, evidence for the presence of a number of soluble carbohydrases in a member of the Phaeophyceae (*Laminaria digitata*), a member of the Rhodophyceae (*Rhodymenia palmata*) and two species of Chlorophyceae (*Cladophora rupestris* and *Ulva lactuca*) is recorded. A preliminary account of part of this work has already been published (Duncan, Manners & Ross, 1954).

METHODS AND MATERIALS

Analytical methods

Paper chromatography. (*a*) *Sugars.* Descending chromatograms were carried out at room temperature with Whatman no. 1 paper and *n*-butanol–pyridine–water–benzene (5:3:3:1, by vol.) as solvent (De Whalley, Albon & Gross, 1951). An alkaline silver nitrate reagent (Trevelyan, Procter & Harrison, 1950) or aniline oxalate reagent (Partridge, 1949) was used to detect the sugars on the chromatograms. The rate of movement of sugars (R_G) was determined by dividing the distance moved by the sugars from the starting line by the distance moved by D-glucose (R_G 1·0) under identical conditions. The R_G value of a particular sugar was found to vary on different chromatograms, e.g. laminaribiose had R_G 0·65–0·76; hence, preliminary identification of a sugar was carried out by placing the sugar and the appropriate reference compound on the same chromatogram, and not by calculation of the R_G value.

(*b*) *Phosphate esters.* Development and detection was effected by the method of Hanes & Isherwood (1949), with *n*-propanol–ammonium hydroxide–water (6:3:1, by vol.) and glucose 1- and 6-phosphates as reference compounds.

Reducing sugars. Reducing sugars were determined by (*a*) the iodometric Shaffer & Somogyi (1933) reagent as modified by Hanes & Cattle (1938), (*b*) the iodometric Somogyi (1945*a*) reagent, or (*c*) the colorimetric Nelson (1944) reagent as modified by Somogyi (1952). The reagents were calibrated, as required, against glucose and maltose. Deproteinization, when necessary, was effected by $ZnSO_4$–$Ba(OH)_2$ (Somogyi, 1945*b*).

Glucose 1-phosphate. A slight modification of the method of Allen (1940) was used.

Nitrogen. Kjeldahl-N was determined by the method of Chibnall, Rees & Williams (1943).

Soluble protein. Soluble protein was extracted from fresh minced seaweed by gentle stirring at room temperature with a suitable solvent; the extract was centrifuged, and the supernatant solution dialysed, freeze-dried and analysed for Kjeldahl-N.

Viscosity measurements. The decrease in viscosity of solutions of certain polysaccharides was measured with an Ostwald viscometer with a 10 cm. capillary and flow time of 222 sec. for 10 ml. of water at 20°. Results are expressed in terms of the specific viscosity (i.e. relative viscosity – 1).

Substrates

Glycosides and sugar phosphates. Phenyl α- and β-D-glucosides were synthesized by the methods of Helferich & Smitz-Hillebrecht (1933) and Nath & Rydon (1954) respectively. Glucose 1- and 6-phosphates were prepared by Maung Khin Maung by the methods of McCready & Hassid (1944) and Viscontini & Olivier (1953) respectively. Dr D. J. Bell kindly provided specimens of phenyl and *n*-butyl β-D-galactosides. The other glycosides were laboratory or commercial specimens.

Di- and oligo-saccharides. Gentiobiose was prepared by the synthetic action of emulsin on glucose as described by Peat, Whelan & Hinson (1952). Isomaltose and isomaltotriose were isolated by charcoal–Celite chromatography of a partial acid hydrolysate of dextran; laminaribiose was prepared by similar means from laminarin. We are indebted to Miss M. Carter for a sample of xylobiose. Maltose was recrystallized thrice from 80% (v/v) aqueous ethanol and further purified by charcoal–Celite chromatography. Dr W. J. Whelan kindly provided a sample of maltotriose. The homogeneity of all other di- and oligo-saccharides was examined by paper chromatography.

Polysaccharides. We are indebted to Dr G. O. Aspinall for samples of esparto xylan (Chanda, Hirst, Jones & Percival, 1950) and ivory-nut mannan A (Aspinall, Hirst, Percival & Williamson, 1953); to Dr D. J. Bell for a sample of leafy-cocksfoot levan (Bell & Palmer, 1952); to Professor C. S. Hanes, F.R.S., for a sample of cellodextrin; to Dr D. H. Northcote for samples of yeast glucan (Bell & Northcote, 1950) and yeast mannan; to Dr B. Lindberg for a sample of pustulan (Lindberg & McPherson, 1954); to Professor I. A. Preece for a sample of barley β-glucosan (cf. Aspinall & Telfer, 1954); to Dextran Ltd. for a sample of dextran; and to Imperial Chemical Industries Ltd. for a sample of Cellofas B (sodium carboxymethylcellulose). Xylan was isolated from *R. palmata* by the method of Barry & Dillon (1940).

Reference carbohydrases

Emulsin. Emulsin (British Drug Houses Ltd., 10·5 g.) was suspended in water (175 ml.) and 0·2M acetate buffer (pH 5·0; 25 ml.), and dialysed against tap water at room temperature for 4 days, to lower the reducing sugar content. After centrifuging, the supernatant liquid was freeze-dried; yield 3·0 g.; N, 11·8%.

Barley 'laminarinase'. Flour (100 g.) from Spratt–Archer barley was extracted with 3% (w/v) KCl solution (350 ml.) by gentle stirring at room temperature for 2 hr. To the supernatant solution, obtained by centrifuging, ammonium sulphate was added to 0·75 saturation. The precipitated protein was suspended in water, and dialysed for 4 days against running tap water. During dialysis a precipitate formed and was removed by centrifuging; yield 1·1 g.; N, 6·4%. The remaining solution was freeze-dried and the laminarinase activity of the resulting powder was determined (Table 4); yield 1·8 g.; N, 10·1%.

Enzymic reactions

Qualitative demonstration of carbohydrase activity. Carbohydrate (approx. 30 mg.) was incubated with freeze-dried extract (approx. 30 mg.) suspended in 0·07–0·20M acetate buffer (pH 5·0; 3 ml.). The digests were examined at intervals by paper chromatography, the results being expressed as follows: (3+) end-products detected within 1–3 days of incubation; (2+) within 3–7 days; (+) after 7 days; (±) very slight activity. By this method, reproducible results have been obtained. Unless otherwise stated, enzymic reactions were carried out at 35°, aseptic conditions being maintained by use of toluene.

Quantitative demonstration of carbohydrase activity. Digests containing carbohydrate (approx. 50 mg.), freeze-dried extract (approx. 50 mg.), 0·2M acetate buffer (pH 5·0) and water to a final volume of 40–50 ml. were set up. The reducing power of 5 ml. portions was determined at intervals; as the extracts (0·1%, w/v) had no reducing power and did not interfere with the Somogyi reagents, deproteinization was unnecessary. In a single experiment (with yeast glucan), 1·3% (w/v) of extract was used, and the samples were accordingly deproteinized. The effect of pH on the 'maltase' and 'laminarinase' activities of the extracts was investigated by incubating substrate (10 mg.), extract (12 mg.), water (10 ml.) and B.D.H. Universal Buffer (pH 4·0–9·0; 10 ml.) at 35°. The reducing power of 5 ml. portions was determined after 24 or 48 hr. In similar experiments on the salicinase activity, the digests comprised salicin (15 mg.), extract (30 mg.), water (7 ml.) and B.D.H. Universal Buffer (pH 4·4–7·0; 5 ml.).

RESULTS

Extraction of soluble protein from marine algae

Although in exploratory experiments a variety of methods were used in attempts to disintegrate algal fronds and stipes (e.g. by homogenization, freezing at −35° and milling, freeze-drying and milling), mechanical mincing in ice water proved to be the most satisfactory and has been used throughout this study. A variety of solvents have been used to extract protein from the minced algae; the results obtained with *R. palmata* are recorded in detail in Table 1; essentially similar results were obtained with *L. digitata* stipe.

Since extraction with 0·25% (w/v) Na_2CO_3 gave material with the highest 'protein' content, this solvent has been used in later extractions, even though extraction with distilled water and 20% ethanol gave a greater total yield of crude protein.

Preparation of whole extracts of marine algae

Several extracts from the four algae have been prepared during the past 3 years. The method of preparation has been modified slightly on occasions, but a typical preparation is described below.

Fresh hand-pressed algae (2–3 kg.), minced with ice water, was extracted by stirring with 0·25 % (w/v) Na_2CO_3 (3–4 l.) at room temperature for 15–18 hr. The pH of the solution, originally approx. 9, fell slowly to a final value of 6–7. (If the pH of a *L. digitata* extract falls below 5, co-precipitation of protein and alginate occurs.) After centrifuging (2000 *g* for 20–30 min.), the extract was dialysed against running tap water for 3–4 days to remove free sugars, amino acids, peptides and other material. Ammonium sulphate was added to 0·7–0·8 saturation, and the precipitated protein was collected by centrifuging at 0°. No further precipitate was formed on increasing the $(NH_4)_2SO_4$ above 0·8 saturation. (If the dialysis stage is omitted, the precipitation of protein by $(NH_4)_2SO_4$ is retarded and is incomplete.) The precipitated protein was redissolved in water, and reprecipitated with $(NH_4)_2SO_4$ at approx. 0·7 saturation. The resulting precipitate was centrifuged, dissolved in water, dialysed until free of $(NH_4)_2SO_4$ and freeze-dried.

The yields of crude protein extracted from the four marine algae, in one series of experiments, are recorded in Table 2; the weights of extracts used in the following experiments refer to these freeze-dried materials. In some extracts, a precipitate of inert material appeared during the dialysis and was discarded; in other experiments, a proportion of the material precipitated by $(NH_4)_2SO_4$ was found to be insoluble in water, and since it had only weak carbohydrase activity it was also discarded. Experiments with *L. digitata* fronds showed that although the residue from the original extraction still contained appreciable amounts of nitrogenous material (N, 1·9 %), it showed little or no carbohydrase activity. Control experiments have shown that the carbohydrase activity of the extract (with the exception of that towards starch) is unaffected by common inorganic ions, and that dialysis does not appreciably diminish the activity.

Table 1. *Kjeldahl-N content of extracts of* Rhodymenia palmata

Minced algae (50 g.) was extracted with 100 ml. of solvent at room temperature for 18 hr. The extracts were then centrifuged, freeze-dried and analysed for Kjeldahl-N.

Solvent	Weight of material extracted (g.)	N content (%)
Distilled water	0·75	3·0
0·25 % (w/v) Na_2CO_3	0·44	3·7
3 % (w/v) KCl	0·69	2·1
20 % (v/v) Ethanol	1·21	1·6
Water, saturated with *n*-butanol	1·00	1·4
0·2 M Borate buffer, pH 9·0	0·41	2·3
0·2 M Phosphate buffer, pH 8·0	0·47	3·1
Solvent A*	0·63	1·0
Solvent B†	1·10	0·9

* Solvent A: 0·95 % (w/v) borax solution–ethanol–ether (10:4:1, by vol.).

† Solvent B: 0·2 M Borate buffer (pH 9·0)–water–ethanol–*n*-butanol (5:5:4:1, by vol.).

Specificity of carbohydrase activity

The seaweed extracts were tested for activity towards various groups of similar carbohydrates. The results, obtained by paper chromatography, were essentially similar for all four extracts; those for *C. rupestris* are as follows, activity being expressed as detailed under Methods.

α-Glucosides. (3+) Isomaltose, isomaltotriose, maltose, maltotriose, phenyl α-glucoside; (2+) sucrose, trehalose; (±) methyl α-glucoside.

β-Glucosides. (3+) Aesculin, amygdalin, arbutin, cellobiose, gentiobiose, helicin, laminaribiose, phenyl β-glucoside, salicin; (±) methyl β-glucoside, phloridzin.

Miscellaneous glycosides. (3+) Xylobiose; (+) lactose; (±) melibiose; no activity towards *n*-butyl and phenyl β-galactosides, hesperidin, methyl α-galactoside, methyl α-mannoside, quercitrin, raffinose and rutin.

Polysaccharides. (3+) Barley β-glucosan, cellodextrin, Floridean starch, glucan (yeast), glycogen, laminarin, lichenin, soluble starch, xylan (*Rhodymenia*); (2+) mannan (ivory nut); (+) carob gum, xylan (Esparto); no activity towards fucoidin, inulin, levan, mannan (yeast) and pustulan.

Table 2. *Extraction of soluble protein from marine algae*

The method of extraction is described in the Results section.

Species	Place and date of collection	Weight of algae extracted (g.)	Weight of soluble extract (g.)	N content (%)
*Cladophora rupestris**	Dunbar (22. i. 54)	2900	42·2	8·8
Laminaria digitata†	North Berwick (1. xii. 53)	3550	2·7	5·5
*Rhodymenia palmata**	North Berwick (24. ix. 53)	2600	2·4	7·2
*Ulva lactuca**	North Berwick (29. vi. 54)	2500	16·0	7·7

* Whole plant minced. † Stipes only minced.

The extracts thus contain α-glucosidase, β-glucosidase, xylobiase, lactase, amylase, mannanase, xylanase and β-glucosanases capable of hydrolysing both 1:3- and 1:4-glucosidic linkages.

Examination of the paper chromatograms from the above experiments has provided evidence for the type of polysaccharase action, since step-wise hydrolysis ('exo' action) of a polysaccharide yields only the constituent monosaccharide(s), whereas random hydrolysis ('endo' action) gives rise, at intermediate stages, to a series of oligosaccharides. During the hydrolysis of laminarin by *L. digitata* extract a series of reducing sugars was produced; the sugar with the highest chromatographic mobility had the same R_G value as authentic glucose. Random hydrolysis of β-1:3-glucosidic linkages had therefore occurred. The xylanase activity of the extracts is also 'endo' action since pentoses with the R_G values of xylose, xylobiose, xylotriose (and higher xylosaccharides) are produced; these di- and oligo-saccharides are not reversion products since incubation of xylose (7 %) with *C. rupestris* or *U. lactuca* extract under identical conditions did not result in the synthesis of xylosaccharides within 21 days.

The enzyme action on maltose, laminarin, starch and xylan does not involve phosphorolysis. After incubation of *C. rupestris* extract with maltose, laminarin or starch, 0·1 M phosphate buffer (pH 6·7), 0·2 % ammonium molybdate (glucose 1-phosphatase inhibitor) and 1·4 mM mercuric chloride or 0·01 M sodium fluoride (phosphoglucomutase inhibitors), for 4–7 days, glucose 1-phosphate could not be detected. Furthermore, paper chromatographic analysis of a digest of *R. palmata* extract, xylan (*Rhodymenia*) and phosphate buffer showed sugar phosphates to be absent.

In control digests from which the algal extract had been omitted, or in those containing boiled extract, free monosaccharides were not liberated on incubation for periods up to 28 days. The observed activity is therefore due to thermolabile factors in the algal extract and not to the adventitious presence of toluene-resistant carbohydrase-producing micro-organisms in the buffer and carbohydrate solutions.

Action on α-glucosides and α-1:4-glucosans

The four algal extracts contain enzymes catalysing the hydrolysis of maltose and other α-glucosides, and of glycogen and starch. Qualitatively, maltose was hydrolysed more readily than the other α-glucosides, and, in dilute solution, enzyme action was essentially complete. Thus, *R. palmata* extract (0·04 %) caused a slow hydrolysis of maltose (0·04 %) at pH 5·1 and 35°, a constant level of 91 % conversion being attained in 10 days.

After prolonged incubation (15 days) of maltose (1 %) with an extract of *L. digitata*, in addition to glucose, the formation of a reducing sugar (hereafter designated M 1) with a chromatographic mobility lower than maltose was observed. In further experiments with *C. rupestris* and *U. lactuca* extracts and with higher concentrations of maltose, the synthesis of two additional reducing sugars (M 1 and M 2) has been shown. For example, in more concentrated solution (7 % maltose) glucose, maltose and traces of M 1 were detected within 4 days, and, after 8 days, glucose, maltose, M 1 and M 2 were present, whilst in a digest containing 17 % maltose chromatographic evidence for the synthesis of M 1 and M 2 was obtained after only 24 hr. incubation at 35°. Control experiments have shown that no such synthesis takes place in digests containing boiled seaweed extracts. On incubation with glucose (7 %) no synthesis of any sugars occurred; the formation of M 1 and M 2 from maltose is therefore due to trans-α-glucosylase activity. A comparison of the R_G values of M 1 and maltotriose, and of their corresponding *N*-benzylglycosylamines (cf. Bayly & Bourne, 1953) suggests that M 1 is maltotriose; this suggestion is supported by the fact that M 1 is completely hydrolysed to glucose and maltose by unpurified salivary amylase. Attempts to induce transglucosylase activity by using maltose as glucosyl donor, and methanol, fructose or galactose as glucosyl acceptors have not yet been successful.

Transglucosylation of α-1:6-linkages has also been demonstrated; in a digest consisting of isomaltose (7 %) and *C. rupestris* extract (0·3 %), chromatographic evidence for the formation of a higher saccharide with R_G 0·14 was obtained after 6 days incubation. On continued incubation, the amount of this sugar slowly increased. Isomaltotriose has R_G 0·14 (cf. maltotriose R_G 0·29).

The effect of pH on the 'maltase' activity of the extracts from *R. palmata*, *C. rupestris* and *U. lactuca* has been investigated, and reducing-sugar estimations show that activity is optimum at about pH 6.

The extracts show marked activity towards α-1:4-glucosans. On incubating soluble starch (0·1 %) with *C. rupestris* extract (0·15 %) at pH 5·1, slow hydrolysis to give 41 % conversion into glucose was observed in 70 hr. Under similar conditions, potato amylose and glycogen gave 32 and 13 % glucose, respectively. In the course of purification of the algal amylase, we have noted that the amylase activity may be differentiated from the α-glucosidase activity. On addition of Cl^- to an extract, the amylase activity is selectively increased; in addition, the amylase is more thermolabile than the α-glucosidase.

Action on β-glucosides and β-glucosans

The preliminary experiments have shown the extracts to possess group-specific hydrolytic activity towards β-glucosidic linkages since they readily hydrolyse aromatic β-glucosides, and disaccharides containing β-1:3-, β-1:4- and β-1:6-glucosidic linkages. In these digests (excepting that with cellobiose) glucose was the sole end product of enzymic action; with cellobiose, however, a series of reducing sugars were produced which will be referred to as C1, C2, C3 in order of decreasing chromatographic mobility. From paper chromatographic evidence, C1 (R_G 0·76) is tentatively identified as laminaribiose, and C_3 (R 0·17) as cellotriose. C_2 has R_G 0·33 (cf. cellobiose, R_G 0·55; gentiobiose, R_G 0·40). Since enzymic synthesis of higher sugars from glucose does not occur under these conditions, the formation of laminaribiose and the cellosaccharides must be ascribed to trans-β-glucosylase activity by the algal extracts.

β-Glucosidase action on salicin has been used to standardize the activity of various β-glucosidase preparations (Veibel, 1950); accordingly, quantitative measurements of the hydrolysis of salicin by the most active seaweed extract (from *C. rupestris*) have been made, and compared with a dilute solution of emulsin under the same conditions. On the basis of equal weights of protein N, the salicinase activity of emulsin was approx. 600 times greater than that of the extract of *C. rupestris*.

The effect of pH on the 'salicinase' activities of the unfractionated extracts from *C. rupestris*, *U. lactuca* and *R. palmata* has been studied; reducing power estimations show the activities to be greatest in the range pH 6–7.

In view of the pronounced hydrolytic activity of the *C. rupestris* extract towards β-glucosans (laminarin, lichenin, glucan), further experiments on the mode of enzyme action have been carried out. Thus in order to decide whether 'laminarinase' activity was due to a single enzyme (capable of hydrolysing both laminaribiose and, in random fashion, laminarin) or to a number of enzymes, the activity of *C. rupestris* towards laminarin (and cellodextrin) under various conditions was examined. Digests containing laminarin or cellodextrin, phosphate buffer (pH 2·9–9·0) and normal, or heated extract were incubated at 35° and examined chromatographically at intervals (Table 3). It is concluded that laminarin is hydrolysed by multi-enzyme action, one component catalysing random scission of β-1:3-linkages, and a second component catalysing stepwise hydrolysis; these components may be differentiated by the greater heat lability of the former. Cellodextrin is also hydrolysed in random fashion, but the enzyme involved differs from the endo-β-1:3-glucosanase since it is relatively heat stable. The extracts of *C. rupestris* thus contain at least three enzymes which can hydrolyse β-glucosidic linkages, viz. an endo-β-1:3-glucosanase, an endo-β-1:4-glucosanase (belonging, therefore, to the cellulase class of hydrolases) and an exo-β-glucosanase. This latter enzyme is probably responsible for the group-specific β-glucosidase activity previously noted.

Examination of the same cellodextrin digests after 7 and 14 days also revealed the presence of a sugar having R_G 0·71 (? laminaribiose) which had presumably arisen by trans-β-glucosylase action similar to that previously observed with cellobiose.

Quantitative estimations of the 'laminarinase' activity (endo and exo activity) of three seaweed extracts have been made, and compared with the

Table 3. *Effect of* pH *and heat on the 'laminarinase' and 'cellodextrinase' activities of* Cladophora rupestris *extract*

Substrates (200 mg.), 0·07 M phosphate buffer (4 ml.), and extract (15 mg.) were incubated at 35°. In digests 5 and 10, the buffer and extract were heated at 60° for 15 min. before addition of the substrate. The digests were examined by paper chromatography after 2 days (cellodextrin) or 7 days (laminarin).

Digest no.	Substrate and conditions	Reducing sugars present* Glucose	R_G 0·65	R_G 0·33	R_G 0·46	R_G 0·08
1	Laminarin, pH 2·9	2+	2+	2+	—	—
2	Laminarin, pH 5·1	3+	2+	+	—	—
3	Laminarin, pH 7·0	2+	+	+	—	—
4	Laminarin, pH 9·0	+	+	+	—	—
5	Laminarin, pH 5·1 (heated)	2+	—	—	—	—
6	Cellodextrin, pH 2·9	2+	—	—	2+	2+
7	Cellodextrin, pH 5·1	3+	—	—	3+	3+
8	Cellodextrin, pH 7·0	2+	—	—	2+	2+
9	Cellodextrin, pH 9·0	+	—	—	+	+
10	Cellodextrin, pH 5·1 (heated)	2+	—	—	2+	2+

* 3+, Intense spot on chromatogram; 2+, moderate spot; +, weak spot; —, no detectable spot.

Table 4. *Enzymic hydrolysis of laminarin*

Digests contained laminarin (16–24 mg.), freeze-dried extract of *R. palmata*, *C. rupestris* or *U. lactuca* (20 mg.) or of barley (5 mg.) and 0·2 M acetate buffer (pH 5·1) and water to 35–50 ml. After incubation at 35°, 5 ml. portions were analysed for reducing sugar.

Time (days)	Apparent percentage conversion into glucose			
	*R. palmata**	*C. rupestris*	*U. lactuca*	Barley
1	49	52	28	70
2	68	74	47	87
3	75	90	62	—
4	—	—	84	91

* After 7 and 12 days' incubation, the percentage hydrolysis was 84 and 90 respectively.

'laminarinase' activity of an extract of Spratt–Archer barley (Table 4). The 'laminarinase' activity of the barley extract is seen to be several times greater than that of the seaweed extracts, on the basis of equal weights of protein N, although in all digests approx. 90 % conversion into glucose was eventually obtained. The 'laminarinase' activities of the extracts of *C. rupestris*, *R.palmata* and *U. lactuca* are optimum at about pH 5·5, 6·0 and 6·3 respectively.

To show that the above activity was not due to microbial contamination of the freeze-dried extracts, the laminarinase activity of an aqueous extract was quantitatively compared with that of extracts from which micro-organisms had been removed. An aqueous solution of *U. lactuca* extract (1 mg./ml.) was divided into three portions which were treated as follows: (1) filtered by gravity, using a Whatman no. 1 paper; (2) filtered through a Seitz bacteriological filter; (3) centrifuged at 90 500 *g* for 30 min. (by courtesy of Dr C. T. Greenwood). Each solution (10 ml.) was then incubated with 0·2 M acetate buffer (pH 5·0; 5 ml.) and an aqueous solution of laminarin (1·7 mg./ml.; 5 ml.) at 35° for 48 hr. The reducing powers of 5 ml. portions of the digests were equivalent to 3·32, 3·35 and 3·35 ml. of 0·01 N sodium thiosulphate respectively, showing that no loss of laminarinase activity occurred during the procedures carried out to remove micro-organisms from the extracts. The above experiment, together with the control experiments previously reported, show that the digests are not contaminated with carbohydrase-producing micro-organisms.

The hydrolysis of yeast glucan by *C. rupestris* extract has also been examined; after incubation of glucan (0·13 %) with extract (1·3 %) at pH 5·1, 73 and 76 % conversion into glucose was observed after 3 and 6 days respectively.

In view of the marked hydrolytic activity of the extracts towards cellodextrin, it was of interest to examine the activity towards a high-molecular-weight cellulose derivative. Sodium carboxymethylcellulose (Cellofas B) was therefore incubated with freeze-dried extracts of *C. rupestris* and *U. lactuca*, and the change in specific viscosity was determined (Fig. 1). Reducing power estimations at 18 hr. indicated an apparent percentage hydrolysis (as glucose) of 8 and 10 respectively. Chromatographic examination of the digests showed the presence of a series of reducing sugars varying from glucose (R_g 1·0) to zero R_g value, providing further evidence for the presence of endo-type β-1:4-glucosanases in the extracts.

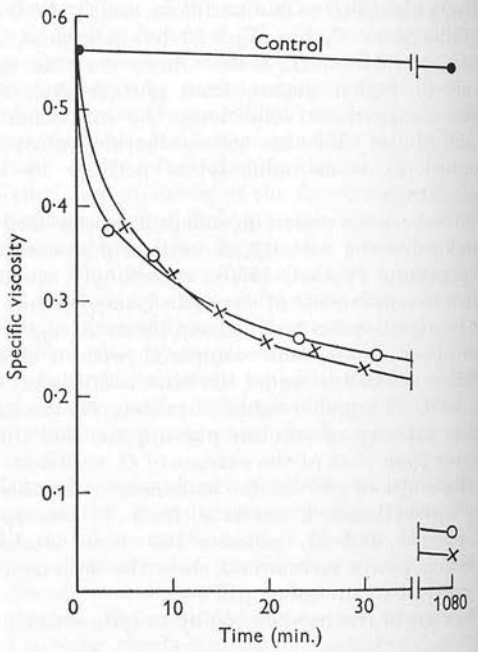

Fig. 1. Viscosity reduction of sodium carboxymethylcellulose by extracts of *C. rupestris* (×) and *U. lactuca* (○). The digests contained 20 ml. of 0·25 % (w/v) Cellofas B and 20 ml. of 0·2 M acetate buffer (pH 5·0) in which 50 mg. of extract was dissolved, and were incubated at 20°. Viscosity measurements were made at intervals.

DISCUSSION

The present preliminary investigation has shown that unfractionated extracts of *L. digitata*, *R. palmata*, *C. rupestris* and *U. lactuca* contain essentially similar complements of carbohydrases, including an α-glucosidase, β-glucosidase, amylase, β-1:3- and β-1:4-glucosanase, xylanase and mannanase; in addition, weak hydrolytic activity towards lactose has been demonstrated. The carbohydrase complements are therefore very similar to those of cereal extracts, although the relative activities are much lower. Attempts (unreported) to detect hydrolytic activity towards alginic acid and pectin by viscometric methods have not been completely successful, although a small decrease in the specific viscosity of the polyuronides after incubation with an extract of *C. rupestris* or *U. lactuca* for 2–7 days has been observed. During this incubation, free uronic acids were not produced. Qualitatively, the activity of the extracts towards the majority of substrates tested increased in the order *L. digitata* < *R. palmata* < *U. lactuca* < *C. rupestris*, and paralleled that of the observed yield of crude protein extracted from the algae (Table 1). It is probable that in *L. digitata*, extraction of protein was retarded by the presence of alginic acid; attempts to remove alginic acid selectively from the extract by precipitation as the calcium salt were unsuccessful, and resulted in coprecipitation of calcium alginate and protein. Smith & Young (1953) observed that under more drastic conditions (0·33 % sodium hydroxide, pH 13; 55°) only about 50 % of the protein-N of *Fucus vesiculosus* could be extracted, and suggested that extraction of protein was impeded by alginate.

For the present discussion, the hydrolytic activity of the extracts towards a number of α- and β-glucosides will be referred to as 'α-glucosidase activity' or 'β-glucosidase activity' respectively; the available data do not yet permit a distinction to be made between activity due to a single group-specific α- (or β-)glucosidase and that of a mixture of closely related α- (or β-)glucosidases with a higher degree of specificity.

The α-glucosidase activity resembles that of yeast α-glucosidase in that the rate of hydrolysis of α-glucosides increases in the order methyl α-glucoside, phenyl α-glucoside and maltose, and is most active at approx. pH 6 (Gottschalk, 1950). The hydrolysis of α-1:6-linked glucosaccharides by the algal extracts is an interesting feature of their activity since 'isomaltase' activity has been reported in only a limited number of biological sources, e.g. cell-free extracts of *Clostridium acetobutylicum* (French & Knapp, 1950) and extracts of brewers' yeast (Manners & Khin Maung, 1955). The specificity of β-glucosidase activity follows that reported for β-glucosidases from other biological sources, methyl β-glucoside being hydrolysed at a lower rate than phenyl β-glucoside or salicin (Veibel, 1950). The algal β-glucosidases differ from emulsin in that they show no synthetic activity towards glucose (cf. Peat, Whelan & Hinson, 1952). Several examples of carbohydrases from plant and mould sources which show both hydrolytic and transglycosylase activity have been reported recently (e.g. Pazur & French, 1952; Buston & Jabbar, 1954), and it is now clear that the algal carbohydrases likewise show dual activity.

Although separation of the 'cellodextrinase' and 'laminarinase' activities of the *C. rupestris* extract has not yet been attempted, evidence for the presence of two distinct endo-type enzymes has been obtained (Table 3); the endo-β-1:3-glucosanase activity is inactivated at 60°, whereas the endo-β-1:4-glucosanase and β-glucosidase are only partly inactivated. Dillon & O'Colla (1950) have noted the similar extreme thermolability of wheat laminarinase.

Hydrolytic activity towards laminarin has been reported with almond emulsin, which yields glucose as the initial and sole product of a step-wise action, and with carbohydrases from wheat, barley and rye, which catalyse random hydrolysis of the laminarin, yielding glucose, laminaribiose and higher β-1:3-glucosaccharides as the initial products (cf. Peat, Thomas & Whelan, 1952; Dillon & O'Colla, 1950, 1951; Manners, 1952, 1955). The algal extracts therefore resemble the cereals in type of laminarinase action. Dillon & O'Colla (1951) reported that extracts of barley, active towards laminarin, had no action on yeast glucan, and would only partially hydrolyse (20 %) an oxidized water-soluble glucan. In contrast, Manners (1952) observed that glucan, although insoluble, was slowly hydrolysed by a barley extract, yielding over 70 % glucose; our extract from *C. rupestris* thus shows a similar 'glucanase' activity.

The activity of the algal extracts towards hemicelluloses is of interest, in view of the limited occurrence of hemicellulases. The algal extracts produce xylosaccharides from xylan, and also slowly hydrolyse xylobiose, although the available evidence does not differentiate between single- and multi-enzyme action. The extracts of the two members of the Chlorophyceae also show appreciable mannanase activity, and hydrolyse the β-1:4-mannosidic linkages in ivory-nut mannan and carob gum; in contrast, no action on the α-mannosidic linkages in the highly branched yeast mannan has been detected.

The separation and purification of the several carbohydrases present in the extract of *C. rupestris*

is now being investigated. Preliminary experiments have shown that, on a micro scale, a partial separation of activities can be effected by paper electrophoresis, and, on a larger scale, by fractionation with ammonium sulphate and organic solvents at low temperatures. Later communications in this series will describe the properties of the purified carbohydrases, the chemical characterization of the oligosaccharides produced by transglucosylase action, and the phosphatase and proteinase activity of the *C. rupestris* extract.

SUMMARY

1. A survey of the carbohydrase activity of unfractionated extracts of four species of marine algae has been carried out.

2. The extracts show hydrolytic activity towards a number of α- and β-glucosides, lactose, mannan, xylan, starch, glycogen, laminarin, lichenin, glucan, cellodextrin and sodium carboxymethylcellulose.

3. Under certain conditions, enzymic synthesis of higher saccharides from maltose, isomaltose and cellobiose has been observed.

4. The hydrolytic activity of an extract of *C. rupestris* towards cellodextrin and laminarin has been shown to be due to separate enzymes.

5. The effect of pH on the hydrolytic activity of the extracts towards maltose, salicin and laminarin has been examined.

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Enzyme Systems in Marine Algae

2. TRANS-α-GLUCOSYLATION BY EXTRACTS OF *CLADOPHORA RUPESTRIS**

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Duncan, Manners & Ross (1956) reported that unfractionated extracts of four species of marine algae (*Cladophora rupestris*, *Laminaria digitata*, *Rhodymenia palmata* and *Ulva lactuca*) showed hydrolytic activity towards a number of carbohydrates, including maltose. In dilute aqueous solution (0·04 %) this sugar was completely hydrolysed to glucose, whereas in concentrated solution (7–17 %) the synthesis of oligosaccharides was observed. By contrast, incubation of an algal extract with a concentrated solution of glucose did not result in oligosaccharide synthesis. The enzymic reaction therefore involves the transfer of α-glucosyl residues to glucosaccharides and not the enzymic polymerization of glucose; in this respect, the algal enzyme systems differ from that in *Aspergillus niger* (strain NRRL 330), which can synthesize disaccharides from glucose (Peat, Whelan & Hinson, 1955).

In the present paper the characterization of the oligosaccharides synthesized from maltose by an extract of *Cladophora rupestris* is described, and the acceptor specificity of the trans-α-glucosylase system is discussed.

* The paper by Duncan, Manners & Ross (1956) is regarded as Part 1.

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METHODS AND MATERIALS

Analytical methods

Chromatography. (*a*) *Qualitative.* Paper chromatography of free sugars was carried out as previously described with either *n*-butanol–pyridine–water–benzene (5:3:3:1, by vol.) (*A*) or ethyl acetate–pyridine–water (10:4:3, by vol.) (*B*) as solvent. The rate of movement of sugars is expressed relative to D-glucose (R_G values) (Duncan *et al.* 1956). The corresponding *N*-benzylglucosylamines were chromatographed by the method of Bayly & Bourne (1953).

(*b*) *Preparative.* Columns containing a 1:1 (w/w) mixture of activated charcoal (British Drug Houses Ltd.) and Celite no. 545 (Johns Manville Co., New York, U.S.A.) were prepared by the general methods of Whistler & Durso (1950), Whelan, Bailey & Roberts (1953) and Bacon (1954). Oligosaccharides were eluted with an increasing concentration of aqueous ethanol; fractions of 700 ml. were collected from the larger columns (70 or 79 cm. × 4·5 cm.), and fractions of 7 ml. from smaller columns.

Electrophoresis. Paper electrophoresis of free sugars was carried out in a water-cooled apparatus (Foster, 1952) with Whatman no. 1 paper in a 0·1 N-borate buffer (pH 10·0), with a 1 hr. period for equilibration. The current (500 V; 12·5 mA) was passed for 3·5 hr., the paper was air-dried and the sugars were located with an aniline oxalate–acetic acid spray reagent.

Periodate oxidation (with Mr F. B. Anderson). The oligosaccharides were oxidized with sodium metaperiodate in the presence of 0·05 M-phosphate buffer (pH 8·0) as described by Hough & Perry (1956). The production of formaldehyde was determined with a phenylhydrazine–ferricyanide reagent (Hough, Powell & Woods, 1956). Under these conditions 1→4-linked oligosaccharides yield 1 mole of formaldehyde/glucose residue, whereas formaldehyde is not liberated from glucose residues which are joined by 1→6 linkages.

Estimation of degree of polymerization (*DP*). The sugars (about 5 mg.) were dissolved in water (10 ml.) and the reducing power of samples (2 ml.) was estimated with the iodometric Shaffer & Somogyi (1933) reagent as modified by Hanes & Cattle (1938). With the exception of M_0, which required 20 min. heating, a heating time of 60 min. was necessary for the full development of reducing power. The reducing powers were calculated as equivalents of maltose. Samples (2 ml.) were hydrolysed with sulphuric acid (36 N; 0·12 ml.) at 100° for 2 hr., neutralized with sodium hydroxide (phenolphthalein), diluted to 5 ml. and the glucose content was determined.

Partial acid hydrolysis. The sugar (1 %, w/v solution; 1 ml.) and sulphuric acid (0·5 N; 0·6 ml.) were heated at 100° for 1 hr., neutralized, concentrated and analysed by paper chromatography. Alternatively, the sugar (1 % solution; 5 ml.), iodine (30 mg.) and potassium hydroxide (1·0 N; 0·2 ml.), mixed in this order, were shaken together, and after the solution had been kept for 30 min. a further portion of alkali (0·1 ml.) was added. After 15 hr. incubation at room temperature, a sample of the aldonic acid solution (1 ml.) was partly hydrolysed as described above.

Enzyme preparations

Extract of C. rupestris. Soluble protein was extracted from fresh minced algae as previously described (Duncan *et al.* 1956). The freeze-dried material contained 8·8 % of nitrogen.

β-Amylase. A commercial preparation from barley was used (Liddle & Manners, 1957).

D-Enzyme. This enzyme was prepared from potato juice by the method of Peat, Whelan & Rees (1956).

EXPERIMENTAL AND RESULTS

Preparation and isolation of oligosaccharides

A digest was prepared containing recrystallized maltose (32·5 g.), 0·1 M-acetate buffer (pH 5·0; 250 ml.), freeze-dried extract (1·25 g.) and toluene. After incubation at 35° for 16 days, the digest was heated to inactivate the enzyme and concentrated. Paper-chromatographic analysis showed the presence of glucose, maltose, and six additional oligosaccharides with R_G values (in solvent *A*) ranging from 0·38 to 0·03 (see Table 1). (R_G values are relative to D-glucose.)

To facilitate separation of the oligosaccharides, the concentrate was chromatographed on a charcoal–Celite column (79 cm. × 4·5 cm.) and the glucose and maltose were eluted with water and 4 % (v/v) aqueous ethanol respectively. The oligosaccharides were then eluted with 30 % ethanol and concentrated.

The resulting syrup was applied to a second column (70 cm. × 4·5 cm.) and the oligosaccharides were eluted with increasing concentrations of ethanol. Fractions (700 ml.) were collected, concentrated and examined by paper chromatography. None of the fractions became acid on concentration.

The oligosaccharides will be referred to as M_0, M_1, M_2, etc., in order of elution from the column; in a preliminary comment on the present work (Duncan *et al.* 1956) the two major sugars were named in order of paper-chromatographic mobility, so that M_1 and M_2 are now redesignated M_2 and M_1 respectively.

The following combined fractions were collected: *A*, glucose; *B*, maltose and M_0 (faint trace); *C*, maltose; *D*, maltose and M_1; *E*, M_1 (1·38 g.); *F*, M_1, M_2, and maltose (trace); *G*, M_2 and maltose (faint trace); *H*, mixed higher oligosaccharides.

Fraction *G* was chromatographed on a third column (65 cm. × 3 cm.), yielding pure M_2 (1·50 g.). Fraction *H* was applied to a fourth column (67 cm. × 3 cm.). Elution with 0–2 and 5 % aqueous ethanol gave chromatographically pure glucose and M_0 (25 mg.) respectively. Elution with increasing concentrations of ethanol (6 % → 18 %) was continued, 7 ml. fractions being collected. The yields of the pure oligosaccharides, after freeze-drying or crystallization from ethanol, are given in Table 1. Mixed fractions were not analysed further, except that chromatography of a mixture

of M_2 and M_3 on Whatman no. 3 MM paper yielded pure M_3 (10 mg.).

A control digest containing maltose (8·1 g.) and 0·1 M-acetate buffer (pH 5·0; 63 ml.) was incubated at 35° for a similar period. After heating, it was concentrated, and applied to a charcoal–Celite column (21 cm. × 4·5 cm.) and developed with increasing concentrations of aqueous ethanol. No sugar other than maltose could be detected. The observed synthesis of oligosaccharides in the main digest is not therefore due to microbial contamination of the buffer and sugar solution. Further, the purity of the maltose used as substrate is confirmed.

Preliminary characterization of the oligosaccharides

The purified oligosaccharides, which were homogeneous by paper chromatography and electrophoresis, were reducing sugars which contained glucose and no other sugar.

The results in Table 1 indicate that M_2 and M_3 are trisaccharides and that M_4, M_5 and M_6 are tetrasaccharides. Further, since the degree of polymerization (DP) values of M_2 and M_5 are very close to whole numbers, these sugars are probably maltosaccharides (cf. Whelan *et al.* 1953).

N-Benzylglycosylamine derivatives of M_1, M_2, maltose and maltotriose were prepared; the R_G values were 0·29, 0·31, 0·57 and 0·31 respectively.

The four main oligosaccharides and the corresponding aldonic acids were partly hydrolysed with acid, and the products were tentatively identified by paper chromatography (Table 2).

Further evidence of the structure of M_1, M_2, M_4 and M_5 was obtained by determining the formaldehyde liberated during 'over-oxidation' with periodate (see Table 1). In a control experiment, maltose gave 1·8 moles/mole (theoretical, 2·0).

In addition to the above observations, molecular rotation and paper-chromatographic mobility [expressed as $\log R_G/(1-R_G)$] data support the suggestion that M_2 and M_5 are maltotriose and maltotetraose respectively. A comparison of the figures with those of maltose shows that a linear relationship exists with the DP of the sugar. This indicates that maltose, M_2 and M_5 belong to the same polymeric series of sugars (cf. Whelan *et al.* 1953). Further, the paper-chromatographic mobilities of M_1 and M_4 are intermediate between those of the maltosaccharides and the isomaltosaccharide series, suggesting that M_1 and M_4 may contain both α-1 → 4- and α-1 → 6-glucosidic linkages (cf. French & Wild, 1953). M_0, M_3 and M_6 have not been examined further.

Characterization of M_1 as panose

M_1 had $[\alpha]_D^{15} + 148°$ in water (*c*, 3·7); cf. $[\alpha]_D + 150°$, reported by Pazur & French (1952) for panose. M_1 had the same R_G in three solvents as an authentic sample of panose, and was not attacked by barley β-amylase or maltase-free saliva. The infrared spectrum of M_1 (panose), kindly determined by Dr D. M. W. Anderson, showed that it was present in the form 2 described recently by Wolfrom & Thompson (1957). M_1 (150 mg.) in water (3 ml.)

Table 1. *Properties of oligosaccharides synthesized from maltose by an extract of* Cladophora rupestris

The periodate-oxidation values are given in moles of formaldehyde/mole of oligosaccharide; theoretical values for the assigned structures are given in parentheses.

Oligosaccharide	Yield (g.)	R_G values *A*	R_G values *B*	DP	Periodate-oxidation value
M_0	0·03	0·38*	0·50*	1·94	—
M_1	2·16	0·18	0·28	—	1·0 (1)
M_2	2·11	0·35†	0·41†	3·04	2·7 (3)
M_3	0·01	0·07	0·14	2·63	—
M_4	0·31	0·11	0·17	3·72	1·8 (2)
M_5	0·47	0·19	0·26	3·95	4·1 (4)
M_6	0·02	0·03	0·09	3·69	—

* Authentic isomaltose had R_G values *A*, 0·38; *B*, 0·48.
† Authentic maltotriose had R_G values *A*, 0·33; *B*, 0·41.

Table 2. *Products of partial acid hydrolysis of the oligosaccharides and of the corresponding aldonic acids*

	Oligosaccharide hydrolysate*	Aldonic acid hydrolysate*	Tentative structure
M_1	M_0, maltose, glucose	M_0, glucose	Panose
M_2	Maltose, glucose	Maltose, glucose	Maltotriose
M_4	M_1, M_2, M_0, maltose, glucose	M_1, M_0, maltose, glucose	6^3-α-Glucosylmaltotriose†
M_5	M_2, maltose, glucose	M_2, maltose, glucose	Maltotetraose

* Unchanged oligosaccharide is not recorded.
† For full systematic name see text.

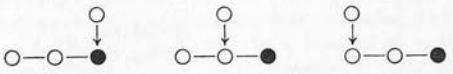
was reduced with potassium borohydride solution (4·5 %, 1 ml.). After 2 hr. at room temperature, the solution was acidified with acetic acid (to decompose excess of borohydride), neutralized and evaporated to dryness. The product (120 mg.) was acetylated with sodium acetate (162 mg.) and acetic anhydride (3 ml.) at 135° for 20 min. The sugar acetate was isolated from ice–water and recrystallized from ethanol [m.p. and mixed m.p. with an authentic sample of panitol dodeca-acetate, 148–149°; $[\alpha]_D^{15}+119°$ in chloroform (*c*, 1·0) (cf. +119° and +120° reported by Peat, Whelan & Edwards, 1955)]. M_1 is therefore identified as panose (6²-α-glucosylmaltose).

Characterization of M_2 *as maltotriose*

M_2 had $[\alpha]_D^{15}+164°$ in water (*c*, 2·8); cf. $[\alpha]_D+160°$, reported by Whelan *et al.* (1953) for maltotriose. M_2 was slowly hydrolysed by maltase-free saliva and by barley β-amylase, giving glucose and maltose, as shown by paper-chromatography and reducing-power measurements. On incubation with D-enzyme, glucose and higher oligosaccharides were formed. The data reported in Tables 1 and 2 and the enzymic studies identify M_2 as maltotriose.

Characterization of M_4 *as* 6³-α-*glucosylmaltotriose*

M_4 had $[\alpha]_D^{15}+177°$ in water (*c*, 0·5) and differed in R_G and M_G (electrophoretic mobility relative to that of D-glucose) values from maltotriose, maltotetraose, isomaltotriose and isomaltotetraose. It was not attacked by α- or β-amylase. On the assumption that M_4 is formed by trans-α-glucosylation from maltotriose, three possible structures have been considered:



where ○ and ● represent respectively non-reducing and reducing glucose residues, and the symbols — and ↓ represent 1 → 4- and 1 → 6-linkages.

On periodate oxidation at pH 8, the structures would yield 0, 1 and 2 moles of formaldehyde repectively. M_4 gave 1·8 moles. Structure (I) and the aldonic acid of compound (II) on partial acid hydrolysis could not yield panose. These results and those reported in Tables 1 and 2 therefore identify M_4 as 6³-α-glucosylmaltotriose [*O*-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 4)-D-glucopyranose].

Characterization of M_5 *as maltotetraose*

M_5 had $[\alpha]_D^{15}+175°$ in water (*c*, 1·3); cf. $[\alpha]_D$ values of +176° and +177° reported by Whistler & Hickson (1955) and Whelan *et al.* (1953) for maltotetraose. M_5 was rapidly hydrolysed by maltase-free saliva to give maltose and glucose, and by barley β-amylase to give maltose. The enzymic studies and the data in Tables 1 and 2 characterize M_5 as maltotetraose.

Attempted transfer of α-glucosyl radicals to other sugars

Digests were prepared containing maltose (300 mg.), acceptor sugar (300 mg.), *C. rupestris* preparation (20 mg.) and 0·1 M-acetate buffer (pH 5·6, 2 ml.), and were examined at intervals, after incubation at 35°, by paper chromatography. The following acceptor sugars were tested: D-xylose, L-xylose, *N*-acetylglucosamine and L-sorbose. In all digests the same series of oligosaccharides were produced as were present in a control digest containing only maltose and extract.

DISCUSSION

The ability of many carbohydrases to catalyse both the hydrolysis and synthesis of oligosaccharides is now widely recognized (cf. Baumann & Pigman, 1957). The reactions may be formulated as follows:

$$G\text{–}O\text{–}X + \text{Enzyme-H} \rightarrow \text{Enzyme-G} + H\text{–}O\text{–}X, \quad (1)$$

$$\text{Enzyme-G} + H\text{–}O\text{–}R \rightarrow \text{Enzyme-H} + G\text{–}O\text{–}R, \quad (2)$$

where G–O–X represents a glucoside (the glucosyl donor) and H–O–R the acceptor molecule. When R is a hydrogen atom, hydrolysis takes place; if R is a univalent radical the synthesis of a new glucoside with the same configuration as the glucosyl donor occurs.

The present study shows that extracts of *C. rupestris* catalyse the synthesis of oligosaccharides from maltose when the concentration of water is sufficiently low. The main products of enzyme action, which have been characterized by chemical and enzymic methods, are panose (6²-α-glucosylmaltose), maltotriose, 6³-α-glucosylmaltotriose and maltotetraose. In addition, very small amounts of other oligosaccharides are also formed.

Although the yields of the oligosaccharides recorded in Table 1 are not quantitative, they are a

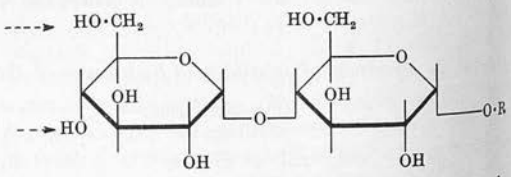


Fig. 1. Carbohydrate acceptors for α-glucosyl radicals transferred from maltose by extracts of *C. rupestris*. The arrows denote possible points of attachment of α-glucosyl radicals. R represents a hydrogen atom (maltose) or a glucose residue (maltotriose).

Table 3. *Trans-α-glucosylation: oligosaccharides synthesized from maltose by various enzyme preparations*

Biological source	Oligosaccharides	Reference
Penicillium chrysogenum Q. 176	Isomaltose, isomaltotriose, panose, 6^2-α-isomaltosylmaltose	Saroja, Venkataraman & Giri (1955)
Aspergillus niger (strain 152)	Isomaltose, isomaltotriose, panose	Barker & Carrington (1953)
Aspergillus oryzae	Isomaltose, isomaltotriose, panose, 6^2-α-isomaltosylmaltose	Pazur & French (1952)
Escherichia coli	Maltotriose and higher maltosaccharides	Barker & Bourne (1952)
Rat liver	Maltotriose, maltotetraose	Giri, Nagabhushanam, Nigam & Belavadi (1955)

measure of the relative proportion of the various sugars present in the equilibrium between synthesis and hydrolysis, under our particular experimental conditions (with 13% maltose solution). They show that the main transfer of α-glucosyl residues from maltose is to the hydroxyl group at $C_{(4)}$ or $C_{(6)}$ of the non-reducing end-group in maltose or maltotriose (Fig. 1). Glucose or panose do not appear to act readily as acceptor molecules.

These results may be compared with similar studies on trans-α-glucosylation by enzyme preparations from other biological sources, in which maltose was used as the α-glucosyl donor (Table 3).

It will be noted that the mould enzymes readily transfer α-glucosyl radicals to the primary alcoholic group in glucosaccharides, including glucose and panose, whereas the animal and bacterial enzyme systems can transfer only to the hydroxyl group at $C_{(4)}$ of the non-reducing end-group of the acceptor.

Experiments in this Laboratory (unpublished work) have also shown that the acceptor specificity for α-glucosyl radicals of the *C. rupestris* enzyme system differs from that found with cell-free extracts of the protozoan *Tetrahymena pyriformis* and with extracts of brewer's yeast.

In a further study of this acceptor specificity, the action of the algal extract on a mixture of maltose and a second sugar has been examined by paper chromatography. If the second sugar functions as an acceptor, new oligosaccharides will be formed in addition to those described here. As reported previously (Duncan *et al.* 1956), trans-α-glucosylation to D-galactose, D-fructose (and methanol) could not be detected. Similar experiments with D-xylose, L-xylose, L-sorbose and *N*-acetylglucosamine were unsuccessful. These six sugars do not therefore fulfil the acceptor-specificity requirements of the maltose trans-α-glucosylase. The results with the pentose sugars are in contrast with those obtained in an analogous study of trans-β-glucosylation in which cellobiose was used as β-glucosyl donor, since β-glucosylpentose disaccharides are formed in the presence of pentoses (D. J. Manners & J. L. Thompson, paper in preparation).

SUMMARY

1. An extract of *Cladophora rupestris* catalysed the synthesis of higher oligosaccharides from maltose.

2. The oligosaccharides were separated by charcoal–Celite chromatography and four sugars characterized by chemical and enzymic methods. They are panose, maltotriose, 6^3-α-glucosylmaltotriose [*O*-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 4)-D-glucopyranose] and maltotetraose.

3. The above and other evidence shows that, with the *C. rupestris* extract, maltose and maltotriose can function as α-glucosyl acceptors, whereas D-glucose, D-fructose, D-galactose, D-xylose, L-xylose, L-sorbose, *N*-acetylglucosamine and panose cannot.

4. The metabolism of maltose by transglucosylases from various biological sources is discussed.

We wish to thank Professor E. L. Hirst, F.R.S., for his advice and encouragement, and Dr W. J. Whelan for the gift of samples of maltotriose, panose and panitol acetate. The work described in this paper forms part of a research programme sponsored by the Institute of Seaweed Research, to whom W.A.M.D. is indebted for a research grant.

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Enzyme Systems in Marine Algae

3. TRANS-β-GLUCOSYLATION BY EXTRACTS OF *CLADOPHORA RUPESTRIS* AND *ULVA LACTUCA**

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(*Received* 30 *January* 1959)

During a study of the hydrolysis of cellodextrin by an extract of *Cladophora rupestris*, Duncan, Manners & Ross (1956) noted that the products of enzyme action, as examined by paper chromatography, included laminaribiose. Since the original substrate did not contain β-1:3-glucosidic linkages, it seemed possible that this sugar had arisen from cellobiose by trans-β-glucosylation involving a double-displacement mechanism:

$$\text{Cellobiose} + \text{enzyme-H} \rightarrow \text{glucose} + \text{glucosyl-enzyme}$$

$$\text{Glucosyl-enzyme} + \text{H·OR} \rightarrow \text{enzyme-H} + \text{glucosyl·OR}$$

where RO·H is an acceptor substrate, in this case D-glucose acting at its C-3 hydroxyl group.

* Part 2. Duncan & Manners (1958).

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This possibility has now been investigated by incubating the algal extract with various concentrations of cellobiose, and with mixtures of cellobiose and other sugars. In particular, the ability of D-xylose to function as a β-glucosyl acceptor has been studied. While this work was in progress, Barker, Bourne, Hewitt & Stacey (1957) reported that cell-free extracts of *Aspergillus niger* (strain 152) could synthesize 3-*O*-β-D-glucopyranosyl-D-xylose from a cellobiose–xylose mixture.

The metabolism of cellobiose by an extract of *Ulva lactuca* has also been examined.

METHODS

Chromatography. (*a*) *Qualitative.* Descending paper chromatograms were prepared at room temperature with Whatman no. 1 or 54 paper and butanol–pyridine–water–benzene (5:3:3:1, by vol.) (*A*), ethyl acetate–acetic acid–formic acid–water (18:3:1:4, by vol.) (*B*) or ethyl acetate–pyridine–water (10:4:3, by vol.) (*C*) as solvent. Aniline phthalate or oxalate, *p*-anisidine hydrochloride, naphtharesorcinol–hydrochloric acid and silver nitrate–sodium hydroxide were used as spray reagents. The rate of movement of sugars is expressed relative to D-glucose (R_G) or to D-xylose (R_X).

(*b*) *Preparative.* Charcoal–Celite columns were prepared as previously described (Duncan & Manners, 1958).

Oxidation by periodate. The reduction of periodate was determined spectrophotometrically (Aspinall & Ferrier, 1957). The remaining methods were those described by Duncan & Manners (1958) and Anderson & Manners (1959).

Enzyme preparations. For the preliminary experiments, unfractionated extracts of *C. rupestris* and *U. lactuca* were used (Duncan *et al.* 1956). In the large-scale digest, an extract of *C. rupestris* was fractionated with ammonium sulphate and the protein precipitated between 0·18 and 0·36 saturation was collected, dissolved in water, dialysed against running tap water for 24 hr. at room temperature and freeze-dried. This fraction had the highest β-glucosidase activity. Toluene was used to maintain aseptic conditions in the enzymic reactions.

EXPERIMENTAL AND RESULTS

C. rupestris *extract*

Action on cellobiose. Digests containing cellobiose (1, 5, 10, 20 %, w/v) and *C. rupestris* enzyme preparation (1 %, w/v) were incubated at 35°. In all digests, glucose was detected after 3·5 hr. The other results were as follows: (*a*) 1 % cellobiose, hydrolysis complete within 96 hr.; (*b*) 5 % cellobiose, oligosaccharides detected after 96 hr.; (*c*) 10 and 20 % cellobiose, oligosaccharides produced within 24 hr. These were identified, by paper chromatography in three solvents, as laminaribiose, gentiobiose and cellotriose. The cellotriose was formed first, and in greatest amount; we are indebted to Dr W. J. Whelan for an authentic sample for comparison.

In a further experiment, cellobiose (6 %, w/v) and extract were incubated for 14 days. In addition to the above oligosaccharides, three sugars with R_g values of 0·12, 0·08 and 0·06 were also produced (solvent *A*).

Since these results were generally similar to those reported with β-glucosidase preparations from various moulds and plants (Crook & Stone, 1957) and, in particular, from barley in which the identity of the laminaribiose and gentiobiose was confirmed by chemical methods (Anderson & Manners, 1959), it was decided to examine the acceptor specificity for β-glucosyl residues in greater detail.

Action on cellobiose–sugar mixtures. Digests were prepared containing cellobiose (15 %, w/v) with D-xylose, L-xylose, L-sorbose or N-acetyl-D-glucosamine (15 %, w/v) and enzyme extract (1 %, w/v) and were incubated at pH 5·6 and 35° for 10–15 days. The last two digests contained the same series of sugars as with cellobiose alone. The pentose-containing digests contained additional sugars which stained pink with aniline oxalate. In solvent *B*, those from the D-xylose digest had R_G values 0·70 and 0·40; in the L-xylose digest, R_G 0·72 and 0·42. The sugars with R_G 0·70 and 0·72 were eluted from paper chromatograms with cold water, hydrolysed with sulphuric acid, neutralized and re-chromatographed. Each hydrolysate contained glucose and pentose in apparently equivalent quantities, and no other sugar. The sugars with R_G 0·40 and 0·42, which were present only in small amounts, were similarly examined; both yielded glucose and pentose. It is possible that these sugars are diglucosylxylose trisaccharides.

The presence of more than one new component in the pentose digests was confirmed by chromatography on small charcoal–Celite columns. Elution with water gave a mixture of monosaccharides, with 5 % aqueous ethanol yielded cellobiose, and with 10 % ethanol the following: with D-xylose as acceptor, R_G 0·74, 0·60 (trace) and 0·42 (solvent *B*); L-xylose as acceptor, R_G 0·73 and 0·45 (solvent *B*), all of which gave pink stains with aniline oxalate.

A comparison of the suitability of other pentose sugars to act as glycosyl acceptors was then carried out by incubating D-lyxose, D-ribose, or L-arabinose (7 %, w/v) with cellobiose (7 %, w/v) and algal extract (0·7 %, w/v). A control cellobiose–D-xylose mixture was also prepared. In all cases, new sugars were formed which appeared to be glucosylpentose disaccharides. The R_X values of the products were: from D-lyxose 0·38 (solvent *B*), from D-ribose 0·44, from L-arabinose 0·28, and from xylose 0·43 and 0·27 (trace). With ribose and xylose, the new sugar could be detected within 24 hr.; with lyxose and arabinose, 8 and 15 days' incubation respectively were required.

Control digests showed that oligosaccharide

synthesis did not occur from glucose or xylose alone.

The greatest trans-β-glucosylation occurred with xylose, and this reaction was therefore examined on a larger scale.

Enzymic synthesis of glucosylxylose. Cellobiose (10·0 g.), D-xylose (10·0 g.) and *C. rupestris* fraction (0·3 g.) in water (40 ml.) were incubated at 35° for 14 days. Paper-chromatographic analysis (solvent *B*) showed the presence of a satisfactory amount of the component with R_X 0·43. The digest was heated to inactivate the enzyme, concentrated under reduced pressure, and placed on a charcoal–Celite column (86 × 6 cm.). The glucose and xylose were eluted with water, and the disaccharides (cellobiose and a sugar shown below to be glucosylxylose) with increasing concentrations of aqueous ethanol (0 → 10 %). Approx. 770 mg. of the glucosylxylose was collected; this was freed from traces of glucose and cellobiose by chromatography on Whatman 3MM paper with solvent *B*, and crystallized from ethanol.

Characterization of glucosylxylose. The sugar was homogeneous by paper chromatography, with R_G 1·02 and R_X 0·64 (solvent *A*), and R_X 0·43 (solvent *B*), and gave a pink stain with aniline phthalate and *p*-anisidine; it had m.p. 120°, $[\alpha]_D - 6{\cdot}4°$ in water (*c*, 2·8) and the reducing power of a disaccharide (xylose standard).

Hydrolysis with 0·67N-sulphuric acid for 6·5 hr. at 100° gave, by Pridham's (1956) method, equimolar proportions of glucose and xylose. After oxidation of the disaccharide with hypoiodite, an acid hydrolysate contained glucose but not xylose. Incubation with emulsin at pH 5·0 and 35° gave glucose and xylose.

In view of the mode of synthesis of the sugar, and of the fact that D-xylose exists in solution in the pyranose form, the above preliminary experiments indicate that it is a β-D-glucopyranosyl-D-xylopyranose. The following evidence shows the presence of a 1:3-linkage:

(1) On heating with sodium acetate–phenylhydrazine hydrochloride, a crystalline osazone was formed; after two recrystallizations from ethyl acetate, the bright-yellow crystals had m.p. 205–206° (cf. m.p. 213–215° reported by Barker *et al.* 1957). An acid hydrolysate of the osazone contained glucose and not xylose. These findings eliminate the possibility of a 1:2-linkage.

(2) On oxidation with sodium metaperiodate in the dark at room temperature, a rapid initial reduction of 3·02 mol.prop. was observed. After 22 hr. this had increased to only 3·07 mol.prop. The theoretical values for a 1:3- and 1:4-linked disaccharide are 3·00 and 4·00 mol.prop., respectively. Under alkaline conditions, the sugar was over-oxidized by periodate, since on prolonged oxidation more than 7 mol.prop. of periodate was reduced. This behaviour is typical of oligosaccharides which give rise to intermediates containing malonaldehyde groups (cf. Hough & Perry, 1956).

(3) Confirmation of a 1:3-linkage was provided by a comparison of R_X values (solvents *B* and *C*) with those of an authentic sample of 3-*O*-β-D-glucopyranosyl-D-xylopyranose kindly given by Dr S. A. Barker (cf. Barker *et al.* 1957).

Ulva lactuca *extract*

A digest containing cellobiose (16 %, w/v) and algal extract (1 %, w/v) was incubated at pH 5·0. The presence of laminaribiose, gentiobiose and cellotriose was detected by paper chromatography within 24 hr. Similar results were obtained after 3, 5 and 7 days' incubation. In contrast, a 1 % solution of cellobiose was completely hydrolysed. During the hydrolysis of cellodextrin (1·7 %, w/v), traces of laminaribiose were detected during the first 3 days; after this time, only cellosaccharides were present.

Attempts to demonstrate trans-β-glucosylation from cellobiose to D-galactose, D-mannose, phenol or methanol were unsuccessful. Digests with cellobiose–acceptor mixtures did not contain new sugars (other than those obtained with cellobiose alone) or glucosides after incubation for periods up to 16 days; the phenol and methanol partly inhibited the enzymic reaction.

DISCUSSION

Recent investigations have shown that cellobiase preparations from various higher plants (e.g. sweet almonds, gentian roots, barley) and moulds (e.g. *Myrothrecium verrucaria, Aspergillus niger, A. aureus, A. flavus*) catalyse the formation of oligosaccharides from concentrated solutions of cellobiose (Crook & Stone, 1957; Anderson & Manners, 1959). The present work shows that marine algae contain similar enzyme systems. The tentative identification of laminaribiose, gentiobiose and cellotriose suggests that, with cellobiose as β-glucosyl donor, both glucose (OH at C-3 and C-6) and cellobiose may function as acceptor substrates. By contrast, D-galactose, D-mannose, L-sorbose, *N*-acetyl-D-glucosamine, phenol and methanol do not appear to satisfy the specificity requirements. In a similar experiment with *A. oryzae* β-glucosidases, Jermyn & Thomas (1953) were able to demonstrate the transfer to methanol of β-glucosyl residues from *p*-nitrophenyl β-D-glucoside, but not from cellobiose.

The synthesis of 3-*O*-β-D-glucopyranosyl-D-xylopyranose from a cellobiose–xylose mixture is of interest since D-xylopyranose has, with the

exception of C-5, the same configuration as D-glucopyranose. The formation of this disaccharide thus parallels that of laminaribiose. This behaviour is similar to that reported by Barker *et al.* (1957) for *A. niger* (152) and differs from that of another strain of this mould (E 19) which was unable to effect appreciable trans-β-glucosylation to D-xylose (Crook & Stone, 1957). It is presumed that the hydroxyl groups at C-2 and C-4 of D-xylose, and at C-2, C-3 and C-4 in the other pentoses are less favourable acceptor sites for the algal cellobiase.

D-Xylose is not an acceptor substrate for trans-α-glucosylation by extracts of *C. rupestris* (Duncan & Manners, 1958).

SUMMARY

1. Extracts of *Cladophora rupestris* and *Ulva lactuca* catalyse the synthesis of oligosaccharides from concentrated solutions of cellobiose. Laminaribiose, gentiobiose and cellotriose have been identified by paper chromatography.

2. On incubation of *C. rupestris* extract with cellobiose–pentose mixtures, glucosylpentose disaccharides are produced. 3-*O*-β-D-Glucopyranosyl-D-xylopyranose has been isolated from a cellobiose–xylose mixture and characterized.

3. The acceptor specificity of cellobiases from various biological sources is discussed.

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The Action of β-Amylase, Muscle Phosphorylase and Potato Phosphorylase on some Glycogens. By A. MARGARET LIDDLE and D. J. MANNERS. (*Department of Chemistry, University of Edinburgh*)

In continuation of structural studies on α-1:4-glucosans, a number of glycogens of known average chain length (determined by potassium periodate oxidation) have been degraded by β-amylase, and by muscle and potato phosphorylases. β-Amylase catalyses a stepwise hydrolysis of *alternate* linkages in exterior chains of branched α-1:4-glucosans, liberating maltose, whilst phosphorylases in the presence of inorganic phosphate, degrade *successive* linkages yielding glucose 1-phosphate. Since neither enzyme can hydrolyse or by-pass the 1:6 inter-chain linkages, the yield of maltose or glucose 1-phosphate is determined, to a first approximation, by the exterior chain length.

Twenty different glycogens had β-amylolysis limits in the range 25–56 %, corresponding to β-limit dextrins (β-dextrins) with average chain lengths of 6–9. Ten different glycogens on treatment with muscle phosphorylase gave 14–35 % conversion to glucose 1-phosphate, corresponding to muscle phosphorylase limit dextrins (φ-dextrins) with average chain lengths of 6–12. Glycogens which had been precipitated by 80 % acetic acid and by ethanol had similar β-amylolysis and phosphorolysis limits (cf. Illingworth, Larner & Cori, 1952). Samples of waxy maize starch had β-amylolysis and phosphorolysis limits of 50–59 % and 40–43 % respectively.

A comparison of the average chain lengths of glycogen β- and φ-dextrins shows that the difference between them is not constant. We interpret this variation as an indication that different glycogens have different degrees of multiple-branching. Assuming (cf. Cori & Larner, 1951; Peat, Whelan & Thomas, 1952) that the A-chain 'stubs' of β- and φ-dextrins contain 2·5 and 1 glucose residue respectively, and that the corresponding B-chain 'stubs' contain n and $(n+4)$ residues, the above variation can be related to the ratio of A- and B-chains in the molecule, i.e. the degree of multiple-branching. (The most probable value of n is 2–3; for definition of A- and B-chains, see Peat, Whelan & Thomas (1952).) The calculated ratios $(\overline{AB})$ vary from approximately 1:1 (*Helix pomatia* glycogen) to 1:3 (foetal sheep liver glycogen).

Potato phosphorylase differs in action pattern from muscle phosphorylase since the former causes only approximately 10 % degradation of 13-unit glycogens.

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Observations on Barley β-Glucosidases. By D. J. MANNERS. (*Department of Chemistry, University of Edinburgh*)

Dillon & O'Colla (1950, 1951) have reported the occurrence of 'laminarinase' in wheat β-amylase preparations and in extracts of barley, oats, potato and hyacinth bulbs, whilst group specific β-glucosidase activity of an enzyme (*Z*-enzyme) isolated from soya-bean β-amylase preparations has been demonstrated by Peat, Thomas & Whelan (1952).

During an investigation of the action of amorphous barley β-amylase on glycogen (Bell & Manners, 1951), it was observed that β-1:3-glucosans (yeast glucan, laminarin) were also hydrolysed by this preparation. Crystalline sweet potato β-amylase, malt α-amylase and salivary α-amylase showed no 'glucanase' or 'laminarinase' activity. Accordingly, the barley β-amylase preparation was treated by the procedure devised by Peat *et al.* (1952) for the isolation of *Z*-enzyme from soya beans; the resulting preparation was free of amylolytic activity, but showed hydrolytic activity towards several β-glucosides.

β-Glucosidase activity has been followed by cuprimetric titration, paper chromatography and manometrically using glucose oxidase. The barley preparation hydrolysed aesculin, amygdalin, arbutin, cellobiose, cellodextrin, gentiobiose, helicin, laminarin, methyl β-glucoside, phenyl β-glucoside, phloridzin, salicin and yeast glucan. Cellodextrin and laminarin were hydrolysed in random fashion yielding glucose and a series of oligosaccharides. The 'laminarinase' activity was optimum at pH 5·0 (cf. Dillon & O'Colla, 1951) and was not inhibited by mercuric chloride (cf. Peat *et al.* 1952).

The available evidence indicates that barley contains at least three different enzymes catalysing the hydrolysis of β-glucosidic linkages, viz. a group specific β-glucosidase, an endo-β-1:4-glucosanase (cellulase) and an endo-β-1:3-glucosanase; the separation of these activities is being investigated.

During enzyme action on a concentrated solution of cellobiose, the formation of higher saccharides was observed; one of these appears to be cellotriose. The barley β-glucosidase preparation, unlike emulsin, cannot synthesize higher saccharides from glucose.

β-Amylase preparations from rye also show β-glucosidase activity (Liddle & Manners, unpublished).

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OBSERVATIONS ON THE SPECIFICITY OF YEAST *iso*AMYLASE

By D. J. Manners and Khin Maung

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In the course of studies on carbohydrate metabolising enzymes in brewer's yeast, we have subjected waxy maize starch to degradation by yeast phosphorylase; 44% conversion to glucose-1-phosphate was observed. The residual dextrin was then treated with β-amylase, resulting in 29% conversion to maltose. This represents 60% total degradation of the original polysaccharide, whereas β-amylase normally removes 50% of the molecule. The phosphorylase was prepared by ammonium sulphate fractionation of an extract of brewer's yeast; it was free of α-amylase, and had a phosphorylase activity similar to that reported[1] for preparations from potatoes and broad beans, namely *ca.* 100 units per g.

Since the action of phosphorylase and β-amylase is confined to the exterior chains of amylopectin and glycogen,[2] we concluded that the phosphorylase preparation contained a debranching enzyme which catalysed the hydrolysis of a proportion of the 1 : 6 inter-chain linkages in waxy maize starch, thereby removing the barriers to β-amylase action.

In 1951, Maruo and Kobayashi[3] reported that autolysates of brewer's yeast contained an enzyme which hydrolysed the inter-chain linkages in glutinous rice starch producing a polysaccharide of lower molecular weight and increased susceptibility to β-amylase. This enzyme, previously known as amylosynthease, was renamed *iso*amylase. It appears probable, therefore, that our phosphorylase preparation contained isoamylase.

By acetone fractionation (at − 10°) of a brewer's yeast extract, we have obtained a protein preparation (hereafter referred to by the provisional name of *iso*amylase) which catalyses the hydrolysis of a proportion of the 1 : 6-linkages in glycogen[4] (from brewer's yeast), β-dextrin (prepared by the action of β-amylase on waxy maize starch) and maltodextrin (a mixture of linear and branched maltosaccharides prepared by the action of malt α-amylase on potato starch). The polysaccharides were incubated with *iso*amylase at pH 7·0 at 20° for various times; after inactivation of the enzyme, the β-amylolysis limit was determined.

Time of incubation with *iso*amylase	β-Amylolysis limit (%)		
	Glycogen	β-Dextrin	Maltodextrin
Nil ..	44	0	67
5 min. ..	49	19	—
2 hr. ..	54	20	—
24 hr. ..	57	32	94

These results demonstrate hydrolytic activity towards non-terminal α-1 : 6-glucosidic linkages. During 24 hr. a 47% increase in the absorption value of the iodine-glycogen complex at 680 mμ was observed. However, attempts to follow enzyme action on β-dextrin by iodine-staining were not successful; this criterion of activity has also been found to be unsatisfactory in similar experiments with the debranching enzyme of higher plants (R-enzyme)[5].

*iso*Amylase action on glycogen is incomplete, and is presumably limited to A-chains (side chains); incubation of BDH glycogen with *iso*amylase for 24, 48, and 72 hours gave a similar increase in β-amylolysis limit, from 45 to 65%. If yeast glycogen and β-dextrin have multiply branched " tier " structures, with 50% of the branch points in the outermost " tier,"[6] then hydrolysis of the outermost branch points (which join A-chains to the rest of the molecule) would increase the β-amylolysis limits to 58 and 32%, respectively.

*iso*Amylase also catalyses the hydrolysis of terminal α-1 : 6-glucosidic linkages, since it hydrolyses *iso*maltose. Furthermore, on incubation with a glycogen of abnormal structure,[7] which contains a number of single glucose residues attached by 1 : 6-linkages to the rest of the molecule, glucose was liberated.

The *iso*amylase preparation was free from α-amylase, phosphorylase and branching enzyme, but contained a trace of maltase.

Debranching enzymes previously reported include R-enzyme[8] (from potatoes and broad beans), and amylo-1 : 6-glucosidase[9] (from rabbit muscle). R-enzyme catalyses the hydrolysis of non-terminal α-1 : 6-glucosidic linkages in amylopectin and its β-dextrin, and in α-dextrins from amylopectin and glycogen; it has no appreciable action on glycogen or on *iso*maltose. By contrast, amylo-1 : 6-glucosidase action is limited to the hydrolysis of *iso*maltose and terminal α-1 : 6-glucosidic linkages in muscle phosphorylase limit dextrins from glycogen and amylopectin. *iso*Amylase has, therefore, a wider specificity than either R-enzyme or amylo-1 : 6-glucosidase.

*iso*Amylase, together with α-1 : 4-glucosidases, is now being used in investigations of the fine structure of amylopectin and glycogen.

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Yeast Branching Enzyme. By D. J. MANNERS and KHIN MAUNG. (*Department of Chemistry, University of Edinburgh*)

During the purification of phosphorylase and isoamylase from brewer's yeast (Manners & Khin Maung, 1955) we observed that certain protein fractions catalysed a rapid decrease in the iodine staining power of amylose. Further investigation has shown that this activity was caused by a branching enzyme, i.e. an amylose → glycogen transglucosidase.

By ethanol-citrate fractionation (at $-5°$) of a brewer's yeast extract, we have obtained a protein preparation which reduced the iodine staining power of amylose, soluble starch, amylopectin, and amylopectin β-dextrin without concomitant liberation of reducing groups. On incubation of amylose (B.V. 1·33; β-amylolysis limit 84 %) with branching enzyme, the iodine absorption value at 680 mμ. decreased by 66 and 81 % within 1 and 4 hr. respectively; the β-amylolysis limit likewise decreased, to 50 and 48 %. After 20 hr. incubation, the end-product stained red-brown with iodine, the absorption spectrum having a maximum at 520 mμ. (cf. amylopectin $\lambda_{max.}$ 540 mμ., glycogen 460–490 mμ.). Yeast branching enzyme has no action on glycogen or glycogen β-dextrin.

Enzyme action is inhibited by mercuric chloride (5×10^{-4} M); it is optimum at pH 7·0 and 20°, these conditions being similar to those for the branching enzymes (*Q*-enzymes) of plants (Hobson, Whelan & Peat, 1950). Unlike the branching enzyme from *Polytomella coeca* (Barker, Bebbington & Bourne, 1953), the yeast enzyme is not activated by maltosaccharides.

The branch points synthesized by yeast branching enzyme are hydrolysed by yeast isoamylase, and are therefore similar to those in amylopectin and glycogen (i.e. α-1:6-glucosidic). Treatment of amylose with branching enzyme for 4·5 hr. gave a branched polysaccharide with a β-amylolysis limit of 45 %. After treatment with isoamylase, the β-amylolysis limit was 65 % (cf. Manners & Khin Maung, 1955).

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MECHANISM OF THE DEGRADATION OF POTATO AMYLOSE BY β-AMYLASE

By J. M. G. Cowie, I. D. Fleming, C. T. Greenwood and D. J. Manners

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Although the general action pattern of β-amylase on amylose is well-established, the specific reaction mechanism is uncertain, and has been the subject of much controversy.

Swanson,[1] on the basis of iodine absorption measurements, and Kerr and Cleveland,[2] using physical measurements, suggested that the mechanism is of the " single-chain " type in which the enzyme attaches itself to one amylose molecule and degrades it completely before attacking another. However, Bourne and Whelan[3] and Hopkins and Jelinek[4] have interpreted their iodine absorption measurements as indicating a " multi-chain " mechanism whereby all the amylose molecules are degraded simultaneously. More recently, Hopkins and his collaborators[5] have also suggested that β-amylase degrades short-chain amylose molecules by multi-chain action.

If the mechanism is of the single-chain type, then under normal experimental conditions, with a large substrate/enzyme ratio, the degree of polymerization ($\overline{D.P.}$) of the residual amylose at any time during the reaction will be the same as that for the original up to the stage when the number of substrate molecules is approximately equal to the number of enzyme molecules. (At this latter point, a multi-chain mechanism is then inevitable.) On the other hand, if a multi-chain mechanism is operative throughout, then the $\overline{D.P.}$ of the amylose will decrease as the reaction proceeds, the reduction being proportional to the percentage conversion to maltose. The reaction mechanism can therefore be established unequivocably from measurements of the molecular weight of the residual amylose at varying stages of β-amylolysis.

Experiments of this type have been carried out by Kerr and Cleveland,[2] who isolated the polymer-product after 50% conversion to maltose and found the $\overline{D.P.}$, iodine affinity, and limiting viscosity number of this to be virtually the same as the original amylose. We have extended this treatment by measuring the $\overline{D.P.}$ of the residual polymer at varying degrees of conversion throughout the reaction, as well as the $\overline{D.P.}$ of a 50% conversion dextrin and a 75% β-limit dextrin.[6]

Amylose (prepared under oxygen-free conditions from potato starch as previously described[7] and having an initial $\overline{D.P.}$ of about 3500) was incubated at pH 4·6 and 35°C. with soya bean and barley β-amylases, which hydrolysed the polysaccharide to give 75% and 100% conversion to maltose, respectively. (The barley enzyme preparation showed Z-enzyme activity.[6]) Portions of each digest were examined at intervals, in 0·2M potassium hydroxide, in an electrically-driven Spinco ultracentrifuge; each fraction was examined at several dilutions in view of the concentration dependence of the sedimentation constant of amylose in alkali.[8]

The sedimentation constant of the original amylose was also determined and comparison of the results indicated that up to 75% conversion to maltose, the residual polymer product in both digests had, within the limits of experimental error, the same sedimentation constant as the original polysaccharide.

Furthermore, a detailed study of the sedimentation constants, limiting viscosity numbers,[7] and iodine affinities[7] of both a 50% conversion product (prepared by using barley β-amylase) and a 75% β-limit dextrin (obtained using soya bean β-amylase) showed that the properties of these polysaccharides differed only slightly from those of the original amylose.

On the basis of these results, it would appear that, in agreement with Kerr and Cleveland,[2] the mechanism of the action of the two β-amylases used here on pure potato amylose of high $\overline{D.P.}$ is of the " single-chain " type.

It is hoped to publish full details of these results and experimental methods elsewhere.

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OBSERVATIONS ON THE SPECIFICITY OF R-ENZYME

By I. D. Fleming and D. J. Manners

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The outermost α-1:6-glucosidic inter-chain linkages in amylopectin and amylopectin β-dextrin are hydrolysed by a " debranching " enzyme, named R-enzyme, isolated from the potato and broad-bean by Hobson, Whelan and Peat.[1, 2] Enzyme action on both substrates results in an increased iodine staining power (measured at 680mμ) and β-amylolysis limit, and a decrease in specific viscosity. By contrast, incubation of rabbit, liver or oyster glycogen (average chain length, $\overline{CL}$ 12-14; cf. 24 for amylopectin) with R-enzyme failed to increase the β-amylolysis limit, although a small increase in iodine staining power (measured at 480mμ) was noted.[3] Peat and his co-workers concluded[3] that the majority of the α-1:6-glucosidic linkages in glycogen were inaccessible to R-enzyme, and that this observation provided a simple means of differentiating between " glycogen " and " amylopectin ".

This Communication describes a comparison of R-enzyme action on a typical glycogen and amylopectin with that on two glycogen samples with intermediate $\overline{CL}$ values. The enzyme was isolated from broad-beans[2, 3] and the β-amylolysis limit of each polysaccharide determined before (*B*) and after (*A*) incubation with R-enzyme (Table I). The detailed experimental conditions were similar to those described in ref. 3. The measurement of $\overline{CL}$ values, exterior chain lengths ($\overline{ECL}$), and interior chain lengths ($\overline{ICL}$) has been reported elsewhere.[4]

Table I

Effect of R-enzyme on the β-amylolysis limit of certain polysaccharides

Polysaccharide	*β-Amylolysis limit (%)*		$\overline{CL}$	$\overline{ECL}$	$\overline{ICL}$
	B	*A*			
Rabbit liver IV glycogen	46	47	13	8–9	3–4
Rabbit liver VI glycogen	51	58	18	11–12	5–6
Trichomonas foetus I glycogen	61	62	15	11–12	2–3
Potato amylopectin	51	71	27	16–17	9–10

The absorption spectrum of the iodine complexes of the rabbit liver glycogens and an amylopectin were also measured, before and after incubation with R-enzyme (Table II). In all cases, a small but significant increase in absorption was noted.

Table II

Effect of R-enzyme on the iodine staining power of certain polysaccharides

Polysaccharide	*Properties of absorption spectrum of iodine complex*		
	λmax.mμ	*Optical density max.*	
		B	*A*
Rabbit liver IV glycogen	460	0·28	0·29
Rabbit liver VI glycogen	470	0·31	0·34
Waxy maize starch	530–540	0·68	0·74

The above evidence indicates that R-enzyme has an appreciable action on 18-unit rabbit liver glycogen. It follows that the susceptibility of a branched α-1:4-glucosan to R-enzyme cannot be used unequivocally to distinguish a " glycogen " from an " amylopectin ". Although the majority of animal glycogens have $\overline{CL}$ values in the range 10-14, several examples of 18-unit glycogen are known.[5] It must also be noted that certain plant polysaccharides (from sweet corn) are resistant to R-enzyme.[6] It is therefore suggested that the term " glycogen " should be confined to the reserve polysaccharide of animal and certain microbial tissues, even though individual samples show variations in molecular structure. The term " phytoglycogen " has been proposed for structurally similar plant polysaccharides.[6]

The results in Tables I and II, and the data of Peat and his co-workers, show that the specificity of R-enzyme is controlled not by the degree of branching in the substrate, or the exterior chain length, but by the average length of the interior chains. It appears that R-enzyme will hydrolyse the outermost inter-chain linkages in a branched α-1:4-glucosan, irrespective of source, in which the branch points are separated by a minimum of five glucose residues.

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ENZYMIC CONVERSION OF AMYLOPECTIN INTO A GLYCOGEN-TYPE POLYSACCHARIDE

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The enzymic synthesis of amylopectin or glycogen from α-D-glucosyl phosphate is catalysed by two separate enzymes.[1] The first, phosphorylase, synthesises linear amylose-type molecules which are converted into branched molecules containing α-1 : 6-glucosidic inter-chain linkages by a "branching enzyme" (systematic name amylo-1 : 4→1 : 6-transglucosidase). There appear to be two classes of branching enzyme: (*a*) those which act on amylose but have no appreciable action on amylopectin, e.g. Q-enzyme from the potato,[2] broad bean,[3] and the flagellate *Polytomella coeca*,[4] (*b*) those capable of introducing branch points into both amylose and amylopectin, e.g. branching enzymes from yeast[5] and liver.[6] Q-enzyme action on amylose yields a branched polysaccharide with many of the chemical and physical properties of amylopectin.[7] In continuation of studies on yeast branching enzyme,[5] we now describe its action on amylopectin.

A sample of amylopectin, prepared by Mr. A. Wright by thymol fractionation of potato starch, was treated at pH 6·8 and 20° with yeast branching enzyme. The latter was free from α-amylase, isoamylase[8] and maltase. Enzyme action resulted in a marked decrease in the absorption value (A.V.) of the polysaccharide-iodine complex (measured at 540 mμ) and a small but significant decrease in β-amylolysis limit (Table 1).

Table 1

Effect of branching enzyme on the A.V. and β-amylolysis limit of amylopectin

Time of incubation (hr.)	*Decrease in A.V. (%)*	*β-Amylolysis limit (%)*
0	—	53·2
2	30	52·0
5	40	51·5
6·5	48	50·5
8	56	49·5
18	92	47·2

After 24 hr., the enzyme was inactivated and the residual polysaccharide isolated and purified. It was soluble in cold water, with $[\alpha]_D + 198°$, and the solution gave a red-brown colouration with iodine. The results of a chemical and enzymic analysis are compared in Table 2 with those of the original amylopectin and a typical animal glycogen.

Table 2

A comparison of the properties of amylopectin-glycogen type polysaccharides

Property	*Potato amylopectin*	*Synthetic polysaccharide*	*Rabbit liver glycogen*
Average chain length* (glucose residues)	22·0	13·5	12·6
Proportion of 1:6-linkages (%)	4·5	7·4	8·0
Iodine complex			
λ max. (mμ)	545	480	460
A.V. max	1·30	0·56	0·41
α-Amylolysis limit (%)	89	79	78
β-Amylolysis limit† (%)			
(*b*)	53	47	46
(*a*)	80	75	78
Exterior chain length (glucose residues)	14·2	8·9	8·3
Interior chain length (glucose residues)	6·8	3·6	3·3
Limiting viscosity number‡	205	12	10

* Determined by potassium periodate oxidation
† (*b*) Before, (*a*) after treatment with isoamylase
‡ Measured in 0·1M sodium chloride, concentration as g./ml.

The periodate-oxidised polysaccharide was reduced with potassium borohydride and hydrolysed with acid. The neutralised hydrolysate did not contain glucose. The branching enzyme had not, therefore, introduced 1 : 2- or 1 : 3-linkages into the amylopectin. This conclusion is supported by the hydrolysis of the outermost inter-chain linkages in the synthetic polysaccharide by yeast isoamylase.[8]

It is concluded that the yeast branching enzyme has introduced a further 2·9% of α-1 : 6-glucosidic inter-chain linkages into amylopectin and that the resulting polysaccharide has a compact and highly branched structure closely resembling that of a normal glycogen.

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135. *Physicochemical Studies on Starches. Part IX.* The Mechanism of the β-Amylolysis of Amylose and the Nature of the β-Limit Dextrin.*

By J. M. G. Cowie, I. D. Fleming, C. T. Greenwood, and D. J. Manners.

The mechanism of the hydrolysis of amylose by (*a*) pure β-amylase, and (*b*) β-amylase and Z-enzyme, has been studied by measuring the $\overline{\text{D.P.}}$ of the residual polymer at varying degrees of conversion into maltose. Amylose fractionated severally in presence and absence of air, and a sub-fraction obtained by aqueous leaching of the granule, have been used as substrates. The molecular properties of the various 50% conversion dextrins and the 77% β-limit dextrins were examined in detail. In all cases, hydrolysis proceeded by an essentially single-chain mechanism as there was no evidence of molecules other than amylose of $\overline{\text{D.P.}} \geqslant$ the original and maltose in the digest. The structure of the β-limit dextrin, which is thought to contain a randomly-situated barrier to β-amylolysis, is discussed.

The action of β-amylase on amylose, which commences at the non-reducing end of the molecule, involves hydrolysis of alternate α-1 : 4-glucosidic linkages with the production of maltose. In a recent paper,[1] we reported the β-amylolysis of an amylose of high molecular weight, prepared [2] by thymol fractionation of potato starch after complete disruption of the granules. Our results confirm that the *pure* enzyme degrades only *ca.* 75% of amylose, and that for complete conversion into maltose a second enzyme (Z-enzyme) is required. The specificity of the latter enzyme was examined, and evidence presented that the β-amylolysis limit for the pure amylose is not an artefact associated with the colloidal instability of the amylose substrate.[1] The nature of the barrier to β-amylolysis is not known.

The specific mode of action of the β-amylase has been in dispute.[3] Maltose may be produced by the enzyme either by (1) attachment to one amylose molecule and then, by stepwise removal of maltose units, complete degradation before attack on another amylose molecule (" single-chain " action), or (2) by removal of one maltose unit on each random collision with an amylose molecule, with the result that all chains in the system will be shortened simultaneously (" multi-chain " action). Reaction mechanisms between (1) and (2) are also possible. However, determination of the molecular weight of the residual polysaccharide at intermediate stages of β-amylolysis will indicate which mechanism is operative. Under normal experimental conditions, with a large substrate : enzyme ratio, the number-average degree of polymerisation ($\overline{\text{D.P.}}$) of the residual amylose at any time during a single-chain reaction will be the same as that for the original up to the stage when the number of substrate molecules is approximately equal to the number of enzyme molecules (at this point, a multi-chain mechanism is inevitable). For multi-chain action throughout, the $\overline{\text{D.P.}}$ of the amylose will decrease as the reaction proceeds, the reduction being proportional to the percentage conversion into maltose. Experiments of this type have been carried out by Kerr and Cleveland,[4] who found that the polymeric product isolated at about 50% conversion into maltose possessed virtually the same iodine affinity, limiting viscosity number, and $\overline{\text{D.P.}}$ as the original amylose. In our work (a preliminary account of which has appeared [5]), we have extended this type of experiment to include the measurement of the $\overline{\text{D.P.}}$ of the residual polymer at varying degrees of conversion into maltose resulting from the action of (*a*) pure β-amylase, and (*b*) β-amylase and Z-enzyme. Amylose fractionated severally in presence and absence of air, and a subfraction obtained by aqueous leaching of the granule, have been used as substrates. The molecular size of various 50% conversion dextrins and 77% β-limit dextrins has been examined, and the structure of the β-limit dextrin is discussed.

* Part VIII, *J.*, 1957, 4640.

Experimental

Preparation of Amylose Samples and their Characterisation.—Potato starch (var. Arran Banner) was fractionated by (1) dispersion in the presence or absence of oxygen, and (2) aqueous leaching at 70°. These methods and those used to characterise the polymers have been described in detail previously in this Series.

Preparation of Enzymes.—Barley β-amylase and soya-bean β-amylase were used. Their preparation and properties have been described elsewhere.[1]

Digest Conditions.—At pH 4·6 and 35° soya-bean β-amylase showed no Z-enzyme activity and converted 77% of amylose samples of high $\overline{D.P.}$ into maltose, whilst under these conditions barley β-amylase hydrolysed all samples completely.[1] Amylose was dissolved directly in water from the well-centrifuged butan-1-ol complex and buffered with acetate to pH 4·6. Enzyme solution [1] was added and the reaction rate followed by withdrawal of aliquot parts at intervals and estimations of the liberated maltose. In all digests, the concentration of enzyme (100 units per mg. of amylose) was such that 50% conversion had occurred within 30 min., and hence retrogradation of amylose was unlikely.

Isolation of and Measurements on β-Amylolysis Products.—The $\overline{D.P.}$ of the polymeric product at different percentage conversions was obtained by withdrawing aliquot parts (2 ml.) of the digest and adding M-potassium hydroxide (0·5 ml.). The resultant 0·2M-potassium hydroxide solution (maximum concentration of amylose, 0·18 g./100 ml.) was examined directly in the Spinco ultracentrifuge. Each aliquot portion was studied at three dilutions; the limiting dilution was 0·02 g./100 ml. The 50% conversion and 77% β-limit dextrins were isolated by adding butan-1-ol, then heating the digest for 2 min. on a boiling-water bath (to complete deactivation of enzyme) and allowing the butan-1-ol complexes to be precipitated at room temperature during 24 hr. After removal by centrifugation, the complexes were thoroughly washed with butan-1-ol-saturated water to remove maltose.

Discussion

A study of the β-amylolysis of amylose is complicated by the fact that the β-limit depends on the method of preparation of the amylose. Our aqueous leaching experiments [1, 2, 6] have shown that potato starch granules contain an easily accessible amylose fraction of relatively low molecular weight, which is completely hydrolysed by pure β-amylase. In this work, the action pattern of β-amylase on the *whole* amylose has been studied in order to investigate the β-limit dextrin and also to use conditions equivalent to those of other workers. The action pattern under these conditions proved to be identical with that for the completely linear amylose prepared by aqueous leaching.

Action of Pure β-Amylase.—(a) *Whole amylose.* Preliminary trial digests showed that the sample of amylose used ($\overline{D.P.}$ 3200) gave 77% conversion into maltose. When aliquot parts were removed at intervals, and studied in the ultracentrifuge, the sedimentation constant (S_{20}) of the residual polymer as a function of the concentration (c) was as shown in Fig. 1*a*. (The sedimentation constant for amylose is concentration-dependent, as previously reported.[7]) All the points lie on the same curve, within experimental error, although there is a tendency for the values in the earlier stages of conversion to be slightly higher. Fig. 1*a* also shows the corresponding plot of S_{20} against $S_{20}.c$ as recommended by Gralén [8] to facilitate extrapolation to infinite dilution. The points again lie on the same curve. The limiting value of S_{20} for all the residual amyloses was therefore independent of the degree of conversion into maltose up to and including the 77% limit. This result would not be expected on the basis of multi-chain action.

In order to confirm that the sedimentation constant of the β-limit dextrin was unchanged, it was isolated from a large-scale digest. A comparison of the properties with those for the original amylose were as shown:

	$[\eta]$	Iodine affinity	$10^{13}(S_{20})_0$
Original amylose	430	19·5	13·1
77% limit	415	19·2	13·1

The sedimentation results are shown in Fig. 1*b*. The agreement in sedimentation constants shows that the liberated maltose does not influence S_{20} when portions of the digest are

studied directly. The properties of the two polymers are identical within experimental error.

Further, paper chromatography of the digest showed that sugars other than maltose were not present. It was apparent that, in the hydrolysis of amylose by pure β-amylase at pH 4·6 and 35°, the hydrolysate contains only amylose with a $\overline{\text{D.P.}}$ greater than or equal to that of the original and maltose. Multi-chain action is therefore excluded.

If amylose is attacked only from the non-reducing end then, as has been stressed by Kerr and Gehman,[9] the rate of maltose production should be proportional to the molarity rather than the actual weight concentration of amylose. Accordingly solutions of amylose of varying $\overline{\text{D.P.}}$ but equal molarities should show the same rate of production of maltose. Results of experiments for two $1{\cdot}4 \times 10^{-6}$M-solutions were as shown:

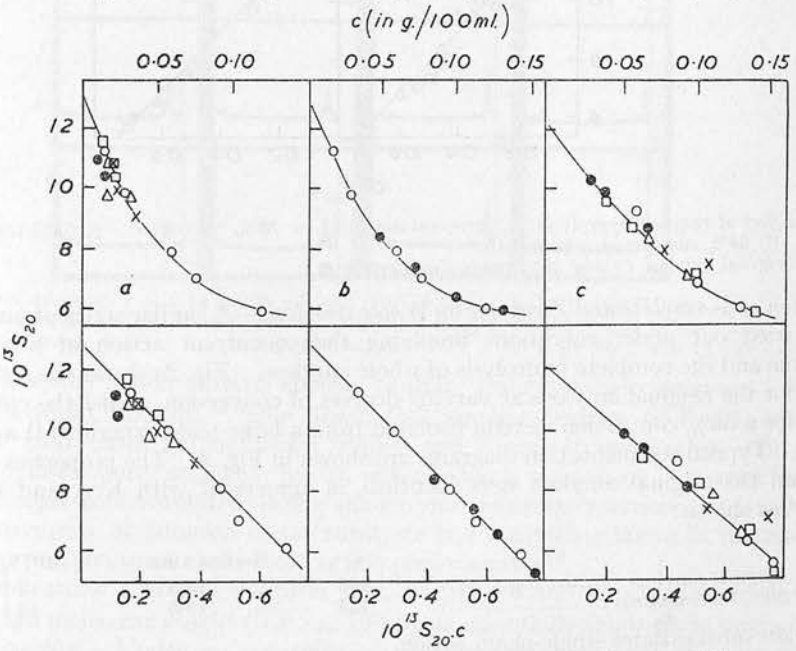
Conversion (%) into maltose at a given time (min.).

$\overline{\text{D.P.}}$ of amylose	5	10	15	30	60
3200	34·9	44·9	55·6	71·0	77·8 (const.)
2000	32·5	44·7	53·0	71·1	75·2 (const.)

These results again substantiate an essentially single-chain action. Similar results have been reported by Kerr and Gehman.[9]

Although it has been reported[10] that oxygen-treatment can introduce barriers to the phosphorolysis of amylose, it was shown elsewhere[1] that amylose prepared in the presence

FIG. 1. S_{20} versus c *and* S_{20} versus $S_{20}.c$ *for amyloses treated with pure β-amylase.*



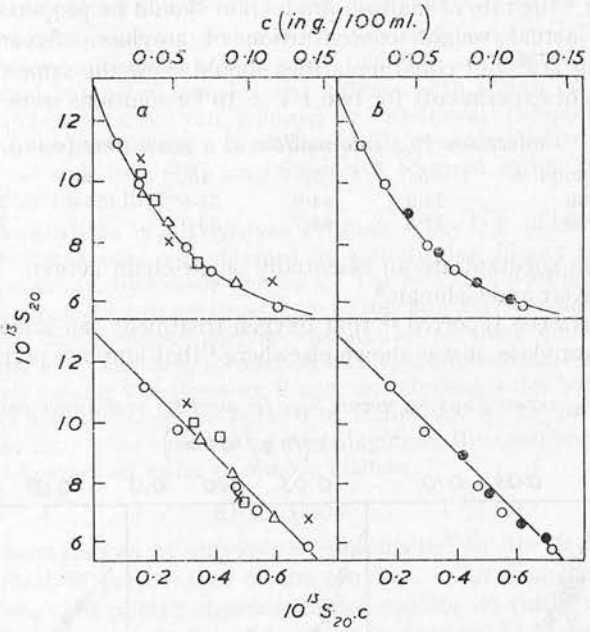
(*a*) *Effect of enzymic hydrolysis*: ○ *original amylose*; × 35% *conversion*; △ 45% *conversion*; □ 55% *conversion*; ● 77% *conversion into maltose.*
(*b*) *Original amylose* ○ *and* 77% *limit dextrin* ●.
(*c*) *Effect of enzymic hydrolysis (oxygen-treated amylose)*: ○ *original amylose*; × 23% *conversion*; △ 34% *conversion*; □ 56% *conversion*; ● 75% *conversion into maltose.*

of oxygen was hydrolysed as far as 75% conversion into maltose. The enzymic degradation of this amylose sample was also studied in detail. Fig. 1*c* shows the plots of S_{20} against c and against $S_{20}.c$ for the residual amylose at varying degrees of conversion into maltose. Again, within experimental error, the limiting value of S_{20} for the residual amylose is independent of the conversion up to, and including, the limit. Typical

sedimentation diagrams are shown in Fig. 3. Essentially single-chain attack is therefore again established.

(b) *Aqueous-leached amylose.* To confirm the above action pattern, a 50% conversion product was prepared from a sample of amylose leached [2,6] at 70°. The limiting viscosity number of this product was the same as that for the original amylose, within experimental error (for the original amylose, $[\eta] = 270$; for the 50% conversion dextrin, $[\eta] = 265$).

FIG. 2. S_{20} versus c *and* S_{20} versus $S_{20}.c$ *for amyloses treated with* β*-amylase and Z-enzyme.*



(*a*) *Effect of enzymic hydrolysis*: ○ *original amylose*; × 35% *conversion*; △ 53% *conversion*; □ 64% *conversion into maltose.*
(*b*) *Original amylose* ○ *and* 50% *conversion dextrin* ●.

Action of β*-Amylase and Z-enzyme on Whole Amylose.*—A similar series of investigations was carried out under conditions involving the concurrent action of β-amylase and Z-enzyme and the complete hydrolysis of whole amylose. Fig. 2*a* shows the sedimentation results for the residual amylose at varying degrees of conversion, whilst the corresponding results for a 50% conversion dextrin (isolated from a large-scale experiment) are shown in Fig. 2*b*. Typical sedimentation diagrams are shown in Fig. 3. The properties of the 50% limit and the original amylose were identical, in agreement with Kerr and Cleveland's results,[4] as shown:

	$[\eta]$	Iodine affinity	$10^{13}(S_{20})_0$
Original amylose	430	19·5	13·1
50% limit dextrin	425	19·6	13·1

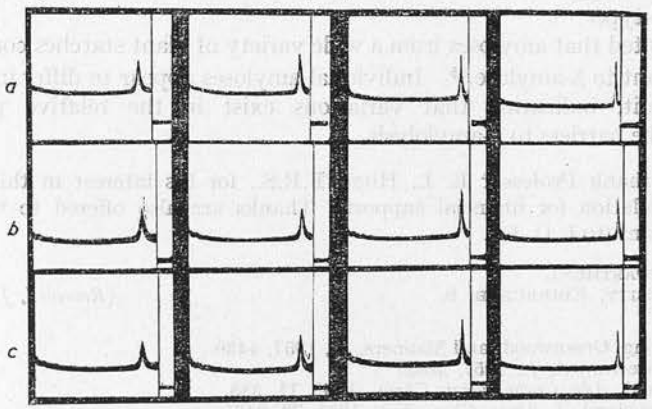
This again substantiates single-chain action.

Effect of β*-Amylase Action on the Absorption Spectra of the Amylose–Iodine Complex.*—Swanson [11] observed that the wavelength of maximum absorption ($\lambda_{max.}$) of the amylose-iodine complex was unaltered during β-amylolysis, thus indicating single-chain action. However, Bourne and Whelan [12] have criticised Swanson's iodine-staining conditions, and found a movement of $\lambda_{max.}$ from 660 to 580 mμ when equal weights of polysaccharide were stained during the formation of 86% of maltose. We have therefore incubated amylose of D.P. 3200 with β-amylase, and stained equal weights of residual amylose at intervals up to the production of 84% of maltose. In all samples, the $\lambda_{max.}$ remained unchanged at *ca.* 660 mμ. It now seems probable that Bourne and Whelan's results are due, in part, to

contamination of amylose with amylopectin (the β-dextrin of amylopectin [13] has $\lambda_{max.}$ *ca.* 540 mμ), and are not the direct result of multi-chain action. (Amylose prepared recently in this Laboratory by the aluminium hydroxide method [14] contained only 81—87% of amylose.) It should also be noted that iodine-staining measurements on amylose of high $\overline{D.P.}$ (*ca.* 3000) do not enable the reaction mechanism to be determined. (With single-chain action, no movement of $\lambda_{max.}$ is to be expected, whilst, with multi-chain action, the residual amylose at 84% β-amylolysis would have a $\overline{D.P.}$ of *ca.* 500; $\lambda_{max.}$ for this would be little altered.[3])

The Action Pattern of β-Amylase.—All the above results are inconsistent with the concept of multi-chain action. Rather, it appears that, under the conditions of our experiments, the amylose after making contact with a substrate molecule hydrolyses it completely before attacking another molecule, in agreement with Kerr and Cleveland's results.[4] This action is consistent with the remarkably high "turn-over number" (250,000) reported [15] for the enzyme.

FIG. 3. *Tracings of typical sedimentation diagrams. Schlieren wire assembly. In all cases, speed = 60,000 r.p.m.; movement is from right to left; times given are those after reaching full speed; the figures in parentheses after the times indicate the angle of the Schlieren wire.*



(*a*) *Original amylose*: c = 0·14 g./100 *ml. at* 5 (70°), 9 (65°), 13 (60°), *and* 17 (60°) *min.*
(*b*) 77% *limit dextrin*: c = 0·13 *g.*/100 *ml. at* 5 (70°), 9 (60°), 13 (60°), *and* 19 (60°) *min.*
(*c*) 50% *conversion dextrin*: c = 0·11 *g.*/100 *ml. at* 5 (70°), 9 (60°), 14 (60°), *and* 18 (60°) *min.*

Nevertheless, the action pattern appears to differ for short-chain amyloses. Recent studies by Bird and Hopkins [16] have shown that amylose-dextrins ($\overline{D.P.}$ 16—30) were degraded by multi-chain action, whilst Bailey and French [17] found that short-chain synthetic amyloses were attacked by an intermediary mechanism, whereby several glucosidic linkages are hydrolysed during the enzyme–substrate reaction. It is probable that the relative rate of diffusion of the substrate is a controlling factor in the reaction, since at higher temperatures multi-chain action predominates.[18]

The present study therefore indicates that, at pH 4·6 and 35°, β-amylase degrades amyloses of high molecular weight ($\overline{D.P.} \approx 10^3$) by an essentially single-chain mechanism.

Order of Reaction.—Under our experimental conditions, the rate of reaction was so fast that a detailed analysis was not possible. However, the reaction in its initial stages was not of a definite zero or first order (cf. refs. 19 and 20), but the value of k altered. For the overall reaction, the plot [21] of $1/\overline{D.P.}$ against t was not linear. This reaction is being investigated further.

Nature of the β-Limit Dextrin and the Structure of Amylose.—As indicated above, amylose in potato starch is heterogeneous both in $\overline{D.P.}$ and in behaviour on β-amylolysis. Our previous results indicate that there is 30—40% of amylose of $\overline{D.P.}$ 1800, which is completely hydrolysed to maltose by *pure* β-amylase. The sample of whole amylose used in this work had a $\overline{D.P.}$ of 3200, and a β-limit of 77%. It therefore follows that the $\overline{D.P.}$ of the

presumably incompletely hydrolysed amylose is of the order of 6000, and that, to the first approximation, it has a β-limit of 50%. (This accounts for a final $\overline{D.P.}$ of *ca.* 3000.) A 50% limit suggests that the barrier to β-amylolysis is randomly distributed throughout the high-molecular-weight amylose.

Although the nature of the barrier has not been established, several possibilities have been considered. The barrier may be situated in the main amylose chain itself, or in a side-chain joined through position 2, 3, or 6 of a constituent glucose residue in the main chain. The former possibility would imply that phosphorylase is not completely specific for α-1 : 4-linked glucopyranose residues.

A side-chain formed by an ester-phosphate group is unlikely, since bone phosphatase, which dephosphorylates starch, does not remove the anomalous linkage.[22] Further, the suggestion by Peat and his co-workers [22] that single glucose residues are attached to a main amylose chain could not be verified experimentally by Hopkins and Bird.[23] An alternative possibility is that the molecule is branched, each branch containing several hundred glucose residues. Kerr and Cleveland [24] have, in fact, suggested that potato and tapioca amylose are singly branched, and contain 1—3 branches per molecule. Our previous studies [1] suggest that, if branching occurs, the interchain linkage is not of the α-1 : 3- or α-1 : 6-type.

It must be noted that amyloses from a wide variety of plant starches contain anomalies which are resistant to β-amylase.[25] Individual amyloses appear to differ in both $\overline{D.P.}$ and β-amylolysis limit, indicating that variations exist in the relative proportion and distribution of the barriers to β-amylolysis.

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Studies on Carbohydrate-Metabolizing Enzymes

TRANS-β-GLUCOSYLATION BY BARLEY ENZYMES

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Recent investigations (reviewed by Gottschalk, 1958) have shown that the enzymic hydrolysis of a glycosidic linkage in an oligosaccharide is, in fact, a transglycosylation reaction in which a glycosyl group is transferred from the substrate to a water molecule. The latter thus acts as a glycosyl acceptor:

$$G\text{–}O\text{–}X + \text{Enzyme–H} \rightarrow \text{Enzyme–G} + H\text{–}O\text{–}X, \quad (1)$$

$$\text{Enzyme–G} + H\text{–}O\text{–}R \rightarrow \text{Enzyme–H} + G\text{–}O\text{–}R, \quad (2)$$

where R is a hydrogen atom, G–O–X represents the substrate (glycosyl donor) and H–O–R the glycosyl acceptor. Other compounds containing hydroxyl groups (e.g. carbohydrates or alcohols) may function as glycosyl acceptors, and with these the synthesis of a new saccharide or glycoside is observed. In the transglycosylation reaction the configuration of the glycosidic linkage is retained. The hydrolytic and 'transferase' activity of a carbohydrase thus represent two examples of the same enzymic reaction, and the enzyme will show a dual specificity towards the glycosyl donor and the acceptor.

During a study of barley β-glucosidases, Manners (1955*a*) found that whereas cellobiose in dilute aqueous solution was completely hydrolysed to glucose, in concentrated solution the production of glucose was accompanied by oligosaccharide synthesis. The latter could arise by the enzymic transfer of β-glucosyl radicals to glucose and cellobiose. This paper describes the characterization of these oligosaccharides, and this has enabled the acceptor specificity of barley cellobiase to be determined. For convenience, the enzyme system catalysing reactions (1) and (2) with cellobiose as β-glucosyl donor will be referred to as cellobiase, although purification has not been attempted and information on the number of active factors is not available.

METHODS AND MATERIALS

Analytical methods

Chromatography. (*a*) Qualitative. Descending paper chromatograms were prepared at room temperature with Whatman no. 1 paper and either ethyl acetate–pyridine–water (10:4:3, by vol.) (*A*) or propanol–ethyl acetate–water (6:1:3, by vol.) (*B*) as solvent. Aniline oxalate or silver nitrate–sodium hydroxide was used as spray reagent. The rate of movement of sugars is expressed relative to D-glucose (R_G values).

(*b*) Preparative. Charcoal–Celite columns were prepared as previously described (Duncan & Manners, 1958).

Electrophoresis. Separation of oligosaccharides on Whatman no. 1 paper was effected in an apparatus similar to that described by Foster (1952) at 750 V and 10 mA. 0·1 N-Borate buffer (pH 10·0) was used.

Acid hydrolysis. Complete acid hydrolysis of oligosaccharides was carried out by heating with 1·5 N-sulphuric acid (0·5% solution) at 100° for 3 hr. Reducing sugars were determined with the Somogyi (1952) reagent calibrated against glucose and cellobiose. Partial acid hydrolysis of oligosaccharides and the derived aldonic acids was effected by the methods of Duncan & Manners (1958).

Periodate oxidation. The consumption of periodate, production of formic acid, and formaldehyde were determined by the methods of Fleury & Lange (1933), Halsall, Hirst & Jones (1947) and Hough, Powell & Woods (1956), respectively.

Acetylation of sugars. The oligosaccharide (about 50 mg.) was heated at 135° with sodium acetate (80 mg.) and acetic anhydride (1·5 ml.) until solution was effected, and then for a further 15 min. The acetylated product was separated by pouring into ice-cold water (5–10 ml.), purified by solution in chloroform and recrystallized from methanol.

Enzyme preparation

Flour produced by hammer-milling Spratt–Archer barley (1954 harvest) provided the enzyme source. A sample (300 g.) was extracted with 0·02M-acetate buffer (pH 5·0; 1 l.) for 2 hr. at room temperature with constant stirring; the grain residue was removed and the solution (750 ml.) dialysed for 2 days against running tap water. The solution (780 ml.) was then cooled to 0°, and absolute ethanol (3 vol.; at 0°) slowly added with constant stirring. The precipitated protein was collected and suspended in M-acetate buffer (pH 3·0; 250 ml.) at 35° for 90 min. to inactivate amylases. The solution was then adjusted to pH 5·0 and insoluble material (*I*) collected by centrifuging, suspended in water and freeze-dried (yield 1·1 g.). The supernatant solution was also freeze-dried (fraction *S*; yield 6·6 g.).

EXPERIMENTAL AND RESULTS

Preliminary experiments

Digests containing cellobiose (0·2 g.), barley fraction *I* or *S* (20 mg.), toluene and 0·04M-acetate buffer (pH 5·0; 1 ml.) were incubated at 35°. Paper-chromatographic analysis (solvent *A*) showed that oligosaccharides were present in both digests within 2 days, with R_G values 0·73 and 0·39 (together with traces of higher oligosaccharides). On continuing the incubation, the cellobiose content decreased and the proportion of oligosaccharides increased. After 18 days, sugars with R_G values 0·73, 0·39, 0·30, 0·25 and 0·13 were present. The R_G values of authentic samples of laminaribiose and gentiobiose were 0·73 and 0·40; cellobiose had R_G 0·53.

A similar digest containing glucose (0·2 g.) was prepared; oligosaccharide synthesis did not take place. Further control experiments showed that the cellobiose substrate was chromatographically pure (it had previously been chromatographed on a charcoal–Celite column) and was not attacked by heat-inactivated barley fraction *S*.

Preparation and isolation of oligosaccharides

A digest containing cellobiose (27 g.), water (350 ml.) and enzyme solution (150 ml.) was incubated at 35° in the presence of toluene and chloroform. The enzyme solution consisted of a 2% (w/v) solution of barley fraction *S* in 0·04M-acetate buffer (pH 5·0). Samples of the digest were examined chromatographically at intervals; the results were identical with those in the preliminary experiment. After 18 days the digest was heated to inactivate the enzyme and coagulated protein removed by filtration.

The solution was concentrated and the oligosaccharides were separated on a charcoal–Celite column (150 cm. × 5 cm.); details of the separation are given in Table 1.

Characterization of oligosaccharides

With the exception of fraction 6, the oligosaccharides were homogeneous by paper chromatography and electrophoresis. On acid hydrolysis glucose was the sole product (paper chromatography).

Fraction 1. This sugar had the same R_G, reducing power and specific rotation (+52°) as D-glucose.

Fraction 2. This fraction had the reducing power of a disaccharide, was hydrolysed by almond emulsin to glucose and had the R_G of gentiobiose. A portion (150 mg.) was acetylated; the product

Table 1. *Chromatographic separation of oligosaccharides synthesized from cellobiose*

Eluent	Vol. of eluent (l.)	R_G value of sugar (solvent *A*)	Yield (mg.)	Fraction no.	Identity
Water	12·5	1·0	20 000	1	Glucose
	10·0	1·0 + mixture	Trace	—	—
	8·0	—	—	—	—
Ethanol (5%)	3·4	—	—	—	—
	4·0	1·0 + mixture	Trace	—	—
	11·0	0·40	1 500	2	Gentiobiose
	5·0	0·40 + 0·55	Trace	—	—
	7·0	0·55	320	3	Cellobiose
	6·0	—	—	—	—
Ethanol (7%)	12·0	0·75	280	4	Laminaribiose
	6·0	0·75	Trace	—	—
	5·0	—	—	—	—
Ethanol (10%)	10·0	Mixture	Trace	—	—
Ethanol (15%)	8·0	0·15	300	5	Gentiotriose
	10·0	0·25 + mixture	50	6	Cellotriose
	6·0	—	—	—	—
	20·0	0·30	350	7	6^2-β-Glucosylcellobiose
Ethanol (20%)	10·0	Mixture	Trace	—	—

(160 mg.) had m.p. 192°; $[\alpha]_D^{20} - 5°$ in chloroform (*c*, 10·0). Gentiobiose β-octa-acetate has m.p. 191–193° and $[\alpha]_D - 5°$ in chloroform (Peat, Whelan & Lawley, 1958).

Fraction 3. This material had the R_G and reducing power of cellobiose. The corresponding acetate (yield 60 mg. from 50 mg.) had m.p. 192–193°, $[\alpha]_D^{20} - 12°$ in chloroform (*c*, 3·3); an authentic sample of cellobiose β-octa-acetate had m.p. 192°, $[\alpha]_D^{20} - 14°$ in chloroform. On oxidation with sodium metaperiodate buffered at pH 8·0, the fraction (5 mg.) gave formaldehyde (2·05 mol.prop.; theoretical value 2·0).

Fraction 4. This fraction had the same R_G and reducing power as laminaribiose, and was completely hydrolysed to glucose by almond emulsin. The sugar was recrystallized from ethanol and had m.p. 160–163°; $[\alpha]_D^{20}$ 28° → 22° in water (*c*, 1·5) [cf. m.p. 160–163°; $[\alpha]_D^{11}$ 23° → 19° in water reported by Connell, Hirst & Percival (1950) for laminaribiose]. The *N*-benzylglycosylamine derivative (cf. Bayly & Bourne, 1953) had the same R_G value as that from an authentic sample of laminaribiose, and the derived osazone had m.p. 194–196° and mixed m.p. 193–194° with laminaribiosazone.

Fraction 5. This sugar had the reducing power of a trisaccharide. Partial acid hydrolysis of both the sugar and the corresponding aldonic acid gave glucose and gentiobiose. On oxidation with sodium metaperiodate at room temperature the initial and final production of formic acid was 4·0 and 5·4 mol.prop. respectively. The theoretical values for gentiotriose are 4·0 and 6·0 mol.prop. Final confirmation of identity was provided by periodate oxidation at pH 8; no formaldehyde could be detected after 2 days (cf. Hough & Perry, 1956).

Fraction 6. The major component of this mixed fraction had the same R_G as cellotriose, kindly supplied by Dr W. J. Whelan (solvents *A* and *B*).

Fraction 7. This material had R_G 0·30 (solvent *A*), the reducing power of a trisaccharide, was hydrolysed by almond emulsin and on partial hydrolysis with acid gave glucose, cellobiose and gentiobiose. After oxidation with hypoiodite, a partial hydrolysate contained glucose and gentiobiose. This indicates that fraction 7 is 6^2-β-glucosylcellobiose [see Crook & Stone (1957) for definition of nomenclature]. The identity has been confirmed by periodate oxidation. At room temperature, the consumption of periodate was 6·3 and 6·7 mol.prop. after 4 and 24 hr.; the theoretical values for the initial and total primary oxidation are 6·0 and 7·0 mol.prop. respectively. The production of formic acid amounted to 3·9, 4·0 and 4·1 mol.prop. after oxidation for 2, 4 and 5 days (theoretical value 4·0). On oxidation in phosphate buffer (pH 8·0) at 35°, 1·03 mol.prop. of formaldehyde was liberated (theoretical value 1·0).

Transfer of β-glycosyl radicals to pentoses

Digests were prepared containing cellobiose (100 mg.), D-xylose, D-lyxose or D-arabinose (100 mg. in 2 ml. of water) and barley fraction *S* (20 mg. in 1 ml. of 0·04M-acetate buffer, pH 5·0) and were incubated at 35°. After incubation for 3 days paper chromatography in solvent *A* showed glucose, cellobiose and a new component. When xylose was present the new component had R_G 0·86, the same as for authentic 3-*O*-β-D-glucopyranosyl-D-xylose, the product from lyxose had R_G 0·90 and from arabinose 0·75. After incubation for 5 days the xylose digest contained a second new component with R_G 0·68. All the new sugars stained pink with aniline oxalate and are presumably glucosyl–pentose disaccharides.

DISCUSSION

The present investigation has shown that extracts of ungerminated barley contain carbohydrase(s) which can transfer β-glucosyl radicals from cellobiose to glucose and glucodisaccharides, and to various pentoses. However, the barley enzymes are unable to synthesize oligosaccharides from glucose itself, and in this respect differ from the β-glucosidases of almond emulsin (Peat, Whelan & Hinson, 1952) and *Aspergillus niger* (Peat, Whelan & Hinson, 1955; Crook & Stone, 1957). A transglycosylation mechanism of the type shown in equations (1) and (2) is therefore indicated.

A solution of cellobiose (5·4 %, w/v) was converted into a mixture of glucose (87 %), gentiobiose (6·5 %), laminaribiose (1·2 %), gentiotriose (1·3 %), 6^2-β-glucosylcellobiose (1·5 %) and cellotriose (0·5 %). Cellobiose (1·4 %) and traces of other oligosaccharides were also present. Since the trisaccharides appear after the disaccharides it is probable that in the initial trans-β-glucosylation reaction part of the cellobiose is hydrolysed to glucose, which can then act as an acceptor molecule. Transfer to the primary alcoholic group (C-6) is predominant. Some of the trisaccharides are then formed by β-glucosyl transfer to the hydroxyl group at C-6 and C-4 of the non-reducing glucose residue of cellobiose or to the similar primary alcohol group of gentiobiose. It seems likely that barley cellobiase is responsible for the observed synthesis of gentiobiose and laminaribiose (glucose acceptor), and of 6^2-β-glucosylcellobiose and cellotriose (cellobiose acceptor).

The formation of gentiotriose could be catalysed by the same enzyme, or by a related 'gentiobiase'

(extracts of barley hydrolyse gentiobiose and amygdalin) with gentiobiose rather than cellobiose as the β-glucosyl donor. The possible identity of barley cellobiase and gentiobiase is being investigated.

The metabolism of cellobiose by β-glucosidase preparations from several biological sources has been investigated recently. Barker, Bourne, Hewitt & Stacey (1955) examined the oligosaccharides produced by *Aspergillus niger* (strain 152); a number of di- and tri-saccharides were isolated and their structures indicated transfer to the hydroxyl groups at C-6, C-4, C-3 and C-2 of glucose, and to cellobiose and gentiobiose. In general, the formation of β-1:6-glucosidic linkages was favoured. Similar observations have been made by Crook & Stone (1957) with enzyme preparations from *A. niger* (E 19), *Helix pomatia*, sweet almonds and gentian roots. Oligosaccharide formation from cellobiose has also been observed with β-glucosidase preparations from *Aspergillus flavus* (Giri, Nigam & Srinivasan, 1954) and marine algae (W. A. M. Duncan, D. J. Manners & J. L. Thompson, to be published).

Buston & Khan (1956) investigated a related enzyme system in *Chaetomium globosum*; the products from cellobiose included cellotriose and other trisaccharides, laminaribiose, gentiobiose and sophorose. However, this system appears to differ from that in barley since trisaccharides were detected in the initial stages, before the appearance of disaccharides. Indeed, an earlier study by Buston & Jabbar (1954) indicated that cellotriose was the major product, formed by β-glucosyl transfer to cellobiose. The disaccharides may therefore result from trans-β-glucosylation to cellobiose, followed by hydrolysis of the β-1:4-linkage adjacent to the reducing group. Buston & Khan (1956) have, in fact, noted that on prolonged incubation cellotriose disappears from the reaction mixture.

The ability of 'cellobiase' enzymes to catalyse oligosaccharide formation from concentrated solutions of cellobiose thus appears to be a general property with preparations from higher plants, marine algae and certain micro-organisms. However, some differences in acceptor specificity are apparent, and the present study shows that the barley enzyme differs from many others in being unable to effect appreciable transfer to the hydroxyl group at C-2 of glucose. In general, trans-β-glucosylases show a lower specificity towards the acceptor molecule than the corresponding trans-α-glucosylases.

Since the reserve β-glucosan of barley contains only 1:3- and 1:4-linkages (Aspinall & Telfer, 1954), it is unlikely that the transglucosylase activity of barley cellobiase is intrinsically of any metabolic significance. The observed oligosaccharide synthesis is merely the result of enzyme action in the presence of a low concentration of water. It follows that in the use of β-glucosidases for the structural analysis of polysaccharides (cf. Manners, 1955*b*), enzyme action should take place in dilute solution, and analysis must be confined to the initial stages in order to avoid any possible ambiguity.

It is of interest to note that pentoses may serve as acceptor substrates during trans-β-glucosylation with barley enzymes. This activity is similar to that of enzyme preparations from *Aspergillus niger* (Barker, Bourne, Hewitt & Stacey, 1957) and *Cladophora rupestris* (W. A. M. Duncan, D. J. Manners & J. L. Thompson, to be published). The characterization of the glucosyl–pentoses will be reported later.

SUMMARY

1. Extracts of ungerminated barley catalyse the synthesis of oligosaccharides from cellobiose.

2. The oligosaccharides have been isolated and characterized. They include gentiobiose, laminaribiose, gentiobiose, cellotriose and 6^2-β-glucosylcellobiose.

3. The acceptor specificity of the barley enzymes and similar enzymes from other biological sources is discussed.

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Studies on Carbohydrate-Metabolizing Enzymes

2. TRANS-α-GLUCOSYLATION BY EXTRACTS OF *TETRAHYMENA PYRIFORMIS**

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In continuation of studies on transglucosylation reactions (Duncan & Manners, 1958; Anderson & Manners, 1959; Duncan, Manners & Thompson, 1959) we now describe an investigation of the metabolism of maltose by cell-free extracts of the ciliate *Tetrahymena pyriformis*. The presence of a maltase in such extracts has been noted by Ryley (1952).

* The paper by Anderson & Manners (1959) is regarded as Part 1.

Since maltase activity may be regarded as a trans-α-glucosylation reaction in which water provides the acceptor substrate:

$$\text{Maltose} + \text{enzyme-H} \rightarrow \text{glucose} + \text{glucosyl-enzyme}$$
$$\text{Glucosyl-enzyme} + \text{H·OR} \rightarrow \text{enzyme-H} + \text{glucosyl·OR}$$

where H·OR is the glucosyl acceptor (Gottschalk, 1958), it follows that, if oligosaccharide synthesis takes place in concentrated solutions of maltose,

the nature of the products provides information on the specificity of the maltase for the acceptor H·OR. This has been obtained in the present study by chromatographic separation and characterization of the oligosaccharides synthesized from 18 % maltose solution. In addition, the ability of other sugars to function as acceptors has been examined with various maltose–monosaccharide mixtures.

MATERIALS AND METHODS

Analytical methods. The methods used have been described by Duncan & Manners (1958) and Anderson & Manners (1959). An ethyl acetate–pyridine–water (10:4:3, by vol.) mixture was used as solvent for paper chromatography.

Organism. A pure culture of *Tetrahymena pyriformis* (GL) was kindly supplied by Dr J. F. Ryley and was maintained at room temperature in a sterile medium, adjusted to pH 7·3, containing 1 % (w/v) of Oxoid brand bacteriological peptone and 0·4 % (w/v) of sodium chloride. Sub-inoculations were made at intervals of 2 weeks (Ryley, 1952).

For large-scale culture, the organism was grown in the dark at 30° in 2 l. conical flasks containing 400 ml. of medium. Maximum culture density was reached after 6 days. The contents of twenty-two such flasks were combined and the cells collected by using a Sharples super-centrifuge. The cells were then dispersed in 150 ml. 0·1 M-sodium citrate buffer (pH 6·0) and freeze-dried to give 7·0 g. of yellow–brown powder. Microscopic examination of an aqueous suspension of this material showed that the cells had disintegrated. Part of the freeze-dried powder (0·35 g.) was suspended in 10 ml. of water and kept, with frequent stirring, at room temperature for 1 hr. Centrifuging at 3500 *g* yielded a yellow, opalescent solution which was free from cells and cell particles.

A further portion of freeze-dried cells (approx. 7 g.) was extracted with 200 ml. of water and centrifuged, and the extract was then freeze-dried (yield, 4·8 g.). This material was stored at 2° over calcium chloride; the maltase activity was not appreciably diminished after 1 year. However, the following experiments were carried out within 4 weeks of preparation of the extract.

The possibility of reducing sugars' being present in this extract, or arising by autolysis of soluble polysaccharide, was examined by paper-chromatographic analysis of a 4 % (w/v) aqueous solution during incubation at 35° for 5 days. A faint trace of glucose was detected initially; this did not increase.

EXPERIMENTAL AND RESULTS

Carbohydrase activity of extract

Digests containing carbohydrate (2 %, w/v) and extract (2 %, w/v) were incubated at 35° for 24 hr. The following carbohydrates were hydrolysed: maltose, isomaltose, panose, maltotriose, starch, cellobiose, sucrose, methyl α-D-glucoside, α-D-glucosyl phosphate. Lactose was not attacked. In view of the considerable α-glucosidase activity, the action was investigated with a concentrated solution of maltose (*a*) alone, (*b*) in the presence of possible α-glucosyl acceptors.

Action on concentrated solution of maltose

A digest containing maltose (20 %, w/v) and extract (1 %, w/v) was incubated at 35° in the presence of toluene. A number of oligosaccharides were produced, as shown in Table 1.

Preparation and isolation of oligosaccharides

Thrice-recrystallized and chromatographically-pure maltose) 30·0 g.), 150 ml. of 0·1 M-sodium citrate buffer (pH 6·0) and 20 ml. of enzyme extract were incubated, with toluene, at 35° for 10 days. The enzyme extract was prepared from 3·8 g. of freeze-dried cells, which were dispersed in 60 ml. of water at room temperature for 2 hr. and then centrifuged (3500 *g* at 2°). Paper-chromatographic analysis at intervals gave the same results as those shown in Table 1. The digest was then heated for 20 min. at 98°, and filtered.

The oligosaccharides were separated by chromatography on a charcoal–Celite column (160 × 7·5 cm.) and elution with increasing concentrations of aqueous ethanol. Fractions (1–2 l.) were collected, concentrated to approx. 5 ml., freed from traces of charcoal and Celite and examined by paper chromatography. Fractions containing like components were combined, and the sugars isolated in the usual manner by crystallization from ethanol or by freeze-drying. The approximate yields are given in Table 2. All the fractions were homogeneous by paper chromatography and electrophoresis, and on acid hydrolysis gave only glucose.

Characterization of oligosaccharides

Fraction 1. The data in Table 2 and the specific rotation in water (+ 52°) characterize this sugar as D-glucose.

Fraction 2. Partial and total acid hydrolysis gave glucose; emulsin had no action. This disaccharide is therefore isomaltose.

Table 1. *Oligosaccharides synthesized from maltose by cell-free extracts of* Tetrahymena pyriformis

+ + +, + +, + and *t* indicate intense, strong, moderate and weak spots, respectively. Glucose, maltose, isomaltose, maltotriose and panose had R_G values of 1·00, 0·62, 0·47, 0·39, 0·28, respectively.

Time of incubation (days)	R_G values					
	1·00	0·62	0·47	0·39	0·28	Others
2	+ +	+ + +		+	+	*t*
4	+ +	+ + +	*t*	+	+	*t*
6	+ +	+ + +	+	+	+	+
8	+ + +	+ + +	+	+ +	+ +	+
10 and 15	+ + +	+ + +	+ +	+ +	+ +	+

Fraction 3. This was identified as maltose by specific rotation (+132°), partial acid hydrolysis and the data in Table 2.

Fraction 4. This trisaccharide had the same R_G as authentic panose and $[\alpha]_D$ +148° in water (*c.* 2·3); cf. $[\alpha]_D$ +148° reported for panose by Duncan & Manners (1958). A partial acid hydrolysate contained glucose, maltose and isomaltose; the corresponding aldonic acid, prepared by hypoiodite oxidation, gave glucose and isomaltose. On periodate oxidation, 0·94 mol.prop. of formaldehyde was released. The theoretical value is 1·00 mol.prop.

Fraction 5. This trisaccharide was characterized as maltotriose by R_G value, $[\alpha]_D$ +161° in water (*c*, 2·7) [cf. $[\alpha]_D$ +160 and +164° quoted by Whelan, Bailey & Roberts (1953) and Duncan & Manners (1958), respectively] and slow hydrolysis to glucose and maltose by salivary α-amylase. Partial acid hydrolysates of the sugar and the corresponding aldonic acid both contained glucose and maltose.

Fraction 6. This sugar was a tetrasaccharide with R_G 0·18 and $[\alpha]_D$ +172° in water (*c*, 0·4); cf. values of R_G 0·17 and $[\alpha]_D$ +177°, reported by Duncan & Manners (1958). Partial hydrolysis with acid gave glucose, maltose, isomaltose, panose and maltotriose. The corresponding aldonic acid gave the same products on partial hydrolysis, except that maltotriose was absent. This finding eliminates 6^1- and 6^2-α-glucosylmaltotriose [see Crook & Stone (1957) for definition of nomenclature] as possible structures. The characterization as the 6^3 isomer is confirmed by the periodate oxidation (Table 2).

Fraction 7 (with Miss Zeenat H. Gunja). This tetrasaccharide had $[\alpha]_D$ +180° in water (*c*, 0·9); cf. $[\alpha]_D$ +177°, quoted by Whelan *et al.* (1953) for maltotetraose. Partial hydrolysis with acid gave glucose, maltose and maltotriose. α- and β-Amylolysis gave a mixture of glucose and maltose, and maltose, respectively.

Trans-α-glucosylation to other sugars

Digests containing maltose (5 %) and a pentose, D-galactose, or D-fructose (5 %) and enzyme extract (1 %) were prepared and incubated at 35°. After 3–5 days' incubation the presence of new components in addition to isomaltose, maltotriose and panose was detected by paper chromatography. The new sugars had R_G values of 0·64 (D-xylose acceptor), 0·69 (D-lyxose acceptor), 0·64 (D-ribose acceptor), 0·72 (L-arabinose acceptor), 0·32 (D-galactose acceptor). The new sugars formed in the digests containing pentoses gave pink stains with aniline oxalate and are presumably α-glucosylpentose disaccharides. Fructose did not function as an acceptor substrate.

Other transglucosylation reactions

Various carbohydrates (20 % solution) and extract (2 %) were incubated for 5 days at 35°. The following results were obtained by paper chromatography: (*a*) isomaltose: products had R_G 1·0, 0·47, 0·19 (? isomaltotriose); a trace of higher oligosaccharides was also present; (*b*) methyl α-D-glucoside: the main product had R_G 1·0; a trace of higher oligosaccharides was produced; (*c*) D-glucose, (*d*) D-xylose, (*e*) α-D-glucosyl phosphate: no synthesis of oligosaccharides.

DISCUSSION

The ability of maltase preparations from a variety of biological sources to catalyse the synthesis of oligosaccharides from maltose has been reported by many workers (see Duncan & Manners, 1958).

The present results (Tables 1 and 2) show that cell-free extracts of the ciliate *Tetrahymena pyriformis* have a similar activity. On incubation with 18 % maltose solution, the main products are panose and maltotriose; smaller quantities of isomaltose, 6^3-α-glucosylmaltotriose and maltotetraose are also formed. Since the extracts are unable to polymerize glucose [in contrast with certain fungal preparations (Peat, Whelan & Hinson, 1955)], it follows that the oligosaccharides are formed by transglucosylation in which the OH groups at both C-4 and C-6 of the non-reducing residue of maltose and maltotriose can function as acceptors for α-glucosyl

Table 2. *Properties of the oligosaccharides*

The degree of polymerization was determined from reducing-power measurements before and after acid hydrolysis. The periodate oxidation value is expressed as moles of formaldehyde per mole of oligosaccharide liberated on oxidation at pH 8. With fractions 5 and 6 a small amount of sugars with R_G 0·31 and 0·15 was also eluted with 8 % (v/v) ethanol.

Fraction no.	Eluent	Yield (g.)	R_G	Degree of polymerization	Periodate oxidation value	Identity
1	Water	—	1·00	1·00	1·01	Glucose
2	Water	0·41	0·47	1·90	0·08	Isomaltose
3	Ethanol (2–6 %, v/v)	5·96	0·62	2·10	1·70	Maltose
4	Ethanol (6 %, v/v)	2·94	0·28	3·20	0·94	Panose
5	Ethanol (6–8 %, v/v)	3·29	0·41	2·94	2·80	Maltotriose
6	Ethanol (8 %, v/v)	0·58	0·18	3·70	1·98	6^3-α-Glucosylmaltotriose
7	Ethanol (10–12 %, v/v)	0·35	0·26	4·08	—	Maltotetraose

radicals. Panose does not appear to be a favourable acceptor, since 6^2-α-isomaltosylmaltose and related sugars are not produced. The ability to transfer to C-4 differentiates the protozoal enzyme system from various fungal maltases which can only synthesize (1 → 6)-linked oligosaccharides (e.g. Pazur & French, 1952).

The acceptor specificity of the protozoal maltase system is generally similar to that observed with extracts of *C. rupestris* (Duncan & Manners, 1958) except that the latter is unable to use monosaccharides as acceptor substrates. Since a number of pentoses and also D-galactose can accept α-glucosyl radicals with the *T. pyriformis* system, it is probable that most of the isomaltose is formed by the interaction of a glucosyl–enzyme complex with the primary alcohol group of a glucose molecule rather than by the hydrolysis of the (1 → 4)-linkage of panose. This view is supported by the fact that isomaltose synthesis and the formation of higher oligosaccharides ($R_G < 0{\cdot}28$) occur simultaneously, showing that the equilibrium between hydrolysis (i.e. water as glycosyl acceptor) and trans-α-glucosylation to maltotriose favours the latter.

Isomaltose itself can function as a glucosyl donor, but the product appears to be isomaltotriose rather than panose or a maltosaccharide. One possible explanation for this is that the maltase and isomaltase activities of the *T. pyriformis* extracts are due to different enzymes. The non-identity of these activities in extracts of yeast and intestinal mucosa (for example, Larner & Gillespie, 1956) has already been established.

SUMMARY

1. Cell-free extracts of the ciliate *Tetrahymena pyriformis* show hydrolytic activity towards maltose, isomaltose, cellobiose, sucrose, methyl α-D-glucoside, α-D-glucosyl phosphate and starch.

2. In concentrated solution, oligosaccharides are synthesized from maltose and from isomaltose, but not from glucose.

3. Oligosaccharide synthesis from maltose results in the formation of isomaltose, maltotriose, panose, 6^3-α-glucosylmaltotriose and maltotetraose.

4. The acceptor specificity for α-glucosyl radicals by the protozoal enzyme system and other maltases is discussed.

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Studies on Carbohydrate-Metabolizing Enzymes

3. YEAST BRANCHING ENZYME*

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The classical researches of Hanes, Peat and their co-workers (for reviews, see Barker & Bourne, 1953; Whelan, 1958) have established that two distinct enzymes are involved in the synthesis of amylopectin from α-D-glucosyl phosphate. Thus P-enzyme (potato phosphorylase) action results in the formation of linear amylose-type molecules which are converted into amylopectin by Q-enzyme. Alternatively, the combined action of P- and Q-enzymes on α-D-glucosyl phosphate also yields a branched polysaccharide, although Q-enzyme alone has no action on the glucoside.

In the animal kingdom a related enzyme system may be partly responsible for glycogen synthesis. Muscle phosphorylase synthesizes amylose-type chains from α-D-glycosyl phosphate and the concurrent action of this enzyme and an enzyme preparation (branching factor or supplementary enzyme) from heart or liver extracts gave a glycogen-type polysaccharide, since the latter gave a red-brown coloration with iodine and, unlike amylose, could function as a primer for muscle phosphorylase (Cori & Cori, 1943). In general, however, the branching enzymes from animal tissues have not been highly purified and the products of enzyme action have not been rigidly characterized.

Glycogen is one of the reserve polysaccharides of brewer's yeast (Manners & Khin Maung, 1955*a*); hence, extracts of this yeast should provide a source of branching enzyme. This enzyme was first encountered as an impurity in yeast-phosphorylase preparations (unpublished work), which, acting on α-D-glucosyl phosphate, synthesized a branched polysaccharide which had a β-amylolysis limit of 44% and gave a red-brown stain with iodine.

This paper describes some of the properties of partly purified yeast branching enzyme and its action on amylose. Furthermore, since yeast glycogen contains 7–8% of 1:6-glucosidic linkages, it was of interest to examine the action of yeast branching enzyme on an amylopectin containing only 4–5% of branch points. Preliminary accounts of some of this work have been published (Manners & Khin Maung, 1956; Gunja & Manners, 1959).

* Part 2: Archibald & Manners (1959).

METHODS AND MATERIALS

Analytical methods. Reducing sugars and protein N were determined by the methods of Somogyi (1952) and Robinson & Hogden (1940) respectively. Enzyme digests containing isoamylase were deproteinized by Nelson's (1944) method. Paper chromatography was carried out as described by Manners & Khin Maung (1955*a*) and Archibald & Manners (1959).

Iodine staining. Polysaccharide sample (0·5–1·0 ml.) was stained with iodine solution [0·2% of I_2 in 2% (w/v) KI; 0·5–1·0 ml.] in a total volume of 50–100 ml. The solution also contained 1 drop of 5N-HCl. The absorption value of the polysaccharide–iodine complex [for definition see Bourne, Haworth, Macey & Peat (1948)] was measured (*a*) by a Hilger Spekker photoelectric absorptiometer in 1 or 4 cm. cells with an Ilford no. 608 (approx. 680 mμ) filter (amylose or starch) or no. 603 (approx. 490 mμ) filter (waxy-maize starch) against an iodine–water blank, or (*b*) with a Unicam SP. 500 spectrophotometer with 1 cm. cells against an iodine–water blank. Amylose, starch and amylopectin solutions were measured at 630, 600 and 540 mμ respectively.

Phosphorylase activity. The conditions of Green & Stumpf (1942) were used; inorganic phosphate was measured by the method of Fiske & Subbarow (1925).

Analysis of polysaccharides

Glucose content. The polysaccharides (0·1%, w/v) were hydrolysed with 2N-H_2SO_4 at 100° for 2 hr. and the reducing-sugar content (as glucose) was determined with the Somogyi reagent. Analytical figures are based on the observed glucose contents.

β-Amylolysis limit. Polysaccharide (25 mg.), 0·2M-sodium acetate buffer (pH 4·6; 4 ml.) and β-amylase (approx. 1250 units) were incubated in a total volume of 25 ml. at 35° for 24 hr. The maltose content of a portion (3 or 5 ml.) was then determined. Under these conditions enzyme action was 93% complete in 4 hr. and the maltose content after 24 and 48 hr. was identical (Liddle, 1956).

β-Amylolysis limit after incubation with isoamylase. Polysaccharide (25 mg.), 0·2M-sodium acetate buffer (pH 6·0; 4 ml.), isoamylase (50 mg.) and water (7 ml.) were incubated at 20° for 24 hr. The digest was heated (3 min. at 98°), cooled and β-amylase solution (approx. 1250 units) and water (to 25 ml.) were added. After 24 hr. at 35°, the maltose content of the digest was determined after deproteinization of a sample.

Simultaneous action of β-amylase and isoamylase. Polysaccharide (20–25 mg.), 0·2M-sodium acetate buffer (pH 6·0; 4 ml.), isoamylase (40 mg.) and β-amylase (1000 units) were incubated for 24 hr. at 20° in a total volume of

25 ml. After deproteinization of a sample (3 ml.) the maltose content was measured.

α-Amylolysis limit. Polysaccharide (30 mg.), 1·0 M-NaCl solution (10 ml.), freeze-dried α-amylase (2·5 mg.) and water (to 50 ml.) were incubated at 35° for 24 hr. Portions (5 ml.) were examined for reducing sugar (expressed as apparent percentage conversion into maltose, P_M).

Limiting viscosity number. By means of a modified Ubbelohde viscometer, the specific viscosity, $\eta_{sp.}$, at 25±0·02° of four concentrations of the polysaccharide in 0·1 M-NaCl or M-KOH was determined. The limiting viscosity number, η, was obtained by extrapolation of $\eta_{sp.}/c$ against c to zero concentration (c as g./ml.).

Enzyme preparations

Pressed brewer's yeast (*Saccharomyces cerevisiae*) was kindly supplied by Wm. Younger and Co. Ltd. It was washed with water, dried at 35° in a current of air and stored in air-tight bottles at 0°. Isoamylase was prepared by the method of Manners & Khin Maung (1955*b*) and Gunja, Manners & Khin Maung (in preparation). α- and β-Amylase were as described by Liddle & Manners (1957); soya-bean β-amylase was also used (Manners & Khin Maung, 1955*a*).

Substrates

Amylose. Various samples, made by the fractionation of potato starch with butanol (H. Baum & G. A. Gilbert, cited by Whelan, 1955), aluminium hydroxide (Bourne, Donnison Peat & Whelan, 1949) or thymol and butanol (Greenwood & Robertson, 1954), were used.

Amylopectin. Two samples of amylopectin were prepared by the thymol fractionation of potato starch (Greenwood & Robertson, 1954). Malted-barley amylopectin was kindly provided by Dr G. O. Aspinall (Aspinall, Hirst & McArthur, 1955). Waxy-maize starch was a commercial sample.

Glycogen. Glycogens isolated from brewer's yeast (Manners & Khin Maung, 1955*a*), rabbit muscle (Liddle & Manners, 1957) and horse-diaphragm muscle (Lawrie, Manners & Wright, 1959) were used. Floridean starch was the sample II described by Fleming, Hirst & Manners (1956).

β-Dextrins. Amylopectin β-dextrin and glycogen β-dextrin were prepared by the prolonged action of β-amylase on waxy-maize starch and *Mytilus edulis* VI glycogen (Liddle & Manners, 1957), followed by dialysis (to remove maltose and inorganic material) and isolation by precipitation with ethanol or freeze-drying.

Preparation of yeast branching enzyme

The starting material was air-dried brewer's yeast. The following procedure has given preparations of yeast branching enzyme of satisfactory activity, although with different batches of yeast the proportion of contaminating enzymes (phosphorylase, isoamylase, isomaltase and maltase) has varied. Dried yeast (100 g.) was extracted with 0·1 M-$NaHCO_3$ solution (500 ml.) at 35° for 2 hr., and the extract centrifuged at 0°. To the solution (about 350 ml.) an equal volume of cold saturated $(NH_4)_2SO_4$ solution was added. The precipitate was collected by centrifuging at 1° and dissolved in 0·1 M-sodium citrate buffer (pH 6·6; 100 ml.). Aq. 50% (v/v) ethanol–0·01 M-sodium citrate buffer, pH 6·8, was added at −5° to give an ethanol concentration of 25%.

The precipitate was removed and the ethanol concentration of the supernatant solution was increased to 30%. This precipitate was collected and triturated with 0·1 M-sodium citrate buffer (pH 6·8; 40 ml.) for 30–60 min. Insoluble material was removed by centrifuging and the solution treated with an equal volume of cold saturated $(NH_4)_2SO_4$ solution. The precipitate was collected, dissolved in 0·1 M-sodium citrate buffer (pH 6·8; 20 ml.) and freeze-dried. Yield 2·0–2·6 g.; N content 1·6–2·3%. The material was free from iodophilic polysaccharide.

A digest containing amylose (2·5 mg.), 0·2 M-sodium citrate buffer (pH 6·8; 1 ml.), yeast branching enzyme (45 mg.) and water (4 ml.) was prepared and incubated at 20°. The absorption values (Spekker units) after 0, 0·5, 1·5 and 20·0 hr. were 0·44, 0·21, 0·15 and 0·04 respectively. The digest did not contain reducing sugars (Somogyi reagent and paper chromatography).

Tests for contaminating enzymes. (*a*) Maltase and isomaltase. Yeast branching enzyme (20 mg.) was incubated with maltose, maltotriose and isomaltose (10 mg.) in 0·2 M-sodium citrate buffer (pH 6·8; 0·5 ml.). Paper-chromatographic analysis after incubation at 20° for 24 hr. showed the presence of both glucose and unchanged oligosaccharide. The enzyme preparation thus contains traces of maltase and isomaltase, although the presence of these enzymes will not interfere with measurements of activity of yeast branching enzyme.

(*b*) α-Amylase. The test is based upon that of Hobson, Whelan & Peat (1950) except that glycogen β-dextrin was used. Amylose (5 mg.), amylopectin β-dextrin or glycogen β-dextrin (10 mg.) was incubated with yeast branching enzyme (60 mg.), 0·2 M-sodium citrate buffer (pH 6·8; 2 ml.) and water (8 ml.). Portions (1 ml.) were removed after 0, 20, 60 and 1080 min. The results are shown in Fig. 1. Since the iodine-staining power of glycogen β-dextrin

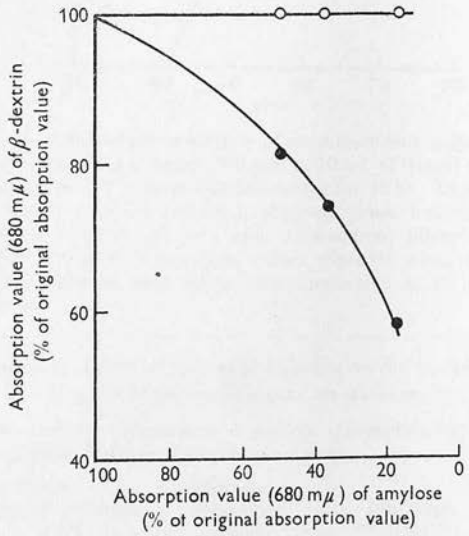

Fig. 1. Detection of α-amylase in preparation of yeast branching enzyme. Relationship between the changes in the absorption value of ○, amylose and glycogen β-dextrin or ●, amylopectin β-dextrin during incubation with yeast branching enzyme.

remained constant it is concluded that yeast branching enzyme is essentially free from α-amylase. The action on amylopectin β-dextrin will be considered later.

(*c*) Isoamylase. Incubation of yeast branching enzyme (1958 preparation) with glycogen gave an insignificant increase in iodine-staining power and β-amylolysis limit. By contrast, preparations made in 1955–56 contained a trace of isoamylase since the β-amylolysis limit of yeast glycogen increased by 4–5% after incubation for 4–14 hr.

(*d*) Phosphorylase. In preliminary experiments the protein precipitated at ethanol concentrations of 17–23 and 23–30% was found to be free from phosphorylase.

RESULTS

Properties of yeast branching enzyme

Effect of enzyme concentration. Although amylose is the normal substrate for branching enzymes, Gilbert & Patrick (1952) have shown that soluble starch may also be used. With this substrate, preliminary experiments showed that with digests containing starch (2%, w/v; 0·5 ml.), 0·2 M-sodium citrate buffer (pH 6·8; 1 ml.), yeast branching enzyme (10–25 mg.) and water to 5 ml., and incubated at 20° for 10 min., activity of yeast branching enzyme as followed by iodine staining was proportional to enzyme concentration. Under these conditions a unit of activity may be defined as the amount of enzyme causing a decrease of 0·1 Spekker absorptiometer unit in the starch–iodine complex. A typical preparation of yeast branching enzyme has an activity of 66 units/g.

Effect of temperature and pH. Figs. 2 and 3 show that action of yeast branching enzyme is optimum at about 20° and pH 7·0.

Effect of possible activators. To determine the effect of maltose, maltotriose, cellobiose and glycogen on the action of yeast branching enzyme on amylose, the following digests were prepared: amylose (2·9 mg.), yeast branching enzyme (15 mg.) 0·1 M-sodium citrate buffer (pH 6·8; 0·5 ml.), activator (5 mg.) and water (3·5 ml.). The reaction was followed by iodine staining. The rate of decrease of absorption value during incubation for 40 min. in a control digest was the same as observed above. The maltosaccharides and cellobiose thus have no effect.

Effect of inhibitors. (*a*) Mercuric chloride. Digests containing amylose (2 mg.), yeast branching enzyme (10 mg. in 0·2 M-sodium citrate buffer, pH 6·8, 0·5 ml.) and mercuric chloride solution (to 2 ml.) were incubated at 20° for 30 min. The results (Table 1) show that yeast branching enzyme

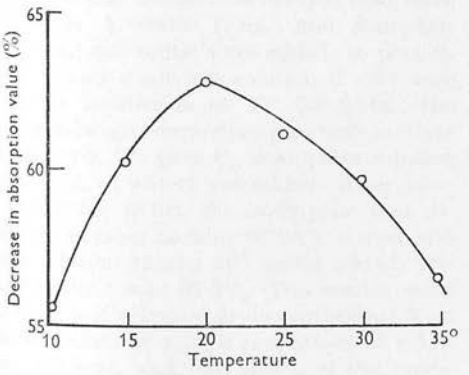

Fig. 2. Effect of temperature on activity of yeast branching enzyme. Amylose (1 mg.) and yeast branching enzyme (20 mg. in 0·1 M-sodium citrate buffer, pH 6·8; 1 ml.) were incubated at various temperatures for 30 min. The reaction was followed by measurements of absorption values with a Spekker absorptiometer.

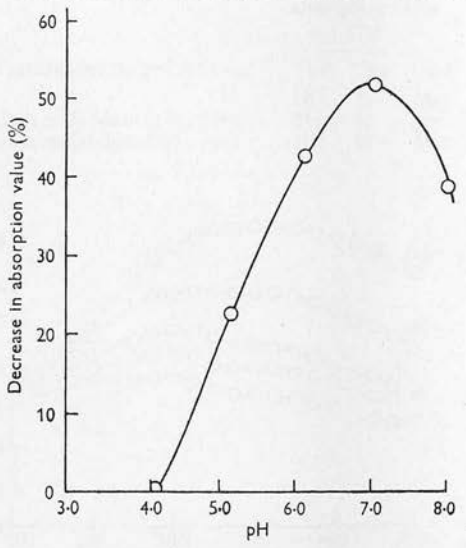

Fig. 3. Effect of pH on activity of yeast branching enzyme. Yeast branching enzyme (100 mg. in 10 ml. of water) was dialysed at 20° against distilled water for 12 hr. Digests contained amylose (2·9 mg.), dialysed yeast branching enzyme solution (1·5 ml.) and 0·2 M-sodium phosphate buffer (1·5 ml.). Absorption values (Spekker absorptiometer readings) were taken after incubation at 20° for 15 min.

Table 1. *Effect of mercuric chloride on the activity of yeast branching enzyme on amylose*

See text for composition of digests. Absorption values are given in Spekker absorptiometer units.

Concn. of mercuric chloride (mM)	Decrease in absorption value	Inhibition (%)
0	0·65	0
0·5	0·00	100
0·2	0·39	40
0·1	0·54	17
0·05	0·60	8

is completely inhibited by 0·5 mM-mercuric chloride.

(*b*) Ammonium molybdate. Amylose (4·5 mg.), 0·2M-sodium citrate buffer (pH 6·8; 1 ml.), yeast branching enzyme (25 mg.) and ammonium molybdate (12 %, w/v) solution (final vol. 5 ml.) were incubated at 20°. The absorption values of the amylose–iodine complexes were measured at intervals. The results, summarized in Table 2, show that yeast branching enzyme is only partly inhibited by 2 % (w/v) ammonium molybdate.

Action on amylose

Amylose (40 mg.), 0·2M-sodium citrate buffer (pH 6·8; 5 ml.), yeast branching enzyme (110 mg.) and water (to 25 ml.) were incubated at 20°. After 1, 2 and 4 hr. enzyme action resulted in a 66, 74 and 81 % decrease in absorption value at 680 mμ, and the β-amylolysis limit fell from 84·1 % to 50·4, 49·3 and 48·6 % respectively. These results differentiate yeast branching enzyme from an α-amylase.

The change in absorption spectrum of the amylose–iodine complex during action of yeast branching enzyme is shown in Fig. 4. After prolonged incubation the residual polysaccharide showed maximum absorption at 520 mμ, the solution giving a brown stain. No increase in reducing power was detected.

The above-described changes indicate the formation of branch points in the amylose, the nature of which was examined enzymically. Amylose (32 mg.), 0·2M-sodium citrate buffer (pH 6·8; 4 ml.) and yeast branching enzyme (80 mg. in 4 ml. of water) were incubated at 20° for 4·5 hr. The enzyme was inactivated by heating (3 min. at 98°) and the digest was cooled and divided into three parts. To part A, water (1 ml.) and β-amylase solution (2 ml.; 800 units) were added; to part B, water (1 ml.) and α-amylase solution (2 ml.) were added. After incubation at 35° for 24 hr., the apparent percentage conversions into maltose were A, 45·4; B, 91·9. To part C, isoamylase solution (25 mg. in 1 ml. of water) was added. After incubation at 20° for 18 hr., the isoamylase was deactivated by heating (3 min. at 98°), cooled and β-amylase solution (2 ml.; 800 units) added. The β-amylolysis limit was 65·3 %. The results show that the branched polysaccharide synthesized from amylose still contains a high proportion of α-1:4-glucosidic linkages, and that many of the newly formed inter-chain linkages are susceptible to isoamylase. Since isoamylase is specific for α-1:6-glucosidic linkages (Manners & Khin Maung, 1955*b*; Gunja, Manners & Khin Maung, in preparation), this characterizes many of the new inter-chain linkages.

The above evidence is consistent with the conversion of amylose by yeast branching enzyme into an amylopectin-type polysaccharide. The properties of the latter were then studied in detail.

Preparation of synthetic amylopectin

Amylose–butanol complex (about 0·5 g.) dissolved in water (250 ml.) was incubated at 20° with yeast branching enzyme (900 mg. in 10 ml. of

Table 2. *Effect of ammonium molybdate on activity of yeast branching enzyme on amylose*

See text for composition of digests. Absorption values are Unicam spectrophotometer readings at 630 mμ.

	Decrease in absorption value (%)		
Time of incubation (hr.)	1·0	2·5	4·5
Control	26	41	58
Ammonium molybdate (1 %, w/v)	21	32	50
Ammonium molybdate (2 %, w/v)	16	22	29

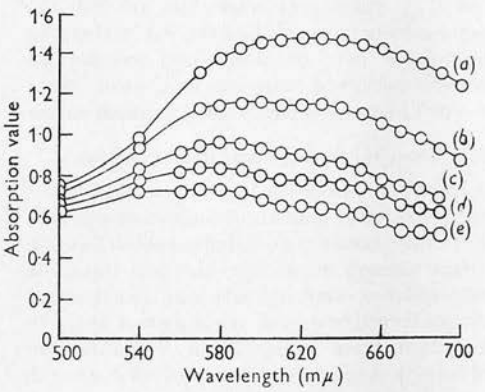

Fig. 4. Light-absorption curves of iodine-stained products during action of yeast branching enzyme on potato amylose. Age of digest (min.): (*a*) 0; (*b*) 10; (*c*) 30; (*d*) 60; (*e*) 120.

Table 3. *Conversion of amylose into an amylopectin-type polysaccharide by yeast branching enzyme*

For composition of digest see text. Absorption values are Unicam spectrophotometer readings at 630 mμ.

Time of incubation (hr.)	Decrease in absorption value (%)	$\lambda_{max.}$ (mμ)	β-Amylolysis limit (%)
0	0	630	97·1
2	20·0	—	78·8
6	29·8	—	73·8
10	38·5	585	70·2
20	61·6	570	60·8
25	63·1	570	59·0
30	67·7	570	58·3
48	77·7	550–555	55·1

Table 4. *Effect of yeast branching enzyme on the absorption value of various amylopectin-type polysaccharides*

Polysaccharide (30–40 mg.), 0·2 M-sodium citrate buffer (pH 6·8; 5 ml.), yeast branching enzyme (20–100 mg.) and water in a final vol. of 25 ml. were incubated at 20°. Portions (0·5 or 1·0 ml.) were stained with iodine, at intervals. A Spekker absorptiometer (filter no. 603) was used for waxy-maize starch and a Unicam SP. 500 spectrophotometer at 540 mμ for malted-barley amylopectin and β-dextrin, and at 555 mμ for synthetic amylopectin, to measure the absorption values.

Time of incubation (hr.)	Absorption value			
	Waxy-maize starch	β-Dextrin	Malted-barley amylopectin	Synthetic amylopectin
0	0·160	0·429	0·295	0·525
2	0·085	—	—	—
3	—	0·392	0·270	0·392
4	0·075	—	—	—
24	0·050	0·375	0·230	0·265
48	—	—	—	0·220

Table 5. *Effect of yeast branching enzyme on the β-amylolysis limit of potato amylopectin*

The digest contained potato amylopectin I (42 mg.), 0·2 M-sodium citrate buffer (pH 6·8; 5 ml.), yeast branching enzyme (70 mg.) and water to 25 ml. Portions (5 ml.) were removed at intervals, heated (98° for 3 min.), cooled and β-amylase solution (3 ml.; 1200 units) was added. After incubation at 35° for 24 hr. the maltose content was determined.

Time of incubation (hr.)	0	1	2	3
β-Amylolysis limit (%)	59·6	53·0	52·2	49·5

water) and 0·2 M-sodium citrate buffer (pH 6·8; 40 ml.). Portions were withdrawn at intervals for measurement of absorption value (0·5 ml.) and β-amylolysis limit (4·5 ml.). The latter solution was heated (3 min. at 98°) to inactivate yeast branching enzyme, cooled and β-amylase solution (500 units in 1 ml. of 0·2 M-sodium acetate buffer, pH 4·6) was added, with water to 50 ml. After incubation at 35° for 24 hr., the maltose content was determined. The results are summarized in Table 3, and indicate the absence of isoamylase from the preparation of yeast branching enzyme.

After 48 hr. the digest was heated (15 min. at 98°), cooled and dialysed against successive changes of distilled water for 48 hr. at room temperature. The polysaccharide–protein mixture was then concentrated *in vacuo* at 35° to one-fifth of the original volume. Since the polysaccharide was only partly soluble, coagulated protein could not be removed by filtration. Deproteinization was therefore effected by the toluene–sodium chloride method (Anderson & Greenwood, 1955). Sodium chloride to 0·1 M-concentration was added and the mixture shaken with freshly distilled toluene (¼ vol.) for 18 hr. The denatured protein was removed from the toluene–water interface and the aqueous solution collected. The deproteinization was repeated three times. The final aqueous solution was dialysed (24 hr., against distilled water) and the polysaccharide isolated by freeze-drying.

The product had the following properties: glucose content, 81 %; α-amylolysis limit, 89 %; β-amylolysis limit, 57 % (before) and 82 % (after) treatment with isoamylase; $[\eta]$ in M-potassium hydroxide, 55·0; the iodine complex showed $\lambda_{max.}$ 550–555 mμ and absorption value $_{max.}$ 1·30. These properties are typical of amylopectin-type polysaccharides (see Table 9). On incubation with yeast branching enzyme a further decrease in iodine-staining power was observed (Table 4).

Action on amylopectin-type polysaccharides

Iodine-staining measurements. Incubation with amylase-free yeast branching enzyme resulted in a marked decrease in iodine-staining power (Table 4), although the rate was much greater with waxy-maize starch and the synthetic amylopectin, both of which have a lower degree of branching than the malted-barley amylopectin and amylopectin β-dextrin. This behaviour distinguishes yeast branching enzyme from the plant Q-enzymes (Peat, Turvey & Jones, 1959).

β-Amylolysis. Under similar conditions an appreciable (10 %) decrease in the β-amylolysis limit of potato amylopectin was observed (Table 5).

In view of these results, the product of action of yeast branching enzyme on potato amylopectin was investigated in detail.

Preparation of synthetic glycogen

Amylopectin (1·3 g.: sample II, prepared by thymol fractionation of potato starch var. Kerr's Pink) was dissolved in water (100 ml.). To the solution after filtration through sintered glass (G. 4) was added 0·2 M-sodium citrate buffer (pH 6·8; 30 ml.) and an aqueous solution of yeast branching enzyme (900 mg.; 20 ml.), and the digest was incubated at 20°. Samples (0·5 or 1·0 ml.) were withdrawn at intervals for determination of absorption

value (Unicam spectrophotometer at 540 mμ) and β-amylolysis limit. The results are shown in Table 6.

After 24 hr. the enzyme was inactivated by heating at 98° for 15 min. Coagulated protein was removed by filtration (G. 3 and G. 4 filters) and the filtrate dialysed against running distilled water at room temperature for 48 hr. The solution was concentrated *in vacuo* and the polysaccharide isolated by freeze-drying.

The product had the following properties: glucose content, 69 %; α-amylolysis limit, 82 %; β-amylolysis limit, 46 %; β-amylolysis limit after pretreatment with isoamylase, 66 %.

The polysaccharide was purified by treatment with ice-cold 4 % trichloroacetic acid. The freeze-dried material was dissolved in water (90 ml.), cooled to 0° and 40 % trichloroacetic acid (10 ml.) was added slowly with stirring. After 18 hr. a small precipitate was removed by centrifuging at 0°, and 2 vol. of cold absolute ethanol was added to the solution. The polysaccharide was dissolved in water, reprecipitated with ethanol, washed with ethanol and dried over P_2O_5. Yield, approx. 0·9 g.

The purified polysaccharide was freely soluble in cold water and the solution gave a red–brown with iodine. It had the following properties: $[\alpha]_D + 198°$ (c, 0·2 in water); glucose content, 95 %; α-amylolysis limit, 79 %; β-amylolysis limit, 47 %; β-amylolysis limit after pretreatment with isoamylase, 75 %; the iodine complex had $\lambda_{max.}$ 480 mμ and absorption value $_{max.}$ 0·56; [η] in 0·1 M-sodium chloride, 11·7. The combined action of isoamylase and β-amylase gave 90 % conversion into maltose; under similar conditions glycogen and amylopectin gave 95 and 84 %.

Table 6. *Conversion of amylopectin into a glycogen-type polysaccharide by yeast branching enzyme*

See text for composition of digest.

Time of incubation (hr.)	Decrease in absorption value (%)	β-Amylolysis limit (%)
0	0	53·2
2	29·6	52·0
5	39·8	51·5
6·5	47·0	50·5
8	56·0	49·5
18	92·0	47·2

The polysaccharide (241 mg.) was oxidized with a saturated solution of potassium metaperiodate (120 ml.) at room temperature. Samples (10 ml.) were analysed for formic acid at intervals; after 10, 12, 15 and 16 days, 0·68, 0·82, 0·86 and 0·86 ml. of 0·01013 N-sodium hydroxide were required for neutralization. The final titre corresponds to an average chain length of 13·5 glucose residues.

The remaining solution of periodate-oxidized glycogen (70 ml.) was filtered and neutralized with ethylene glycol (5 ml.). After dialysis against running tap water at room temperature for 3 days the solution was concentrated (5 ml.). A part (1 ml.) of the solution was hydrolysed with 2 N-sulphuric acid at 100° for 2 hr., neutralized with barium carbonate, concentrated and deionized with Amberlite IR-4B and IR-120 resins. No glucose could be detected by paper chromatography. The remaining solution was treated with potassium borohydride (20 mg.) at room temperature for 3 days. The polyalcohol was precipitated with ethanol, washed with ethanol and then hydrolysed with 2 N-sulphuric acid (2 ml.). The neutralized deionized hydrolysate did not contain glucose (tested by paper chromatography).

Action on glycogen-type polysaccharides

Yeast branching enzyme had no significant effect on the iodine-staining power or β-amylolysis limit of glycogen or Floridean starch [the latter is an algal polysaccharide closely resembling glycogen in many properties (Fleming *et al.* 1956)], as shown in Table 7. The results also confirm the absence of isoamylase from the preparation of yeast branching enzyme.

DISCUSSION

This investigation shows that extracts of brewer's yeast contain an active branching enzyme (systematic name amylo-1:4 → 1:6-transglucosidase) in addition to the previously reported phosphorylase and isoamylase. This finding has been confirmed

Table 7. *Effect of yeast branching enzyme on the absorption value and β-amylolysis limit of glycogen-type polysaccharides*

For composition of digests see Table 4. (*a*) Absorption value; (*b*) β-amylolysis limit.

Time of incubation (hr.)	Rabbit-muscle glycogen		Horse-muscle glycogen		Floridean starch	
	(*a*) (460 mμ)	(*b*) (%)	(*a*) (480 mμ)	(*b*) (%)	(*a*) (540 mμ)	(*b*) (%)
0	0·105	40	0·119	55	0·227	35
3	0·098	41	—	—	0·230	34
24	0·101	41	0·117	56	0·226	35
48	0·108	—	0·118	—	—	—

independently by Hopkins (1955). In the course of purification of yeast phosphorylase (D. J. Manners & Khin Maung, unpublished work), protein fractions precipitated with ethanol showed the least phosphorylase activity and the greatest activity of yeast branching enzyme. Fractionation of yeast proteins with ethanol–sodium citrate at low temperature, as used by Gilbert & Patrick (1952) for the purification of potato Q-enzyme, was therefore carried out. Although all the fractions showed some activity, that obtained at 25–30% ethanol concentration was most active; the partly purified enzyme was finally isolated by freeze-drying in the presence of sodium citrate buffer, pH 6·8. This method has been applied to the related potato enzymes (Barker, Bourne, Wilkinson & Peat, 1950). The majority of the preparations of yeast branching enzyme were substantially free from phosphorylase and isoamylase but contained traces of maltase and isomaltase, the presence of which does not affect the interaction between yeast branching enzyme and starch-type polysaccharides. The detection of traces of α-amylase is extremely difficult; for example, some preparations of β-amylase contain an α-amylase (Z-enzyme) which appears to attack amylopectin β-dextrin but not glycogen β-dextrin (. . Cunningham, D. J. Manners, A. Wright & I. D. Fleming, unpublished work). With this proviso, the results in Fig. 1 and Table 7 suggest that a normal α-amylase is not a significant contaminant of the preparation of yeast branching enzyme. Hopkins (1955) has also concluded that α-amylase is absent from brewer yeasts.

The action of yeast branching enzyme on both amylose and amylopectin results in a decrease in both iodine-staining power and β-amylolysis limit (Tables 3–6) without the concomitant production of reducing groups. The introduction of branch points into the substrate rather than random hydrolysis is therefore indicated. Enzyme action on amylose is optimum at pH 7·0 and 20°, these conditions being similar to those of other branching enzymes (Table 8). Yeast branching enzyme, like potato Q-enzyme, is completely inhibited by mercuric chloride, but only partially by ammonium molybdate and does not require a carbohydrate activator or co-substrate. In this respect, *Polytomella coeca* Q-enzyme, the action of which is markedly stimulated by certain carbohydrates (Barker, Bebbington & Bourne, 1953), would appear to be unique (see also Larner & Uwah, 1956).

Action on amylose

The action of yeast branching enzyme results in a marked change in iodine-staining properties, shown by: (1) a large and rapid decrease in absorption value at all wavelengths, and especially at 680 mμ; (2) a displacement in $\lambda_{max.}$ from 630 to about 520–550 mμ. These changes are accompanied by a 50% decrease in β-amylolysis limit. Since some of the newly formed inter-chain linkages can be hydrolysed by isoamylase, this indicates that they are α-1:6-glucosidic in nature. These results may be compared with the similar action of potato Q-enzyme, and the apparent inability of the animal branching enzymes to attack this polysaccharide (Barker & Bourne, 1953; Cori & Cori, 1943).

Under our experimental conditions amylose is converted into an amylopectin-type polysaccharide. This was difficult to purify, and analysis was confined to iodine-staining and enzymic degradation, since protein impurities interfere with periodate oxidation. Nevertheless, the results obtained (Fig. 5, and Table 9) clearly show the close structural relationship between the synthetic and a natural (potato) amylopectin, particularly with regard to P_M values on α-amylolysis, which are directly related to the degree of branching.

The synthetic amylopectin represents an intermediate in the conversion of amylose into glycogen since on reincubation with yeast branching enzyme further branching takes place. The relative proportion of branching enzyme is therefore a major factor in controlling the degree of branching, in accord with the findings of Barker, Bourne, Peat & Wilkinson (1950).

Action on amylopectin

In contrast with plant Q-enzymes, yeast branching enzyme has a marked action on amylopectin-type polysaccharides. On incubation with waxy-maize starch (or its β-dextrin), potato amylopectin or malted-barley amylopectin, the iodine-staining power or β-amylolysis limit of the substrate or both are appreciably reduced (Tables 4–6). The end-product of action of yeast branching enzyme on potato amylopectin appears to be a glycogen-type polysaccharide since the extent of

Table 8. *Properties of branching enzymes*

Source	Optimum pH	Optimum temperature	Reference
Potato Q-enzyme	7·0	21±1°	Barker, Bourne & Peat (1949)
Broad-bean Q-enzyme	7·25–7·5	20	Hobson, Whelan & Peat (1950)
Green-gram Q-enzyme	7·0	24	Ram & Giri (1952)
Polytomella coeca Q-enzyme	7·3	25–33	Bebbington, Bourne, Stacey & Wilkinson (1952)
Yeast branching enzyme	7·0	20	—

Table 9. *Properties of amylopectin-glycogen type polysaccharides*

Property	Potato amylopectin	Synthetic amylopectin	Rabbit-liver glycogen	Synthetic glycogen	Brewer's-yeast glycogen
Average chain length (glucose residues)	22·0	—	12·6	13·5	13·2
Proportion of 1:6-linkages (%)	4·5	—	8·0	7·4	7·6
Iodine complex					
$\lambda_{max.}$ (mμ)	545	550–555	460	480	430
Absorption value$_{max.}$	1·30	1·30	0·41	0·56	0·34
α-Amylolysis (P_M value)	89	89	78	79	79
β-Amylolysis limit (%)*					
Before pretreatment	53	57	46	47	44
After pretreatment	80	82	78	75	68
Limiting viscosity number					
In 1 M-potassium hydroxide	205	55	—	—	—
In 0·1 M-sodium chloride	—	—	10	12	7

* 'Before' and 'after' refer to pretreatment with yeast isoamylase.

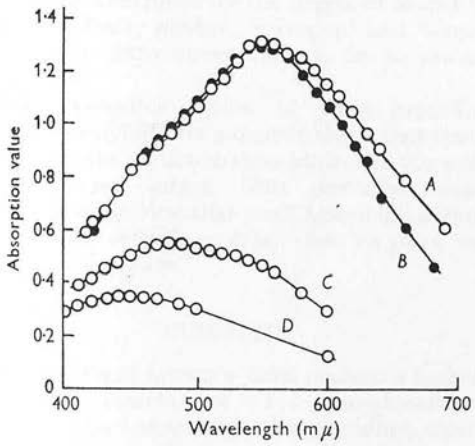

Fig. 5. Light-absorption curves of iodine-stained polysaccharides. Solutions contained 0·01% of polysaccharide (*A*, synthetic amylopectin; *B*, potato amylopectin; *C*, synthetic glycogen) and 0·02% of iodine in 2·0% potassium iodide, and were read against an iodine–iodide reference solution in a Unicam SP. 500 spectrophotometer. The curve for rabbit-liver glycogen (*D*) is taken from Liddle (1956).

enzymic degradation, end-group content and physical properties (especially limiting viscosity number) are very similar to those of brewer's-yeast glycogen and a normal sample of rabbit-liver glycogen (Table 9). It follows that two classes of 'branching enzyme' may be distinguished: (*a*) those that have no action on amylopectin, e.g. potato Q-enzyme (Peat *et al.* 1959); (*b*) those acting on amylopectin, e.g. liver branching enzyme (Larner, 1955). For this reason it is suggested that the term 'Q-enzyme' be confined to the former class of enzymes (cf. Hopkins, 1955).

The present results suggest that the enzymic conversion of amylose into glycogen occurs through an intermediary amylopectin-type polysaccharide, in agreement with generally accepted views, as reviewed by Barker & Bourne (1953). However, Erlander (1958) has proposed that the starch components are produced by the action of a debranching enzyme (not yet detected or isolated) on glycogen, and that amylopectin is a partly debranched glycogen. This theoretical reaction is virtually the reverse of that now demonstrated *in vitro*. It is clear that the detailed mechanism of glycogen and amylopectin synthesis *in vivo* requires further investigation, and that the pathways for synthesis and degradation may, in fact, involve separate enzyme systems [cf. Villar-Palasi & Larner (1958) Robbins, Trant & Lipmann (1959)].

On the basis of partial acid-hydrolysis (Wolfrom & Thompson, 1956, 1957) and periodate-oxidation studies (Abdel-Akher, Hamilton, Montgomery & Smith, 1952; Hamilton & Smith, 1956) it has been suggested that both amylopectin and glycogen contain a small proportion of α-1:3-glucosidic linkages. In view of this, the nature of the inter-chain linkages introduced by yeast branching enzyme into amylopectin was examined, as follows: (*a*) periodate-oxidized synthetic glycogen was converted into the corresponding polyalcohol and hydrolysed with acid: glycose could not be detected; (*b*) the outermost inter-chain linkages of the synthetic glycogen are, like those of the parent amylopectin, susceptible to isoamylase, which has no action on α-1:3-glucosidic linkages; (*c*) the combined action of β-amylase and isoamylase causes 90% degradation. In view of the relatively small amount of synthetic glycogen available for analysis (the chemical studies by the workers cited above

required 10–100 g. quantities), our results are considered to provide qualitative evidence against the synthesis of a significant proportion of α-1:3-glucosidic linkages.

Action on other polysaccharides

The results in Table 7 show that yeast branching enzyme has no significant action on the β-amylolysis limit and iodine-staining properties of normal glycogen (muscle glycogen was used since it is more iodophilic than yeast glycogen). A polysaccharide containing 8% of α-1:6-glucosidic linkages probably represents the highest degree of branching obtainable under these conditions *in vitro*. However, the failure of yeast branching enzyme to act on horse-muscle glycogen (average chain length 17) and ability to react slowly with malted-barley amylopectin (average chain length 18) suggests that the specificity of yeast branching enzyme is not entirely controlled by the degree of branching in the substrate; further, 'glycogen' and 'amylopectin' may differ structurally in an as yet unknown way.

The appreciable action of yeast branching enzyme on amylopectin β-dextrin shows that chains carrying 'stubs' of two to three glucose residues can be transferred intact. This provides further evidence for the view that yeast branching enzyme has a rather wider specificity than its plant and animal counterparts.

SUMMARY

1. Extracts of brewer's yeast contain a branching enzyme (amylo-1:4 → 1:6-transglucosidase) which may be isolated by ethanol–sodium citrate fractionation at −5°.

2. Enzyme action on amylose is optimum at pH 7·0 and 20°, and is inhibited by mercuric chloride (0·5 mM).

3. The reaction is characterized by a marked decrease in iodine-staining power and β-amylolysis limit of the substrate without the concomitant production of free reducing groups.

4. Enzyme action on amylose yields initially an amylopectin-type polysaccharide; on incubation with further enzyme, amylopectin is converted into a glycogen-type polysaccharide.

5. The mode of synthesis of glycogen and the properties of related animal and plant branching enzymes are discussed.

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Studies on Carbohydrate - Metabolizing Enzymes. Part IV.* The Action of Z-Enzyme on Starch-type Polysaccharides

By W.L. Cunningham, D.J. Manners, A. Wright and (in part) I.D. Fleming

The Z-enzyme impurity in a barley β-amylase preparation has no action on the anomalous linkages in amylose, but causes random hydrolysis of a small number of α-1:4-glucosidic linkages. Amylopectin and amylopectin β-dextrin are also slowly attacked, but under similar conditions, the rate of hydrolysis of glycogen and glycogen β-dextrin is not measurable. The activity, which is optimum at pH 5.6, is stabilized by calcium ions, and partly inhibited by EDTA and mercuric chloride, is attributed to a minute trace of α-amylase.

The Z-enzyme activity of soya bean β-amylase preparations, and of almond emulsin, are also due to the presence of very small traces of α-amylase.

*Part III, Gunja, Manners, and Khin Maung Biochem. J., 1960 (in the press).

The methods available for the detection of α-amylase impurities in carbohydrase preparations are discussed. The most sensitive assay is that using amylose β-limit dextrin as substrate and viscometry as the method of analysis.

THE action of purified β-amylase on most samples of amylose is incomplete, since only 65-80% conversion into maltose is observed[1]. This indicates the presence of a small number of resistant or anomalous linkages in the substrate. Further studies[2] have shown that amylose is heterogeneous with respect to both degree of polymerization ($\overline{DP}$) and behaviour on β-amylolysis; with potato amylose 40% (by weight) of $\overline{DP}$ *ca.* 2,000 is completely hydrolysed by β-amylase, and the anomalous linkages occur only in the remaining material of $\overline{DP}$ *ca.* 6,000. For complete amylolysis a second enzyme, named Z-enzyme[3], is required; this occurs together with β-amylase in soya-beans[3,4] and barley[5], is inactivated at pH3.6[4], and has no action on α-1:3 or α-1:6-glucosidic linkages or on β-glucosidic linkages. We now report evidence that Z-enzyme action involves the hydrolysis of a small number of non-terminal α-1:4-glucosidic linkage in amylose rather than the selective hydrolysis of the anomalous linkages and is due to the presence of a minute trace of α-amylase in the β-amylase preparation. Z-enzyme (α-

amylase) also slowly degrades amylopectin and its β-dextrin.

In our earlier studies on α-1:4-glucosans, a highly active preparation of barley β-amylase (Wallerstein Analytical reagent) was used. By conventional tests, α-amylase could not be detected:- (a) the iodine staining power of amylopectin β-dextrin measured at 680 mμ. did not decrease within 24 hr.[7], (b) the β-amylolysis limit of glycogen was independent of enzyme concentration[7], (c) the molecular weight of glycogen β-limit dextrin (13×10^6) was in good agreement with that calculated from the molecular weight and β-amylolysis limit (29×10^6, 49%)[9], (d) during enzyme action on amylose, the intermediate 50% limit dextrin had the same molecular size as the original substrate[10]. The same enzyme preparation was considered to contain Z-enzyme since complete degradation of amylose at pH 4.6 but not at 3.6 was observed[2].

The first indication that the apparent Z-enzyme activity might be due to a trace of α-amylase impurity was obtained during studies on the β-amylolysis of amylopectin, when an apparent relationship between enzyme concentration and β-amylolysis limit was found[11]. With digests containing 33, 66, 99 and 128 units [12] of β-amylase per mg. polysaccharide, the apparent β-amylolysis limits were 64, 65, 68 and 71 after 92 hr. incubation at pH 4.9 and 37°. Such a relationship is characteristic of the α-amylases[13]. Since this finding was at variance with the previous results, the enzymic homogeneity

of the β-amylase preparation was investigated.

The Degradation of Amylopectin and β-Dextrin by Barley Z-enzyme - The above observation has been examined in detail using amylopectin β-dextrin as substrate, and (a) iodine staining, (b) viscosity, (c) reducing power measurements to follow enzyme action.

Peat, Pirt and Whelan[4] carried out iodine staining by measuring the decrease in absorption value (A.V.) of the polysaccharide-iodine complex at 680 mμ, the wave length used for 'blue-value' (B.V.) determinations. In our experiments, the wavelength of maximum absorption (λmax) has been used, namely 530-540 mμ. Under these conditions, and with an increase in the relative enzyme concentration and time of incubation, a marked decrease in iodine staining power was observed. For example, with 0.1% substrate and 0.2% barley preparation (equivalent to ca 250 β-amylase units [12]/mg polysaccharide) at pH 4.6, an 87% decrease in A.V. occurred in 70 hr., and the residual polysaccharide then showed λmax at 420 mμ. This indicates random hydrolysis of non-terminal α-1:4-glucosidic linkages (the degradation of α-1:6-linkages in β-dextrin results in an increase in A.V.[14]). Under similar conditions the A.V. of glycogen β-dextrin at 470, 430, 420 and 410 mμ was unchanged.

The decrease in A.V. of amylopectin β-dextrin could be

detected with only 0.03% barley preparation; at 480, 520 and 560 mμ, the A.V. fell by 17, 30 and 40% respectively after 70 hr. Further experiments (Table I) showed that this activity was increased by pre-incubation of the enzyme with $5x10^{-3}$M calcium sulphate for 30 min., and was decreased by a similar treatment with $5x10^{-2}$M ethylene diamine tetra acetic acid (EDTA). The function of the calcium appears to be that of an enzyme stabiliser rather than a specific activator. The presence of this ion decreased the rate of inactivation of the enzyme at pH 4.6 and 37° during incubation for 67 hr. in the absence of substrate, and did not restore the activity of a partly inactivated enzyme preparation. EDTA would appear to lower the activity by removal of the calcium. (cf. ref. 15) The protection of other α-amylases, including malt α-amylase, from inactivation by calcium ions has been noted by several workers.[16]

The activity towards β-dextrin was greater in acetate buffer pH 5.6 than at pH 4.8 or 6.5 (see Fig. 1 and 2); none was detected at pH 3.6. At pH 5.6, the activity in acetate and B.D.H. Universal buffer was identical, in contrast to Cladophora rupestris amylase which is more active in the latter buffer.[17] The addition of $5x10^{-3}$M borate, which inhibits isoamylase[18] and activates Cladophora amylase[17], to the acetate buffer had no effect; phenylacetate ($7x10^{-3}$M) and phosphate

(7×10^{-3}M) also did not alter the rate of decrease of A.V. (540 mμ).

In contrast to the above results (with ca 250 units β-amylase /mg. substrate), the enzyme preparation had no appreciable effect on the iodine staining power of amylopectin when the experimental conditions similar to those employed by Peat, Pirt and Whelan[4] were used (ca 40 units /mg. substrate; A.V. (680 mμ)) as shown in Table 2. This result illustrates the importance of varying the enzyme concentration in testing for contaminating enzymes.

A slow and limited degradation of 0.5% amylopectin β-dextrin solution by 0.5% barley preparation at pH 5.6 and 25° was also shown by viscosity measurements. After 5, 40 and 70 min., the specific viscosity (η_{sp}) values were 0.194, 0.186 and 0.179 respectively. At pH 3.6, the viscosity was unchanged.

Despite the limited extent of the degradation of β-dextrin in the above experiments, the effect of this initial α-amylolysis can be magnified by the presence of an excess of β-amylase. The hydrolysis of only a small number of interior α-1:4-linkages liberates non-reducing end-groups which are susceptible to this enzyme. The resultant increase in reducing power is now appreciable whereas β-amylase and low concentrations of α-amylase acting separately on β-dextrin do not yield reducing sugars. The results summarised in Table 3 show the apparent percentage conversion into maltose (P_M)

observed during the degradation of β-dextrin by the barley preparation. In a further experiment, the relative P_M values at pH 3.6, 5.6 and 6.5 were 4, 45 and 33 respectively.

The Degradation of Amylose and β-Dextrin by Barley Z-enzyme - Although the highly branched amylopectin β-dextrin may be used to detect relatively high concentrations of Z-enzyme, the use of a linear substrate is preferable since the hydrolysis of only a small number of linkages will produce a correspondingly greater change in physical properties.

Using amylose β-limit dextrin (prepared by the prolonged action of β-amylase at pH 3.6 on potato amylose), the effect of certain inhibitors on barley Z-enzyme was investigated. The results are summarised in Table 4.

Peat, Thomas and Whelan[3] reported that Z-enzyme was not inhibited by low concentrations of mercuric chloride (ca 1.5×10^{-6} M) and concluded that this fact distinguished Z-enzyme from α- and β-amylase. Although the extreme sensitivity of β-amylase for mercury compounds and related -SH reagents is well known[19], there is evidence that -SH groups are not essential for the activity of α-amylases.[20,21] We have examined the effect of various concentrations of mercuric chloride on the activity of a number of α-amylases and the results (Table 5) show that only partial inactivation occurs

with concentrations of 10^{-5} to 10^{-6}M. Furthermore, the action of barley Z-enzyme is only partially inhibited by mercuric chloride (Table 4 and 5). Notwithstanding the qualitative nature of these studies (the concentration of α-amylase was not identical in the various assays), it is concluded that the use of mercuric chloride shows, in fact, a similarity between Z-enzyme and α-amylase.

p-Chloromercuribenzoate (PCMB; 1×10^{-5}M) causes complete inhibition of β-amylase[19] and yet has less effect on barley Z-enzyme than similar concentrations of mercuric chloride (Table 4). This reagent has therefore been used for the selective inhibition of β-amylase. On incubation of amylose (0.2%) with barley preparation (0.07%, corresponding to ca 45 β-amylase units/mg) and PCMB, a marked decrease in specific viscosity was observed, but without a concomitant decrease in iodine staining power or increase in reducing power. This is attributed to the random hydrolysis of a small number of non-terminal linkages. Since the iodine staining power of amylose as obtained by 'blue-value' or λ_{max} measurements is approximately the same for samples of $\overline{DP}$ 500 or 2,000,[23] this result emphasises the caution required in following limited α-amylolysis by iodine staining. Measurements of the change in reducing power,[13] or of the sedimentation constant[10] of the residual amylose are also unsatisfactory when the concentration

of α-amylase is extremely low. It is clear that viscometry provides the only sensitive method when concentrations of β-amylase contaminated by Z-enzyme similar to those used in our previous studies[6-8] and by Peat and co-workers[3,4] are employed.

The presence of traces of α-amylase in unpurified barley β-amylase preparations has been noted by earlier workers including Hopkins, Murray, and Lockwood.[24] The amount of α-amylase appears to depend upon the condition of the grain, and the variety of the barley.[25] Part of the α-amylase may represent precursors of the enzyme which develops in quantity during germination, and part may arise from contamination of the barley husk by amylase-secreting bacteria.[26] The α-amylase constituents of ungerminated and germinated barley are undoubtedly closely related, and we have found that the initial action of malt α-amylase on amylose β-dextrin is also not activated by calcium ions, and is partially inhibited by mercuric chloride (1.5×10^{-8} and 1.5×10^{-6}M). Furthermore, the optimum pH of unpurified malt α-amylase is ca 5.4,[21] a value similar to that shown in Fig. 2 and different from that of bacterial α-amylase[27] (ca 6.5).

The Z-enzyme Activity of Soya-bean β-Amylase and Almond Emulsin - Since Z-enzyme was originally detected in unpurified ('stock') preparations of soya-bean β-amylase[3,4] and in almond emulsin[3,28] (a complex mixture of carbohydrases

including β-glucosidases), samples of these have been examined for contamination with α-amylases.

Both preparations had no significant effect on the production of maltose from amylopectin - β-amylase or Floridean starch - β-amylase systems (cf. Table 2 and ref. 6); by this criterion[13] gross contamination with α-amylase could be ruled out. However, using amylose, amylose β-dextrin or amylopectin β-dextrin as substrates, and assay by iodine staining and reducing power or viscosity methods, the presence of a <u>trace</u> of α-amylase was established. Slight random degradation of the substrates occurred; for example, incubation of amylase (0.1%) with emulsin (0.5%) at pH 4.6 for 24 hr. reduced η by 88% and increased the β-amylolysis limit from 75 to 95%. This α-amylolytic activity was increased by calcium ions (which again act as a stabiliser rather than activator), and partly inhibited by EDTA and mercuric chloride (<u>ca</u> 10^{-4} - 10^{-6}M), and was optimum in the region pH 5.8-6.1 (cf. Peat, Thomas, and Whelan[8] who reported that the action of soya-bean Z-enzyme on amylose β-dextrin was optimum at pH 6). Typical results are shown in Table 6 and Figs. 3 and 4. Under similar conditions, the extent of hydrolysis of a glycogen β-dextrin was very low (P_M 2-4). The properties of the α-amylase present in soya-beans and almond elulsin are therefore generally similar to, although not necessarily

identical with, those of barley Z-enzyme.

Discussion and Conclusions - The recognition of the presence of a trace of α-amylase in the barley and soya-bean β-amylase preparations, and in emulsin, provides an explanation for the observed increase in the β-amylolysis of amylose. The slight random hydrolysis will expose sufficient new non-reducing end groups to enable further β-amylolysis to take place. Assuming the presence of only one anomalous structure per amylose molecule, the random hydrolysis of only one α-1:4-glucosidic linkage will cause a 10-15% increase in β-amylolysis limit, e.g., from ca 75 to ca 87%. The ability of Z-enzyme to increase the phosphorolysis limit of amylose, from 70 to 95% conversion into glucose-1-phosphate[3], can now also be explained in terms of slight α-amylolytic activity.

The nature of the structural anomalies in amylose is not yet known.* These may include one or more of the following possibilities (a) an anomalous linkage (i.e. a glucosidic linkage other than the α-1:4-type) present either in the amylose chain, or as a branch point, (b) an anomalous

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*Since the proportion of these is extremely low (less than 1%), their presence in enzymic hydrolysates of amylose cannot be revealed by available methods of analysis, and is inferred from the know specificity requirements of α- and β-amylase.

residue i.e. an α-1:4-linked hexose residue derived from D-glucopyranose by the substitution of a phosphate group probably at $C_{(6)}$, or by acylation or oxidation at $C_{(2)}$, $C_{(3)}$ or $C_{(6)}$, (c) an anomalous residue and linkage. Recent evidence[39] suggests that a small number of glucose residues in amylose may become modified by oxidation during isolation of the polysaccharide. These modified residues are resistant to β-amylase and phosphorylase. Since Z-enzyme is an α-amylase, its action will involve the 'by-passing' of such structural anomalies rather than their removal by selective hydrolysis. Similarly, any anomalous linkages which are present as branch points will not be hydrolysed by Z-enzyme (or any other α-amylase) i.e. Z-enzyme does not act as a 'debranching' enzyme.

Some anomalous structures are present in unfractionated starch since the β-amylolysis limits with purified and 'stock' soya-bean β-amylase are 53 and 61% respectively.[4] This difference was attributed to the action of Z-enzyme on the amylose component. However, Hopkins and his co-workers[24] had previously shown that barley β-amylase prepared by a method involving pretreatment at pH 3.4 caused 56% conversion of soluble starch into maltose, and that if this treatment was omitted, or a minute trace of bacterial α-amylase was added to the purified preparation, the β-amylolysis limit was 63%. These findings are in accord with our observations, and the

view that 'Z-enzyme' is a trace of α-amylase provides an adequate explanation for the effect of pH on the β-amylolysis limit of unfractionated starch (cf. ref. 13).

The amount of α-amylase present in the barley preparation is too small to be assessed accurately, but in comparative experiments, a salivary α-amylase solution containing 34 units[27] was diluted 50,000 times and found to cause a slow decrease in the iodine staining power of both amylose and amylopectin and to have a negligible effect on glycogen. This suggests that the α-amylase activity of the barley preparation is of the order of 10^{-4} units/mg. A further indication of the minute degree of contamination is shown by a comparison of the turnover number[30] of the related malt α-amylase, equivalent to the hydrolysis of 19,000 bonds per min. per mol., with the limited degradation of amylose β-dextrin observed in our experiments during incubation for some hours. It is suggested that the inability of barley Z-enzyme to attack glycogen is a consequence of (a) the low concentration of enzyme, (b) the lowered affinity of α-amylases in general for glycogens,[31] rather than to an absolute specificity requirement (cf. R-enzyme which hydrolyses 1:6-linkages in amylopectin but not in normal 12-unit glycogen[14]). The hydrolysis of glycogen and its β-dextrin with normal concentrations of other α-amylases can readily be detected.[32]

Our general conclusions are in accord with the findings of Hopkins and Bird,[33] who have emphasised the difficulty in

detecting traces of α-amylase using amylopectin rather than amylose as a substrate, and with the recent results of Baba and Kojima,[34] and of Banks, Greenwood, and Jones.[35] The former workers also showed the presence of α-amylase in emulsin, whilst the latter have independently shown random hydrolysis of starch components, using light scattering and viscosity measurements, by the Z-enzyme contaminant of several unpurified β-amylase preparations.

It must be noted that the present results do not alter our earlier conclusions[2,6,7,8] on the molecular structure of starch and glycogen-type polysaccharides, or on the mechanism of β-amylase action[10], which are derived, in part, from results obtained using the Wallerstein barley β-amylase preparation.

EXPERIMENTAL

Analytical Methods - The general methods used were those described in earlier papers.[2,6-8] For viscometry, digests were prepared in either modified Ubbelohde or Ostwald viscometers, and the viscosity at 25° measured at intervals. Since the activity of an α-amylase is related to $d\left(\frac{1}{(\eta s_p)}\right)$,[36] graphs of $\left(\frac{1}{(\eta s_p)}\right)$ against t (mins.) were prepared.

With identical enzyme and substrate concentrations, the effect of added reagents could then be observed by a comparison of the slopes The polysaccharide solutions were filtered through sintered glass (G4) before analysis.

In the iodine staining experiments using amylopectin β-dextrin measurements at 540 mμ increased the A.V. to ca 0.5 in the most sensitive region of the spectrophotometer (Unicam S.P. 500) cf. A.V. of ca 0.1 at 680 mμ.

Enzyme Preparations - The properties of the barley β-amylase are reported in ref. 7. 'Stock' and purified soya-bean β-amylase were prepared by the methods of Bourne, Macey and Peat[37] and Peat, Pirt and Whelan.[4] Sweet almond emulsin was isolated by Tauber's method[38]; a weighed amount was suspended in the stated volume of water, centrifuged, and insoluble material discarded. Wallerstein malt diastase was used as a source of malt α-amylase, with short incubation periods to minimise the effect of β-amylase.

Substrates - (a) Amylose. Various samples made by the fractionation of potato starch (var. Kerr's pink) with thymol and butanol were used, together with amylose VI, VII and VIII.[2] Amylose β-dextrin was prepared by incubating amylose VIII (500 mg) with barley β-amylase (100 units/mg) at pH 3.6 for 24 hr. The β-amylolysis limit was 72%. The digest was heated for 10 min., cooled, filtered (G4 sinter) and the pH adjusted to 5.6 with sodium hydroxide. The dextrin was stored under toluene at room temperature; the maltose present did not interfere with subsequent measurements.

(b) Amylopectin. Fractionation of potato starch with thymol or pyridine gave samples I and II respectively. Waxy maize and sorghum starches were commercial samples. Amylopectin β-dextrin was prepared from waxy maize starch I (5 g.) treated with purified β-amylase (6,000 units) in a total volume of 250 ml, at 35° for 48 hr. The β-amylolysis limit was 53%.

After dialysis, the dextrin was isolated by freeze-drying.

(c) Glycogen β-dextrin. This was isolated from a digest of Ascaris ~~lumbricoides~~ lum glycogen and β-amylase.

Action of Barley Z-Enzyme on Amylopectin β-dextrin -

(a) Iodine staining measurments : Preliminary experiments had shown that the decrease in A.V. (540 mμ) of β-dextrin was proportional to enzyme concentration. Polysaccharide (25 mg), barley preparation (52 mg), 0.2M - acetate buffer (pH 4.6; 3ml) and water to 20 ml. were incubated at 35° for 70 hr. Samples (2 ml.) were removed heated to inactivate the enzyme and stained with iodine solution (2.5 ml) in a total volume of 25 ml. The A.V. (540 mμ) of amylopectin β-dextrin decreased from 0.740 to 0.097 and the product showed λ_{max} 420 mμ and $A.V._{max}$ 0.210. With glycogen β-dextrin, the initial and final A.V.'s were: 470 mμ, 0.044 and 0.042; 430 mμ, 0.073 and 0.074; 420 mμ, 0.075 and 0.078; 410 mμ, 0.067 and 0.069. Using only 6.25 mg. barley preparation; the following results were obtained:-

λ(mμ)	480	500	520	540	560	580
Initial A.V.	0.545	0.622	0.674	0.680	0.630	0.552
Final A.V.	0.446	0.469	0.472	0.440	0.377	0.319

Barley preparation (ca 50mg) pretreated with $5x10^{-3}$M calcium sulphate or $5x10^{-2}$M EDTA (pH 4.7) for 30 min. at 37° was incorporated into similar digests. Samples (3 ml) were removed after 72 hr.; the results are reported in Table 1.

In a further experiment with 0.67 mg. EDTA-treated enzyme, only a slight decrease in iodine staining power was noted:-

λ(mμ)	480	500	520	540	560	580
Initial A.V.	0.529	0.607	0.675	0.665	0.610	0.531
Final A.V.	0.529	0.573	0.600	0.575	0.517	0.436

The optimum pH for EDTA-calcium complex formation is 7.5;[39] hence, in the above experiments, the calcium ions may not have been completely removed.

(b) Reducing power measurements: Digests were prepared containing amylopectin β-dextrin (19.2 mg.), barley β-amylase (3,800 units), buffer solution (3 ml) and water to 20 ml. Samples (5 ml) were removed at intervals, deproteinised, and and the apparent maltose content determined. The results are shown in Table 3.

(c) Viscometry: Amylopectin β-dextrin (1% filtered solution, 10 ml), 0.2M-acetate buffer pH 3.6 or 5.6 (5 ml) and barley β-amylase (100 mg in 5 ml water) were mixed in a viscometer. The η_{sp} was determined over a 2 hr. period. At pH 5.6, $\frac{d(\frac{1}{\eta_{sp}})}{dt}$ indicated a relative activity of 7.8×10^{-3} units, and in in presence of mercuric chloride ($1 \times 5 \times 10^{-5}$M), of 7.2×10^{-3} units. At pH 3.6, there was no change in viscosity.

Effect of pH and various Ions on Activity - Amylopectin β-dextrin (ca. 30 mg), barley preparation (6,000 units) buffer (3 ml) and water to 25 ml. were incubated at 35°. The buffers

used were (a) 0.2 M-acetate pH 3.6, (b) pH 4.8, (c) pH 5.6, (d) pH 6.5 and (e) pH 5.6 containing borate to give a final concentration of 0.005M. The A.V. of samples (2 ml) was measured at intervals. The results obtained at 540 mμ are shown in Fig. 1. Similar results were obtained over the range 460 - 680 mμ.

For the pH-activity curves, β-dextrin (10 mg.) was incubated with β-amylase (1,250 units) and 0.2M-acetate buffer (pH 4.6 - 7.6; 5 ml.) in a total volume of 15 ml. Samples (3 ml.) were removed after 6 and 24 hr. and the A.V. at both 540 and 680 mμ determined. At 6 hr. and 540 mμ, the maximum decrease was at pH 5.5; at 24 hr., over the range pH 6.1 - 6.4 (see Fig. 2). The small change in pH is attributed to the decreased stability of the enzyme in acetate buffer in the pH region 4-6. The same results were obtained from A.V. (680 mμ). The experiment was repeated using phosphate-citrate buffer (pH 5.2 - 7.3 ; 0.1M citric acid and 0.2M disodium hydrogen phosphate ; 3 ml.) in a 10 ml. digest. The maximum fall in A.V. (540 mμ) occurred at pH 5.6 (after 8.75 hr.) and pH 5.8 (after 27 hr.)

Digests containing β-dextrin (10 mg.), barley preparation (2,000 units), buffer (5 ml.) in a total volume of 15 ml. were incubated at 35°. The following results were obtained.

Buffer	Acetate pH 5.6	B.D.H. Universal pH 5.6	Phenylacetate*	Borate*	Phosphate*
in A.V. (540 mμ)					
(a) 6 hr.	34	33	34	33	33
(b) 27 hr.	70	69	70	70	70
after 99 hr.	12.6	12.9	13.1	12.5	12.6

[*These digests contained 5 ml. acetate buffer pH 5.6 and 5 ml. of 2×10^{-2} M anion].

The function of the calcium ion was examined by incubating enzyme solution (2 ml.) with β-dextrin (10 mg.), acetate buffer (pH 5.8, 3 ml.) in a total volume of 10 ml. Digest no. 1 contained barley preparation preincubated at 37° and pH 5.8 for 67 hr.; digest no. 2, enzyme solution as above, but containing 5×10^{-2} M calcium acetate; digest no. 3; as digest no. 1, except that the enzyme was added to a mixture of β-dextrin and calcium acetate.

Fall in A.V. (540 mμ), %

Time of incubation (hr.)	Digest no. 1	Digest no. 2	Digest no. 3
4.5	13	31	13
7	18	45	19
24	49	83	51

Action of Barley Z-Enzyme on Amylose β-Dextrin -

(a) Iodine staining measurements - Digests containing amylose β-dextrin solution (0.46 mg/ml by acid hydrolysis, 14 ml), and

β-amylase (14 mg. in 0.5 ml. water, preincubated at 20° for 20 min. with 0.5 ml. inhibitor solution) were incubated at 35°. Samples (4 ml.) were withdrawn after 2.5 and 4.5 hr. and stained with iodine solution (1 ml.) and diluted with water to 25 ml. A.V. was measured at 560, 580, 600 and 640 mμ. The trend of results was the same at all wavelengths; the results at 600 mμ are given in Table 4.

The effect of mercuric chloride was examined in digests containing amylose β-dextrin (10 mg.), β-amylase (15 mg.), 0.2M-acetate buffer (pH 5.5, 3 ml), mercuric chloride solution (1 ml.) and water (6 ml.). Samples (3 ml.) were removed after 2.5 hr.

Concentration of mercuric chloride (M)	10^{-4}	10^{-5}	10^{-6}	0
Decrease in A.V. (600 mμ), %	7	15	47	49

(b) Viscometry - A digest containing amylose solution (70 mg.; 20 ml.; PCMB 1×10^{-5}M), and β-amylase (25 mg. in 15 ml. 0.2M acetate buffer pH 4.6; PCMB 1×10^{-5}M) was prepared in a viscometer. The following results were obtained:-

Time (min.).	15	30	60	97	120	155	205	20hr.	45hr.
$\frac{1}{\eta_{sp}}$	3.13	3.32	3.73	3.94	4.20	4.48	4.65	6.25	6.76

Samples were also removed for the measurement of A.V. at both 540 and 680 mμ, and the reducing power. No change could be detected within 24 hr. A control experiment showed that PCMB

(1×10^{-5}M) had no effect on the reaction of maltose with the Somogyi reagent. In the absence of PCMB the viscosity change is accompanied by a marked decrease in iodine staining power and the rapid production of reducing sugars.

Action of Normal Concentrations of β-Amylase on Amylopectin - Digests were prepared containing waxy maize starch I (30 mg.), 0.2M-acetate buffer (pH 4.6, 10 ml.), barley β-amylase or stock soya-bean β-amylase (1300 units) and water to a final volume of 50 ml. Samples (2 ml. for iodine-staining; 3 ml. for reducing power measurements) were removed at intervals. The results are shown in Table 2.

Action of 'Stock' Soya-bean β-Amylase on Amylopectin β-Dextrin - Polysaccharide (23.9 mg.), 0.2M-acetate buffer (pH 4.6, 3 ml.), enzyme solution (3 ml.) and water (19 ml.) were incubated at 35°. [The enzyme solution was prepared by dissolving 50 mg. powder (activity ca 100 units/mg.) in 5 ml. buffer and centrifuging.] Samples (2 ml.) were removed after 27 and 72 hr.: the results after 27 hr. were:-

λ(mμ)	480	500	520	540	560	580	680
Initial A.V.	0.528	0.612	0.687	0.700	0.649	0.574	0.198
Final A.V.	0.034	0.039	0.039	0.038	0.038	0.038	0.020

Similar results were obtained after 72 hr.

The effect of pH was examined in digests containing β-dextrin (5 mg.), phosphate-citrate buffer (pH 4.6-7.6; 5 ml.), β-amylase solution (1%, 2 ml.) and water (3 ml.). The P_M

values of 3 ml. portions were determined after 25 hr. at 35° (see Fig. 3).

Amylopectin β-dextrin (10 mg. in 5 ml. water) was added to β-amylase solution (0.3%, 4 ml.) pre-incubated as follows:- (a) with 0.2M-acetate buffer (pH 6.1, 10 ml. containing $5x10^{-3}$M calcium acetate); (b) with buffer containing $5x10^{-3}$M EDTA; (c) with buffer alone. The total volume was 24 ml. The percentage decrease in A.V. (540 mμ) of samples (2 ml.) measured after 1, 13 and 42 hr. was:- (a) 7, 66 and 92; (b) 0,6 and 13; (c) 1, 24 and 50. The P_M values after 42 hr. were 19, 7 and 14 respectively.

Digests containing β-dextrin (10 mg.), 0.2M-acetate buffer (pH 6.0, 5 ml.), β-amylase solution (0.25%, 2 ml.) and water and mercuric chloride (to give final concentrations of $1.5x10^{-5}$, $1.5x10^{-6}$M) in a total volume of 15 ml. were prepared. The change in A.V. (540 and 680 mμ) is shown in Fig. 4. The P_M values determined after 8.5 and 27.5 hr. were:- (a) without mercuric chloride, 15 and 18, (b) $1.5x10^{-6}$M, 14 and 18, (c) $1.5x10^{-5}$M, 5 and 9.

Action of 'Stock' Soya-bean β-Amylase on Other Polysaccharides - Potato amylopectin II (40 mg.), 0.2M-acetate buffer (pH 4.6, 9 ml) β-amylase solution (0.2%, 1 ml.) and water to 30 ml. were incubated at 35°. The β-amylolysis limit was 49 (0.5 hr.), 50 (1 hr.), 53 (4 hr.) and 53 (24 hr.). Under similar conditions soluble starch had a β-amylolysis limit of 62% but with purified soya-bean β-amylase, the value was 57%. Using potato amylose VI, β-amylolysis limits of 77% were found after 4 and 22 hr.

incubation with ca 5 units/mg. polysaccharide at pH 4.6; with higher enzyme concentrations, complete degradation occurred.

Action of Emulsin on Amylopectin - Waxy maize starch (20 mg.), β-amylase (50 units/mg.), 0.2M-acetate buffer pH 5.0 (4 ml.) and water to 25 ml. was incubated at 35° for 48 hr. The β-amylolysis limit was 57%. Emulsin (20 mg.) was added and after a further 24 hr. the β-amylolysis limit was 58%. In a second digest in which β-amylase and emulsin acted together on waxy maize starch, the β-amylolysis limit was 56 and 56% after 24 and 48 hr.

Action of Emulsin on Amylose - Amylose VIII (30 mg.) was incubated at pH 3.6 with barley β-amylase (100 units/mg.) in a total volume of 50 ml. for 24 hr. The β-amylolysis limit was 75%. The enzyme concentration was then doubled, and after 24 hr. the β-amylolysis limit was 76%. The pH of the digest was then adjusted to 4.8, and to a 15 ml. portion, emulsin solution (1%, 5 ml.) was added. After 1 and 24 hr., the β-amylolysis limits were 88 and 95% and the A.V. (680 mμ) (measured on a 3 ml. sample stained with 1 ml. iodine solution and diluted to 25 ml.) was 0.005 and 0.002 respectively compared with an original A.V. of 0.200.

A second 15 ml. portion of the digest was incubated with emulsin and 0.01M-mercuric chloride (0.5 ml.) in a total volume of 25 ml. (final concentration 2×10^{-4}M). The A.V. (680 mμ) was 0.202, 0.195 and 0.165 after 0, 1 and 24 hr.

Using amylose VI, β-amylolysis limits of 73% before, and 101% after the addition of emulsin were obtained; the A.V. (680 mμ) of a sample fell from 0.21 to 0.08.

Amylose VII solution (2 mg./ml.; 25 ml.) was then incubated at pH 4.6 with emulsin solution (2%, 12.5 ml.) in a total volume of 50 ml. After 24 hr. the digest was heated, cooled, and denatured protein removed at the centrifuge. The residual polysaccharide was precipitated with ethanol, washed, and dried. The specific viscosity at 25° of 25 mg. of polysaccharide dissolved in 20 ml. 0.2N-potassium hydroxide was 0.025, and the β-amylolysis limit, measured at pH 3.6, was 93%. Under similar conditions, amylose VII has a β-amylolysis limit of 75%.[2] In a control experiment using heat-denatured emulsin, the residual polysaccharide had a specific viscosity of 0.212.

Effect of pH on Activity - Digests containing amylose β-dextrin (2.4 mg. in 2 ml. water), phosphate-citrate buffer (pH 4.6-7.6; 2 ml.), emulsin solution (15 mg. in 1 ml. water), were incubated at 37°. Control digests (a) without enzyme, (b) without β-dextrin, were also prepared. After 27 hr. samples (2.5 ml.) were withdrawn, heated, and centrifuged. Iodine solution (1 ml.) was added to 2 ml. solution and the A.V. at 640 mμ measured. The results are shown in Fig. 3. No correction was required for the enzyme control.

Effect of calcium ions and inhibition on emulsin -

(a) Iodine staining measurements. Digests were prepared

containing amylose solution (0.4% 5 ml.), emulsin (0.5% in 0.2M-acetate buffer pH 5.6) and calcium acetate (5×10^{-3}M; 0.5 ml.) or water (0.5 ml). Digest (a) contained newly prepared enzyme and substrate and water; (b) contained enzyme preincubated at 37° for 40 hr.; (c) enzyme preincubated with calcium and (d) preincubated enzyme added to calcium. Samples (2 ml.) were withdrawn at intervals, heated, coagulated protein removed, and 1 ml. portion used for A.V. (680 mμ) measurements.

Fall in A.V. (%)

Time of incubation (hrs.)	(a)	(b)	(c)	(d)
5	11	-	-	-
6.5	-	2	4	2
16	35	-	-	-
21.5	-	9	29	12
40	52	-	-	-
47.3	-	12	47	15

The results show the stabilising effect of the calcium ions rather than an activating action.

(b) Viscosity Measurements. Digests containing amylase β-dextrin (13.5 mg.), emulsin solution (approximately 2% 4.5 ml. preincubated for 20 min. with reagent, and water (total volume 15 ml.) were incubated in a viscometer. The relative activities in two series of experiments were (a) 6.5 and 5.7×10^{-3} units with water and mercuric chloride

(1.5×10^{-6}M) respectively, (b) 9.3, 8.2 and 6.5×10^{-3} units with water, calcium sulphate (2×10^{-4}M) and EDTA (2×10^{-4}M) respectively.

Action of Emulsin on Amylopectin - and Glycogen - β-Dextrin - Digests were prepared containing either amylopectin β-dextrin (30 mg. in 15 ml. of 0.2M-acetate buffer pH 5.8) or glycogen β-dextrin (20 mg. in 5 ml. buffer) and emulsin (0.5% solution, 5 ml.). The following results were obtained.

	Amylopectin β-dextrin		Glycogen β-dextrin
	Decrease in	P_M	P_M
Time of incubation (hr.)	A.V.% (540 mμ)		
4	7	-	-
24	26	4.2	-
30	-	-	2.4
48	35	7.8	3.7

Action of Malt α-Amylase on Amylose β-Dextrin - β-Dextrin (5 mg.), 0.2M-acetate buffer (pH 5.6, 9 ml.), diastase solution (0.01%; 0.5 ml.) and water or reagent (0.5 ml) were incubated at 37° for 30 min. The A.V. (640 mμ) of a sample (3 ml.) was then determined. The following results were obtained (expressed as % fall in A.V.); control, 68 ; calcium acetate (2.5×10^{-4}M), 67; EDTA (2.5×10^{-3}M), 66; mercuric chloride (1.5×10^{-4}M), 2; (1.5×10^{-5}M), 25; (1.5×10^{-6}M), 41, PCMB (1×10^{-5}M), 51. The latter result shows the effect caused solely by the α-amylase and the results in

Table 5 are calculated on this basis.

Action of Salivary α-Amylase on β-Dextrins.- Freeze-dried salivary α-amylase (34 units[27], 1 mg. in 1 ml. water) was diluted 50,000 times, and sodium chloride added to a final concentration of 0.05M. Digests were prepared containing various β-dextrins (4.8-12.0 mg.) dissolved in 0.2M-acetate buffer pH 5.8 (6 ml.) and diluted salivary amylase (1 ml.). Samples (1 or 3 ml.) were removed for analysis by iodine staining or reducing power measurements. After incubation for 23 and 42.5 hr., the following results were obtained; with amylose β-dextrin (4.8 mg.) the A.V. (640 mμ) fell by 25 and 53%; with amylopectin β-dextrin (12.0 mg.), the A.V. (540 mμ) fell by 24 and 39%; with glycogen β-dextrin (12.0 mg.) the P_M values were 0.9 and 1.8 respectively.

In additional digests containing amylopectin β-dextrin (17.0 mg.) and glycogen β-dextrin (17.8 mg.), and either (a) 6.8×10^{-4} units α-amylase or (b) 13.6×10^{-4} units, in a total volume of 16 - 27 ml., the extent of degradation was as follows:-

	Time of incubation (hr.)	
Amylopectin β-dextrin	24.5	48.5
(a) Fall in A.V. (540 mμ), %	10	22
P_M	3.0	3.2
(b) Fall in A.V. (540 mμ), %	22	42
P_M	6.2	7.0
Glycogen β-dextrin		
(a) P_M	0.5	1.4
(b) P_M	1.4	3.2

Under similar conditions, the diluted salivary α-amylase thus hydrolyses approximately twice as many bonds in amylopectin β-dextrin as in glycogen β-dextrin.

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30. Schwimmer and Balls, J. Biol. Chem., 1949, 179, 1063.

31. Schwimmer, J. Biol. Chem., 1950, 186, 181; Hanrahan and Caldwell, J. Amer. Chem. Soc., 1953, 75, 2191.

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33. Hopkins and Bird, Nature, 1953, 172, 492.

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Table 1. The effect of the barley β-amylase preparation on the A.V. of amylopectin β-dextrin

Barley preparation	Fall in A.V. (%)		
	Normal	Pretreated with calcium ions	Pretreated with EDTA
λ (mμ)			
480	67	80	14
500	75	85	24
520	82	89	27
540	85	91	33
560	86	92	36
580	88	92	38
Original λ_{max} (mμ)	530	530	530
Original A.V.	0.67	0.66	0.68
Final λ_{max} (mμ)	430	430	510
Final A.V.	0.22	0.23	0.49

Digests contained 0.1% substrate and 0.2% enzyme preparation and were incubated at pH 4.6 and 35° for 72 hr.

Table 3. Action of barley preparation on amylopectin β-dextrin

	Apparent percentage conversion into maltose*		
Age of digest (hr.)	20	70	86
Digest conditions:			
Acetate buffer pH 4.6	25	33	37
" " pH 5.6	35	54	55
" " pH 5.6	35	54	56
Containing 5×10^{-3}M borate			
B.D.H. Universal buffer pH 5.6	31	49	52

*For composition of digests, see Experimental

Table 4. Effect of various inhibitors* on the action of barley Z-enzyme on amylose β-dextrin

Inhibitor	Concentration	Inhibition (%)†
Iodoacetate	($1x10^{-2}$N)	100
Mercuric chloride	($1.5x10^{-5}$M)	80
Phenylmercuric acetate	(1.10^{-4}M)	79
	($1x10^{-5}$M)	73
PCMB	($1x10^{-5}$M)	7
Silver nitrate	($1x10^{-4}$M)	86

* All inhibitors caused 100% inhibition of β-amylase.

† Based on A.V. (600 mμ) measurements; see Experimental.

Table 5. Effect of mercuric chloride on the activity of α and β-amylases

Concentration of mercuric chloride	Inhibition (%)			
	10^{-3}M	10^{-4}M	10^{-5}M	10^{-6}M
Barley β-amylase*	100	100	100	100
Salivary α-amylase†	84	66	44	22
Bacterial α-amylase†	87	67	35	26
Malt α-amylase††	-	96	50	19
Barley Z-enzyme††	-	86	70	7

*Assay under the conditions of Hobson, Whelan, and Peat[12]

†Assay under the conditions of Bernfeld[22]

††See Experimental

Table 6. Effect of emulsin on the iodine staining power of amylose β-dextrin

	Fall in A.V. (%)			
Wavelength (mμ)	580	600	640	680
Digest conditions*				
(a) Control	56	60	65	69
(b) Mercuric chloride				
(1.5×10^{-5} M)	33	35	38	41
(1.5×10^{-6} M)	44	47	55	51
(c) Pretreated with calcium sulphate†	72	74	79	82
(d) Pretreated with EDTA††				
(i) alone†	23	24	26	26
(ii) diluted with water	27	28	31	32
(iii) diluted with calcium sulphate solution†	47	50	51	55

* incubated for 25 hr. at 35°

† final concentration 2×10^{-4} M

†† see Experimental section

Fig. 1. Effect of barley Z-enzyme on the iodine staining power of amylopectin β-dextrin

[Enzymic reactions carried out at pH 3.6 (A), 4.6 (B), 5.6 (C), 6.5 (D) and at 5.6 in presence of 5×10^{-3}M borate (E)]

Fig. 2. Effect of pH on barley Z-enzyme activity

[Substrate : amylopectin β-dextrin, acetate buffer -O- analysed after 6 and 24 hrs. (curves A and B), and phosphate-citrate buffer -●- analysed after 8.75 and 27 hrs. (curves C and D)]

Fig. 3. Effect of pH on the Z-enzyme activity of emulsin and 'stock' soya-bean β-amylase preparation

[Curve A represents action of soya-bean preparation on amylopectin β-dextrin (reducing power measurements) and B, the effect of emulsin on the A.V. (640 mμ) of amylose β-dextrin]

Fig. 4. Effect of soya-bean β-amylase on the A.V. (540 mμ, -O- or 680 mμ, -●-) of amylopectin β-dextrin

[The concentration of mercuric chloride in the digests was zero (curve A), 1.5×10^{-6}M (B) and 1.5×10^{-5}M (C)]

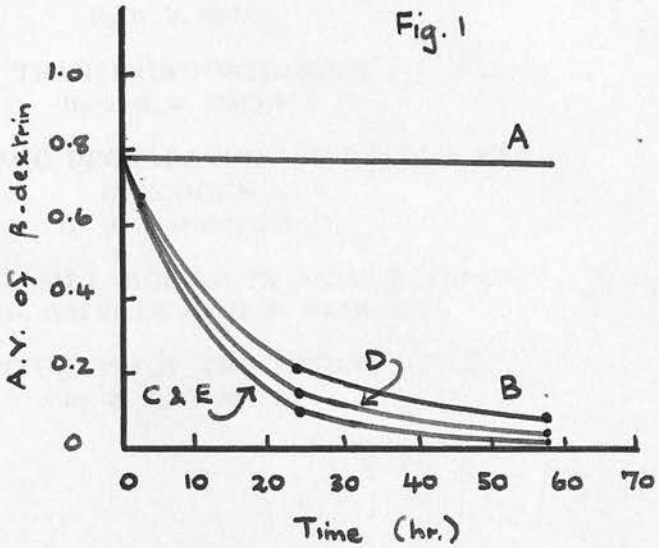
Fig. 1
A.V. of β-dextrin
1.0
0.8
0.6
0.4
0.2
0
A
C & E
D
B
0 10 20 30 40 50 60 70
Time (hr.)

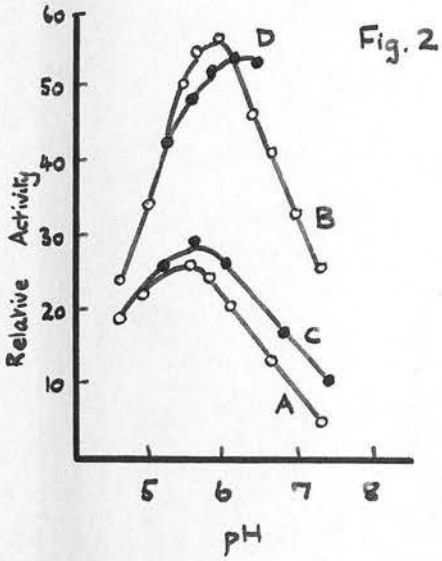
Fig. 2
Relative Activity
60
50
40
30
20
10
D
B
C
A
5 6 7 8
pH

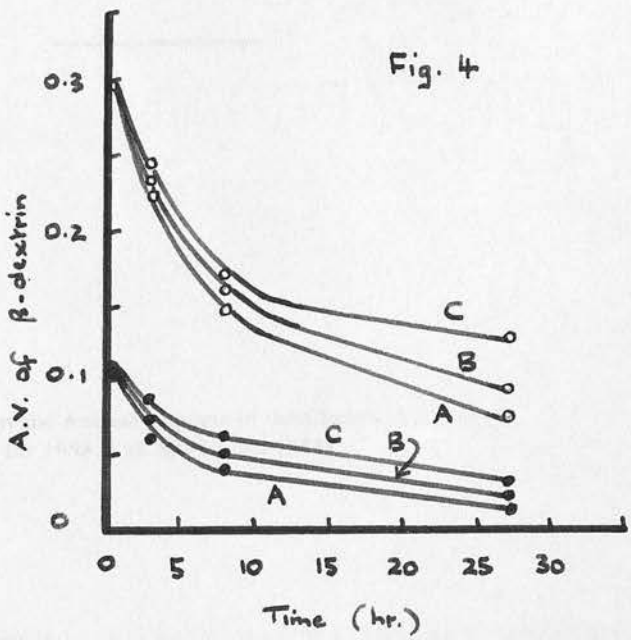
Fig. 4
A.V. of β-dextrin
0.3
0.2
0.1
0
C
B
A
C
B
A
0 5 10 15 20 25 30
Time (hr.)

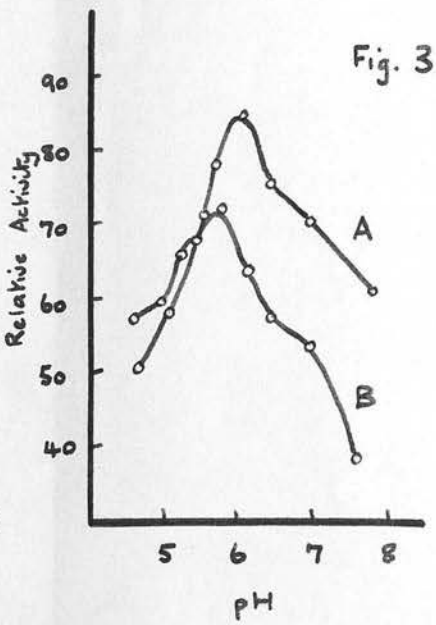
Fig. 3
Relative Activity
90
80
70
60
50
40
A
B
5 6 7 8
pH

41.

BIOCHEMISTRY

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BIOCHEMISTRY.

1. INTRODUCTION.

FOUR fields of extremely active research in biological chemistry are covered in the following Report. The hitherto unexpected "transferring" activity of the invertases is of both theoretical and practical interest. Carbohydrate chemists are more and more making use of highly purified enzymes as tools to aid the unravelling of amylosaccharide structures; here the degradative action of enzymes follows paths which are inaccessible by present chemical methods.

A vast amount of new knowledge regarding the chemistry of fatty acid metabolism has recently become available. Much of it originates in almost simultaneous reports from the laboratories of Green, Lipmann, Lynen, and Ochoa, and is on the whole available, so far, only from preliminary notes and reviews. To assign priorities would be difficult and perhaps meaningless.

Interest throughout the world in utilisation of resources provided by the marine algæ is active. The Institute of Seaweed Research, in this country, is the focal point of much of the discovery summarised below.

In many of the biological fields, chemical development is so rapid that the Reporters have been forced, for obvious reasons, to refer in a few instances to presently unpublished work. It seems reasonable to expect that such work will be available by the time this Report reaches its readers.

D. J. B.

2. TRANSFRUCTOSYLATION.

During the last three years paper partition chromatography, applied to the action of hydrolytic enzymes on disaccharides, has revealed the presence of hitherto unsuspected reaction products. Their formation can be interpreted as the result of the transference of sugar residues from the disaccharide, acting as donor, to the various sugars present in the reaction mixture, acting as acceptors.

Transglycosidation.—This process of enzymic transfer of sugar residues is named "transglycosidation" by Rabaté, who observed transfer of glucose residues from various glycosides, both artificial and natural, to such acceptors as ethanol, catalysed by preparations from leaves of a number of species of plants.[1] Miwa and his collaborators have named this enzyme "glucotransferase."[2] They were unable to differentiate it from β-glucosidase, and from an examination of apricot-emulsin preparations of various degrees of purity, and also of β-glucosidases from various other sources, concluded that the transferring action is "an inherent character of β-glucosidase,"[3] a view in keeping with the results reported below.

Hehre has suggested[4] that the term "transglycos*yl*ation" describes

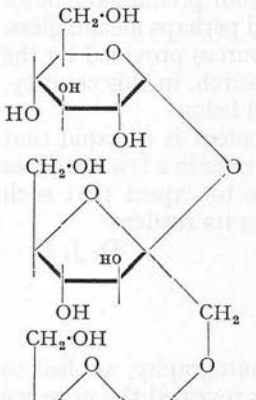
[1] Cf. J. Rabaté, *Compt. rend.*, 1937, **204**, 153.
[2] T. Miwa, K. Takano, K. Mafune, and S. Furutani, *Proc. Japan. Acad.*, 1949, **25**, 111.
[3] K. Takano and T. Miwa, *Biochem. J., Japan*, 1950, **37**, 435.
[4] E. J. Hehre, *Adv. Enzymology*, 1951, **11**, 330.

more accurately the nature of the group transferred, in those cases (phosphorylases) where the mechanism of the reaction has been studied with the use of ^{18}O-labelled phosphate. Koshland and Stein have investigated the action of yeast invertase on sucrose in presence of ^{18}O-labelled water,[5] but have so far shown only that the glucose produced does not contain ^{18}O.

Despite present uncertainty, however, it seems reasonable to follow Hehre, and to refer to the transfer of fructose residues as "transfructosylation," and no longer as "transfructosidation." [6]

An excellent review of transglycosylation reactions, from the aspect of polysaccharide synthesis, has been given by Barker and Bourne.[7] The mechanism of levan synthesis by various bacteria is fully discussed, and will not be further reported here; it is evidently explicable in terms of transfructosylation, but the early stages in the synthesis of a molecule of the polysaccharide have yet to be fully investigated.[8]

Enzymes of the Jerusalem Artichoke.—Dedonder [9] and Bacon and Edelman [10] discovered independently that the carbohydrates of the tubers and other organs of the Jerusalem artichoke appeared on a paper chromatogram as a regular series of spots extending from the position of sucrose to the starting line, each being non-reducing and yielding both glucose and fructose on hydrolysis. Dedonder [11] isolated the four lower members of this series of "inulides" as syrups, by partition chromatography on cellulose powder, but neither he nor Bacon and Edelman made any structural investigations. They inferred from the presence of glucose and the lack of reducing power that each component was a derivative of sucrose, and that the series was produced by successive additions of a β-fructofuranose residue to the fructose residue of this disaccharide. The fructose-to-fructose linkages were assumed to be 2 : 1′, as in inulin. The trisaccharide would thus have the structure (I). It still remains to be proved that this is the case, although further circumstantial evidence has since appeared to support it.



(I)

While examining the possibility that sucrose might be the substrate for inulin synthesis, Edelman and Bacon found that tuber extracts catalysed a transfer of fructose residues from inulin to sucrose to produce a substance (or substances) having the same R_F as the tuber trisaccharide, "spot 2." [6] They did not demonstrate satisfactorily that the substance synthesised was homogeneous, or that it was identical with "spot 2." Very little free glucose was liberated from inulin–sucrose mixtures by their preparations, and quantitative paper chromatography showed that most, if not all, of the fructose appearing in the trisaccharide could be accounted for by loss of

[5] D. E. Koshland and S. S. Stein, *Fed. Proc.*, 1953, **12**, 233.
[6] J. Edelman and J. S. D. Bacon, *Biochem. J.*, 1951, **49**, 529.
[7] S. A. Barker and E. J. Bourne, *Quart. Reviews*, 1953, **7**, 56.
[8] G. Kohanyi and R. Dedonder, *Compt. rend.*, 1951, **233**, 1142.
[9] R. Dedonder, *ibid.*, 1950, **230**, 549.
[10] J. S. D. Bacon and J. Edelman, *Biochem. J.*, 1949, **45**, xxvii; 1951, **48**, 114.
[11] R. Dedonder, *Compt. rend.*, 1951, **233**, 1134.

ctose from the inulin, the fructose residues of sucrose moving " passively " the trisaccharide position :

$$(\text{Fructose})_n + \text{Sucrose} \longrightarrow (\text{Fructose})_{n-1} + \text{Trisaccharide}$$

ppreciable amounts of free fructose were formed by all their preparations, id these were increased by raising the inulin concentration from 0·5 to 0%; on the other hand, increasing the sucrose concentration from 1·0 to 4% had no effect. Their preparations would also transfer fructose residues free fructose, or to the trisaccharides, raffinose and melezitose, but not, parently, to glucose, maltose, lactose, or $\alpha\alpha'$-trehalose.

Dedonder [12] found an increased synthesis of higher oligosaccharides when ulin as well as sucrose was added to tuber extracts, but considered that the nificant amounts of free glucose liberated by his preparations indicated at sucrose itself was the principal donor of fructose residues in the system. e believes that the chief fructose-transferring enzyme in the tuber is an nulosucrase," catalysing reactions of the type :

$$\text{Sucrose} + \text{Sucrose} \longrightarrow \text{Trisaccharide} + \text{Glucose}$$
$$\text{Sucrose} + \text{Trisaccharide} \longrightarrow \text{Tetrasaccharide} + \text{Glucose}$$

e oligosaccharides formed being those of the inulide series. A similar stem in the stems differed in requiring the presence of inorganic osphate.[12]

Mould Invertases.—Edelman[13] investigated the action of takadiastase on crose, to compare it with yeast invertase, and found that it produced series of non-reducing oligosaccharides, similar in composition and R_F to e lower inulides. Extracts of the mycelia of many species of mould ve this action, and the same mixture of oligosaccharides appears in the lture medium when moulds are growing on sucrose as a source of rbohydrate.[14]

By " gradient elution " [15] with aqueous ethanol from charcoal–Celite lumns the trisaccharide component of the oligosaccharide mixture formed takadiastase acting on sucrose was found to consist of two substances; e was isolated as a syrup.[16] Methylation followed by hydrolysis led to the ntification of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose, 1 : 3 : 4 : 6-tetra-*O*-thyl-D-fructose, and 3 : 4 : 6-tri-*O*-methyl-D-fructose in equal proportions. e products of partial hydrolysis were sucrose, fructose, and glucose. Since e trisaccharide is completely hydrolysed by yeast invertase (considered to drolyse only β-fructofuranosides), it probably has the structure (I). The ne trisaccharide has been isolated and obtained crystalline by Barker and rrington from the action of extracts of *Aspergillus niger* on sucrose.[17] ey also isolated from this source another non-reducing trisaccharide with e same general composition, *i.e.*, 2 fructose : 1 glucose.

Studies by Bealing have supported the view that the enzyme responsible oligosaccharide formation is that hitherto called " mould invertase." [18] wever, Pazur has given the name " transfructosidase " to an oligo-

[12] R. Dedonder, *Bull. Soc. Chim. biol.*, 1952, **34**, 171.
[13] J. Edelman, Thesis, Univ. Sheffield, 1950.
[14] F. J. Bealing and J. S. D. Bacon, *Biochem. J.*, 1953, **53**, 277.
[15] R. S. Alm, R. J. P. Williams, and A. Tiselius, *Acta Chem. Scand.*, 1952, **6**, 826.
[16] J. S. D. Bacon and D. J. Bell, *J.*, 1953, 2528.
[17] S. A. Barker and T. R. Carrington, *J.*, 1953, 3588.
[18] F. J. Bealing, *Biochem. J.*, 1953, **55**, 93.

saccharide-forming enzyme preparation from *Aspergillus oryzae*, which he apparently regards as distinct from invertase.[19] He isolated a trisaccharide from the reaction products, assigning to it the structure (I), on the basis of paper chromatography of the partial hydrolysis products. It seems probable that he was in fact dealing with the same enzyme, although his trisaccharide had a specific rotation considerably lower than that recorded later by other workers.

The inhibition of mould invertase by glucose, regarded by Kuhn[20] as an indication that it was an α-glucosidase, has been shown by Edelman and Bealing to be due to the acceptance of fructose residues by glucose.[21] Radioactive glucose added to the reaction mixture becomes incorporated rapidly into sucrose, and then into the other oligosaccharides.

The formation of oligosaccharides by mould invertase preparations has been noted by others.[22,23] No evidence for a separation of the transferring activity from the hydrolytic has been found,[14] but the possibility of achieving this has not been tested exhaustively.

Pazur found that a reducing difructose was formed by the enzyme from mixtures of raffinose and fructose.[19] Bealing has confirmed this, and has also shown that the enzyme will transfer fructose to various primary alcohols.[18]

Yeast Invertase.—The discovery that oligosaccharides are formed by yeast invertase preparations was made independently by Blanchard and Albon,[24] and by Bacon and Edelman,[25] in 1950; their observations have since been confirmed in several laboratories.[26–28] A claim that a certain commercial enzyme preparation did not form oligosaccharides[29] has been shown to be due to the low concentration of sucrose employed for the test.[30] The ratio of transference to hydrolytic action by yeast invertase preparations is much smaller than that for mould preparations; oligosaccharide formation is favoured by high sucrose concentrations in both cases.[14,31]

A paper chromatogram of the products at an intermediate stage of the reaction showed, in addition to sucrose, glucose, and fructose, several other spots numbered I to V.[25,27] Of these components, IV is a tetrasaccharide in nature, while the others occupy the positions of trisaccharides (II, III) and disaccharides (I, V).

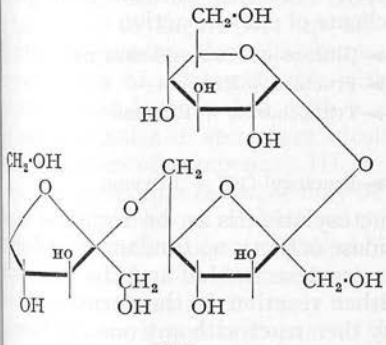
Blanchard and Albon first showed that a trisaccharide fraction from the reaction mixture had the approximate composition 2 fructose : 1 glucose. White and Secor showed that components II and III separately had this composition, and also that component I was a reducing disaccharide formed from glucose and fructose.[27] Kestose, a trisaccharide with the R_F of com-

[19] J. H. Pazur, *J. Biol. Chem.*, 1952, **199**, 217.
[20] R. Kuhn, *Z. physiol. Chem.*, 1923, **129**, 57.
[21] J. Edelman and F. J. Bealing, *Biochem. J.*, 1953, **53**, ii.
[22] K. Wallenfels and E. Bernt, *Angew. Chem.*, 1952, **64**, 28.
[23] H. Kurasawa, S. Saito, N. Honma, and Y. Yamamoto, *Bull. Fac. Agric., Nigata Univ., Nigata, Japan*, 1953, **4**, 51.
[24] P. H. Blanchard and N. Albon, *Arch. Biochem.*, 1950, **29**, 220.
[25] J. S. D. Bacon and J. Edelman, *ibid.*, 1950, **28**, 467.
[26] E. H. Fischer, L. Kohtès, and J. Fellig, *Helv. Chim. Acta*, 1951, **34**, 1132.
[27] L. M. White and G. Secor, *Arch. Biochem.*, 1952, **36**, 490.
[28] J. W. White, *ibid.*, 1952, **39**, 238. [29] S. Aronoff, *ibid.*, 1951, **34**, 484.
[30] S. Aronoff and J. S. D. Bacon, *ibid.*, 1952, **41**, 476.
[31] J. S. D. Bacon, *Biochem. J.*, 1954, in the press.

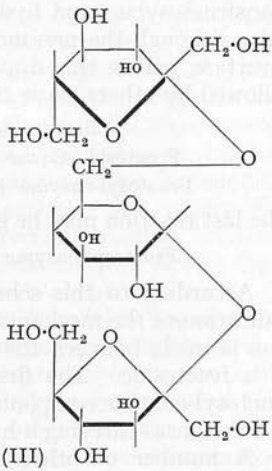
ponent III, isolated by partition chromatography on cellulose powder, was shown to have the structure (II) by methylation, hydrolysis, and analysis of the products.[32]

Component I was isolated by the same method. Its hypoiodite oxidation product no longer yielded glucose on hydrolysis; the fructose content was virtually unchanged.[31] Whelan and Jones isolated a similar substance from the products of action of yeast invertase on a mixture of methyl β-fructofuranoside and glucose.[33] After reduction with sodium borohydride it was hydrolysed by yeast invertase to a mixture of fructose and sorbitol (D-glucitol); treatment with sodium metaperiodate gave almost the 4 mols. of formic acid expected from *O*-6-[β-D-fructofuranosyl]-D-glucose.[33]

The use of gradient elution from charcoal–Celite has shown that component II consists of two substances, named II_1 and II_2 in order of their elution.[31] The former was indistinguishable from the trisaccharide (I) produced by mould invertase; it required the same concentration of ethanol for elution, and had the same R_F, the same mobility on filter-paper electrophoresis in borate buffer,[34] and the same infra-red absorption spectrum. Component II_2 has been shown by methylation studies to have the structure (III).[35]



(II)



(III) OH

Gradient elution also provided a means of separating component V from sucrose.[31] The pure material had seven-tenths of the reducing power of its hydrolysis products, which consisted only of fructose. Filter-paper electrophoresis showed two major components and a minor one.[34]

Radioactive glucose added to the reaction mixture was incorporated only into component I, radioactive fructose into component V.[36]

The fructose residue of methyl β-fructoside was transferred to glucose, mannose, galactose, sorbitol, and mannitol, but not to sugars without a primary alcoholic group (arabinose, ribose, rhamnose).[33]

[32] N. Albon, D. J. Bell, P. H. Blanchard, D. Gross, and J. T. Rundell, *J.*, 1953, 24.
[33] W. J. Whelan and D. M. Jones, *Biochem. J.*, 1953, **54**, xxxiv.
[34] D. Gross, *Nature*, 1954, **173**, 487.
[35] D. Gross, P. H. Blanchard, and D. J. Bell, *J.*, 1954, in the press.
[36] J. Edelman, *Biochem. J.*, 1954, **57**, 22.

Addition of various primary alcohols to a mixture of enzyme and sucrose gave rise to additional fructose-containing substances;[37,38] that formed in the presence of methanol is apparently methyl β-fructofuranoside.[37] The glycerol added to stabilise commercial invertase preparations gives rise to substance first noticed by Bacon and Edelman on phenol–water paper chromatograms,[25] and later by Gross during paper-electrophoresis examination of the reaction mixture;[34] it is presumed to be a monofructosylglycerol.

Some of the above-mentioned oligosaccharides have been noticed during the growth of yeasts on sucrose-containing media.[39] Their possible importance in the sugar industry has been discussed.[40]

The Significance of Transfructosylation.—In the Jerusalem artichoke transfructosylation has been suggested as the means by which the carbohydrate composition changes during the winter months, leading to an increase in the proportion of the lower terms of the inulide series.[41] Alternatively, transfructosylation from some fructosyl derivative as yet undiscovered, or Dedonder's inulosucrase acting upon sucrose, may be the means by which the Compositae synthesise inulin and inulides.

In the case of the invertases it remains to be proved conclusively that the transfructosylase and hydrolase activity belong to the same enzyme molecule, although the presence of transferring activity in highly purified yeast invertase makes this appear more likely.[26] Fischer and his colleagues,[26] followed by others, have suggested a scheme of enzyme action :

$$\text{Sucrose} + \text{Enzyme} \longrightarrow \text{Glucose} + \text{Fructosyl-enzyme}$$
$$\text{Fructosyl-enzyme} + \text{Water} \longrightarrow \text{Fructose} + \text{Enzyme}$$
$$\text{Fructosyl-enzyme} + \text{Sucrose} \longrightarrow \text{Trisaccharide} + \text{Enzyme}$$

The last reaction may be generalised :

$$\text{Fructosyl-enzyme} + \text{HO·R} \longrightarrow \text{Fructosyl-O·R} + \text{Enzyme}$$

According to this scheme, transfructosylation is to be regarded as an indication of the mechanism of fructosidase action; no fundamental distinction is made between the transfer of a fructose residue and the hydrolysis of a fructoside. The first step in either reaction is the formation of a fructosyl-enzyme compound, which may then react with any one of a number of substances carrying a hydroxyl group.

A number of other carbohydrases,[42] and other kinds of hydrolases (phosphatases, peptidases), have been found to catalyse group-transfer reactions, and Morton, reviewing these,[43] has suggested that all hydrolytic enzymes capable of attacking more than one substrate may be found to act as transferring enzymes. (Edelman and Bacon discussed the possibility that their "transfructosidase" was identical with the hydrolytic fructosidase of the artichoke tuber.[6,44])

[37] J. S. D. Bacon, *Biochem. J.*, 1952, **50**, xviii.
[38] A. I. Oparin and M. S. Bardinskaya, *Doklady Akad. Nauk S.S.S.R.*, 1953, **89**, 531.
[39] E. C. Barton-Wright and G. Harris, *Nature*, 1951, **167**, 560.
[40] H. C. S. de Whalley, *Int. Sugar J.*, 1952, **54**, 127; B. Freed and D. Hibbert, 1953, Paper to Sixth Technical Conference, British Sugar Corp., Ltd.
[41] J. S. D. Bacon and R. Loxley, *Biochem. J.*, 1952, **51**, 208.
[42] K. Wallenfels, in "Biologie und Wirkung der Fermente," 4th Colloquium der Gesellschaft für Physiologische Chemie, Springer, Berlin, 1950, p. 160.
[43] R. K. Morton, *Nature*, 1953, **172**, 65.
[44] J. Edelman and J. S. D. Bacon, *Biochem. J.*, 1951, **49**, 446.

Most results so far reported are consistent with Fischer's scheme, but ere is no direct evidence that a fructosyl-enzyme compound is formed. is intermediate should arise whatever substrate is being acted upon, and e transfer reactions which follow its formation should depend only upon e nature and concentrations of the acceptors present. In the case of yeast vertase a fructosylglucose is formed in the presence of glucose, whether crose or methyl β-fructoside is the substrate, but it remains to be proved at the same fructosylglucose is formed in each case. Edelman has plied this test to mould invertase;[36] here the product of fructose nsfer from sucrose to glucose is sucrose, but he could detect no sucrose rmation when the mould enzyme acted on a mixture of methyl β-fructoside d glucose. If it could be proved more conclusively that sucrose does not pear during the latter reaction the fructosyl-enzyme hypothesis in its mple form would have to be abandoned.

In every fructose-transferring system discovered, including levan-crase, free fructose is produced.[45] Among the enzymes attacking *glucosidic* kages there is one, sucrose phosphorylase, that evidently forms an rmediate glucosyl-enzyme compound; this enzyme, however, shows no drolytic activity.[46] Dextransucrase, the analogue of levansucrase, may so produce no free glucose.[4] It will be interesting to see whether any nsfructosylases exist that do not have some hydrolytic action.

Progress in the isolation and characterisation of the products of trans-ctosylation to sugars has up till now shown the transference to be stricted to primary alcoholic groups. However, the isolation from mould zyme action of a trisaccharide with the same R_F and the same hexose mposition as kestose, but with a different specific rotation,[47] suggests at some transfer to secondary alcoholic groups occurs. The apparently ltiple nature of component III of the yeast invertase–sucrose reaction xture suggests that the same may be true of the yeast enzyme, as also does e existence of various unidentified minor components of this mixture.[31]

The evidence already available shows qualitative differences between the ichoke, mould, and yeast enzymes, with respect to the products of transfer. e differences do not necessarily mean that the acceptor molecule has to be ivated before it can take part in the reaction, but must mean that the ecific action of the enzyme extends to groupings other than the β-fructo-anosyl radical itself, a concept already well established for some carbo-drases.[48]

The recognition of transfructosylase activity where it was not hitherto pected must lead to a fresh consideration of the relation between plant vertases and sucrose synthesis. It is also relevant to the discovery of uctose-containing oligosaccharides in the leaves and stems of barley[49] d in wheat flour.[50]

J. S. D. B.

[45] S. Hestrin and S. Avineri-Shapiro, *Biochem. J.*, 1944, **38**, 2.
[46] W. Z. Hassid and M. Doudoroff, *Adv. Carbohydrate Chem.*, 1950, **5**, 46.
[47] E. J. Bourne, S. A. Barker, and T. R. Carrington, personal communication.
[48] S. Veibel, in J. B. Sumner and K. Myrbäck, "The Enzymes," Vol. I, Part I, ademic Press, New York, 1950, p. 583.
[49] H. K. Porter and J. Edelman, *Biochem. J.*, 1952, **50**, xxxiii.
[50] L. M. White and G. Secor, *Arch. Biochem.*, 1953, **43**, 60.

3. THE ENZYMIC DEGRADATION OF STARCH AND GLYCOGEN.

This section of the Report is confined to recent developments in the chemistry of the enzymic degradation of starch and glycogen; the literature to the end of 1950 has been adequately reviewed elsewhere.[1-5] During the past few years, important advances have been made, particularly in studies of the enzymes hydrolysing the inter-chain linkages in branched amylosaccharides. Progress has been facilitated by the development of improved procedures for the preparation of highly purified substrates,[6, 7] by improved methods of protein chemistry resulting in the crystallisation of several enzymes, and applications of chromatographic analysis to the products of enzyme action.[8-10] However, detailed knowledge of the fine structures of starch and glycogen is still incomplete, and until more progress has been made by purely chemical methods the exact specificity and mode of action of certain amylases cannot be fully understood. Reports of the presence of "anomalous" linkages in amylose,[11] amylopectin,[12] and glycogen,[12, 13] and the occurrence of fructose in certain samples of glycogen [14] and waxy maize starch [8] require further chemical investigation. In this connection, the new procedure devised by F. Smith and his co-workers [12] for the determination of the fine structure of polysaccharides should be noted.

The biological synthesis of starch and glycogen has been reviewed in detail,[15] and, apart from a consideration of the reversible action of the phosphorylases, will not be considered further here.

The General Structure of Starch and Glycogen.—Before discussing the results of enzymic degradations of starch and glycogen, a brief account of their structures will be given. Attention is drawn to recent reviews.[16, 17]

Starch contains two distinct components—an essentially linear polymer (amylose) and a branched polymer (amylopectin), both consisting of D-glucopyranose units. Evidence available from chemical and enzymic studies [18]

[1] K. Myrbäck and G. Neumüller in "The Enzymes," by J. B. Sumner and K. Myrbäck, Academic Press, New York, 1951, Vol. I, p. 653.
[2] M. L. Caldwell and M. Adams, *Adv. Carbohydrate Chem.*, 1950, **5**, 229.
[3] P. Bernfeld, *Adv. Enzymology*, 1951, **12**, 379.
[4] R. W. Kerr and H. Gehman, *Die Stärke*, 1951, **3**, 271.
[5] K. H. Meyer, *Angew. Chem.*, 1951, **63**, 153.
[6] P. N. Hobson, S. J. Pirt, W. J. Whelan, and S. Peat, *J.*, 1951, 801.
[7] R. S. Higginbotham and G. A. Morrison, *Shirley Inst. Mem.*, 1948, **22**, 148; K. H. Meyer and P. Rathgeb, *Helv. Chim. Acta*, 1948, **31**, 1533; E. H. Fischer and W. Settele, *ibid.*, 1953, **36**, 811.
[8] W. J. Whelan and P. J. P. Roberts, *Nature*, 1952, **170**, 748.
[9] *Idem*, *J.*, 1953, 1298.
[10] J. H. Pazur, D. French, and D. W. Knapp, *Iowa State Coll. J. Sci.*, 1950, **57**, 203.
[11] S. Peat, S. J. Pirt, and W. J. Whelan, *J.*, 1952, 705, 714; S. Peat, G. J. Thomas, and W. J. Whelan, *ibid.*, p. 722.
[12] M. Abdel-Akher, J. K. Hamilton, R. Montgomery, and F. Smith, *J. Amer. Chem. Soc.*, 1952, **74**, 4970.
[13] D. J. Bell, *J.*, 1948, 992.
[14] S. Peat, P. J. P. Roberts, and W. J. Whelan, *Biochem. J.*, 1952, **51**, xvii.
[15] S. A. Barker and E. J. Bourne, *Quart. Reviews*, 1953, **7**, 56; E. J. Bourne, *Biochem. Soc. Symp.*, 1953, **11**, 3.
[16] R. W. Kerr, "Chemistry and Industry of Starch," Academic Press, New York, 1950, 2nd edn.; E. J. Bourne, *Chem. and Ind.*, 1951, 1047; K. H. Meyer and G. C. Gibbons, *Adv. Enzymology*, 1951, **12**, 341; W. Z. Hassid in "Organic Chemistry," Vol. IV, Ed. by H. Gilman, Wiley, New York, 1953, p. 901.
[17] K. H. Meyer, *Experientia*, 1952, **8**, 405.
[18] R. H. Hopkins, *Nature*, 1953, **171**, 429; B. Lindberg, *Acta Chem. Scand.*, 1953, **7**, 237.

oes not support the suggestion by J. Blom and B. Schwarz [19] that starch ontains glucofuranosidic linkages. Most starches contain *ca.* 20% of mylose; [16] the amylose content of waxy cereal starches, however, is less than % [16] whilst that of wrinkled pea starches is 65—80%.[20]

Amylose molecules usually consist of a linear chain of several hundred -1:4-linked glucose residues, the number depending on the source,[21, 22] nd may vary with the method of extraction from the starch granule.[23] here is evidence that some samples of amylose contain β-glucosidic link-ges,[11] and that others may have a low degree of branching [22, 24-26] (see 293).

Amylopectin is composed of several hundred unit-chains, each consisting f 20—25 α-1 : 4-linked glucose residues; * each chain is linked to an adjacent hain by a 1 : 6-glucosidic linkage. Amylopectins from different sources ary in chain length,[21, 27] in molecular weight,[24] and in the position of branch-g in the unit-chains (see Table 2). Certain amylopectins appear to be eterogeneous, and can be separated into fractions of different degrees of anching and molecular weight.[28] Other amylopectins, however, show no ariation in degree of branching on fractionation.[29] A few higher plants .g., *Zea mais*) contain branched amylosaccharides with 12-unit chains; [30, 31] hese polysaccharides should be described as amylopectins, and not as lycogens, as hitherto.[30]

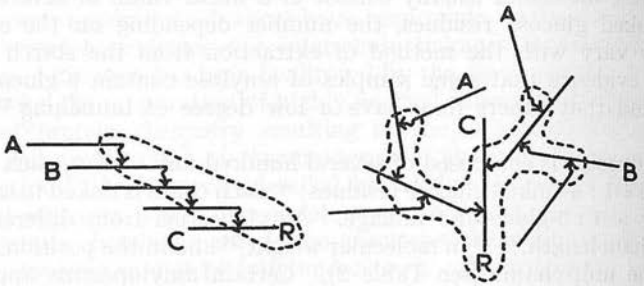
Although several molecular structures have been proposed for amylo-ectin,[16] only two will be considered here, namely, the singly-branched aminated) structure proposed by W. N. Haworth and E. L. Hirst [32] as the implest structure in accord with data from methylation studies, and the ultiply-branched (tree) structure suggested by K. H. Meyer.[33] Both are lustrated in the Figure. Various types of "unit-chain" can be distin-uished,[34] *viz.*: *A-type* (side chain)—joined to the rest of the molecule only y a linkage from the reducing group; *B-type* (main chain) to which are ttached other chains; and *C-type* terminated by the sole reducing group f the molecule. Furthermore, A-chains and those parts of B- and C-chains etween the branch point and the non-reducing terminal group will be eferred to as "exterior" chains; those parts between two branch points ill be regarded as "interior" chains (see Figure).

Acta. chem. Scand.

[19] J. Blom and B. Schwarz, *Nature*, 1952, **6**, 697.
[20] A. L. Potter, V. Silveira, R. M. McCready, and H. S. Owens, *J. Amer. Chem. Soc.*, 953, **75**, 1335; D. M. W. Anderson and C. T. Greenwood, personal communication.
[21] A. L. Potter and W. Z. Hassid, *J. Amer. Chem. Soc.*, 1948, **70**, 3488.
[22] *Idem, ibid.*, 1951, **73**, 593.
[23] R. T. Bottle, G. A. Gilbert, C. T. Greenwood, and K. N. Saad, *Chem. and Ind.*, 953, 541.
[24] A. L. Potter and W. Z. Hassid, *J. Amer. Chem. Soc.*, 1948, **70**, 3774.
[25] R. W. Kerr and F. C. Cleveland, *ibid.*, 1952, **74**, 4036.
[26] B. N. Stepanenko and E. M. Afanas'eva, *Chem. Abs.*, 1953, **47**, 2282.
[27] D. J. Bell, *Ann. Reports*, 1947, **44**, 223.
[28] K. H. Meyer and W. Settele, *Helv. Chim. Acta*, 1953, **36**, 197.
[29] A. L. Potter and W. Z. Hassid, *J. Amer. Chem. Soc.*, 1951, **73**, 997.
[30] W. Dvonch and R. L. Whistler, *J. Biol. Chem.*, 1949, **181**, 889.
[31] K. H. Meyer and M. Fuld, *Helv. Chim. Acta*, 1949, **32**, 757.
[32] W. N. Haworth, E. L. Hirst, and F. A. Isherwood, *J.*, 1937, 577.
[33] K. H. Meyer and P. Bernfeld, *Helv. Chim. Acta*, 1940, **23**, 875.
[34] S. Peat, W. J. Whelan, and G. J. Thomas, *J.*, 1952, 4546.

* All figures quoted for unit-chain lengths, which are given to the nearest whole umber, represent *mean* values.

Glycogen, the reserve carbohydrate of animals, and certain yeasts,[35, 36] bacteria,[37] and protozoa,[38] has a highly branched structure comprising several hundred unit-chains of *ca.* twelve α-1 : 4-linked glucose residues; the inter-chain link is of the 1 : 6-glucosidic type.[16, 39] The molecular weight



Singly- and multiply-branched structures for amylopectin.

——— Linear chain of α-1 : 4-linked glucose units.

⌇ 1 : 6-Inter-chain linkage.

R Free reducing group.

- - - Extent of β-amylolysis.

A, B, C Types of unit-chain.

of glycogens (10^6—10^7) varies with the source.[40] Whilst most glycogens have a chain length of 12 ± 2 glucose units,[39, 41-43] samples have been isolated with chain lengths of *ca.* 6 [39, 41] or 15—23.[41, 44, 45] In many instances these specimens have been assayed by more than one method. The existence of different average chain-lengths presumably reflects differences in equilibria between the enzymes: phosphorylase, branching enzyme, amylo-1 : 6-glucosidase and glucose-6-phosphatase.[46] Glycogen appears to have a "tree" structure similar to that proposed by Meyer for amylopectin, but with shorter unit-chains.[16, 47]

Degradation of α-1 : 4-Linkages by Hydrolytic Enzymes (Amylases).—*A. Amylases producing maltose* (β-*amylases*). β-Amylases catalyse the stepwise hydrolysis of chains of 1 : 4-linked α-glucose units by attacking alternate linkages, with the liberation of β-maltose. This action, involving a Walden inversion, commences at the non-reducing end of the chain and is arrested by the presence of glucosidic linkages other than α-1 : 4. β-Amylolysis is

[35] D. H. Northcote, *Biochem. J.*, 1953, **53**, 348.
[36] D. J. Manners and K. Maung, unpublished work.
[37] C. Barry, R. Gavard, G. Milhaud, and J. P. Aubert, *Ann. Inst. Pasteur*, 1953, **84**, 605.
[38] D. J. Manners and J. F. Ryley, *Biochem. J.*, 1952, **52**, 480.
[39] D. J. Manners, *Biochem. J.*, 1953, **55**, xx.
[40] C. T. Greenwood, *Adv. Carbohydrate Chem.*, 1952, **7**, 300; B. S. Harrap and D. J. Manners, *Nature*, 1952, **170**, 419.
[41] D. J. Bell and D. J. Manners, *J.*, 1952, 3641; unpublished work.
[42] M. Abdel-Akher and F. Smith, *J. Amer. Chem. Soc.*, 1951, **73**, 994.
[43] M. Morrison, A. C. Kuyper, and J. M. Orten, *ibid.*, 1953, **75**, 1502.
[44] B. Illingworth, J. Larner, and G. T. Cori, *J. Biol. Chem.*, 1952, **199**, 631.
[45] M. Schlamowitz, *ibid.*, 1951, **188**, 145.
[46] G. T. Cori and C. F. Cori, *ibid.*, 1952, **199**, 661.
[47] J. Larner, B. Illingworth, G. T. Cori, and C. F. Cori, *ibid.*, p. 641.

herefore confined to the exterior chains of amylopectin and glycogen.[1,3-5] -Amylase has no action on the intact starch granule.[17]

β-Amylases occur only in higher plants; soya-bean β-amylase has been ighly purified,[11] whilst those from barley,[48] sweet potato,[49] and wheat [50] ave been crystallised. Crystalline sweet-potato β-amylase has been sub-cted to a full physicochemical analysis by S. Englard and T. P. Singer [51] ho, with S. Sorof,[52] have shown that free –SH groups are essential for nzymic activity—a conclusion reached independently by N. I. Proskurya-ov.[53] β-Amylases are inhibited by a variety of reagents, including eparin,[54] sodium dodecyl sulphate,[55] sodium cyanide,[56] and ascorbic acid.[57] lthough the crystallisation of an enzyme is indicative of a high degree of rity, it is not a criterion of enzymic homogeneity. Certain crystalline -amylases contain minute traces of α-amylase [58] and/or Z-enzyme and, unless e enzyme concentration and pH of the digestion are controlled, these con-minating enzymes interfere with true β-amylolysis.

The limit of β-amylolysis of amylose (see Table 1) is dependent on *a*) the sample of amylose and (*b*) the enzyme concentration. Some amyloses,

TABLE 1. *The degradation of amyloses by crystalline sweet potato β-amylase.*

Source of amylose	D.P.* of amylose	β-Amylolysis limit †	Ref.
ople	545	90	59
laize	—	75	60
laize	—	68	11
laize	490	90	61
tato	—	88	60
tato	—	68	11
tato	—	75	41
go	1200	70	11, 62
go	1200	97	63
apioca	3500	70	11, 62

* Degree of polymerisation. † Percentage conversion into maltose.

pecially those of low molecular weight, are completely hydrolysed by -amylase, and are presumed to be linear α-1 : 4-glucosans.[17] Other samples, owever, are incompletely degraded, particularly at low enzyme concen-tions. Sago amylose, for example, has β-amylolysis limits of 70 [11] and % [63] with low and high concentrations of enzyme respectively. This pparent discrepancy is due to the presence of anomalous linkages in the mylose,[11] and to traces of Z-enzyme in the crystalline β-amylase. The tivity of Z-enzyme, which hydrolyses these anomalous linkages, is insig-

[48] K. H. Meyer, E. H. Fischer, and A. Piguet, *Helv. Chim. Acta*, 1951, **34**, 316.
[49] A. K. Balls, M. K. Walden, and R. R. Thompson, *J. Biol. Chem.*, 1948, **173**, 9.
[50] K. H. Meyer, P. F. Spahr, and E. H. Fischer, *Helv. Chim. Acta*, 1953, **36**, 1924.
[51] S. Englard and T. P. Singer, *J. Biol. Chem.*, 1950, **187**, 213.
[52] S. Englard, S. Sorof, and T. P. Singer, *ibid.*, 1951, **189**, 217.
[53] N. I. Proskuryakov, V. Y. Voronkova, and E. S. Mikhaïlova, *Chem. Abs.*, 1952, 10215.
[54] K. Myrbäck and B. Persson, *Arkiv Kemi*, 1953, **5**, 477.
[55] *Idem, ibid.*, 1952, **4**, 531.
[56] D. K. Roy and L. A. Underkofler, *Cereal Chem.*, 1950, **27**, 404.
[57] N. I. Proskuryakov and L. S. Kholopova, *Chem. Abs.*, 1953, **47**, 3903.
[58] R. H. Hopkins and R. Bird, *Nature*, 1953, **172**, 492.
[59] A. L. Potter, W. Z. Hassid, and M. A. Joslyn, *J. Amer. Chem. Soc.*, 1949, **71**, 3.
[60] A. L. Potter, personal communication.
[61] S. Nussenbaum and W. Z. Hassid, *J. Biol. Chem.*, 1951, **190**, 673.
[62] W. J. Whelan, personal communication.
[63] T. G. Halsall, Thesis, Manchester, 1948.

nificant at low concentrations of crystalline β-amylase; at higher concentrations, however, the amount of Z-enzyme is sufficient to affect the β-amylolysis limit.

These lower β-amylolysis limits are due neither to impurities in the amylose—as suggested by Meyer [17]—nor to retrogradation of the substrate, since on the addition of Z-enzyme (a β-glucosidase), or emulsin, complete β-amylolysis occurs.[11] It is assumed that such amyloses contain one or more β-glucosidic linkages which arrest β-amylase action; [11] the exact situation of such linkages is not known and the tentative suggestion that they join single glucose residues to the main chain has not been confirmed experimentally.[58] (Recent additional evidence confirms that Z-enzyme is a β-glucosidase and not a weak α-amylase; [64] it occurs in soya beans,[11] wheat,[65] barley,[41] rye,[66] and other ungerminated cereals; its presence in amorphous preparations of β-amylase accounts for the ability of such preparations completely to hydrolyse amylose, and slowly to attack laminarin and yeast glucan.) From a study of the rate of β-amylolysis of various samples of amylose, it has been concluded that potato and tapioca amyloses contain one or two and two or three branch points respectively, per molecule.[25]

Crystalline β-amylase will rapidly hydrolyse short-chain maltosaccharides to maltose or to a mixture of maltose and maltotriose, according to the chain length of the substrate; [67] whether or not maltotriose is a substrate for β-amylase has not yet been finally decided.[68]

E. J. Bourne and W. J. Whelan have reviewed the mechanism of β-amylolysis of amylose.[69] Two theories of enzymic action have been proposed. The "single-chain" theory postulates that β-amylase completely degrades one amylose molecule to maltose before attacking a second molecule; at intermediate stages of hydrolysis, only maltose and unchanged amylose are thus present. According to the "multi-chain" theory, enzyme action involves a simultaneous attack of all the amylose molecules, each of which is therefore progressively shortened. Recent work has shown that enzyme action is normally intermediate between these two mechanisms, but that at higher temperatures it approximates to a multi-chain action.[68, 70] β-Amylase is a remarkably efficient catalyst; the turn-over number in terms of glucosidic linkages ruptured per minute at 30° and pH 4·8 is *ca.* 250,000 [52] (not 2,370,000 as reported previously [51]).

The products of β-amylolysis of branched amylosaccharides are maltose and a limit-dextrin of high molecular weight (β-dextrin).[1, 3-5] This dextrin differs from the original polysaccharide only in that the exterior chains comprise two or three glucose residues [34, 44] (not one or two as originally suggested by Meyer [5]). The original exterior chain lengths of amylopectins can therefore be calculated from the β-amylolysis limit and chain length, as shown in Table 2. Amylopectins do not contain Z-labile linkages.[11]

[64] S. Peat and W. J. Whelan, *Nature*, 1953, **172**, 494.
[65] T. Dillon and P. O'Colla, *Chem. and Ind.*, 1951, 111.
[66] A. M. Liddle and D. J. Manners, unpublished work.
[67] W. J. Whelan, J. M. Bailey, and P. J. P. Roberts, *J.*, 1953, 1293; cf. also R. H. Hopkins and B. Jelinek, *Biochem. J.*, 1954, **56**, 136; R. Bird and R. H. Hopkins, *ibid.*, p. 140; R. W. Kerr and F. C. Cleveland, *J. Amer. Chem. Soc.*, 1951, **73**, 2421.
[68] W. J. Whelan, *Biochem. Soc. Symp.*, 1953, **11**, 17.
[69] E. J. Bourne and W. J. Whelan, *Nature*, 1950, **166**, 258.
[70] D. French, D. W. Knapp, and J. H. Pazur, *J. Amer. Chem. Soc.*, 1950, **72**, 1866.

TABLE 2. *The degradation of amylopectins by crystalline β-amylase.*

Source	Chain length	A	B	C	Ref.
heat	18, *e*	11	13—14	3—4	47
axy maize	19, *p*	13	15—16	2—3	28
nthetic	20—21, *e*, *p*	10—11	13	7	61
axy maize	22 *m*, *p*	11—12	14	7	71
apioca	22, *p*	15	17—18	3—4	28
axy maize	23, *p*	16	18—19	3—4	28
aple	24, *p*	15	17—18	5—6	59
aize	24, *e*	15—16	18	5	47
axy sorghum	25, *m*, *p*	13	15—16	8—9	71

e = Enzymic assay.
p = Periodate oxidation assay.
m = Methylation assay.
A = No. of glucose units removed on β-amylolysis.
B = Exterior chain length (A + 2·5).
C = Interior chain length (chain length − B − 1).

The discovery of Z-enzyme has provided an explanation for the variation the β-amylolysis limit of whole starches. Crystalline or acid-treated amylases, which are free from Z-enzyme, yield *ca.* 53% of maltose, but ther preparations, which contain Z-enzyme, yield *ca.* 60%, the difference ing due to the extent of hydrolysis of the amylose component.

A critical study of the β-amylolysis of glycogen has been made recently [41] nd typical results are recorded in Table 3. Animal [41] and yeast [35] glycogens o not contain Z-labile linkages.

ABLE 3. *The degradation of glycogens by crystalline sweet-potato β-amylase.*

Source	Chain length	A	B	C	Ref.
dix pomatia	7, *p*	2	4—5	1—2	41
east	12, *p*, *m*	6	8—9	2—3	35
trahymena pyriformis .	13, *p*	6	8—9	3—4	38
abbit liver	13, *p*, *m*	5—6	8	4	41
t liver	13, *p*	6—7	9	3	41
abbit liver	15, *e*	7	9—10	4—5	47
ytilus edulis	17, *p*, *m*	8	10—11	5—6	41
abbit liver	18, *m*	9—10	12	5	71

* See Table 2 for footnotes.

From Tables 2 and 3 it can be seen that glycogens and amylopectins show nsiderable variation in molecular structure; differences in degree and sition of branching can be noted between (*a*) samples from different urces, and (*b*) different samples from the same source, whilst amylo-charides with the same chain length vary in the position of branching in e chain.

B. Amylases producing more than one sugar (*α-amylases*). α-Amylases talyse random hydrolysis of α-1 : 4-linkages in both starch and glycogen.[1-5] ey also attack the intact starch granule [17] (cf. β-amylases). The purified zymes cannot hydrolyse the 1 : 6-inter-chain linkages in branched amylo-charides, but can by-pass them so that both exterior and interior chains n be degraded. α-Amylolysis is characterised by a rapid decrease of the ine staining power,[1] turbidity,[72] and viscosity [1] of the substrate (a trinisation action), followed by the slow production of reducing sugars saccharification action). Random hydrolysis of linkages during the tial stages of α-amylolysis yields achroic α-dextrins (D.P. 6—10) which are

[71] T. G. Halsall, E. L. Hirst, L. Hough, and J. K. N. Jones, *J.*, 1949, 3200.
[72] S. Schwimmer, *J. Biol. Chem.*, 1950, **188**, 477.

then slowly hydrolysed to mixed saccharides, the nature of which depends on the substrate and the source of α-amylase. Different α-amylases show different affinities for the same substrate. For example, salivary amylase readily attacks starch, maltohexaose, and maltohexaonic acid, with approximately the same initial velocity. With malt α-amylase, however, the initial rates of degradation are in the ratio 50 : 9 : 1 respectively.[73]

α-Amylases are widely distributed in Nature, and many have now been purified and crystallised, *e.g.*, those from malt,[17, 74] human saliva,[17] human pancreas,[17] swine pancreas,[17, 75] *B. subtilis*,[17] *B. mesentericus*,[76] *Aspergillus oryzae*,[17, 77, 78] and *A. candidus* var. *amylolyticus*.[79] The chemical and physical properties of several crystalline α-amylases have been reviewed by K. H. Meyer.[5, 17] It has been reported that crystalline malt α-amylase contains traces of another enzyme, a " dextrinase." [80] Purified α-amylases have been isolated from *Clostridium butyricum* (from pig cæcum [81] and sheep rumen [82]), *Cl. acetobutylicum*,[83] a variant of *Cl. pasteurianium*,[84] and a strain of *Streptococcus* from sheep rumen.[82]

The nature of the " active group " in α-amylases is not yet known, although there is some evidence that a free –OH group (tyrosine) is concerned; –SH groups are not essential.[3, 85] The suggestion [86] that inositol is an active constituent of α-amylases has now been disproved.[74, 87, 88] Certain α-amylases (from *B. subtilis*, and mammalian pancreatic or salivary secretions) require Cl^- for maximal activity.[2, 5, 17] The effects of urea, ammonium phosphate, and temperature variation on the activation of salivary amylase by Cl^- have been studied by L. H. Schneyer.[89] Malt α-amylase is activated by Ca^{++},[17, 74, 75] whilst *A. oryzae* α-amylase is unaffected by either of these ions.[17] α-Amylase inhibitors studied recently include heparin,[90] 1-fluoro-2 : 4-dinitrobenzene,[91] and caffeine.[92]

α-Amylolysis of amylose has been studied by many workers.[1–5, 9, 17, 93] α-Amylases catalyse a random hydrolysis of the non-terminal linkages in amylose (cf., however, ref. 10). The enzymes have a high affinity for amylose, but a much lower affinity for short-chain linear molecules, the differences being greatest with malt α-amylase. A study of salivary amylolysis of linear maltodextrins has provided further evidence of random attack,

[73] K. Svanborg and K. Myrbäck, *Arkiv Kemi*, 1953, **6**, 113.
[74] S. Schwimmer and A. K. Balls, *J. Biol. Chem.*, 1949, **179**, 1063.
[75] M. L. Caldwell, M. Adams, J. T. Kung, and G. C. Toralballa, *J. Amer. Chem. Soc.*, 1952, **74**, 4033.
[76] B. Hagihara, *Proc. Japan. Acad.*, 1951, **27**, 346.
[77] S. Akabori, B. Hagihara, and T. Ikenaka, *ibid.*, p. 350.
[78] L. A. Underkofler and D. K. Roy, *Cereal Chem.*, 1951, **28**, 18.
[79] K. Takaoka, H. Fuwa, and Z. Nikuni, *Mem. Inst. Sci. Res. Osaka*, 1952, **10**, 199.
[80] S. Schwimmer, *Cereal Chem.*, 1951, **28**, 77.
[81] W. J. Whelan and H. Nasr, *Biochem. J.*, 1951, **48**, 416.
[82] P. N. Hobson and M. Macpherson, *ibid.*, 1952, **52**, 671.
[83] D. Scott and L. R. Hedrick, *J. Bact.*, 1952, **63**, 795.
[84] N. I. Proskuryakov and N. V. Dmitrievskaya, *Brit. Abs.*, 1952, AIII, 1775.
[85] L. H. Schneyer, *Arch. Biochem. Biophys.*, 1952, **41**, 345.
[86] R. L. Lane and R. J. Williams, *Arch. Biochem.*, 1948, **19**, 329.
[87] E. H. Fischer and P. Bernfeld, *Helv. Chim. Acta*, 1949, **32**, 1146.
[88] M. L. Caldwell, N. Larson, and B. Huston, *Cereal Chem.*, 1952, **29**, 463.
[89] L. H. Schneyer, *J. Dental Research*, 1952, **31**, 767; *Arch. Biochem. Biophys.*, 1952, **39**, 65.
[90] K. Mrybäck and B. Persson, *Arkiv Kemi*, 1952, **5**, 177.
[91] K. Benner and K. Myrbäck, *ibid.*, 1952, **4**, 7.
[92] D. Vincent and R. Lagreu, *Compt. rend. Soc. Biol.*, 1950, **144**, 1658.
[93] J. T. Kung, V. M. Hanrahan, and M. L. Caldwell, *J. Amer. Chem. Soc.*, 1953, **75**, 5548; R. Bird and R. H. Hopkins, *Biochem. J.*, 1954, **56**, 86.

has shown that the susceptible linkages in a particular substrate are drolysed at the same rate.[9]

The composition of the end-products of such α-amylolyses appears to y with the particular enzyme preparation used. Amorphous and crystal- salivary amylase and crystalline *A. oryzae* amylase (all maltase-free) grade amylose to maltose (87%) and glucose (13%).[94, 95] Maltose itself ot hydrolysed by α-amylases since it is a non-competitive inhibitor;[96] glucose presumably arose from the slow hydrolysis of maltotriose. H. Pazur has shown that maltotriose is slowly hydrolysed by unpurified ivary amylase.[97] Whelan and Roberts, however, using a purified ivary α-amylase, found maltose and maltotriose in the molar ratio of 0:1, to be the sole end products.[9] Svanborg and Myrbäck, likewise, re reported that maltotriose is not attacked by salivary amylase.[73] These crepancies may be due to differences in the relative concentrations of yme and substrate, or may imply the existence of a specific "malto- se" as an impurity in certain α-amylase preparations.

α-Amylolysis of amylopectin is slower than that of amylose, and relatively her enzyme concentrations are required to degrade the α-dextrins to ducing sugars.[1-5] The end-products include glucose, maltose, and non- mentable dextrins, when crystalline *A. oryzae*,[95] pancreatic, or malt mylase is used,[98] but maltose, maltotriose, and dextrins when amorphous ivary amylase is used.[8] The structures of the dextrins which contain one more 1 : 6-linkages, have been discussed in detail.[1, 8, 99] The smallest nched dextrin from salivary amylolysis is a pentasaccharide, indicating t the three 1 : 4-linkages adjacent to a 1 : 6-linkage are resistant to enzyme ion.[8]

α-Amylolysis of whole starch yields a mixture of maltose (*ca.* 70%), cose, and dextrins, the exact proportion of each, and the D.P. of the trins depending on the source and concentration of enzyme and the time digestion. The residual dextrins usually have a D.P. of 5—8, and contain 1:6-linkage, since they arise from the amylopectin component of rch.[1, 73, 95, 100]

Although all α-amylases catalyse similar reactions, their modes of action pear to differ in some respects (cf. also ref. 93). There is evidence that, ring their respective actions, salivary amylase combines with the exterior ins of a branched α-dextrin, whilst malt α-amylase combines with the hain (see Figure).[73] Furthermore, malt α-amylase liberates glucose ring the dextrinisation stage of amylolysis, in contrast to *A. oryzae*[95] d pancreatic amylases[2] which liberate glucose only during the sacchari- tion stage, and salivary amylase which produces little or no glucose at y stage.[73, 100] The action pattern of salivary amylase has now been ined in detail;[8, 9] similar studies on other α-amylases which are in pro- ss[62] should help to eliminate some of the inconsistencies in present data α-amylolysis.

[94] K. H. Meyer and W. F. Gonon, *Helv. Chim. Acta*, 1951, **34**, 294.
[95] V. M. Hanrahan and M. L. Caldwell, *J. Amer. Chem. Soc.*, 1953, **75**, 2191.
[96] S. Schwimmer, *J. Biol. Chem.*, 1950, **186**, 181.
[97] J. H. Pazur, *ibid.*, 1953, **205**, 75.
[98] K. H. Meyer and W. F. Gonon, *Helv. Chim. Acta*, 1951, **34**, 308.
[99] K. Myrbäck, *Arkiv Kemi*, 1952, **4**, 433.
[100] K. Myrbäck and B. Persson, *ibid.*, 1953, **5**, 365.

Studies on glycogen α-amylolysis are as yet incomplete. B. Carlquist [101] has shown that glycogen is degraded by malt α-amylase to a heterogeneous mixture of maltose and linear and branched dextrins. Oyster glycogen on *A. oryzae* amyolysis gave 57% of maltose, 4% of glucose, and 39% of dextrin (D.P. 8).[95] Roberts and Whelan have examined quantitatively the salivary α-amylolysis of pregnant-doe liver glycogen. The products were maltose, maltotriose, and dextrins containing 1 : 6-linkages. Further examination of these dextrins provided evidence in favour of a random enzyme action and indicated that the glycogen had a multiply branched structure.[8] Bell and Manners [102] found that some of the linkages in the interior chains of rabbit-liver glycogen were resistant to malt α-amylase, but were readily hydrolysed by salivary amylase—further proof that these two α-amylases have different affinities for the *same* substrate.

Peat *et al.*[14] have claimed that a sample of rabbit-liver glycogen contained *ca.* 2·5% of fructose, since on α-amylolysis small quantities of maltulose (*ca.* 5%) and fructose-containing α-dextrins could be isolated. Other workers have been unable to detect fructose in various samples of mammalian-liver glycogen, and a discussion of the significance of this finding must be postponed until more experimental results are available.

C. "Amylases" producing glucose. Recently a group of "amylases" which produce glucose as the primary product of amylolysis has been discovered. These enzymes are not mixtures of α- or β-amylase and maltase. Glucose-forming amylases attack their substrates from the non-reducing terminal groups, and, in stepwise fashion, catalyse the hydrolysis of *every* 1 : 4-gluco-sidic linkage.

R. W. Kerr and his co-workers [103] have purified "amyloglucosidase" from *Aspergillus niger*. This enzyme had no true α-amylase activity, but converted maize amylose almost quantitatively into glucose; with maize amylopectin *ca.* 80% conversion to glucose occurred. The rate of hydrolysis of amylopectin was greater than that of amylose, owing to the larger number of non-reducing terminal groups present in the amylopectin molecule. A study of the rate of hydrolysis of amylose suggested (*a*) a "single-chain" mechanism,[103] and (*b*) the existence of a low degree of branching in the amylose.[25]

The so-called "maltase" of *Clostridium acetobutylicum* has been purified by D. French and D. W. Knapp.[104] This enzyme slowly hydrolysed maltose, maltoheptaose, *iso*maltose, starch, α- and β-dextrin, and a Schardinger dextrin, to give nearly quantitative yields of glucose. Dextran, however, was not attacked. An examination of the products formed during the hydrolysis of maltoheptaose led these workers to postulate a "multi-chain" action for this enzyme.

From the mould *Rhizopus delemar*, L. L. Phillips and M. L. Caldwell [105] obtained a purified "gluc-amylase," which catalysed the hydrolysis, to glucose, of maize amylose and amylopectin, waxy maize starch and its β-dextrin, glycogen, and maltose. It had no action on *iso*maltose, dextran,

[101] B. Carlquist, *Acta Chem. Scand.*, 1948, **2**, 770.
[102] D. J. Bell and D. J. Manners, *Biochem. J.*, 1951, **49**, lxxvii.
[103] R. W. Kerr, F. C. Cleveland, and J. Katzbeck, *J. Amer. Chem. Soc.*, 1951, **73**, 3916.
[104] D. French and D. W. Knapp, *J. Biol. Chem.*, 1950, **187**, 463.
[105] L. L. Phillips and M. L. Caldwell, *J. Amer. Chem. Soc.*, 1951, **73**, 3559, 3563.

r cyclic Schardinger dextrins. Their results suggest that gluc-amylase annot split 1 : 6-inter-chain linkages, but can by-pass them (cf. α-amylases).

"γ-Amylase" isolated (in partly purified form) from *Aspergillus awamori* ppears to be a unique glucose-producing amylase.[106] This enzyme (also resent in *A. batatae* and *A. cinnamomeus*) liberates β-glucose together with a mall amount of maltose from soluble starch; it apparently has no action on altose. Further work is necessary on the amylolytic systems of this group f moulds.

D. B. macerans "*amylase.*" This amylase is unique in that it degrades tarch by a transference rather than a hydrolytic mechanism, to yield a ixture of cyclic dextrins. The isolation, purification, properties, and mode f action of *B. macerans* "amylase" have been described recently,[15, 107-109] nd will not be discussed further in this Report.

Degradation of α-1 : 4-Linkages by Transferring Enzymes.—*Phosphorylases.* The phosphorylases catalyse the reversible transfer of a glucosyl adical from α-D-glucose 1-phosphate to a chain of α-1 : 4-linked glucose esidues, according to the equation :

$$[G]_n + m\text{G.1.P} \rightleftharpoons [G]_{n+m} + m\text{HO.P}$$

here $[G]_n$ or $[G]_{n+m}$ represents a chain of n or $n + m$ glucose residues, .1.P. = glucose 1-phosphate, and HO.P = inorganic phosphate. Although he equilibrium position of the reaction is in the direction of synthesis, in the resence of an excess of inorganic phosphate, degradation of an α-1 : 4-lucosan occurs.

Phosphorylases have been isolated from many sources; those from nimal cells will be referred to as "phosphorylases" and those from plants s "P-enzymes," since they differ in several respects, even though they atalyse similar reactions.

Muscle phosphorylase has been crystallised,[110] several recrystallisations eing necessary to remove traces of other enzymes.[111] It is a conjugated rotein; studies on the prosthetic group [112] and amino-acid composition [113] ave been reported, but the nature of the "active groups" and the mechanism of its activation by adenylic acid remain unknown. Liver phosphorylase as been purified by E. W. Sutherland,[114] the active enzyme differing from uscle phosphorylase in its physical properties, *e.g.*, solubility. Liver hosphorylase also occurs in two inactive forms, only one of which is active the presence of adenylic acid (cf. muscle phosphorylase).

In the presence of inorganic phosphate, phosphorylases cause partial egradation of maize amylose, glycogen, and amylopectin, the limits being t. 70, 28—49, and 36—57% respectively.[44, 61, 115] The reason for the incomplete action on amylose may be due to the presence of anomalous linkges, or to a low degree of branching. With glycogen and amylopectin,

[106] K. Kitahara and M. Kurushima, *Mem. Res. Inst. Food Sci. Kyoto*, 1952, 15.
[107] S. Schwimmer and J. A. Garibaldi, *Cereal Chem.*, 1952, **29**, 108.
[108] S. Schwimmer, *Arch. Biochem. Biophys.*, 1953, **43**, 108.
[109] E. Norberg and D. French, *J. Amer. Chem. Soc.*, 1950, **72**, 1202.
[110] A. A. Green and G. T. Cori, *J. Biol. Chem.*, 1943, **151**, 21; G. A. Kritsky and B. Kuvaeba, *Brit. Abs.*, 1951, A, III, 1284.
[111] G. T. Cori and J. Larner, *J. Biol. Chem.*, 1951, **188**, 17.
[112] M. V. Buell, *Fed. Proc.*, 1952, **11**, 192.
[113] S. F. Velick and L. F. Wicks, *J. Biol. Chem.*, 1951, **190**, 741.
[114] E. W. Sutherland, "Phosphorus Metabolism," 1951, Vol. I, p. 58. Johns Hopkins Press, Baltimore.
[115] S. Hestrin, *J. Biol. Chem.*, 1949, **179**, 943.

phosphorylase action is limited to the exterior chains, since the pure enzyme cannot rupture or by-pass 1 : 6-linkages.[115] Furthermore, the affinity of the enzyme for the substrate decreases as the exterior chains are shortened. Phosphorolysis of a B-type chain in glycogen (or amylopectin) ceases at about the sixth residue from the branch point, whereas an A-type chain is completely degraded except for a single glucose residue which remains attached to the B-chain. It is this residue which arrests phosphorolysis of the B-chain.[111]

Potato P-enzyme has been purified by several groups of workers [116, 117] and has now been crystallised.[118] P-Enzymes do not require adenylic acid for activity, but the nature of their active centres are as yet unknown. In presence of inorganic phosphate, P-enzyme degrades certain amyloses completely to glucose 1-phosphate; [119] with other samples, only 70% degradation occurs.[11] On addition of Z-enzyme, complete degradation follows, affording additional evidence of β-linkages in these amyloses.[11] The degradation of amylose by P-enzyme proceeds by a multi-chain mechanism.[120] The action of P-enzyme on amylopectins is confined to their exterior chains, the limiting conversion to glucose 1-phosphate (36—57%) depending on the sample examined,[119] and is unaltered by Z-enzyme.[11] P-Enzyme has very little action on glycogen, presumably owing to its low affinity for the relatively short exterior chains (*ca.* 8 glucose units as compared with *ca.* 14 units in amylopectins).[117]

In presence of arsenate, amylosaccharides are converted by P-enzymes into glucose.[121] The enzymes transfer a glucosyl unit to arsenate, giving the rapidly decomposing glucose 1-arsenate. The limits of P-enzyme arsenolysis and β-amylolysis of amylopectins have been compared by K. H. Meyer *et al.*[122] and found to be identical. The speeds, however, differed considerably, β-amylolysis occurring *ca.* 1000 times as fast as arsenolysis (or *ca.* 100 times as fast as phosphorolysis). Since muscle phosphorylase, but not P-enzyme, limit dextrins are further hydrolysed by β-amylase, P-enzymes and phosphorylases differ in their affinities for the same branched amylosaccharides. Related differences with respect to specificity of " primer " molecules during the synthesis of such polysaccharides have been noted elsewhere.[15, 115]

Other transglucosylases. Several enzymes are known which can reversibly transfer a glucosyl unit from a suitable donor to a chain of 1 : 4-α-linked glucose units, *e.g.*, amylomaltase, amylosucrase, Schardinger dextrinogenase (*B. macerans* amylase), and D-enzyme. Since studies on these enzymes have been directed mainly to their synthetic, rather than to their degradative, activities, they will not be discussed further in this Report.

Degradation of 1 : 6-Linkages by Hydrolytic Enzymes.—For some time it has been known that extracts of certain plants, yeasts, or moulds could hydrolyse the inter-chain linkages in starch-type polysaccharides. Only

[116] S. A. Barker, E. J. Bourne, I. A. Wilkinson, and S. Peat, *J.*, 1950, 84; G. A. Gilbert and A. D. Patrick, *Biochem. J.*, 1952, **51**, 186.
[117] E. H. Fischer and H. M. Hilpert, *Experientia*, 1953, **9**, 177.
[118] H. Baum and G. A. Gilbert, *Nature*, 1953, **171**, 983.
[119] J. Katz, W. Z. Hassid, and M. Doudoroff, *ibid.*, 1948, **161**, 96.
[120] J. M. Bailey and W. J. Whelan, *Biochem. J.*, 1952, **51**, xxxiii.
[121] J. Katz and W. Z. Hassid, *Arch. Biochem.*, 1951, **30**, 272.
[122] K. H. Meyer, R. M. Weil, and E. H. Fischer, *Helv. Chim. Acta*, 1952, **35**, 247.

uring the past few years, however, have purified " debranching " enzymes een isolated.

R-Enzyme. From the potato and broad bean, S. Peat and his lleagues [123] isolated an enzyme (R-enzyme) which hydrolysed the inter-ain linkages in amylopectin and its β-dextrin. Treatment of these sub-rates with R-enzyme increased β-amylolysis by *ca.* 20 and 60% respectively, hilst a mixture of R-enzyme and α-amylase caused complete degradation linear oligosaccharides.[8] R-Enzyme has no action on amylose, *iso*maltose, xtran, or glycogen, and has no synthetic activity.

Amylo-1 : 6-*glucosidase.* G. T. Cori and J. Larner [111] isolated from uscle extracts an enzyme, amylo-1 : 6-glucosidase, which, acting together ith phosphorylase, caused complete digestion of glycogen and amylopectin. mylo-1 : 6-glucosidase hydrolysed the single unit remaining from the chain in the appropriate phosphorylase limit dextrin, liberating one mol. glucose. The combined action of phosphorylase and amylo-1 : 6-cosidase therefore gives glucose 1-phosphate (*ca.* 95%) together with cose, the amount of the latter depending on the number of 1 : 6-linkages the molecule. The ratio glucose : glucose 1-phosphate is therefore a easure of the degree of branching in the polysaccharide, and the combined tion of these two enzymes has been used to assay chain lengths of amylo-ctins and glycogens with results in good agreement with methylation and tassium periodate oxidation assays of the *same* samples. Less good greement was obtained with those from low temperature sodium periodate says.[44, 111] Amylo-1 : 6-glucosidase slowly hydrolyses *iso*maltose but has action on glycogen or amylopectin.

A. N. Petrova [124] has described the presence in rabbit muscle of an zyme " amylose isomerase " which catalyses the degradation and synthesis 1 : 6-linkages in glycogen. It seems probable that this preparation is mixture of amylo-1 : 6-glucosidase and muscle branching enzyme, although trova has repeatedly claimed that " amylose isomerase " has a dual nction. It has been suggested that " amylose isomerase " requires a rmostable non-protein co-factor for full degradative activity.[125]

Yeast " debranching " enzymes. In 1940 it was reported [33] that β-amylase the presence of a brewer's yeast extract, could further hydrolyse β-dextrins m glycogen and amylopectin. This impure yeast enzyme, called " amylo-cosidase," also hydrolysed maltose and *iso*maltose.[126] More recently, B. aruo and T. Kobayashi [127] isolated from autolysed brewer's yeast an enzyme amylase (formerly known as amylosynthease) which hydrolysed the inter-ain linkages in glutinous-rice starch, and an amylopectin (cf. R-enzyme).

" *Limit dextrinase.*" Although the existence of various " limit xtrinases " has been reported, full details of their specificities are not yet ailable. That from *A. oryzae* has been crystallised.[128]

Structural Analysis of Amylosaccharides by Enzymic Degradations.—mylolysis is proving a useful method of investigating the structures of cosans. Hydrolysis of a glucosan by α-amylase implies the presence of a

[123] P. N. Hobson, W. J. Whelan, and S. Peat, *J.*, 1951, 1451.
[124] A. N. Petrova, *Chem. Abs.*, 1948, **42**, 7807; 1952, **46**, 2103.
[125] *Idem, ibid.*, 1953, **47**, 1809.
[126] K. H. Meyer and P. Bernfeld, *Helv. Chim. Acta*, 1942, **25**, 399.
[127] B. Maruo and T. Kobayashi, *Nature*, 1951, **167**, 606.
[128] L. A. Underkofler and D. K. Roy, *Cereal Chem.*, 1951, **28**, 18.

number of α-1 : 4-glucosidic linkages; examination of the hydrolysis products will assay the number of these, and will reveal the existence of other types of linkage if present, *e.g.*, 1 : 6 in glycogen,[8] and 1 : 3 in *iso*lichenin.[129] Degradation by β-amylase indicates that the molecule contains one or more non-reducing chains of α-1 : 4-linked glucose residues; quantitative studies provide a method for determining the length of such chains. Polysaccharides from protozoa [38, 130] and micro-organisms [131] have been characterised in this way. Furthermore, the activity of branching enzymes can be followed by examining the action of β-amylase on the products formed from amylose, since these products will have lower β-amylolysis limits than the amylose.[132]

Several enzymic methods of end-group assay of α-1 : 4-glucosans have been developed. The combined use of phosphorylase and amylo-1 : 6-glucosidase has already been discussed (p. 299). A method developed by W. J. Whelan [8, 68] involves the preparation of α-dextrins from glycogen or amylopectin and subsequent treatment with R-enzyme. The number of reducing groups liberated on " debranching " equals the number of 1 : 6-linkages present in the original glucosan. A further method of enzymic assay is based on the determination of maltotriose obtained on degrading an amylopectin with a mixture of R-enzyme and β-amylase. It is assumed that, on a statistical basis, one-half of the chains present in the molecule contain an odd number of glucose residues and will therefore yield maltotriose.[68]

The successive action of phosphorylase and amylo-1 : 6-glucosidase has led to the postulation of a " tier " (tree) structure for amylopectin and glycogen.[47] Each enzyme action removes one " tier " of exterior chains, and the resulting limit dextrin can be isolated and characterised. There seems to be little doubt that glycogens are multiply-branched molecules as originally suggested by K. H. Meyer.

An alternative method of structural analysis involves the action of R-enzyme on a β-dextrin.[34] A polysaccharide with singly branched unit-chains would yield one molecule of maltose or maltotriose from its sole A-chain, together with linear saccharides of D.P. $\geqslant 6$ from the B-chains. A polysaccharide with a multiply-branched structure would give a much larger amount of maltose and maltotriose. In the case of waxy maize starch the observed yield of these sugars was greater than that expected from a singly branched structure, and Peat and his colleagues have concluded " that multiple branching is an intrinsic part of the amylopectin structure." It can be shown [133] from their experimental data that the waxy maize starch contains one A-chain to every four B-chains—compare a Meyer " tree " structure which requires equal numbers of A- and B-chains. It appears therefore that glycogens and amylopectins differ in *degree of multiple branching*. This suggestion is supported by physico-chemical evidence, *e.g.*, viscosity [71, 134] and iodine-binding power.[135] These indicate fundamental

[129] N. B. Chanda, unpublished work.
[130] E. J. Bourne, M. Stacey, and I. A. Wilkinson, *J.*, 1950, 2694.
[131] S. A. Barker, E. J. Bourne, and M. Stacey, *J.*, 1950, 2884; P. N. Hobson and H. Nasr, *J.*, 1951, 1855.
[132] A. Bebbington, E. J. Bourne, and I. A. Wilkinson, *J.*, 1952, 246; S. Peat, W. J. Whelan, and J. M. Bailey, *J.*, 1953, 1422.
[133] E. L. Hirst and D. J. Manners, *Chem. and Ind.*, 1954, 224.
[134] R. W. Kerr, F. C. Cleveland, and W. J. Katzbeck, *J. Amer. Chem. Soc.*, 1951, 73, 111.
[135] D. M. W. Anderson and C. T. Greenwood, *Chem. and Ind.*, 1953, 642; also unpublished work.

fferences in the arrangement of the unit-chains in glycogen and amylo-ctin; these polysaccharides should therefore not be regarded as variants the same basic structure, differing only in chain length.

The use of enzymes of known action pattern in structural investigations of anched α-1 : 4-glucosans therefore provides methods for the determination the degree and type of branching, and for the location of the branch points the unit-chains. Caution is necessary, however, when using a mixture two purified enzymes since a synergic reaction may occur; it has been ported that α-amylases, in the presence of Schardinger dextrinogenase,[107] R-enzyme,[68] behave abnormally, the specificity of the enzymes being tered and considerable amounts of glucose liberated. In general, enzymic gradation studies provide, with certain reservations, a valuable means for dying the fine structure of starch and glycogen, the results so obtained being ed to confirm and supplement those obtained by purely chemical methods.

D. J. M.

4. FATTY ACID METABOLISM IN ANIMAL TISSUES.

Abbreviations used are: AcAc, acetoacetyl; AcCoA, *S*-acetyl-coenzyme ; AcAcCoA, *S*-acetoacetyl-CoA; ATP, ADP, AMP, adenosine tri-, di-, nd mono-phosphate; CoA or CoA'·SH, coenzyme A;[1] DPN_{ox}, DPN_{red}, idised and reduced coenzyme I; FAD, flavin–adenine dinucleotide; PN_{red}, reduced coenzyme II. *C denotes an isotopically labelled carbon tom.

Great advances have been made in the understanding of fatty acid etabolism since the last review in these Reports,[2] but much remains iscure, particularly regarding branched-chain fatty acids and those con-aining an odd number of carbon atoms (odd-C acids). Intermediates have sually been studied by photometric, isotopic, and enzymic methods, and hemical characterisations are mostly still to be undertaken.

Fatty Acid Oxidation.—That many tissues besides liver can oxidise fatty ids, although at considerably differing rates, has been confirmed *in vitro* ith isotopically labelled substrates and tissue slices.[3] By three successive dvances in technique, fatty acid metabolism has been studied with cell-ee particle-preparations, soluble systems, and separated enzymes. Fatty id oxidation by washed-liver particles was first achieved by Leloir and Muñoz [4] and Lehninger.[5–8] Observations were later extended to kidney [10]

[1] G. D. Novelli, *Physiol. Reviews*, 1953, **33**, 525; *Fed. Proc.*, 1953, **12**, 675.
[2] F. Dickens, *Ann. Reports*, 1945, **42**, 197.
[3] (*a*) R. P. Geyer, L. W. Matthews, and F. J. Stare, *J. Biol. Chem.*, 1949, **180**, 1037; S. Weinhouse, R. H. Millington, and M. E. Volk, *ibid.*, 1950, **185**, 191; (*c*) M. E. Volk, H. Millington, and S. Weinhouse, *ibid.*, 1952, **195**, 493.
[4] (*a*) J. M. Muñoz and L. F. Leloir, *ibid.*, 1943, **147**, 355; (*b*) L. F. Leloir and J. M. uñoz, *ibid.*, 1944, **153**, 53; (*c*) L. F. Leloir, *Enzymologia*, 1947, **12**, 263.
[5] A. L. Lehninger, (*a*) *J. Biol. Chem.*, 1944, **154**, 309; (*b*) 1945, **157**, 363; (*c*) **161**, (*d*) 437; (*e*) 1946, **164**, 291, (*f*) **165**, 131; (*g*) 1951, **190**, 345; (*h*) *Biochem. Soc. mp.*, 1952, **9**, 66. [6] A. L. Lehninger and E. P. Kennedy, *J. Biol. Chem.*, 1948, **173**, 753.
[7] E. P. Kennedy and A. L. Lehninger, *ibid.*, (*a*) 1948, **172**, 847; (*b*) 1949, **179**, 957; 1950, **185**, 275; (*d*) 1951, **190**, 361.
[8] *Idem*, " Phosphorus Metabolism," Johns Hopkins Press, Baltimore, 1952, Vol. II, 253. [9] A. Kornberg, *op. cit.*, p. 245.
[10] (*a*) A. L. Grafflin and D. E. Green, *J. Biol. Chem.*, 1948, **176**, 95; (*b*) W. E. Knox, N. Noyce, and V. H. Auerbach, *ibid.*, p. 117; (*c*) R. J. Cross, J. V. Taggart, G. A. ovo, and D. E. Green, *ibid.*, 1949, **177**, 655.

and heart muscle.[5f] Fatty acid oxidation takes place in isolated mitochondria and not in other cell fractions.[7a, b, 11] Particle oxidising systems are unspecific, attacking lower [10a] and higher [7c] fatty acids, enoic, dienoic, odd-C, branched-chain, β-oxo-, and D- and L-β-hydroxy-acids, but not ynoic, 2- or 3-substituted or dicarboxylic acids.[10a] Effects of various factors on fatty acid oxidation by such systems have been thoroughly studied, and the findings well reviewed.[12, 5h, 8] These studies showed that fatty acids must be "activated" before being oxidised, and, together with isotopic evidence, indicated that the 2C-units formed by β-oxidation [13] are the same as those arising from oxidative decarboxylation of pyruvate, *i.e.*, AcCoA.[14] A scheme similar to that shown below [(1)] was suggested by Barker and his colleagues [15, 16] as a result of work with *Clostridium kluyveri* extracts, and by Lynen [17] and his co-workers after discovery of acetylcoenzyme A. That coenzyme A is involved in fatty acid oxidation in animal tissues was proved by Drysdale and Lardy,[18, 19] and by Green and his co-workers,[20] who obtained CoA-dependent oxidation of butyrate to acetoacetate in soluble systems from acetone-dried rat-liver mitochondria and from ox-liver respectively. Finally, confirmation of sequence (1) was supplied by observations with separated enzymes in the laboratories of Green, Lipmann, Lynen, and Ochoa.

$$\begin{aligned} &\mathrm{R{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CO_2H} \overset{\mathrm{I}}{\rightleftharpoons} \mathrm{R{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}S{\cdot}CoA'} \overset{\mathrm{II}}{\rightleftharpoons} \\ &\mathrm{R{\cdot}CH_2{\cdot}CH{:}CH{\cdot}CO{\cdot}S{\cdot}CoA'} \left(\rightleftharpoons \mathrm{R{\cdot}CH{:}CH{\cdot}CH_2{\cdot}CO{\cdot}S{\cdot}CoA'}\right) \\ \overset{\mathrm{III}}{\rightleftharpoons} &\mathrm{L\text{-}R{\cdot}CH_2{\cdot}CH(OH){\cdot}CH_2{\cdot}CO{\cdot}S{\cdot}CoA'} \overset{\mathrm{IV}}{\rightleftharpoons} \mathrm{R{\cdot}CH_2{\cdot}CO{\cdot}CH_2{\cdot}CO{\cdot}S{\cdot}CoA'} \\ \overset{\mathrm{V}}{\rightleftharpoons} &\mathrm{R{\cdot}CH_2{\cdot}CO{\cdot}S{\cdot}CoA' + CH_3{\cdot}CO{\cdot}S{\cdot}CoA'} \quad . \ . \ . \ . \ . \ . \ . \ . \ . \ . \ . \quad (1) \end{aligned}$$

Fatty acid is "activated" by conversion into the coenzyme A derivative, and then by stages II—V yields acetyl-coenzyme A, and acyl-coenzyme A with two carbon atoms less. The latter passes through II—V again, and so on. This is essentially Dakin's scheme [21] applied to coenzyme A derivatives instead of free acids.

Lynen has used *S*-acyl derivatives of 2-acetamidoethanethiol † instead of coenzyme A as model substrates. Wave-lengths (mμ) of absorption maxima of these and some coenzyme A derivatives,[22, 23] together with structural features to which they are attributed, are: $CH_3{\cdot}CO{\cdot}CH_2{\cdot}CO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}NHAc$ 233 (–CO·S–), 303 (in alkaline solution, intensified by Mg^{++}, enolate ion);

[11] W. C. Schneider, *J. Biol. Chem.*, 1948, **176**, 259.
[12] D. E. Green, *Biol. Reviews*, 1950, **26**, 410.
[13] S. Weinhouse, G. Medes, and N. F. Floyd, *J. Biol. Chem.*, 1944, **155**, 143.
[14] S. Korkes, A. del Campillo, and S. Ochoa, *ibid.*, 1952, **195**, 541.
[15] H. A. Barker, in "Phosphorus Metabolism," Johns Hopkins Press, Baltimore, 1951, Vol. I, p. 204.
[16] E. R. Stadtman, *Fed. Proc.*, 1953, **12**, 692.
[17] F. Lynen, E. Reichert, and L. Rueff, *Annalen*, 1951, **574**, 1.
[18] G. R. Drysdale and H. A. Lardy, in "Phosphorus Metabolism," Johns Hopkins Press, Baltimore, 1952, Vol. II, p. 281.
[19] G. R. Drysdale and H. A. Lardy, *J. Biol. Chem.*, 1953, **202**, 119.
[20] See H. A. Mahler, in "Phosphorus Metabolism," Johns Hopkins Press, Baltimore, 1952, Vol. II, p. 286.
[21] H. D. Dakin, "Oxidations and Reductions in the Animal Body," Longmans, Green and Co., London, 1912.
[22] F. Lynen, *Fed. Proc.*, 1953, **12**, 683.
[23] F. Lynen and S. Ochoa, *Biochim. Biophys. Acta*, 1953, **12**, 299.

† *Added in proof.* In the nomenclature proposed by Z. M. Bacq, J. Baddiley, L. Eldjarn, F. Lipman, and F. Lynen (*Science*, 1954, **119**, 163) this is *N*-acetylcysteamine.

$CHMe{:}CH{\cdot}CO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}NHAc$, 263 (?–CO·S–), 224 (C:C); *S*-acyl- and β-hydroxyacyl-coenzyme A, 233* (–CO·S–); *S*-acetoacetyl-coenzyme A, 303 (as with acetamidoethanethiol compound); *S*-crotonyl-coenzyme A, 263* (?–CO·S–), 224* (C:C). In addition, the coenzyme A derivatives absorb strongly around 260 mμ (adenine), which obscures maxima marked *; these are, however, measured with an equivalent amount of alkali-hydrolysed material in the spectrophotometer reference cell (alkali hydrolyses thiol-esters).

Some thiol-esters needed for enzyme studies have been prepared chemically: (i) From thiols and diketen ($CH_3{\cdot}CO{\cdot}CH_2{\cdot}CO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}NHAc$ and the coenzyme A derivative [23]); (ii) from coenzyme A and acid anhydride (*S*-succinyl-,[24] acetyl-,[25] and crotonyl derivatives [26]); (iii) from coenzyme A and acyl(thiophenol) [27] (*S*-acetoacetyl and β-hydroxybutyryl derivatives); (iv) from the lead salt of acetamidoethanethiol and acyl chloride (*S*-crotonyl derivative [28]); and (v) from coenzyme A and thiol-acids (*S*-acetyl-,[25] *S*-butyryl,[29] and *S*-palmityl derivatives [30b]). *S*-Derivatives of coenzyme A have also been prepared by using various enzymes described below (see summary [31a] and refs. 32, 30*a*, and 31*b*).

Reactions applied to determination of acyl derivatives of coenzyme A formed enzymically include: (*a*) Hydroxamic acid formation,[33] usually with hydroxylamine present during the enzyme reaction.[34] (*b*) Acetylation of sulphanilamide [35, 17] or aminoazobenzenesulphonate [36] (pigeon-liver fraction added). (*c*) For acetyl-coenzyme A, arsenolysis in presence of transacetylase.[29] (*d*) Nitroprusside reaction, to measure disappearance of the thiol group of coenzyme A. (*e*) Oxaloacetate + acetyl-coenzyme A ⟶ citrate (condensing enzyme added; citrate determined).[37] (*f*) Malate + DPN_{ox} ⟶ oxaloacetate + DPN_{red}; oxaloacetate + acetyl-coenzyme A ⟶ citrate (malic dehydrogenase and condensing enzyme added; DPN_{red} determined).[38] Acyl-thiols [39] and also hydroxamic acids [40] may be separated by paper-chromatography.

I. *Fatty-acid Activation.*—(i) *Acetate.* Acetate is "activated" by extracts of animal tissues in presence of ATP [41, 35] and coenzyme A,[42–44]

[24] E. J. Simon and D. Shemin, *J. Amer. Chem. Soc.*, 1953, **75**, 2520.
[25] I. B. Wilson, *ibid.*, 1952, **74**, 3205.
[26] J. R. Stern and A. del Campillo, *ibid.*, 1953, **75**, 2277.
[27] T. Wieland and L. Rueff, *Angew. Chem.*, 1953, **65**, 186.
[28] W. Seubert and F. Lynen, *J. Amer. Chem. Soc.*, 1953, **75**, 2787.
[29] E. R. Stadtman, *J. Biol. Chem.*, 1953, **203**, 501.
[30] A. Kornberg and W. E. Pricer, *ibid.*, 1953, **204**, (*a*) 329, (*b*) 345.
[31] H. Beinert, (*a*) *Fed. Proc.*, 1953, **12**, 681; (*b*) *J. Biol. Chem.*, 1953, **205**, 575.
[32] H. Beinert and P. G. Stansly, *ibid.*, 1953, **204**, 67.
[33] F. Lipmann and L. C. Tuttle, *ibid.*, 1945, **159**, 21.
[34] T. C. Chou and F. Lipmann, *ibid.*, 1952, **196**, 89.
[35] F. Lipmann, *ibid.*, 1945, **160**, 173.
[36] M. E. Jones, S. Black, R. M. Flynn, and F. Lipmann, *Biochim. Biophys. Acta*, 1953, **12** 141.
[37] S. Ochoa, J. R. Stern, and M. C. Schneider, *J. Biol. Chem.*, 1951, **193**, 691.
[38] J. R. Stern, B. Shapiro, E. R. Stadtman, and S. Ochoa, *ibid.*, p. 703.
[39] E. R. Stadtman, *ibid.*, 1952, **196**, 535.
[40] K. Fink and R. M. Fink, *Proc. Soc. Exp. Biol., N.Y.*, 1949, **70**, 654; E. R. Stadtman and H. A. Barker, *J. Biol. Chem.*, 1950, **184**, 769.
[41] D. Nachmansohn and A. L. Machado, *J. Neurophysiol.*, 1943, **6**, 397.
[42] N. O. Kaplan and F. Lipmann, *J. Biol. Chem.*, 1948, **174**, 37.
[43] M. Soodak and F. Lipmann, *ibid.*, 1948, **175**, 999.
[44] J. R. Stern and S. Ochoa, *ibid.*, 1949, **179**, 491; 1951, **191**, 161.

forming acetyl-coenzyme A.[34] The reaction catalysed by preparations from yeast,[45,36] pigeon liver,[45,36] and mammalian heart muscle[46-48] involves pyrophosphoryl scission of ATP (cf. ref. 49) :

$$AcO^- + CoA + ATP \rightleftharpoons AcCoA + AMP + pyrophosphate \quad . \quad (2)$$

(Pyrophosphate breakdown by contaminating pyrophosphatase[50] may be prevented by fluoride.[45,36]) Reaction (2) is reversible,[36,47,48,51] the equilibrium constant [AcCoA][AMP][pyrophosphate]/[AcO$^-$][ATP][CoA] being approximately 2·5,[51] in conformity with calculations[38,39] that the free energy of hydrolysis of the –C(O)–S– bond of acetyl-coenzyme A is comparable with that of each pyrophosphate bond of ATP. The enzymes from yeast[36,52] and pig heart[48] have been partially purified, and also that from ox-heart particles.[47] Only acetate and propionate are activated;[48,52,47] Mg^{++} is necessary;[36,48] K^+, NH_4^+, and Rb^+ stimulate, whilst Na^+ and Li^+ inhibit strongly.[53] The formulation of reaction (2) with coenzyme A–pyrophosphate as intermediate[45,54] could not be confirmed,[36,48,52] and the one now proposed[55] is (E = enzyme) :

$$E + ATP \rightleftharpoons E\text{–}AMP + Pyrophosphate \quad . \quad . \quad . \quad (3)$$

$$E\text{–}AMP + HS\cdot CoA' \rightleftharpoons E\text{–}S\cdot CoA' + AMP \quad . \quad . \quad . \quad . \quad . \quad . \quad (4)$$

$$E\text{–}S\cdot CoA' + AcO^- \rightleftharpoons E + Ac\cdot S\cdot CoA' \quad . \quad . \quad . \quad . \quad . \quad . \quad (5)$$

Evidence for this, obtained with purified yeast enzyme, follows. For reaction (3) : ATP equilibrates rapidly with ^{32}P-pyrophosphate in absence of coenzyme A (excluding coenzyme A–pyrophosphate as intermediate). For (4) : coenzyme A inhibits this exchange reaction, presumably by lowering the E–AMP concentration. For (5) : labelled acetate equilibrates with acetyl-coenzyme A, at a rate unaffected by AMP addition. Mg^{++} is needed for (3), but not for (5).

(ii) *Lower fatty acids.* Mahler and his co-workers[56-58] have separated from ox-liver mitochondria an enzyme ("fatty acid activating enzyme") which activates C_4—C_{11} fatty acids (optimum C_7) by a reaction analogous to (2). It appeared homogeneous in the ultra-centrifuge, but not on electrophoresis. Mg^{++} (or Mn^{++}) is needed; Na^+ does not inhibit the reaction. The equilibrium constant with heptanoate is about 1, indicating again that the free energy of hydrolysis of acyl-coenzyme A is comparable with that of ATP. Enzyme activity increases linearly with pH. Unsaturated, branched-chain, phenyl-substituted, and β-hydroxy-acids are activated; dicarboxylic

[45] F. Lipmann, M. E. Jones, S. Black, and R. M. Flynn, *J. Amer. Chem. Soc.*, 1952, **74**, 2384; *J. Cell. Comp. Physiol.*, 1953, **41**, Suppl. 1, 109.
[46] D. E. Green, *Science*, 1952, **115**, 661.
[47] M. P. Hele, *Fed. Proc.*, 1953, **12**, 216; *J. Biol. Chem.*, 1954, **206**, 671.
[48] H. Beinert, D. E. Green, P. Hele, H. Hift, R. W. Von Korff, and C. V. Ramakrishnan, *ibid.*, 1953, **203**, 35.
[49] W. K. Maas and G. D. Novelli, *Arch. Biochem. Biophys.*, 1953, **43**, 236.
[50] K. Bailey and E. C. Webb, *Biochem. J.*, 1944, **38**, 394.
[51] M. E. Jones, *Fed. Proc.*, 1953, **12**, 708.
[52] F. Lynen, *Bull. Soc. Chim. biol.*, 1953, **35**, 1061.
[53] R. W. Von Korff, *J. Biol. Chem.*, 1953, **203**, 265.
[54] D. M. Needham, *Ann. Reports*, 1952, **49**, 275.
[55] M. E. Jones, F. Lipmann, H. Hilz, and F. Lynen, *J. Amer. Chem. Soc.*, 1953, **75**, 3285.
[56] S. Wakil and H. R. Mahler, *Fed. Proc.*, 1953, **12**, 285.
[57] H. R. Mahler, *ibid.*, p. 694.
[58] H. R. Mahler, S. J. Wakil, and R. M. Bock, *J. Biol. Chem.*, 1953, **204**, 453.

d amino-acids are not. Formation of coenzyme A derivatives from fatty ids,[18] crotonic, and D- and L-β-hydroxybutyric acids [59] has been shown so in rat-liver mitochondria extracts. Butyrate is said to be activated by action with succinyl-coenzyme A.[57]

(iii) *Higher fatty acids.* Kornberg and Pricer [9, 30a] have found that luble and small-particle fractions of guinea-pig liver convert higher fatty ids (*e.g.*, myristic, palmitic, stearic) into coenzyme A derivatives, again by reaction analogous to (2). Mono-, di-, and tri-enoic C_{18} acids were also tivated.

II. *Acyl-coenzyme A Dehydrogenation.*—This is reversible, and in their zyme assay Seubert and Lynen [28] measured oxidation of leuco-safranine ith 2-acetamido-1-crotonylthioethane as model substrate. Their enzyme, om sheep liver, was yellow and a FAD-protein. It was named "ethylene ductase" by analogy with the FAD-protein "Fumarat-Hydrase."[60] otonate was not activated; DPN_{red} or TPN_{red} would not replace leuco-dye. ahler and his co-workers.[57, 61, 62] prepared from ox-liver mitochondria butyryl-coenzyme A dehydrogenase," a vivid green FAD-protein, homo-eneous on electrophoresis and ultra-centrifugation. It catalyses dehydro-nation of coenzyme A derivatives of fatty acids from C_3 to C_7 (C_4 optimum; ble activity with C_8). It is uncertain whether butyryl-coenzyme A yields otonyl- or vinylacetyl-coenzyme A; probably it is the former, since opionyl-coenzyme A is acted upon. The enzyme, reduced by sodium hionite or by butyryl-coenzyme A, is rapidly re-oxidised by crotonyl-enzyme A. Absorption maxima are at 265, 355, 432, and 685 mμ. On dition of butyryl-coenzyme A the extinctions at 355, 432, and 685 mμ are creased. The enzyme contains bound copper (1 Cu : 2 flavin); on dialysis ainst potassium cyanide solution the 685-mμ peak, not seen with other roproteins, disappears; it is attributed to copper–enzyme bonding. anide-treated enzyme catalyses dehydrogenation of butyryl-coenzyme A ith 2 : 6-dichlorophenolindophenol (a two-electron acceptor), but not with tochrome *c* or ferricyanide (one-electron acceptors) unless the cyanide-ated enzyme is first incubated with Cu^{++}. Mahler implies that the zyme-bound copper probably transfers electrons from reduced flavin to unidentified, one-electron acceptor in the cell. A yellow "acyl-coenzyme dehydrogenase," also from ox-liver mitochondria, catalyses dehydrogen-on of coenzyme A derivatives of higher fatty acids (C_5 and above).[57, 62]

III. *Enoyl-coenzyme A Hydration.*—Existence of an enzyme ("croton-e"[26] "αβ-unsaturated acyl-coenzyme A hydrase"[57]) which catalyses rotonyl-CoA + $H_2O \rightleftharpoons$ S-β-hydroxybutyryl-CoA was indicated by periments [26] with heart and liver fractions. The ox-liver enzyme has en partially purified in Green's [62, 57] and Ochoa's [23, 52] laboratories. In ays with crotonyl-coenzyme A as substrate, hydration was measured by DPN reduction in presence of β-hydroxyacyl-coenzyme A dehydrogenase

[59] A. L. Lehninger and G. D. Greville, *J. Amer. Chem. Soc.*, 1953, **75**, 1515; *Biochim. ophys. Acta*, 1953, **12**, 188.
[60] F. G. Fischer and H. Eysenbach, *Annalen*, 1937, **530**, 99; K. P. Harrison, *Nature*, 3, **172**, 509.
[61] H. R. Mahler, *J. Amer. Chem. Soc.*, 1953, **75**, 3288; *J. Biol. Chem.*, 1954, **206**, 13; E. Green, S. Mii, H. R. Mahler, and R. M. Bock, *ibid.*, p. 1.
[62] H. Beinert, R. M. Bock, D. S. Goldman, D. E. Green, H. R. Mahler, S. Mii, P. G. asly, and S. J. Wakil, *J. Amer. Chem. Soc.*, 1953, **75**, 4111.

(see reaction 6),[57] or (*b*) decrease in absorption at 263 mμ,[23, 52] which corresponds to a maximum for crotonyl- but not for β-hydroxybutyryl-coenzyme A (p. 302). The enzyme hydrated the coenzyme A derivatives of all 2 : 3-unsaturated acids tested (C_4—C_{12}),[57] but not 2-acetamido-1-crotonylthioethane, crotonate,[23, 52] or *cis*-but-2-enoyl-coenzyme A [62] (*cis*-but-2-enoate is, however, oxidised by liver particles [10a]). It is specific for the coenzyme A derivative of L-(+)-β-hydroxybutyric acid,[57] and presumably of the L-isomers of other β-hydroxy-acids. Vinylacetyl-coenzyme A reacts,[57] so an equilibrium must be set up between crotonyl, vinylacetyl, and β-hydroxybutyryl derivatives. At equilibrium at pH 9·0 [total butenoyl-coenzyme A]/[L-β-hydroxybutyryl coenzyme A] = *ca.* 0·7.[62]

IV. β-*Hydroxyacyl-coenzyme A Dehydrogenation.*—The enzyme ("β-ketoreductase," [63] "β-hydroxyacyl-coenzyme A dehydrogenase" [62]) which catalyses

$$S\text{-}\beta\text{-Hydroxybutyryl-CoA} + DPN_{ox} \rightleftharpoons AcAc\text{-}CoA + DPN_{red} \quad . \quad (6)$$

was purified from sheep liver by Lynen and his co-workers,[63] using as assay oxidation of DPN_{red} by 2-acetamido-1-acetoacetylthioethane. With the enzyme, acetyl-CoA, and β-ketothiolase contained in a liver fraction (see below), acetoacetyl-coenzyme A was reduced to the β-hydroxybutyryl derivative at the expense of the nucleotide. The same reduction was effected by a pig-heart fraction.[64b] In Green's laboratory, the enzyme was purified from ox-liver mitochondria; [57, 62] the coenzyme A derivatives of the β-hydroxy-acids tested (C_4—C_{12}) were all oxidised at about the same rate; β-oxoacyl-coenzyme A derivatives were formed. Acetoacetate [23] and *S*-acetoacetylglutathione [65] were not activated. DPN can be replaced by coenzyme III,[66] but not by TPN.[62] The enzyme is specific for the coenzyme A derivative of L-(+)-β-hydroxybutyric acid,[59, 62] and presumably of the L-isomers of the other β-hydroxy-acids. β-Hydroxybutyric dehydrogenase is specific for the D-(−)-isomer of the free acid.[67] Rat liver converts L-β-hydroxybutyrate into a coenzyme A derivative, so particulate preparations oxidise it *via* the tricarboxylic acid cycle, whilst the D-salt is converted largely into acetoacetate.[59] The equilibrium position of reaction (6) is dependent on hydrogen-ion concentration,[63] as with other DPN-linked dehydrogenase reactions, owing to changing ionisation of DPN_{red} over a certain pH range. Thus it is strongly on the side of hydroxy-compound at pH 7, but shifts in favour of acetoacetyl-coenzyme A as the pH rises.[63, 57, 23]

V. β-*Oxoacyl-coenzyme A Cleavage* (*Thiolysis*).—The finding that the coenzyme-A-catalysed synthesis of acetoacetate from acetate [43] involves condensation of two "activated" 2C-units,[68] and evidence that acetoacetate forms citrate *via* acetyl-coenzyme A,[44] indicated the reversible reaction:

$$AcAc\text{-}S\text{-}CoA' + CoA'\cdot SH \rightleftharpoons 2Ac\text{-}S\text{-}CoA' \quad . \quad . \quad . \quad . \quad (7)$$

The names "β-ketothiolase," [63] "acetoacetate condensing enzyme," [64] and "AcAcCoA cleavage enzyme" [62] have been proposed for an enzyme cata-

[63] F. Lynen, L. Wessely, O. Wieland, and L. Rueff, *Angew. Chem.*, 1952, **64**, 687.
[64] J. R. Stern, M. J. Coon, and A. del Campillo, (*a*) *Nature*, 1953, **171**, 28; (*b*) *J. Amer. Chem. Soc.*, 1953, **75**, 1517. [65] T. Wieland and H. Köppe, *Annalen*, 1953, **581**, 1.
[66] T. P. Singer and E. B. Kearney, *Biochim. Biophys. Acta*, 1952, **8**, 700; 1953, **11**, 290. [67] D. E. Green, J. G. Dewan, and L. F. Leloir, *Biochem. J.*, 1937, **31**, 934.
[68] E. R. Stadtman, M. Doudoroff, and F. Lipmann, *J. Biol. Chem.*, 1951, **191**, 377.

sing this reaction. Acetoacetyl-coenzyme A formation by a sheep-liver action was studied by Lynen and his co-workers [63] as indicated under (IV). etoacetate formation from acetyl-coenzyme A was shown with an ox-liver eparation.[64] Thiolysis has been studied in conjunction with the transase (see section VI below) which supplies acetoacetyl-coenzyme A by action between acetoacetate and succinyl-coenzyme A.[46, 69, 70, 64b] Thiosis has been measured by decrease in absorption at 305 mμ due to disppearance of acetoacetyl-coenzyme A,[64b, 23] increase in absorption at 0 mμ accompanying formation of a second –CO–S– bond,[23] citrate formion (method *e*, p. 303),[64b] and DPN reduction (method *f*, p. 303).[57] rmation of acetoacetyl-coenzyme A from the acetyl derivative at pH 9 is own by increase in absorption at 305 mμ.[52] An enzyme purified from pig art was most active with acetoacetyl-, but inactive with β-oxohexanoylenzyme A; [23] an enzyme purified from ox-liver mitochondria functioned ith coenzyme A derivatives of all β-keto-acids tested (C_4—C_8).[69, 57] The uilibrium position of reaction (7) is far towards cleavage: [63, 64b] at equiliium $[AcCoA]^2/[AcAcCoA][CoA] = 5 \times 10^4$ at pH 8·1, and 1×10^4 at H 9·0.[23]

Evidence has been provided [22, 23] that β-ketothiolase is an " SH-enzyme," d the following mechanism (HS·E = enzyme) has been suggested:

$$R{\cdot}CH_2{\cdot}CO{\cdot}CH_2{\cdot}CO{\cdot}S{\cdot}CoA' + HS{\cdot}E \rightleftharpoons R{\cdot}CH_2{\cdot}CO{\cdot}S{\cdot}E + CH_3{\cdot}CO{\cdot}S{\cdot}CoA' \quad (8)$$

$$R{\cdot}CH_2{\cdot}CO{\cdot}S{\cdot}E + HS{\cdot}CoA' \rightleftharpoons R{\cdot}CH_2{\cdot}CO{\cdot}S{\cdot}CoA' + HS{\cdot}E \quad (9)$$

me support for reaction (9) is given by the finding that ^{35}S enters propionyl-coenzyme A when this is incubated with $H^{35}S{\cdot}CoA'$ and enzyme. A ssible formulation of reaction (8), when $R{\cdot}CH_2 = Me$, is:

$$E{\cdot}S{\cdot}\underset{Me}{C^+}{-}O^- + H^+{-}C^-H_2{\cdot}CO{\cdot}S{\cdot}CoA' \rightleftharpoons E{\cdot}S{\cdot}\underset{Me}{C}(OH){\cdot}CH_2{\cdot}CO{\cdot}S{\cdot}CoA' \rightleftharpoons$$

$$E{\cdot}SH + Me{\cdot}CO{\cdot}CH_2{\cdot}CO{\cdot}S{\cdot}CoA' \quad (10)$$

is conforms with Lynen's view [22] of the methyl activation of acetylenzyme A, for which he finds support in the low pK for enol ionisation acetoacetyl-coenzyme A. Beinert and Stansly,[32] unaware of the thiol oup in the enzyme, have proposed a mechanism somewhat different from t indicated by reactions (8) and (9) (p. 315).

VI. *Acetoacetate Formation.*—There is said [57] to be in liver a specific AcCoA-" deacylase " which catalyses hydrolytic formation of acetoetate; details have not been given. In heart, and probably in skeletal scle and kidney, but not in liver, acetoacetate may be " activated " 313) by the reaction,[46, 70, 69, 64] $AcAcO^-$ + succinyl-CoA $\rightleftharpoons$ AcAcCoA + cinate, catalysed by " succinyl-CoA–$AcAcO^-$ transferase." The equiliium is greatly in favour of acetoacetyl-coenzyme A formation. The pigart enzyme has been considerably purified and freed from β-ketothiolase; [23] ctivates C_4—C_6 (not C_8) β-keto-acids.

" *Reconstructed Systems.*"—Butyrate has been converted into acetylenzyme A, and thence into citrate, by a mixture of the enzymes described

[63] D. Goldman, *Fed. Proc.*, 1953, **12**, 209.
[64] D. E. Green, D. S. Goldman, S. Mii, and H. Beinert, *J. Biol. Chem.*, 1953, **202**, 137.

in sections I—V, together with ATP, coenzyme A, malate, malic dehydrogenase, condensing enzyme, DPN, diaphorase, pyocyanine, and triphenyltetrazolium salts (final electron-acceptor).[62] Synthesis of butyryl-coenzyme A from the acetyl derivative has been achieved with an appropriate mixture of enzymes, and DPN_{red} and reduced " benzyl viologen " as hydrogen donors for (IV) and (II) respectively.[71]

" Sparking " and " Priming." [12, 5h, 8] Washed-particle preparations oxidise fatty acids through the tricarboxylic acid cycle and also convert them into acetoacetate. If increasing concentrations of an intermediate in the cycle (*e.g.*, malate) are added, acetoacetate formation becomes progressively less and oxidation *via* the cycle greater,[6] since more oxaloacetate is provided to react with the acetyl-coenzyme A from the fatty acid (" sparking "—Green). Particularly with aged or exhaustively washed particles, fatty acids are not attacked until tricarboxylic acid cycle intermediates are added in low concentration, whereupon acetoacetate is formed.[6, 10b] This phenomenon is also called " sparking " by Green, but should perhaps be distinguished as " priming " (Lehninger). Several findings indicate that priming is concerned with initial " activation " of fatty acids, *i.e.*, conversion into acyl-coenzyme A with the aid of ATP: (i) 2:4-Dinitrophenol and gramicidin, which inhibit oxidative formation of ATP, also stop fatty acid oxidation [10c] at concentrations which scarcely affect oxidation of intermediates in the cycle. (ii) Priming is achieved by addition of DPN_{red},[7d] oxidation of which results in ATP formation.[72, 5g] (iii) Fatty acid oxidation results in ATP formation,[6] and continues after all the DPN_{red} has been oxidised in (ii) (" self-priming " [8, 12]). (iv) Priming is needed for oxidation of β-keto-, αβ-unsaturated,[12] and β-hydroxy-acids.[59] Priming is necessary despite presence of added ATP (see, however, ref. 73), and is not required in soluble systems, which are unaffected by dinitrophenol.[18] It seems, therefore, likely that priming provides ATP at some site in mitochondria usually inaccessible to external ATP. Addition of ATP is necessary for most metabolic reactions with mitochondria, but possibly to maintain their integrity, since it prevents [74] and reverses [75] swelling. Fluoride inhibits fatty acid oxidation in particles [4a] and not in soluble systems; [18] it may also affect priming (by preventing pyrophosphate scission?).

Fatty Acid Synthesis.—The first evidence by that carbohydrate ⟶ fat conversion in animals [76] occurs by condensation of 2C-units came from the use of isotopes,[77] which also showed that a considerable part of dietary carbohydrate is normally converted into fat.[78] Incorporation of *C from acetate into fatty acids *in vitro* has been shown with slices of liver,[79, 80, 81a]

[71] P. G. Stansly and H. Beinert, *Biochim. Biophys. Acta*, 1953, **11**, 600.
[72] M. Friedkin and A. L. Lehninger, *J. Biol. Chem.*, 1949, **178**, 611.
[73] J. D. Judah and K. R. Rees, *Biochem. J.*, 1953, **55**, 664.
[74] J. Raaflaub, *Helv. Physiol. Pharmacol. Acta*, 1953, **11**, 142, 157.
[75] J. B. Chappell, personal communication.
[76] J. B. Lawes and J. H. Gilbert, *Phil. Mag.*, 1866, **32**, 439.
[77] R. Schoenheimer, " The Dynamic State of Body Constituents," Harvard Univ. Press, Cambridge, Mass., 1946, p. 3.
[78] DeW. Stetten and G. E. Boxer, *J. Biol. Chem.*, 1944, **155**, 231.
[79] K. Bloch, E. Borek, and D. Rittenberg, *ibid.*, 1946, **162**, 441.
[80] K. Bloch and W. Kramer, *ibid.*, 1948, **173**, 811.
[81] R. O. Brady and S. Gurin, (*a*) *ibid.*, 1950, **186**, 461; (*b*) 1950, **187**, 589.

idney, heart, spleen, testis,[82] and mammary gland; [83] so has incorporation ^{14}C from glucose with liver, kidney, diaphragm,[84] and mammary gland,[85, 86] nd of deuterium from D_2O in adipose tissue.[87] Incorporation of ^{14}C-acetate to long- and short-chain fatty acids has been shown in homogenates,[88, 89a] nd in pigeon-[90] and rat-liver [91] soluble systems obtained by mixing " cell-ap " material with the soluble components from lysed mitochondria. In ntrast, with lactating rat mammary tissue the complete incorporation stem seems to be in the " cell sap." [89b, c] Finally, butyryl-coenzyme A s been formed from the acetyl derivative in a system containing purified zymes (p. 308).[71]

Mechanism.—Addition of a 2C-unit to $C_{(1)}$ of $^{14}C_{(1)}$-myristate has been own in rats,[92] and of $CH_3{\cdot}^{14}CO_2^-$ to $C_{(1)}$ of endogenous palmitate *in vivo* nd *in vitro*.[93] Evidence that short-chain fatty acids derived from $H_3{\cdot}^{14}CO_2^-$ are formed by addition of 2C-units to the $C_{(1)}$-position was tained with mammary gland; [94, 95] $C_{(\omega-1)}$ and $C_{(\omega-3)}$ had equal activities, wer than those of $C_{(1)}$, $C_{(3)}$, etc., probably as a result of dilution of :3-^{14}C]acetoacetyl-coenzyme A by endogenous unlabelled β-hydroxy-tyryl-coenzyme A.

Earlier suggestions that a 3C-unit may be incorporated as such into fatty ids during synthesis [96, 97] have not been confirmed: (1) $*C_{(3)}$- and $*C_{(2)}$-ruvate yield the same isotope distribution in fatty acids as do $*CH_3{\cdot}CO_2^-$ $CH_3{\cdot}*CO_2^-$; [85, 95] and $CH_3{\cdot}CH(OH){\cdot}^{14}CO_2^-$ contributes no ^{14}C to fatty ids.[98] (2) Appearance of [1-^{14}C]glucose almost entirely in even-numbered rbon atoms of octanoate [95, 86] suggests glycolysis followed by pyruvate carboxylation. (3) Synthesis of ^{14}C-butyryl-coenzyme A from $H_3{\cdot}^{14}CO{\cdot}S{\cdot}CoA'$ (p. 308) [71] leaves little doubt that the key reaction in fat nthesis is repeated condensation of acetyl-coenzyme A with R·CO·S–, and at pyruvate merely provides acetyl-coenzyme A, unless two synthetic thways exist, one using acetyl-coenzyme A and the other the pyruvate. ccasionally $*C\text{-}AcO^-$ is not incorporated into fatty acid, but it is when ruvate is added as well; [80, 99] in these cases acetate might not be activated the absence of energy available from pyruvate oxidation. Pyruvate ight also act by enlarging the acetyl-coenzyme A pool, with consequent duction of $^{14}CO_2$ formation by the tricarboxylic acid cycle, if the size of e pool affects rate of fatty acid synthesis more than that of carbon dioxide rmation.

[82] G. Medes, A. Thomas, and S. Weinhouse, *J. Biol. Chem.*, 1952, **197**, 181.
[83] J. H. Balmain, S. J. Folley, and R. F. Glasscock, *Biochem. J.*, 1952, **52**, 301.
[84] S. S. Chernick, E. J. Masoro, and I. L. Chaikoff, *Proc. Soc. Exp. Biol., N.Y.*, 50, **73**, 348. [85] T. H. French and G. Popják, *Biochem. J.*, 1951, **49**, iii.
[86] G. Popják, G. D. Hunter, and T. H. French, *ibid.*, 1953, **54**, 238.
[87] B. Shapiro and E. Wertheimer, *J. Biol. Chem.*, 1948, **173**, 725.
[88] N. L. R. Bucher, *J. Amer. Chem. Soc.*, 1953, **75**, 498.
[89] G. Popják and A. Tietz, (*a*) *Biochem. J.*, 1954, **56**, 46; (*b*) *ibid.*, 1954, p. xxiii; *Biochim. Biophys. Acta*, 1953, **11**, 587.
[90] R. O. Brady and S. Gurin, *J. Biol. Chem.*, 1952, **199**, 421.
[91] F. Dituri and S. Gurin, *Arch. Biochem. Biophys.*, 1953, **43**, 231.
[92] H. S. Anker, *J. Biol. Chem.*, 1952, **194**, 177. [93] I. Zabin, *ibid.*, 1951, **189**, 355.
[94] G. Popják, G. D. Hunter, and T. H. French, *Biochem. J.*, 1953, **54**, 238.
[95] G. Popják, *Biochem. Soc. Symp.*, 1952, **9**, 37.
[96] I. Smedley-MacLean, " The Metabolism of Fat," Methuen, London, 1943, p. 21.
[97] K. Bloch, *Cold Spring Harbor Symp.*, 1948, **13**, 29.
[98] J. M. Felts, I. L. Chaikoff, and M. J. Osborn, *J. Biol. Chem.*, 1951, **191**, 683.
[99] W. Shaw and S. Gurin, *Arch. Biochem. Biophys.*, 1953, **47**, 220.

The mechanism of carbohydrate ⟶ fat conversion may be reviewed in terms of the hypothetical equation :

$$4\text{Hexose (24C)} \xrightarrow{(a)} 8CO_2 \xrightarrow{(b)} 1\text{Palmitate (16C)} + 8CO_2$$

$$+\begin{bmatrix}8\text{CoA}\\8\text{ADP}\\16\text{DPN}_{ox}\end{bmatrix} \quad +8\text{AcCoA} \quad +\begin{bmatrix}8\text{ATP}\\16\text{DPN}_{red}\end{bmatrix} \quad +\begin{bmatrix}8\text{CoA}\\8\text{ATP}\\14\text{DPN}_{ox}+2\text{DPN}_{red}\end{bmatrix} \quad (11)$$

Coenzyme A, ATP, and DPN are needed [100] for incorporation of $CH_3{\cdot}^{14}CO_2^-$; with acetyl-coenzyme A as substrate instead of acetate, ATP is unnecessary.[71] The overall change may be divided into two steps : (*a*) catabolic, furnishing units and energy for synthesis, and (*b*) anabolic, with C–C bond formation and hydrogenation, giving the paraffinic acid. The citrate required by soluble systems [90, 91, 100] is possibly used for reduction of DPN_{ox}. In the reconstituted system DPN_{red} does not react with butyryl-coenzyme A dehydrogenase,[71] but some intermediary may exist in the cell.

Effect of Fasting and Diabetes.—(i) Short fasts (*e.g.*, 1 day) suppress incorporation of *C-acetate into fatty acids in rats [101] and in rat [97, 102, 82] (but not cat [81b])-liver slices. (ii) [^{14}C]Glucose fails to be incorporated with slices from rats fed on a high-fat or protein diet.[103, 104] (iii) Uptake of ^{14}C from acetate and glucose into fatty acids is impaired in liver slices from depancreatised cats [81b, 105] and alloxan-diabetic rats.[81b, 106] With the latter, oxidation of [^{14}C]glucose to carbon dioxide is also suppressed.[106] Insulin pre-treatment of the rats restores the oxidative and lipogenic pathways;[107] but addition of insulin to the slice has no effect,[81b] although it accelerates incorporation in normal slices.[80, 81a] (Incorporation is reduced in liver slices of " Houssay " cats injected with pituitary growth-hormone.[105]) (iv) Feeding fructose (but not glucose) to diabetic rats for three days before killing them restores the ability of the liver slice to incorporate ^{14}C into fatty acids from acetate and lactate, but not from glucose.[108] These findings, taken with other evidence,[109] indicate that one defect in fat synthesis from glucose in the diabetic or fasted [110] rat arises from failure of glucose to reach or to be activated by hexokinase (fructose seems to be less affected).

A second defect is indicated by two findings : (*a*) [^{14}C]fructose, added to diabetic rat-liver slices, is not incorporated into fatty acids, even though it is converted into carbon dioxide at a normal rate;[109] and (*b*) prolonged fasting (2 and 3 days) of normal rats impairs incorporation by liver slices of ^{14}C from fructose into fatty acids, but not into glycogen or carbon dioxide.[110] In (*b*), glycolysis and the tricarboxylic acid cycle are (pre-

[100] J. Van Baalen and S. Gurin, *J. Biol. Chem.*, 1953, **205**, 303.
[101] J. T. Van Bruggen, T. T. Hutchens, C. K. Claycomb, W. J. Cathey, and E. S. West, *ibid.*, 1952, **196**, 389; J. G. Coniglio, C. E. Anderson, and C. S. Robinson, *ibid.*, 1952, **198**, 525.
[102] I. Lyon, M. S. Masri, and I. L. Chaikoff, *ibid.*, 1952, **196**, 25.
[103] E. J. Masoro, I. L. Chaikoff, S. S. Chernick, and J. M. Felts, *ibid.*, 1950, **185**, 845.
[104] I. L. Chaikoff, Harvey Lectures, 1952, Vol. XLVII, p. 99.
[105] R. O. Brady, F. D. W. Lukens, and S. Gurin, *J. Biol. Chem.*, 1951, **193**, 459.
[106] S. S. Chernick, I. L. Chaikoff, E. J. Masoro, and E. Isaeff, *ibid.*, 1950, **186**, 527.
[107] S. S. Chernick and I. L. Chaikoff, *ibid.*, p. 535.
[108] N. Baker, I. L. Chaikoff, and A. Schusdek, *ibid.*, 1952, **194**, 435.
[109] S. S. Chernick, I. L. Chaikoff, and S. Abraham, *ibid.*, 1951, **193**, 793; W. C. Stadie, *Physiol Reviews*, 1954, **34**, 52.
[110] G. H. Wyshak and I. L. Chaikoff, *ibid.*, 1953, **200**, 851.

ably) normal, so there must be a defect beyond acetyl-coenzyme A, in the anabolic step of reaction (11). This defect is overcome by cose injection in fasted rats,[110] and by fructose, but not glucose, feeding diabetic ones.[108] It apparently results from insufficient supply of colysable substrate *in vivo*; after alloxan treatment the inhibition of cose utilisation is probably more severe than after fasting,[106, 110] so that ctose must be fed to prevent the second defect. In a *soluble* system from loxan-diabetic liver, incorporation of acetate into fatty acids is restored by dition of glycogen or hexose phosphates;[99] the second defect in diabetes d that after fasting, if this be the same) may, therefore, possibly be altered meability of some cell component.

Some evidence suggests that incorporation of acetate is dependent on colysis *in vitro*: for incorporation of acetate is proportional to the cogen content of liver slices and also to their glycogen utilisation;[111a] and agon and adrenalin, known to be glycogenolytic *in vitro*,[112] when added liver slices, decrease acetate incorporation.[111b] The findings with the ble system (previous paragraph) are in conformity with this possibility.

Incorporation of ^{14}C from acetate into fatty acids by liver slices is unually great when rats fasted for short periods are given a large dose of cose,[102, 82] and when alloxan-diabetic rats are treated with insulin.[113] der the latter conditions an accelerated incorporation of ^{14}C from ruvate [114] into fatty acids in the slices after four days of insulin treatment paralleled by a transient *rise* in liver fat *in vivo* which, may, therefore, be to net accumulation of fat from synthesis. Unlike normal ones, the es incorporate ^{14}C from pyruvate [115] and acetate [113] into fatty acid much re extensively than into carbon dioxide, despite normal production of al carbon dioxide. These observations suggest increase in rate of fat thesis without a comparable increase in that of oxidation (cf. effect of ruvate on incorporation of acetate, p. 309).

Fat Synthesis.—Free fatty acids may not be intermediates in fat synthesis, direct evidence is lacking. Esterification of glycerol-α phosphate (*e.g.*, give dipalmitophosphatidic acid) in a fraction from guinea-pig liver is to reaction between R·CO·S·CoA′ and R′·OH with elimination of A′·SH.[30b] Fats are therefore possibly generated by transesterification. optimum chain length for R is 15—17 atoms, and Professor Lynen suggested to the Reporters that this specificity may account for the ponderance of C_{16} and C_{18} acids in animal lipids.

Ketogenesis and Ketolysis.—We define certain terms used in the text as ows: (a) *ketogenesis*, formation of ketone bodies; (b) *antiketogenesis*, vention of their formation; (c) *ketolysis*, their removal; (d) *ketosis* and a., ketone body accumulation in animals and *in vitro* respectively, when ogenesis exceeds ketolysis.

Ketogenesis.—This depends on the relative rates at which acetyl-coenzyme oxidised *via* the tricarboxylic acid cycle and converted into acetoacetate.

(a) *Liver.* Here the k.b.a. depends mainly on the rate of ketogenesis,

[111] E. S. Haugaard and W. C. Stadie, (*a*) *J. Biol. Chem.*, 1952, **199**, 741; (*b*) 1953, 753.
[112] E. W. Sutherland, " Recent Progress in Hormone Research," 1950, Vol. V, p. 441.
[113] J. M. Felts, I. L. Chaikoff, and M. J. Osborn, *J. Biol. Chem.*, 1951, **193**, 557.
[114] M. J. Osborn, J. M. Felts, and I. L. Chaikoff, *ibid.*, 1953, **203**, 173.
[115] M. J. Osborn, I. L. Chaikoff, and J. M. Felts, *ibid.*, 1951, **193**, 549.

ketolysis being small (p. 313). Slices from fed rats show aerobically a slight k.b.a., those from fasted rats a greater.[116a] Addition of even-C fatty acids causes considerable k.b.a. with slices from fed rats, again greater with those from fasted animals [116a] (confirmed with labelled fatty acids [117]). With perfused livers the k.b.a. is inversely proportional to the glycogen content,[118] and with slices it is reduced by addition of glycogen.[119] Ammonium chloride produces ketosis in rats [120] and k.b.a. with liver slices.[116a] This is due to suppression of the tricarboxylic acid cycle by the removal of α-oxoglutarate as glutamate, so that 2C-fragments from fatty acids fail to be oxidised and are diverted to acetoacetate.[121] Malonate increases k.b.a. in slices,[122] formation of carbon dioxide from fatty acids being at the same time diminished,[123] again by blockage of the cycle.[5d] 2 : 4-Dinitrophenol ($4{\cdot}5 \times 10^{-5}$M) increases the respiration of rat-liver slices and the ratio of acetoacetate to β-hydroxybutyrate,[124] possibly as a result of accelerated oxidation of DPN_{red}.

Pyruvate, as a carbohydrate metabolite, should be antiketogenic, and indeed it is in the whole animal (cf., *e.g.*, ref. 125). However, since it is rapidly oxidised to acetyl-coenzyme A [14] it is potentially ketogenic. Rat-liver particles, particularly in presence of malonate or absence of intermediates from the tricarboxylic acid cycle, convert pyruvate almost quantitatively into acetoacetate.[5e] Conversion into acetoacetate also occurs in slices (see, *e.g.*, refs. 116*a*, 124) (but not always [126]), and to a slight extent in perfused [127] and minced liver.[128] Variations between different preparations may be related to the relative rates of (i) oxidation of pyruvate to acetyl-coenzyme A, and (ii) carboxylation of pyruvate to yield oxaloacetate which catalyses oxidation of acetyl-coenzyme A. Reaction (ii), observed in rat liver,[129] is likely to be more rapid in intact cells, particularly with a high carbon dioxide tension,[130] than with washed-particle preparations.[131] If the availability of glycogen determines the level of pyruvate, and hence that of oxaloacetate, the antiketogenic action of glycogen and the effect of fasting (see above) may be partly explained.

(b) *Extrahepatic tissues.* With rat-kidney slices, (i) there is a small k.b.a. aerobically from butyrate and hexanoate (also with spleen and testis),[122] (ii) labelled fatty acids (C_5—C_9 and C_{12}) yield acetoacetate only one-tenth as fast as they form carbon dioxide,[132] (iii) some acetoacetate is formed without added substrate,[122, 126] (iv) although net formation of aceto-

[116] N. L. Edson, *Biochem. J.*, 1935, **29**, (*a*) 2082, (*b*) 2498.
[117] S. Weinhouse, R. H. Millington, and B. Friedman, *J. Biol. Chem.*, 1949, **181**, 489.
[118] N. Blixenkrone-Møller, *Z. physiol. Chem.*, 1938, **252**, 117.
[119] B. G. Bobbitt and H. J. Deuel, *J. Biol. Chem.*, 1942, **143**, 1.
[120] V. B. Wigglesworth, *Biochem. J.*, 1924, **18**, 1203.
[121] R. O. Recknagel and V. R. Potter, *J. Biol. Chem.*, 1951, **191**, 263.
[122] M. Jowett and J. H. Quastel, *Biochem. J.*, 1935, **29**, 2181.
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[124] P. Fantl, G. J. Lincoln, and J. F. Nelson, *Biochem. J.*, 1951, **48**, 96.
[125] I. Shapiro, *J. Biol. Chem.*, 1935, **108**, 373.
[126] S. Weinhouse and R. H. Millington, *ibid.*, 1951, **193**, 1.
[127] G. Embden and M. Oppenheimer, *Biochem. Z.*, 1912, **45**, 186.
[128] E. Annau, *Z. physiol. Chem.*, 1934, **224**, 141.
[129] R. K. Crane and E. G. Ball, *J. Biol. Chem.*, 1951, **188**, 819.
[130] D. E. Green, W. F. Loomis, and V. H. Auerbach, *ibid.*, 1948, **172**, 389.
[131] M. F. Utter and H. G. Wood, *Adv. Enzymology*, 1951, **12**, 41.
[132] R. P. Geyer and M. Cunningham, *J. Biol. Chem.*, 1950, **184**, 641.

etate from acetate has not been found, isotopic acetate is readily incorated into added acetoacetate,[133] (v) fasting does not seem to increase b.a.,[134] and (vi) malonate reduces formation of carbon dioxide from belled fatty acids;[135] but we find no statement that it increases k.b.a. ren in presence of malonate,[5f] cardiac-muscle homogenates fail to form tone bodies from octanoate,[5f] and rapidly oxidise acetoacetate.[5f, 136] owever, malonate causes accumulation of succinate and α-oxoglutarate in is tissue, unlike liver, which mainly accumulates ketone bodies, unless gh concentrations of dicarboxylic acids from the tricarboxylic acid cycle e present.[6] We find no evidence for ketogenesis in skeletal muscle.

Ketolysis.—(a) *Extrahepatic.* Eviscerated animals rapidly utilise injected etoacetate.[137] Kidney,[136, 138] heart,[5f] diaphragm,[138] skeletal muscle,[139] leen, omentum, and brain [138] oxidise ketone bodies *in vitro*. That acetoetate is oxidised *via* the tricarboxylic acid cycle first appeared probable 1943 [140] and was established later.[141, 136] The key reactions seem to be tivation of acetoacetate by attachment to coenzyme A and cleavage of e product to acetyl-coenzyme A. Activation occurs in heart muscle, d probably skeletal muscle and kidney, by transfer of coenzyme A to etoacetate from succinyl-coenzyme A (p. 307). The latter arises by idative decarboxylation of α-oxoglutarate,[142] or from interaction of enzyme A, ATP, and succinate.[143] More direct activation of acetoacetate coenzyme A and ATP also occurs in kidney preparations.[57] The comrative absence of acetoacetyl-coenzyme A deacylase (p. 307) in extrapatic tissues [57] favours these activating reactions and may be responsible r difficulty in detecting ketogenesis.

(b) *Liver.* The liver has a much greater capacity for producing ketone dies than for utilising them. Lehninger [5e] could detect no removal of acetoetate by rat-liver particle preparations even in presence of malate. Cohen d Stark,[144] however, claimed a rapid disappearance of ketone bodies when etoacetate was incubated with slices of liver from various rodents, less th starved than with fed animals. Oxidation to carbon dioxide of the tone bodies disappearing (*e.g.*, 140 μmoles per g. of dry tissue per hr. for a fed t) would demand a remarkably high respiration. Recent measurements isotopic carbon in the respiratory carbon dioxide proved that rat-liver es oxidise labelled acetoacetate slowly (about 12 μmoles per g. per hr. th fed,[126 145] 7 μmoles with fasted rats [126]). Acetoacetate, when removed t not oxidised to carbon dioxide, largely appeared as β-hydroxybutyrate.[126]

[133] G. Medes, S. Weinhouse, and N. F. Floyd, *J. Biol. Chem.*, 1945, **157**, 751.
[134] R. P. Geyer, E. J. Bowie, and J. C. Bates, *ibid.*, 1953, **200**, 271.
[135] R. P. Geyer, L. W. Matthews, and F. J. Stare, *ibid.*, 1950, **182**, 101.
[136] H. A. Krebs and L. V. Eggleston, *Biochem. J.*, 1948, **42**, 294.
[137] I. L. Chaikoff and S. Soskin, *Amer. J. Physiol.*, 1928, **87**, 58.
[138] R. A. Shipley, *ibid.*, 1944, **141**, 662.
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[140] H. Wieland and C. Rosenthal, *Annalen*, 1943, **554**, 241; F. L. Breusch, *Science*, 1, 97, 490.
[141] J. M. Buchanan, W. Sakami, S. Gurin, and D. W. Wilson, *J. Biol. Chem.*, 1945, , 695.
[142] S. Kaufman, C. Gilvarg, O. Cori, and S. Ochoa, *ibid.*, 1953, **203**, 869; D. R. zadi and J. W. Littlefield, *ibid.*, 1953, **201**, 103.
[143] S. Kaufman, in "Phosphorus Metabolism," Johns Hopkins Press, Baltimore, l, Vol. I, p. 370. [144] P. P. Cohen and I. E. Stark, *J. Biol. Chem.*, 1938, **126**, 97.
[145] R. W. Chen, D. D. Chapman, and I. L. Chaikoff, *ibid.*, 1953, **205**, 383.

^{14}C from $CH_3 \cdot {}^{14}CO \cdot CH_2 \cdot CO_2^-$ appears in higher fatty acids [145] and cholesterol.[146, 147]

Whether liver oxidises acetoacetate by a route similar to that in other tissues must be considered. Liver contains but little of the enzymes which convert it into acetoacetyl-coenzyme A.[57] Acetoacetate decomposes non-enzymically to acetone and carbon dioxide at an appreciable rate,[148, 126] but the acetone is probably not oxidised to carbon dioxide by liver slices.[147] During the preparation of sodium acetoacetate by hydrolysis of the ethyl ester some acetate is formed,[147] but this could yield only a small part of the respiratory carbon dioxide attributed to acetoacetate.[145] When $CH_3 \cdot {}^{14}CO \cdot CH_2 \cdot CO_2Et$ was added to liver slices some labelled acetate was formed; further addition of unlabelled acetate diminished the ^{14}C content of the respiratory carbon dioxide,[147] but by an amount which suggests that oxidation of acetoacetate cannot occur mainly *via* free acetate. Both carbonyl- and carboxyl-labelled acetoacetate undergo little or no randomisation of the label by rat-liver slices; [149, 145] this might indicate that acetyl-coenzyme A is not formed, but it is not decisive in view of the many outlets for that compound. To summarise, acetoacetate is oxidised slowly by liver tissue, probably through acetyl-coenzyme A, but possibly in part through acetate.

Whole Animals.—(a) *Normal.* It has been widely held [150] that even in normal fed animals the liver converts fatty acids largely into ketone bodies which are oxidised to carbon dioxide and water in the extrahepatic tissues. When, however, Crandall, Ivy, and Ehni [151] analysed blood entering and leaving the liver in dogs they did not detect production of ketone bodies by the liver unless the animals were fasting. There is nevertheless a small concentration of ketone bodies in normal circulating blood, and Breusch,[150b] making certain assumptions, calculated that the extrahepatic tissues of a fasting 75-kg. man utilise about 21 g. of ketone bodies (equivalent to about 14 g. of fat) per day. Since a normal fat intake is 100—120 g. per day, this suggests that, at the most, 15% of fat catabolism takes place *via* hepatic ketone-body production. The rate of fat oxidation in liverless animals can approach 50% of that of normal animals, but this does not exclude the possibility that the liver is the principal site of fat oxidation in the intact animal.[152]

(b) *Abnormal.* Factors, including starvation,[153] which induce ketosis seem more usually to increase ketogenesis than to decrease ketolysis. Feeding with fat [151, 153, 154] or fatty acid [155] rapidly causes ketosis, particularly

[146] R. O. Brady and S. Gurin, *J. Biol. Chem.*, 1951, **189**, 371.
[147] G. L. Curran, *ibid.*, 1951, **191**, 775.
[148] H. A. Krebs and L. V. Eggleston, *Biochem. J.*, 1945, **39**, 408.
[149] J. M. Buchanan, W. Sakami, and S. Gurin, *J. Biol. Chem.*, 1947, **169**, 411.
[150] (*a*) W. C. Stadie, *Physiol. Reviews*, 1945, **25**, 395; (*b*) F. L. Breusch, *Adv. Enzymology*, 1948, **8**, 343; (*c*) E. S. West and W. R. Todd, "A Textbook of Biochemistry," Macmillan, New York, 1951, p. 929; (*d*) G. G. Duncan, "Diseases of Metabolism," W. B. Saunders, Philadelphia, 1952, p. 221.
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[152] D. S. Goldman, I. L. Chaikoff, W. O. Reinhardt, C. Entenman, and W. G. Dauben, *J. Biol. Chem.*, 1950, **184**, 719; R. P. Geyer, W. R. Waddell, J. Pendergast, and G. S. Yee, *ibid.*, 1951, **190**, 437.
[153] J. P. Peters and D. D. Van Slyke, "Quantitative Clinical Chemistry," Bailliere, Tindall and Cox, London, 1946, Vol. I, Part 1.
[154] L. A. Crandall, *J. Biol. Chem.*, 1941, **138**, 123.
[155] C. H. Beatty and E. S. West, *ibid.*, 1951, **190**, 603.

previously fasted animals; but whether ketosis persists with high-fat ets depends on the other constituents. Ketosis produced by high-fat ets subsides after a few days;[120, 156] horse meat, but not casein, added to se diets prolongs the ketosis.[157, 158] A nitrogen-free substance in the id fraction of horse muscle is effective.[158] There may be a ketogenic ctor in dried liver as well.[159] Various extracts of anterior-pituitary gland oduce ketosis when injected,[160] but the responsible constituents have not en unequivocally identified.

Unsymmetrical Labelling of Acetoacetate.—Acetoacetate is formed by mbination of pairs of acetyl-coenzyme A molecules. If those derived by idation from a carboxyl-labelled fatty acid combine randomly,[13] the etoacetate should be equally labelled in the carbonyl and the carboxyl oup (*CO/*CO_2H = 1). Most recorded values of *CO/*CO_2H, obtained en rodent liver and kidney slices,[5h, 8] washed liver-particles,[5h, 8, 161] and tochondrial extracts [19] act on carboxyl-labelled fatty acids, are, however, s than 1.[5h, 8] Despite critical attention to analytical methods, quotients y greatly.[162] The shorter the fatty acid chain, the lower is the quotient; d $C_{(\omega-1)}$-labelled fatty acids give quotients up to 4.[5h, 8, 163] This and other idence [5h, 8] led to the postulate that two species of 2C-fragment arise ring fatty acid oxidation: (1) the acetylating type [CH_3·CO–], derived m the terminal CH_3·CH_2–, forms the acetyl group of acetoacetate; (2) the ferentially acetylated type [–CH_2·CO–], derived from the other 2C-units, ms the –CH_2·CO_2H. This concept may be abandoned as a result of ent studies with β-ketothiolase (p. 307), which suggest that unsymmetrical belling is a consequence of the mechanism of action of this enzyme exchange hypothesis").

During oxidation of a carboxyl-labelled fatty acid, there will be present labelled AcCoA, (*b*) labelled AcAcCoA (*CO/*CO_2H = 1), and (*c*) unbelled AcAcCoA from the four atoms $C_{(\omega)}$—$C_{(\omega-3)}$. It has been sug-sted [22, 23, 52] that (*c*) will react with β-ketothiolase (HS·E) (p. 307) as llows:

$$Ac\cdot CH_2\cdot CO\cdot S\cdot CoA' + HS\cdot E \rightleftharpoons Ac\cdot S\cdot E\cdot + Ac\cdot S\cdot CoA' \quad . \quad . \quad (12)$$

ing acetyl-coenzyme A which will mix with the labelled acetyl-enzyme A. By the right-to-left reaction, the labelled atom will then enter CH_2·CO·S·CoA′ in the 1-position. Acetoacetate formed by the fatty id oxidation will thus become preferentially labelled in the carboxyl oup. Beinert and Stansly [32] suggest that unsymmetrical labelling arises m the reactions (Enz = enzyme)

$$\text{Enz} + Ac\cdot CH_2\cdot CO\cdot S\cdot CoA' \rightleftharpoons \text{Enz-}Ac\cdot CH_2\cdot CO\cdot S\cdot CoA' \overset{\pm CoA'\cdot SH}{\rightleftharpoons} \text{Enz-}Ac\cdot S\cdot CoA' + Ac\cdot S\cdot CoA' \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (13)$$

e mechanisms are similar in principle; with both it is implicit that the should remain in combination with enzyme or as AcAcCoA (and not as

[156] C. L. Gemmill and E. G. Holmes, *Biochem. J.*, 1935, **29**, 338.
[157] H. P. Marks and F. G. Young, *J. Endocrinol.*, 1939, **1**, 470.
[158] H. B. Stewart and F. G. Young, *Nature*, 1952, **170**, 976.
[159] H. J. Deuel, J. S. Butts, and L. Hallman, *Proc. Soc. Exp. Biol. N.Y.*, 1935, **32**, 897.
[160] For references see J. Tepperman and H. M. Tepperman, *Ann. N.Y. Acad. Sci.*, , **54**, 707; A. L. Greenbaum and P. McLean, *Biochem. J.*, 1953, **54**, 413.
[161] V. H. Cheldelin and H. Beinert, *Biochim. Biophys. Acta*, 1952, **9**, 661.
[162] D. S. Goldman, G. W. Brown, H. R. Matheson, and I. L. Chaikoff, *J. Biol. Chem.*, , **195**, 415. [163] M. J. Coon and N. S. B. Abrahamsen, *ibid.*, p. 805.

AcCoA or acetoacetate) long enough for significant incorporation of isotope into $C_{(1)}$ of AcAcCoA. They differ in that thiolysis is by CoA'·SH in (13) and by E·SH in (12). Beinert and Stansly [32] found that when $Me\cdot{}^{14}CO\cdot S\cdot CoA'$ and acetoacetate were added to a soluble system containing β-ketothiolase and acetoacetate–succinyl-coenzyme A transferase, the acetoacetate became unsymmetrically labelled ($*CO/*CO_2H < 1$). They suggest exchange and cleavage reactions by which Enz-AcAc·S·CoA' might be formed; unsymmetrical labelling would then follow by reactions (13) or (10) (p. 307).

Some evidence advanced [5h, 8] in favour of the two-species hypothesis should be reviewed in the light of the "exchange hypothesis." (i) $*CO/*CO_2H$ is less than 1 when liver particle preparations oxidise unlabelled octanoate and labelled acetate simultaneously.[164] Since the former should yield acetoacetyl- and the latter labelled acetyl-coenzyme A, the conditions of Beinert and Stansly's experiment (see above) are in effect reproduced. (ii) The longer the fatty acid the higher is the ratio of carbon dioxide produced to acetoacetate formed,[3b, 7c] which may imply [5h, 8] that the $CH_3\cdot CH_2-$ forms acetoacetate more readily, and carbon dioxide less readily, than the other 2C-units. With Lynen's mechanism [22] of β-ketothiolase action, the $CH_3\cdot CO-$ is the only 2C-unit which combines with the enzyme before passing through the acetyl-coenzyme A pool. (iii) The $CH_3\cdot CH_2-$ unit forms liver glycogen less readily than the other 2C-units.[165] This may be considered in conjunction with (ii); if the $CH_3\cdot CH_2-$ unit yields acetoacetate more readily, and is oxidised *via* the tricarboxylic acid cycle less readily, than the other 2C-units, then it will form liver glycogen less readily since acetoacetate is but slowly utilised by liver (p. 313).

The observation [164, 166] that addition of unlabelled pyruvate raises the $*CO/*CO_2H$ quotient obtained with carboxyl-labelled octanoate is difficult to explain by the two-species hypothesis, since pyruvate might be expected to yield $[CH_3\cdot CO-]$-type fragments.[164] It is explicable by the exchange hypothesis, since unlabelled pyruvate will lower the isotope concentration of the acetyl-coenzyme A pool.

Chaikoff *et al.*[167] found that livers from rats injected with [5-^{14}C]- and [11-^{14}C]-palmitate gave, *in vitro*, acetoacetate with $*CO/*CO_2H$ 1·2—1·3, and devised a hypothesis [5h, 8] to explain this unexpected result.

Fixation of Carbon Dioxide in Acetoacetate.—When rat-liver homogenates formed acetoacetate from intrinsic substrates in presence of $^{14}CO_2$, the ^{14}C appeared in the carboxyl group.[168] Addition of acetone or acetoacetate caused no increase in carboxyl-labelling (although with $CH_3\cdot{}^{14}CO\cdot CH_3$ the ^{14}C appeared in the $CH_3\cdot CO\cdot CH_2-$ portion of the acetoacetate); but with pyruvate and certain fatty acids, ^{14}C appeared in the carboxyl group of the extra acetoacetate formed. Labelling might arise from the reaction: $CH_3\cdot CO\cdot CH_2\cdot CO_2H \rightleftharpoons CH_3\cdot CO\cdot CH_3 + CO_2$. Energy ($\Delta F^\circ = 16{,}300$ cal.) would be needed for the resynthesis; labelling accompanying fatty acid and pyruvate breakdown might then result through utilisation of the energy derived from oxidation. Against this is the absence of ^{14}C from acetoacetate

[164] D. I. Crandall and S. Gurin, *J. Biol. Chem.*, 1949, **181**, 829.
[165] V. Lorber, M. Cook, and J. Meyer, *ibid.*, p. 475.
[166] D. I. Crandall, R. O. Brady, and S. Gurin, *ibid.*, p. 845.
[167] I. L. Chaikoff, D. S. Goldman, G. W. Brown, W. G. Dauben, and M. Gee, *ibid.*, 1951, **190**, 229.
[168] G. W. E. Plaut and H. A. Lardy, *ibid.*, (*a*) 1950, **186**, 705; (*b*) 1951, **192**, 435.

rmed by oxidation of DL-β-hydroxybutyrate in presence of $^{14}CO_2$, even th *cis*-aconitate as an additional source of energy; more probably, fatty d and pyruvate yield some intermediate to which carbon dioxide is added form acetoacetate. Both yield acetyl-coenzyme A, which has been shown give labelled acetoacetate in presence of $*CO_2$.[19] Since the *iso*propyl iety of *iso*valerate, following dehydrogenation adds carbon dioxide to form toacetate (p. 321), it may possibly be that a similar 3C-group is formed, ectly or indirectly, from acetyl-coenzyme A. Fixation of carbon dioxide acetoacetate may account for the finding [169] that, when $Na_2{}^{14}CO_3$ is fed rats, ^{14}C appears in the fatty acids of the carcass fats.

Propionic Acid.—Propionate is glycogenic [170] and unlike acetate and yrate is not ketogenic.[171] Both *in vivo* [172] and *in vitro* [173] administration propionate leads to accumulation of lactate and pyruvate. Isotopically rked propionate fed to animals [174] or added to liver slices [173, 175] gives to labelled glucose units in glycogen. Three hypotheses have been anced to explain this utilisation of propionate: (1) it is directly carb-lated in the β-position, to give succinate; (2) it undergoes β-oxidation, ming malonic semialdehyde and malonate; and (3) it undergoes α-oxid-n, giving acrylate and then lactate and pyruvate.

Distribution of isotope in glucose units of rat-liver glycogen derived *in* [174, 176] and *in vitro* [175] from propionate differs from that from lactate. example, with $CH_3{\cdot}{*}CH(OH){\cdot}CO_2^-$, labelling of $C_{(1)}$ equals that of $C_{(6)}$, of $C_{(2)}$ equals that of $C_{(5)}$, and the quotient $(*C_{(2)} + *C_{(5)})/(*C_{(1)} + *C_{(6)}) =$ whereas with $CH_3{\cdot}{*}CH_2{\cdot}CO_2^-$ the quotient is 1·1. Thus, whilst some lomisation of label, attributed to partial equilibration with symmetrical arboxylic acids of the tricarboxylic acid cycle, accompanies formation of ose from lactate, it is much greater when glucose is formed from pro-nate. Again, lactate formed from $*CH_3{\cdot}CH_2{\cdot}CO_2^-$ by liver slices shows al amounts of *C in positions 2 and 3.[173b] These observations imply mmetrical intermediate between propionate and lactate. The distri-ion of isotopic carbon in glucose formed from propionate rules out malon-as intermediate [174] and therefore restricts consideration to pathways (1) (3). The former obligatorily provides a symmetrical intermediate; latter would not seem to without further assumptions (see below).

Lardy [177] has shown that $*CO_2$ can be fixed into propionate to form inate anaerobically by extracts of acetone-dried mitochondria supple-ted with ATP. If propionate were to yield pyruvate (pathway 3), lled succinate might arise through the sequence pyruvate ⟶ oxalo-

[169] J. Schubert and W. D. Armstrong, *Science*, 1948, **108**, 286.
[170] (*a*) A. I. Ringer, *J. Biol. Chem.*, 1912, **12**, 511; (*b*) H. J. Deuel, J. S. Butts, Hallman, and C. H. Cutler, *ibid.*, 1935, **112**, 15.
[171] (*a*) M. Jowett and J. H. Quastel, *Biochem. J.*, 1935, **29**, 2159; (*b*) E. M. MacKay, Wick, and C. P. Barnum, *J. Biol. Chem.*, 1940, **136**, 503.
[172] L. Blum and P. Woringer, *Bull. Soc. Chim. biol.*, 1920, **2**, 88.
[173] (*a*) A. Hahn and W. Haarmann, *Z. Biol.*, 1930, **90**, 231; (*b*) L. Daus, M. Meinke, M. Calvin, *J. Biol. Chem.*, 1952, **196**, 77.
[174] V. Lorber, N. Lifson, W. Sakami, and H. G. Wood, *ibid.*, 1950, **183**, 531.
[175] W. W. Shreeve, *ibid.*, 1952, **195**, 1.
[176] V. Lorber, N. Lifson, H. G. Wood, W. Sakami, and W. W. Shreeve, *ibid.*, 1950, 517.
[177] (*a*) H. A. Lardy and R. Peanasky, *Physiol. Reviews*, 1953, **33**, 560; (*b*) H. A. y, *Proc. Nat. Acad. Sci.*, 1952, **38**, 1003; H. A. Lardy and J. Fischer, Abstr. 123rd ing, Amer. Chem. Soc., 1953, p. 10c.

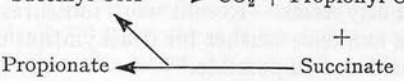
acetate ⟶ malate ⟶ fumarate ⟶ succinate; but this must be excluded since no *C appears in malate or fumarate. It could not arise through the sequence: [178] 4 Pyruvate + $^{14}CO_2$ ⟶ 2 Citrate ⟶ α-Hydroxyglutarate + [1-^{14}C]succinate, since CO_2-carbon would not appear in succinate. Participation of the symmetrical succinate explains (i) the isotope distribution data of the previous paragraph, (ii) the even distribution of ^{14}C in trapped acetate derived from $CH_3{\cdot}^{14}CH_2{\cdot}CO_2^-$ in rat-liver slices [175] (*via* succinate ⟶ pyruvate ⟶ acetyl-coenzyme A), and (iii) the failure to find deuterium in acetate derived from deuterated propionate in the rat,[179] since the deuterium of the succinate will be lost on the way through fumarate ⟶ malate ⟶ oxaloacetate ⟶ pyruvate.

The pathway proposed by Green's associates,[180a] propionate ⟶ acrylate ⟶ L-lactate ⟶ D-lactate ⟶ pyruvate, conforms with their concept [180] that L-lactate is converted into pyruvate by liver mitochondria only after optical inversion by a soluble "racemase." (The latter depends on oxidative phosphorylation.) To reconcile their mechanism with the isotopic findings cited above, they suggest an unusual rearrangement of the lactate ion, involving a symmetrical intermediate, under the action of the "racemase." Their main evidence for the α-oxidation pathway is: (*a*) Carbon from labelled propionate appeared in lactate and pyruvate.[181] However, the interconvertability of succinate and pyruvate makes this equally consistent with pathway (1). (*b*) To obtain propionate oxidation by cyclophorase preparations, a soluble liver-fraction had to be added, the function of which was ascribed to its "racemase" activity [180b] (similar activation has, however, been obtained with excess of succinate [182]). If "racemase" is necessary, the propionate oxidation path must pass through lactate. However, there is no evidence that the factor in the soluble fraction is "racemase." The factor is possibly part of the system for propionate–succinate conversion; it may well have been washed out of the cyclophorase particles but retained in Lardy's acetone-dried mitochondria. It is noteworthy that propionate–succinate conversion is greatly reduced in Lardy's preparations when made from biotin-deficient rats, but is restored by addition of "protein fractions from normal animals." [177a]

Whilst there is as yet no need to recognise the α-oxidation pathway for propionate in animals, it seems that in a micro-organism, *Clostridium propionicum*, this path may be traversed in reverse.[183] Thus acrylate yields propionate, and lactate is converted into propionate without shift of isotopic carbon from the α- to the β-position [184] and without carbon dioxide fixation.[185] In other bacteria, however, carbon dioxide is fixed into propionate to form succinate,[186] and acrylate is not metabolised.[187]

[178] C. Martius, in "Symposium sur le Cycle Tricarboxylique," IInd Int. Congr. Biochem., Sedes, Paris, 1952, p. 28.
[179] K. Bloch and D. Rittenberg, *J. Biol. Chem.*, 1944, **155**, 243.
[180] F. M. Huennekens, H. R. Mahler, and J. Nordmann, *Arch. Biochem.*, 1951, **30**, (*a*) 66, (*b*) 77. [181] H. R. Mahler and F. M. Huennekens, *Biochim. Biophys. Acta*, 1953, **11**, 575.
[182] K. Lang and K. H. Bässler, *Biochem. Z.*, 1953, **324**, 401.
[183] B. P. Cardon and H. A. Barker, *Arch. Biochem.*, 1947, **12**, 165.
[184] F. W. Leaver, *Fed. Proc.*, 1953, **12**, 471.
[185] A. T. Johns, *J. Gen. Microbiol.*, 1952, **6**, 123.
[186] (*a*) E. A. Delwiche, E. F. Phares, and S. F. Carson, *Fed. Proc.*, 1953, **12**, 194; (*b*) H. Larsen, *J. Biol. Chem.*, 1951, **193**, 167; S. Barban and S. Ajl, *ibid.*, 1951, **192**, 63.
[187] H. A. Barker and F. Lipmann, *Arch. Biochem.*, 1944, **4**, 361.

Studies with cell-free bacterial preparations have thrown light on the chanism of propionate–succinate interconversion. Whiteley [188] has nd that, with extracts of *Micrococcus lactilyticus* depleted of coenzyme A d ATP, addition of these substances in "catalytic" amounts evokes arboxylation of succinate. He gives evidence for the mechanism:



d for priming of the system by initial generation of succinyl-coenzyme A m succinate, ATP, and coenzyme A. With *Propionibacterium pentosum* extracts, Delwiche, Phares, and Carson [186a] found that coenzyme A d ATP are needed for succinate decarboxylation, but apparently only nzyme A is needed for carboxylation of propionate. CO_2-carbon was rporated into excess of unlabelled succinate much more slowly than was pionate-carbon. They infer that a propionate derivative combines with ne one-C derivative (possibly of coenzyme A [189]), obtained from succinate, preference to carbon dioxide. At present, however, the evidence does not m to demand any such derivative. If compounds A and B react under influence of enzyme E to form AB, so that, for instance, $E + A + B \rightleftharpoons E\text{-}B \rightleftharpoons E\text{-}AB \rightleftharpoons E + AB$, and if A leaves and returns to E more idly than does B, then unlabelled AB takes up from solution labelled A re rapidly than labelled B (cf. ref. 131).

Other Odd-carbon Fatty Acids.—These are metabolised,[21, 190, 191] and to mall extent deposited in the fat depots,[192] with some 9 : 10-desaturation.[193] d-C acids (C_5—C_{13}) are ketogenic both *in vitro* [171a, 194] and *in vivo*,[171b] ugh less so than even-C acids; addition of malonate greatly increases the d of acetoacetate from liver slices metabolising odd-C acids (C_5—C_9).[123] d-C acids (C_3—C_{11}), unlike even-C acids, are glycogenic as well.[170] –C_{17} acids are as rapidly oxidised as even-C acids by liver particulate parations, but the yield of acetoacetate is smaller.[7c] Kidney cyclo-rase preparations, oxidising odd-C acids, consumed oxygen in amount icating the final formation of carbon dioxide, water, and propionate.[10a] rmation of propionate (and, probably, traces of acetate) from *n*-valerate der these conditions was established by counter-current distribution.[195] ce accumulation of acetoacetate,[171b, 7c] enhanced by malonate,[123] implies idation with liberation of acetyl-coenzyme A, odd-C acids are probably rtened by β-oxidation two carbons at a time until a 3C-unit remains ch behaves as propionate (see above). The subsequent formation of icarboxylic acid cycle intermediate which catalyses oxidation of acetyl-

[188] H. R. Whiteley, *Proc. Nat. Acad. Sci.*, 1953, **39**, 772, 779.
[189] H. G. Wood and F. W. Leaver, *Biochim. Biophys. Acta*, 1953, **12**, 207.
[190] F. Knoop, *Beitr. Chem. Physiol. Path.*, 1905, **6**, 150.
[191] (*a*) W. Keil, H. Appel, and G. Berger, *Z. physiol. Chem.*, 1939, **257**, 1; (*b*) H. el, G. Berger, H. Böhm, W. Keil, and G. Schiller, *ibid.*, 1940, **266**, 158; (*c*) R. Emm- and E. Nebe, *ibid.*, p. 174.
[192] K. Thomas and G. Weitzel, in "FIAT Review of German Science, 1939—1946," hemistry, Part I, Office of Military Government for Germany Field Information ncies, Technical, Wiesbaden, 1947, p. 1.
[193] H. Appel, H. Böhm, W. Keil, and G. Schiller, *Z. physiol. Chem.*, 1947, **282**, 220.
[194] R. P. Geyer, M. Cunningham, and J. Pendergast, *J. Biol. Chem.*, 1950, **185**, 461.
[195] W. A. Atchley, *ibid.*, 1948, **176**, 123.

coenzyme A explains the low yield of ketone bodies. Since the ultimate fragment contains three rather than two carbon atoms, the low $*CO/*CO_2H$ quotient in the " acetoacetate " analysed after *n*-valerate catabolism [194, 161] is not explained by current hypotheses (p. 315). If $CH_3{\cdot}CH_2{\cdot}CO{\cdot}CH_2{\cdot}*CO_2H$ were to accumulate, however, it would yield a low $*CO/*CO_2H$ quotient by the analytical method used. Similar considerations apply to higher odd-C acids.

Shorter Branched Fatty Acids.—Recent work indicates that these undergo β-oxidation, and gives evidence neither for dealkylation nor for α- or γ-oxidation at one time considered as possible.[196]

*iso*Butyric acid arises from *iso*-acids with an odd number of carbon atoms in the straight chain, and from valine by oxidative decarboxylation of its keto-analogue.[197] Atchley [195] showed by counter-current methods that kidney " cyclophorase " preparations (which unsupplemented failed to oxidise propionate) oxidised 4-methylpentanoate to *iso*butyrate. Also *iso*butyrate gave propionate, probably by β-oxidation of one methyl group to the aldehyde level, followed by decarboxylation and completion of the oxidation. This is supported by the observation that [2-^{14}C]valine yields $^{14}CO_2$ in kidney homogenates, whilst ^{14}C from [4 : 4′-$^{14}C_2$]valine appears in lactate, equally distributed between the methyl and the carboxyl group, in *iso*butyrate and propionate, but does not appear in carbon dioxide.[198] Isotope distribution in the glucose units of glycogen from rats given $C_{(3)}$-labelled valine was almost identical with that after administration of $CH_3{\cdot}*CH_2{\cdot}CO_2^-$.[199] Hence odd-C fatty *iso*-acids are converted by β-oxidation into *iso*butyrate, and then by β-oxidation and decarboxylation into propionate.

α-Methylbutyric acid also appears to be attacked by β-oxidation. Administration of α-methyl-γ-phenylbutyrate to dogs leads to excretion of phenylacetate; [200] and Carter [201] believed that β-oxidation occurs along the longer chain, resulting in a 3C-fragment and a straight-chain fatty acid. This is supported by liver-slice experiments [163] with α-methyl-[*carboxy*-^{14}C]- and -[γ-^{14}C]-butyrate; the carboxyl group contributed virtually no carbon to the acetoacetate formed, while the [3-^{14}C]-compound yielded acetoacetate with $*CO/*CO_2H$ about 1. These findings suggest that $C_{(1)}$, $C_{(2)}$, and a methyl group separate as one unit which is metabolised without direct formation of acetyl-coenzyme A, while $C_{(3)}$ and $C_{(4)}$ form acetyl-coenzyme A and hence acetoacetate. Since *iso*leucine, which is metabolised to α-methylbutyrate, is slightly glycogenic,[202] the 3C-fragment probably forms pyruvate, possibly through propionate.

*iso*Valeric acid is apparently an intermediate in leucine catabolism.[197] Leucine and *iso*valerate are ketogenic *in vivo* [202, 203] and *in vitro*.[116b, 204]

[196] K. Lang and F. Adickes, *Z. physiol. Chem.*, 1940, **263**, 227.
[197] S. J. Bach, " The Metabolism of Protein Constituents in the Mammalian Body," Oxford, Univ. Press, 1952, Chap. IV.
[198] D. Kinnory and D. M. Greenberg, *Fed. Proc.*, 1953, **12**, 230.
[199] E. A. Peterson, W. S. Fones, and J. White, *Arch. Biochem. Biophys.*, 1952, **36**, 323.
[200] H. D. Kay and H. S. Raper, *Biochem. J.*, 1924, **18**, 153.
[201] H. E. Carter, *Biol. Symp.*, 1941, **5**, 47.
[202] J. S. Butts, H. Blunden, and M. S. Dunn, *J. Biol. Chem.*, 1937, **120**, 289; L. C. Terriere and J. S. Butts, *ibid.*, 1951, **190**, 1.
[203] J. Baer and L. Blum, *Arch. exp. Path. Pharmak.*, 1906, **55**, 89; **56**, 92; A. N. Wick, *J. Biol. Chem.*, 1941, **141**, 897.
[204] G. Embden, H. Salomon, and F. Schmidt, *Beitr. Chem. Physiol. Path.*, 1906, **8**, 129; M. J. Coon and S. Gurin, *J. Biol. Chem.*, 1949, **180**, 1159.

liver slices, *iso*valerate undergoes β-oxidation yielding (*a*) a 2C-fragment[205] from $C_{(1)}$ and $C_{(2)}$ which can form acetoacetate[206] or, presumably, oxidised through the tricarboxylic acid cycle, and (*b*) an *iso*propyl moiety[207] which by fixation of carbon dioxide yields acetoacetate,[206, 168b] $C_{(4)}$ and $C_{(4')}$ (the Me-carbon atoms) giving rise to the α- and the γ-carbon atom of acetoacetate and $C_{(3)}$ to its carbonyl group. Leucine seems to be non-ketogenic.[208, 202] The above mechanism conforms with this, unless acetone can yield carbohydrate,[209] since there is no proved route by which 2C-units at the level of oxidation of acetic acid can give a net yield of glycogen.

β-Ethylbutyric acid is also ketogenic, but is not analogous to the methyl derivative since it yields acetone and not ethyl methyl ketone.[196]

Higher Branched-chain and Dicarboxylic Acids.—There has been much work of a more physiological nature on these because of their importance in synthetic fat manufacture. In general the acid has been fed as ester or glyceride, and metabolic products isolated from the urine. Interpretation of results is rendered difficult by uncertainty regarding the degree of absorption, ease of penetration to sites of metabolic activity, extent of storage in depots and liver, and existence of excretion routes other than urine. The finding[210] that the amount of ω-oxidation can depend on the glycogen reserves indicates a further uncertainty. With these reservations the following generalisations appear to be consistent with the literature examined.

(1) 2-, 3-, and 4-Methyl fatty acids are well utilised.[192, 201, 211, 212] When there is a methyl group on an even-numbered carbon atom of the chain β-oxidation probably occurs, with separation of a 3C-fragment. The fate of acids with a methyl group on an odd-numbered carbon atom is less clear, except in the case of *iso*valerate (see above). Acids with alkyl branches larger than methyl are less well utilised. When the main chain is from C_3—C_7, branched-chain acids are largely excreted in the urine unchanged; when the main chain is from C_7 to C_{12} the branch (Et, Pr, or Bu) prevents complete β-oxidation, although the chain may be shortened by one or two 2C-units before excretion.[211, 213] With still longer 2-alkyl fatty acids (C_{14} and C_{18}) utilisation appears to be much better, less than 10% of the absorbed acid appearing in the urine as unchanged or ω-oxidised material; β-oxidation possibly occurs with separation of one short- and one long-chain normal fatty acid.[212]

(2) Of the unbranched dicarboxylic acids, succinic and glutaric appear to be well utilised,[215] and adipic, pimelic, suberic, azelaic, and sebacic to be excreted unchanged; the higher acids are sometimes shortened by one or

[205] K. Bloch, *J. Biol. Chem.*, 1944, **155**, 255. [206] M. J. Coon, *ibid.*, 1950, **187**, 71.
[207] I. Zabin and K. Bloch, *Fed. Proc.*, 1949, **8**, 267.
[208] O. Simon, *Z. physiol. Chem.*, 1902, **35**, 315; J. T. Halsey, *Amer. J. Physiol.*, 1904, **10**, 229.
[209] H. G. Wood, in "Isotopes in Biochemistry" (Ciba Foundation Conference), Churchill, London, 1951, p. 227.
[210] P. E. Verkade, J. van der Lee, and M. Elzas, *Biochim. Biophys. Acta*, 1948, **2**, 38.
[211] W. Keil, *Z. physiol. Chem.*, 1942, **274**, 175. [212] G. Weitzel, *ibid.*, 1951, **287**, 254.
[213] W. Keil, *ibid.*, 1942, **276**, 26; 1947, **282**, 137.
[214] R. Emmrich, *ibid.*, 1939, **261**, 61; R. Emmrich, P. Neumann, and I. Emmrich-Esser, *ibid.*, 1941, **267**, 228; K. Bernhard, *ibid.*, **269**, 135; K. Thomas, G. Weitzel, and P. Neumann, *ibid.*, 1947, **282**, 192; H. Bodur, *ibid.*, p. 206; G. Weitzel, H. Queckenstedt, H. Grellmann, and H. Lautner, *ibid.*, 1950, **285**, 58; K. Bernhard and H. Lincke, *Helv. Chim. Acta*, 1946, **29**, 1457.
[215] K. Thomas and G. Weitzel, *Deut. med. Woch.*, 1946, **71**, 18.

two 2C-units, presumably by β-oxidation.[192, 216] Blocking one carboxyl group by ester, amide, or substituted amide formation prevents appearance in the urine of the administered acid, but its fate is unknown.[217]

(3) Acids obtained by alkylation of malonic, succinic, glutaric, and adipic acids are not extensively utilised, and if the alkyl substituent is less than six carbon atoms in length they appear in the urine in large quantities. With longer alkyl substituents, excretion of the acids in the urine diminishes or even ceases, and they are probably utilised.[214]

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5. CONSTITUENTS OF THE MARINE ALGÆ.

Although occasional reference has been made in these Reports to constituents of marine algæ, no collective review has appeared previously. This summary attempts, therefore, to emphasise modern work on the main constituents of the brown, red, and green algæ. As it has not been possible to consult many Japanese journals, and as work carried out in Japan during the last ten years is only now appearing in *Chemical Abstracts*, this Report cannot be regarded as complete. It is hoped, however, that T. Mori will bring the Japanese literature up to date in his forthcoming review on seaweed polysaccharides.[1]

Carbohydrates of Brown Marine Algæ (Phæophyceæ).—In recent years considerable work has been carried out in Canada,[2] Japan,[3] and Britain[4] on factors affecting the chemical composition of the brown algæ. The chemical composition depends on species, season of the year, habitat, depth at which the alga grows, and stage of development.[4] Concentration gradients are found in both the Fucaceæ [5] and the Laminariaceæ.[6] Furthermore, a correlation exists between the chemical composition of the algæ and the composition of the sea-water in which they grow.[7] The work [4, 6, 7] has required methods of analysis for mannitol, alginic acid, laminarin, and combined fucose,[8] and improved methods for alginic acid [9] and combined L-fucose.[10]

Mannitol. This is present in solution in the cell sap and increases from about 5% of dry Laminariaceæ fronds in spring to over 35% in late summer,[4] and shows considerable variation with depth of growth [4] and also along the frond of the same plant.[6] Laboratory-scale isolations have been worked out aiming at ultimate large-scale production.[11] These comprise solvent

[216] R. Emmrich and I. Emmrich-Glaser, *Z. physiol. Chem.*, 1940, **266**, 183; K. Bernhard, *Helv. Chim. Acta*, 1941, **24**, 1412.
[217] B. Flaschenträger, *Z. physiol. Chem.*, 1926, **159**, 297; B. Flaschenträger and K. Bernhard, *ibid.*, 1936, **240**, 19; K. Bernhard, *ibid.*, 1937, **246**, 133; R. Kuhn and I. Löw, *ibid.*, 1939, **259**, 182.
[1] T. Mori, *Adv. Carbohydrate Chem.*, 1954, **8**, in the press.
[2] M. G. Macpherson and E. G. Young, *Canad. J. Bot.*, 1952, **30**, 67.
[3] N. Suzuki, *Bull. Fac. Fish. (Hokkaido Univ. Japan)*, 1953, **3**, 68.
[4] W. A. P. Black, *Nature*, 1948, **161**, 174; *J. Soc. Chem. Ind.*, 1948, **67**, 165, 355; 1949, **68**, 183; 1950, **69**, 161; *J. Marine Biol. Assoc.*, 1950, **29**, 45.
[5] B. L. Moss, *Ann. Bot.*, 1950, **14**, 395.
[6] W. A. P. Black, *J. Marine Biol. Assoc.*, 1954, **33**, 49.
[7] W. A. P. Black and E. T. Dewar, *ibid.*, 1949, **27**, 673.
[8] M. C. Cameron, A. G. Ross, and E. G. V. Percival, *J. Soc. Chem. Ind.*, 1948, **67**, 161.
[9] E. G. V. Percival and A. G. Ross, *ibid.*, p. 420.
[10] W. A. P. Black, W. J. Cornhill, E. T. Dewar, E. G. V. Percival, and A. G. Ross, *ibid.*, 1950, **69**, 317.
[11] W. A. P. Black, E. T. Dewar, and F. N. Woodward, *J. Appl. Chem.*, 1951, **1**, 414.

extraction of dried algæ and complete evaporation of dilute acid extracts of fresh or dried algæ, followed by isolation of mannitol by (*a*) solvent-extraction, (*b*) precipitation as the water-insoluble tri*iso*propylidene or diethylidene derivative, or (*c*) the use of ion-exchange resins. Recent chromatographic work on extracts of *F. vesiculosus* has shown the presence, in small amounts, of D-mannitol 1-acetate, 1-(β-D-glucopyranoside), and 1:6-di-(β-D-glucopyranoside).[12]

Laminarin. Parke [13] has shown that there are two periods of growth in the sea. The period of slow growth coincides with deficiency of nitrate and phosphate in our waters [7] when laminarin accumulates to form as much as 36% of the dry frond of *L. cloustoni*. Laminarin [14, 15] appears to be a reserve carbohydrate of the brown algæ; it is, however, absent from the stipe of the Laminariaceæ at all times of the year and from the actively growing section of the frond proximal to the stipe, but makes up 32% of the dry matter of distal sections.[6] It exists in two forms; *L. cloustoni* frond and, to a lesser extent, *L. saccharina* frond, give the so-called insoluble laminarin (insoluble in cold water but readily soluble in hot water), while *L. digitata* frond gives the form soluble in cold water. Both modifications have recently been studied,[16, 17] but no fundamental chemical differences have been observed between them. The general structure is a chain of about twenty 1 : 3-linked β-D-glucopyranose units. During the present year, however, evidence for the presence of branch linkages in laminarin has been provided [18] by the isolation, after column-chromatography, of small quantities of gentiobiose and ββ-trehalose from the partial hydrolysis of insoluble laminarin. The breakdown of laminarin with lime-water also favours a branched structure; [19] if laminarin is composed exclusively of an unbranched chain of 1 : 3-β-linked glucose units it would be completely broken down from end to end whereas lime-water treatment resulted in only 50% cleavage.

"Laminaritol" has been prepared by reducing laminarin with sodium borohydride; [20] recent *X*-ray studies have shown that the two forms of laminarin, when prepared under identical conditions, give similar diagrams [21] which are, however, different from that of the paramylon of *Euglena*.[22]

Methods for isolating and purifying laminarin from brown marine algæ have been described [23] and the quantitative production of D-glucose from laminarin has recently been studied.[24] From an autoclaved solution of the polysaccharide (18% w/w) in 0·05N-hydrochloric acid (at 135° for one hour) D-glucose monohydrate is obtained crystalline by a process similar to its commercial production from starch.

[12] B. Lindberg, *Acta Chem. Scand.*, 1953, **7**, 1119.
[13] M. Parke, *J. Marine Biol. Assoc.*, 1948, **27**, 651.
[14] H. N. Rydon, *Ann. Reports*, 1950, **47**, 247; 1951, **48**, 237.
[15] E. J. Bourne, *ibid.*, 1952, **49**, 242.
[16] J. J. Connell, E. L. Hirst, and E. G. V. Percival, *J.*, 1950, 3494.
[17] E. G. V. Percival and A. G. Ross, *J.*, 1951, 720.
[18] S. Peat, W. J. Whelan, and H. G. Lawley, *Biochem. J.*, 1953, **54**, xxxiii.
[19] W. M. Corbett, J. Kenner, and G. N. Richards, *Chem. and Ind.*, 1953, 462.
[20] M. Abdel-Akher, J. K. Hamilton, and F. Smith, *J. Amer. Chem. Soc.*, 1951, **73**, 4691. [21] E. Nicolai and R. D. Preston, unpublished work.
[22] D. R. Kreger and B. J. D. Meeuse, *Biochim. Biophys. Acta*, 1952, **9**, 699.
[23] W. A. P. Black, W. J. Cornhill, E. T. Dewar, and F. N. Woodward, *J. Appl. Chem.*, 1951, **1**, 505.
[24] W. A. P. Black, E. T. Dewar, and F. N. Woodward, *J. Sci. Food Agric.*, 1953, **4**, 58.

Fucoidin. Brief mention has been made in a previous Report [14] of fucoidin. This polysaccharide sulphate is believed to occur in the intercellular mucilage of the brown algæ and to undergo marked seasonal fluctuation which varies with the depth of immersion of the alga.[25] Thus, in more tidally exposed algæ like *Pelvetia canaliculata* it makes up over 20% of the dry matter. Its function, in view of its hygroscopic nature, may be to prevent desiccation on prolonged exposure. In the permanently submerged Laminariaceæ, on the other hand, it makes up only about 5%. The isolation and purification of fucoidin have been studied by (the late) E. G. V. Percival and A. G. Ross,[26] who consider that its repeating unit is derived from a calcium fucose monosulphate $(C_6H_9O_4,SO_3Ca_{0\cdot5})_n$. Hydrolysis of methylated fucoidin (*F. vesiculosus*) gave L-fucose, 3-*O*-methyl-L-fucose, and 2 : 3-di-*O*-methyl-L-fucose, roughly in the proportions 1 : 3 : 1.[27] The predominant radical in fucoidin was, therefore, believed to be a 1 : 2-α-fucopyranose unit sulphated on $C_{(4)}$. Two theories were advanced to account for the free fucose and the 2 : 3-di-*O*-methyl-L-fucose residues in methylated fucoidin : (1) some fucose residues might carry two sulphate groups, whereas others (linked 1 : 4) are unsubstitituted, or (2) the free fucose might originate from branching points at $C_{(3)}$, and the dimethyl derivative from terminal groups having free hydroxyl groups on $C_{(2)}$ and $C_{(3)}$.

Larger-scale isolations of fucoidin,[28] involving treatment of the algæ with hydrochloric acid at 70° for one hour at pH 2·0—2·5, fractional precipitation with alcohol, and final purification with formaldehyde, have been devised. Laboratory-scale preparations of L-fucose from dried milled algæ have been studied [29] through the following stages : (1) acid hydrolysis; (2) formation of fucose phenylhydrazone; and (3) decomposition of the phenylhydrazone. Isolation of L-fucose from fucoidin [29] involves heating a 16% (w/w) solution of the polysaccharide in 0·25N-hydrochloric acid at 135° for 2 hours, treatment with ion-exchange resins, purification with ethanol and charcoal, and direct crystallisation of the fucose from ethanol. Fucoidin from *Pelvetia canaliculata* gave crystalline fucose in 56·6% yield and of 95·1% purity.

From the " seed mucilage " of *Ascophyllum nodosum* a polysaccharide resembling fucoidin has recently been isolated [30] as the salt of a polysaccharide sulphuric ester consisting of fucose and galactose units in the ratio of about 8 : 1, with a sulphate hexose ratio of 1 : 1.

Alginic acid. Alginic acid, which with cellulose makes up the cell wall of brown algæ, was recently reviewed in an investigation to evaluate the common brown algæ as a source of alginate.[31] Viscosities of 0·25% sodium alginate in 0·1N-sodium chloride at 25° were determined for *Pelvetia canaliculata, F. spiralis, F. vesiculosus, F. serratus, Ascophyllum nodosum, Laminaria cloustoni, L. saccharina,* and *L. digitata.* Factors affecting the grade of the alginic acid were also studied. Recent work has confirmed the main structural feature of the molecule as repeating 1 : 4-linked β-D-mannuronic

[25] W. A. P. Black, unpublished work.
[26] E. G. V. Percival and A. G. Ross, *J.*, 1950, 717.
[27] J. Conchie and E. G. V. Percival, *J.*, 1950, 827.
[28] W. A. P. Black, E. T. Dewar, and F. N. Woodward, *J. Sci. Food Agric.*, 1952, **3**, 122.
[29] W. A. P. Black, W. J. Cornhill, E. T. Dewar, and F. N. Woodward, *ibid.*, 1953, **4**, 85.
[30] T. Dillon, K. Kristensen, and C. O hEochdha, *Proc. Roy. Irish Acad.*, 1953, **55**, *B*, 189.
[31] W. A. P. Black, W. J. Cornhill, and E. T. Dewar, *J. Sci. Food Agric.*, 1952, **3**, 542.

acid radicals; the chain length of the alginic acid used was about 100.[32] The methylated alginic acid was hydrolysed (by formic acid), the products were esterified by methanol, and the resulting esters were reduced to the corresponding methyl mannosides by lithium aluminium hydride. The methylated mannoses, formed after aqueous-acid hydrolysis, were separated chromatographically; 2 : 3-di-*O*-methylmannose was found to be the main component with traces of 2 : 3 : 4-tri-*O*-methyl- monomethyl-mannose, and dimethyl-glucose. It is not known whether the glucose forms part of the structure or is present as an impurity.

Mention may be made of Japanese chemical studies on sodium alginate as a blood-plasma substitute;[33,34] relations between the degree of polymerisation, viscosity, concentration, and colloidal osmotic pressure in 0·9% saline solution, and the permeability of blood vessel walls, have been determined. The mutual coagulation of sodium alginate with protein and its agglomerating action on red cells have been studied, while a new method has been worked out for its determination in serum and urine.[33] In America, alginic acid sulphate and its salts have been tested as blood anti-coagulants.[35]

Cellulose. Cellulose isolated from various species of marine algæ has been shown to be essentially the same as cotton cellulose.[36] Hydrolysis with 72% sulphuric acid gave only D-glucose, and cellobiose octa-acetate has been prepared by acetolysis, indicating the presence of 1 : 4-β-linkages. *X*-Ray diagrams of algal cellulose have the characteristic pattern of normal cellulose.[37]

In brown marine algæ, a marked seasonal variation in the cellulose content is found in the common Laminariaceæ and Fucaceæ [38] which may contain, *e.g.*, less than 1% of cellulose (in *Ascophyllum nodosum*) to 10% in *L. cloustoni* stipe (dry basis)].

Kylin has reviewed the biochemistry of the Phæophyceæ.[39]

Carbohydrates of Red Marine Algæ (Rhodophyceæ).—Although mention has frequently been made in these Reports of constituents of the Rhodophyceæ, especially agar [40,41,42] and carragheenin,[43,44,45] the chemistry of the red marine algæ has not been so thoroughly investigated as that of the brown algæ. Sufficient is known, however, to show that little similarity exists between the two groups. Ross [46] recently analysed 26 species of red algæ for ash, total sulphate, nitrogen, carbon tetrachloride- and ethanol-soluble material, uronic acid, cellulose, and total reducing sugars after hydrolysis; the various sugars obtained were identified on the paper chromatogram. In the majority of the species, galactose was the predominant sugar with small amounts of glucose, xylose, mannose, and fucose; in *Rhodymenia palmata* and *Rhodochorton floridulum* the main sugar was xylose, while *Dilsea edulis* had a comparatively high glucose content. Galactose in the red algæ is

[32] S. K. Chanda, E. L. Hirst, E. G. V. Percival, and A. G. Ross, *J.*, 1952, 1833.
[33] K. Inokuchi, *Mem. Fac. Sci. Kyushu Univ.*, 1950, Ser. C, Chem. 1, 109.
[34] H. Matsubayashi, *Igaku Kenkyuu*, 1953, **23**, 187.
[35] H. E. Alburn, U.S.P. 2,612,498/1952; 2,638,469/1953.
[36] E. G. V. Percival and A. G. Ross, *J.*, 1949, 3041. [37] *Idem*, *Nature*, 1948, **162**, 895.
[38] W. A. P. Black, *J. Marine Biol. Assoc.*, 1950, **29**, 379.
[39] H. Kylin, *Kgl. fysiogr. Sällsk. Lund Forh.*, 1944, **14**, 226.
[40] E. L. Hirst and S. Peat, *Ann. Reports*, 1936, **33**, 251.
[41] S. Peat, *ibid.*, 1939, **36**, 269. [42] *Idem*, *ibid.*, 1941, **38**, 153.
[43] F. W. Norris, *ibid.*, 1940, **37**, 425. [44] J. K. N. Jones, *ibid.*, 1946, **43**, 196.
[45] H. N. Rydon, *ibid.*, 1950, **47**, 247. [46] A. G. Ross, *J. Sci. Food Agric.*, 1953, **4**, 333.

present chiefly as a galactan or galactan sulphuric ester, such as agar or carragheenin; xylose in *Rhodymenia palmata* is present as a xylan, while most of the glucose obtained on mild hydrolysis is considered to be derived from Floridean starch. These constituents will be discussed below.

Hexitols and simple glycosides. D-Mannitol, the well-known hexitol of the brown seaweeds, has not been detected in the red algæ, but dulcitol (galactitol) and sorbitol (D-glucitol) have been isolated from *Bostrychia scorpioides*,[47] while dulcitol has been shown to be present in *Iridaea laminarioides*.[48] Nothing is known of the quantities present, or whether they undergo seasonal variation; their presence in species common to Great Britain, such as *Gigartina stellata* or *Rhodymenia palmata*, has not been proved.

The glycoside "floridoside" was discovered in *R. palmata* by Kylin,[49] who concluded it was trehalose; this was later disproved.[50] *R. palmata* is reported [51] to contain 15% of floridoside, and its presence has been demonstrated in 19 species of red algæ.[52] Colin [53] considered this substance to be glycerol 2-(α-D-galactoside). *Polysiphonia fastigiata* and *P. fructiculosa* have been shown to contain sodium L-glycerate α-D-mannoside.[54]

Floridean starch. Granular material which gives a colour with iodine has been observed histologically in various red algæ (for references to 1937 and 1945 respectively, see Kylin [55] and Fritsch [56]). The iodine colour varies from deep violet to brown. In the isolation of Floridean starch from *Dilsea edulis*,[57] the weed is first extracted with cold dilute hydrochloric acid to remove the galactan sulphuric ester, and the Floridean starch is then dissolved with boiling water and precipitated by alcohol. The purified product, $[\alpha]_D^{15}$ +156° (in water), gave D-glucose (96%) on hydrolysis and resisted attack by crystalline β-amylase. Oxidation with periodate showed that it is structurally different from normal starches and glycogens in that it contains a large proportion of 1 : 3- as well as 1 : 4-linked units. During the past year, Floridean starch from *Dilsea edulis* has been further investigated; [58] extracted as before [57] and treated with ion-exchange resins, it could not, however, be freed completely from galactan sulphate. The specific rotation (+166° in water) indicated a predominance of α-links, and hydrolysis with a wheat β-amylase extract gave a 50% yield of maltose (cf. ref. 58). The polysaccharide was acetylated and methylated, but the methoxyl content could not be raised above 28·2%. Fractionation of the hydrolysed ether on a hydrocellulose column gave 2 : 3 : 6-tri-*O*-methyl- (42%) and 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose (3·3%), thereby proving that about 50% of the molecule consists of 1 : 4-linked α-D-glucopyranose units.

[47] P. Haas and T. G. Hill, *Biochem. J.*, 1931, **25**, 1470; 1932, **26**, 987.
[48] W. Z. Hassid, *Plant Physiol.*, 1933, **8**, 480.
[49] H. Kylin, *Z. physiol. Chem.*, 1915, **94**, 337.
[50] H. Colin and E. Guéguen, *Compt. rend.*, 1930, **190**, 653; **191**, 163.
[51] H. Kylin, *Z. physiol. Chem.*, 1918, **101**, 236.
[52] H. Colin and E. Guéguen, *Compt. rend.*, 1933, **197**, 1688.
[53] H. Colin, *Bull. Soc. chim.*, 1937, **4**, 277.
[54] H. Colin and J. Augier, *Compt. rend.*, 1939, **208**, 1450.
[55] H. Kylin, Linsbauer "Handbuch der Pflanzenanat.," Vol. VI, 2B, Borntraeger, Berlin, 1937.
[56] F. E. Fritsch, "The Structure and Reproduction of the Algæ," Cambridge Univ. Press, 1945, Vol. II, p. 409.
[57] V. C. Barry, T. G. Halsall, E. L. Hirst, and J. K. N. Jones, *J.*, 1949, 1468.
[58] P. O'Colla, *Proc. Roy. Irish Acad.*, 1953, **55**, *B*, 321.

Agar. The production and uses of this important polysaccharide have been extensively reviewed in recent years.[59, 60, 61] Early work on the constitution of agar is amply covered by Tseng,[59] while elucidation of its structure by methylation is described in a number of excellent reviews.[62, 63, 64] The recent article by Araki [64] summarises the large amount of work done by the Japanese in this field. When agar was last reviewed in these Reports,[42] the constitution was represented by a chain of nine 1 : 3-β linked D-galactopyranose units, terminated by an L-galactopyranose 6-sulphate residue linked through $C_{(4)}$. During methylation, the terminal sulphate residue was believed to be split off with the formation of a 3 : 6-anhydro-ring, and this theory was put forward to account for the presence of 3 : 6-anhydro-L-galactose residues among the hydrolysis products of methylated agar.[65] This view on the structure of agar has been contested [66, 67] on the ground that the sulphur content of natural agar is too low to account for the yields of 3 : 6-anhydro-L-galactose derivatives isolated, although Percival [63] admitted that the 3 : 6-anhydro-ring could have been formed by cleavage of a sulphate group at some earlier stage in the elaboration of the polysaccharide.

Little work of a structural nature has been done on agar during the past ten years. A disaccharide, agarobiose, has been obtained by partial hydrolysis, and methanolysis yielded methyl α-D-galactopyranoside and 3 : 6-anhydro-L-galactose dimethyl acetal.[68] Agarobiose was shown [69] to be 4-O-[β-D-galactopyranosyl]-3 : 6-anhydro-L-galactose by methylation and hydrolysis, thus confirming the view [65, 70] that the 3 : 6-anhydro-L-galactose is attached to the chain through $C_{(4)}$.

Carragheenin. Carragheenin, first isolated by Schmidt,[71] is the dried aqueous extract obtained from *Chondrus crispus* or *Gigartina stellata*, and these two species, either individually or together, are known as "carragheen" or "Irish Moss." The production and uses of carragheenin ("British Agar") are similar to those of agar, and are reviewed by Tseng [59] and Marshall, Newton, and Orr.[60] Chemically, carragheenin is not identical with true agar, the most obvious difference being the very much higher organic sulphate content (25—35%). Early work on the constitution of carragheenin is summarised by Tseng,[59] and structural studies are reviewed by Jones and Smith [62] and Percival.[63] The main structural feature was shown to consist of a chain of 1 : 3-linked α-D-galactopyranose units with the sulphate group located on $C_{(4)}$.[72, 73] Isolation of 2 : 4 : 6-tri-*O*-methyl-

[59] C. K. Tseng, in "Colloid Chemistry," Reinhold Publ. Corp., New York, 1946, Vol. VI, p. 629.
[60] S. M. Marshall, L. Newton, and A. P. Orr, "A Study of Certain British Seaweeds and their Utilisation in the Preparation of Agar," H.M.S.O., 1949.
[61] H. J. Humm, in "Marine Products of Commerce," by D. K. Tressler and J. McW. Lemon, Reinhold Publ. Corp., New York, 1951, p. 47.
[62] J. K. N. Jones and F. Smith, *Adv. Carbohydrate Chem.*, 1949, **4**, 275.
[63] E. G. V. Percival, *Quart. Reviews*, 1949, **3**, 376.
[64] C. Araki, *Mem. Coll. Sci. Tech., Kyoto*, 1953, **2**, *B*, 17.
[65] W. G. M. Jones and S. Peat, *J.*, 1942, 225.
[66] V. C. Barry and T. Dillon, *Chem. and Ind.*, 1944, **63**, 167.
[67] E. G. V. Percival, *Nature*, 1944, **154**, 673.
[68] C. Araki, *J. Chem. Soc. Japan*, 1944, **65**, 533. [69] *Idem, ibid.*, p. 627.
[70] E. G. V. Percival and T. G. H. Thomson, *J.*, 1942, 750.
[71] C. Schmidt, *Ann. Chem. Pharm.*, 1844, **51**, 29.
[72] J. Buchanan, E. E. Percival, and E. G. V. Percival, *J.*, 1943, 51.
[73] E. T. Dewar and E. G. V. Percival, *J.*, 1947, 1622.

D-galactose from the hydrolysis of methylated, partially degraded, desulphated carragheenin has confirmed this view.[74, 75] Much still remains to be done, however, before the full constitution is settled, for the yield of galactose obtained on hydrolysis represents only about two-thirds of the organic matter present. The isolation of a crystalline derivative of 2-oxo-D-gluconic acid has been reported,[76] but it is by no means certain that this product is present as such in the original material.[63] The problem has been further complicated by the isolation of L-galactose derivatives from methylated carragheenin.[74]

The electrophoretic, sedimentation, and diffusion properties of carragheenin have recently been studied.[77] Four samples with different intrinsic viscosities were examined and the results showed that the molecular weight, axial ratio, and estimated diameter of the ellipsoid all increased with the intrinsic viscosity of the sample. All samples were polydisperse and two components were revealed by sedimentation, the amount of the more rapidly sedimenting minor component increasing with the viscosity from nil to about 12% of the most viscous sample. The mean values obtained were comparable with those reported for certain cellulose preparations, indicating that the major constituent has a linear structure. It is suggested that the minor component with the higher rate of sedimentation may have a different size or shape and may be branched.

Iridophycin. This name was suggested by Tseng [59] for the galactan sulphuric acid ester from *Iridaea laminarioides*.[78] From methylation studies[79] Hassid concluded that iridophycin consists of 1 : 4-linked β-D-galactopyranose units sulphated on $C_{(6)}$. Since all other galactan sulphates from red algæ have subsequently been found to be 1 : 3-linked, Hassid's evidence has been re-examined and re-interpreted by Jones and Smith.[62] Mori [80] has proved conclusively the presence of 1 : 3-linkages by isolating 2 : 4 : 6-tri-*O*-methyl-D-galactose from hydrolysed, methylated, desulphated iridophycin, and considers from triphenylmethylation experiments [81] that the sulphate group is attached to $C_{(6)}$ and that the glycosidic linkage is α.[82]

Other polysaccharides. A galactan sulphate, similar to carragheenin but with a much lower sulphate content, has been isolated [83] from *Dilsea edulis*. The methylated, partially degraded, sulphate-free mucilage on hydrolysis gave a high yield of 2 : 4 : 6-tri-*O*-methyl-D-galactose, thereby showing a preponderance of 1 : 3-linked galactose residues, as in carragheenin.[84] The sulphate group was assigned to $C_{(4)}$. The mucilage contained a uronic acid residue, and the ratio of galactose : sulphate : uronic acid was estimated to be 9 : 2 : 1.

A very similar mucilage, with galactose : sulphate : uronic acid in the ratio 9 : 4 : 1, has been extracted from *Dumontia incrassata*, which belongs to the same family (Dumontaceæ) as *Dilsea edulis* but is commoner.[85] Periodate oxidation indicates the majority of the inter-galactose links to be 1 : 3.

[74] R. Johnston and E. G. V. Percival, *J.*, 1950, 1994.
[75] T. Dillon and P. O'Colla, *Proc. Roy. Irish Acad.*, 1951, **54**, *B*, 51.
[76] E. G. Young and F. A. H. Rice, *J. Biol. Chem.*, 1946, **164**, 35.
[77] W. H. Cook, R. C. Rose, and J. R. Colvin, *Biochim. Biophys. Acta*, 1952, **8**, 595.
[78] W. Z. Hassid, *J. Amer. Chem. Soc.*, 1933, **55**, 4163.
[79] *Idem, ibid.*, 1935, **57**, 2046. [80] T. Mori, *J. Agric. Chem. Soc. Japan*, 1943, **19**, 297.
[81] *Idem, ibid.*, 1949, **23**, 81. [82] T. Mori and T. Fumoto, *ibid.*, 1949, **23**, 81.
[83] V. C. Barry and T. Dillon, *Proc. Roy. Irish Acad.*, 1945, **50**, *B*, 349.
[84] T. Dillon and J. McKenna, *ibid.*, 1950, **53**, *B*, 45. [85] *Idem, Nature*, 1950, **165**, 318.

The structure of the xylan [86, 87] of *Rhodymenia palmata* has recently been ıvestigated.[88, 89] Periodate oxidation and methylation showed both :4- and 1 : 3-links between the D-xylose units. It therefore differs from sparto xylan.[90] The isolation of a mannan from *Porphyra umbilicalis* by opper precipitation of a hot 20% sodium hydroxide extract has recently een reported.[91] Hydrolysis of the completely methylated derivative ave chiefly 2 : 3 : 6-tri-*O*-methyl-D-mannose, indicating a predominating :4-linkage. The outer membrane of *Porphyra tenera* is reported to contain polysaccharide which on hydrolysis gives chiefly mannose.[92]

Funorin, a gluey substance present in species of Gloeopeltis which has ng been used in the Orient as sizing material for textiles and paper,[59] s not been examined chemically to any extent, but the limited information ailable points to its being a galactan sulphate.[93, 94] Other references to the esence of galactan sulphates in red algæ have been collected by Jones and mith [62] but no structural studies have been carried out.

Little work appears to have been carried out on seasonal variations in mposition of red algæ or on any of the other factors which have been own to affect the chemical composition of the brown algæ. Macpherson id Young,[95] in their study of the chemical composition of marine algæ of ova Scotia, included five red algæ collected at one time of the year and alysed them for water, nitrogen, ash, lipids, calcium, phosphorus, and on. The biochemistry of the Rhodophyceæ has been reviewed by Kylin.[96]

Carbohydrates of Green Marine Algæ (Chlorophyceæ).—No complete view has yet been given for the green algæ, probably since very little emical work has been carried out on these plants. Reference was made in 37 to evidence presented by S. Endo that glucose was the first product of otosynthesis in *Codium latum* whereas in *Cladophora wrightiana* fructose as the first-detected sugar,[97] and in 1942 to the rhamnose-containing lysaccharide from *Ulva lactuca*.[98] In Japan a similar type of polyccharide, considered to be a polymer of L-rhamnose and uronic acid, has en isolated from *Ulva pertusa* [99] and *Enteromorpha compressa*.[100] Kylin [101] so studied the polysaccharides of these two algæ and isolated two pectinke materials, one (ulvin) water-soluble, and the other (ulvacin) waterisoluble. Ulvin he showed to be a sulphuric acid ester containing a methylntose, while the composition of ulvacin was undetermined. From *Cladoora rupestris*, Kylin [102] isolated what he regarded as a reserve carbodrate, resembling the paramylon of the Euglenaceæ. Also, an extract this alga, on acid hydrolysis, showed the presence of sulphuric acid, and

[86] C. Sauvageau and G. Denigès, *Compt. rend.*, 1922, **174**, 791.
[87] V. C. Barry and T. Dillon, *Nature*, 1940, **146**, 620.
[88] E. G. V. Percival and S. K. Chanda, *ibid.*, 1950, **166**, 787.
[89] V. C. Barry, T. Dillon, B. Hawkins, and P. O'Colla, *ibid.*, p. 788.
[90] S. K. Chanda, E. L. Hirst, J. K. N. Jones, and E. G. V. Percival, *J.*, 1950, 1289.
[91] J. K. N. Jones, *J.*, 1950, 3292. [92] T. Miwa, *Japan. J. Botany*, 1940, **11**, 41.
[93] K. Aoki, *Bull. Jap. Soc. Fish.*, 1935, **3**, 359; 1937, **6**, 88, 145, 182; 1938, **7**, 25, 8.
[94] A. G. Ross, unpublished work.
[95] M. G. Macpherson and E. G. Young, *Canad. J. Res.*, 1949, **27**, *C*, 73.
[96] H. Kylin, *Kgl. fysiogr. Sällsk. Lund Forh.*, 1943, **13**, 51.
[97] A. G. Pollard, *Ann. Reports*, 1937, **34**, 452. [98] F. W. Norris, *ibid.*, 1942, **39**, 235.)
[99] S. Miyake, K. Hayashi, and Y. Takimo, *J. Soc. Trop. Agr. Tohoku Imp. Univ.*, 8, **10**, 232. [100] S. Miyake and K. Hayashi, *ibid.*, 1939, **11**, 269.
[101] H. Kylin, *Kgl. fysiogr. Sällsk. Lund Forh.*, 1946, **16**, 102.
[102] *Idem, ibid.*, 1944, **14**, 221.

galactose was confirmed, indicating that the substance was probably a galactan sulphate. Recent analyses of *Ulva lactuca* and *Enteromorpha flexuosa* for crude proteins, ash, fats, and fibre,[103] and of *Ulva lactuca* and *Enteromorpha intestinalis* for nitrogen, fats, ash, calcium, phosphorus, and iron,[95] have been reported while cell-wall studies in the Chlorophyceæ have been carried out.[104]

Work on the evolution of dimethyl sulphide from the algæ has been extended to *Enteromorpha intestinalis*, and 2-carboxyethyldimethylsulphonium chloride has been isolated from this alga.[105]

A further study of *Ulva pertusa* has led to the isolation from it by steam-distillation of a new acidic compound, ulvaic acid ($C_{18}H_{36}O_3$).[106]

Other Constituents of Marine Algæ.—As great similarity occurs in the groups of algæ already discussed in regard to the following constituents, a brief review will now be given of the proteins, lipids, sterols, vitamins, and pigments in these algæ.

Proteins, amino-acids and peptides. Lemberg and Legg [107] have reviewed the earlier work on algal chromoproteins and their photosynthetic role has been considered.[108, 109, 110] A review by Lugg [111] has dealt with the earlier work on the amino-acid composition of various marine algæ. A concise review of peptide studies and amino-acid composition of marine algæ occurs in a recent paper by Channing and Young [112] and the literature on the free amino-acids, iodo-amino-acids, and isolated proteins has been surveyed.[113, 114]

Amino-acids. Results of previous work [115] on algal amino-acid composition are now known to be erroneous [112, 116] and the protein amino-acids of different algæ are qualitatively similar [111, 112, 114] although the free and peptide amino-acids of various algæ differ.[112, 113] Roche and Lafon [117] originally reported the detection of 3 : 5-di-iodotyrosine in *Laminaria saccharina* and *L. flexicaulis*, but conflicting evidence has arisen.[114] More recent work [118] on iodo-amino-acid stability and iodine lability under conditions of barium hydroxide hydrolysis, in which ^{131}I was used, seems to indicate that all iodo-amino-acids, except thyroxine, are unstable, their iodine being labile. It has also been shown [118] that *Rhodymenia palmata*, *Laminaria digitata* frond, and *Ulva lactuca* have the ability to synthesise iodo-amino-acids and other iodine compounds in the dark though this ability seems to be absent in *L. digitata* stipe and *Fucus vesiculosus*.

Peptides. An examination of the peptides of *Pelvetia canaliculata* [112] using 1-bromo-2-fluoro-3 : 5-dinitrobenzene followed by a counter-current

[103] J. H. Axtmayer and H. Estremera, *El Crisol*, 1950, **4**, 19.
[104] E. Nicolai and R. D. Preston, *Proc. Roy. Soc.*, 1952, *B*, **140**, 244.
[105] R. Bywood and F. Challenger, *Biochem. J.*, 1953, **53**, xxvi.
[106] T. Katayama and T. Tomiyama, *J. Fac. Agr. Kyushu Univ.*, 1950, **9**, 271.
[107] R. Lemberg and J. W. Legg, "Hematin Compounds and Bile Pigments," Interscience Publ., New York, 1949, pp. 127, 145, 571.
[108] S. Granick, *Ann. Rev. Plant Physiol.*, 1951, **2**, 115.
[109] E. I. Rabinowitch, *ibid.*, 1952, **3**, 229; "Photosynthesis and Related Processes," Interscience Publ., New York, 1945, pp. 417, 476.
[110] H. H. Strain, in "Photosynthesis in Plants," Iowa State College Press, Ames, 1949, p. 163.
[111] J. W. H. Lugg, *Adv. Protein Chem.*, 1949, **5**, 229.
[112] D. M. Channing and G. T. Young, *J.*, 1953, 2481.
[113] C. B. Coulson, *Chem. and Ind.*, 1953, 971.
[114] *Idem, ibid.*, p. 997.
[115] A. Mazur and H. T. Clarke, *J. Biol. Chem.*, 1938, **123**, 729; 1942, **143**, 39.
[116] J. W. H. Lugg, *Adv. Protein Chem.*, 1949, **5**, 249.
[117] J. Roche and M. Lafon, *Compt. rend.*, 1949, **229**, 481.
[118] R. Scott, unpublished work.

artition of the bromodinitrophenyl-peptides showed that glutamic acid was he major peptide component.

Proteins. Photosynthesis experiments,[119] following those of Haxo and links,[120] show that phycocyanin and chlorophyll of the Rhodophyceæ robably obtain energy from phycoerythrin-absorbed light. Swingle and iselius, using phycoerythrin from *Ceramium rubrum*, demonstrated that ricalcium phosphate columns could be used for chromatographic separ-ion.[121] Ultra-centrifuge and electrophoretic studies [122] of phycoerythrin *Callithamnion* spp.) isolated chromatographically on tricalcium phosphate ggest the presence of two protein components. The liberated phyco-rythrobilin is oxidised in air to a blue product similar to phycocyanobilin.[122] *ina pertusa* proteins have been fractioned [123] with water, sodium chloride lution, and weak alkaline extractants, their isoelectric points determined nd their spectrographic properties examined. The amino-acids of isolated roteins of various marine algæ [114] and *Oscillatoria* phycocyanin [124] have een examined by paper-chromatography; those of dried algæ appear to be eficient in histidine,[114] and it is suggested that phycocyanin may contain ydroxyglutamic acid.[124] The presence of alginic acid in the Phæophyceæ as, so far, prevented the isolation of a relatively pure protein.[114] It has een found that, as more protein nitrogen is precipitated, it is associated ith more alginate.[125] An alginate–protein complex is said to be formed etween their two isoelectric points, at high alginate : protein ratios,[126] hen casein, gelatine, and egg albumen are used, although electrophoretic vidence of this has not been found for ovalbumin in less acid solutions and ith lower alginate : protein ratios.[127]

Lipids. Although in the 1942 Reports [128] reference was made to a eview on the lipoid constituents, this dealt entirely with the carotenoids and erols. No review of the algal fats has yet appeared in these Reports. sujimoto in 1925 isolated the fatty acids from seven species of algæ common Japan [129] but no work of a chemical nature was carried out for several ears thereafter. Russell-Wells in 1932 determined the fats in the brown lgæ common to Britain and established a relation between the fatty con-ituents and the depth of immersion of the algæ; the percentage of crude ats decreased with the depth of immersion while the unsaponifiable residue nversely increased.[130] During the next few years a systematic study of e fats and their properties was carried out in Japan.[131, 132] The fatty acids

[119] C. S. French and V. K. Young, *J. Gen. Physiol.*, 1952, **35**, 873.
[120] F. T. Haxo and L. R. Blinks, *ibid.*, 1950, **33**, 389.
[121] S. M. Swingle and A. Tiselius, *Biochem. J.*, 1951, **48**, 171.
[122] A. A. Krasnovskii, V. B. Evstigneev, G. B. Brin, and V. A. Gavrilova, *Doklady kad. Nauk S.S.S.R.*, 1952, **82**, 947.
[123] M. Takagi, *Bull. Fac. Fish. Hokkaido Univ.*, 1950, **1**, 35.
[124] E. C. Wassink and H. W. J. Ragetli, *Proc. Acad. Sci. Amsterdam*, 1952, **55**, *C*, 462.
[125] D. G. Smith and E. G. Young, 1st Int. Seaweed Symp., 1952, 54.
[126] V. C. E. Le Gloahec, U.S.P. 2,430,180/1947.
[127] D. G. Smith and E. G. Young, *J. Biol. Chem.*, in the press.
[128] F. W. Norris, *Ann. Reports*, 1942, **39**, 229.
[129] M. Tsujimoto, *Chem. Umschau*, 1925, **32**, 125.
[130] B. Russell-Wells, *Nature*, 1932, **129**, 654.
[131] E. Takahashi, K. Shirahama, and S. Tase, *J. Chem. Soc. Japan*, 1933, **54**, 619; 35, **56**, 1250.
[132] E. Takahashi and K. Shirahama, *ibid.*, 1936, **57**, 411; E. Takahashi, K. Shira-ma, and N. Ito, *ibid.*, 1938, **59**, 662; E. Takahashi, K. Shirahama, and N. Togasawa, d., 1939, **60**, 56.

identified were palmitic, stearic, myristic, decanoic, octanoic, hexanoic, linolenic, and oleic. The unsaponifiable matter gave a sterol " pelvesterol" (later identified with fucosterol), hydrocarbons such as $C_{18}H_{36}$, $C_{20}H_{34}$, and unsaturated terpenes.[133] Recently the composition of the oil of *Dictyopteris divaricata* has been examined; [134] steam-distillation gave about 1% of an oil consisting of sesquiterpenes and sesquiterpene alcohols, and from these cadinene and (−)-cadinol have been isolated.

Sterols. At intervals reviews of algal sterols have appeared in these reports.[134a] Since the last, the constitution and stereochemical configuration of fucosterol, as given by MacPhillamy,[135] have been confirmed by the isolation of acetaldehyde and 24-oxocholesterol on ozonolysis.[136] Bergmann and Klosty [137] independently verified these results by converting fucosterol into 24-oxocholesterol by way of *iso*fucosteryl methyl ether which was transformed into 24-oxocholesteryl acetate. A method is described for the determination of fucosterol in algæ; [138] crude fats, extracted with ether, are saponified and the fucosterol is determined colorimetrically in the unsaponifiable fraction by a method based on the Liebermann–Burchardt reaction. A correlation was found between the percentage of fucosterol and the total crude fats, the sterol being highest in the most exposed alga, *Pelvetia canaliculata* (0·28% on the dry basis), and decreasing with the degree of immersion of the alga to less than 0·1% in *L. cloustoni* frond.

Vitamin A. Considerable work, particularly in Japan, has been carried out on the vitamin content of the algæ. Presence of vitamin A has not been confirmed although Freudenthal [139] has shown that algæ in very small doses can supply all the vitamin A and D requirements of the rat. He investigated three species of algæ, *Furcellaria fastigiata*, *Fucus serratus*, and *F. vesiculosus*, by feeding increasing amounts to rats kept on a diet deficient in vitamins A, D, and E.

β-Carotene is widely distributed in the algæ; according to Seybold and Egle [140] the total carotenoid content is from 29 to 190 mg. per 100 g. of dry material in the Phæophyceæ, 93—406 in the Chlorophyceæ, and 12—158 in the Rhodophyceæ.

Vitamin B complex. Thiamine, determined in a number of algæ, varies considerably in the different portions of the plant, particularly in the Laminariae.[141] In *L. saccharina* and *L. digitata* the thiamine content varied from 7·25 and 9·35 in the growing portion of the frond to 1·31 and 2·56 μg. per g. of dry weight in the upper part of the fronds respectively.

The riboflavin content of a number of red, brown, and green algæ common to Japan has recently been reported.[142] *Alaria crassifolia*, *Laminaria japonica*, *Porphyra tenera*, and *Enteromorpha linza*, for example, contained respectively 1000, 940, 3700, and 2500 μg. per 100 g. of the dried samples.

[133] K. Shirahama, *J. Agric. Chem. Soc. Japan*, 1935, **11**, 980; 1936, **12**, 521; 1937, **13**, 705; 1938, **14**, 349, 415, 421, 743.
[134] M. Takaoka and Y. Ando, *J. Chem. Soc. Japan*, 1951, **72**, 999.
[134a] C. W. Shoppee, *Ann. Reports*, 1947, **44**, 175.
[135] H. B. MacPhillamy, *J. Amer. Chem. Soc.*, 1942, **64**, 1732.
[136] D. H. Hey, J. Honeyman, and W. J. Peal, *J.*, 1950, 2881; *J.*, 1952, 4836.
[137] W. Bergmann and M. Klosty, *J. Amer. Chem. Soc.*, 1951, **73**, 2935.
[138] W. A. P. Black and W. J. Cornhill, *J. Sci. Food Agric.*, 1951, **2**, 387.
[139] P. Freudenthal, "Om Vitamin I Alger," Nyt. Nordisk Forlag, Copenhagen, 1949.
[140] A. Seybold and K. Egle, *Jahrb. wiss. Bot.*, 1938, **86**, 50.
[141] G. Gerdes, *Arch. Mikrobiol.*, 1951, **16**, 53.
[142] M. Tsujimura, K. Tabei, and T. Wada, *J. Agric. Chem. Soc. Japan*, 1952, **26**, 11.

Although there is no certain evidence of the presence of vitamin B_{12} in terrestrial plants, Ericson [143] has detected it in several algæ, *e.g.*, *Polysiphonia nigrescens* and *Pelvetia canaliculata* gave B_{12} (cobalamin) activity corresponding respectively to 1·0 and 0·5 μg. of B_{12} per g. of dry weight. In order to determine whether the B_{12} was synthesised by the alga, or was of bacterial origin and then concentrated by it, Ericson [143] studied the uptake of radioactive cobalt and B_{12} by some marine algæ, and concluded that the B_{12} was in fact of bacterial origin. Chromatographic, ionophoretic, and spectrophotometric methods for the examination of vitamin B_{12} and other growth factors have recently been studied,[144, 145] and growth factors related to B_{12} and folinic acid in some brown and red algæ have been estimated by the agar cup plate method.[146] The bio-autographic separation of vitamin B_{12} and the various forms of folinic acid in these algæ has, in addition, been accomplished.[147] Also, by use of paper-chromatographic and bio-autographic methods, at least nine growth factors for *Streptococcus faecalis* have been identified in aqueous extracts of dried samples of algæ such as *L. saccharina*, *F. vesiculosus*, *Polysiphonia nigrescens*, and *Rhodomela subfusca*.[148]

Vitamin C. Considerable work done on ascorbic acid in algæ has been reviewed by Tsuchiya [149] who studied seasonal variation and effects of temperature, pH, and chlorine content on ascorbic acid in *Ulva pertusa*, *Enteromorpha* spp., and *Gracilaria confervoides*. In *U. pertusa* it reached a maximum of 241·23 mg. per 100 g. of dry weight at the beginning of January, in *Enteromorpha* spp. a maximum of 238·85 at the end of the same month, but in *Gracilaria confervoides* it stayed at a level of 148·63—97·62 from January to May. It was also found [149] that the ascorbic acid content was influenced by physical and chemical conditions in a manner similar to that in the higher green plants. The ascorbic acid content (indophenol determination) of marine algæ common to Hokkaido has also been reported: [150] it was low in two species of Chlorophyceæ and fifteen species of Rhodophyceæ (4—65 mg. per 100 g. of dry wt.) but greater in fourteen species of Phæophyceæ (309—888 mg.).

Vitamin D. Doubt still exists as to the occurrence of the antirachitic vitamins in plants and Rygh [151] stresses that determinations should be restricted to biological tests. He extracted the vitamin D fraction from a series of plants with ether and obtained values corresponding to 2·5—20 microunits of vitamin D per g. of dry wt. of plant; with drifting green algæ he obtained still higher values in agreement with Johnson and Levring [152] who determined the vitamin D content of a number of brown and green algæ by the rat assay method. *F. vesiculosus* gave an antirachitic effect corresponding to a content of 5 microunits per g. of dry matter. Johnson

[143] L.-E. Ericson, *Chem. and Ind.*, 1952, 829.
[144] *Idem*, *Acta Chem. Scand.*, 1953, **7**, 703.
[145] L.-E. Ericson and A. G. M. Sjöström, *ibid.*, p. 704.
[146] L.-E. Ericson and Z. G. Bánhidi, *ibid.*, p. 167.
[147] Z. G. Bánhidi and L.-E. Ericson, *ibid.*, p. 713.
[148] L.-E. Ericson, E. Widoff, and Z. G. Bánhidi, *ibid.*, p. 974.
[149] Y. Tsuchiya, *Tohoku J. Agric. Research*, 1950, **1**, 97.
[150] Y. Ishihara, S. Umemoto, and Y. Matsubara, *Mem. Fac. Agr. Hokkaido Univ.*, 1953, **1**, 83. [151] O. Rygh, *Research*, 1950, **3**, 577.
[152] N. G. Johnson and T. Levring, *Svenska Hydrografisk. Biol. Komm. Skrifter*, 1947, No. 3, 1.

and Levring [152] found that the higher algæ, growing just below the surface or floating in the sea and exposed to the active ultra-violet radiation of the sun, did possess some antirachitic activity and also concluded that provitamin in varying amounts is present in many marine organisms. Further analysis made it very probable that the provitamin is 7-dehydrocholesterol or the D_3 provitamin.

Vitamin E. α-, γ-, and δ-Tocopherols have been found in the three littoral brown algæ examined, *F. vesiculosus*, *Ascophyllum nodosum*, and *Pelvetia canaliculata*, while only α-tocopherol has been found in *L. cloustoni*.[153] This vitamin appears to undergo considerable seasonal variation, increasing from 13·8 mg. per 100 g. of dry matter in *F. vesiculosus*, 15·6 in *Ascophyllum nodosum*, and 22·9 in *Pelvetia canaliculata* in the January samples to 27·2, 29·8, and 34·7 in the September samples.

Pigments. Brief reference has been made to the algal pigments in previous reports.[128, 154, 155, 156] An extensive review and bibliography up to 1945 have been prepared by A. H. Cook; [157] work to 1951 has been reviewed by Strain.[157a] Since then, Karrer and Tappi [158] chromatographically separated the carotenoid pigments from *Cladophora glomerata*, by absorption spectra showing presence of β-carotene, xanthophyll, xanthophyll epoxide, and violaxanthin. In a review by Sir Ian Heilbron and A. H. Cook,[159] the structure, synthesis, and physiological significance of the carotenoids and vitamin A are discussed: the authors comment on the appearance of unusual carotenoids in individual organs, such as that of α-carotene in the spermatozoids of the Fucus species, and how this has prompted the suggestion that pigmentation is associated with motility or the chemotactic sensitivity. Carotenoid biogenesis has been recently reviewed by Goodwin; [160] no work appears to have been carried out on carotogenesis in the algæ.

Cook, Elvidge, and Heilbron [161] have studied chemotaxis between the gametes of the Fucaceæ. Cell-free preparations of *F. serratus* and *F. vesiculosus* eggs have been obtained which exert a chemotactic attraction on the sperms of *F. serratus*, *F. vesiculosus*, and *F. spiralis*. The chemotactic principle was found to be easily expelled from aqueous solution by a stream of inert gas, and could be recovered in a cooled receiver, but it was not fully identified.[162] Dilute solutions in sea-water of a number of simple organic compounds (hydrocarbons, ethers, and esters) stimulate the cell-free preparations from Fucus eggs and cause attraction of the spermatozoa of *F. serratus* and *F. vesiculosus* in the same manner. The results indicated that *n*-hexane or a closely related hydrocarbon might be the chemotactic principle.

In view of the ability of algæ to concentrate elements present in sea-water, the importance of this in the disposal of radioactive waste materials into the sea is manifest. Black and Mitchell [163] have reviewed this field

[153] F. Brown, *Chem. and Ind.*, 1953, 174.
[154] A. W. Stewart, *Ann. Reports*, 1914, **11**, 144.
[155] A. G. Pollard, *ibid.*, 1937, **34**, 453. [156] R. A. Morton, *ibid.*, 1949, **46**, 247.
[157] A. H. Cook, *Biol. Reviews*, 1945, **20**, 115.
[157a] H. H. Strain, "Manual of Phycology," 1951, Waltham, Mass., U.S.A., p. 243.
[158] P. Karrer and G. Tappi, *Helv. Chim. Acta*, 1950, **33**, 2211.
[159] Sir Ian Heilbron and A. H. Cook, *Endeavour*, 1951, **10**, 175.
[160] T. W. Goodwin, *J. Sci. Food Agric.*, 1953, **4**, 209.
[161] A. H. Cook, J. A. Elvidge, and Sir Ian Heilbron, *Proc. Roy. Soc.*, 1948, *B*, **135**, 293.
[162] A. H. Cook, J. A. Elvidge, and R. Bentley, *ibid.*, 1951, *B*, **138**, 97.
[163] W. A. P. Black and R. L. Mitchell, *J. Marine Biol. Assoc.*, 1952, **30**, 575.

nd have determined trace elements in the common brown algæ and in ea-water. A seasonal variation was noted as well as considerable variation in he content of these elements among different species from the same habitat. The extent to which the algæ concentrate these elements has been calcul-ted; *e.g.*, *F. spiralis* contains 10,000 times more titanium than the sur-ounding seawater. Kelly,[164] and Roche and Yagi,[165] have studied the ptake of radioactive ^{131}I. The thalli of *L. flexicaulis* and *L. saccharina* ere immersed in sea-water containing $Na^{131}I$. Radio-autographs showed he ^{131}I fixed in certain regions of the tissue.[165] At least 80% remained in norganic form while some was converted into mono- and di-iodotyrosine.

In Japan, recent interest has been directed to use of seaweed as a growth edium for yeast. Two suitable species of yeast which were isolated *Candida* spp. No. 1 and 2) had high mannitol-assimilating powers of 87·4 nd 61·8% respectively [166] (cf. *Torula utilis* 19%). The brown alga, *Eckeloria ma*, was a suitable substrate, and optimum conditions were worked out r the growth of the yeast on hydrolysates of this alga.[167] On the other and, an *Ulva* sp. was found unsuitable for yeast production, as also were he alkaline extracts and waste liquors from the alginate industry. The acid each, however, before extraction of the alginic acid could be utilised.[168]

Extracts of several species of Chlorophyceæ, Rhodophyceæ, and Phæo-hyceæ common to the central Californian coast have been found to inhibit he growth *in vitro* of one or more species of pathogenic bacteria.[169] The ude ether-extract of *Rhodomela larix* [170] contains iodine 0·05, bromine 7·7, chlorine nil, total halogen 37·6, and total ash 1·0%. Further tests dicated that a brominated phenolic compound may be the active antibiotic.

Blinks has recently reviewed work on the physiology and biochemistry f the algæ,[171] and a recent monograph by Fogg gives valuable information n their metabolism.[172]

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[164] S. Kelly, *Biol. Bull.*, 1953, **104**, 138.
[165] J. Roche and Y. Yagi, *Compt. rend. Soc. Biol.*, 1952, **146**, 642.
[166] Y. Tomiyasu and B. Zenitani, *J. Agric. Chem. Soc. Japan*, 1951—52, **25**, 406.
[167] *Idem, ibid.*, p. 479. [168] *Idem, ibid.*, 1952, **26**, 6.
[169] R. Pratt, H. Mautner, G. M. Gardner, Y.-H. Sha, and J. Dufrenoy, *J. Amer. harm. Assoc.*, 1951, **40**, 575.
[170] H. G. Mautner, G. M. Gardner, and R. Pratt, *ibid.*, 1953, **42**, 294.
[171] L. R. Blinks, " Manual of Phycology," Waltham, Mass., U.S.A., 1951, p. 263.
[172] G. E. Fogg, " The Metabolism of Algæ," Methuen & Co., Ltd., London, 1953.

42.

THE ENZYMIC DEGRADATION OF POLYSACCHARIDES

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THE ENZYMIC DEGRADATION OF POLYSACCHARIDES *

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Introduction

THE main structural features of a polysaccharide can usually be determined by the chemical methods of methylation and periodate oxidation.[1] Certain limitations in these methods have, however, become apparent ; it is, for example, difficult to effect complete methylation of a highly branched polysaccharide,[2] or to avoid " over-oxidation " of the reducing group of a polysaccharide during periodate oxidation.[3] Additional methods of structural analysis have therefore been sought. One possible method is a study of the degradation of the polysaccharide by purified *hydrolytic* enzymes. This Review will outline recent progress in the chemistry of such enzymes (polysaccharases), with particular reference to their activity *in vitro*, since in many instances this has yielded new information on the fine structure of a polysaccharide. Physiological aspects of polysaccharase action will not be discussed here.

Before reviewing the various classes of polysaccharases, the methods of polysaccharase chemistry will be considered. Enzymic action involves the hydrolysis of a glycosidic linkage ; in Fig. 1 the scission of a β-1 : 4-manno-

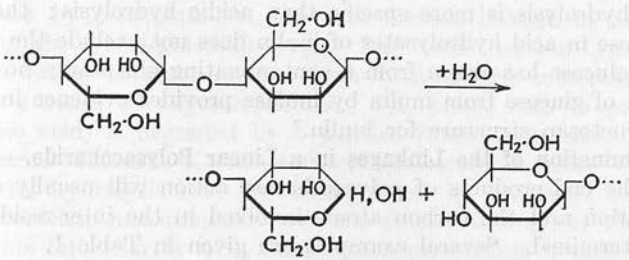


FIG. 1

Hydrolysis of a β-1 : 4-mannosidic linkage.

sidic linkage is shown. The course of hydrolysis of a soluble polysaccharide can be followed by means of (*a*) the decrease in viscosity or turbidity of the polysaccharide, (*b*) the change in optical rotation, (*c*) the increase in reducing power, (*d*) chromatography of the polysaccharide–enzyme mixture.

[1] See, for example, Percival, " Structural Carbohydrate Chemistry ", Garnet Miller, London, 1953, 2nd edn. ; Pigman and Goepp, " Chemistry of the Carbohydrates ", Academic Press, New York, 1948 ; Whistler and Smart, " Polysaccharide Chemistry ", Academic Press, New York, 1953.

[2] Cf. Bell, *Ann. Reviews Biochem.*, 1949, **18**, 87 ; Bell and Manners, *J.*, 1954, 1891.

[3] Cf. Head and Hughes, *J.*, 1954, 603.

* The Enzymic Synthesis of Polysaccharides has been reviewed recently by Barker and Bourne (*Quart. Reviews*, 1953, **7**, 56).

Paper chromatography has been widely used for preliminary identification of the products of polysaccharase action. The degradation of an insoluble polysaccharide, *e.g.*, cellulose, can be examined by methods (*c*) and (*d*). Ideally, the mode of action (action pattern) of a polysaccharase is determined by investigating the degradation of a polysaccharide of known structure; the enzyme may then be used in structural investigations of related polysaccharides. For example, the action pattern of salivary α-amylase [4] and of the cereal β-amylases [5] has been determined by using amylose and amylopectin (the linear and branched components of starch) as substrates; with this knowledge, the degradation of various bacterial and protozoal α-1 : 4-glucosans by these enzymes has given structural data which confirm and extend those obtained by chemical methods. Unfortunately, present knowledge of the action pattern of many polysaccharases is far from complete; accordingly reference to the enzymic degradation of mucopolysaccharides, plant gums and mucilages, and many other heteropolysaccharides will be omitted from this Review. In the following sections certain aspects of enzymic structural analysis will be discussed; a general account of several classes of polysaccharases then follows.

Determination of the Component Monosaccharides in a Polysaccharide.—The complete acid hydrolysis of a polysaccharide is usually a satisfactory method for determining the component monosaccharides. In a number of instances, enzymic experiments have been of value, *e.g.*, the presence of L-galactose in an enzymic hydrolysate of snail galactogen has confirmed the presence of both isomers of galactose in this polysaccharide.[6] In general, enzymic hydrolysis is more specific than acidic hydrolysis; the presence of D-glucose in acid hydrolysates of inulin does not exclude the possibility that the glucose has arisen from a contaminating glucosan; however, the liberation of glucose from inulin by inulase provides evidence in favour of a glucofructosan structure for inulin.[7]

Determination of the Linkages in a Linear Polysaccharide.—Identification of the end-products of polysaccharase action will usually enable the configuration and the carbon atoms involved in the inter-residue linkage to be determined. Several examples are given in Table 1.

The end-products will depend on the action pattern of the polysaccharase. An enzyme catalysing the *stepwise* hydrolysis of *every* glycosidic linkage will produce only the constituent monosaccharide(s): *e.g.*, glucose is the

[4] Whelan and Roberts, *Nature*, 1952, **170**, 748; *J.*, 1953, 1298.

[5] See Manners, *Ann. Reports*, 1953, **50**, 288; Myrbäck and Neumüller, in "The Enzymes", by Sumner and Myrbäck, Academic Press, New York, 1950, Vol. I, Part 1, p. 653.

[6] Weinland, *Biochem. Z.*, 1953, **324**, 74.

[7] Dedonder, *Bull. Soc. Chim. biol.*, 1952, **34**, 157.

[8] Tracey, *Biochem. Soc. Symp.*, 1953, **11**, 49.

[9] Jeanes, Wilham, Jones, Tsuchiya, and Rist, *J. Amer. Chem. Soc.*, 1953, **75**, 5911.

[10] Duncan, Manners, and Ross, *Biochem. J.*, 1954, **57**, xviii, and unpublished work.

[11] McCready and McComb, *Agric. Food Chem.*, 1953, **1**, 1165.

[12] Jones and Reid, *J.*, 1954, 1361; Altermatt and Deuel, *Helv. Chim. Acta*, 1954, **37**, 770.

TABLE 1. *Products of polysaccharase action*

Polysaccharide	Enzyme	Major product(s)	Main repeating linkage	Ref.
Cellulose .	Cellulase	Cellobiose	β-1 : 4-Glucosidic	8
Dextran .	Dextranase	*iso*Maltose *iso*Maltotriose	α-1 : 6-Glucosidic	9
Laminarin .	β-Glucosanase	Glucose Laminaribiose Laminaritriose	β-1 : 3-Glucosidic	10
Pectic acid .	Polygalacturonase	Galacturonic acid Digalacturonic acid Trigalacturonic acid	α-1 : 4-Galacturonidic	11, 12
Starch . .	α-Amylase	Maltose	α-1 : 4-Glucosidic	5
Xylan . .	Xylanase	Xylose Xylobiose Xylotriose	β-1 : 4-Xylosidic	10

ole product of " amyloglucosidase " (a polysaccharase from *Aspergillus iger*) action on amylose.[13] If, however, a polysaccharase catalyses a *stepwise* hydrolysis of *alternate* glycosidic linkages, the sole end-product will e a disaccharide containing the same glycosidic linkage as the polysaccharide. Thus, certain samples of amylose are quantitatively converted nto maltose by β-amylase ; [14] the inter-residue linkage in amylose is herefore of the α-1 : 4-glucosidic type. The most common type of polysaccharase action involves *random* hydrolysis of the substrate thereby roducing a series of oligosaccharides : *e.g.*, pectic acid (a polymer of alacturonic acid) is degraded by a purified fungal polygalacturonase to ive a mixture of mono-, di-, tri-, and tetra-galacturonic acids.[11] Random nd stepwise hydrolysis may be distinguished by means of viscometric nd reductometric measurements ; in the former action, the viscosity of he substrate falls sharply with concomitant production of only a few reducing groups, whereas in stepwise hydrolysis, reducing groups are quickly liberated, and the viscosity decreases slowly and regularly.

Determination of the Linkages in a Branched Polysaccharide.—Branched olysaccharides contain more than one type of glycosidic linkage : the majority of these are " inter-residue " linkages which unite the monoaccharides in the " unit-chains " ; * the remainder are " inter-chain "

[13] Kerr, Cleveland, and Katzbeck, *J. Amer. Chem. Soc.*, 1951, **73**, 3916.
[14] Meyer, *Experientia*, 1952, **8**, 405.

* Chemical analysis of a branched polysaccharide of high molecular weight may reveal that, on a statistical basis, the ratio of non-terminal to terminal monosaccharide esidues is, for example, 15 : 1. The polysaccharide is thus composed of a large number f repeating units of 15 monosaccharide residues. For convenience, these units may e referred to as " unit-chains " ; it must be noted, however, that figures for unithain lengths (which are given to the nearest whole number) represent mean values, nd that individual unit-chains vary considerably in length.

*

linkages. Thus amylopectin contains *ca.* 4% of α-1 : 6-linkages which inter-link the constituent unit-chains, each of which comprises *ca.* 20 α-1 : 4-linked glucose residues.[5] Random enzymic hydrolysis will therefore produce a mixture of oligosaccharides belonging to more than one homologous series. The majority of these will contain inter-residue linkages; the remainder will consist either of a disaccharide containing the inter-chain linkage, or higher saccharides containing this linkage and one or more inter-residue linkages, depending on the specificity of the polysaccharase. Salivary α-amylase, for example, cannot hydrolyse α-1 : 4-linkages which are adjacent to 1 : 6-linkages in amylopectin : accordingly, the products of salivary amylolysis include a series of branched oligosaccharides (α-dextrins) which contain a 1 : 6-linkage and three or more α-1 : 4-linkages.[4]

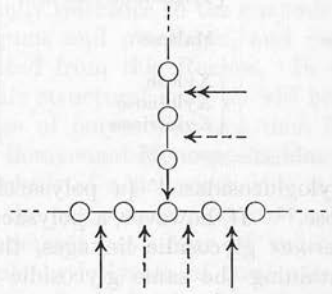


FIG. 2

α-Amylolysis of branched chain in amylopectin.

○ Glucose residue. — α-1 : 4-Linkage. → α-1 : 6-Linkage.
--→ Linkage susceptible only to *A. oryzæ* α-amylase.
⟶→ Linkage susceptible to salivary and to *A. oryzæ* α-amylase.

In contrast, *Aspergillus oryzæ* α-amylase can hydrolyse α-1 : 4-linkages adjacent to a 1 : 6-linkage,[15] and the end-products of amylolysis therefore include *iso*maltose (see Fig. 2). These types of enzymic structural analysis have been greatly facilitated by the development of chromatographic methods for the separation of oligosaccharides.[16]

Determination of the Degree and Type of Branching in a Polysaccharide.—This type of investigation requires a detailed knowledge of the action pattern of highly purified polysaccharases, and is, at present, limited to starch-type polysaccharides since the requisite knowledge for polysaccharases other than amylases is not yet available. Details of the methods developed for the determination of the degree of branching (*i.e.*, end-group assay) and type of branching (*i.e.*, single or multiple) in amylopectins will be described on p. 84—87.

Criteria of Purity and Activity of Polysaccharases.—For the structural analysis of polysaccharides by enzymic methods, highly purified enzyme

[15] Montgomery, Weakley, and Hilbert, *J. Amer. Chem. Soc.*, 1949, **71**, 1682.
[16] *E.g.*, see Whelan, Bailey, and Roberts, *J.*, 1953, 1293; Derungs and Deuel, *Helv. Chim. Acta*, 1954, **37**, 657.

reparations are essential. Recent progress in the development of fraconation procedures for proteins (for reviews see ref. 17) has enabled olysaccharases of a high degree of purity to be prepared. In addition, f the conclusions drawn from enzymic studies are to be valid, the *enzymic omogeneity* of the polysaccharase must be ensured ; other carbohydrases r transglycosylases are often associated with polysaccharases. The preence of cellobiase in cellulase or of maltase in amylase preparations led arly workers to conclude that glucose was the primary product of " cellulse " or " amylase " action on cellulose or starch respectively. The ossibility of transglycosylase impurities has only recently been realised.[18] ransglycosylases catalyse the transfer of a glycosyl unit from a donor to suitable acceptor : *e.g.*, *Aspergillus niger* extracts convert maltose into mixture of glucose and panose (4-α-*iso*maltosyl-D-glucose) [19] (cf. Fig. 3).

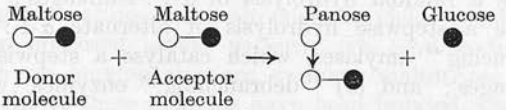


FIG. 3

Transglucosylation of maltose by A. niger *extracts.*

Symbols as for Fig. 2. ● Reducing glucose molecule.

uch amylolytic extracts cannot be used, therefore, in experiments aimed t the characterisation of the inter-chain linkages in starch-type polyccharides.

Artefacts may arise during polysaccharase action in two additional ays. First, some polysaccharases, under certain conditions, show *synthetic* tivity. Thus, emulsin when incubated with high concentrations of glose, catalyses the synthesis of gentiobiose and other β-linked disaccharides.[20] he same enzyme hydrolyses certain β-glucosans, *e.g.*, laminarin and yeast ucan ; [10] the presence of disaccharides in these hydrolysates cannot be ken as unambiguous evidence for the nature of the inter-residue and ter-chain linkages since the possibility of " reversion " from glucose mains. All polysaccharases must therefore be examined for synthetic tivity. Secondly, if a mixture of two purified enzymes is used in a ructural analysis, a " synergic " reaction may occur. In such reactions, e specificity of one enzyme is altered by the presence of the second, and normal hydrolysis occurs. Two examples of a synergic reaction with amylase have been discovered recently [21, 22] and are discussed on p. 87 ;

[17] Schwimmer and Pardee, *Adv. Enzymology*, 1953, **14**, 375.

[18] *E.g.*, the transglucosylase (transglucosidase) activity of various amylase preations had been determined by Pan, Nicholson, and Kolachov, *Arch. Biochem. ophys.*, 1953, **42**, 421.

[19] *Idem*, *J. Amer. Chem. Soc.*, 1951, **73**, 2547 ; Wolfrom, Thompson, and Galkowski, id., p. 4093.

[20] Peat, Whelan, and Hinson, *Nature*, 1952, **170**, 1056.

[21] Whelan, *Biochem. Soc. Symp.*, 1953, **11**, 17.

[22] Schwimmer and Garibaldi, *Cereal Chem.*, 1952, **29**, 108.

the possibility of similar reactions with other polysaccharases must not be overlooked.

Enzymic Degradation of Starch

Starch, the reserve carbohydrate of many plants, contains two distinct polysaccharides—amylose and amylopectin. The amylose content of starch is usually *ca.* 20%, an amylose molecule consisting essentially of a linear chain of several thousand glucose residues united by α-1 : 4-linkages. Amylopectin is a highly branched molecule, composed of several hundred unit-chains, each of which comprises 20—25 α-1 : 4-linked glucose residues; the unit-chains are inter-linked by glucosidic linkages from the reducing group to $C_{(6)}$ of a glucose residue in an adjacent chain.[23]

Four main groups of *hydrolytic* enzymes attack starch : (*a*) α-amylases, which catalyse a random hydrolysis of α-1 : 4-linkages; (*b*) β-amylases, which catalyse a stepwise hydrolysis of alternate α-1 : 4-linkages; (*c*) "glucose-producing" amylases, which catalyse a stepwise hydrolysis of all α-1 : 4-linkages; and (*d*) "debranching" enzymes, which hydrolyse α-1 : 6-linkages.

Starch is also degraded by *Bacillus macerans* amylase (Schardinger dextrinogenase); this enzyme, by a transference action, converts starch into a mixture of cyclic dextrins.[5]

α-Amylases.—α-Amylases have been isolated in purified form from many sources, *e.g.*, barley malt, mammalian pancreatic and salivary secretions, and several bacterial and fungal extracts.[5, 14] Several α-amylases have been crystallised.[24] Superficially, all α-amylases catalyse a similar reaction, *viz.*, random hydrolysis of α-1 : 4-linkages, shown initially by a rapid decrease in the viscosity, turbidity, and iodine-staining power of the substrate and, later, by the production of oligosaccharides. The initial enzyme action (dextrinisation) involves degradation of the substrate into α-dextrins which contain 6—10 glucose residues. α-Dextrins from amylose are linear molecules, whereas those from amylopectin have branched structures, since α-amylases cannot hydrolyse α-1 : 6-linkages. In the later stages of α-amylolysis (the saccharification stage), α-dextrins are further broken down to reducing sugars. Recent investigations have revealed important differences in the mode of action of different α-amylases. During the α-amylolysis of amylose, the achroic stage of hydrolysis is reached when 23% of the linkages have been hydrolysed by swine pancreatic α-amylase, whereas with human salivary or *Aspergillus oryzæ* amylase, only 15 or 12% of the linkages are broken at this stage.[25] These differences reflect some variation in dextrinisation action. Specificity differences during saccharification have also been demonstrated. Malt α-amylase and *B. subtilis* amylase, unlike salivary amylase, can hydrolyse the linkage adjacent to

[23] For reviews of starch chemistry see Bourne, *Chem. and Ind.*, 1951, 1047; Meyer and Gibbons, *Adv. Enzymology*, 1951, **12**, 341; Hassid in "Organic Chemistry", Vol. IV, Ed. by Gilman, Wiley, New York, 1953, p. 901.

[24] Meyer, *Angew. Chem.*, 1951, **63**, 153.

[25] Tung Kung, Hanrahan, and Caldwell, *J. Amer. Chem. Soc.*, 1953, **75**, 5548.

a reducing group, thereby liberating glucose ; [26] they can also hydrolyse maltotriose.[4, 26] Since the composition of the end-products of α-amylolysis is dependent on the enzyme source, further discussion will be limited to salivary amylolysis, since the action pattern has been most clearly defined, by Whelan and Roberts.[4]

Potato amylose, on complete salivary amylolysis, gave an apparent conversion into maltose (from reducing-power determinations) of 91% ; the end-products[4] were maltose and maltotriose in the molar ratio of 2·39 : 1. Since glucose was not produced, terminal α-1 : 4-linkages must be resistant to enzyme action. If α-amylolysis were completely random, and if all susceptible linkages were hydrolysed at the same rate, the calculated molar ratio of maltose and maltotriose would be 2·35 : 1. Whelan and Roberts therefore postulated that salivary amylolysis of amylose is a random hydrolysis of non-terminal linkages. Experiments using linear maltosaccharides of DP * 4—7 gave maltose and maltotriose in yields which were in agreement with the proposed action pattern. The end-products of salivary amylase action on amylopectin are maltose, maltotriose, and branched α-dextrins ; eight of these dextrins have been isolated, the smallest being a pentasaccharide, and the largest an octasaccharide.[4] These findings

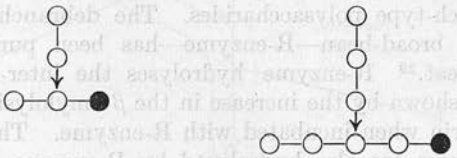


FIG. 4

α-Dextrins from amylopectin.

suggest that the three α-1 : 4-linkages adjacent to a 1 : 6-linkage are resistant to enzyme action. Salivary amylolysis thus involves random hydrolysis of non-terminal α-1 : 4-linkages except those adjacent to an inter-chain linkage.

β-Amylases.—The action pattern of β-amylase, which is known only in certain plants (*e.g.*, wheat, barley, and soya bean) does not appear to depend on the enzyme source. β-Amylolysis consists of stepwise hydrolysis of alternate linkages in a chain of α-1 : 4-linked glucose residues, from the non-reducing end, with the liberation of β-maltose.[5] Enzyme action is arrested by the presence of anomalous linkages in the chain ; these may be inter-chain linkages or ester-phosphate linkages. Unlike α-amylases, β-amylase cannot by-pass such linkages, since interior † chains in branched α-1 : 4-glucosans are not attacked. Linear amylose molecules on β-amylolysis are completely degraded ; other samples (as shown in Table 2) have low β-amylolysis limits and presumably contain a small number of anomalous

[26] Bird and Hopkins, *Biochem. J.*, 1954, **56**, 86.

* DP = Degree of polymerisation.

† Those parts of a unit-chain between two branch-points (cf. Fig. 5).

linkages. β-Amylolysis of amylopectin yields maltose and a β-dextrin of high molecular weight which differs from amylopectin in that the exterior chains (*i.e.*, those parts of a unit-chain between the branch point and the non-reducing terminal group) contain only two or three glucose residues.

Glucose-producing Amylases.—In 1951, the existence of two amylases which yield glucose as the *primary* product of their action on starch was reported. These enzymes catalyse a stepwise hydrolysis of every linkage in a chain of α-1 : 4-linked glucose residues, beginning at the non-reducing terminal linkage. One such amylase, from the mould *Rhizopus delemar*, liberates over 90% of the glucose from amylose, amylopectin, glycogen, and a β-dextrin.[27] This enzyme cannot hydrolyse 1 : 6-linkages, but can by-pass them, thereby attacking interior chains. A second glucose-producing amylase has been isolated from *Aspergillus niger* and named " amyloglucosidase ".[13] The so-called " maltase " from *Clostridium acetobutyricum* also appears to be a glucose-producing amylase since it converts maltose, maltoheptaose, *iso*maltose, and starch almost quantitatively into glucose; it differs from the above mould amylases in that it can hydrolyse both α-1 : 4- and 1 : 6-linkages.[28]

" Debranching " Enzymes.—Important advances have been made recently in studies of enzymes catalysing the hydrolysis of α-1 : 6-inter-chain linkages in starch-type polysaccharides. The debranching enzyme from the potato and broad-bean—R-enzyme—has been purified by Hobson, Whelan, and Peat.[29] R-enzyme hydrolyses the inter-chain linkages in amylopectin, as shown by the increase in the β-amylolysis limits of amylopectin or β-dextrin when incubated with R-enzyme. The 1 : 6-linkages in branched α-dextrins are also hydrolysed by R-enzyme, giving a mixture of linear maltosaccharides.[4] R-enzyme cannot hydrolyse the inter-chain linkages in glycogen—a branched α-1 : 4-glucosan which forms the reserve carbohydrate of animals and differs from amylopectin in that the unit-chains usually comprise only *ca.* 12 glucose residues.

From a brewer's yeast autolysate, a debranching enzyme known as *iso*amylase has been obtained, which hydrolyses the inter-chain linkages in glutinous rice starch producing a more linear polysaccharide of lower molecular weight.[30] *iso*Amylase and R-enzyme are therefore similar in that they hydrolyse *non-terminal* α-1 : 6-linkages.

A second type of debranching enzyme which can only hydrolyse *terminal* α-1 : 6-linkages has been isolated from rabbit muscle, and named amylo-1 : 6-glucosidase; it has no action on amylopectin or glycogen, but can hydrolyse those 1 : 6-linkages which are exposed by the action of muscle phosphorylase on these polysaccharides.[31] Phosphorylases [5, 32] catalyse the degradation of chains of α-1 : 4-linked glucose residues by transferring a

[27] Phillips and Caldwell, *J. Amer. Chem. Soc.*, 1951, **73**, 3559, 3563.
[28] French and Knapp, *J. Biol. Chem.*, 1950, **187**, 463.
[29] Hobson, Whelan, and Peat, *J.*, 1951, 1451; Peat, Whelan, Hobson, and Thomas, *J.*, 1954, 4440.
[30] Maruo and Kobayashi, *Nature*, 1951, **167**, 606.
[31] Cori and Larner, *J. Biol. Chem.*, 1951, **188**, 17.
[32] Bernfeld, *Adv. Enzymology*, 1951, **12**, 379.

ucosyl residue from the chain to inorganic phosphate, according to the quation :

$$[G]_n + m\text{HO.P} \rightleftharpoons m\text{G.1P} + [G]_{n-m}$$

here $[G]_n$ or $[G]_{n-m}$ represents a linear chain of n or $(n - m)$ α-1 : 4-linked ucose residues, HO.P = inorganic phosphate, and G.1P. = α-glucose phosphate. The reaction is reversible. Amylopectin or glycogen contains three types of unit-chain, each of which is linear and composed of 1:4-linked glucose residues, *viz.*, *A-chain*, linked to the molecule only y a 1 : 6-linkage to an adjacent chain ; *B-chain*, to which one or more -chains are attached, and which is itself linked by a 1 : 6-linkage from e reducing group to an adjacent chain ; *C-chain*, to which other chains e attached and which carries a free reducing group.[33] Muscle phosphorylse shows a different specificity towards glucosidic linkages in the three pes of unit-chain.[31] Phosphorolysis of an A-chain is complete except

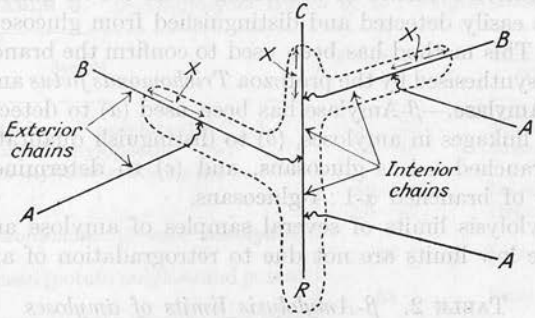


FIG. 5

Phosphorolysis of amylopectin or glycogen.

— Linear chain of α-1 : 4-linked glucose residues.
1 : 6-linkage. A, B, C : Types of unit-chain.
R : Free reducing group. ····· Extent of phosphorolysis.
X : *ca.* 6 glucose residues.

a single glucose residue which remains attached, by a 1 : 6-linkage, to B-chain, whereas phosphorolysis of a B- or a C-chain ceases at approximately the sixth residue from the 1 : 6-linkage (see Fig. 5). Amylo-1 : 6-ucosidase action is limited to the hydrolysis of the 1 : 6-linkages which ach single glucose residues to B(or C)-chains in a phosphorylase limit-xtrin of amylopectin or glycogen, thereby yielding glucose.[31]

Structural Analysis of α-1 : 4-Glucosans using Hydrolytic Enzymes

Use of Salivary α-Amylase.—The salivary amylolysis of a glucosan may e used to detect α-1 : 4-glucosidic linkages. Resistance of a glucosan to mylase implies that the polysaccharide contains few, if any, sequences *ca.* 3 or more adjacent α-1 : 4-linkages. Conversely, linear α-1 : 4-ucosans are completely degraded to a mixture of reducing sugars ; thus,

[33] Peat, Whelan, and Thomas, *J.*, 1952, 4546.

potato amylose on α-amylolysis has a Rm value * of *ca.* 91%.[4] The polysaccharide synthesised by the yeast *Cryptococcus neoformans* (*Torula histolytica*), on α-amylolysis, has Rm 93 and therefore has an essentially unbranched structure.[34] Since branched α-1 : 4-glucosans, on α-amylolysis, yield α-dextrins in addition to reducing sugars, the Rm value is correspondingly lower ; † *e.g.*, glycogen [35] (rabbit liver or oyster) has Rm 73. The polysaccharide synthesised by a strain of *Neisseria perflava*, on α-amylolysis, has Rm 72 ; it is therefore a branched α-1 : 4-glucosan.[35] A sample of liver glycogen from a case of von Gierke's disease had Rm 31, indicating that an unusually small proportion of the α-1 : 4-linkages were susceptible to salivary amylase ; [36] further evidence of an abnormal structure was obtained by chemical end-group assay which revealed a chain length of only 6 glucose residues.

Paper-chromatographic examination of an α-amylolytic digest will also provide qualitative evidence of branching in an α-1 : 4-glucosan, since the α-dextrins are easily detected and distinguished from glucose, maltose, and maltotriose. This method has been used to confirm the branched nature of the glucosans synthesised by the protozoa *Trichomonas fœtus* and *T. gallinæ*.[37]

Use of β-Amylase.—β-Amylase has been used (*a*) to detect the presence of anomalous linkages in amyloses, (*b*) to distinguish qualitatively between linear and branched α-1 : 4-glucosans, and (*c*) to determine the exterior chain lengths of branched α-1 : 4-glucosans.

The β-amylolysis limits of several samples of amylose are recorded in Table 2. The low limits are not due to retrogradation of amylose during

TABLE 2. *β-Amylolysis limits of amyloses*

Sample	DP	β-Amylolysis limit *	Ref.
Apple	545	90	39
Maize	—	75	40
Maize	—	68†	38
Maize	490	90	41
Potato	—	88	40
Potato	—	68†	38
Potato	—	76†	42
Rubber seed (*Hevea brasiliensis*)	1500	79	43
Sago	1200	70†	38
Tapioca	3500	70†	38
Wheat	540	84	40

* Percentage conversion into maltose produced by crystalline sweet potato β-amylase (free from α-amylase and Z-enzyme).

† Completely hydrolysed by a mixture of β-amylase and a β-glucosidase.

[34] Hehre, Carlson, and Hamilton, *J. Biol. Chem.*, 1949, **177**, 289.
[35] Hehre, *ibid.*, p. 267 ; Carlson and Hehre, *ibid.*, p. 281.
[36] Manners, *J.*, 1954, 3527.
[37] Manners and Ryley, *Biochem. J.*, 1955, **59**, 369.

* Apparent percentage conversion into maltose.

† The following analysis is only semi-quantitative, since certain unpurified salivary amylase preparations are contaminated with maltotriase which causes a small increase in Rm, owing to hydrolysis of maltotriose to maltose and glucose.

zyme action, or to the presence of contaminating branched glucosans. some cases, the addition of a β-glucosidase, *e.g.*, emulsin or Z-enzyme, a β-amylolytic digest results in complete saccharification, suggesting at these amyloses contain one or more β-glucosidic linkages which prevent mplete β-amylolysis.[38] The nature of the " barriers " to β-amylase in her samples of amylose has not yet been determined.

The main product of β-amylolysis of amylose is maltose (70—100%), hereas amylopectin gives maltose (40—70%) and a β-dextrin of high olecular weight. The determination of the β-amylolysis limit and the ture of the end-products of enzyme action is therefore a convenient method r differentiating between amylose and amylopectin-type polysaccharides en though the chain length of the polysaccharide is unknown. Examples this type of analysis are given in Table 3.

TABLE 3. *β-Amylolysis limits of* α-1 : 4-*glucosans*

Sample	β-Amylolysis limit	Structural type	Ref.
Clostridium butyricum " amylopectin " (s)	57—71	Branched	44
Neisseria perflava " amylopectin " (cs)	35—40	Branched	35
Corynebacterium diphtheriæ starch (g)			
Fraction A	85—86	Linear	35
Fraction B	64—67	Branched	35
Cryptococcus neoformans (*Torula histolytica*) " amylose "	> 86	Linear	34
Synthetic glucosan (potato amylose and potato Q-enzyme)	58	Branched	45
Synthetic glucosan (potato amylose and bean Q-enzyme)	55	Branched	46
Synthetic glucosan (potato amylose and Q-enzyme from *Polytomella cæca*)	33—34	Branched	47
Polytomella cæca starch			
Fraction V	89	Linear	48
Fraction VI	52	Branched	48
Synthetic " amylose " (glucose 1-phosphate and muscle phosphorylase)	97	Linear	49

(s) Synthesised from sucrose.
(cs) Synthesised by cell-free system from sucrose.
(g) Synthesised from glucose 1-phosphate.

[38] Peat, Pirt, and Whelan, *J.*, 1952, 705, 714; Peat, Thomas, and Whelan, *J.*, 2, 722.
[39] Potter, Hassid, and Joslyn, *J. Amer. Chem. Soc.*, 1949, **71**, 4075.
[40] Potter, personal communication.
[41] Nussenbaum and Hassid, *J. Biol. Chem.*, 1951, **190**, 673.
[42] Bell and Manners, *J.*, 1952, 3641.
[43] Greenwood and Robertson, *J.*, 1954, 3769.
[44] Hobson and Nasr, *J.*, 1951, 1855.
[45] Barker, Bourne, Peat, and Wilkinson, *J.*, 1950, 3022.
[46] Hobson, Whelan, and Peat, *J.*, 1950, 3566.
[47] Bebbington, Bourne, and Wilkinson, *J.*, 1952, 246.
[48] Bourne, Stacey, and Wilkinson, *J.*, 1950, 2694.
[49] Hassid, Cori, and McCready, *J. Biol. Chem.*, 1943, **148**, 89.

The exterior chain lengths of branched α-1 : 4-glucosans can be calculated from the β-amylolysis limit and chain length, as shown in Table 4. The mean lengths of the interior chains can then be obtained. The lengths of the exterior chains cannot be determined, as yet, by any chemical method.

TABLE 4. *Determination of exterior and interior chain lengths of α-1 : 4-glucosans*

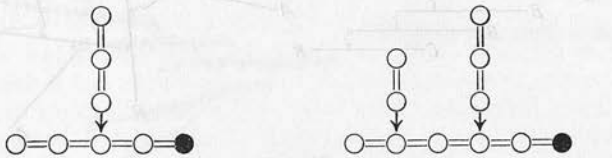
Sample	Chain length	β-L.	E.C.L.	I.C.L.	Ref.
Amylopectins					
Maize	25 (p)	63	18	6	50
Wheat	23 (p)	62	16—17	5—6	50
Easter lily	27 (p)	60	18—19	7—8	50
Tapioca	23 (p)	62	16—17	5—6	50
Sago	22 (p)	62	16	5	50
Potato	27 (p)	59	18—19	7—8	50
Barley	26 (p)	59	18	7	51
Malted barley	17—18 (m)	44	10	6—7	51
Sweet corn 1	12 (p)	47	8	3	52
Sweet corn 2	11 (p)	45	7—8	2—3	52
Waxy maize	22 (m.p.)	53	14	7	53
Waxy sorghum	25 (m.p.)	52	15—16	8—9	53
Glycogens					
Rabbit liver	12—13 (m.p.)	43	8	3—4	42
Rabbit liver	18 (m.p.)	53	12	5	53
Mytilus edulis	16—18 (m.p.)	47	10—11	5—6	42
Helix pomatia	7 (p)	37	5	1	42
Brewer's yeast	13 (p)	44	8	4	54
Baker's yeast	12 (m.p.)	50	8—9	2—3	55
Bacterial and protozoal polysaccharides					
Neisseria perflava	11—12 (m)	57	9	1—2	56
Tetrahymena pyriformis	13 (p)	44	8	4	57
Trichomonas fœtus	15 (p)	60	11—12	2—3	37
Trichomonas gallinæ	9 (p)	51	7	1	37
Bacillus megatherium	11 (m.p.)	46	7—8	2—3	58

β-L. = β-Amylolysis limit.
E.C.L. = Exterior chain length (no. of glucose residues removed by β-amylase + 2·5).
I.C.L. = Interior chain length (chain length − E.C.L. − 1).
p = Periodate oxidation assay. m = Methylation assay.

Use of α-Amylase and R-Enzyme.—By examination of the products produced by the *successive* action of salivary α-amylase and R-enzyme on rabbit-liver glycogen, Whelan and Roberts determined the chain length of

[50] Katz and Potter, personal communication.
[51] Aspinall, Hirst, and MacArthur, unpublished work.
[52] Dvonch and Whistler, *J. Biol. Chem.*, 1951, **181**, 889.
[53] Halsall, Hirst, Hough, and Jones, *J.*, 1949, 3200.
[54] Manners and Maung, *J.*, 1955, 867.
[55] Northcote, *Biochem. J.*, 1953, **53**, 348.
[56] Barker, Bourne, and Stacey, *J.*, 1950, 2884.
[57] Manners and Ryley, *Biochem. J.*, 1952, **52**, 480.
[58] Barry, Gavard, Milhaud, and Aubert, *Ann. Inst. Pasteur*, 1953, **84**, 605.

the polysaccharide, and obtained evidence for a multiply branched structure.[4] α-Amylolysis of the glycogen gave maltose and maltotriose (from the linear portions of the molecule) and a series of branched α-dextrins, which were isolated by charcoal column chromatography. These α-dextrins were then treated with R-enzyme, the 1 : 6-inter-chain linkages being hydrolysed and a series of linear maltosaccharides produced. Since the number of reducing groups liberated by R-enzyme action is dependent on the number of 1 : 6-linkages in the molecule, experimental determination of this number will give the percentage of 1 : 6-linkages and, hence, of non-reducing terminal groups in the molecule. By this method, Whelan and Roberts obtained a chain length of 12·5 for the glycogen; periodate oxidation gave 13·6. Further examination of the linear maltosaccharides obtained by " debranching " the α-dextrins showed that a small proportion of these were hexa- or hepta-saccharides. The largest maltosaccharide which can arise from a singly branched α-dextrin is maltopentaose; the



Singly branched α-dextrin. Doubly branched α-dextrin.

FIG. 6

For symbols see Fig. 3. = α-1 : 4-Linkage, resistant to α-amylase.

presence of these higher saccharides suggests that some of the α-dextrins contained two 1 : 6-linkages (see Fig. 6). This finding affords further evidence for multiple branching in glycogen (see also p. 86).

Use of β-Amylase and R-Enzyme.—The successive action of β-amylase and R-enzyme on amylopectin has provided evidence of a multiply branched structure.[33] Methylation studies of amylopectin led Haworth and Hirst [59] to postulate a singly branched " laminated " structure for this polysaccharide, whereas Meyer [60] considered a multiply branched " tree " structure to be the simplest representation of the amylopectin molecule (see Fig. 7). The " laminated " and the " tree " structure differ in the ratio of A : B chains; in the former, the ratio is $1 : (n - 2)$ where n is the number of chains in the molecule, whereas in the " tree " structure there are approximately equal numbers of A- and B-chains.

β-Amylolysis of an amylopectin, as previously mentioned, is confined to the exterior chains, which are degraded to leave " stubs " composed of two or three glucose residues. Treatment of a β-dextrin with R-enzyme will therefore liberate maltose or maltotriose from the A-chain stubs, whereas B-chains will yield linear saccharides of a much higher molecular weight; experimentally, the smallest of these has been shown to be maltohexaose. Hence, by determining the amount of maltose and maltotriose

[59] Haworth, Hirst, and Isherwood, *J.*, 1937, 577; Haworth, *Nature*, 1947, **160**, 901.
[60] Meyer and Bernfeld, *Helv. Chim. Acta*, 1940, **23**, 875.

produced by the action of R-enzyme on a β-dextrin, it is possible to assess the proportion of A-chains therein, and hence, distinguish between singly branched "laminated" and multiply branched "tree" structures.

Peat and his co-workers[33] treated a β-dextrin of waxy maize starch (of DP *ca.* 3000) with R-enzyme; the observed molar percentage of maltose and maltotriose was 5·3% whereas a singly branched molecule would yield only 0·083%. These authors therefore concluded that "multiple branching is an intrinsic part of the amylopectin molecule". Hirst and Manners [61] have shown that an amylopectin with a ratio of A : B chains of *ca.* 1 : 4 would yield 5·3% of maltose and maltotriose. Thus, in the waxy maize starch examined by Peat *et al.*, only one unit-chain in every

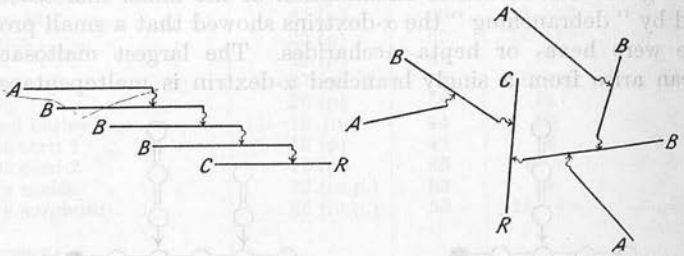


FIG. 7

Singly and multiply branched structures for amylopectin.
For symbols see Fig. 5.

five contained more than one branch point, the molecular structure being therefore intermediate between a "laminated" and "tree" model.

The *combined* action of β-amylase and R-enzyme has been used to determine the unit-chain length of waxy maize starch.[21] A linear chain containing an odd number of glucose residues is degraded by *dilute* solutions of β-amylase into maltose and one molecule of maltotriose, the latter arising from the reducing end of the chain. A polysaccharide sample may be assumed to contain equal numbers of even and odd chains; hence, by treating amylopectin with β-amylase and R-enzyme (to hydrolyse the 1 : 6-linkages which prevent complete β-amylolysis), and determining the amount of maltotriose produced, the number of odd chains in the molecule can be assessed. By this method, waxy maize starch had a chain length of 26; periodate oxidation gave a value of 24—25.

Use of Amylo-1 : 6-Glucosidase and Phosphorylase.—An alternative method of enzymic end-group assay involves the *combined* action of phosphorylase and amylo-1 : 6-glucosidase on amylopectin or glycogen in the presence of inorganic phosphate.[31, 62] Glucose 1-phosphate and glucose are produced; the latter arises from the hydrolysis of 1 : 6-linkages by amylo-1 : 6-glucosidase and, by estimating the molar percentage of glucose, the number of 1 : 6-linkages and the chain length can be calculated. This

[61] Hirst and Manners, *Chem. and Ind.*, 1954, 224.
[62] Illingworth, Larner, and Cori, *J. Biol. Chem.*, 1952, **199**, 631.

ethod has been applied to several samples of amylopectin and glycogen ; he results are in good agreement with those obtained by methylation or otassium periodate oxidation assays.

Stepwise degradation of amylopectin and glycogen by phosphorylase nd amylo-1 : 6-glucosidase has provided evidence of multiple branching n these glucosans.[63] If a phosphorylase limit dextrin (LD.1) is treated ith amylo-1 : 6-glucosidase and the latter then inactivated, the dextrin ill be susceptible to further attack by phosphorylase ; this phosphorolysis,

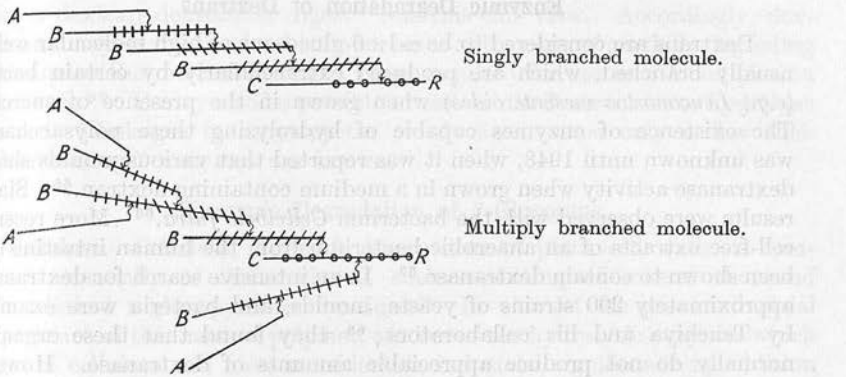


FIG. 8

The stepwise degradation of a singly and a multiply branched molecule by muscle phosphorylase and amylo-1 : 6-glucosidase.

owever, is incomplete. A second dextrin (LD.2) can be isolated, and on eatment with amylo-1 : 6-glucosidase, it again becomes susceptible to hosphorylase action. Each successive action of phosphorylase and amylo- : 6-glucosidase thus causes the removal of the A-chains from the phos- horylase limit dextrin. Furthermore, about 50% of the branch points re removed during the first combined treatment, but only about 20% uring the second. Since a singly branched molecule would lose only one f the branch points at each treatment, the above experiments provide vidence for multiply branched structures (see Fig. 8).

Synergic Reactions.—In 1952 Schwimmer and Garibaldi [22] reported that, though glucose was not a major product of the action of malt α-amylase n starch, the addition of *Bacillus macerans* amylase to an enzymic digest starch and malt α-amylase increased the rate and extent of hydrolysis d resulted in the formation of considerable amounts of glucose. In the

[63] Larner, Illingworth, Cori, and Cori, *J. Biol. Chem.*, 1952, **199**, 641.

following year, Whelan [21] observed that when glycogen or amylopectin is degraded by a mixture of R-enzyme and α-amylase, the end-products of this action included glucose. These two enzymes acting together also degraded panose (4-α-*iso*maltosyl-D-glucose) to give a mixture of glucose and maltose; neither enzyme acting separately liberated glucose from amylopectin or glycogen, nor attacked panose. It appears, therefore, that certain polysaccharase-substrate "complexes" can be modified by the presence of a second enzyme so that an abnormal reaction occurs.

Enzymic Degradation of Dextrans

Dextrans are considered to be α-1 : 6-glucosans of high molecular weight, usually branched, which are produced extracellularly by certain bacteria (*e.g.*, *Leuconostoc mesenteroides*) when grown in the presence of sucrose.[64] The existence of enzymes capable of hydrolysing these polysaccharides was unknown until 1948, when it was reported that various moulds showed dextranase activity when grown in a medium containing dextran.[65] Similar results were observed with the bacterium *Cellvibrio fulva*.[66] More recently, cell-free extracts of an anaerobic bacterium from the human intestine have been shown to contain dextranase.[67] In an intensive search for dextranases, approximately 200 strains of yeasts, moulds, and bacteria were examined by Tsuchiya and his collaborators; [68] they found that these organisms normally do not produce appreciable amounts of dextranase. However, certain moulds (*e.g.*, *Aspergillus niger* and some strains of *Penicillium*), when grown in submerged culture in the presence of dextran, produce dextranases extracellularly. Dextranase production is therefore an adaptive process.

The first detailed study of dextranase action was made by Jeanes and co-workers [9] who degraded a dextran (synthesised by *L. mesenteroides*) with culture filtrates of two strains of the mould *Penicillium funiculosum*. 95% of the linkages in the dextran were of the α-1 : 6-type. One dextranase gave *iso*maltose (45%), *iso*maltotriose (22%), and higher saccharides, whilst the second dextranase yielded glucose (10%), *iso*maltose (53%), *iso*maltotriose (7%), and a series of higher saccharides; separation of these sugars was effected by charcoal column chromatography.

Dextrans from various strains of *L. mesenteroides*, which contain different proportions of α-1 : 6-glucosidic linkages, are hydrolysed at different rates by a particular dextranase.[68] Since adaptively formed dextranases are free from other polysaccharases,[66] the presence in the dextran of linkages other than α-1 : 6 will interfere with hydrolysis; the relative rates of hydrolysis of different dextrans by a dextranase may therefore be used qualitatively to detect structural differences in these polysaccharides.

[64] For reviews of dextran chemistry see Stacey and Ricketts, *Fortschr. Chem. Org. Naturstoffe*, 1951, **8**, 28; Evans and Hibbert, *Adv. Carbohydrate Chem.*, 1946, **2**, 209.

[65] Nordström and Hultin, *Svensk Kem. Tidskr.*, 1948, **60**, 283; Hultin and Nordström, *Acta Chem. Scand.*, 1949, **3**, 1405.

[66] Ingelman, *ibid.*, 1948, **2**, 803.

[67] Hehre and Sery, *J. Bact.*, 1952, **63**, 424.

[68] Tsuchiya, Jeanes, Bricker, and Wilham, *ibid.*, 1952, **64**, 513.

Furthermore, quantitative studies similar to those of Jeanes and co-workers should enable the inter-chain linkages to be identified. Recent chemical studies [69] have indicated that certain dextrans contain 1 : 3-linkages, in addition to, or in place of, the 1 : 4-linkages previously reported ; accordingly, further work in this field is necessary.

From a study of the change in viscosity of dextran during enzymic degradation, Hultin and Nordström,[65] and Ingelman,[66] have concluded that dextranase catalyses a random rather than a stepwise hydrolysis of the polysaccharide. The isolation of glucose, *iso*maltose, and *iso*maltotriose from a dextran–dextranase digest [9] confirms this view. Accordingly, dextranases may have considerable importance as a means of partially degrading dextrans to the molecular-weight range required for use as a blood-plasma substitute.[66] It may also be possible to use certain dextranases to hydrolyse the α-1 : 6-linkages in amylopectin or glycogen, since only a few such "debranching" enzymes are known.

Enzymic Degradation of β-Glucosans

Cellulose.—Cellulose, the main constituent of plant cell walls, is composed essentially of linear chains, each consisting of several thousand β-1 : 4-linked D-glucopyranose residues.[70] In view of the great industrial importance of this natural polymer, studies of the hydrolytic enzymes which degrade cellulose will have considerable industrial significance in addition to physiological interest. Cellulases are widely but erratically distributed in Nature ; they are present in certain higher plants (*e.g.*, barley) and in invertebrate digestive secretions, and are produced extracellularly by many bacteria and fungi ; they are absent from mammalian digestive secretions, although herbivores maintain populations of symbiotic micro-organisms in their intestinal tracts, which produce cellulases.[8, 71]

In general, cellulases have not been extensively purified, and our knowledge of the mechanism of enzyme action is accordingly limited. Many cellulase preparations show cellobiase activity ; separation of these enzymes has been achieved by Grassmann and his collaborators [72] who removed cellobiase from *Aspergillus oryzæ* cellulase by selective adsorption of the former on aluminium hydroxide, and by Whistler and Smart [73] who used columns of powdered cellulose to adsorb cellobiase from an *Aspergillus niger* cellulase preparation. An important advance in this field has been made by Whitaker who has purified the cellulase from the mould *Myrothecium verrucaria* ; [74] the purified enzyme was homogeneous in the ultra-centrifuge and on electrophoresis, and has a molecular weight of 63,000.[75]

[69] Barker, Bourne, Bruce, Neely, and Stacey, *J.*, 1954, 2395 ; Sloan, Alexander, Lohmar, Wolff, and Rist, *J. Amer. Chem. Soc.*, 1954, **76**, 4429.

[70] See Aspinall, *Biochem. Soc. Symp.*, 1953, **11**, 42.

[71] Pigman in "The Enzymes", by Sumner and Myrbäck, Academic Press, New York, 1951, Vol. I, Part 2, p. 728.

[72] Grassmann, Zechmeister, Tóth, and Stadler, *Annalen*, 1933, **503**, 167.

[73] Whistler and Smart, *J. Amer. Chem. Soc.*, 1953, **75**, 1916.

[74] Whitaker, *Arch. Biochem. Biophys.*, 1953, **43**, 253.

[75] Whitaker, Colvin, and Cook, *ibid.*, 1954, **49**, 257.

Since the natural substrate for cellulases is insoluble in water, enzyme action cannot be determined viscometrically ; in view of the simplicity and convenience of such methods, soluble derivatives of cellulose (*e.g.*, carboxymethylcellulose) have been used as substrates in many cases. The latter are hydrolysed *ca.* 10—30 times more rapidly than cellulose, under identical conditions, as shown by measurements of reducing power, the actual ratio depending on the enzyme source.[8] This variation in the relative rates of hydrolysis of cellulose and its soluble derivatives has led to the postulation of a multienzyme mechanism for cellulase action. According to Reese and his co-workers [76] cellulase preparations contain an enzyme (C_1) which alters the physical state of cellulose fibres and liberates the polymeric chains, whilst a second enzyme (Cx) catalyses the hydrolysis of these chains to reducing sugars. The enzyme Cx, acting alone, is supposed to be able to degrade carboxymethylcellulose but not native cellulose. The ratio of the rates of hydrolysis of these substrates would therefore depend on the relative concentrations of C_1 and Cx in a particular cellulase preparation. Quantitative data to test the validity of this hypothesis have, so far, been obtained only with the cellulase from *Myrothecium verrucaria*.[74] This cellulase showed the same activity towards five substrates ranging in degree of polymerisation (and hence solubility) from cellulose to cellobiose, throughout purification from the crude culture filtrate of this mould, to the final purified state.

A recent observation by Whitaker [77] on the effect of proteins on *M. verrucaria* cellulase is of considerable interest. He showed that cellulase action on insoluble substrates is stimulated by the addition of small amounts of certain proteins (*e.g.*, bovine plasma albumin and β-lactoglobulin) which are adsorbed on the cellulose. No such effect was obtained with a soluble substrate. The mechanism of this " activation " is, as yet, unknown, but the apparent differences in activity of various " cellulases " towards soluble and insoluble cellulose may well have been due to the presence of protein impurities in the various enzyme preparations, rather than to the presence of different proportions of two distinct cellulolytic enzymes.

The nature of the end-products of cellulase action has been investigated by several workers. Whistler and Smart [73] have isolated glucose and cellobiose from the hydrolysate of cellulose by *A. niger* cellulase. Sugars other than these were absent, and cellobiose was not formed on prolonged incubation of glucose with the enzyme ; hence the cellobiose was preformed in the substrate. Whether glucose is a primary product of *A. niger* cellulase action or arises solely from cellobiose (the cellulase contained traces of cellobiase) is not yet known. The products of the action of *Clostridium thermocellulaseum* cellulase are glucose, cellobiose, and probably cellotriose ; [78] in this case, the glucose is a primary product of cellulase action. Whitaker [74] has shown that *M. verrucaria* cellulase liberates both glucose

[76] Reese, Siu, and Levinson, *J. Bact.*, 1950, **59**, 485 ; Reese and Levinson, *Physiologia Plantarum*, 1952, **5**, 345. [77] Whitaker, *Science*, 1952, **116**, 90.

[78] Enebo, " Studies in Cellulose Decomposition by an Anaerobic Thermophilic Bacterium and Two Associated Non-Cellulolytic Species ", Stockholm, 1954.

and cellobiose—in roughly equimolar proportions—from both a soluble and an insoluble cellulose. This cellulase, and that from *Cl. thermocellulaseum*, thus catalyse random hydrolytic cleavage of the β-1 : 4-linkages in cellulose. In contrast, cellobiose is the sole product from the degradation of " hydrocellulose " by a purified cellulase from the fungus *Irpex lacteus* and enzyme action is believed to involve the release of cellobiose molecules from the ends of long cellulose chains.[79] It is evident that the term " cellulase " comprises a group of closely related enzymes, and that differences in action pattern exist between cellulases from different organisms. The recent demonstration [80] that cell-free filtrates of certain cellulolytic moulds contain several chromatographically distinct cellulases is particularly significant.

Cellulase action *in vitro* is usually incomplete. Apart from the heterogeneous nature of the action, the physical state of the substrate is of prime importance. The surface potential of cellulose, upon which depends the rate of formation of the enzyme–substrate complex, is an important factor—as shown by the stimulating effect on cellulase action, at certain pH's, of basic dyes (but not acidic dyes) which are adsorbed on the substrate.[81] Furthermore, the degree of crystallinity of the cellulose is important, since the available evidence indicates that cellulase activity is limited to the amorphous regions where the cellulose chains in the fibre are more readily accessible.[82]

Cellulases are a group of enzymes of considerable importance. Attempts have been made to use cellulose breakdown and fermentation as an industrial method for the production of alcohols and acids, and as a means of saccharifying plant fibres.[78] In the laboratory, cellulases provide a method for investigating the structural relations between cellulose, the hemicelluloses, and lignin in woody plant tissues.

Lichenin, Laminarin, and Glucan.—Extracts of some higher plants (*e.g.*, barley [42, 83]), seaweeds,[10] and snail secretions [84] contain enzyme systems which hydrolyse the β-glucosans lichenin, laminarin, and glucan (yeast " cellulose "). Early investigations in this field were hampered by lack of structural knowledge of these substrates; recent chemical studies have now shown lichenin to be a linear β-glucosan containing both 1 : 3- and 1 : 4-linkages,[85] laminarin to be *essentially* a linear β-1 : 3-glucosan,[86] and glucan to be a branched β-1 : 3-glucosan containing *ca.* 11% of β-1 : 2-interchain linkages.[87]

[79] Nishizawa and Kobayashi, *Symp. Enzyme Chem. (Japan)*, 1953, **8**, 123; *Chem. Abs.*, 1953, **47**, 12438.

[80] Jermyn, *Austral. J. Sci. Research*, 1952, *B*, **5**, 433; Reese and Gilligan, *Arch. Biochem. Biophys.*, 1953, **45**, 74. [81] Basu and Whitaker, *ibid.*, 1953, **42**, 12.

[82] Cf. Nickerson, *Adv. Carbohydrate Chem.*, 1950, **5**, 103.

[83] Dillon and O'Colla, *Chem. and Ind.*, 1951, 111.

[84] Pigman, ref. 71, p. 725—744.

[85] Meyer and Gürtler, *Helv. Chim. Acta*, 1947, **30**, 751; Boissonnas, *ibid.*, p. 1703; N. B. Chanda, Ph.D. Thesis, Edinburgh, 1952.

[86] Connell, Hirst, and Percival, *J.*, 1950, 3494; Percival and Ross, *J.*, 1951, 720.

[87] Bell and Northcote, *J.*, 1950, 1944.

The above-mentioned extracts are known to contain several enzymes (*e.g.*, cellulase and β-glucosidase) in addition to the enzyme(s) responsible for the β-1 : 3-glucosanase activity.

Lichenase and cellulase are distinct enzymes, as shown by their different pH optima (5·9 and 4·7 respectively for fungal enzymes) and by the separation of the two activities by fractional precipitation with ether and ethanol.[88] The end-product of snail lichenase action (of unestablished homogeneity) is glucose ; [89] similar information with purified lichenases from other sources is not yet available.

The enzymic hydrolysis of laminarin has been studied by several workers. From a hydrolysate with snail juice, Barry [90] isolated laminaribiose (3-β-D-glucopyranosyl-D-glucose)—further evidence for the presence of β-1 : 3-glucosidic linkages in laminarin. Plant enzymes hydrolyse laminarin initially to a mixture of glucose, laminaribiose, and higher oligosaccharides ; the final end-product is glucose.[83] In marine algæ, at least two enzymes are involved in this action ; one enzyme hydrolyses in random manner non-terminal β-1 : 3-linkages, yielding a series of oligosaccharides, whilst a second enzyme hydrolyses terminal β-linkages, thereby producing glucose.[10]

Yeast glucan is hydrolysed by almond emulsin and seaweed extracts to give glucose,[10] and by cereal extracts to give a mixture of glucose and laminaribiose, thus confirming the configuration of the glucosidic linkages.[83]

Until complete separation of these β-glucosanases has been achieved, little progress can be made in enzymic investigations of the fine structure of lichenin and other β-glucosans, since their action patterns are, as yet, unknown. In particular a specific β-1 : 3-glucosanase would be of value in detecting the presence of linkages other than β-1 : 3 in laminarin, in view of the reported presence of gentiobiose and 1-β-glucosylmannitol in partial acid hydrolysates of this polysaccharide.[91]

Enzymic Degradation of Hemicelluloses

Mannans.—Present knowledge of the enzymic degradation of mannans is limited and, although mannanase (mannase) activity has been reported in a number of protein preparations, these enzymes have not been purified or characterised.

Mannanases appear to be erratically distributed in Nature ; they occur in malt,[92] in the culture filtrate of an agar-agar splitting bacterium,[93] in the mould *Neurospora sitophila*,[94] and in the marine alga *Cladophora rupestris*.[10] The mould *Chætomium globosum* can synthesise mannanase adaptively.[95]

[88] Freudenberg and Ploetz, *Z. physiol. Chem.*, 1939, **259**, 19.

[89] Karrer and Staub, *Helv. Chim. Acta*, 1924, **7**, 518.

[90] Barry, *Sci. Proc. R. Dublin Soc.*, 1941, **22**, 423 ; laminaribiose has been synthesised by Bächli and Percival, *J.*, 1952, 1243.

[91] Peat, Whelan, and Lawley, *Biochem. J.*, 1953, **54**, xxxiii ; *Chem. and Ind.*, 1955, 35.

[92] Klages and Maurenbrecher, *Annalen*, 1938, **535**, 175.

[93] Ishimatsu and Kibesaki, *Symp. Enzyme Chem. (Japan)*, 1950, **4**, 75 ; *Chem. Abs.*, 1952, **46**, 2124.

[94] Takai, *Jap. J. Nutrition*, 1950, **8**, 131 ; *Chem. Abs.*, 1951, **45**, 8058.

[95] Sørensen, *Physiologia Plantarum*, 1952, **5**, 183 ; *Nature*, 1953, **172**, 305.

The algal mannanase appears to catalyse random hydrolytic cleavage f ivory-nut mannan A (essentially a β-1 : 4-mannosan [96]) since the end- roducts include mannose, mannobiose, and other saccharides. It has no ction on the branched yeast mannan. Ivory-nut mannan is also hydro- ysed by a malt extract to give a mixture of mannose and a disaccharide resumably mannobiose) ; [92] this extract contains two enzymes—a true annanase and a mannobiase, since mannose is the sole end-product from he digestion of salep mannan by a normal extract, whereas an aged pre- aration yields only the disaccharide.[97] Salep mannan is known to be a :4-mannosan,[98] and the above findings suggest that the mannosidic nkages have a β-configuration.

Galactomannans.—Studies on the enzymic degradation of galacto- annans have so far been limited to gum gatto (carubin [99]) and guaran ; [100] n both cases, information on the structure of the polysaccharide has been btained.

Gum gatto, the galactomannan from the carob bean *Ceratonia siliqua*, ontains D-mannose residues (*ca.* 84%) together with D-galactose (*ca.* 16%).[101] lethylation studies indicate that it comprises a main chain of 1 : 4-linked annose units and that one-fifth of these have a single galactose residue ttached at $C_{(6)}$. It has been degraded [99] by a commercial polysaccharase reparation " Helisol " which contains an alkali-labile mannanase and an lkali-stable galactosidase. At alkaline pH's, galactose is liberated, but he viscosity of gum gatto is only slightly reduced, whereas at more acid H's the viscosity quickly decreases, with the liberation of a mixture of educing substances ; these include galactomannose saccharides, mannose- ontaining oligosaccharides, and free galactose. These findings are in ccord with the structure suggested for the gum. Since certain mannanase reparations attack both ivory-nut mannan and gum gatto, it is probable hat the mannose residues in the latter also have a β-configuration.

Guaran, a reserve polysaccharide found in the endosperm of guar seeds, n acid hydrolysis gives D-mannose (64%) and D-galactose (36%)[102]. hemical studies have shown that guaran consists essentially of a chain f 1 : 4-linked D-mannose residues ; to alternate residues are attached, $C_{(6)}$, single D-galactose residues.[102] The characterisation of the oligo- ccharides produced during the enzymic hydrolysis of guaran has enabled he identity of the constituent glycosidic linkages to be confirmed.

An enzyme preparation from germinated guar seeds brought about 65% ydrolysis of the guaran. The hydrolysate was fractionated by a charcoal- romatographic procedure and found to contain the following sugars :

[96] Aspinall, Hirst, Percival, and Williamson, *J.*, 1953, 3184.
[97] Pringsheim and Genin, *Z. physiol. Chem.*, 1924, **140**, 299.
[98] Klages and Niemann, *Annalen*, 1936, **523**, 224.
[99] Deuel, Leuenberger, and Huber, *Helv. Chim. Acta*, 1950, **33**, 942.
[100] Whistler and Stein, *J. Amer. Chem. Soc.*, 1951, **73**, 4187 ; Whistler and Smith, d., 1952, **74**, 3795.
[101] Hirst and Jones, *J.*, 1948, 1278.
[102] Heyne and Whistler, *J. Amer. Chem. Soc.*, 1948, **70**, 2249 ; Ahmed and Whistler, d., 1950, **72**, 2524 ; Palmer and Ballantyne, *ibid.*, p. 736.

monosaccharides (65% including most of the galactose originally present), mannobiose (7%), a galactomannose (0·5%), and a mannotriose (7·5%).[10] No higher saccharides were produced by incubation of the enzyme with mannose. Methylation experiments showed that the mannosidic linkages in mannobiose and mannotriose were of the β-1 : 4-type. Guaran must therefore contain a main chain of β-1 : 4-linked D-mannose units. The galactomannose was shown to be 6-α-D-galactopyranosyl-β-D-mannopyranose —further confirmation for the presence of galactose residues attached to $C_{(6)}$ of mannose residues in the main chain of guaran.

Xylans.—Little information is available at present on the enzymes catalysing the hydrolytic degradation of xylans. Xylanase activity has been reported in extracts of germinated barley,[84] in snail secretions,[84] in moulds (*e.g.*, *Aspergillus niger* and *A. oryzæ*) and in extracts of some seaweeds.[10]

Sørensen [95] has shown that the mould *Chætomium globosum* can produce a xylanase when grown in a medium containing xylan (*i.e.*, it is an "adaptive" enzyme). This "xylanase" has optimal activity at pH 6·5 (cf. pH 4·6 for barley xylanase [103]), and appears to contain two components—an enzyme capable of hydrolysing xylan in random fashion, and a less stable enzyme which hydrolyses xylobiose. The combined action of these enzymes produces xylose and a series of oligosaccharides, whilst the former liberates only oligosaccharides from xylan. A related enzyme system occurs in certain seaweeds ; [10] esparto xylan (a β-1 : 4-xylosan [104]) is degraded by the algal xylanases to give a mixture of xylose, xylobiose, and xylotriose ; the same enzyme preparation will also slowly hydrolyse xylobiose and xylotriose.

Enzymic studies have yielded information on the structure of hemicellulose A of oak wood,[105] and a wheat-straw xylan.[95] Hemicellulose A of oak sapwood, on treatment with Taka-diastase (a commercial preparation containing several polysaccharases), yielded glucose and a "xylan" which was identical with that found in the heartwood.[105] Prolonged incubation of this "xylan" with Taka-diastase gave xylose (3 parts) and a water-soluble polysaccharide (2 parts) ; on acid hydrolysis, the latter yielded xylose and a monomethylhexuronic acid, and analysis indicated these sugars to be present in the molar ratio of 6 : 1. An enzyme-resistant "core" has also been prepared from wheat-straw xylan.[95] Incubation of this xylan with a mould xylanase (from *Chætomium globosum*) gave xylose, a small quantity of arabinose, and a water-soluble enzyme-resistant polysaccharide which was composed of xylose, arabinose, glucose, and uronic acids. Methylation studies of such enzyme-resistant "cores" would be of great interest, provided the homogeneity of the xylan is established.

To summarise, the widespread use of hemicellulases as "analytical tools" must await further separation and purification of the individual enzymes and the determination of their action patterns. An interesting

[103] Voss and Butter, *Annalen*, 1938, **534**, 161, 185.
[104] Chanda, Hirst, Jones, and Percival, *J.*, 1950, 1289.
[105] O'Dwyer, *Biochem. J.*, 1939, **33**, 713.

example of hemicellulase action has been reported by Bishop and Whitaker [105a] who have obtained a series of arabinose–xylose oligosaccharides by hydrolysing wheat-straw xylan with an enzyme preparation from *Myrothecium verrucaria*. This observation is further evidence for the existence of arabinose as a minor constituent of wheat-straw xylan.

Enzymic Degradation of Fructosans

Although several investigations of the enzymic degradation of inulin essentially a β-1 : 2-fructosan [1]) have been reported, little attention has been paid to the enzymic breakdown of other fructosans.

An early investigation by Pringsheim and Ohlmeyer [106] showed that extracts of the mould *A. niger* had inulinase (inulase) activity ; complete degradation of the inulin occurred, with the liberation of fructose (over 90%) and glucose (1·5%). These extracts also hydrolysed sucrose. For some time it appeared that inulinase and invertase were identical enzymes, but recent findings are in conflict with this hypothesis. Hudson and his collaborators [107] studied the action of purified yeast " invertase " on sucrose and inulin ; the optimum pH's were found to be 5·2 and 3·6 respectively, suggesting the presence of two distinct enzymes. Furthermore, a comparative study of the inulinase activity of several " invertase " preparations revealed large differences in the ratios of the two activities. More recently Legrand and Lewis [108] have obtained a yeast inulinase preparation devoid of invertase activity and a yeast invertase which had no action on asphodeloside (an inulin-type fructosan), whilst Edelman and Bacon [109] have shown that extracts of Jerusalem artichokes, which have relatively little invertase activity, rapidly hydrolyse inulin.

Inulinases have been detected in a variety of sources including yeast,[110] moulds (*e.g.*, *Penicillium notatum* [111]), and those plants which store inulin as a carbohydrate reserve.[112]

A study of inulinase action has provided evidence of a glucofructosan structure for inulin.[7] Inulinase from the mould *Sterigmatocystis nigra* hydrolysed inulin to give, at intermediate stages of hydrolysis, a series of glucofructosyl oligosaccharides (D, C, B) in accordance with the scheme :

$$\text{D} + H_2O \longrightarrow \text{C} + \text{fructose} \qquad \text{C} + H_2O \longrightarrow \text{B} + \text{fructose}$$

$$\text{D} + H_2O \longrightarrow \text{Sucrose} + \text{fructose} \qquad \text{Sucrose} + H_2O \longrightarrow \text{glucose} + \text{fructose}$$

Enzyme action therefore involves a stepwise hydrolysis of β-1 : 2-fructofuranosidic linkages, beginning from the non-reducing terminal fructose group, and this experiment provides evidence that inulin is a linear molecule

[105a] Bishop and Whitaker, *Chem. and Ind.*, 1955, 119.
[106] Pringsheim and Ohlmeyer, *Ber.*, 1932, **65**, 1242 ; 1933, **66**, 1292.
[107] Adams, Richtmyer, and Hudson, *J. Amer. Chem. Soc.*, 1943, **65**, 1369.
[108] Legrand and Lewis, *Compt. rend.*, 1951, **232**, 1439.
[109] Edelman and Bacon, *Biochem. J.*, 1951, **49**, 446.
[110] Hongô, *J. Agric. Chem. Soc. Japan*, 1942, **18**, 981 ; *Chem. Abs.*, 1951, **45**, 4753.
[111] Mori, *J. Japan Soc. Food Nutrition*, 1951, **3**, 209 ; *Chem. Abs.*, 1952, **46**, 7139.
[112] Shibuya and Tsukamoto, *J. Japan Biochem. Soc.*, 1950, **22**, 189 ; *Chem. Abs.*, 1951, **45**, 9090.

composed of β-1 : 2-fructofuranose residues, at the reducing end of which is attached a single α-glucosyl residue.

Holden and Tracey [113] have reported that the digestive juices of the snails *Helix pomatia* and *H. aspersa* hydrolyse inulin, grass and bacterial levans (which contain 2 : 6-fructofuranosidic linkages [1]), and irisin; no purification or separation of these activities was recorded. The hydrolysis of grass levan and irisin by extracts of Jerusalem artichoke is incomplete, only *ca.* 20% of the fructoside linkages being broken.[109] Since irisin contains both 1 : 2- and 2 : 6-fructofuranosidic linkages,[1] further enzymic studies should enable the branched nature of this fructosan to be characterised.

The existence of an enzyme catalysing the hydrolysis of bacterial levan has been reported recently.[114] The enzyme (levanpolyase) is produced by levan-forming bacteria, *e.g.*, *Azotobacter chroöcoccum*, when grown on levan; enzyme action is incomplete, the main product being fructosaccharides of mean DP 11. If the same bacteria are grown on sucrose, the culture fluids contain levanpolyase and a second enzyme "levanoligase" which hydrolyses the fructosaccharides to fructose. Levanoligase, but not levanpolyase, also hydrolyses sucrose. Since the enzymic hydrolysis of 2 : 6-fructofuranosidic linkages is uncommon, details of the action pattern of these adaptively produced bacterial enzymes will be awaited with interest.

Enzymic Degradation of Pectic Substances

The pectic substances comprise a group of closely related plant polysaccharides which are polymers of galacturonic acid; the carboxyl groups of the acid residues may be partly neutralised by bases, or may be partly esterified by methyl groups.[115] The most important pectic substance is pectin, a water-soluble polygalacturonide which usually contains about 12% of ester methoxyl and forms gels with sugar and acids under suitable conditions. Pectin occurs in many fruits and vegetables, and is prepared on an industrial scale from citrus fruits. Pectic acid is produced by the de-esterification of pectin with acid or alkali, or with the enzyme pectinesterase (PE; also known as pectase). Chemical studies have shown that the galacturonide linkages in pectic acid are of the α-1 : 4-type.

Pectinesterase occurs in higher plants (*e.g.*, tomato, citrus fruits) and in certain moulds.[116, 117] In the latter sources it is usually associated with polygalacturonase (PG; also known as pectinase), an enzyme catalysing the hydrolysis of the glycosidic linkages in pectic acid but having no action on glycosidic or methyl ester linkages in pectin. Pectinesterase activity may be followed either by determination of the liberated methanol, or by continuous titration of the liberated carboxyl groups with dilute alkali.

[113] Holden and Tracey, *Biochem. J.*, 1950, **47**, 407.

[114] Hestrin and Goldblum, *Nature*, 1953, **172**, 1046.

[115] For reviews see Hirst and Jones, *Adv. Carbohydrate Chem.*, 1946, **2**, 235; Kertesz, "The Pectic Substances", Interscience Publ., New York, 1951; McCready and Owens, *Econ. Bot.*, 1954, **8**, 29.

[116] Kertesz and McColloch, *Adv. Carbohydrate Chem.*, 1949, **5**, 79.

[117] Kertesz, ref. 71, p. 745.

The pure enzyme does not depolymerise pectin and has no action on methyl galacturonate or methyl galacturonide methyl ester. Enzymic demethylation of pectin is rapid, and appears to take place by a stepwise rather than a random action, in contrast to acid or alkali demethylation. Differences have been reported in the physical and chemical properties of pectinesterases from different sources; thus mould pectinesterase has an optimum pH range of 4·5—5·0 and is very thermolabile, whilst tomato pectinesterase is most active at pH 7—8 and is more resistant to inactivation by heat.[118]

Several enzymes are now known which can hydrolyse the α-1 : 4-galacturonide linkages in pectic substances. Recent work has established the existence of two main groups of such enzymes: (*a*) polygalacturonases (PG), which degrade pectic acid to the disaccharide level; and (*b*) pectin depolymerases (PD),* which degrade pectin and pectic acid to polyuronides of low molecular weight. These polyuronides are however susceptible to attack by polygalacturonase. In addition, certain polygalacturonase preparations may contain a galacturonidase capable of hydrolysing di- and tri-galacturonic acid to galacturonic acid.[119]

Polygalacturonase activity has been detected in several moulds, in certain bacteria, in barley malt, and in snail digestive juices.[116, 117] In the mould *Aspergillus fœtidus*, polygalacturonase is associated with a pectin depolymerase.[119] Fungal polygalacturonase has been purified by several workers, and has been widely studied. It is a highly specific enzyme; it does not attack pectin or the Pneumococcus Type I polysaccharide (which contains 60% of α-1 : 4-D-galacturonic acid residues).[120] It catalyses random hydrolysis of pectic acid, the viscosity of the substrate being halved at only 2% hydrolysis, and at intermediate stages of hydrolysis, tri-, tetra-, and penta-galacturonic acids are produced.[121]. The composition of the end-products of polygalacturonase action appears to depend on the particular enzyme preparation used; di- and tri-galacturonic acid are the main products when an *Aspergillus fœtidus* preparation is used,[119] whereas other purified fungal polygalacturonases yield galacturonic acid as the sole product.[11] The rates and extents of hydrolysis of three pectic acids ranging in molecular weight from 1900 to 35,000 have been found to be identical.[11] A limited number of yeasts (*e.g.*, *Saccharomyces fragilis*) produce an exocellular polygalacturonase which resembles the fungal enzyme in that the extent of hydrolysis of partially demethylated pectins is inversely proportional to the methoxyl content of the pectin; it differs, however, since the hydrolysis of pectic acid is incomplete, the end-products being mono- and di-galacturonic acids.[122] Physicochemical studies of purified yeast

[118] McColloch and Kertesz, *Arch. Biochem.*, 1947, **13**, 217; Calesnick, Hills, and Salaman, *ibid.*, 1950, **29**, 432.

[119] Ayres, Dingle, Phipps, Reid, and Solomons, *Nature*, 1952, **170**, 834.

[120] Lineweaver, Jang, and Jansen, *Arch. Biochem.*, 1949, **20**, 137.

[121] Altermatt and Deuel, *Helv. Chim. Acta*, 1952, **35**, 1423; Rahman and Joslyn, *Food Research*, 1953, **18**, 308.

[122] Luh and Phaff, *Arch. Biochem. Biophys.*, 1954, **48**, 23; Demain and Phaff, *Biol. Chem.*, 1954, **210**, 381.

* This abbreviation is preferable to DP as suggested by Kertesz (ref. 117).

polygalacturonase support the hypothesis that the activity is due to a single enzyme.[123]

Certain fungal polygalacturonase preparations appear to be heterogeneous. Schubert [124] observed that culture extracts of *Aspergillus niger* contained four different polygalacturonase enzymes, which were differentiated by selective inactivation or selective adsorption. Full details of the specificity of these four enzymes were not recorded. Polygalacturonase preparations from *Aspergillus fœtidus* contain two enzymes; one of these appears to be a type of pectin depolymerase since it degrades pectin or pectic acid to polyuronides of low molecular weight, whilst the second enzyme shows true polygalacturonase activity in that it degrades pectic acid (and the polyuronides of low molecular weight) to a mixture of di- and trigalacturonic acid.[119, 125]

The existence of pectin-depolymerases has only recently been verified, although their presence in pectolytic preparations has been suspected for some time. Thus, pectolytic preparations from tomatoes contain an enzyme which differs from fungal polygalacturonase in that it is relatively insensitive to heat, has an optimum pH of 4·5 (that of fungal polygalacturonase is 3·5), and causes only partial breakdown of pectic acid, the end-products being polyuronides of DP > 5.[116, 126] The mould *Neurospora crassa* produces an extracellular enzyme which partly degrades pectin and pectic acid; [127] the end-product of this action, at *ca.* 30% hydrolysis, is a polygalacturonide of molecular weight 4000. A similar depolymerising enzyme has been obtained from a commercial enzyme preparation and named polymethylgalacturonase (PMG).[128] Purified polymethylgalacturonase, which has negligible pectinesterase activity, catalyses a 26% hydrolysis of pectin and pectic acid. Enzyme action was random, since at 0·5% hydrolysis the viscosity of the substrates was halved. The end-product of this action had $[\alpha]_D + 221°$ (those of pectin and galacturonic acid are $+ 235°$ and $+ 56°$ respectively), and was immobile on a paper chromatogram. Cell-free extracts of *Bacterium aroideæ* also contain a depolymerising enzyme which rapidly reduces the viscosity of pectic acid or pectin (OMe 9%) but with little increase in reducing power.[129] Pectin depolymerases thus differ from polygalacturonases in (*a*) ability to degrade pectin without previous de-esterification, (*b*) inability to effect complete hydrolysis, (*c*) chemical and physical properties, *e.g.*, optimum pH.

It is now apparent that the field of pectolytic enzymes is as complex as that of amylolytic enzymes, and future studies must be directed towards the preparation of homogeneous enzymes which are essential for detailed investigations of enzyme action.

[123] Demain and Phaff, *Nature*, 1954, **174**, 515.
[124] Schubert, *Nature*, 1952, **169**, 931.
[125] Dingle, Reid, and Solomons, *J. Sci. Food Agric.*, 1953, **4**, 149.
[126] McColloch and Kertesz, *Arch. Biochem.*, 1948, **17**, 197.
[127] Roboz, Barratt, and Tatum, *J. Biol. Chem.*, 1952, **195**, 459.
[128] Seegmiller and Jansen, *ibid.*, p. 327.
[129] Wood, *Nature*, 1951, **167**, 771.

Summary and Conclusions

In this Review, present knowledge of the enzymes catalysing the hydro-tic cleavage of many polysaccharides has been summarised. Since early ork in this field has been adequately reviewed elsewhere, attention has een focused on the more recent developments, with particular reference o the use of enzymes in structural investigations of polysaccharides.

Enzymic studies have been used to determine the nature of the repeating nit in many polysaccharides (Table I), and have also been used to char-cterise the branching-linkages in a number of branched polysaccharides. n all cases, the results have confirmed those obtained by purely chemical ethods. Furthermore enzymic studies provide the only available method r determining the degree of multiple branching in starch-type polysac-harides, and for locating the mean position of the branching points in the onstituent chains.

It seems probable that during the next decade similar enzymic structural nalyses of other polysaccharides will be possible as the appropriate poly-accharases are isolated, purified, and characterised. In the search for dditional sources of polysaccharases, further study of the mammalian igestive systems and the enzyme systems of many higher plants would e of value. Perhaps the greatest promise in this field is in the adaptive roduction of polysaccharases by certain bacteria and moulds. Amylase, ellulase, dextranase, and polygalacturonase, essentially free from other olysaccharases, have been produced in this way. Although an extensive earch may well be necessary to find a micro-organism which could be duced to grow in a medium containing, for example, araban as sole rbohydrate source, the specific arabanase(s) so produced would be an valuable tool for the chemist engaged in structural investigations of the veral plant gums and mucilages which contain L-arabinose.

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Enzymological Section

The properties of P-Enzyme, Glycogen phosphorylase, Q-Enzyme and Branching Enzyme

P-ENZYME

P-enzyme catalyses a similar reaction to glycogen phosphorylase [Hanes, C.S. (1940), Proc.Roy.Soc.B, 129, 174]. Acting with Q-enzyme, P-enzyme action on G 1-P results in starch synthesis in photosynthetic plants, especially tubers and species of Gramineae, Leguminoseae and fresh-water algae.

Potato P-enzyme has been studied in most detail, and was crystallised after fractionation of potato juice (to remove α-amylase, maltase, phosphatase, D-, Q- and R-enzyme) with $(NH_4)_2SO_4$ (300-fold purification) [Fischer, E.H. and Hilpert, H.M. (1953), Experientia, 9, 176] or ethanol in presence of amylose [Baum, H. and Gilbert, G.A. (1953), Nature, 171, 983]. P-enzyme is assayed from the inorganic phosphate liberated during amylose synthesis from G 1-P [Green, D.E. and Stumpf, P.K. (1942), J.biol.Chem. 142, 355]. Activity is optimum at pH 6.0 and is greatly reduced at temperatures $> 50^{\circ}$.

P-enzyme is specific for G 1-P but can use maltotetraose (and higher maltodextrins), amylose, amylopectin or glycogen as primer molecules. In presence of phosphate, maltodextrins and the starch components are degraded by multi-chain action to G 1-P [Whelan, W.J. and Bailey, J.M. (1954), Biochem.J. 58, 560]. In presence of arsenate, slow degradation to D-glucose via an unstable α-D-glucosyl arsenate occurs [Katz, J. and

Hassid, W.Z. (1951), Arch.Biochem. 30, 272].

The equilibrium between inorganic phosphate and G 1-P is pH-dependent; the ratio varies from 10.8 at pH 5.0 to 3.1 at pH 7.0 although the ratio $[HPO_4]^{2-}$ / $[C_6H_{11}O_5.O.PO_3]^{2-}$ remains constant at 2.2 (Hanes 1940). The free-energy change during amylose synthesis is -1460 cal./mole. The respective K_m values for P-enzyme and inorganic phosphate, G 1-P, arsenate and starch are 6.2 x 10^{-3}M, 2.6 x 10^{-3}M, 6 x 10^{-3}M and 0.97 g./l. [Weibull, C. and Tiselius, A. (1945), Arkiv. Kemi, Mineral Geol. A19, No.19; Katz and Hassid, 1951].

Unlike muscle phosphorylase, potato P-enzyme does not require AMP for activity, is not inhibited by phlorhizin, has little action on glycogen and can degrade maltodextrins. The active groups in P-enzyme are not known, although it is inhibited by Ag^+, Cu^{2+} and F^-, but not by $HgCl_2$ (cf. amylases and Q-enzyme).

A related enzyme from baker's yeast has also been studied [Meyer, K.H. and Bernfeld, P. (1942), Helv.chim.Acta, 25, 399].

GLYCOGEN PHOSPHORYLASE

Phosphorylase catalyses the synthesis and degradation of amylose-type molecules by transglucosylation from α-D-glucosyl phosphate

(G 1-P):

$$mG\ 1\text{-}P + [G]_n \rightleftharpoons [G]_{n+m} + mH_3PO_4 \qquad (1)$$

where $[G]_n$ or $[G]_{n+m}$ represent a chain of n or n + m α-1:4-linked glucose residues (usually part of a glycogen molecule). This enzyme largely controls the metabolism of glycogen in animal cells. Phosphorylases from rabbit muscle [Cori, C.F., Cori, G.T. and Green, A.A. (1943), J.biol.Chem. 151, 21, 31, 39, 57; (1945), J.biol.Chem. 158, 315, 321, 333, 341; Madsen, N.B., Cori, C.F. and Gurd, F.R.N. (1956), J.biol.Chem. 223, 1055, 1067, 1075; (1957), J.biol.Chem. 224, 899], dog-heart muscle [Rall, T.W., Wosilait, W.D. and Sutherland, E.W. (1956), Biochim.biophys.Acta, 20, 69] and dog liver [Sutherland, E.W. and Wosilait, W.D. (1956), J.biol.Chem. 218, 459 et seq.] have been extensively studied.

Phosphorylase from resting rabbit muscle (phosphorylase a) has been purified ca. 40-fold by $(NH_4)_2SO_4$ fractionation and crystallized [Cori, G.T., Illingworth, B. and Keller, P.J. (1955), Methods in Enzymology, 1, 200]. It is assayed by the amount of inorganic phosphate liberated from G 1-P during glycogen synthesis, under first-order conditions. The crystalline enzyme has an activity of ca. 3500 units per mg. at pH 6.7 (optimum pH range 6.5-6.9) and 30°, equivalent to a

turnover number of 40,000 G 1-P moles. The activation energy is ca. 25,000 cal./mole.

Phosphorylase is specific for G 1-P [Campbell, P.N., Creasey, N.H. and Parr, C.W. (1952), Biochem.J. 52, 448], for which K_m is 5.7×10^{-3}M (Cori et al. 1943). In presence of inorganic phosphate, glycogen, amylopectin and amylose are partly degraded to G 1-P; linear maltodextrins are not attacked [Hestrin, S. (1949), J.biol.Chem. 179, 943]. [For the complete metabolism of glycogen, branching enzyme and amylo-1:6-glucosidase are required.] K_m for glycogen is 0.20 g./l.

The equilibrium, which favours synthesis, is pH-dependent; the ratio inorganic phosphate:G 1-P is 3.6 at pH 6.8 and 6.1 at pH 6.0. For maximum activity, adenosine 5'-phosphate (AMP; activator) and traces of cysteine or EDTA are required. In absence of AMP, only 65% maximum activity is obtained; 4 moles AMP are bound/mole enzyme. Glucose is a competitive inhibitor, while phlorhizin and p-chloromercuribenzoate are non-competitive inhibitors. The latter combines with 18 SH groups/enzyme molecule which is split into 4 'monomeric units' of mol. wt. 135,000 (Madsen et al. 1956, 1957). Phosphorylase a may therefore have 4 active centres.

Phosphorylase a (mol. wt. 495,000) is converted by trypsin

or phosphorylase-rupturing (PR) enzyme from rabbit muscle into a more soluble b form (mol. wt. ca. 250,000). Phosphorylase b, inactive in the absence of AMP, is present in resting muscle, and is converted into the a form by an enzyme system requiring ATP and Mn^{2+} [Fischer, E.H. and Krebs, E.G. (1958), J.biol.Chem. 231, 65, 73]. Crystalline phosphorylase a and b contain 4 and 2 moles of pyridoxal 5-phosphate respectively per mole, which does not appear to participate directly in the enzymic reaction [Illingworth, B., Jansz, H.S., Brown D.H. and Cori, C.F. (1958), Proc.nat.Acad.Sci. 44, 1180; Kent, A.B., Krebs, E.G. and Fischer, E.H. (1958), J.biol.Chem. 232, 549].

The enzymic inactivation and reactivation of dog-liver phosphorylase (LP) has been studied (Wosilait and Sutherland, 1956). A soluble liver enzyme converts LP into an inactive form with the release of inorganic phosphate, but without altering the sedimentation constant of the LP-protein molecule. Inactive-LP is not affected by AMP, but is reactivated by the enzyme dephosphophosphorylase kinase in presence of ATP and Mg^{2+}. This system incorporates inorganic phosphate into the LP-protein molecule, and is stimulated by glucagon and epinephrine [Rall, T.W., Sutherland, E.W. and Berthet, J. (1957), J.biol.Chem. 224, 463].

It is of interest to note that phosphorylases from different

organs of the same animal, and from the same organ of different species, are immunologically different [Henion, W.F. and Sutherland, E.W. (1957), J.biol.Chem. 224, 477].

Q-ENZYME AND BRANCHING ENZYME

These enzymes catalyse the synthesis of α-1:6-glucosidic linkages in amylose-type chains with the formation of amylopectin and glycogen, respectively. The concurrent action on G 1-P of P- and Q-enzymes [Barker, S.A., Bourne, E.J., Peat, S. and Wilkinson, I.A. (1950), J.chem.Soc. p.3022] or of glycogen phosphorylase and branching enzyme [Cori, G.T., and Cori, C.F. (1943), J.biol.Chem. 151, 57] also results in amylopectin, or glycogen, synthesis. The mechanism of action has been reviewed [Barker, S.A. and Bourne, E.J. (1953), Quart.Reviews, 7, 65]. Enzymes with similar functions are present in all animal and plant cells which store glycogen and starch.

Q-ENZYME

This has been crystallized after fractionation of potato juice with ethanol [Gilbert, G.A. and Patrick, A.D. (1952), Biochem.J. 51, 181]. Enzymic activity, optimum at pH 7 and 20°, is measured by the decrease in iodine-staining power of

amylose or starch; it is activated by salts, e.g. $(NH_4)_2SO_4$, and is not inhibited by phlorhizin. However, most Q-enzyme preparations are contaminated with D-enzyme [Peat, S., Whelan, W.J. and Rees, W.R. (1956), J.chem.Soc. p.44], although this does not affect Q-enzyme action.

The activity of Q-enzyme is irreversible; it has little or no action on amylopectin or on amylose chains of DP (degree of polymerization) $<$ 40 [Peat, S., Whelan, W.J. and Bailey, J.M. (1953), J.chem.Soc. p.1422]. Q-enzyme from broad bean and wrinkled pea has similar properties to potato Q-enzyme [Hobson, P.N., Whelan, W.J. and Peat, S. (1950), J.chem.Soc. p.3566].

The existence of isophosphorylase, a potato enzyme reputed to catalyse the reversible formation of α-1:6-linkages in amylopectin has been disproved [Bailey, J.M. and Whelan, W.J. (1950), J.chem.Soc. p.3573].

The flagellated protozoon Polytomella coeca contains a Q-enzyme [Bebbington, A., Bourne, E.J., Stacey, M. and Wilkinson, I.A. (1952), J.chem.Soc. p.240], which was purified via a lead complex and $(NH_4)_2SO_4$ fractionation; it showed optimum activity at pH 7.3 and 25-33°.

BRANCHING ENZYME

Branching enzyme (amylo-1:4 $\longrightarrow$ 1:6-transglucosidase) from rat or rabbit liver has been purified by acid precipitation of a

homogenate and adsorption of contaminating α-amylase on starch [Larner, J. (1955), Methods in Enzymology, 1, 222]. This enzyme has little action on amylose, but increases the degree of branching of amylopectin.

A related enzyme, from brewer's yeast, was purified by ethanol-citrate fractionation at -5° [Gunja, Z.H., Manners, D.J. and Khin Maung (1960), Biochem.J. in the press]. Enzyme action is optimum at pH 7 and 20° and is inhibited by $HgCl_2$. Yeast-branching enzyme introduces α-1:6-linkages into both amylose and amylopectin.

Polytomella coeca Q-enzyme is activated by short-chain maltosaccharides including maltose, which probably function as acceptor molecules [Barker, S.A., Bebbington, A. and Bourne E.J. (1953), J.chem.Soc. p.4051]. In contrast, branching enzymes from rat liver, potato, broad bean, wrinkled pea [Larner, J. and Uwah, D.N. (1956), J.Amer.chem.Soc. 78, 3647] and yeast (Gunja et al., 1960) are not activated by maltose.

The branching enzymes are, in general, unstable; they are inactivated (wholly or partly) in aqueous solution, on dialysis or on precipitation with organic solvents, and are extremely thermolabile. Purification, particularly the separation from α-amylase, is difficult. Information on the chemistry of the enzyme-proteins is not therefore available.

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TRANS α- and β-GLUCOSYLATION REACTIONS

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Although the enzymic synthesis of starch and dextran-type polysaccharides by transglucosylation reactions is widely known, the fact that certain glucosides and oligosaccharides may be synthesised by related types of group-transfer reactions is a more recent discovery in carbohydrate biochemistry. The reactions are of the general type:-

$$G\text{-}O.X + H\text{-}O.R \longrightarrow G\text{-}O.R + H\text{-}O.X \qquad (1)$$

where G-O.X represents a glucoside (glucosyl donor), and H-O.R an acceptor substrate; typical examples are shown in Table 1.

Since the synthesis of polysaccharides and the role of UDPG in transglucosylation reactions are discussed elsewhere, the present review will be confined to the enzymic synthesis of oligosaccharides and glucosides. These appear to be formed by two classes of enzymic reaction; firstly, the synthesis of some polysaccharides e.g., dextran, nigeran, cellulose by micro-organisms is accompanied by the formation of small amounts of oligosaccharides. By varying the experimental conditions, the latter synthesis can be increased at the expense of polysaccharide production e.g. in the formation of dextran from sucrose by Leuconostoc Mesenteroides, small quantities of leucrose are produced (i.e. glucosyl transfer to fructose rather than to low-molecular weight dextran) and the formation of this disaccharide is favoured by a high sucrose concentration. [1]

Secondly, a number of hydrolytic enzymes (carbohydrases) which normally catalyse the hydrolysis of glucosidic linkages (i.e. H-O.R is a water molecule in eqn. 1) may, with high substrate concentrations (e.g., 10-30% w/v) or in the presence of added acceptors, transfer glucosyl residues to a carbohydrate substrate rather than to water, resulting in the formation of a new glucosyl derivative. The nature of the product will depend upon the acceptor specificity of the carbohydrase.

The experimental methods involve incubation of glucosyl donor and possible acceptor substrates with the appropriate enzyme preparation, followed by chromatographic separation (usually on charcoal - Celite [2]) of the resulting mixed carbohydrates, and characterisation by chemical and enzymic methods. The enzyme preparations include cell-free extracts of micro-organisms, and protein fractions from various animal or plant extracts. Unfortunately, the majority of these preparations are not enzymically homogeneous so that deductions with regard to acceptor specificity require caution. For this reason, the term "enzyme-system" will be used when dealing with this aspect of transglucosylation reactions. Since many carbohydrases and polysaccharide-synthesizing enzymes are microbial in origin, an alternative technique involves the growth of the organism on a synthetic medium

to which potential glucosyl donor and acceptor substrates have been added. In such cases, sterilisation of media by Seitz filtration rather than by autoclaving is advisable since the latter process may result in the chemical formation of small quantities of oligosaccharide impurities [3].

Trans-α-glucosylation reactions

(a) Polysaccharide-metabolizing enzymes. The ability of four such enzymes (dextransucrase, amylomaltase, D-enzyme and Bacillus macerans amylase) to synthesize oligosaccharides by the transfer of one or more α-glucosyl residues will be discussed; in all cases, the configuration at the anomeric C atom is retained.

Dextransucrase preparations from L. mesenteroides [4], Betacoccus arabinosaceous (a related strain) [5], and Streptococcus bovis [6] catalyse the synthesis of dextran from sucrose:-

Sucrose + Acceptor ⟶ Fructose + α-Glucosyl acceptor ——②

where the normal acceptor is a chain of α-1:6-linked glucose residues. However, in the presence of various mono- and disaccharides, the yield of dextran is lowered, the reaction rate is increased, and oligosaccharides are the main products [7]. This suggests that the added sugars are functioning as alternative acceptors and act as 'primers' for the formation of short chains of α-1:6-linked glucose residues. The

enzyme-system appears to be completely specific for the glucosyl donor (sucrose) but to be less specific for the acceptor substrate; nevertheless, the α-glucosyl group is always transfered to a primary alcohol group on the acceptor e.g., with 3-O-methyl D-glucose, a homologous series of oligosaccharides based on 6-O-α-D-glucopyranosyl-3-O-methyl-D-glucose is produced [8].

The synthesis and degradation of α-1:4-glucosans by certain strains of Escherichia coli from maltose is catalysed by amylomaltase [9]:-

$$n \text{ maltose} \rightleftharpoons n \text{ glucose} + (\text{glucose})^n \qquad (3)$$

At equilibrium, the product of the trans α-glucosylation reaction is a series of maltosaccharides containing 3-6 glucose residues. [10] In the presence of glucose oxidase, this equilibrium is altered and the synthesis of blue-iodine-staining amylose-type material occurs. The transfer is therefore limited to the hydroxyl group at C(4) of the non-reducing end-group of a maltosaccharide.

A related enzyme which catalyses the transfer of α-glucosyl residues to and from various maltosaccharides has been isolated from potato juice, and named D-enzyme [11]. A typical disproportionating action, which is freely reversible, is illustrated in eqn. 4.

$$\text{maltotriose} + \text{maltotriose} \rightleftharpoons \text{maltopentaose} + \text{glucose} \qquad (4)$$

Unlike other trans α-glucosylases, the transfer of two or more

α-glucosyl residues may be effected, e.g., eqn. 4 shows the transfer of a maltosyl residue. Maltotriose and higher maltosaccharides, amylose or the outer chains of an amylopectin molecule can function as donor substrates, but glucose and maltose are inactive. The specificity towards acceptor substrates is lower; glucose is the most effective, but nine other carbohydrates show activities ranging from 5-37% that of glucose, including methyl α-D-glucoside, leucrose and D-mannose. In all cases, transfer is to the hydroxyl group at C(4). The equilibrium shown in eqn. 4 may be altered by the presence of a glucose-removing enzyme system (e.g., hexokinase and ATP) so that iodine-staining amylose-type polysaccharide is produced [12]. D-Enzyme may thus provide an alternative system for the synthesis of amylose.

The final enzyme in this group, Bacillus macerans amylase, has been known for many years [13]. Enzyme action on starch normally results in the formation of cyclic Schardinger dextrins (DP 6-8), but recent studies have shown that in the presence of a suitable co-substrate, the cyclodextrins are converted into linear molecules containing the co-substrate at the reducing-end e.g., with cyclohexa-amylose and methyl α-D-glucoside, methyl α-D-maltoheptaoside is produced. Furthermore, this amylase can transform a linear maltosaccharide into a mixture of linear and cyclic compounds e.g., with maltoheptaose $[G]^7$:-

$$[G]^7 + [G]^7 \longrightarrow [G]^4 + [G]^{10}$$

$$[G]^{10} \longrightarrow [G]^4 + \text{cyclohexa-amylose}$$

The ability of B. macerans amylase to catalyse oligosaccharide formation from maltose provides a further difference from D-enzyme.

(b) Maltases. A large number of maltase preparations catalyse the synthesis of oligosaccharides from concentrated (10-20%) solutions of maltose. In such cases, either glucose or maltose acts as an initial acceptor. The acceptor specificity of these enzyme systems appears to vary with the biological source (see Table 2). Mould enzymes transfer predominantly to the primary alcohol group at C(6) of glucose, or of the terminal non-reducing glucose residue of maltose, isomaltose and panose. By contrast, transfer by bacterial or mammalian liver preparation is confined to the hydroxyl group at C(4) of the terminal non-reducing residue of maltose or a maltosaccharide with the production of higher maltosaccharides. Intermediate between these types are the maltase-systems present in extracts of marine algae, brewers' yeast and the protozoan Tetrahymena pyriformis which can use hydroxyl groups at both C(4) and C(6) as acceptor sites. Whether or not this activity is due to the presence of two distinct 'maltase' enzymes is not yet known.

The transfer of α-glucosyl groups from maltose to hydroxyl groups at C(2) and C(3) of glucose does not appear to be common, although the synthesis of nigerose (α-1:3-linkage) by an A. oryzae preparation has been reported [23]. It is possible,

however, that such transfer reactions have been overlooked, since Kojibiose (α-1:2-linkage) and nigerose (and related oligosaccharides) have similar paper chromatographic mobilities to maltose (and maltotriose).

The enzymic synthesis of riboflavinylglucoside from maltose and riboflavin by rat liver provides a further example of trans α-glucosylation. With maltose, maltulose or turanose as donor substrates, riboflavin and certain other isoalloxazine derivatives can function as acceptor substrates [24]. Riboflavinylisomaltosaccharides may also be produced by further transfer to riboflavinylglucoside using related enzyme preparations from A. oryzae and E. Coli [25]. The relationship of these enzyme-systems to those reported in Table 2 is not known.

Trans β-glucosylation reactions

(a) Cellobiose and Cellulose-metabolizing enzymes. The metabolism of cellobiose by enzyme preparations from higher plants, marine algae, and various micro-organisms has been widely studied [26]. With concentrated solutions of cellobiose, oligosaccharides are synthesised including some of the following:- gentiobiose, laminaribiose, sophorose, gentiotriose, cellotriose, 6^2-β-glucosylcellobiose and 3^2-β-glucosylcellobiose (for nomenclature, see ref. 26). Transfer of a β-glucosyl residue to hydroxyl groups at C(6), C(3) or C(2) of glucose or to C(4), C(6) or C(3) of the non-reducing residue of

cellobiose is indicated. In general, the trans β-glucosylases appear to show a lower specificity towards the acceptor molecule than the corresponding trans α-glucosylases, although transfer to a primary alcohol group may predominate. Furthermore, differences in acceptor specificity between enzyme preparations from different biological sources are not so marked.

The synthesis of cellulose from glucose by certain Acetobacter species is accompanied by oligosaccharide formation [27]. These include cellobiose, cellotriose, cellotetraose and oligosaccharides containing fructose residues and phosphorylated sugar residues. Since mutant organisms which are unable to synthesize cellulose did not produce these oligosaccharides when grown on the same synthetic medium, cellulose synthesis and oligosaccharide formation may be inter-related. Other species of Acetobacter which do not produce cellulose, when grown in media containing glucose as sole carbon source, synthesized gentiobiose, sophorose and higher oligosaccharides [28]. This suggests that in these micro-organisms, enzymes capable of polymerizing glucose are present, in addition to trans β-glucosylases.

(b) Aryl β-glucosidases. Although extracts of many plants and moulds show carbohydrase activity towards cellobiose, and aryl β-glucosides, recent studies have shown that these activities are due to distinct enzymes. Aryl β-glucosidase

preparations devoid of cellobiase activity have been isolated from Stachybotrys atra [29], apricot emulsin, A. niger and P. chrysogenum [30] and Myrothecium verrucaria [31]. These preparations catalyse reactions of the type first discovered by Rabaté in 1935:-

Aryl β-glucoside + Aliphatic Alcohol ⟶ Alkyl β-glucoside + Aryl hydroxide ——————— ⑤

Using p-nitrophenyl β-glucosides as donor, a whole range of primary aliphatic alcohols (but not glycerol and secondary and tertiary alcohols) will act as acceptors, with the M. verrucaria enzyme system [31]. The latter shows a marked preference for alcohol rather than water as an acceptor substrate; in only 3 M-methanol, 64% of the glucose is transferred to the alcohol.

The fungus Irpex lacteus contains a related enzyme which acting on p-nitrophenyl β-cellobioside in the presence of methanol, catalyses the formation of methyl β-cellobioside [32]. This demonstration of cellobiosyl transfer may have in vivo significance.

The Mechanism of Transglucosylation Reactions

It is now generally accepted that transfer reactions of the type shown in eqn. 1 involve the formation of an intermediate glucosyl-enzyme complex:-

$$G\text{—}O.R + Enz.\text{—}H \rightleftharpoons G\text{—}Enz. + H\text{—}O.R \quad \text{——————} \quad (6a)$$

$$G\text{—}Enz. + H\text{—}O.X \rightleftharpoons G\text{—}O.X + Enz.\text{—}H \quad \text{——————} \quad (6b)$$

This view is supported inter alia, by (a) the effect of

glucose oxidase on the amylomaltase system [9], and of hexokinase-ATP on D-enzyme action [12], (b) kinetic studies of β-glucosidase activity [33] and (c) the results of ^{14}C-tracer studies using mould or rat liver trans-α-glucosylases [15, 17, 23].

In the reactions so far discussed, the configuration of the glucosidic linkage is retained. One possible explanation is that reactions 6a and 6b occur by a double-displacement mechanism involving two successive Walden inversions [34], although direct evidence for the formation of an intermediary β-glucosyl-enzyme complex during trans α-glucosylation is not yet available.

The occurrence of a small number of reactions involving inversion of configuration has been reported. These include the hydrolysis of starch by β- and γ- amylase [35], and A. niger amyloglucosidase [36], the hydrolysis of maltose and cyclohexaamylose by taka-diastase [37], and the maltose and cellobiose phosphorylase reactions catalysed by preparations from Neisseria meningitidis [38], and Ruminococcus flavefaciens [39] respectively:-

maltose + inorganic phosphate $\rightleftharpoons$ β-D-glucosyl 1-phosphate + glucose

cellobiose + inorganic phosphate $\rightleftharpoons$ α-D-glucosyl 1-phosphate + glucose

These reactions may occur by single displacement mechanisms [34].

The use of "unatural" acceptor and ^{14}C labelled donor substrates has facilitated studies on the specificity and mechanism of transglucosylation reactions. Examples of the former are given in Table 3 where substrates lacking the normal acceptor site are incubated with the transglucosylase and a glucosyl donor. With A. niger enzyme systems, which transfer predominantly to primary alcohol groups, trans β-glucosylation to C(3) and trans α-glucosylation to C(2) are favoured with pentose or uronic acid acceptor substrates, respectively [42, 43]. The first syntheses of 'branched' trisaccharides has been achieved in this way. When cellobiose or lactose rather than maltose is the acceptor, transfer to C(2) of the reducing glucose residue is favoured, by dextran sucrase [45]. The mechanism of this reaction [48] and those catalysed by D-enzyme [49], B. macerans amylase [13], mould trans α-glucosylase [17] and rat liver trans α-glucosylase [15] have been investigated by the use of ^{14}C-tracer techniques.

Conclusions

The study of transglucosylation reactions is becoming of increased importance. It is yielding information on carbohydrase-specificity, and possible metabolic pathways for the formation of glucosides and oligosaccharides; the latter may represent intermediates or 'primers' in the synthesis of

polysaccharides. Furthermore, transglucosylation reactions may provide a method for the formation of branch-points in highly branched polysaccharides. In addition, these reactions enable a number of oligosaccharides including leucrose [1], panose [50], glucosyl-pentose disaccharides [40, 41, 47], pseudoaldobiouronic acids [43], maltosaccharides [49], methyl α- and β-maltosaccharides [51] and methyl α-isomaltosaccharides [52] to be conveniently prepared. With the development of synthetic methods [53] for the hitherto 'rare' sugars gentiobiose, nigerose and kojibiose, the range of potential donor and acceptor substrates is being increased. Finally, improved methods of protein fractionation (especially chromatographic techniques) should lead to the isolation of homogeneous enzymes, which in turn, will yield more accurate and unambiguous information on the specificity and mode of action of these important group-transferring enzymes.

Table 1. Types of trans α- and β-glucosylation reaction

Glucosyl Donor	Glucosyl Acceptor	Product	Enzyme Source
D-Glucosyl phosphate	Maltosaccharide	Amylose*	Higher plants, mammalian liver and muscle.
crose	Low molecular weight dextran	High molecular weight dextran*	Bacteria
D-Glucosyl phosphate	Fructose	Sucrose	Bacteria
PG	Fructose	Sucrose	Higher plants
ltose	Maltose	Panose	Moulds
omaltose	Isomaltose	Isomaltotriose	Moulds
llobiose	Cellobiose	Cellotriose	Moulds
Nitrophenyl β-D-ucoside	Methanol	Methyl β-D-glucoside	Moulds

*Produced by repeated transfer of glucosyl residues to the same acceptor molecule.

Table 2. Oligosaccharides synthesized from maltose by various enzyme preparations

Oligosaccharides	Enzyme Source	Ref.
Maltotriose and higher maltosaccharides	Rat liver	14, 15
	Bovine plasma	16
	Escherichia coli	10
Isomaltose, isomaltotriose, panose and related oligosaccharides	*Aspergillus oryzae*	17
	Aspergillus niger	18
	Penicillium chrysogenum	19
Maltotriose, panose, maltotetraose and 6^3-α-glucosylmaltotriose	*Cladophora rupestris*	20
	Saccharomyces cerevisae	21
	Tetrahymena pyriformis	22

Table 3. Transglucosylation to acceptors other than glucosaccharides

Donor	Acceptor	Product*	Enzyme Preparation	Ref.
ellobiose	Xylose	G.1.β.3.X	A.niger / C. rupestris	40 / 41
altose	Sucrose	G.1.α.6.G.1.αβ.2F.	A.niger	42
altose	D-glucurone	G.1.α.2.GU (G.1.α.6.G.1.α.2.GU)	A.niger	43
altose	Fructose	G.1.α.6.F.	Yeast	44
ucrose	Cellobiose	G.1.β.4 / G.1.α.2)G	B.arabinosaccous	45
ucrose	Lactose	Gal.1.β.4) / G.1.α.2)G	B.arabinosaccous	45
ucrose	Raffinose	Gal.1.α.6) / G.1.α.2)G.1.αβ.2F	L.mesenteroides	46
-D-glucosyl hosphate	Xylose	G.1.α.4.X≠	N.meningitidis	47

*The following abbreviations are used: G, D-glucose; F, D-fructose; X, D-xylose; GU, D-glucuronic acid; Gal, D-galactose. The figures and α or β define the glycosidic linkages.

≠ Inversion of configuration.

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