BIOCHEMICAL STUDIES ON STARCH-TYPE POLYSACCHARIDES.

- by -

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ABSTRACT OF THESIS

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Title of Thesis "Biochemical Studies on Starch-Type Polysaccharides".

In this thesis, chemical and enzymic studies on a number of starch-type polysaccharides are described, with special reference to the possible presence of anomolous linkages or other structural modifications.

The action of hot dilute hydrochloric acid on maltose in concentrations usually employed for the partial acid hydrolysis of polysaccharides has been shown to give rise to nigerose, a disaccharide containing an a-1,3-linkage in amounts that would account for the presence of this disaccharide in partial acid hydrolysates of some starch-type polysaccharides. Some evidence has been adduced for the retention of configuration of the glucosidic linkage during this acid catalysed transglucosidation. There is therefore no satisfactory evidence for the occurrence of a-1,3-linkages in starch-type polysaccharides.

A preliminary survey of the enzymic degradation products of some polysaccharides has been carried out for fructosyl containing disaccharides. The isolation and partial characterisation of maltulose from an a-amylolysate of waxy-maize starch suggests that fructosyl residues occur in the original polysaccharide.

An extract from sweet-corn flour was subjected to ammonium sulphate fractionation followed by continuous electrophoresis. Further fractions were obtained that contained branching activity. Incubation of the sweet-corn branching enzyme (SCBE) with amylopectin gave rise to a glycogen-like polysaccharide which was characterised by the analytical use of enzymes. The SCBE yielded a branched polysaccharide on incubation with amylose. Some of the properties of the SCBE were characterised: viz., pH- and temperature optimum, susceptibility to inorganic salts. Evidence was also obtained for the presence of a sweet-corn debranching enzyme (SCDBE), and its pH-optimum determined.

Biopsy specimens from seven suspected cases of glycogen storage disease were examined for glycogen content, phosphorylase and glucose-6-phosphatase activity. The glycogen structure was partially characterised in most of these, and an estimation of the erythrocyte glycogen was made in two of the cases. Data was obtained which enabled two of the cases to be discounted as suffering from glycogenosis, and four others for a clear diagnosis to be made of the glycogenosis type. The remaining case was not very clearly defined either from a biochemical or clinical point of view.

Use other side if necessary.

To my Father.

ACKNOWLEDGMENTS.

I wish to thank Professor Sir Edmund Hirst, C.B.E., F.R.S., for the interest taken in this work, and for the provision of laboratory facilities.

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GENERAL INTRODUCTION.

In the structural investigation of polysaccharides, a number of purely chemical techniques have been developed. For example, the treatment of wood cellulose (the most abundant polysaccharide) with strong mineral acid yields the information that this material is composed entirely of D-glucose. Similar treatment of potato starch, another typical polysaccharide, yields identical information. Wood cellulose and potato starch are classified as homopolysaccharides, i.e. they are both composed of a single constituent sugar. The biological function of each of these two materials, however, is entirely different; the cellulose in wood is a structural feature of the plant and is virtually metabolically inert, whereas the starch of potato is metabolically very active and has no structural significance. It is therefore fundamentally unlikely that the molecular structure of these two materials is the same. In contrast to homopolysaccharides, heteropolysaccharides are composed of two or more simple sugars; for example, certain cereal hemicelluloses have been shown to consist of both D-xylose and L-arabinose.

More advanced chemical techniques have been used to reveal the nature of the basic differences between cellulose and starch (Honeyman, 1959). For example, permethylation of cellulose followed by hydrolysis shows that each D-glucose unit /

unit is linked to two others by positions 1 and 4 of its carbon skeleton. Methylation analysis of starch shows that 1,4-linkages are present, but in addition there is also a small percentage of 1,6-linkages. Optical rotation studies on cellulose derivatives have shown further that the configuration of the glucosidic linkage is β-, whilst that of starch is α-. These results have been confirmed by infra-red analysis.

Thus on the foregoing evidence, it would appear that the cellulose molecule consists of β-D-glucose units linked in a linear array through 1,4-glucosidic bonds, whereas starch is largely composed of α-1,4-linked D-glucose residues.

Although prior to 1940, starch was regarded as a single polysaccharide, most native starches of plant origin can be fractionated into at least two chemically distinct components (Schoch, 1942). The higher molecular weight fraction called amylopectin, contains the small proportion of 1,6-linkages referred to above. Methylation analysis suggests that these occur wherever two chains of glucose residues are joined together. Quantitative examination of the products of methylation indicate that the average chain-length ($\overline{CL}$) of the amylopectin molecule is approximately 25, i.e. that there is one 1,6-linkage per 25 glucose residues. The other starch component amylose is linear, and has a high affinity for iodine.

Since /

Since amylose forms insoluble complexes with some alcohols, e.g. n-butanol, a convenient method of separation from amylopectin is available. Although amylopectin has a lower affinity for iodine, both compounds exhibit characteristic iodine absorption spectra. Amylose, unlike amylopectin, is relatively insoluble in water. The two main starch components are accordingly very easily distinguished from each other by simple chemical tests. In the plant, starch occurs in characteristic, bi-refringent granules. Evidence for the way in which the chains of glucose residues in the amylopectin molecule are arranged, has not been obtained by chemical analysis alone; the use of enzymic methods is essential.

Partial acid hydrolysis (Evans _et al_, 1959), i.e. controlled treatment of a polysaccharide with dilute acid such that complete fragmentation does not occur, is usually sufficient in order to show that a branched heteropolysaccharide may be composed of several distinguishable types of chain. The resultant molecular fragments can be placed in order rather in the same way that amino acid sequences in proteins have been determined. In a homopolysaccharide, there is evidently no such "internal" means of identification of molecular fragments produced by fragmentation procedures.

The branching characteristics of the starch-type polysaccharides have been determined by the analytical use of two classes /

classes of hydrolytic enzyme - (a) the amylases and phosphorylases that attack α-1,4-linkages, and (b) the "debranching" enzymes or α-1,6-glucosidases that hydrolyse branch points.

In addition to starches of plant origin, glycogen (Bernard, 1857), a product typical of animal metabolism, has a structure that is similar to the branched starch component amylopectin in some respects. The structure of glycogen is considered in Part Four of this Thesis. In Part Three, some properties of phytoglycogen, a starch-type polysaccharide isolated from sweet-corn (Zea Mays) are described.

Accordingly a starch-type polysaccharide may be defined as a high molecular weight polymer of D-glucose, in which the α-1,4-linkage predominates, but which also may contains appreciable quantities of α-1,6-linkages, forming the branch-points of an arborescent structure. Some starch degrading enzymes are considered next (Manners, 1962).

β-Amylase: One of the amylases, β-amylase, characteristically liberates β-maltose fróm starch by rupture of the C-1-O bond, (Meyer & Larner, 1959); the only other major product is an iodophilic polysaccharide (that resists further enzymic action) referred to as a β-limit dextrin. It is convenient to consider the action of β-amylase (an enzyme found in many higher plants) on linear substrates and branched substrates separately. When β-amylase degrades amylopectin, maltose is released /

released to the extent of ca. 60%. The remaining portion of the amylopectin molecule constitutes the β-limit dextrin, the enzyme being unable to circumvent α-1,6-linkages. In fact, β-amylolytic attack stops short of the branch-points in an amylopectin molecule by 2-3 glucose residues. If the action of β-amylase is followed by a debranching enzyme (an α-1,6-glucosidase) the short or "stub" chains are released, and β-amylolysis can proceed until a second β-limit dextrin is reached. By stepwise degradation of this type, it is possible to build up a structural model for amylopectin (Peat et al, 1956). Studies of the action of β-amylase on amylose have had a chequered history. In the first instance, most β-amylase preparations are contaminated with a second enzyme, identified as Z-enzyme (Pirt et al, 1952), which resulted in the complete conversion of amylose into maltose irrespective of the method used to isolate the amylose. This point is important, since an amylose isolated under anaerobic conditions has been shown to exhibit a higher β-amylolysis limit (percentage conversion to maltose) than an aerobically isolated amylose. However, Z-enzyme was later re-identified as small traces of α-amylase (Cunningham et al, 1960), and the barriers to β-amylase action as α-1,6-linkages (Kjolberg & Manners, 1963a). The successive action of β-amylase and isoamylase (a debranching enzyme specific for α-1,6-linkages) resulted in the complete conversion of amylose to maltose.

α-Amylase /

α-Amylase: In contrast to the orderly release of maltose from suitable substrates by β-amylase, the action of α-amylase (Roberts & Whelan, 1960) is characteristically random, and takes place in both the exterior and interior regions of starch-type polysaccharides. Less than complete α-amylolysis of starch produces a wide range of α-dextrins. A convenient method for following the reaction is by measurement of viscosity which decreases sharply, or alternatively by the change in colour of the iodine/polysaccharide complex from blue to red to colourless. At this stage of the reaction, the achroic point, the α-dextrins have an average D.P. (degree of polymerisation being the number of glucose units present) of ca. 8. The second stage characteristic of α-amylolytic action then proceeds relatively slowly, during which the α-dextrins are hydrolysed to maltotriose, maltose and glucose. The configuration of the anomeric carbon atom is retained.

Recent work has shown that low concentrations of α-amylase hydrolyse amylose to maltotriose and maltose, and that higher concentrations are required to effect the further slow hydrolysis of maltotriose to maltose and glucose. Further, the action of the enzyme on maltosaccharides is not completely random (Pazur, 1953), e.g. maltotetraose yields maltotriose and glucose on salivary α-amylolysis. Again, when branched substrates are hydrolysed a low concentration of the enzyme gives /

gives rise mainly to maltose and maltotriose, and to oligosaccharides of DP> 3. whereas a higher enzyme concentration affords maltose and glucose, and oligosaccharides of DP> 4. According to the enzyme source, glucosidic linkages adjacent to branch-points in amylopectin have been shown to be more or less resistant to hydrolysis. The enzyme is widely distributed in the animal kingdom and amongst higher plants (Bird & Hopkins, 1954). Neither α- nor β-amylase have any synthetic ability.

<u>Phosphorylase</u>: The enzyme phosphorylase (Cori <u>et al</u>, 1939), abundantly present in muscle, is considered in detail in Part Four of this Thesis. The enzyme catalyses the following reaction:

$$\text{G-1-P} + (\text{G})_n \rightleftharpoons (\text{G})_{n+1} + \text{P}_i$$

The action of the enzyme is either to effect the <u>de novo</u> synthesis of a linear polymer resembling amylose, or in presence of inorganic phosphate to degrade such a linear substrate to glucose-1-phosphate. The degradative action of the enzyme has been extensively employed in the elucidation of the branching characteristics of amylopectins and glycogens.

<u>P-enzyme</u>: The action of P-enzyme (Pirt <u>et al</u>, 1952) found in higher plants is in many ways analogous to phosphorylase; the same type of equilibrium reaction is catalysed, and the degradation of an amylopectin to a phosphorylase limit dextrin is similar. However, a "primer" molecule consisting of a maltodextrin /

maltodextrin of $DP > 4$ is required before any synthesis of amylodextrins can take place.

Amylo-1,6-glucosidase. (Larner *et al.* 1952) an enzyme typical of animal metabolism debranches glycogen and amylopectin phosphorylase limit dextrins with the production of a small amount of glucose. The simultaneous action, therefore, of phosphorylase and amylo-1,6-glucosidase leads to the complete degradation of a branched substrate to α-glucose-1-phosphate and glucose; the amount of the latter present after enzymic digestion is directly proportional to the degree of branching of the substrate.

R-enzyme. (Hobson *et al.* 1951) a debranching enzyme found in higher plants, for example, potato or broad-bean, can hydrolyse the branch-points of amylopectin or the limit dextrins of P-enzyme or β-amylase. The enzyme has no action on normal glycogens or their limit dextrins. Used in conjunction with β-amylase and α-amylase, R-enzyme has provided structural information on the branching characteristics and chain-lengths of branched substrates. Early preparations of R-enzyme were contaminated with a debranching enzyme having a slightly different substrate specificity. This enzyme, limit dextrinase (MacWilliam & Harris (1959)), hydrolyses the branch-points of amylopectin in α-limit dextrins, but has no action on the intact polysaccharide or β-limit dextrin.

Isoamylase /

Isoamylase. A debranching enzyme from yeast, isoamylase (Gunja et al, 1961; Maruo & Kobayashi, 1951), hydrolyses the branch-points of amylopectin and glycogen and their respective β-limit-dextrins: the enzyme thus has a broader substrate specificity compared with R-enzyme.

The present work is concerned with the application of biochemical techniques (exemplified by the analytical use of starch-degrading enzymes) to the determination of the structure of starch-type polysaccharides. Whilst the basic structure of most of the known polysaccharides that fall within this classification is well known, structural features that are not included in the broad definition of starch-type polysaccharides, have been reported in the literature. Parts One and Two of this Thesis deal with an investigation of these structural anomalies. In part Three, an investigation of a branching enzyme from sweet-corn is described, and some of its properties with regard to starch-type polysaccharides investigated. In Part Four, some cases of glycogen storage disease (where metabolic disorders affect human carbohydrate synthesis and degradation), have been examined with respect to the structure of liver and muscle glycogens, and also with respect to the endogenous levels of enzymic activity in the liver.

GENERAL EXPERIMENTAL PROCEDURES.

Drying of Polysaccharides

Samples in small tubes fitted with cotton-wool plugs were placed in a pistol-drier at 60°, and dried overnight under reduced pressure over phosphorus pentoxide. Sugar samples were stored in an evacuated desiccator over phosphorus pentoxide.

Distillation

The volume of polysaccharide and other solutions was reduced either (when the initial volume did not exceed 1 l.) in a Shandon rotary film evaporator at a temperature not exceeding 40°, or (for very much larger volumes) in a steam-heated cyclone apparatus.

Dialysis

The dialysis tube was placed in distilled water which was stirred mechanically. Dialysing enzyme solutions were placed in a cold room (0-5°). When the diffusible products were not required, dialyses were carried out against running tap-water at 18°.

Paper Chromatography - Solvents

The following solvent systems were used and are quoted throughout this Thesis according to the volumetric proportions of their constituents.

Solvent /

Solvent	Volumetric Proportions
(1) Ethyl acetate:pyridine:water	10/4/3
(2) Ethyl acetate:acetic acid: formic acid:water	18/3/1/4
(3) n-Butanol:ethanol:water	40/11/19
(4) n-Butanol:acetic acid: methanol:water	517/100/219/166
(5) n-Butanol:pyridine:water	6/4/3
(6) n-Butanol:acetic acid:water	4/1/5 (top phase only)
(7) n-Propanol:ethyl acetate:water	14/2/7

Solvent (1) was extensively used to separate glucose and individual maltodextrins (DP = 1-5) from one another, (development time 24-36 hr). Solvents (2) and (4) were used to separate nigerose from maltose (development time 2-3 days). Solvents (3) and (6) separated most glucose disaccharides adequately, especially when a multiple development technique was used, (development time 4-5 days). Solvent (5) was used to separate maltose, isomaltose and maltotriose, (development time 48 hr).

Paper Chromatography - Spray Reagents

Alkaline Silver Nitrate This reagent was prepared from saturated silver nitrate solution (1 ml) and acetone (200 ml). The slight precipitation that occurred on mixing these two solutions was removed by the drop-wise addition of water. The dried, developed chromatographic and electrophoresis papers were dipped into the acetone:silver nitrate reagent and air-dried. The dry papers were sprayed with an ethanolic solution of /

of sodium hydroxide, prepared by making up a sodium hydroxide solution (5ml, 10N) to ca. 40ml with methylated spirits. Colour was allowed to develop first at air-temperature, and then over a steam bath. This treatment enhanced the sensitivity of the reagent. Finally the background (dark brown) was removed by immersion of the papers in a saturated solution of sodium thiosulphate, followed by a thorough washing in tap-water. On the dry papers, black spots against a white background were visible: both reducing and non-reducing carbohydrates were revealed by this method, (Trevelyan et al, 1950).

Aniline Oxalate Dried chromatography papers were sprayed with a saturated solution of aniline oxalate in methylated spirits, and the colour revealed by heating for ca. 10 min at 120°. Aldoses produced a yellow:brown spot, and pentoses a pink spot against an initially white background. The reagent was generally less sensitive than alkaline silver nitrate, but specific for reducing sugars, (Partridge, 1949).

Dimedone The most successful ketose reagent tried was dimedone (1,1-dimethyl-3,5-diketocyclohexane). This reagent was prepared by dissolving dimedone (300 mg) in ethanol (90ml) to which was added o-phosphoric acid (10ml). The dry chromatography papers were sprayed with the reagent, air-dried, and then heated at 110° for 5 min. A brown spot (which tended to fade) was produced against a white background. The reagent was /

was sensitive to μg quantities of fructose in the presence of a large excess of glucose, (Adachi, 1964).

Resorcinol-Hydrochloric Acid This reagent was also tried, but it tended to produce rather fragile papers with a heavy background coloration. Ketoses gave pink spots that tended to fade, (Forsyth, 1948).

Urea-Hydrochloric Acid The use of an ethanolic solution of this reagent was limited to the detection of relatively large amounts of ketoses (Dedonder, 1952).

Periodate-Permanganate This reagent was prepared before use by the addition of potassium permanganate solution (1 part, 015% in 2% sodium carbonate) to sodium metaperiodate solution (4 parts, 1%). Dry chromatography papers were sprayed with the newly mixed reagent and the colour allowed to develop at room temperature for ca. 30 min. The pink background was then washed away with tap-water, leaving a brown spot against a white background. This reagent was used to detect the presence of α- and β-methylglucosides, (Lemieux & Bauer, 1954).

Aniline-Diphenylamine-Phosphoric Acid This reagent was used to distinguish between maltose and nigerose: the α-1,4-linked disaccharide gave a blue spot, and α-1,3-linked disaccharide a grey spot. The papers were rendered very fragile by this reagent, (Schwimmer & Bevenue, 1956).

Paper /

Paper Chromatography - Techniques

Qualitative Paper Chromatography Whatman No. 1 paper was used generally and was developed in 10/4/3 solvent. The sugars were revealed by the alkaline silver nitrate reagent.

Preparative Paper Chromatography Whatman 3MM paper was used for this method. Typically, 100-200 mg of sugar mixture (as a syrup) was streaked 5 in. from and parallel to the shorter edge of a 16in x 18in paper sheet. The paper was developed from 24-46 hr in 10/4/3 solvent. Bands of the separated components were located by guide strips, i.e. narrow strips of paper (cut in the direction of solvent flow) taken from either edge and the centre of the chromatogram, and which were sprayed with alkaline silver nitrate reagent. Individual bands were eluted with water. The eluate was concentrated and a portion checked for purity by re-chromatography.

Quantitative Paper Chromatography The same procedure as above was employed, except that Whatman No. 1 paper was used. The guide strips were spotted separately on the same sheet of paper beside the streak of mixed sugars. The bands of individual components, located by guide strips, were eluted with water, filtered, and estimated by the phenol sulphuric method.

The Estimation of Sugars

The Somogyi (1952) Method Sugar solution (5ml) containing not more than 2mg of glucose or 4mg of maltose and Somogyi reagent (5ml) were mixed in a stoppered tube, and heated in a boiling water bath for 20 min. The cooled solution was titrated against sodium thiosulphate (0.01N) solution after the addition of 2N sulphuric acid (3ml). The end-point was detected with starch indicator (2-4 drops). Generally, a titre difference of 1.0ml was equivalent to 0.51mg maltose or 0.29mg glucose. The concentration of standard sugar solutions (in saturated benzoic acid) was determined by polarimetry.

The Phenol Sulphuric Method A sugar solution (1.0ml) containing not more than 90 μg carbohydrate material was mixed with phenol solution (1.0ml, 5.0%). Sulphuric acid (A.R.) (5.0ml) was added from a burette with the end shortened (to increase the flow-rate). The phenol sulphuric solutions were allowed to cool for ca. 30 min, and then the optical density of each was read at 490m. in 1 cm cells. Each measurement was carried out in triplicate and reagent blanks were made for each determination (Dubois et al, 1956).

The Estimation of Polysaccharides

Acid Hydrolysis A suitably diluted polysaccharide solution was made 2N with respect to sulphuric acid and heated in a boiling water bath for 135 min. The cold solution was made alkaline to /

to phenolphthalein with sodium hydroxide (2N) and the pink colour removed by the drop-wise addition of sulphuric acid (0.01N). The neutral hydrolysate was made up to a standard volume and estimated by the Somogyi (1952) method.

<u>The Phenol Sulphuric Method</u> The method described above was used. It was found that the ratio of the optical densities obtained for equal weights of glucose and glycogen was 0.9.

<u>The Estimation of Protein Nitrogen</u>

<u>The Kjeldahl Method</u> The sample to be estimated was digested overnight with sulphuric acid (A.R., 2.0ml) and a catalyst (sodium sulphate: copper sulphate:sodium selenate, 80:20:1 w/w). The clear digest solution was transferred to a Kjeldahl steam distillation apparatus, and the ammonia liberated by the addition of sodium hydroxide (10N, 10ml) estimated by titration with standard hydrochloric acid, Chibnall <u>et al</u>, 1943).

<u>The Biuret Method</u> This method (Robinson and Hogden, 1940) is described in Part Three, this Thesis.

<u>Paper Electrophoresis</u>

<u>Small Scale Electrophoresis</u> Electrophoresis was carried out in a Shandon apparatus using borate buffer (0.05 or 0.1M, pH 10.0) and 10-30 volts per cm for 1.5 to 2 hr. (Foster, 1953).

β-Amylolysis Limits

A β-amylase preparation supplied by Wallerstein laboratories (New York) was used for all experiments. The enzyme contained no maltase activity, but did show residual traces of α-amylase (Z-enzyme). Generally, 50 units (Hobson *et al.* 1950) were used per mg. polysaccharide. The maltose liberated was estimated by Somogyi (1952) reagent.

PART ONE:

Studies on Acid Reversion in Relation to the Fine Structure of Starch-Type Polysaccharides.

INTRODUCTION.

The fragmentation of a polysaccharide by graded treatment with dilute acid has proved a powerful analytical tool in the determination of structure. (Bouveng & Lindberg, 1960). Analysis of the products of a complete acid hydrolysis enables the identity of the simple monosaccharide unit or units comprising the polysaccharide to be established. The products of a partial acid hydrolysis include some undegraded fragments, and the identification of these, for example, by comparison with known di- or tri-saccharides demonstrates the configuration of the glycosidic bond linking the monosaccharide units. It should therefore be possible using this technique to build up a structural model of any polysaccharide that is correct on two counts: the identity of the basic structural unit or units, and the type of glycosidic bond or bonds linking these units.

A number of factors require careful consideration before evidence from acidic fragmentation studies can be unequivocably accepted. From a purely practical point of view it is important that none of the monomeric polysaccharide fragments is destroyed, (Wolfrom <u>et</u> <u>al</u>, 1948) by treatment with acid that is too strong, or by excessively prolonged exposure to acidic conditions. Pentoses are liable on acidic treatment to be degraded to 2-furfuraldehyde, and keto- and aldo-hexoses can be degraded to 5-hydroxy-2-furfuraldehyde which is further degraded to levulinic acid /

acid and formic acid (Newth, 1951). The production of anhydrides also occurs (Wolfrom et al. 195^4). It is also important that the experimental conditions remain acidic or neutral, since carbohydrates are usually alkali-sensitive (Whistler & BeMiller, 1959). This applies particularly during neutralisation procedures. For example, alkaline treatment of glucose gives rise to fructose and mannose (Speck, 1959), or to a series of saccharinic acids (Sowden, 1958). This type of difficulty can be avoided, however, by careful selection of experimental conditions.

Of the artifacts that can arise, one example has already been described, viz: the interconversion of monosaccharides or alterations of their structure. Another type of artifact, with which Part One of this thesis is primarily concerned, arises as a consequence of the de-novo synthesis of oligosaccharides including disaccharides under the conditions of partial acid hydrolysis. This phenomenon, termed "acid reversion", leads to the recombination of polysaccharide fragments with glycosidic bonds which may or may not be the same as in the original polysaccharide.

Acid hydrolysis can in this sense be considered as a reversible process, and the condensation of monosaccharides in an acidic environment may lead eventually to the formation of non-dialysable polymers. The conditions favouring this type of reaction /

reaction, however, are not generally encountered during partial acid hydrolysis experiments. The polycondensation of D-glucose (50% w/v) in acid has been shown (Pacsu & Mora, 1950), and also under anhydrous acidic conditions in dimethyl sulphoxide (Micheel & Gesser, 1958). Strongly acidic cationic resins can also catalyse polymerisation reactions of D-glucose (O'Colla & Lee, 1956). Early examples (Dobereiner, 1832) of "acid reversion" include the isolation by Fischer (1890) of isomaltose as an osazone after treatment of maltose with hydrochloric acid, and the work of Moelwyn Hughes (1928), who explained some of the results he obtained from optical rotation studies on acidic solutions of D-glucose in terms of the formation of a "di-glucose".

A number of model experiments have been described in the recent literature in which different concentrations of monosaccharides or pairs of monosaccharides have been treated with dilute acid. These are exemplified by L-arabinose which yields primarily the β-1,3- and β-1,4- and the β,β-linked disaccharides (Jones & Nicholson, 1958). The β,β-linked disaccharide is also produced from D-arabinose (Rice, 1956). Acid-treatment of D-xylose (Ball & Jones, 1958) gives rise to a series of disaccharides linked by α-1,2-, α-1,3- and α and β-1,4-, and also β,β-bonds. D-mannose on similar treatment yields α-1,6-, β-1,3-, β-1,4- and β-1,6-linked disaccharides (Jones & Nicholson, 1958). Mixtures of D-xylose and galactose have also been studied (Täufel, <u>et</u> <u>al</u>, 1956). D-glucose yields a variety of reversion /

reversion products with both α- and β- configurations (Anno, et al. 1959). O'Colla et al. (1962) have reported a high incidence of 1,3- and 1,6-linked disaccharides among those isolated from reactions in which molten glucose was treated with cationic resin. Wolfrom et al. (1953) stated that the predominant products of an acid reversion of D-glucose were 1,6-linked disaccharides; a small quantity of levoglucosan was also isolated (Wolfrom et al. 1954). In contrast to this observation, the anhydro-sugar has been shown to constitute 70% of the reversion products of D-glucose (Peat et al. 1958), under different experimental conditions.

An interesting confirmation of the multiplicity of reversion products is provided by a number of analyses that have been conducted on "hydrol", an industrial starch conversion liquor resulting from the acid treatment of starch (Sowden & Spriggs, 1956). All the theoretically possible ways of linking two D-glucose molecules have been shown in the disaccharides isolated from this source except the α,β-linked trehalose (Sato, 1957, 1958). With the exception of maltose and isomaltose, where the glucosidic linkage is preformed in the starch molecule, the other disaccharides, viz: gentiobiose (β-1,6-), nigerose (α-1,3-), laminaribiose (β-1,3-), cellobiose (β-1,4-), kojibiose (α-1,2-), sophorose (β-1,2-) and the α,α, and the β,β-linked trehaloses, must result from the acid treatment of starch.

In /

In summary then, acids can catalyse the dehydration of monosaccharides by: (1) intermolecular condensation resulting in the formation of oligo- and polysaccharides (2) intramolecular elimination of water yielding anhydro-sugars and (3) the formation of unsaturated furfural derivatives particularly from ketoses.

Evidence for the presence of α-1,6-linkages in amylopectin has been obtained from acidic fragmentation studies. The disaccharide isomaltose has been isolated from partial acid hydrolysates in greater quantities than would be expected from acid reversion alone. In control experiments in which an equivalent amount of glucose was treated in the same way as the polysaccharide, it was shown that the amount of isomaltose produced by reversion was <u>ca.</u> 0.5% of that isolated from the hydrolysed polysaccharide (Wolfrom <u>et</u> <u>al</u>, 1953). Similar evidence has been adduced for glycogens (Bacon & Bacon, 1954) (Peat <u>et</u> <u>al</u>, 1955). In any case, less equivocable evidence is provided by enzymic studies (Larner <u>et</u> <u>al</u>, 1952), methylation analysis (Haworth <u>et</u> <u>al</u>, 1937) and also indirectly through a knowledge of the <u>in</u> <u>vivo</u> synthesis of branched starch-type polysaccharides (Manners, 1962).

In addition to the isolation of di- and tri-saccharides containing α-1,4- and α-1,6-linkages from partial acid hydrolysates, the isolation of nigerose, a disaccharide containing an α-1,3-linkage, has been reported. Accordingly, the α-1,3-linkage has been /

been suggested as a structural feature of glycogen (Wolfrom & Thompson, 1957), amylopectin, (Wolfrom & Thompson, 1956) and Floridean starch (Peat, et al, 1959). Confirmatory evidence in the form of periodate-resistant units (Hamilton & Smith, 1956) in these polysaccharides is not satisfactory (Manners & Mercer, 1963), although the isolation of nigerose from Floridean starch by enzymic hydrolysis has been reported (Peat et al, 1959).

Whilst D-glucose has been shown to yield most of the eleven theoretically possible disaccharides on reversion, it has been suggested that monosaccharides are not the most suitable control sugars for model reversion experiments (Peat et al, 1958). These authors suggest that when a disaccharide is hydrolysed, the energy associated with the original bond becomes available for the formation of a new glycosidic bond if the resultant monosaccharides recombine. The use of a disaccharide that contains the same linkage as the polysaccharide in control experiments is therefore preferable. When maltose and glucose are heated in an acidic medium, the yield of nigerose is of the order of 1%, (Pazur & Budovitch, 1956). The minute quantities of nigerose that have been isolated from partial acid hydrolysates (0.001-0.28%) appear to be accountable in terms of a limited acid reversion, i.e. they are experimental artifacts (Manners & Mercer, 1963)[a]. The use of a radioactive monomer in reversion experiments has been suggested (Jones & Nicholson, 1958). By adding a radioactive monosaccharide /

monosaccharide to a polysaccharide in dilute acid, any reversion products are labelled. The results of preliminary studies with maltose and radioactive glucose have been published (Manners et al, 1965), and the production of radioactive oligosaccharides was shown.

In view of the large amounts of nigerose obtained by acid reversion of glucose and maltose, compared with the yields obtained when glucose alone is used as a control sugar (Stroczyuiki & Boruch, 1964), a number of model reversion experiments have been devised in Part One as an extension of the work initiated by Mercer (Ph.D. Thesis, 1962).

The conditions selected were those used for the partial acid hydrolysis of amylopectin and glycogen (Peat et al, 1958, 1959a). In the first series of experiments, confirmation was sought of an observation of Mercer (Ph.D. Thesis, 1962), that approximately equal quantities of isomaltose and nigerose were produced by the action of dilute acid on maltose. A 1% solution of maltose in 0.33 N sulphuric acid was heated at 100° for two hours, the conditions used by Peat et al, (1959a) for the hydrolysis of Floridean starch. A 0.4% solution of polysaccharide heated in 0.1 N hydrochloric acid for 8 hr. was favoured by Wolfrom and Thompson (1956) to hydrolyse amylopectin (waxy maize starch), and so another experiment has been carried out in which a 0.4% solution of maltose in 0.1 N hydrochloric acid was heated for 8 hr. The results of a quantitative paper chromatographic analysis of these reversion /

reversion experiments are discussed later.

The production of isomaltose by acid hydrolysis from glycogen has also been studied in view of a report by Kügler and Wilkinson (1962) that this disaccharide was not detected in partial acid hydrolysates prepared from glycogen obtained from starved rabbits. The production of isomaltose from maltose has been followed kinetically in a paper chromatographic study, and in several other model experiments, the action of dilute acid on α-methyl glucoside and β-methyl glucoside has been examined. Different techniques for the neutralisation of acid reversion experiments have been compared, and finally when the present author had become more adept with the technical problems that arose in these studies, the action of dilute hydrochloric acid on maltose was re-examined.

EXPERIMENTAL.

(1) The Preparation of Chromatographically Pure Maltose (I)

A chromatographic analysis of the maltose sample available showed the presence of a small amount of maltotriose and other maltosaccharides. These impurities were removed by displacement chromatography on a charcoal:Celite column, prepared as follows. Celite 545 (60 g) was washed in a 1:1 dilution of hydrochloric acid (500 ml), and then washed with water until neutral. The Celite suspensions were stirred electrically for ca. 15 min., in five separate washings of water (800 ml). Charcoal (B.D.H., 60 g) was twice washed with water, the fines being decanted each time. The washed charcoal and Celite were mixed and more fines decanted. A 2.5 in. diameter tube fitted with a graphite-lubricated tap was used for the column. A glass-wool plug was placed in the bottom, followed by a layer (1 in.) of Celite. The charcoal:Celite mixture was added in small batches, and a filter-paper disc placed on top of the prepared column. The column was washed with 8 l. water. Mercuric chloride (200 mg) was then added as an antiseptic.

Maltose (2.0 g) was dissolved in water (30 ml) and placed on the column and eluted with a step-wise ethanol:water gradient, viz: water, 5%, 10% and 30% ethanol. The final ethanol concentration was used to empty the column which was then washed free /

free of alcohol. The contents of each fraction were assayed by paper-chromatography.

A second and third batch of maltose were each similarly purified, but using a different gradient elution system, viz: water, 4%, 8%, and 24% ethanol. The second system was found to give a better yield of pure maltose, namely, 1.4 g in the first experiment, and 1.9 g in the second.

Table I shows the results of the chromatographic examination of the fractionation obtained by the second elution system. Each fraction was evaporated to a syrup under reduced pressure, and spotted on to a 6 in. strip of Whatman No. 1 paper, and developed overnight in solvent 10/4/3 against suitable controls. The second, third and fourth fractions were combined.

TABLE I.

Maltose Elution Scheme.

			Ethanol Concentration						
Fraction	Water		4%				8%	24%	Water
Volume	1	1	1.5	1.5	1.5	1.5	2	2	8 litres
Glucose	++				(+)				
Maltose	+	++	++	++	+	(+)			
Maltotriose					(+)				

(+), trace; +, small spot; ++, large spot.

(2) Acid Reversion Experiment A 2: the Action of Dilute Sulphuric Acid on Maltose.

Maltose (579 mg) was dissolved in 58 ml sulphuric acid (0.366 N) contained in a 250 ml flask fitted with a water reflux condenser, and heated for 2 hr. on a boiling water-bath. The flask was cooled and the hydrolysate neutralised by the addition of barium carbonate (12 g). The well-stirred precipitate was centrifuged and washed twice with water. Further washings of the precipitate, examined by paper chromatography proved to be free of carbohydrate material. The supernatants were combined and the total volume adjusted to 50 ml.

(3) Acid Reversion Experiment C 2: the Action of Dilute Hydrochloric Acid on Maltose (I).

Maltose (528 mg) was dissolved in 133 ml hydrochloric acid (0.102 N) and heated under reflux for 8 hr. on a boiling water bath. The hydrochloric acid was removed from the cooled hydrolysate by ion-exchange chromatography. The Duolite A-4 resin (50 ml rest-volume) was regenerated in 0.1 N sodium hydroxide (500 ml) by stirring the resin suspension for 30 min. The resin, placed on a 1 in. diameter column was back-washed to remove the smaller particles to the top. 2 l. water were required to remove all traces of alkalinity.

Reversion mixture C 2 was passed through the column and eluted /

eluted with 4 bed-volumes of water. The neutral eluate volume was then adjusted to 50 ml.

(4) Acid Reversion Experiment D 1: the Action of Dilute Hydrochloric Acid on Maltose (II).

Maltose (535 mg) was dissolved in 133 ml hydrochloric acid (0.102 N) and heated under reflux for 8 hr. on a boiling water bath. The cooled hydrolysate was neutralised by the addition of silver carbonate (21 g). The silver carbonate was prepared by dissolving A.R. silver nitrate (50 g) in water (250 ml) contained in a dark-glass bottle. A.R. sodium carbonate (15 g) in water (250 ml) was added slowly to this solution. The supernatant was decanted and the precipitate washed with water (2 x 250 ml) until free from carbonate, i.e. until the washings gave no colouration with phenol-phthalein. The silver carbonate was collected on a Büchner filter and washed _in situ_ with ethanol (2 x 250 ml) and then with water (2 x 250 ml). The product was dried first by suction on the filter, and then at 60° in a pistol-drier. The yield (of a light tan coloured powder) was 36.1 g., and decomposed at 218°.

The precipitate obtained by the addition of silver carbonate to reversion mixture D 1 was centrifuged, washed with water and the volume of the combined supernatants adjusted to 50 ml.

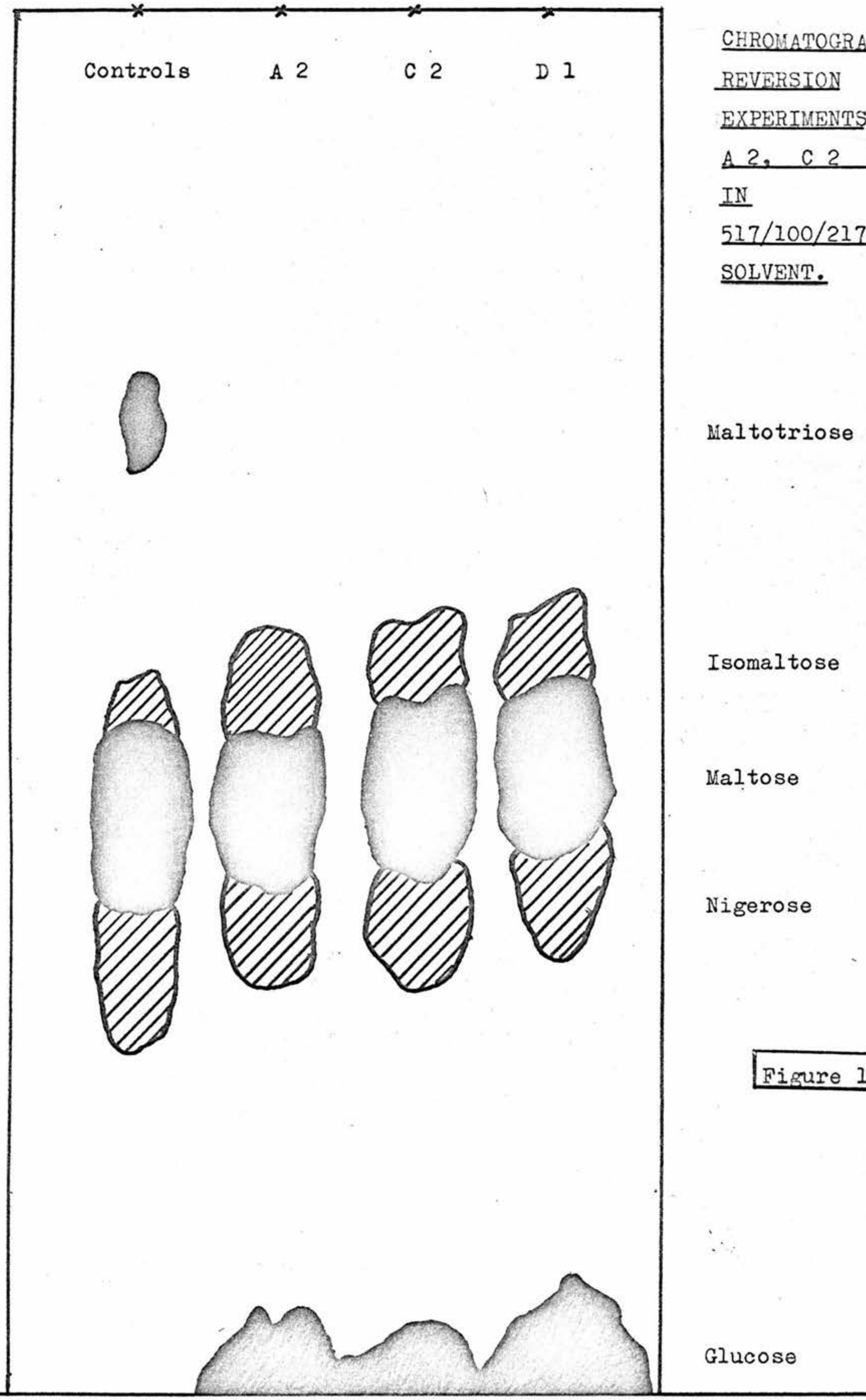
(5) Chromatographic Examination of the Reversion Components.

A portion (20 ml) of the neutral solution from each reversion experiment was concentrated to a syrup under reduced pressure, and spotted onto Whatman No. 1 sheets, together with suitable markers. The chromatograms were developed for three days in solvent 517/100/217/166. On spraying with alkaline silver nitrate reagent, the presence of nigerose, maltose, and isomaltose in the disaccharide region of the papers was clearly shown (see p. 31).

(6) Determination of Nigerose in A 2 and C 2.

Neutral solution (0.20 ml) from reversion experiments A 2 and C 2 was separately streaked on two sheets (16 in. x 18 in.) of Whatman 3 mm paper. The chromatograms were each developed for 70 hr. in solvent 517/100/217/166, and the nigerose band located by guide strips. Each band was eluted by placing it between two microscope slides, and allowing water to travel down the band into a tared beaker. The volume of each eluate was obtained by weight, and the carbohydrate content of 1 ml portions estimated by the phenol sulphuric method. These results are shown in Table II.

TABLE II /



CHROMATOGRAPHY OF REVERSION EXPERIMENTS A 2, C 2 & D 1 IN 517/100/217/166 SOLVENT.

Figure 1.

TABLE II.

Estimation of Nigerose.

Reversion Conditions	Volume of Eluate (ml.)	μg per ml.	Nigerose (%)
C 2	3.73	1.70	3.0
A 2	6.70	7.90	2.3

Each value shown is the average of three determinations less the paper blank reading.

(7) Estimation of Mono- and Disaccharide Components.

Three separations were carried out in order to estimate the amount of glucose, nigerose, maltose and isomaltose present in A 2, C 2, and D 1. In the first, the ratio of isomaltose to maltose + nigerose was determined by a separation on Whatman 3 mm paper developed in 10/4/3 solvent. The appropriate bands were located by guide strips, eluted with water (50 ml.), and 2 ml portions of the filtered eluate estimated by the phenol sulphuric method, against paper blanks. These results are shown in Table III. In the second separation ,using the same chromatographic paper and solvent system, the ratio of glucose to maltose + nigerose was determined. These results are shown in Table IV.

TABLE III

Estimation of the Isomaltose and Maltose + Nigerose.

Reversion Conditions	Isomaltose (μg/ml.)	Maltose + Nigerose (μg/ml.)
A 2	24.6	84.5
C 2	31.1	72.0
D 1	29.8	84.5

TABLE IV.

Estimation of Glucose and Maltose + Nigerose.

Reversion Conditions	Glucose (μg/ml.)	Maltose + Nigerose (μg/ml.)
A 2	67.5	18.0
C 2	70.5	15.0
D 1	66.5	8.2

(8) In the third separation, the solvent 517/100/217/166 was used to separate nigerose and maltose. These results, obtained by phenol sulphuric estimation of aliquots of filtered eluates, are tabulated below.

TABLE V.

Estimation of Maltose and Nigerose.

Reversion Conditions	Maltose (μg/ml.)	Nigerose (μg/ml.)
A 2	17.2	7.2
C 2	10.3	6.7
D 1	14.7	5.6

All readings given in the preceding three tables are the average results of three determinations from which paper blank readings have been subtracted.

In /

In Table VI the relative proportions of the main products of reversion in this series of experiments are expressed on a per cent. basis.

TABLE VI.

Percentage Composition of A 2, C 2, and D 1.

Components.	Reversion Conditions.		
	A 2	C 2	D 1
Glucose	74	76	84
Maltose	14	10	8
Isomaltose	6	7	4
Nigerose	6	7	4

(9) Acid Reversion Experiment C3: the Prolonged Action of Dilute Hydrochloric Acid on Maltose (III).

The sample of maltose used in this series of experiments was from the same preparation that was used in reversion experiments A 2, C 2 and D 1.

Maltose (1008 mg) was dissolved in hydrochloric acid (250 ml, 0.1022N) at 0-5°. Three reversion experiments were carried out on the acid maltose solution. In the first, 50 ml were heated in a 250 ml flask fitted with a water reflux condenser in a boiling water bath for 1 hr. In the second, heating was continued for 2 hr, and in the third, for 8 hr. At the end of each period of heating, the appropriate flask was cooled, and the acid removed by ion-exchange chromatography. The preparation of the /

the Duolite A-4 column used is described on p.28 . The columns were washed through with 250 ml water, which was then evaporated to a syrup under reduced pressure.

(10) Determination of Mono- and Di-saccharides Present.

The syrups obtained in the preceding section were each streaked separately onto Whatman 3 mm sheets, and developed overnight with solvent 10/4/3. The glucose, maltose, and isomaltose bands were located by guide strips, cut into thin strips, and eluted by immersion in 25 ml water. Portions of the filtered eluates were estimated by the phenol sulphuric method. The experimental readings for two determinations are presented in Table VII.

TABLE VII.

Estimation of Mono- and Di-saccharides.

Component	60 min.		120 min.		480 min.	
(μg/ml.)	(1)	(2)	(1)	(2)	(1)	(2)
Glucose	11.1	33.0	33.0	16.2	31.4	23.0
Maltose	53.4	85.0	27.6	18.2	8.1	6.1
Isomaltose	1.9	7.1	12.1	2.6	6.6	3.6

These results are shown in Table VIII expressed as percentage of each component present at each time given.

TABLE VIII.

The Production of Isomaltose with Time.

Time	Reversion Components (%)					
(Min.)	Maltose		Glucose		Isomaltose	
	(1)	(2)	(1)	(2)	(1)	(2)
0	100	-	0	-	0	-
60	67.8	67.9	25.6	28.6	2.7	2.5
120	38.0	49.0	45.5	44.0	7.0	16.0
480	17.6	18.4	68.0	70.0	11.0	14.3

(11) The Preparation of Chromatographically Pure Maltose (II).

The maltose sample was shown to contain a trace of trisaccharide material, identified by paper chromatography as maltotriose (See Experimental section P. 26).

Maltose (15 g) was dissolved in 4% ethanol:water 2 l.) and the solution passed through a charcoal:Celite column (1:1 W/W) dimensions 2.2 x 10.5 cm. The column eluate was evaporated to dryness under reduced pressure (at 40°) in a rotary film evaporator and the maltose recrystallised from acetone:ethanol solution. The yield of trisaccharide-free maltose was 14 g. The oligosaccharide material remaining on the column was eluted with 25% ethanol:water and the eluate evaporated as before. Chromatography in solvent 10/4/3 revealed the presence of maltotriose and higher maltosaccharides.

(12) /

(12) Acid Reversion Experiment H: the Action of Dilute Hydrochloric Acid on Maltose (IV).

Maltose (400 mg) was placed in a 250 ml flask containing 100 ml of 0.1025 N hydrochloric acid maintained at 100° in a boiling water bath. A water reflux condenser was fitted, and the hydrolysis allowed to continue for 8 hr.

(13) Preparation of the Duolite A-4 Column.

Approximately 500 ml (rest-volume) of resin were placed in a large column fitted with a glass-wool plug. 0.1 N sodium hydroxide solution (4 l.) was passed through, followed by water until the effluent was neutral. The column was back-washed, and 25 ml of the fine-particles from the top of the column resin-bed were removed and placed in a 2.2 cm dia., column fitted with a glass-wool plug.

(14) Neutralisation of the Hydrolysate H.

After 8 hr the flask containing the hydrolysed maltose was rapidly transferred to, and cooled in a refrigerator (0-5°). The cold acid solution was placed on the column described above, and the column washed through with 5 bed-volumes of water at ca. 5 ml per minute. The final pH of the eluate was 6.5 (Johnson Papers).

(15) /

(15) Chromatography of H.

The column eluate was evaporated to 2-3 ml under reduced pressure at 35° and kept frozen when not required. Paper chromatography in 10/4/3 showed the presence of glucose, maltose, and isomaltose (against appropriate markers). A very heavily spotted chromatogram run for two days in solvent 10/4/3 showed the presence in trace amounts of trisaccharide material, and higher maltosaccharides. The reagent spray used in each case was alkaline silver-nitrate.

(16) The Quantitative Estimation of Components.

A sheet of Whatman No. 1 paper (16 in. x 18 in.) was streaked twice along the shorter side, 5 inches from the edge with "H" and developed for one day in 10/4/3. The maltose and isomaltose bands were located by guide strips and eluted as follows: the maltose bands (duplicates) were cut into small strips, placed in a crystallising dish and immersed in 10 ml water delivered from a pipette. The dishes were each covered with a clock-glass and agitated gently several times during 30 min. The isomaltose bands were similarly treated. The eluates obtained in this way were filtered through glass-wool plugs, and 1 ml portions estimated by the phenol-sulphuric method,(see Table IX).

TABLE IX.

The Determination of the Maltose to Isomaltose Ratio.

$O.D._{490}$	Maltose (a)	Isomaltose (b)	Ratio $_a/b$
A	0.151	0.057	3.2
B	0.067	0.032	2.9
Blank	0.014		

(17) Determination of the Disaccharide to Monosaccharide Ratio.

A second quantitative separation was carried out similarly to the first in order to determine the disaccharide to the monosaccharide ratio. The disaccharide band was eluted with 10 ml water, and the glucose band with 50 ml. 1 ml portions of the disaccharide eluate and 0.5 ml portions of the glucose eluate were estimated as before:- the optical density readings given in Table X were taken against a paper blank.

TABLE X.

The Determination of the Di- to Mono-saccharide Ratio.

$O.D._{490}$	Disaccharides	Glucose	Ratio
	0.117	0.635	1.2:63.4

(18) The Estimation of Nigerose.

A 16 in. x 18 in. sheet of Whatman No. 1 paper was streaked as previously described (p 37) with "H" and developed in the solvent 18/3/1/4 for 7 days. The maltose and nigerose bands were then located by spraying the guide strips with alkaline silver nitrate reagent. The sugars were eluted with 10 ml water, the eluates filtered and 1 ml portions estimated by the phenol sulphuric method as described on p. 15 . The results are tabulated below in Table XI.

TABLE XI.

The Determination of the Maltose to Nigerose Ratio.

$O.D._{490}$	Maltose	Nigerose	Ratio
A	0.037	0.084	-
B	0.037	0.083	2.2:1

From the foregoing data, the percentage composition in terms of the predominating components of "H" was calculated, (see Table XII).

TABLE XII.

Composition of Reversion Mixture H.

Sugar	Per cent.
Glucose	98.0
Isomaltose	0.56
Nigerose	0.99
Maltose	0.45

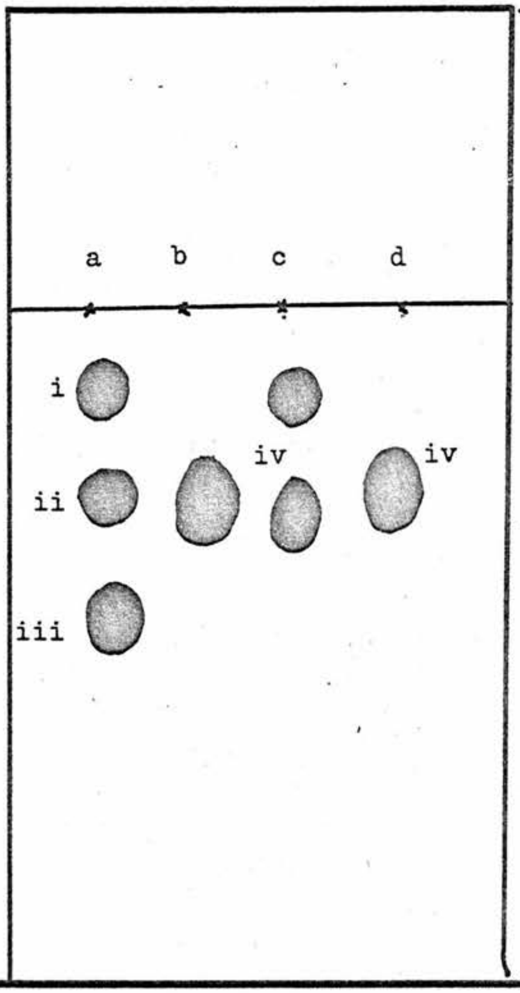
(10) The Isolation of Components: Thick-paper Chromatography.

The remainder of the neutralised reversion "H" syrup was placed on two Whatman 3 mm sheets as a streak, and chromatographed for 24 hr in solvent 10/4/3. After drying, three areas of the paper, cut into small strips each located by guide strips were eluted by agitation in 200-300 ml water. The three areas were: (i) glucose, (ii) disaccharides, (iii) higher saccharides. Each paper-area was eluted twice, and the eluates filtered through glass-wool plugs.

(i) The glucose fraction was concentrated to a syrup under reduced pressure and checked for purity by re-chromatography.

(ii) The disaccharide fraction was also evaporated to a syrup, and then streaked on two further sheets of Whatman 3 mm paper. The chromatogram was developed for 36 hr in solvent 10/4/3. /

BORATE-ELECTROPHORESIS OF NIGEROSE



a,b & d controls.

i - maltose
ii-isomaltose
iii-glucose
iv-nigerose + trace
isomaltose

c- fraction 2b.

Figure 2.

10/4/3.

(iii) The "higher" saccharide fraction was re-chromatographed after concentration as a single spot on Whatman No. 1 paper for 48 hr in solvent 10/4/3. Traces of five oligosaccharides of $R_G < 0.3$ were shown by the alkaline silver nitrate spray reagent.

(20) The Disaccharide Fraction (ii).

This fraction (see (ii) above) was further examined as follows.

The isomaltose (2a) and the maltose + nigerose (2b) bands were located by guide strips and eluted separately as previously described (see p.40). The purity of the isomaltose fraction was checked by re-chromatography. Fraction 2b was streaked on a Whatman 3 mm sheet and chromatographed for seven days in solvent 18/3/1/4. The separation was not as clear cut as had been obtained on No. 1 paper, so the nigerose band and the leading edge of the maltose band were re-eluted, and the filtered eluate analysed by borate electrophoresis.

(21) The Electrophoresis of Nigerose.

Fraction 2b was electrophoresed against authentic nigerose glucose and maltose and isomaltose markers on a 3 inch wide strip of Whatman No. 1 paper (see tracing on previous page). The conditions used were 0.1 M borate buffer, pH 10.0; 300 v; 15 mA for 1 hr. Spray reagent, alkaline silver nitrate. Fraction 2b was shown to contain a component with the same electrophoretic mobility as authentic nigerose.

(22) /

(22) Paper-Chromatography of Nigerose.

A Whatman No. 1 paper-chromatogram, spotted similarly to the electrophoretogram described above was developed in solvent 18/3/1/4 for 5 days. The presence of nigerose was confirmed by R_G-value.

(23) Acid Reversion Experiment E 1: the Action of Dilute Hydrochloric Acid on Isomaltose.

Preparation of Materials: the isomaltose sample used was chromatographically pure, and the same hydrochloric acid described on p. 33 was used.

The Treatment of Isomaltose with Dilute Acid: isomaltose (67.7 mg) was dissolved in 16.8 ml hydrochloric acid (0.102 N) and heated under reflux on a boiling water-bath for 4 hr. The cooled hydrolysate was neutralised on a Duolite A-4 column prepared as described on p. 28 . The column was washed through with 500 ml water, and the eluate concentrated to a syrup under reduced pressure.

(24) Chromatographic Separation of Di- and Trisaccharide Components.

The syrup obtained in the preceding section was streaked on a Whatman 3 mm sheet which was developed overnight in solvent 10/4/3. The trisaccharide band was located by guide strips, eluted, filtered, concentrated, and chromatographed against authentic /

authentic isomaltotriose and panose in 18/3/1/4 solvent for three days. The trisaccharide component had the same R_G-value as isomaltotriose.

(25) Borate Electrophoresis.

A disaccharide component obtained in the preceding section displayed the same mobility as maltose on borate electrophoresis.

(26) Acid Reversion Experiments F and G: the Action of Dilute Hydrochloric Acid on the Methylglucosides.

Preparation of Materials: The α- and β-methyl D-glucosides were recrystallised from aqueous ethanol, and checked for purity by paper-chromatography. Alkaline silver nitrate, and periodate-permanganate spray reagents were used to reveal the glucosides.

(27) Hydrolysis of α- and β-methylglucoside.

α-methyl glucoside (500 mg.) was dissolved in 25 ml hydrochloric acid (2.01 N) at 70°, and refluxed for 2 hr at this temperature. (Reversion Experiment F). The condenser was removed and the flask and contents rapidly cooled in an ice-water mixture. The hydrolysate was neutralised on a De-Acidite FF column prepared as follows: resin (rest-volume 75 ml) was regenerated by stirring in sodium hydroxide solution (2 N) for one hr, and then freed from alkali by repeated washings with /

with distilled water. The resin was placed in 22 mm dia. column fitted with a glass-wool plug. The column was eluted with 4 bed-volumes of water, and the eluate concentrated under reduced pressure. Residual acidity in the concentrated eluate was removed by the addition of more resin which was filtered off before reducing the volume to 2-3 ml. The neutral syrup was kept at -15°.

β-methyl glucoside was treated in exactly the same way. (Reversion Experiment G).

(28) Chromatographic Separation of Components.

Approximately half the material obtained was streaked onto a 16 in. x 18 in. Whatman 3 mm sheet, which was developed overnight in 10/4/3 solvent. Guide strips were used to locate the 1,6-linked disaccharides, which were cut out, and eluted with 25 ml water. 4 Mg were obtained.

(29) Enzymic Hydrolysis of Disaccharides.

Micro-scale enzyme digests were prepared (see p. 63), using an emulsin preparation, shown previously to preferentially attack β-linked glycosides. Authentic isomaltose and gentiobiose control digests were incubated at the same time. After incubation overnight at 37°, the digests were examined by paper-chromatography.

(30) /

(30) Paper Chromatographic Examination of Digests.

The enzyme digests were spotted onto Whatman No. 1 paper with appropriate markers, and the paper developed overnight in 10/4/3 solvent. No glucose was apparent after incubation with emulsin, either in the control isomaltose digest, or the isolated disaccharide from the α-methyl glucoside hydrolysate digest, whereas gentiobiose and the disaccharide from the other digest had been considerably hydrolysed.

(31) Borate Electrophoresis of Disaccharides.

On borate electrophoresis (0.05 M, pH 10.0) for 50 min. at 450 v. and 8-24 mA, the sugar from methyl α-glucoside had the same mobility as authentic isomaltose. The eluates were treated with Bio-deminrolit before electrophoresis.

(32) The Action of Dilute Acid on Glycogen.

The Acid Hydrolysis of Glycogen: approximately 90 mg of glycogen (rabbit liver, 1954 preparation) was dissolved in 10 ml sulphuric acid (0.988 N). Aliquots of 1 ml of this solution were pipetted into 8 stoppered test-tubes. The tubes were placed in a boiling water bath, and two tubes withdrawn at 2, and then at 4, 6, and 8 hr. The hydrolysates were neutralised by the addition of sodium hydroxide solution (2 N) until a faint pink colouration was observed in the presence of phenol-phthalein. This colour was removed by the addition of a few drops of ca. 0.05 N sulphuric acid. The neutralised hydrolysates were made up to 25 ml /

25 ml in standard flasks, and the amount of hydrolysis that had taken place was measured by the Somogyi cuprimetric method for reducing sugars.

The polysaccharide concentration was determined by hydrolysis of a 1 ml aliquot in sulphuric acid (2 N) for 2.25 hr in a boiling water-bath, and determination of the liberated glucose by the Somogyi method.

(33) Paper Chromatography of the Hydrolysis Products.

The remainder of the neutral hydrolysates was concentrated under reduced pressure, and the salts present precipitated by the addition of 4 volumes of ethanol. The "salt-free" concentrates were then developed on Whatman No. 1 paper for 24 hr using 10/4/3 solvent.

Table XIII shows the percentage hydrolysis with time of the glycogen samples, and also the results of the chromatographic analysis.

TABLE XIII.

The Hydrolysis of Glycogen (1% Solution in N-sulphuric Acid).

Time (hr.)	mg Glycogen Hydrolysed	Hydrolysis (%)	Appearance of Isomaltose
0	(wt. of glycogen present per tube, 85.6 mg).		
2	78.8 mg	92.0	+
4	81.5	95.2	++
5	83.0	97.0	+++
8	84.8	99.0	++++

+ → ++++ increasing amounts of isomaltose.

DISCUSSION.

The action of hot dilute acids on chromatographically pure maltose has been studied under a variety of conditions. In the first series of experiments A 2, C 2 and D 1, maltose has been hydrolysed under the conditions generally used for the partial acid hydrolysis of polysaccharides, in which the production of experimental artifacts due to reversion from glucose is minimal. Under the conditions selected (see Introduction) the production of approximately equal amounts of isomaltose and nigerose was demonstrated by paper chromatography, confirming earlier work (Mercer, 1962). In a large-scale qualitative experiment, Mercer found that treatment of maltose (12.5 g) with hot dilute sulphuric acid (0.33 N, 1.25 l.) gave a mixture of sugars including isomaltose and nigerose which he characterised chemically. The present work was concerned with more quantitative aspects of the rate of formation of these disaccharides, and in view of Mercer's results, our products have been characterised only by electrophoresis and paper chromatography against authentic compounds.

Although the present work was done on a milligram scale, the production of nigerose was clearly detectable by paper chromatography, in sharp contrast to reversion experiments in /

in which glucose had been used as a reversion "control" (Wolfrom et al. 1956).

In respect to the rapid production of reversion artifacts, the results of Kügler and Wilkinson are surprising. These authors have attributed structural significance to their failure to find isomaltose in a "partial acid hydrolysate" of liver glycogen from starved rabbits. Their experimental conditions (6-8 hr hydrolysis in normal sulphuric acid) are sub-optimal for the formation of a maximum yield of isomaltose (Wolfrom et al. 1951). It was of interest therefore to repeat the hydrolysis of glycogen under the conditions specified by Kügler and Wilkinson and to follow the course of the reaction both by paper-chromatography and also by measurement of reducing power. Wolfrom et al. (1951) in an extension of the work by Kuhn and his collaborators (1932) have shown that maximum production of isomaltose from glycogen occurs at 89% hydrolysis. In Table XIII it is shown that after 2 hr more than 90% of the glycogen has been hydrolysed. Paper chromatography showed also that isomaltose production was apparently still increasing during the later stages of the hydrolysis. This could be partly an artifact due to reversion. Thus, even assuming that the structural deductions made by these authors were correct, they should still have found isomaltose during their hydrolysis experiments due to acid reversion.

In the reversion experiments, A 2, B 2, and D 1, different techniques of neutralisation were used, and the hydrolysis artifacts examined by paper-chromatography. Qualitatively, apparently equal amounts of isomaltose and nigerose appeared in each experiment and on quantitative analysis this observation was confirmed (see Table VI). Re-chromatography of the nigerose fractions showed that an incomplete separation from maltose had been obtained in the quantitative experiment, so that the high yield of nigerose shown (4-7%) has to be reconsidered. The method of calculation used assumes a complete recovery of all components after neutralisation of the hydrolysates. If, for example, a 10% loss were assumed for each component, the adjustment necessary to the glucose percentage yield in the D 1 column (Table VI q.v.) would result in a yield of the reversion disaccharides of the order of 2%. This conclusion is borne out in a separate experiment in which a known volume of the neutral reversion solution was chromatographed and the nigerose component separately eluted and estimated (see Table II). However, the difficulty in obtaining a complete separation of nigerose from maltose does not invalidate the observation that the primary reversion artifact (disaccharides) are present in approximately equivalent amounts. The neutralisation techniques employed were shown not to yield significantly different compositions of reversion products.

In /

In the reversion experiment C 3, the hydrolysis of maltose and the concomitant formation of reversion disaccharides was followed kinetically by quantitative paper-chromatography. The results given in Table VIII ~~are presented graphically on the opposite page.~~ The ratio [maltose/isomaltose] suggests that under the given conditions there is a tendency to an equilibrium condition after eight hours. The observation of Overend *et al.* (1962) that on hydrolysis α-methyl glucoside yielded approximately 10% of disaccharide products is in harmony with this view. However, these authors identified their disaccharide unexpectedly as gentiobiose; the action of dilute acid on both methyl glucosides was therefore investigated.

In the methyl glucoside experiments, evidence was obtained that is in accordance with the suggestion (Mercer, 1962) that maltose reversion products are preponderantly linked by bonds with the α-configuration. The reversion disaccharide from β-methyl glucoside was hydrolysed by emulsin, the almond β-glucosidase, whereas the equivalent disaccharide from α-methyl glucoside was not. (cf. Armstrong 1903). The fact that when a glucose and maltose mixture is treated with dilute acid the reversion products are predominantly α-linked disaccharides suggests that acid catalysed transglucosidation in which maltose is the donor of glucosyl is a sufficiently rapid process for the products of reaction to retain the configuration of the glucoside. The /

The mechanisms generally accepted for the hydrolysis of glucosides (see Vernon et al.,(1961) and Overend et al.,(1962)) either yield products where anomerisation has occurred, or no such prediction is made. Hurd and Cantor (1938) made the observation that on methylation, maltose hydrolysed slightly, and that the presence of α-methyl glucoside could be shown as a product of reaction.

The action of hot dilute acid on isomaltose was also examined. The major products of reaction included maltose, and a trisaccharide identified as isomaltotriose. The identification of isomaltotriose in partial acid hydrolysates of glycogen should therefore be treated with caution (Wolfrom et al, 1957). The acid catalysed transglucosidation reactions of the type under discussion are therefore not limited to maltose or the methyl glucosides.

Finally, the experiment in which the action of dilute hydrochloric acid on maltose was re-examined (reversion H) is considered. The products of reaction were examined by an improved, quantitative paper chromatographic technique, and showed (see Table XII) that reversion of a maltose "control" can yield ca. 1% nigerose. In conclusion, it would appear that a limited amount of acid catalysed transglucosidation could account for the nigerose that has been isolated from various amylopectins and glycogen (see Introduction). The occurence of isomaltotriose as a pre-formed unit in glycogen is also questioned /

questioned.

French (1964) has pointed out that during acidic fragmentation of highly branched polysaccharides, the local carbohydrate concentration is very high compared to the bulk concentration, even in very dilute solution. The role of "control" reversion experiments should therefore be considered rather differently. As a basis for calculation, French has assumed that the polysaccharide has an ideal Meyer (1943) structure, with three glucose units 5 Å long each between each pair of interior branch points. Accordingly the branch may be considered to move around in a sphere of radius 7.5 Å. and there will be four glucose units to each branch point. The molar concentration of monosaccharide units is, therefore:

$$\frac{4}{6.02 \times 10^{23}} \times \frac{10^{24} \times 10^{3}}{4/3 \, . \, \pi (7.5)^{3}} = 4.3 \text{ M}$$

Viscosity measurements suggest that the polysaccharide is hydrated to the extent of 2 g. water per g. of polysaccharide, and this provides the basis for calculating the molar concentration of glucose units in a different way, viz:

$$\frac{1/162}{2.6} \quad 10^{-3} = 2.4 \text{ M}$$

By assuming the limiting glycogen spherical model (see p. 120), a /

a third method of calculating the local concentration of monosaccharide units follows:

$$\frac{61{,}000}{0.602 \times 10^{24}} \;/\; 4/3 \,.\, \pi (180)^3 \times 10^{-27} = 4.3\ M$$

The internal concentration of glucose units in a glycogen molecule therefore corresponds to between 30-60% w/v which compared with the apparent concentration used in acidic fragmentation studies is very high indeed. The relevance of control "reversion" experiments is therefore doubtful (Cf: Wolfrom <u>et</u> <u>al</u>. 1963), especially when they are used to prove that reversion has not taken place in another rather different set of experimental conditions. In addition the extreme proximity of the chains of glucose units in a branched polysaccharide such as glycogen, coupled with the fact that α-1,4-glucosans even of very short chain-length tend to form helices which may intertwine, together offer unusual opportunities for transglucosidation. The experiment of Pazur and Budovitch (1956) where glucose (13% w/v) and maltose (27% w/v) were heated in decinormal hydrochloric acid conditions under which <u>ca</u>. 1% nigerose was formed by transglucosidation from maltose (donor) to glucose would appear to have a greater comparative value than the "control" experiments generally quoted.

SUMMARY.

(1) The action of dilute hydrochloric and sulphuric acid on maltose has been studied under the conditions generally used for the partial acid hydrolysis of polysaccharides.

(2) The ready formation of experimental artifacts, mainly isomaltose and nigerose has been shown, in yields of the order of 1%.

(3) Further evidence has been obtained that favours the hypothesis that acid catalysed transglucosidation is partly responsible for the α-linked "reversion" products of maltose.

(4) The methods used to neutralise the acid hydrolysates have been shown, in three instances, not to effect the composition of the mixture of reversion disaccharides.

(5) The action of dilute acid on isomaltose has been shown to yield isomaltotriose amongst other reversion products. The action of dilute acid on α-methylglucoside has been shown to give rise to isomaltose, and the action of dilute acid on β-methyl glucoside similarly gives rise to gentiobiose.

(6) The rate of hydrolysis of glycogen by hot dilute acid has been studied, and the rate of production of reversion artifacts has /

has been measured. The results obtained have been discussed in relation to the existing "reversion" literature.

An account of some of this work has been published in the Journal of the Chemical Society, March, 1965, pp 2150-2156. See Appendix I.

PART TWO:

Studies on the Enzymic Degradation of Starch-Type Polysaccharides with reference to their Fine-Structure.

INTRODUCTION.

In addition to the possible occurrence of anomalous α-1,3-linkages in starch-type polysaccharides (see Part One of this Thesis) reports have appeared in the literature of two other types of anomalous structure. All native starches contain phosphorus (Parrish & Whelan, 1961) and the unequal distribution of chemically bound phosphorus (Schoch, 1942) between the two major starch components (Baldwin, 1930; Posternak, 1935) enabled Samec and Haerdtl (1920) to devise an electrophoretic separation of amylose, the linear starch component virtually free from phosphorus, and the branched amylopectin component that contains the major proportion of phosphorus. Posternak (1935) isolated glucose-6-phosphate from a phosphorylated oligosaccharide prepared from starch by digestion with pancreatic α-amylase, and demonstrated the alkali lability of the esterified phosphate. Hodge _et al_ (1948) found that the inorganic phosphate remained in the limit dextrin obtained on β-amylolysis. Posternak later (1951) showed that the phosphate esterified to a maltohexaose and a tetraose isolated from starch was not linked either to the reducing or the non-reducing terminal glucose residues. The hexaose was not degraded by β-amylase, nor was it an effective primer for the synthetic action of phosphorylase. The location of the esterified phosphate in the phosphomaltotetraose of Posternak (1951) was established by Parrish and Whelan /

Whelan (1961) by a periodate technique (Parrish & Whelan, 1959; Ellis et al, 1950) as on the third glucose residue from the reducing end group. The same authors adduced electrophoretic evidence for some of the phosphate groups in starch being separated by fewer than three glucose residues; such a structure would be resistant to α-amylolysis as in the case of nearly vicinal α-1,6-linkages (Roberts & Whelan, 1960). Radomski and Smith (1963) have suggested an association between the esterified phosphorus of starch and the α-1,6-linkages of the amylopectin component; this would explain the distribution of phosphorus in the starch fractions obtained on α- and β-amylolysis. Such phosphate groups may well constitute a barrier to the hydrolytic action of β-amylase (MacWilliam & Harris, 1963).

The second anomalous structure that may occur to a small extent in starch-type polysaccharides has been suggested by the isolation of maltulose from enzymic digests of glycogen and waxy maize starch (Whelan & Roberts, 1952). Radomski and Smith (1962) also report the occurrence of maltulose in α-amylolysates of waxy maize starch. Peat et al. (1952) showed the presence of fructose-containing α-limit dextrins in enzymic digests prepared from glycogen extracted from the livers of pregnant doe rabbits. The occurrence of maltulose has also been reported from the enzymic degradation of phytoglycogen (Turvey et al, 1956). Bacon and Bacon (1954) reported /

reported the isolation of an "isomaltose fraction" containing a ketose function, from Mytilus edulis glycogen.

The possibility that some starches and glycogens contain a small percentage of fructose residues raises the question of amylase specificity. If the disaccharide maltulose is a natural product of the α-amylolysis of, for example, waxy maize starch, then the enzyme cannot have an absolute specificity for an α-1,4-linkage between two glucose residues. The reported presence of nigerose in β-amylolysates of Floridean starch (Peat et al, 1959) raises a similar question for the specificity of β-amylase.

In the preparation of dextran from sucrose by the synthetic action of the microorganism Leuconostoc mesenteriodes (Jeanes et al, 1948) a levan (a fructose polymer) is entrained with the dextran. It is also possible that free fructose or sucrose in the medium may be carried down (Wise, et al, 1955). Fructose units might occur as a structural part of the dextran molecule, as "easily hydrolysable" terminal fructosyl units (Hehre, cited by Wise et al, 1955).

A number of enzymic degradation experiments have been designed in Part Two of this Thesis to investigate (a) the possibility of isolating the disaccharide nigerose from several polysaccharides including Floridean starch, and (b) the possible occurrence of fructose residues in the same starting materials.

For this series of experiments a new preparation of the enzyme /

enzyme isoamylase was required, and a slightly modified method of extraction from baker's yeast (Saccharomyces cerevisiae) is described in the experimental section (Kjolberg, Ph. D. Thesis, 1962).

EXPERIMENTAL.

(1) The Preparation of Isoamylase from Baker's Yeast (I)

Dried baker's yeast (D.C.L., 100g) was stirred for 2.5 hr at room temperature in bicarbonate buffer (0.1M, 700ml). The extract was centrifuged for 20 min at 2,000 r.p.m. and the supernatant solution (600ml) was cooled to 0-5° and an equal volume of re-distilled acetone (A.R.) added and the resulting precipitate was stirred for 2 hr at 0-5°. The acetone was distilled at 56° over potassium permanganate acidified with sulphuric acid). The acetone precipitate was centrifuged at 2,000 r.p.m. for 20 min, and extracted overnight at 0-5° with McIlvaine buffer (0.1M, pH 6.0).

The buffer extract was centrifuged and redistilled acetone (88ml) was stirred into the supernatant solution (460ml) at 0-5°. The precipitate (fraction 1) was centrifuged at 2,000 r.p.m. for 20 min, and then dissolved in ca. 30 ml water. The supernatant solution (525ml) was treated with acetone (88ml) as before, yielding fraction 2. The fractionation procedure was repeated on the third supernatant solution (560ml) /

(560ml) by the addition of acetone (140ml). The three fractions, dissolved in water, were then dialysed for 36 hr at 0-5° against water (10 l.)

(2) Examination of the Properties of Fractions 1,2,3.

Dialysed enzyme preparation (1 ml) and glycogen (2ml, 1mg/ml in 0.1M citrate, pH 6.0) was incubated at room temperature. The iodine staining power of a 1 ml aliquot and of iodine solution (5ml) was measured on a P.E. 137 recording spectrophotometer at 0 and 3 hr in 2 cm cells. The iodine reagent was prepared by diluting 0.2% solution of iodine in 2.0% potassium iodide (10ml) to 200ml. This solution was acidified by the addition of hydrochloric acid (3ml, 6N). The results are tabulated below (Table I).

TABLE I.

The Isoamylase Activity of Fractions 1, 2 and 3.

Fraction	1	2	3	O.D. 480
Acetone (%)	0 - 16	17 - 28	29-50	
T = 0	0.325	0.425	0.300	
T = 3	0.325	0.510	0.315	
Difference (%)	0.0	20.0	5.0	

Half of fraction 2 was freeze dried in McIlvaine buffer (0.01M pH 7.0) and the other half fractionated further by continuous electrophoresis.

(3) /

THE CONTINUOUS ELECTROPHORESIS OF ISOAMYLASE: PROTEIN ELUTION CURVE.

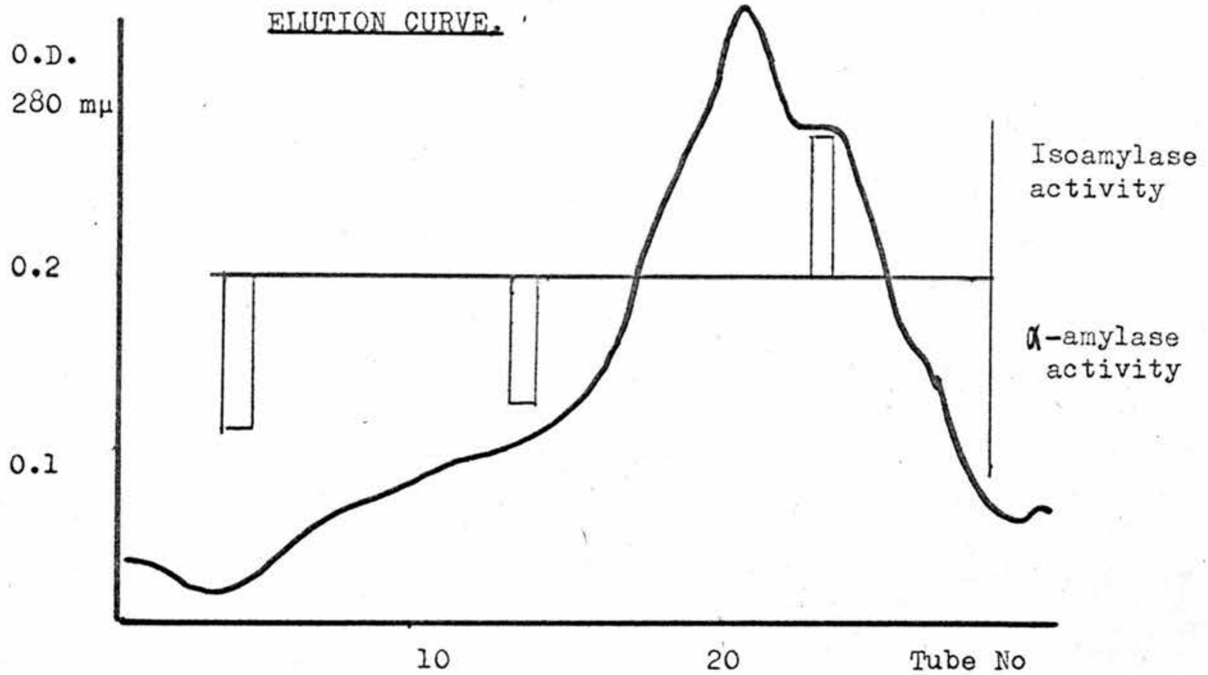


Figure 1.

THE ELUTION OF WAXY-MAIZE STARCH β-LIMIT DEXTRIN & MALTOSE FROM A SEPHADEX G-75 COLUMN BY WATER.

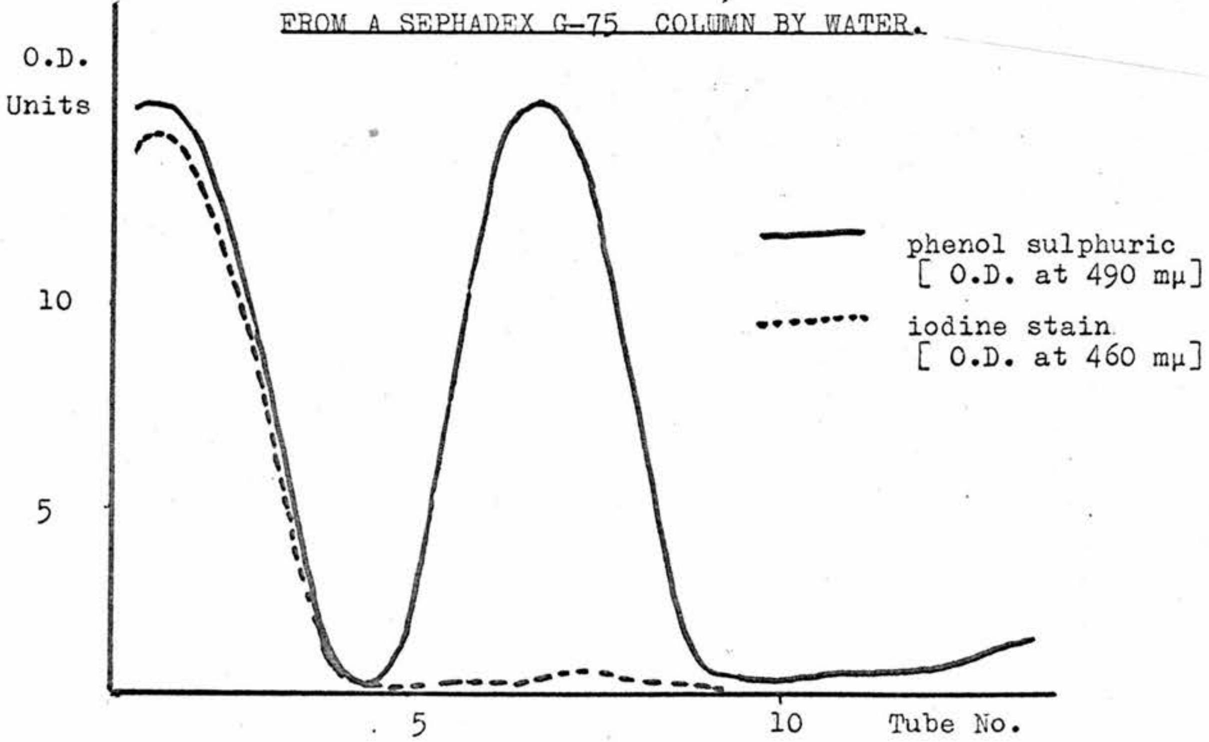


Figure 2.

(3) The Continuous Electrophoresis of Isoamylase Fraction 2.

Enzyme solution (25ml) was fractionated over a 24 hr period on a Spinco continuous electrophoresis cell at 600 V and 75 mA in citrate buffer pH 6.3, 0.01M. Individual tubes were assayed for protein content at 280mμ, and tubes representative of the main areas under the protein elution curve assayed for enzymic activity.

(a) Digest - isoamylase activity:
Enzyme fraction (1ml)
Substrate (2ml), 2mg glycogen in citrate buffer, pH6.0,

(b) Digest - α)amylase activity: as for digest (a), but with amylopectin in place of glycogen.

The digests were incubated at room temperature for 3 hr, and portions (1ml) were stained with iodine reagent (5ml digest (a), 10ml digest (b) at 0 and at 3 hr. The results of this experiment are shown in Fig. 1 (opposite).

(4) The Freeze-Drying of Isoamylase.

The freeze-drying of the first half of fraction 2 yielded 855mg of active material, tested by its activity towards a glycogen substrate. The purest electrophoresis fractions were batched (numbers 22-32) and freeze-dried, yielding 337mg. However, on testing this final fraction proved to be virtually inactive; loss of activity having occurred during the freeze-drying process. A large scale isolation of isoamylase was next attempted, using a slightly modified method.

(5) /

(5) The Preparation of Isoamylase from Baker's Yeast (II)

Dried baker's yeast (D.C.L., 500g) was extracted with bicarbonate buffer (0.1M, 3.5l.) by stirring for 2.5 hr in two 5 l. beakers at room temperature. The extract was centrifuged for 20 min. at 2,000 r.p.m. giving a final volume of 2.6 l. An equal volume of redistilled acetone was added slowly at 0-5° to the supernatant solution (2.6 l.) which was then stirred for 3 hr in the cold room. The acetone (A.R.) was distilled at 56° from a 2 l. flask containing 250g potassium permanganate and sulphuric acid (10ml). The redistilled acetone was stored in a brown glass bottle at -15°.

The acetone-precipitate was centrifuged at 2,000 r.p.m. for 15 min and the supernatant solution discarded. The residue was extracted with McIlvaine buffer (pH 6.0, 0.1M 1 l.) over-night (18 hr) at 0-5°. The buffer extract was centrifuged at -10° for 20 min at 2,000 r.p.m. and the supernatant (950ml) fractionated as follows:

Redistilled acetone (181ml) was slowly added with stirring to the supernatant solution which was placed in a beaker surrounded by an ice-salt mixture (0-5°). The stirring was continued for 15 min after the addition of the acetone. The precipitate was centrifuged at -10° for 20 min at 2,000 r.p.m. and the residue discarded. The supernatant solution (1050ml) was further fractionated by the addition of redistilled acetone (230ml), and stirring continued for 15 min after its addition, under /

under the same conditions as before. The precipitate was similarly centrifuged, dissolved in ca. 100ml water, and dialysed against water (5 l.) overnight at 0.5°. The dialysate was made 0.1M with respect to citrate buffer (pH 6.0) by the addition of an equal volume of 0.2M buffer, and freeze-dried. The yield of isoamylase preparation (between 16 and 31% acetone) was 5.5g.

(6) Examination of the Active Isoamylase Fraction.

(i) α-Amylase Activity. An enzyme solution (1ml) containing 10mg/ml was prepared, and its activity tested towards amylopectin and glycogen substrates (see para. 2 above) by an iodine staining technique. The action of the enzyme towards amylose was also examined. The optical density of iodine stained aliquots taken from the glycogen digest increased by 14% after 3 hr and by 120% after 17 hr. No decreases in optical density were observed for either the amylopectin and amylose experiments after a 3 hr incubation at room temperature. The isoamylase fraction was therefore devoid of α-amylase activity.

(ii) Maltase Activity. The micro-enzymic techniques of Hoban and Parker (1954) was used to test for the activity of maltase in the presence of ribitol, erythritol, glycerol and Tris at pH 6.0 at room-temperature. After 24 hr., the ribitol and erythritol digests showed that slight hydrolysis had taken place on examination by paper chromatography; none was observed for the Tris and glycerol digests. After 48 hr, more hydrolysis had /

had occurred in the erythritol and ribitol digests, and the glycerol digest showed hydrolysis. Only a trace of glucose was present in the Tris digest, indicating almost complete inhibition of the contaminating maltase under these conditions. (Keleman & Whelan, 1965).

(iii) *Effect of pH on Maltase Activity*. A differential maltase response was shown at pH 6.0 and pH 8.0 towards Tris buffer. A trace of activity was present after 24 hr incubation at pH 6.0, whilst none at all could be shown under the same conditions at pH 8.0.

(iv) *Maltotriase Activity*. No activity could be shown after a 24 hr incubation at pH 6.0 in citrate buffer (0.1M) at room-temperature.

(v) The enzyme preparation was shown to be devoid of phosphatase activity (the Cori (1952) method was used).

(7) The Salivary α-Amylolysis of Waxy Maize Starch.

Saliva (200ml) was collected, after chewing a piece of paraffin-wax, diluted with an equal volume of water, and stored under toluene. The activity of a series of decimally diluted solutions of the enzyme was determined according to the method of Fischer and Stein (1954). Enzyme (1ml. 1:1 dilution) activity was equivalent to 3.5 units, where 1 unit is defined as the amount of enzyme that will liberate 1 mg. of maltose from a 1% solution of starch in 3 min.

A preliminary experiment in which 100mg of waxy maize starch /

starch at pH 7.0 were treated with 0.1 units of enzyme per 20 mg polysaccharide showed that 60-70% apparent conversion into maltose was produced after a 20 hr incubation period at 37°. A large scale digest was set up as follows: Waxy maize starch (100 g) was dissolved in water (4 l.) with gentle heat. McIlvaine buffer (0.2M, pH 7.0, 500ml) was added to the filtered starch solution, and also sodium chloride (500mg). Enzyme solution (150ml) was then added and the digest incubated at 37° under toluene for 18 hr. Determination of the reducing power of an aliquot showed that 62.8% apparent conversion into maltose had been effected. The enzyme action was arrested by heating the digest to *ca.* 80° for 10 min, and the whole digest concentrated to 250ml under reduced pressure.

(8) Charcoal:Celite Chromatography of Waxy Maize Starch α Dextrins

A large charcoal:Celite column was prepared from 2 kg Ultrasorb charcoal and 2 kg Celite. Half the enzyme digest (125 ml) was placed on the column as a 3-fold dilution, and the elution commenced with water. The first 3 l. of the eluate were discarded. The following results were obtained with the technical assistance of Mr. J. Chalmers.

TABLE /

TABLE II.

Column Fractions.

Fraction Nos	Eluant(%)	Volume of Eluate(l)	Sugars Present (Shown by Paper Chromatography)
0-1	Water	8.3	None
2-8	Ethanol 4	32.1	Glucose
8-9	Ethanol 8	3.8	Glucose
10	Ethanol 8	1.7	None
11-12	Ethanol 8	5.4	Maltose
13-14	Ethanol 8	10.0	Maltose, Glucose (Trace)
15-24	Ethanol 8	51.1	Maltose
25-31	Ethanol 8	59.0	Maltose (Trace)
32	Ethanol 12	7.3	Maltose (Trace)
33-41	Ethanol 12	46.3	None
41-43	Ethanol 20	8.3	None
44	Ethanol 20	3.9	Maltotriose
45-58	Ethanol 20	59.9	Maltotriose,Higher Oligosaccharides
59-61	Ethanol 30	11.3	Maltotriose,Higher Oligosaccharides
62-65	Ethanol 40	9.8	Maltotriose,Higher Oligosaccharides
66-74	Ethanol 50	21.6	Maltotriose,Higher Oligosaccharides
75-77	Ethanol 50	-	None

Fractions 11-14 were combined, and also fractions 15-30; these were labelled F-I and F-2. Fractions 40-63 constituted F-3 and finally fractions 64-74 (F-4) were also combined. The remaining fractions were discarded.

These fractions were obtained in the following yields: F-1 4.8g, F-2 11.7g, F-3 7.5g, and F-4 7.3g.

(9) /

(9) Examination of the Column Fractions for Ketoses.

Solutions of the column fractions containing ca. 10mg/ml were examined by the procedure of Heyrovsky (1956). Portions (1ml) were pipetted into stoppered tubes, and β-indolylacetic acid (0.25ml, 0.5% solution in 96% ethanol) added together with hydrochloric acid (8.0ml). The well-mixed solutions were allowed to stand overnight (18 hr) at room temperature. The optical density of the resulting violet coloured solutions was read in 1 cm cells at 530 mμ. A standard calibration curve for fructose was prepared (10-100 μg) and also controls in which an equal weight of maltose (chromatographically pure) was estimated. The exact concentration of each solution was determined by the phenol-sulphuric method. The results obtained are expressed in Table III as fructose equivalents.

TABLE III

Ketose Content of Column Fractions (as Fructose Equivalent).

Column Fraction	Ketose Content (μg/ml)	Control* Ketose Content (μg/ml)	Net Ketose Content (μg/ml)
F-1	12	7	-
F-2	126	29	96
F-3	30	24	-
F-4	27	32	-

* these figures are taken as accurate to within 5 μg.

Since /

Since fraction F-2 was the only fraction containing carbohydrate with a significant ketose function, a portion (600mg) was chromatographed on a sheet of Whatman 3 MM paper, and developed for 36 hr in 14/2/7 solvent. The leading edge of the maltose band on the guide strips appeared pink when sprayed with the resorcinol-hydrochloric acid spray reagent, and accordingly, this part of the chromatogram was eluted with water and re-chromatographed after concentration under reduced pressure. Chromatography in three different solvents (10/4/3, 18/3/1/4, and 14/2/7) against an authentic maltulose marker convincingly demonstrated that this disaccharide containing a ketose group has been isolated from waxy maize starch. The dimedone spray reagent was used; this method detects as little as 1 g fructose, and is unaffected by up to 300 g glucose.

(10) Studies on the Enzymic Degradation of Some Polysaccharides.

The following polysaccharides were examined by some or all of the methods described in this section.

Floridean starch, potato amylopectin, phytoglycogen A1 (prepared by Mr. J.C. Patterson (see p.79), and phytoglycogen A2 (prepared by Dr. J.R. Turvey, see Peat et al, 1956). Waxy maize starch, waxy sorghum starch and rabbit liver glycogen (extracted from the livers of pregnant does, and kindly provided by Dr. J.R. Turvey) were also examined.

In /

ENZYMIC DEGRADATION OF POLYSACCHARIDES.

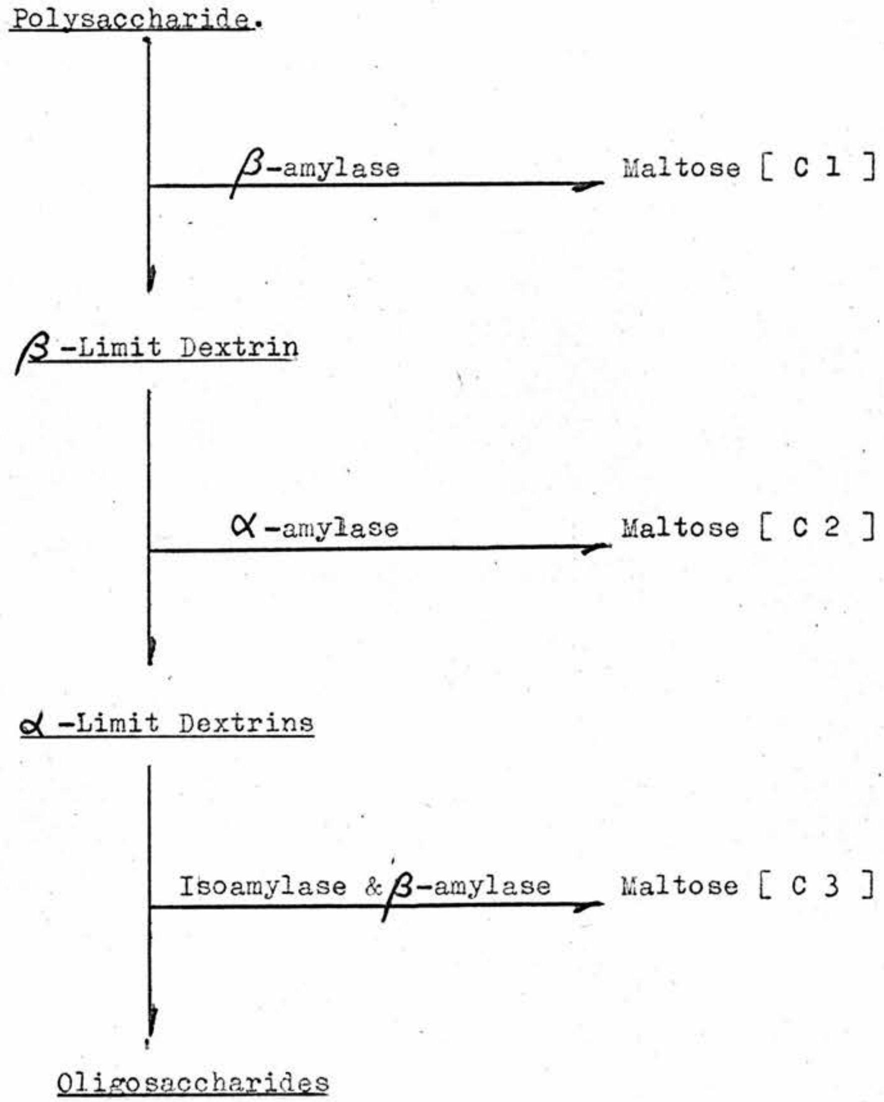


Figure 3 .

In Figure 3, the experimental procedure is shown schematically.

(a) The β-limit dextrins used in the following series of experiments were prepared by dissolving the polysaccharide (500mg) in water, and after the addition of acetate buffer (100ml, 0.1M, pH 4.7) and β-amylase (250mg) the total volume was adjusted to 500ml. The incubation was carried out under toluene for 24 hr at 37°. The enzyme was deactivated by raising the temperature of the digest to 100° for 10 min. The volume was reduced to ca. 50ml, and the denatured protein removed by centrifugation. The digest was dialysed against water (5 l.), after which the β-limit dextrin was precipitated by the addition of ethanol (3 vol.). The dialysate was concentrated under reduced pressure. Continued incubation of the limit dextrin with β-amylase did not yield any additional maltose.

(b) A convenient method for the isolation of β-limit dextrins was afforded by gel-filtration on a Sephadex G-75 column (1 in. x 9 in.). A β-amylase digest (containing ca. 100mg polysaccharide) was placed on the column and eluted with water. Fractions (5ml) were collected and the progress of the separation was followed by the phenol-sulphuric method and by an iodine staining technique. The β-dextrin appeared first, shown by both iodine stain and the phenol-sulphuric method. The maltose followed, detected only by the phenol-sulphuric method. The /

The separation was clear cut, as shown by paper chromatography and the profile of the elution curves, and had the advantage that each component was eluted by a total volume of ca.50ml water (see Fig. 2).

(ii) Enzymic Degradation of the β-limit Dextrins.

Digests: Enzyme (Saliva, 1:10 dilution, 2ml)
Polysaccharide (200mg)
Buffer (Citrate, 0.2M, pH 7.0, 5ml)
Total volume, 50ml.

Incubation under toluene at 37° for 24 hr.

The concentration of saliva used was found to yield mainly maltose and maltotriose under these conditions. After incubation, the digest was concentrated under reduced pressure, and chromatographed on Whatman 3 MM sheets (development for 20 hr in 10/4/3 solvent). The maltose band was located by guide strips and eluted, and the α-dextrins then eluted separately. The maltose obtained at this stage was labelled C2, C1 being the maltose liberated during the initial β-amylolysis.

The α-dextrins (in 25ml water) were incubated with isoamylase (50mg) in citrate buffer (pH 6.0, 0.1M, 5ml) for 48 hr at room temperature. The enzyme was deactivated by heat (10 min. at 100°). & β-amylase (50mg acetate buffer, 10ml 0.1M) added to a total volume of 50ml. Incubation was continued for 24 hr at 37°, at pH 4.7.

The products of enzymolysis were separated on Whatman 3 MM sheets under the same conditions as before, and the maltose band /

band (C3) eluted. The three maltose bands (C1, C2, and C3) were examined for the presence of (a) nigerose and (b) maltulose as follows: Sheets of Whatman No. 1 paper (6in.) spotted with C1, C2, and C3 and authentic nigerose markers were developed for 2-3 days in 18/3/1/4 solvent and examined with the alkaline silver nitrate spray reagent for the possible presence of nigerose. Corresponding chromatograms spotted with authentic maltulose markers were also developed for 2 days in 14/2/7 solvent and sprayed with the dimedone reagent (Adachi, 1964).

The following polysaccharides were examined: Floridean starch, potato amylopectin, phytoglycogen A1, waxy sorghum starch and waxy maize starch. Nigerose was detected only in the C3 band of Floridean starch, and maltulose was not detected at any stage by this method.

(12) α-Amylolysis of Several Polysaccharides.

Phytoglycogen A1, phytoglycogen A2, waxy maize starch and rabbit liver glycogen were subjected to α-amylolysis under the conditions described above for the α-amylolysis of β-limit dextrins. The products of enzymic degradation were separated by paper chromatography in solvent 14/2/7 and the disaccharide band eluted with water (15ml).

The filtered eluates were assayed by the phenol sulphuric method (0.2ml) and by the β-indolyl acetic acid method (10ml) described above. The results indicated a maximum possible ketose /

ketose content in each of the order of 0.2%. A paper-chromatographic examination of the disaccharide eluates suggested that only the phytoglycogen A2, and the waxy maize starch contained appreciable quantities of fructose.

DISCUSSION.

Evidence has been obtained that supports the observation of Peat et al (1959) that nigerose exists preformed in Floridean starch (Dilsea edulis). That the disaccharide was detected only after the α-amylolysis of the polysaccharide β-limit dextrin suggests that the α-1,3-linkages occur only within the interior of the molecule: the work of Peat et al indicated, in contrast, that some of these linkages were in the outer chains. The fact that an analagous experiment on potato amylopectin carried out at the same time failed to yield any nigerose rules out the possibility of enzymic transglucosidation being responsible for the formation of nigerose in the Floridean starch experiment. The experimental conditions were either at pH 4.7, pH 6.0 or pH 7.0 which would rule out acid catalysed transglucosidation (see Part One, this Thesis).

The presence of a small proportion of α-1,3-linked glucose residues in Floridean starch would suggest that there are some differences between the starch synthesising systems in Dilsea edulis /

Dilsea edulis and the higher land plants. Neither starch-UDPglucosyl transferase nor phosphorylase, are, as far as it is known (Manners, 1962) able to synthesise any glucosidic linkage but the α-1,4-type.

The isolation of maltulose from an α-amylolysate of waxy maize starch confirms the qualitative evidence already obtained by Whelan and Peat (1952) and Radomski and Smith (1962). The yield in the present experiment was of the order of 0.5%. The α-glucosan structure that could incorporate a fructosyl residue is not yet known; this must await the isolation of a larger fragment than a disaccharide. Paper chromatographic evidence and also evidence by colorimetric assay has also been obtained for the possible presence of maltulose in two phytoglycogens (Turvey *et al*, 1956) and also in pregnant doe liver glycogen (Peat *et al*, 1952). These observations also have implications with regard to the biosynthesis of starch-type polysaccharides.

A modified preparation of isoamylase from baker's yeast has been devised which yielded a pure, active isoamylase fraction: an attempt to fractionate this enzyme by continuous electrophoresis was partly successful, but the lability of the enzyme prevented any further work on the electrophoresis fractions.

SUMMARY.

(1) A pure fraction of the enzyme isoamylase has been obtained from baker's yeast.

(2) A large scale salivary α-amylolysis experiment on waxy maize starch yielded *ca*. 0.5% of a fructose-containing disaccharide, tentatively identified as maltulose.

(3) An enzymic degradation experiment of Floridean starch produced evidence for α-1,3-linkages in this polysaccharide, probably in the interior of the molecule.

(4) Two phytoglycogens and a rabbit liver glycogen were also examined for the possible occurrence of fructose, and also nigerose. The latter was not detected, and the presence of a fructose-containing disaccharide was shown in one of the phytoglycogens.

(5) A modified method for the extraction of isoamylase from baker's yeast has been devised.

PART THREE.

Studies on the Isolation and Properties of the Branching Enzyme of Sweet-Corn (Zea Mays).

INTRODUCTION.

Sweet-corn (Zea Mays) differs from most higher plants in its ability to synthesise both a two-component starch and a glycogen-type polysaccharide (phytoglycogen). Morris and Morris (1939) on the basis of iodine staining observations and cupric chloride crystallisation patterns first characterised this sweet-corn component as "glycogen-like". Their material was obtained by an acetic acid fractionation. The name "phytoglycogen", however, was suggested by Sumner and Somers (1944). A chemical characterisation by methylation analysis (Hassid & McCready, 1941) established that phytoglycogen had an average chain length ($\overline{CL}$ = 12) typical of glycogens. Whilst some authors (Peat et al 1956) regard phytoglycogen as indistinguishable from animal glycogens, Dvonch and Whistler (1949) prefer to describe the plant polysaccharide as a highly branched amylopectin. This point of nomenclature would appear to be covered by the description of this polysaccharide as a phytoglycogen (Cf. Frydman & Cardini, 1965). The suggestion has been made (Whelan, 1955) that the potentiometric iodine titration method of Anderson and Greenwood (1953) might be used to distinguish phytoglycogens from other starch-type polysaccharides.

A small percentage of an iodophilic glucan has sometimes been found to contaminate phytoglycogen preparations (Peat et al, 1956 /

1956 : Dvonch & Whistler, 1949) and can be removed by adsorption on cellulose following a suggestion made by Pacsu and Mullen (1941). The latter authors, noting the everyday use of starch in the sizing of linen (and tracing this procedure to the sizing of papyrus cf. Hadley, 1953) successfully used a column packed with cotton for the adsorption of amyloses.

The characteristically highly branched structure of phytoglycogen suggests that enzymes mediating the <u>in vivo</u> synthesis of this polysaccharide might differ from those already described for the amylopectin component of plant starches. For example, the plant branching enzyme (Q-enzyme) has been isolated from the potato (Peat <u>et al</u>, 1944, 1945a, 1945b; Barker <u>et al</u> 1950) and has been prepared in a crystalline state by Gilbert & Patrick (1952) and also by Baum & Gilbert (1953). Q-enzyme also occurs in the broad bean and pea (Hobson <u>et al</u>, 1950) and green gram (Ram & Giri, 1952). The isolation of a branching enzyme from animal tissues was earlier reported by Cori & Cori (1943), and since then, branching enzymes have also been found in yeast (Gunja <u>et al</u>, 1960; Manners & Khin Maung, 1956), the flagellated protozoan <u>Polytomella coeca</u> (Barker <u>et al</u>, 1953) and <u>Neisseria perflava</u> (Hehre <u>et al</u>, 1947, 1949), and also in <u>Arthrobacter</u> (Ghosh & Preiss, 1965). The branching enzyme of rat-liver and muscle has been studied by Larner (1953).

The animal enzyme differs from the plant Q-enzyme in substrate specificity, since the latter displays no activity towards amylopectin /

amylopectin. The animal enzyme will convert this substrate into a synthetic glycogen, a reaction that provides a simple assay method based on the decrease in iodine staining power (Larner, 1955). The plant enzyme can be assayed by use of a linear substrate, for example amylose, into which it will introduce branch points (Barker et al, 1949) with concomittant decrease in iodine staining power. The action of the enzyme is probably irreversible (Barker et al, 1950). A decrease in β-amylolysis limit values of suitable substrates is also a useful measure of branching activity. The plant enzyme introduces new branch-points into the outer chains of certain branched substrates; this has been demonstrated by Larner (1953). By using muscle phosphorylase (a) to degrade the outer chains of a rabbit liver glycogen and (b) to re-synthesise them in the presence of labelled D-glucose-1-phosphate, this author obtained a glycogen in which most of the outer chains were abnormally long and were radioactive. By Q-enzyme action, the polysaccharide was further branched, and then by the successive action of phosphorylase and a debranching enzyme, the new branch points were liberated and found to be radioactive. The nature of the branch points as being α-1,6-glucosidic in type has also been demonstrated by methylation studies on synthetic amylopectins (Bourne et al, 1950, 1952). The transfer of short branched chains by the yeast branching enzyme has been reported (Kjolberg & Manners, 1963).

The /

The branching enzyme of sweet corn has been examined in Part III of this thesis. During the completion of this work, we were informed by Dr. C.E. Cardini that his colleagues (Lavintman & Krisman, 1964) had obtained evidence for the presence of a branching enzyme in sweet corn (See Manners & Rowe, 1964, and also Appendix 1).

EXPERIMENTAL.

Analysis of Phytoglycogen A.

(1) Preparation

The extraction and fractionation were carried out by Mr. J.C. Patterson.

Mature Zea Mays (400 g. var. Golden Bantam) was ground and steeped in 0.01 M mercuric chloride (1 l.) for 18 hr. The solid was re-extracted four times (2 hr) with 0.001 M mercuric chloride (1.5 l.). The combined extracts and steep liquor were passed through a Sharples centrifuge, and the volume reduced to 1.2 l. under reduced pressure. The supernatant solution was passed through a pad of Celite, and ethanol (1.5 l.) added at 0°. The precipitate was centrifuged, washed with acetone and ether, yielding 41 g. of polysaccharide. The polysaccharide was fractionated by dissolving 20 g. in water (300 ml) at 0° and adding 600 ml acetic acid slowly, with continued stirring. The mixture was left overnight at 0°. The precipitate was centrifuged, washed free of acid with acetone, and redissolved in water. The pH was adjusted to 6.5 (sodium hydroxide) and the polysaccharide precipitated by the addition of ethanol at 0°. Yield of Phytoglycogen A: 16 g. (See Peat et al, 1956, and Greenwood & DasGupta, 1958).

(2) /

(2) Periodate Oxidation of Phytoglycogen A.

Phytoglycogen A (200 mg) was dissolved in 50 ml water. A portion of this solution (20 ml) was added to 20 ml of sodium metaperiodate solution (8%), and the volume made up to 50 ml. The preparation of the reaction mixture and the subsequent oxidation experiment were carried out at 0-2°. Aliquots (10 ml) were taken at 24 hr intervals and titrated against sodium hydroxide to pH 5.8 on a Pye Universal pH-meter. Titration readings are given in Table I.

TABLE I.

Time (hr)	Titration (ml)	
24	0.44	The sodium hydroxide solution was standardised against potassium hydrogen tartrate, and all titrations were carried out in an atmosphere of N_2.
48	0.46	
72	0.48	
96	0.51	
Blank	0.00	

The exact concentration of the polysaccharide solution was determined by hydrolysis in 2N sulphuric acid for 2.25 hr., and estimation of the liberated glucose by the Somogyi method. The formic acid yield was obtained by extrapolation to zero time.

(3) /

(3) β-amylolysis Limit of Phytoglycogen A.

Polysaccharide solution (5 ml) plus β-amylase (10 mg 1000 units) in 5 ml acetate buffer (pH 4.6, 0.2 M) were made up to 25 ml, and the digest incubated for 24 hr at 37°. Aliquots (5 ml) were estimated by the Somogyi method.

(4) α-amylolysis Limit of Phytoglycogen A.

Polysaccharide solution (5 ml) plus α-amylase (10 mg "calibrated" enzyme) in 0.25 M citrate buffer, pH 7.0, and sodium chloride 2.5 ml (0.5%) were made up to 25 ml, and incubated for 24 hr at 37°. Portions, (2.0 ml) were estimated by the Somogyi method. These results are incorporated in Table II.

TABLE II.

Analysis of Phytoglycogen A: Results.

Periodate Oxidation	Moles Formic acid per	Average
wt p/s 52.4 mg/50ml	Anhydrohexose Unit	Chain-Length
(10 ml aliquots)	0.0668	15.0
α-amylolysis Limit	Maltose released	α-Limit Value (Pm)
wt p/s 13.1 mg	11.95 mg	87.0%
β-amylolysis Limit	Maltose released	β-Limit Value
wt p/s 13.1 mg	7.03 mg	52.8%

(5) /

(5) Extraction of Enzymes.

Sweet-corn flour (500 g.) was stirred at room temperature (18°) for 1 hr in citrate buffer (2 l., 0.05 M, pH 7.0). The extract was centrifuged for 10 min. at 2,000 r.p.m. The milky supernatant solution was passed twice through a Sharples centrifuge at 200 ml/min. The final volume of the extract was 1465 ml.

(6) Fractionation.

Solid ammonium sulphate (May & Baker) was stirred into the extract. The fractionation was carried out at 4°. After each addition, stirring was continued for a further 15 min. The resulting fractions were each centrifuged for 20 min. at 2,000 r.p.m. The details of the fractionation procedure are given in Table III. Fractions I-IV were then taken up in water, and dialysed separately in a cold room (0-5°) for 48 hr against running tap-water. The dialysates were centrifuged for 10 min. at 2,000 r.p.m. after each had been made 0.1 M with respect to citrate buffer at pH 7.0.

TABLE III /

TABLE III.

Fractionation Protocol and Composition.

Fraction Number:	I	II	III	IV
Percent. Salt (w/v).	22	32	55	70
Volume Centrifugate (ml)	1465	1650	1830	432
Wt. added amm. sulphate (g.)	366	212	432	200
Fraction Weight (g.)	6.9	6.1	84.0	1.7
Percent. Protein (Kjeldahl)	3.4	2.2	7.6	1.3
Percent. Carbohydrate (Phenol-sulphuric)	12.0	11.5	71.5	20.0

(7) Relative Activity of Fractions.

Digest: Enzyme, 10 mg in 3 ml buffer, 0.5 M citrate,
pH 6.0 Substrate, amylopectin (10 mg) in 2 ml.

Digests were incubated at 37°. Aliquots (1 ml) were taken at 0, 1, 2, and 3 hr, and stained with standard iodine solution; 3 drops of 6 N hydrochloric acid were added and the solution diluted by the addition of water (40 ml). The optical density at 550 mμ was read in 1 cm cells. The results are given in Table IV.

TABLE IV /

TABLE IV.

Relative Activity of Fractions.

Time (hr)	f-I	f-II	f-III	f-IV
0	0.638	0.650	0.704	0.614
1	-	0.645	0.625	-
2	-	0.575	0.550	-
3	0.600	0.590	0.532	0.580
Percent. Decrease at T = 3 hours	6.0	9.2	24.0	5.5

(8) Amylolytic Activity in f-1 - f-IV.

Digest (I): 3 ml enzyme (7.5 mg) in 0.25 M citrate pH 6.0. 2 ml polysaccharide solution (glycogen 20 mg).

The digest was incubated at room-temperature (18°).

Digest (II): As above (digest I) but with amylopectin in place of glycogen.

Digest II was incubated at 37°.

Aliquots (1 ml) were taken at 0, 1, 2, and 24 hr, stained with 1 ml iodine standard solution, and diluted with 20 ml water (glycogen digests) and 40 ml water (amylopectin digests), together /

together with 3 drops of 6 N hydrochloric acid. Optical density readings were taken at the wave-lengths of maximum absorption in 2 cm cells digest (I) and 1 cm cells digest (II). The results of these determinations are given in Table V, and were calculated by working out the change in optical density per unit weight of polysaccharide at T=2 hr., i.e. in column f-I (digest I) at T=2 the optical density reading difference is 0.018, and (for digest II) 0.054. But in digest I, there is four times the "amount" of polysaccharide present therefore the corresponding figure is divided by 4.

(c) Acetone Fractionation of f-III.

f-III (10 g) was dissolved in 0.05 M sodium bicarbonate (250 ml) and stirred for 15 min. The temperature was then lowered to -5° and the stirring continued for a further 15 min. The resulting solution was centrifuged for 10 min. at 2,000 r.p.m. and the supernatant solution fractionated as shown in Table VI. The acetone (distilled over potassium permanganate, acidified with sulphuric acid) was added with stirring over 10 min., and the stirring then continued for a further 10 min. The temperature was maintained at ca. -8°.

TABLE V/

TABLE V.

Digest I, O.D.$_{470}$	f-I	f-II	f-III	f-IV
T = 0 (hr)	0.758	0.772	0.902	0.752
T = 1	-	0.769	0.882	0.661
T = 2	0.740	-	0.885	0.621
T = 24	0.698	0.778	0.850	-
Digest II, O.D.$_{550}$				
T = 0 (hr)	0.775	0.780	0.784	0.732
T = 1	0.760	0.760	0.755	0.712
T = 2	0.721	0.722	0.700	0.715
T = 24	0.711	0.708	0.552	0.688
Amylase + Branching Activity	0.054	0.060	0.084	0.017
Amylase Activity	0.005	0.000	0.005	0.033

TABLE VI /

TABLE VI.

Fractionation Protocol and Composition.

Fraction Number:	f-III/I	f-III/2	f-III/3	f-III/4
Percent. Acetone	15	26	47	75
Volume Centrifugate (ml)	250	300	360	470
Volume acetone added (ml)	45	55	150	400
Fraction Weight (g)	0.4	4.5	0.6	0.7
Protein Percent. (Kjeldahl)	4.3	7.5	19.7	17.2

Each fraction was centrifuged for 10 min. at 2,000 r.p.m. and the precipitate taken up in water, and dialysed against distilled water for 3 hr at 0°. Each dialysate was made 0.1 M with respect to citrate at pH 6.0, and freeze dried. The detailed results are given in Table VI.

(10) Amylolytic Activity in f-III Sub-fractionation.

Digest I: Enzyme 7.5 mg in 3 ml 0.5 M citrate, pH 6.0.
Glycogen 40 mg in 2 ml.

The digest was incubated at room temperature.

Digest II: As above, but with amylopectin in place of glycogen.
The digest was incubated at 37°, and aliquots (1ml) were treated as before.

The /

The results of this assay are given in Table VII.

TABLE VII.

Digest I, O.D.$_{470}$	f-III/1	f-III/2	f-III/3	f-III/4
T = 0	0.830	1.050	0.888	0.815
T = 1	0.739	0.941	0.782	0.699
T = 2	0.610	0.751	0.624	0.528
T = 24	0.710	0.880	0.708	0.515
Digest II, O.D.$_{550}$				
T = 0	0.920	0.948	0.942	0.924
T = 1	0.840	0.848	0.848	0.816
T = 2	0.750	0.658	0.774	0.750
T = 24	0.558	0.680	0.714	0.521
Amylase + Branching Activity	0.170	0.299	0.168	0.174
Amylase Activity	0.055	0.073	0.066	0.072

(11) /

(11) Action on β-Limit Dextrin.

The following digest was set up:

Enzyme 5 ml (40 mg f-II)
Polysaccharide solution 20 ml (40 mg)
Buffer, 5 ml citrate (1.0 M, pH 6.0)

The reaction was carried out for 3 hr at 37°, and then stopped by heating for 10 min. at 100°. An identical digest was similarly treated without incubation to afford a blank. The β-amylolysis limit of each digest was determined on 20 ml portions of the digests by the addition of β-amylase (80 mg) in 5 ml water. The β-amylolysis digests were made up to 50 ml with acetate buffer pH 4.1 and 0.1 M., and incubated for 24 hr at 37°. The liberated maltose was estimated by the Somogyi method. The results are tabulated below:

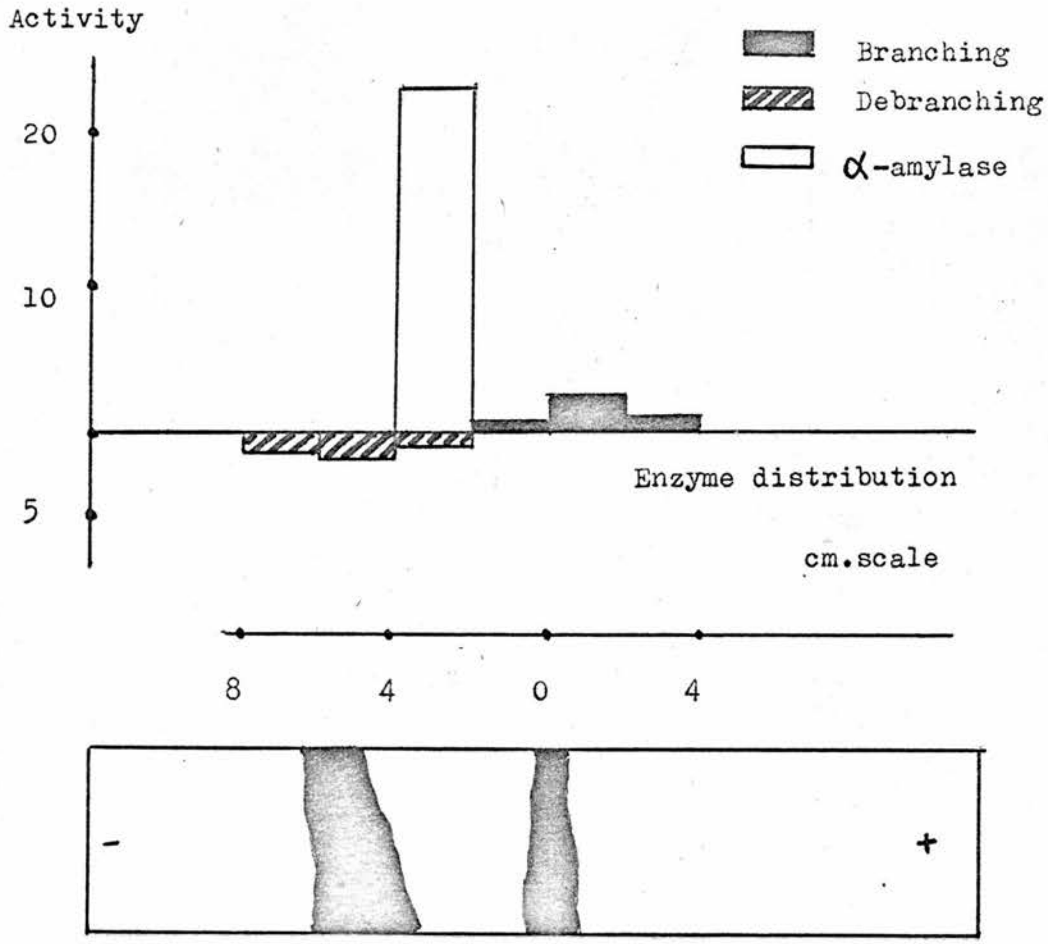
Reagent Blank 9.43 and 9.44 ml.
T = 0 Control 7.95 and 7.94 ml.
T = 3 β-Limit 7.74 and 7.73 ml.

The change in β-amylolysis limit was calculated as follows: the titre difference between the control and the branching enzyme experiment was 1.70 - 1.49 ml = 0.21 ml. Since a titre difference of 1.0 ml = 0.51 mg maltose (calibration experiment, see general methods section), 1.07 mg maltose or 1.02 mg polysaccharide have been liberated per 50 ml digest, and the increase in the β-amylolysis limit of the β-dextrin due to the action of the branching enzyme is:

$$\underline{1.02 \times 100/27 = 3.8\%}$$

(12) /

PRELIMINARY ELECTROPHORESIS OF EXTRACT OF SWEET-CORN.



Protein distribution on stained electrophoretogram.
[See Grassmann & Hunnig, Naturwissenschaften (1950) 37, 496].

Figure 1.

(12) Iodine Staining.

The action of the branching enzyme on the waxy-maize starch β-limit dextrin was also followed by an iodine staining technique. The absorption of 1.0 ml portions of the control and enzyme digests was measured at 535 mμ by staining with 1.0 ml standard iodine solution, and diluting with 20 ml water (and 3 drops 6 N HCl). The optical density readings were initially 0.920, and after 3 hr, 0.875. The percentage decrease was therefore,

<u>5.0%</u>.

The action of the branching enzyme on β-limit dextrin was therefore accompanied by an increase in the β-amylolysis limit, and a small decrease in the absorption of the polysaccharide:iodine complex.

(13) Electrophoresis of f-III.

A preliminary small-scale electrophoresis was first carried out. Enzyme (ca. 10 mg) was dissolved in 0.5 ml buffer (0.01 M citrate, pH 6.3) and streaked onto a piece of "wick" paper S & S 470 (see Fig 1). The experimental conditions were:

Paper 4.6 x 24.0 cm.: Volts 440: Amps: 6-8 mA.: Buffer 0.01 M citrate, pH 6.3: Time 2.25 hr.

The electrophoresis was run in duplicate, one paper being stained /

stained to show areas of migration, and the other assayed as follows. The paper was divided longitudinally, and each half cut into 2 cm strips numbered from the datum line. Each strip was immersed in 5 ml of the following buffer/substrate solution: 4 ml glycogen (10 mg/ml) 25 ml citrate 0.25 M, pH 6.0, 25 ml water. Corresponding strips were immersed in 5 ml of a buffer/substrate solution in which the glycogen was replaced by amylopectin (2 ml, 10 mg/ml).

Aliquots (1 ml) were removed at T = 0 and T = 2 hr, and added to 20 ml iodine reagent (amylopectin digests) and 10 ml (glycogen digests). The iodine reagent was prepared by diluting 10 ml of standard iodine solution and 3 ml of 6 N hydrochloric acid to 200 ml.

A similar preliminary electrophoresis experiment was also carried out using 0.02 M citrate at pH 7.0, with less success. The results of the experiment described are given in Table VIII.

TABLE VIII /

TABLE VIII.

Preliminary Electrophoresis of f-III.

Strip Number	Glycogen Substrate		Amylopectin Substrate	
	T = 0	T = 2	T = 0	T = 2
-5	0.188	0.185	0.401	0.401
-4	0.188	0.192	0.401	0.401
-3	0.188	0.193	0.401	0.401
-2	0.188	0.190	0.401	0.378
-1	0.188	0.183	0.401	0.406
+1	0.188	0.177	0.401	0.401
+2	0.188	0.180	0.401	0.401
+3	0.188	0.188	0.401	0.401
+4	0.188	0.188	0.401	0.401
+5	0.188	0.188	0.401	0.395

(14) The Continuous Electrophoresis of f-III.

f-III (4.0 g) were dissolved in water (20 ml) and dialysed against running tap-water for 3 hr. The dialysis was then continued at 0° against 0.01 M citrate at pH 6.3 overnight. A Spinco model CP continuous electrophoresis cell was used for the fractionation of the dialysed material over a 42 hr period. Individual tubes were qualitatively assayed for protein content by measuring their U.V. absorption at 280 mμ. These results are /

(14) THE CONTINUOUS ELECTROPHORESIS OF f-III: PROTEIN ELUTION CURVES.

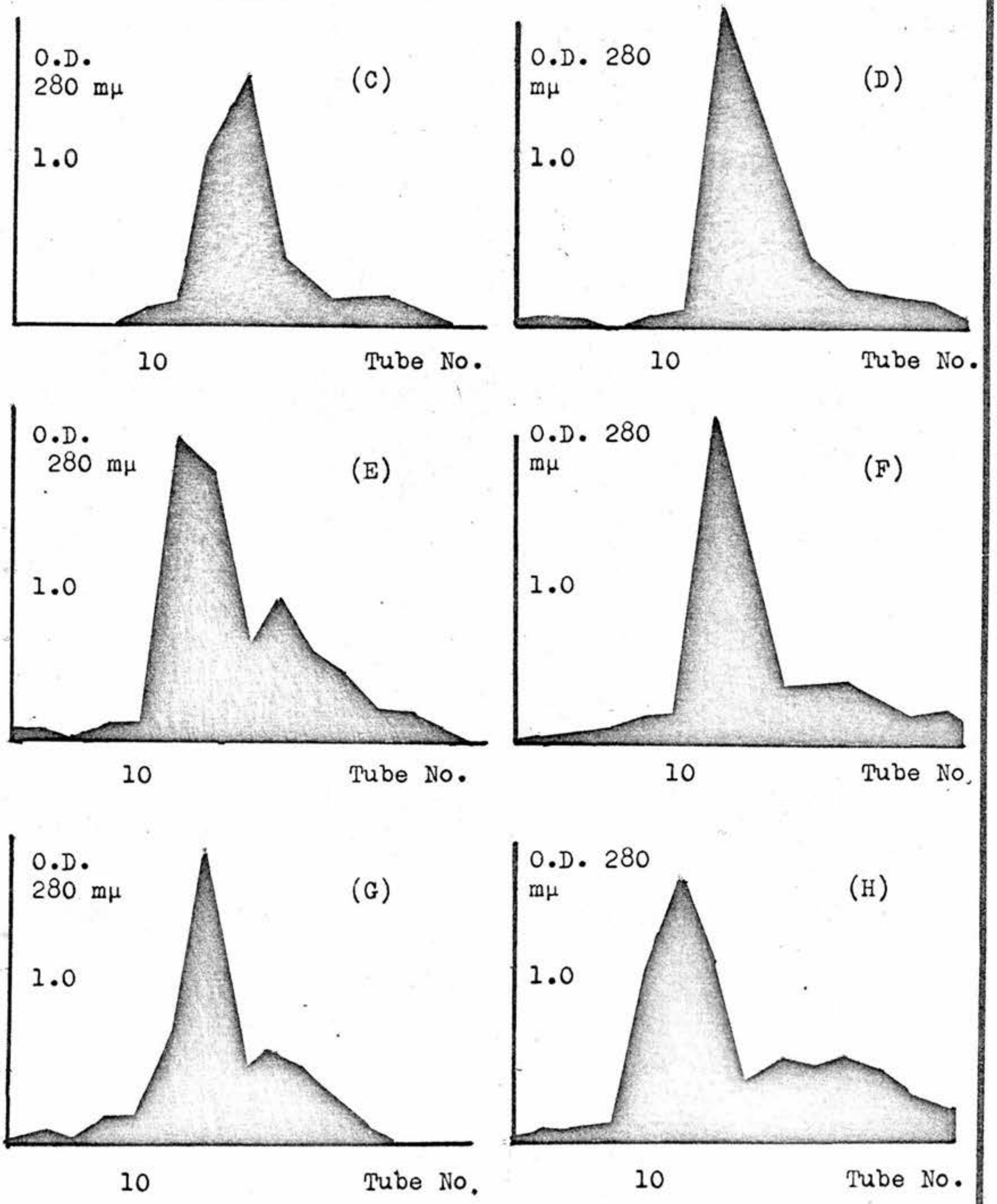


Figure 2.

are presented graphically in fig. 2 .

The experimental conditions were: Volts 600: Amps. 69 mA: Buffer, 0.01 M citrate, pH 6.3.

(15) Assays of the Electrophoresis Fractions.

Tube numbers 9, 17, and 25 were selected from each rack as being representative of each of the three main areas of the elution curve, and were assayed using amylose, amylopectin, and glycogen as substrates under standard conditions as follows:

Digest: 1 ml enzyme fraction. 2 ml substrate (2 mg) in 0.25 M citrate, pH 6.0.

The digests were incubated for 2 hr at 37°. Aliquots (1 ml) were removed at T = 0, and T = 2 hr and pipetted into iodine reagent (5 ml for glycogen digests, and 10 ml for amylopectin and amylose digests). The results of these assays, expressed as percent. decrease in optical density readings are given in Table IX. The glycogen readings were taken at 470 mμ, the amylopectin readings at 550 mμ, and the amylose readings at 630 mμ.

For further experiments with the "debranching enzyme", fractions B9, C9, and D9 were combined, and for further experiments with the "branching" enzyme, fractions C 25, D 25, and F 25 were combined and kept frozen.

(16) /

TABLE IX.

Enzyme Activity in Electrophoresis Fractions.

Sample		Substrate.											
		Amylopectin				Glycogen				Amylose			
Rack	Tube	T=0	T=2	Diff.	%	T=0	T=2	Diff.	%	T=0	T=2	Diff.	%
B	9	678	675	0	0%	081	096	+15	+19%	-	-	-	-
(M)	17	721	720	0	0%	194	180	14	7%	-	-	-	-
	25	682	484	198	29%	105	96	9	6%	-	-	-	-
C	9	690	631	(59)	0%	097	101	+4	+4%	218	215	0	0%
(M)	17	724	718	6	1%	220	212	8	2%	238	252	0	0%
	25	684	444	240	35%	102	103	0	0%	177	063	113	64%
D	9	701	702	0	0%	081	097	+15	+19%	241	238	0	0%
(M)	17	784	702	82	10%	282	283	0	0%	264	250	0	0%
	25	699	449	250	36%	102	105	0	0%	238	083	115	65%
E	9	689	678	11	2%	084	092	+8	+10%	243	241	0	0%
(M)	17	778	682	96	12%	266	267	0	0%	261	260	0	0%
	25	692	474	281	32%	103	112	+9	+9%	238	092	146	61%
F	9	692	682	10	2%	105	112	+7	+7%	245	243	0	0%
(M)	17	850	661	189	22%	397	364	33	8%	277	254	23	8%
	25	699	461	238	34%	179	180	0	0%	238	086	152	64%
G	9	692	678	20	3%	088	-	-	-	238	-	-	0%
(M)	17	782	639	143	18%	257	242	15	6%	260	240	20	8%
	25	721	434	287	40%	114	124	+10	+9%	239	084	145	61%
H	9	690	668	22	3%	091	108	+17	+19%	238	236	0	0%
(M)	17	740	654	86	12%	174	176	0	0%	253	240	13	5%
	25	696	444	252	36%	399	118	+19	+19%	235	085	150	64%

(M) Maltase activity present.

(16) Assays of the Combined Electrophoresis Fractions.

All the remaining fractions were combined as follows: tubes 1 and 2 from each rack, and tubes 3 and 4 from each rack &c, were combined, and the resulting 16 fractions were further assayed for branching activity. The digests used were:

Digest: 1 ml enzyme fraction: 2 ml substrate in 0.25 M citrate, pH 6.0.

The substrate was amylopectin or glycogen (1 mg/ml). Each digest was incubated at 37° for 2 hr, and 1 ml aliquots were diluted with 20 ml iodine reagent (amylopectin digests) and 5 ml (glycogen digests). These results are given in Table X and are also presented graphically.

TABLE X.

Percent. Optical Density Decrease.

Glycogen Assay **Amylopectin Assay**

Tube	$T_0=0$	$T_2=2$	T_0-T_2	$\frac{T_0-T_2}{T_0}$100%	Original Fraction Number	$T_0=0$	$T_2=2$	T_0-T_2	$\frac{T_0-T_2}{T_0}$ 100%
1	.258	.194	.064	25%	1,2	.517	.520	.000	0%
2	256	185	071	27%	3,4	552	530	022	4%
3	257	188	069	26%	5,6	559	532	027	5%
4	263	194	069	26%	7,8	553	533	020	4%
5					9,10				
6	264	207	057	22%	11,12	552	527	025	5%
7	264	202	062	24%	13,14	578	548	030	5%
8	377	300	077	21%	15,16	578	548	030	5%
9	530	393	137	26%	17,18	618	552	066	11%
10	339	272	087	26%	19,20	604	542	062	10%
11	284	212	072	25%	21,22	560	512	048	9%
12	273	223	050	18%	23,24	551	481	070	13%
13	276	228	046	17%	25,26	551	444	107	19%
14	270	230	040	15%	27,28	559	438	121	22%
15	268	238	030	11%	29,30	559	480	079	14%
16	275	247	028	10%	31,32	559	472	087	16%

O.D.$_{460}$ mμ : 1 cm cells
5 ml Iodine Reagent
1 ml aliquots.

O.D.$_{550}$ mμ : 1 cm cells
20 ml Iodine Reagent
1 ml aliquots.

(17) Assay of Electrophoresis Fractions for Polysaccharide.

Because the large amount of polysaccharide (70%, see Table I) in f-III appeared in some of the electrophoresis fractions, these were examined for iodine staining power. Aliquots were taken (1 ml), and stained with iodine reagent (5 ml). The results are given in Table XI.

TABLE XI.

Enzyme Iodine Staining Power.

Tube	O.D. 460 mμ	O.D. 550 mμ	O.D. 630 mμ.
1	0.000	0.000	0.000
2	0.010	0.003	0.003
3	0.009	0.007	0.003
4	0.013	0.066	0.000
5	0.027	0.006	0.004
7	0.026	0.009	0.007
8	0.238	0.142	0.012
9	0.476	0.310	0.156
10	0.362	0.258	0.135
11	0.125	0.080	0.045
12	0.072	0.037	0.024
14	0.066	0.023	0.011
15	0.039	0.007	0.003

(18) /

THE ACTION of f III ON POTATO AMYLOPECTIN AT 37°.

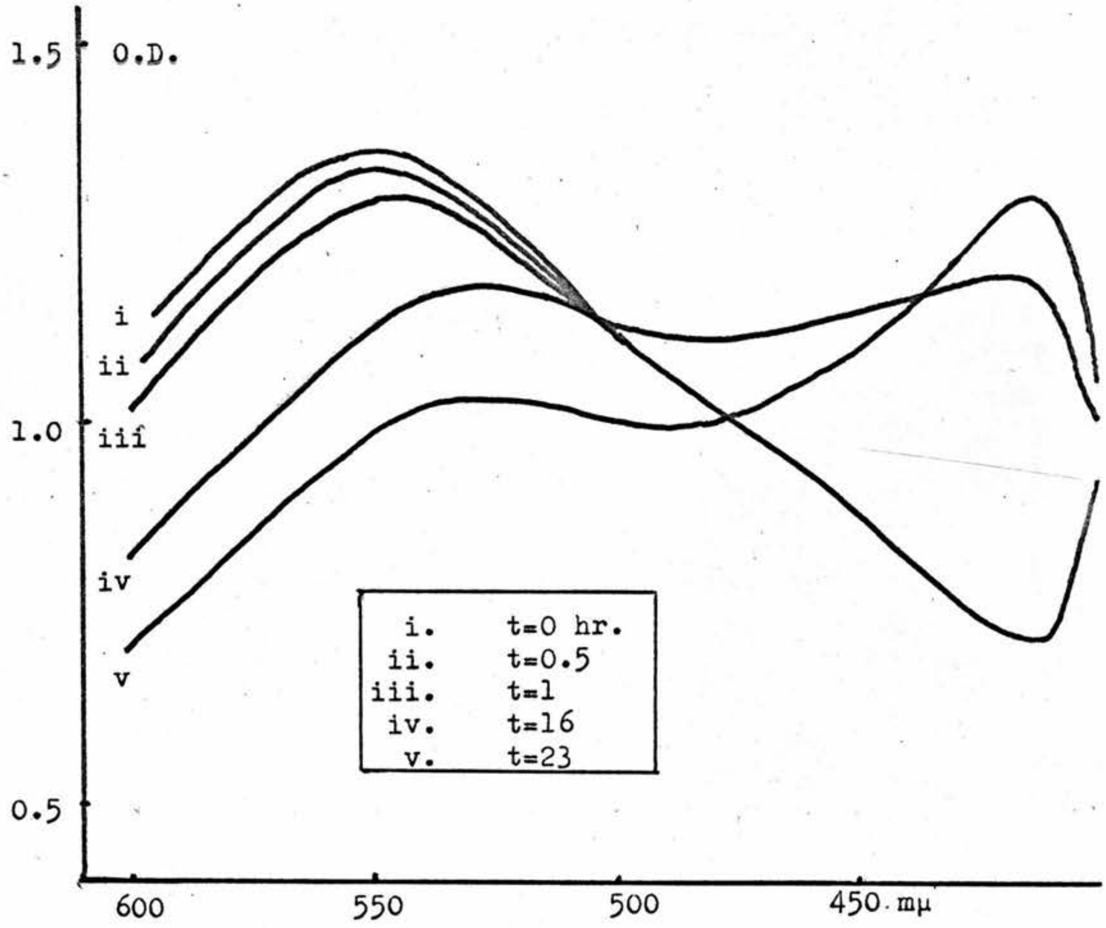


Figure 3 .

ASSAY OF COMBINED ELECTROPHORESIS FRACTIONS FOR BRANCHING AND α-AMYLASE ACTIVITY.

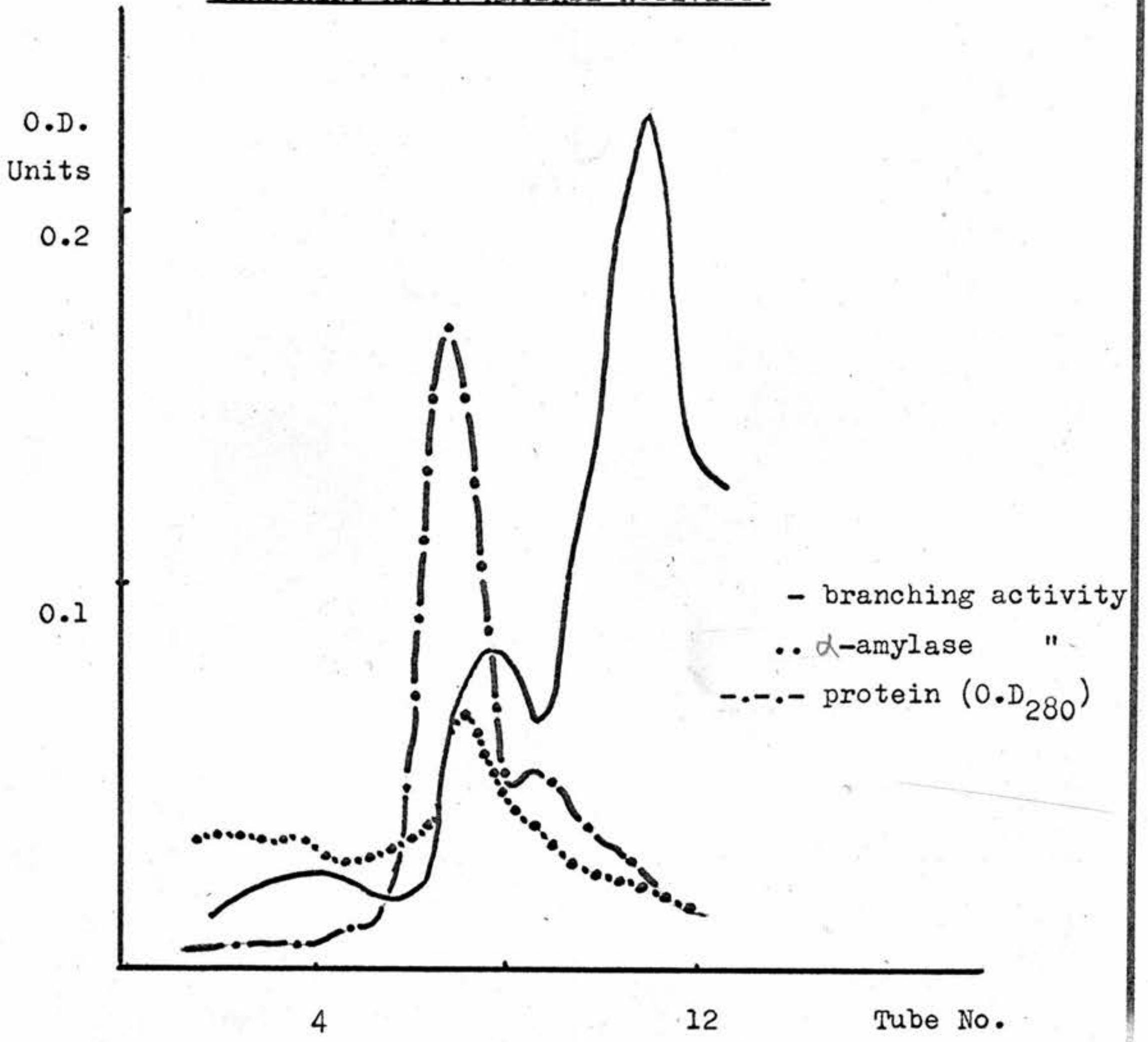


Figure 4.

(18) The Action of Branching Enzyme on Amylopectin and Amylose.

Digest: 5 ml enzyme (pooled fractions C25, D25, F25):
20 ml polysaccharide (1 mg/ml) in buffer:
Buffer, 0.5 M citrate, pH 6.0.

Portions, (1 ml) were taken at T = 0, 1, 3, 6, and 24 hr and stained with iodine reagent (5 ml). The results are given in Table XII.

TABLE XII.

T	Amylopectin		Amylose	
(hr)	λ Max	O.D.	λ Max	O.D.
0	554	0.870	630	1.220
1	546	0.770	594	0.610
3	541	0.670	578	0.580
6	535	0.570	565	0.765
24	516	0.350	556	0.710

(19) Estimation of Protein by the Biuret Method.

Standard Protein Solution: Tryptone (900 mg) was made up to 100 ml, and a Kjeldahl determination of 2 ml gave 2.16 mg nitrogen. The Tryptone therefore contained 75% protein.

The Biuret Method.

Tryptone solution (0.5 to 2.0 ml) was made up to 3.0 ml in centrifuge tubes. The total volume was brought up to ca.9 ml with 3% sodium hydroxide solution, and 0.25 ml 20% copper sulphate solution added. The tubes were each shaken for 1 min. and then allowed to stand for 15 min. The tubes were then centrifuged and the volume adjusted to 10 ml. Electrophoresis fractions (3 ml) /

(3 ml) 9, 17 and 25 were similarly treated. The optical densities of the centrifuged solutions were read at 550 mμ in 1 cm. cells. The standard solution (1.0 ml) gave a Biuret optical density of 0.115.

TABLE XIII.

Protein (%) in Electrophoresis Fractions.

O.D.$_{550}$	f-9	f-17	f-25
	0.000	0.104	0.016
mg Protein per ml	0.02	0.3	0.06

(20) Studies on the Effects of Salts on the Branching Enzyme.

Digests incubated at 37° were sampled at T = 0, 15, 30 and 60 min.

Digest: 1.5 ml enzyme (pooled fractions C25, D25, F25):
3.0 ml polysaccharide (6.0 mg amylopectin) in
0.25 M citrate, pH 6.0: 0.5 ml salt solution.

Aliquots (1 ml) were stained with iodine reagent (10 ml). In the following tables, the final salt concentration is given. Optical densities at 550 mμ were read in 2 cm cells. The results so obtained are tabulated in Tables XIV, XV, and XVI.

TABLE XIV /

TABLE XIV.

Mercuric Chloride.

$HgCl_2$	T = 0	T = 15	T = 30	T = 60
5×10^{-6} M	0.850	0.820	0.815	0.810
5×10^{-5} M	0.850	0.850	0.840	-
5×10^{-4} M	0.850	0.850	0.845	-
5×10^{-3} M	0.850	0.850	0.850	-
Control	0.850	0.820	0.812	0.790

Experiment (22)

TABLE XV

Ammonium Molybdate.

Concentration Percent.	T = 0	T = 15	T = 30	T = 60
0.5	0.850	0.818	0.805	0.799
1.0	0.850	0.830	0.820	0.808
2.0	0.850	0.835	0.820	0.808
4.0	0.850	0.835	0.820	0.804
Control	0.850	0.820	0.812	0.790

(23) /

Experiment (23)

TABLE XVI

Sodium Borate.

Concentration	T = 0	T = 15	T = 30	T = 60
0.001 M	0.850	0.818	0.808	-
0.002 M	0.850	0.818	0.808	-
0.004 M	0.850	0.818	0.795	0.790
0.008 M	0.850	0.818	0.795	0.790
Control	0.850	0.820	0.812	0.790

Sodium borate therefore caused no inhibition, ammonium molybdate slight inhibition above 1.0%, and mercuric chloride complete inhibition above 5 x 10^{-5}M.

(24) Determination of the Branching Enzyme pH-Optimum.

A series of digests, each at a different pH, was set up.

Digest: Enzyme solution (1 ml): polysaccharide solution (1 ml, 4 mg amylopectin): buffer solution (1 ml Molar citrate, pH 4 to 8).

The digests were incubated at 37° and sample (1 ml) withdrawn at 0 and 3 hr., and stained with iodine reagent (20 ml). The optical density readings were measured in 2 cm cells at 550 mμ. The pH-values shown in Table XVII were measured at the end of the incubation period.

TABLE XVII /

TABLE XVII.

pH-Optimum of Branching Enzyme.

pH	T = 0	T = 3	Difference	Percent. Decrease
4.32	0.772	0.681	0.091	12
5.35	0.765	0.508	0.157	20
5.80	0.770	0.500	0.270	35
6.10	0.772	0.500	0.270	35
6.41	0.772	0.520	0.252	33
6.50	0.775	0.532	0.240	31
6.60	0.755	0.551	0.204	27
7.40	0.780	0.655	0.125	16

(25) Determination of the Debranching Enzyme pH-optimum.

A series of digests was set up, each digest adjusted to a different pH.

Digest: Enzyme (pooled enzyme B9, C9, D9) 2 ml: polysaccharide solution (1 ml, 4 mg glycogen): buffer (2 ml, 0.5 M citrate).

Samples (1 ml) were withdrawn at T = 0 and T = 2 hr., and stained with 5 ml iodine reagent. The pH given in Table XVIII is that of each digest after 2 hr. The optical density at 470 mμ was read in 2 cm cells.

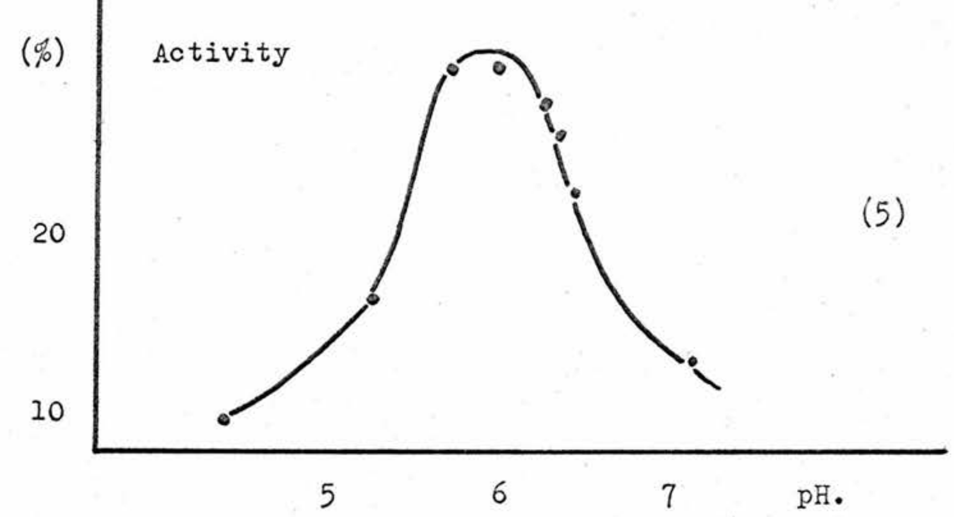
TABLE XVIII /

TABLE XVIII.

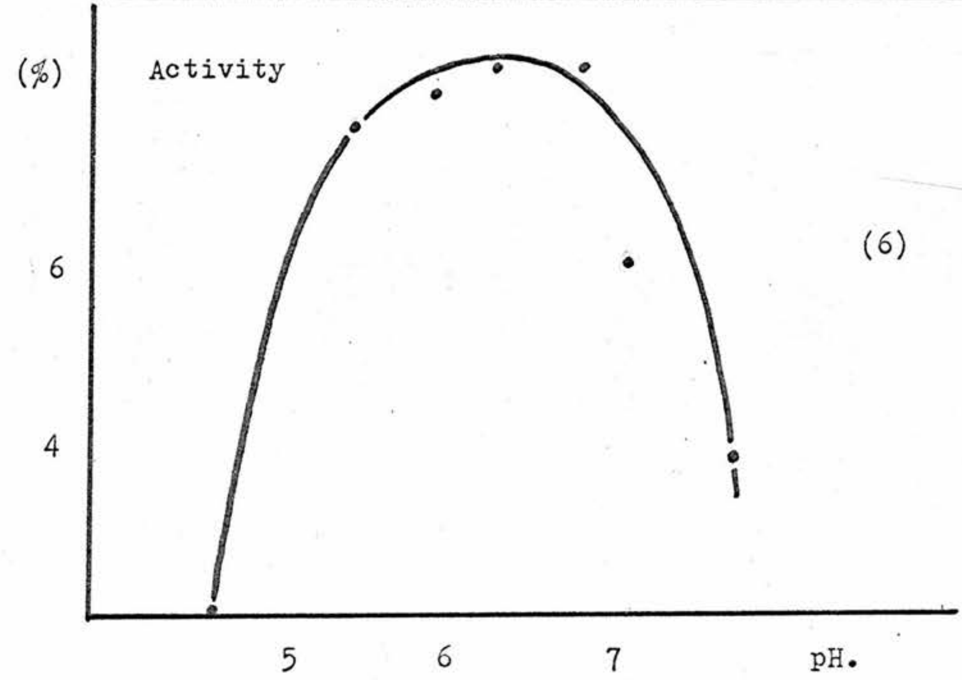
pH-Optimum of Debranching Enzyme.

pH	T = 0	T = 2	Difference	Percent. Increase
4.25	0.220	0.220	0.000	0.0
5.19	0.220	0.234	0.014	6.4
5.76	0.220	0.235	0.015	6.8
6.08	0.220	0.236	0.016	7.2
6.72	0.220	0.236	0.016	7.2
6.92	0.220	0.226	0.006	4.5
7.66	0.220	0.224	0.004	2.0

THE SWEET-CORN BRANCHING ENZYME pH-OPTIMUM at 37°.



THE SWEET-CORN DEBRANCHING ENZYME pH-OPTIMUM at 18°.



Figures 5 & 6 .

The Action of Branching Enzyme on Maize Amylose.

(The amylose was prepared by Dr. J.R. Stark).

(26) Isolation of Amylose from Butanol Complex.

The complex was stirred and washed into two tubes, which were then centrifuged. More complex was added and centrifuged. The precipitate was stirred with butanol and re-centrifuged. The stirring and washing process was repeated eight times with ethanol and finally once with ether. The ether-washed amylose was placed in a desiccator over phosphorous pentoxide and paraffin wax shavings. The yield was 100 mg.

(27) The solution of Amylose.

The dry amylose was dissolved in 5 ml 0.3 N sodium hydroxide by gentle heating, and the solution, neutral to phenol-phthalein, made up to ca. 25 ml. The digests described on the following pages utilised this solution; an aliquot of which 5 ml was retained in order to determine the exact concentration of the polysaccharide by acid hydrolysis.

(28) The following digest was set up:

> Enzyme solution (25 ml): Maize amylose solution (20 ml): adjusted to 50 ml with 0.5 M citrate, pH 6.0.

The digest was incubated at 37°, and 5 ml aliquots (a) for the determination of β-amylolysis limit values, and (b) for the combined isoamylase and β-amylolysis limit values, taken.

(29) /

(29) β-amylolysis.

Aliquots (5 ml) were taken at T = 0 and T = 24 hr and inactivated by immersion in a boiling water bath for 10 min. β-amylase (1000 units) was added in 0.2 M acetate buffer (5 ml, pH 4.6), and the maltose content estimated after a 24 hr incubation at 37° by the Somogyi method.

(30) Isoamylolysis.

Aliquots (5 ml) were taken at T = 0 and T = 24 hr and inactivated by immersion in a boiling water bath. Isoamylase (10 mg) was added in citrate buffer (pH 6.0, 0.25 M) and incubation at room-temperature (18°) carried out for 24 hr. The inactivated digest was then further treated with β-amylase (1000 units) for 24 hr at 37° and finally the combined action of both enzymes continued for 48 hr at room-temperature by the further addition of 10 mg isoamylase and 1000 units of β-amylase at pH 6.0. The results are tabulated below. (Table XIX).

TABLE XIX

	Wt p/s as maltose (mg)	Wt maltose released at T = 0 (mg)	Wt maltose released at T = 24 (mg)
β-amylolysis Limit	5.99	6.10 101.0%	4.59 77.0%
Isoamylolysis Limit	3.59 -		3.54 i.e. 98.0%

(31) /

(31) The Action of Branching Enzyme on Amylopectin.

The following digest was used in this experiment:

Enzyme solution (25 ml): Amylopectin (20 ml):
adjusted to 50 ml with 0.5 M citrate, pH 6.0.

The digest was incubated at 37°, and 5 ml aliquots withdrawn for the determination of β-amylolysis limit, and 3 ml aliquots for both α-amylolysis and combined β- and iso-amylolysis limit determinations. The results are tabulated below. The polysaccharide concentration was determined by acid hydrolysis.

TABLE XX.

	Wt p/s as maltose (mg)	Maltose released at T=0 (mg)	Maltose released at T=24 (mg)	Wt of Maltose released (mg)
α-amylolysis Limit	4.82 -	4.33 90%	4.05 84.0%	
β-amylolysis Limit	8.04 -	4.85 59.0%	4.47 54.0%	
Combined Iso- and β-amylolysis Limits	8.04			6.84 i.e.85.0%

(32) The Combined Action of β- and Iso-amylase on Phytoglycogen A.

A solution of Phytoglycogen A was prepared containing approximately 2 mg/ml. A portion of this was reserved for the determination /

determination of the polysaccharide concentration by acid hydrolysis. 10 ml of the solution was treated first with β-amylase (1000 units) and then with isoamylase (10 mg). The separate action of these enzymes was followed by their combined action at room temperature. As a result of this treatment, phytoglycogen A (equivalent to 23.9 mg maltose) released maltose (18.15 mg), giving a limit value of 76.0%.

(33) The Action of Heat on Branching Enzyme.

The activity of the branching enzyme was examined at 20, 30, 40 and 50°. Each digest (described below) was incubated for 30 min., and an aliquot (1 ml) stained with standard iodine reagent (10 ml). The optical density readings were taken on the Perkin Elmer Recording spectrophotometer in 2 cm cells, at 550 mμ.

Digest: Enzyme solution (2 ml): polysaccharide (2 ml, containing 2 mg amylopectin): buffer (1 ml citrate, pH 6.0, 0.25 M).

The results of this experiment are incorporated in Table XXI.

TABLE XXI.

Temperature Optimum of the Branching Enzyme.

Temperature °C	$O.D._{550}$ at T = 0	$O.D._{550}$ at T = 30	Percent. Decrease
20	0.630	0.595	5.5
30	0.620	0.595	4.0
40	0.610	0.495	18.8
50	0.610	0.580	4.8

DISCUSSION.

A branching enzyme has been isolated from sweet-corn flour and some of its properties investigated. This enzyme, unlike other plant branching enzymes (Q-enzymes) which are unable to introduce branch-points into amylopectins, has been shown in this respect to resemble the yeast branching enzyme described by Gunja *et al* (1960). Thus evidence for the presence of a new, atypical plant branching enzyme has been obtained.

Ammonium sulphate fractionation followed by continuous electrophoresis, the method used to fractionate the extract prepared from sweet-corn flour in this work, has been found to be satisfactory. In preliminary experiments, the flour was extracted in a citrate buffer solution, and the ammonium sulphate fractionation yielded two highly active protein precipitates, and also two rather less active fractions (see Table III). The protein that precipitated between 32 and 55% ammonium sulphate constituted the most active fraction, and was further fractionated by continuous electrophoresis, since an acetone fractionation experiment failed to separate branching from amylase activity.

The assay methods that were used are enumerated below:

(1) Detection of α-amylase (glycogen substrate, assayed by decrease in iodine staining power)

(2) Detection of branching enzyme (amylopectin substrate, assayed /

assayed by decrease in iodine staining power)

(3) Detection of debranching enzyme (glycogen substrate, assayed by increase in iodine staining power).

A measure of the branching activity in each fraction was obtained from the difference in optical density change between (1) and (2) above under standard conditions (Krisman, 1962). The method finally adopted of adding a dilute standard iodine "reagent" solution to each digest aliquot was found to be reproducible and rapid.

In figure 2 , the results of an assay of each electrophoresis fraction for protein content is given. The variation in readings obtained for corresponding tubes in successive racks is attributable to the polysaccharide component accompanying f-III (see Table III). This migrated down the paper curtain of the electrophoresis cell more slowly than the enzyme protein, and showed a tendency to spread out from the point of application of the sample (shown by iodine staining) thus effectively "spreading" subsequent paths of the separated components as the electrophoresis progressed. Examination of these results also suggests that the enzyme has a tendency to remain associated with the polysaccharide.

By assaying the electrophoresis fractions individually, tubes containing uniquely one kind of enzymic activity were distinguished and combined, and such combined fractions were the basis of further study. These fractions, however, tended to lose /

lose their activity on storage at -15°. Some evidence for a debranching enzyme was obtained both from the preliminary electrophoresis experiments (see p. 92) and also from the main electrophoretic separation experiment, since some fractions were found that could cause an increase in the iodine staining power of a glycogen substrate. Sufficient material was obtained to carry out a determination of the pH-optimum of this enzyme. An unsuccessful attempt was made to obtain a large yield of the debranching enzyme in a second electrophoresis experiment. Some of f-III was taken, and fractionated by continuous electrophoresis as before. The fractions so obtained were assayed and the presence of a debranching enzyme indicated in a number of fractions which were then combined. The combined fractions were re-run on the electrophoresis machine, after moving the point of application of the sample in order to obtain a better distribution of the enzyme, i.e. the debranching fractions were found to occur to the left (anode) side of the point of application, so by applying the sample further to the right (cathode) side of the paper curtain, the debranching activity would be expected to occur in the central tubes of the fraction collector. However, no debranching activity could be detected.

Some/

The Synthetic Polysaccharide Preparation.

When the sweet-corn branching enzyme was incubated with amylopectin, a polysaccharide was produced that closely resembled a glycogen in several respects. The properties of this glycogen-type polysaccharide are compared with amylopectin and also a phytoglycogen isolated from sweet-corn in the Table below.

TABLE XXIII.

A Comparison of the Properties of Amylopectin-Glycogen-Type Polysaccharides.

Property	Amylopectin	Synthetic Polysaccharide	Glycogen
α-amylolysis Limit P_M(%)	90	84	87
Proportion of 1,6-Linkages, $P_{1,6}$(%)	5.4	6.6	6.0
Average Chain Length (Glucose Residues)	19	15	16-17
Exterior Chain Length	14	10-11	11
β-amylolysis Limit after isoamylolysis	80	85	-

The /

The results tabulated above show the similarities that exist between the synthetic polysaccharide, and phytoglycogen with regard to average chain length and β-amylolysis limit. By way of comparison, the branching enzyme of yeast (Gunja **et al.** 1960) was able to synthesise a polysaccharide (from potato amylopectin) that very closely resembled a brewers yeast glycogen, comparative figures for which are given below:-

TABLE XXIV

Properties of Amylopectin-Glycogen-Type Polysaccharide.

Properties	Amylopectin	Synthetic Polysaccharide	Yeast Glycogen
α-amylolysis Limit P_M (%)	89	79	79
Proportion 1,6-Linkages $P_{1,6}$(%)	4.5	7.4	7.6
Average Chain Length	22.0	13.5	13.2
β-amylolysis Limit	53	47	44
Exterior Chain Length	14	9	8-9
β-amylolysis Limit after Isoamylolysis	80	75	68

The /

The Action of the Branching Enzyme on an Amylose Substrate.

Proof of the nature of the new branch points synthesised by the branching enzyme was obtained by the analytical use of enzymes. A sample of maize amylose was incubated with the branching enzyme. The β-amylolysis limit of the maize amylose was initially shown to be 101.0%. However, after treatment with branching enzyme, this value fell to 77.0%, indicating the introduction of glucosidic linkages into the linear substrate that were not susceptible to β-amylase attack. When the branched amylose was first treated with isoamylase, an enzyme that is specific for α-1,6-glucosidic linkages (Gunja *et al.*, 1961) and secondly with β-amylase, the β-amylolysis limit was shown to have risen to 98.0%, confirming the identity of the new branch points.

The pH-Optimum and Temperature-Optimum of the Branching Enzyme.

The branching enzyme was shown to be most active towards an amylopectin substrate at approximately pH 6.2 in citrate buffer. The temperature-optimum (at pH 6.0) was found to occur between 35-40°. In Table XXV these properties are compared with values published for other branching enzymes.

TABLE XXV /

TABLE XXV.

Properties of Branching Enzymes.

Source	Optimum pH	Optimum Temperature	Reference
Potato Q-enzyme	7.0	21	Barker *et al* (1949)
Broad-bean Q-enzyme	7.3-7.5	20	Hobson *et al* (1950)
Green Gram Q-enzyme	7.0	24	Ram & Giri (1952)
Yeast Branching enzyme	7.0	20	Gunja *et al* (1960)

A high temperature optimum plateau 25-33° has been reported for the branching enzyme isolated from *Polytomella coeca* by Bebbington *et al* (1952), which compares with the optimum of ca. 35° obtained for the sweet-corn branching enzyme. The pH-optimum of 6.2 is also not typical of a plant branching enzyme.

The Effect of Various Salts on the Branching Enzyme.

The branching activity of the sweet-corn enzyme was completely inhibited in the presence of 5×10^{-5}M mercuric chloride whereas the yeast branching enzyme (Gunja *et al*, 1960) required 5×10^{-4} M mercuric chloride before complete inhibition was observed. Similarly the effect of ammonium molybdate, which partially inhibited the sweet-corn enzyme in 1% solution, was less on the yeast enzyme, which required at least 2% ammonium /

ammonium molybdate before partial inhibition was observed. The effect of sodium borate in concentrations from 0.001 to 0.008 M was negligible on the sweet-corn enzyme.

In conclusion, a new type of plant branching enzyme has been found in sweet-corn. This enzyme (SCBE) synthesises from an amylopectin substrate a polysaccharide that closely resembles the phytoglycogen found in sweet-corn: the branching enzyme of yeast (YBE) similarly mediates the *in vitro* synthesis of a polysaccharide that is very similar to the glycogen that has been isolated from yeast. The action of SCBE on amylopectin is completely atypical of plant branching (Q-) enzymes. In this connection it is of interest to note that Frydman and Cardini (1965) have isolated a "phytoglycogen-synthetase" which is very similar to the "starch-synthetase" of Leloir (1961) and unlike the glycogen synthetase of Leloir and Goldemburg (1960) which sediments with particulate glycogen. Frydman and Cardini propose a synthetic pathway for phytoglycogen in which their synthetase and SCBE participate.

SUMMARY.

(1) A branching enzyme has been isolated from sweet-corn flour, and purified by the combined techniques of ammonium sulphate fractionation and continuous electrophoresis.

(2) The action of this enzyme towards branched and linear substrates has been studied.

(3) The pH- and temperature optimum of the enzyme have been determined, and the effect of different salts at various concentrations on the branching activity of the enzyme have been examined.

(4) The enzyme has been shown to be atypical of plant branching enzymes.

(5) Evidence has been obtained for the presence of a debranching enzyme in extracts of sweet-corn flour.

A short account of this work has been published in Chemistry and Industry, (1964) pp. 1834-1835. See Appendix I.

PART FOUR:

Biochemical Studies on Some Cases
of
Glycogen Storage Disease

INTRODUCTION.

The study of cases of glycogen storage disease presents a number of different problems to the biochemist. For example, the biopsy specimens available for examination are very small. Since it is desirable to measure more than one type of enzymic activity on each specimen, the development of semi-micro assay techniques is essential. If in addition, a structural analysis of the glycogen is to be attempted, the analysis has to be performed on milligram quantities or less of the material, again demanding adaptation of existing experimental techniques. The biochemical information thus obtained can be used to complement the clinicians' findings.

Much work on glycogen storage disease has been reported (over the past decade) in the literature, and has been extensively reviewed (Stetten & Stetten, 1960; Illingworth, 1961; Larner, 1962; Whelan, 1964; Hers, 1964a). The combination of the biochemical approach together with observations on the clinical manifestations of the disease has shed new light on the understanding of the disease itself, and has led to a better understanding of the intermediary metabolism of glycogen.

The biochemical approach has, however, a number of serious limitations. Apart from the standardisation of experimental techniques, which is discussed in the Experimental Section, little /

little is known about what constitutes "normal" human metabolism. While one might expect a severe disruption of normal metabolism to take place because one "key" enzyme is completely absent, the correlation between partial enzymic defects and associated pathological manifestations is not usually very clear.

In this section, glycogen and its metabolism will be reviewed briefly and then discussed in relation to glycogen storage disease.

The Structure of Glycogen.

Glycogen consists primarily of D-glucose residues linked mutually between positions 1 and 4, and also containing some 6-10% of linkages between positions 1 and 6. This has been shown by methylation studies (Haworth *et al*, 1937), acidic fragmentation analysis (Wolfrom *et al*, 1951), periodate oxidation (Halsall *et al*, 1947) and degradative enzymic techniques (Cori & Larner, 1951).

Hydrolysis of the permethylated polysaccharide yields 2,3-dimethyl glucose in addition to 2,3,6-trimethyl glucose, the major product. Tetramethyl glucose has also been isolated. These results suggest a branched structure for glycogen, i.e. the molecule is built up from chains of 1,4-linked glucose residues, 10-18 units long (depending on the source of the glycogen) mutually joined by 1,6-linkages or branch-points.

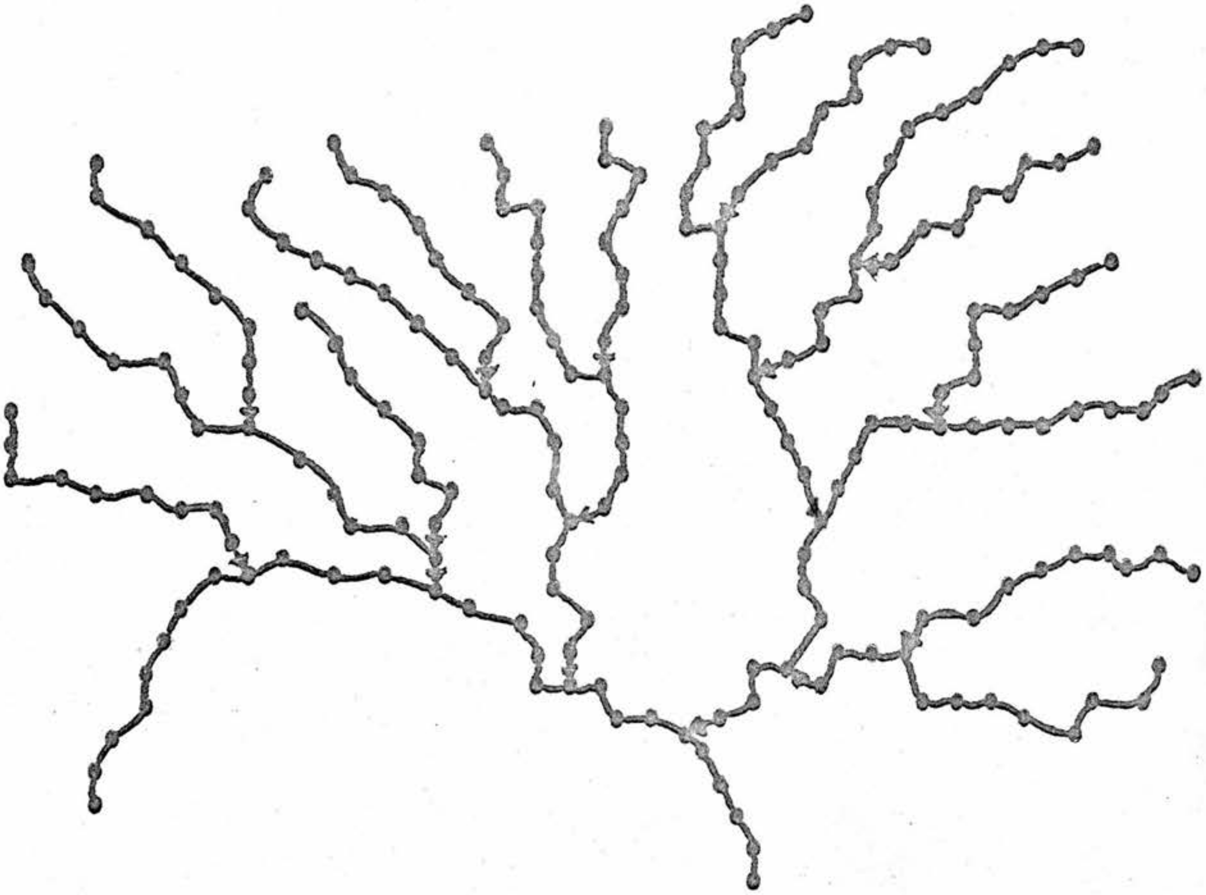
When glycogen is partially hydrolysed by dilute mineral acid /

acid, the isolation of isomaltose (Wolfrom et al, 1951; Bacon & Bacon, 1954) and other maltosaccharides (Peat et al, 1955) containing an α-1,6-linkage confirms the nature of the branch points. The configuration of the linkage occurring within the chain, as well as at the branch-point, is determined by this technique. The difficulties of obtaining a fully methylated glycogen are great (Bell, 1948; Bell & Manners, 1954) and demethylation can occur during fragmentation procedures (Bell, 1948; Lindberg et al, 1960). The action of acid on polysaccharides is, in general, likely to give rise to experimental artifacts (see Part One, this Thesis). The structural information obtained by these two methods alone is therefore not complete, although the basic structure of any polysaccharide may be determined by these means.

Oxidation by periodic acid has been used to obtain reliable values for the average chain lengths of different glycogens (Manners & Wright, 1961), and provides an additional method for the determination of the glycosidic linkages in the molecule (Bell & Manners, 1954). The technique has also been adapted for the detection of linkages other than 1,4- or 1,6- between glucose residues (Hirst et al, 1948).

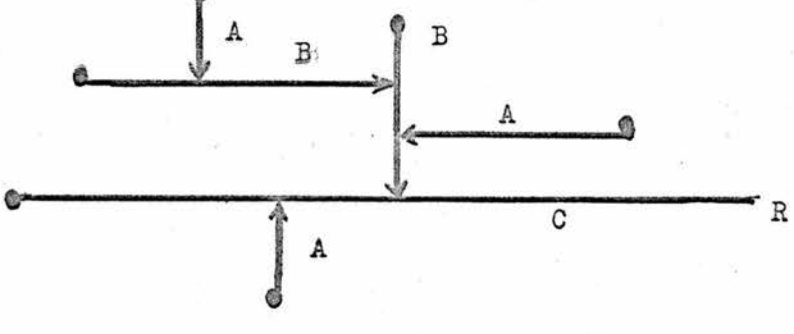
The early models for glycogen structure, based only on chemical evidence, were not satisfactory (see Haworth et al, 1937; Staudinger & Huseman, 1937). As a result of methylation and /

Figure 1.



MODEL OF A SEGMENT OF THE GLYCOGEN MOLECULE (See Cori [1953], Manners [1957]).

[KEY: ▬ -1,4-linkage, ➔ 1,6-linkage, ● glucose residue.]



TYPES OF CHAIN WITHIN A MULTIPLY-BRANCHED STRUCTURE (Peat *et al*,[1952]) and enzymic studies Meyer and Fuld (1941) introduced the concept of multiple branching, and this feature forms the basis of the currently accepted model of glycogen structure (see Fig. I). Meyer and Bernfeld (1940) originally applied this concept to amylopectin.

A multiply branched molecule (see Fig. I) contains interior chains that are situated between two branch points, and exterior chains which are attached to rest of the molecule by one branch point. The latter have been described as A-chains (Peat <u>et al</u>, 1952), and one or more A-chains may be linked to a B-chain. When a molecule contains approximately equal numbers of both kinds of chain, the B-chain, on the average, comprises one exterior and two interior chains. The C-chain is characterised by its terminal reducing group, of which there is probably only one per molecule.

Unambiguous experimental proof of the multiply branched structure of glycogen was established when by alternate use of a debranching enzyme (amylo-1,6-glucosidase) and muscle phosphorylase it was shown that successive tiers of side-chain branches of the glycogen molecule could be removed (Larner <u>et al</u>, 1952).

Glycogen molecules are typically very highly branched indeed, and it has been calculated that steric limitations on its ultimate structure would fix the molecular weight at 4-20 x 10^6, depending on /

on the degree of hydration. The calculation, due to French (1964) assumes a volume for each glucose unit from X-ray measurement, and an average chain length of 12 units. The highest experimental values, determined by osmometry (Carter & Record, 1939), light-scattering (Harap & Manners, 1952) and ultracentrifugation (Greenwood & Manners, 1957; Polglase <u>et al</u>, 1952) indicate a maximum molecular weight between 10^6 and 10^7 (Greenwood, 1952 & 1956). Glycogen, however, as extracted from animal tissues possesses a wide range of molecular weight values which can be shown in part to depend on the method of preparation. In general, glycogen of high molecular weight can only be extracted by use of very mild techniques (Orrell <u>et al</u>, 1964): for example, cold alkaline glycine buffer. There is at present no conçensus on which method yields a glycogen most representative of the native material; a high molecular weight is in itself no criterion.

In summary, therefore, the established model for glycogen structure that emerges is that of a polydisperse, high molecular weight polymer of D-glucose. The glucose residues are joined together primarily by α-1,4-linkages into chains that on average, vary from 10 to 18 units in length, but also at the points in the molecule where individual chains interconnect, by α-1,6-linkages, to form a multiply-branched, polymolecular structure. The description by Meyer (1943) of glycogen as a biological conception /

conception rather than a chemical entity still seems apt.

The Metabolism of Glycogen.

The majority of the chemical reactions that occur in the living cell are catalysed by enzymes. Anabolism, where a sequence of reactions leads to a net synthesis, and catabolism, where the enzyme action exerted is primarily degradative, together constitute metabolism. In glycogen metabolism, simple monosaccharides such as glucose or galactose are converted by enzyme action into the complex polysaccharide, glycogen. Overall the process is one that stores the energy not immediately required by the cellular economy (anabolism) and can make available the stored energy when needed (catabolism), for example, to exert muscular effort. The metabolism of proteins and lipids is intimately linked to that of the carbohydrates (Baldwin, 1959) and the interconversion of all three types of cellular constituent is possible; thus when a defect in glycogen metabolism is being considered the possibility of related side-effects should not be ignored.

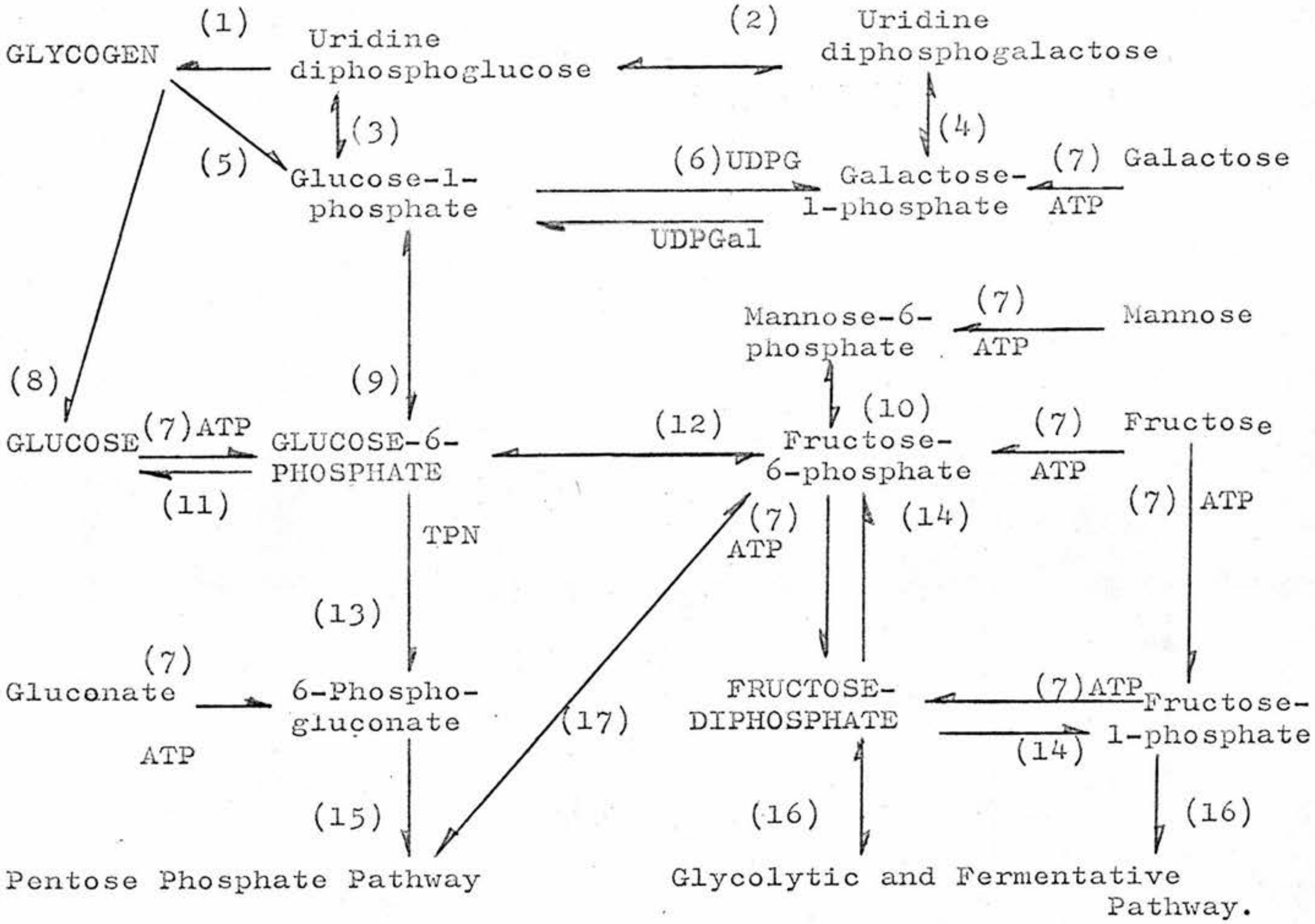
Systems in Nature that are concerned with energy transfer are closely linked with phosphate metabolism, and the synthesis and degradation of glycogen is no exception. To take the simplest case first, before it can participate in the synthesis of glycogen, glucose must first be phosphorylated at position 6. This reaction is mediated by the enzyme hexokinase and adenosine triphosphate /

triphosphate (ATP). The bonds linking the phosphoric acid residues are not all identical in this compound: two of them are spoken of as energy rich (George & Rutman, 1960). This is because $-\Delta F^0$ for the second and third bonds, shown thus below, is approximately 5 Cal/mole greater than for the standard ester. ATP itself can be regarded as a store of energy, as is illustrated in Fig. 2a.

The energy derived by the hydrolysis of ATP drives intracellular endergonic reactions, i.e. those reactions where $\Delta F > 0$. The phosphorylation of ADP is linked to respiration through electron transport from substrates along a mitochondrial system to molecular oxygen (Ernster & Luft, 1964) and affords a control over energy production that has the net effect of maintaining ATP levels within the cell.

The glucose-6-phosphate now undergoes isomerisation to glucose-1-phosphate, and can be incorporated into the glycogen molecule. A summary of the intermediary reactions involving glycogen metabolism is given on the next page. Whilst the metabolic inter-conversions that take place in the hexose monophosphate pool will not be considered in any detail here (since the main purpose of this review is to show how some of the enzymes that have been studied in relation to glycogen storage disease operate in the <u>in vivo</u> synthesis and degradation of glycogen), it can be seen, however, that glucose-6-phosphatase occupies /

METABOLIC INTERCONVERSIONS OF HEXOSES AND HEXOSE PHOSPHATES

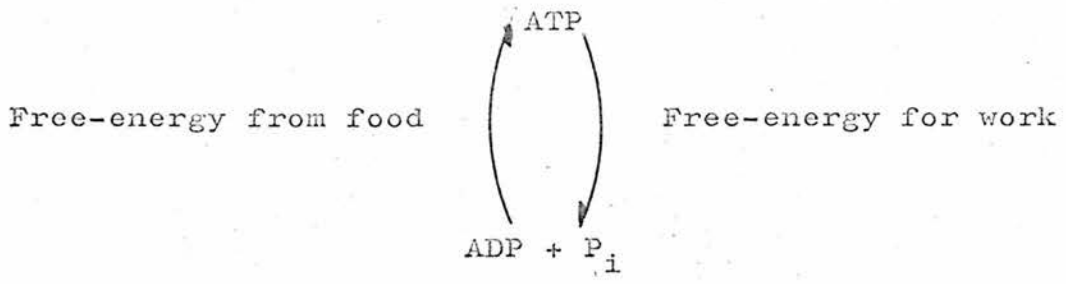


Key to Enzymes.
(1) Glycogen synthetase
(2) Uridine Diphosphogalactose-4-epimerase
(3) Uridine Diphosphoglucosepyrophosphorylase
(4) Uridine Diphosphogalactosepyrophosphorylase
(5) Phosphorylase
(6) Phosphogalactose-uridyl transferase
(7) (various) Hexokinases and phosphohexokinases
(8) Acid maltase
(9) Phosphoglucomutase
(10) Phosphomannose isomerase
(11) Glucose-6-phosphatase
(12) Phosphoglucoseisomerase
(13) Glucose-6-phosphatase dehydrogenase
(14) Fructosediphosphatases 2
(15) 6-Phosphogluconate dehydrogenase
(16) Aldolase
(17) Transaldolase and transketolase.

occupies an important position at a point where several sequences of synthetic and degradative reactions converge. Absence of the enzyme glucose-6-phosphatase would curtail, for example, the conversion of glycogen into blood glucose. This was, in fact, the observation of Mason and Sly (1943), who suggested a causal connection between von Gierke's disease and an inability of the liver to dephosphorylate hexose monophosphate.

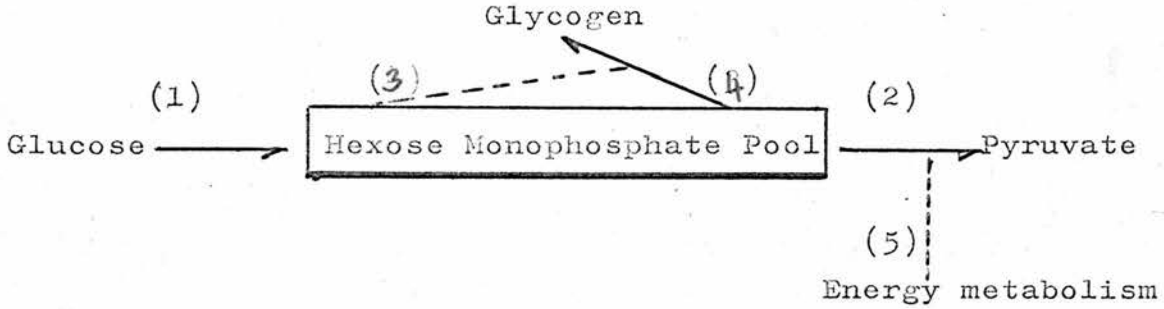
Glucose-6-phosphate can leave the hexose monophosphate pool in two ways: in the first, the rate-limiting step is mediated by the enzyme glycogen synthetase, and in the second, the dominant enzyme, leading to glycolytic breakdown is 6-phosphofructokinase (Newsholme, 1965). Glucose-6-phosphate is a powerful activator of glycogen synthetase activity (Kornfeld & Brown, 1962) and so when glycolysis is proceeding at a reduced rate, a net synthesis of glycogen occurs, i.e. when an accumulation of glucose-6-phosphate is possible. On the other hand, the enzyme 6-phosphofructokinase limits the rate of glycolysis, and hence the production of ATP: but small quantities of ATP in turn inhibit the activity of 6-phosphofructokinase. However, fructose-1,6-diphosphate, adenosine-monophosphate (AMP) or inorganic phosphate can reverse the inhibition caused by ATP (Poissoneau & Lowry, 1962). Thus the rate-limiting step of glycolysis, viz. the conversion of fructose-6-phosphate to fructose-1,6-diphosphate is controlled by factors related to energy metabolism.

These /



A-R-P∼P∼P. the energy-rich bonds of adenosine triphosphate

Figure 2a.



KEY
(1) hexokinase. (2) 6-phosphofructokinase.
(3) synthetase control by glucose-6-phosphate.
(4) glycogen synthetase.
(5) phosphofructokinase control by energy factors.

Figure 2b.

These observations are summarised in Fig. 2b.

Williamson (1965) has shown that kinase mediated processes, including the phosphofructokinase reaction are far displaced from equilibrium whereas other glycolytic reactions are not, and adduces evidence for variations in the citrate level, rather than that of the adenosine nucleotides being responsible for the control of phosphofructokinase activity. Wu (1965) has listed a number of other rate-limiting factors in glycolysis: e.g. glucose transport, and also the availability of inorganic phosphate and enzymic co-factors.

It has already been pointed out that the cellular level of ATP tends to remain constant: the enzyme adenylate kinase, which catalyses an equilibrium between ATP and AMP and also adenosine diphosphate (ADP) occurs very widely, and Krebs (1964) suggested that because of the ATP, ADP and AMP equilibrium, AMP acts as a signal of ATP concentration changes.

Alternative metabolic pathways are common in Nature. To take a well known example the breakdown of glucose-6-phosphate itself can proceed aerobically by the Warburg Dickens route, referred to as the pentose phosphate pathway in Fig(p123), the carbon flow-sheet for which can be summed up as (Harrison, 1959):

$$\text{G-6-P} + 12\ \text{TPN} + 6\,H_2O \text{–} 6\ CO_2 + 12\ \text{TPNH}_2 + P_i.$$

Alternatively, glucose-6-phosphate can be broken down anaerobically by the glycolytic and fermentative pathway due to Embden /

Embden and Meyerhof, summarised as follows (Harrison, 1959):

$$G\text{-}OH + 2\ DPN + 2\ ADP + 2\ P_i - $$
$$2\ \text{Pyruvic acid} + 2\ DPNH_2 + 2\ ATP + H_2O.$$

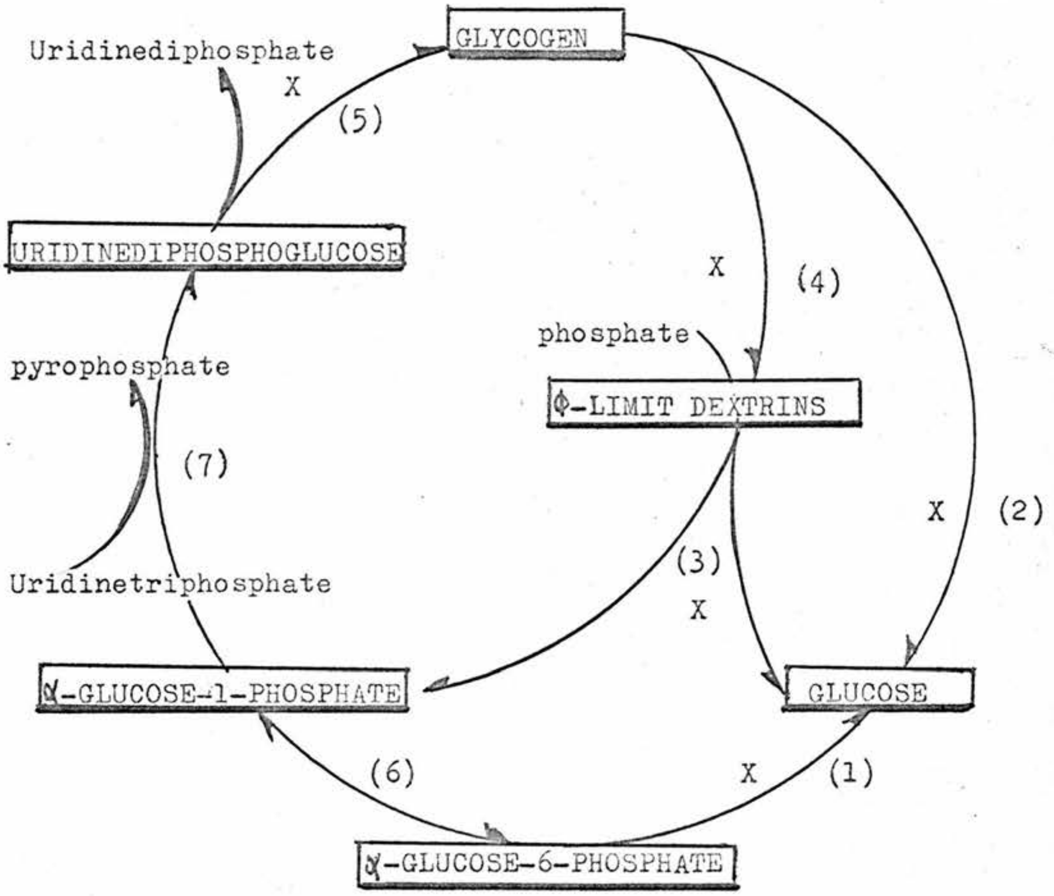
The conversion of pyruvic acid to lactic acid by the enzyme lactic dehydrogenase completes the picture. The oxidative decarboxylation of pyruvic acid via the citric acid cycle (Baldwin, 1959) links carbohydrate metabolism directly with lipid and protein metabolism.

A number of phosphorylytic enzymes have been implicated in the synthesis and degradation of glycogen, and these working in conjunction with branching and debranching enzymes can cause extensive modifications of glycogen structure. However, none of these appear to be able to increase the number of glycogen molecules already present in the cell. The amyloiytic digestion of glycogen (Rutter & Brosemer, 1961; Olivarria, 1960) could give rise to suitable precursors for the synthesis of "fresh" glycogen, and it has also been suggested that trans-glycosylation reactions are involved (Stetten & Stetten, 1960).

The metabolic dysfunction most likely to cause glycogen storage disease, which is characterised by an accumulation of glycogen, is a block in the catabolic process: the enzymes involved here are accordingly considered next. The main synthetic and degradative routes are summarised in Fig. 3.

<u>Phosphorylase</u>: The action of phosphorylase has been considered briefly in the General Introduction. It is now generally accepted /

THE SYNTHESIS AND DEGRADATION OF GLYCOGEN IN RELATION TO GLYCOGEN STORAGE DISEASE



[KEY: (1) glucose-6-phosphatase, (2) α-glucosidases, including "acid-maltase", (3) phosphorylase + oligo-1,4->1,4-glucantransferase + amylo-1,6-glucosidase, (4) phosphorylase, (5) glycogen synthetase + branching enzyme, (6) phosphoglucomutase, (7) uridyltransferase. X marks the sites of genetically determined enzyme "lesions" causing excessive glycogen deposition.]

Figure 3.

accepted that in vivo this enzyme exerts solely a degradative function; the ratio of inorganically linked to glycosidically linked phosphate (which determines the equilibrium position) has been shown to preclude synthesis (Hanes, 1940, see also Stetten & Stetten, 1960). However, in some cases of glycogen storage disease (see later) where this enzyme is demonstrably absent, the synthesis of glycogen persists. In addition, a synthetic route implicating uridine diphosphoglucose (Leloir & Cardini, 1957) is thermodynamically very favorable. The effect of adrenaline, which activates phosphorylase (Rall et al, 1957) (this point is discussed later), and hence glycogenolysis, furnishes further evidence for the in vivo action of the enzyme phosphorylase.

At least two forms of phosphorylase have been shown to exist in mammalian muscle and liver. They are characterised by their requirement for adenylic acid, and in muscle are interconvertible according to the following scheme (Krebs & Fischer, 1962).

2 phosphorylase b + 4 ATP → phosphorylase a + 4 ADP ...(1)

phosphorylase a + 4 H_2O → 2 phosphorylase b + 4 P_i .....(2)

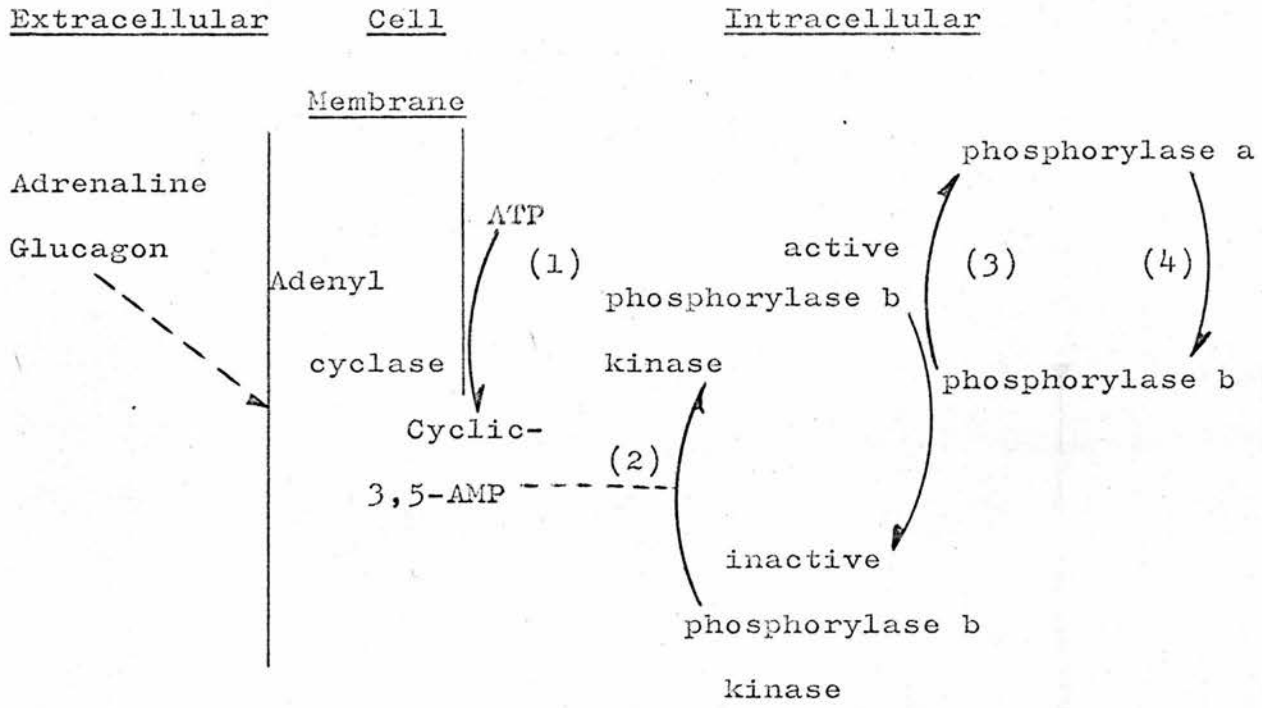
Most of the work on phosphorylase has concerned the enzyme extracted from rabbit-muscle, and so the work described usually applies only to rabbits: for example primarily the b form has been found in resting muscle (Krebs & Fischer, 1957). Immunological differences exist between muscle and liver phosphorylase (Henion & Sutherland, 1957).

Phosphorylase /

Phosphorylase b is less active than phosphorylase a, and the interconversion (1) above, effected by the enzyme phosphorylase b kinase, has a requirement for magnesium ions and ATP. In addition, the kinase is inert as extracted from muscle, and activation can be induced by incubation with calcium ions, as well as with ATP and magnesium ions. Reaction (2) is catalysed by the enzyme phosphorylase a phosphatase, an enzyme reversibly inhibited by low concentrations of AMP.

Adrenaline activates phosphorylase (Posener _et al_, 1962) by stepping up the conversion rate of form (a) from form (b) by increasing the concentration of cyclic-3,5- AMP (a nucleotide that has been shown by Rall _et al_ (1957) to enhance the activation effect of ATP and magnesium ions) during which a phosphate group from ATP is transferred to phosphorylase b kinase. The hormonal control (Sutherland, 1962) of phosphorylase activity is shown schematically in Fig. 4. The key enzyme, adenyl cyclase, catalyses the formation of cyclic-3,5- AMP from ATP, and so the link between hormone and glycogenolysis is complete. Phosphorylase b has been shown to be inhibited by ATP and glucose-6-phosphate, an effect that is reversed by AMP and inorganic phosphate (Morgan & Parmeggiani, 1964). Pyridoxal-5-phosphate has been identified in stoichiometric amounts, attached to phosphorylase a (4 moles) and to the b form (2 moles) (Baranowski _et al_,1957) and inactivation of the enzyme by removal of this "prosthetic group" by /

Hormonal Control of Phosphorylase Activity.



KEY:
(1) adenylcyclase catalysed formation of cyclic-3,5-AMP
(2) Phosphorylase b activation
(3) conversion of phosphorylase b to a by phosphorylase b kinase
(4) conversion of phosphorylase a to b by phosphorylase a phosphatase.

Figure 4.

by acid hydrolysis can be reversed by re-incubation in the presence of pyridoxal-5-phosphate for 30 min. (Cori & Illingworth, 1957). The properties of muscle phosphorylase have been reviewed by Krebs and Fischer (1962) and the structure of phosphorylase by Fischer, Krebs and Appleman (1964).

<u>Glycogen Synthetase</u>: Like phosphorylase, the enzyme glycogen synthetase has been found to exist in two forms (Rosell-Perez <u>et al</u>, 1962); these are termed I and D. The D-form is dependent for its activity on the presence of glucose-6-phosphate, whereas the I-form, is independently active. The two forms are interconvertible according to the following scheme (Larner, 1964)

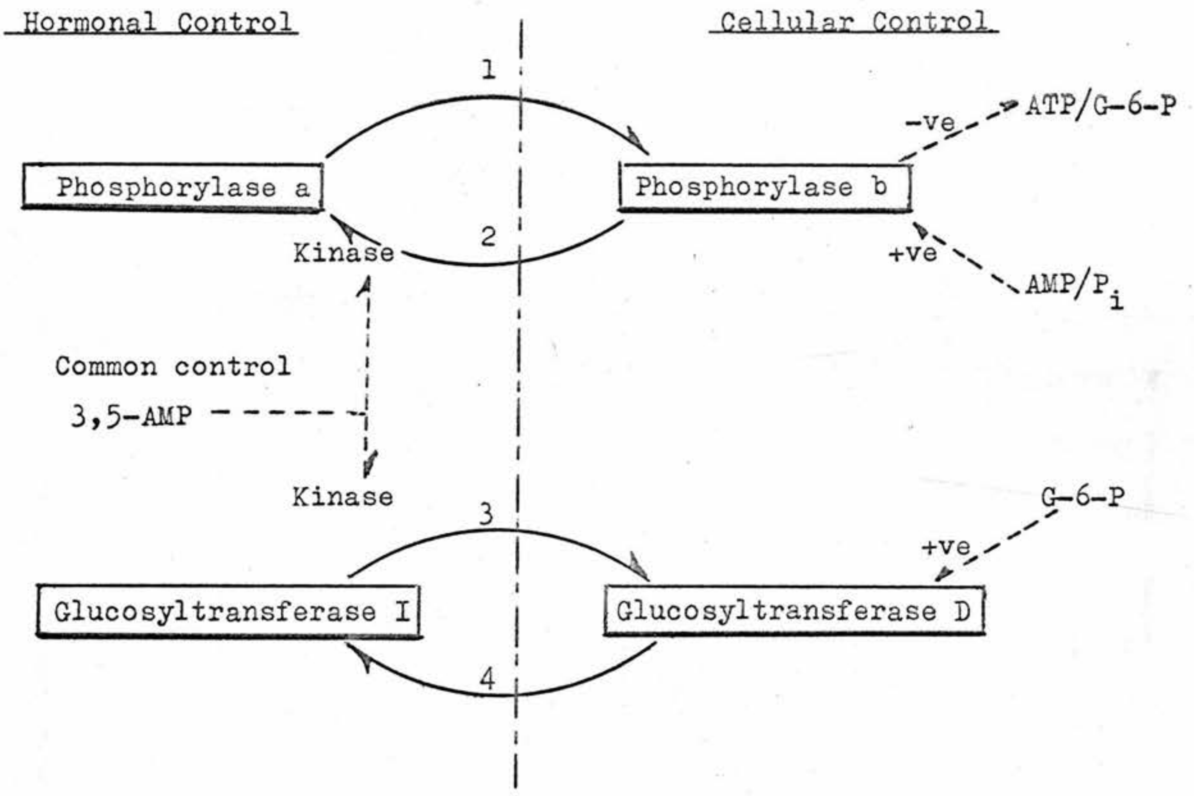
glucosyltransferase I + ATP - glucosyltransferase D + ADP

glucosyltransferase D + H_2O - glucosyltransferase I + P_i.

The presence of insulin increases the conversion of D- to the I-form. The effects of hormones, and cellular constituents on the activities of phosphorylase and glycogen synthetase are summarised in Fig. 5 given in Newsholme (1965).

Both forms of phosphorylase and both forms of glycogen synthetase are interconvertible by way of phosphorylation and dephosphorylation reactions, and as suggested in Fig. 5 there is a strong possibility that <u>in vivo</u> these enzymes are subject to common control mechanisms. There is evidence (Belocopitow, 1961) that glycogenolysis is activated at the expense of glycogenesis: adrenaline inhibits synthesis, but simultaneously activates /

GLYCOGEN METABOLISM: A SUMMARY OF POSSIBLE CONTROL MECHANISMS



KEY: (1) Phosphorylase a phosphatase. (2) Phosphorylase b kinase.
(3) Glucosyltransferase I kinase. (4) Glucosyltransferase - D kinase.

+ve --------> activation effect; -ve -------> inhibition effect;

----------- hormonal control

Figure 5.

activates phosphorolytic action. In addition, cyclic-3,5- AMP appears to exert a controlling action on the kinases that mediate the interconversions of both enzymes (Craig & Larner, 1964).

The synthetic action of the synthetase enzyme can be summarised as follows (Villar-Palasi & Larner, 1958)

$$\text{Uridine triphosphate} + \alpha\text{-D-G-(1)-P} \xrightleftharpoons{\text{UDPG pyrophosphorylase}} \text{Uridine 5-(D-glucosyl pyrophosphate} + \text{pyrophosphate}$$

$$\text{Uridine 5-(D-glucosyl pyrophosphate)} + (G)_n \xrightarrow{\text{glycogen synthetase}} \text{Uridine 5-pyrophosphate} + (G)_{n+1}$$

<u>Amylo-1,6-glucosidase</u>: The debranching enzyme, amylo-1,6-glucosidase liberates free glucose from the phosphorylase limit dextrins of branched substrates, first shown by Cori and Larner (1951). The enzyme is accordingly implicated in the *in vivo* degradation of glycogen (see Fig. 3)in conjunction with phosphorylase. The enzyme does not act on glycogen itself (Larner *et al* 1952), but can effect complete degradation acting with phosphorylase. However, Walker and Whelan (1960) have shown that R-enzyme (a plant-debranching enzyme) acting on a phosphorylase limit dextrin prepared from amylopectin liberated maltotetraose and other oligosaccharides, and suggested that conversion of the dextrin molecule by transglucosylation could explain the liberation of free glucose observed by Cori and Larner (1951). Brown and Illingworth (1962) have shown that a small

amount /

amount of transglycosylase does contaminate even very pure preparations of the debranching enzyme. Recent work by Abdullah and Whelan (1963) and Brown et al (1963) on the debranching of glycogen by preparations of rabbit muscle amylo-1,6-glucosidase is also explicable in terms of the presence of transferase. Hers (1964b) has adduced evidence for a 1,4-1,4-glucan transferase accompanying the debranching enzyme by use of a radioactive technique. The conclusion is that the Walker-Whelan structure of a phosphorylase limit dextrin is correct (see Fig. 6).

Acid Maltase: An acid maltase (Lejeune 1963) may also be involved in glycogen breakdown, and its significance is discussed in the next section.

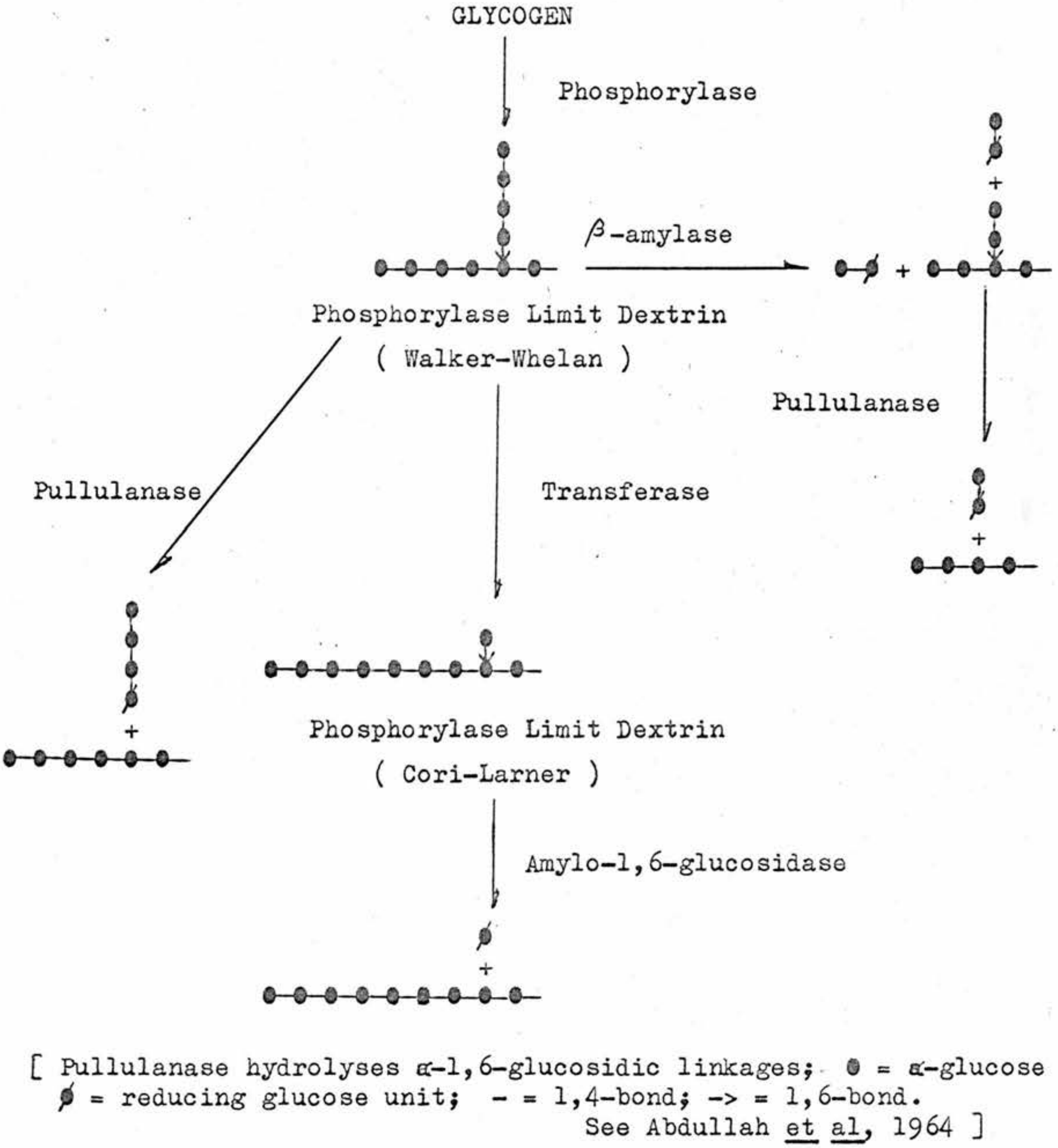
Phosphofructokinase: Tarui (1965) has identified a type of glycogen storage disease characterised by a lack of this enzyme.

Classification of Glycogen Storage Disease.

In the Blackadder Lecture (1963), van Creveld used the name glycogen disease to indicate "a congenital and sometimes familial error of carbohydrate metabolism leading to abnormal accumulation of glycogen in certain organs".

Although a number of different cases have been described since van Creveld first reported on an infant with a glycogen disease (1928), it was not until Cori and Cori (1952) related a specific enzyme defect (i.e. virtual absence of glucose-6-phosphatase) to a particular type of glycogen disease that a reliable /

THE STRUCTURE OF GLYCOGEN PHOSPHORYLASE LIMIT DEXTRIN.



[Pullulanase hydrolyses α-1,6-glucosidic linkages; ● = α-glucose
ø = reducing glucose unit; – = 1,4-bond; -> = 1,6-bond.
See Abdullah *et al*, 1964]

Figure 6 .

reliable classification of these diseases became possible. Classification on clinical grounds alone can be misleading because the same biochemical defect can lead to a wide range of symptoms and also, different metabolic disturbances may produce similar pathological conditions (Gitzelmann, 1957). The development of biochemical techniques of diagnostic value is therefore very important and so far has distinguished six basically different types of glycogen storage disease - a convenient "umbrella" term used to include all the known types of the disease.

The six basic types as enumerated by Cori (1957) and Field (1960) can be considered under two main headings: the first where the most obvious clinical abnormality is the massive enlargement of the liver with glycogen, viz:

I. The Hepatomegalic Types.

Type I, or von Gierke's disease classified by Cori and Cori (1952) is characterised by a deficiency or virtual absence of the microsomal enzyme, glucose-6-phosphatase. Although it now seems unlikely that the original cases described by von Gierke and van Creveld were Type I glycogenosis, the name von Gierke's disease has remained, and has been and is often erroneously used to cover all types of glycogenosis. The glycogen that accumulates in the liver and kidneys, where the deficient enzyme is normally abundantly present, is of normal structure.

Type III, or limit dextrinosis, classified by Illingworth et al (1956), is due to a lack of the debranching enzyme, amylo-1,6-glucosidase, an enzyme normally accompanied by a 1,4-1,4-glucantransferase. According to whether the metabolic defect concerns one or other or both of these enzymes, three further subgroups of Type III glycogenosis have been proposed (Manners et al (1961). In addition, the debranching defect can occur either in the liver or the muscle tissue. The structure of the accumulated glycogen usually approximates to that of a phosphorylase limit dextrin.

Type VI, due to Hers (1959), a relatively common type of glycogenosis is due to a deficiency of liver phosphorylase: there is no other enzymic lesion. The structure of the deposited glycogen is normal.

Type IV, or Andersen's disease is characterised by the accumulation of a glycogen-type polysaccharide that very closely resembles amylopectin. Only two cases have been reported in the literature, the first by Andersen (1956).

2. The Muscular Type.

Type II, or Pompe's disease (Pompe, 1932) is thought to be due to a lack of the enzyme acid maltase (Hers, 1963). The deposition of a glycogen of normal structure is generalised throughout the body. The heart is often greatly enlarged.

In Type V glycogenosis, muscle phosphorylase is absent. The first description is due to McArdle (1951).

The /

The main features of the different types of glycogen storage disease according to the Cori classification are presented in Fig. 7. (Schmidt, 1964).

Some Aspects of Glycogenosis.

In Type I glycogenosis, the liver may be sufficiently enlarged to induce a curvature of the spine (lordosis), and adiposity is not uncommon, suggesting a disturbance of the normal lipid metabolism.

Dwarfism is often characteristic of Type I glycogenosis. The disease usually becomes apparent during the first year of life, although cases of late development have been reported (Bernedetti et al, 1962). If the patient survives the fourth year, the prognosis is generally good, and the symptoms become milder: death frequently occurs in infancy due to hypoglycemic brain damage, or as a result of some other infection. Diagnosis of Type I glycogenosis is made on the grounds that (I) the liver is enormously enlarged, and the structure of the deposited glycogen is normal, and (2) the virtual absence of the enzyme glucose-6-phosphatase from the liver and kidney tissue; (3) the fasting blood sugar level is low, generally around 50mg/100ml, and the normal transient hyperglycemia following administration of galactose (Schwartz, 1957) or fructose is absent; (4) no response is shown after the administration of either adrenaline or glucagon; there is no evidence for an accumulation of glucose-6-phosphate occurring (Hers, 1964a); (5) a high level of urate and lactate is characteristic of hepatomegalic glycogenosis.

A /

Classification of Glycogen Storage Diseases.

Cori Type	Eponyms	Enzyme Defect	Glycogen Structure	Organs Involved	Clinical Manifestations.
I	von Gierke's disease	Glucose-6-phosphatase	Normal	Liver, kidney	Hepatomegaly, hypoglycaemia acidosis and ketonuria
II	Pompe's disease	Acid Maltase	Normal	Generalised	Cardiomegaly, cardio respiratory failure and death
III	Cori's disease, Limit-dextrinosis	Amylo-1,6-glucosidase (debranching)	Abnormal, short outer chains.	Liver, heart muscle.	Hepatomegaly, moderate hypoglycaemia acidosis
IV	Andersen's disease	Amylo-(1,4-1,6)-transglucosidase (branching)	Abnormal, few branch points	Liver, heart muscle erythrocytes	Cirrhosis of the liver, progressive hepatic failure and death.
V	McArdle's disease	Muscle phosphorylase	Normal	Muscle	Muscle cramps on exercise
VI	Hers's disease	Liver phosphorylase	Normal	Liver leucocytes	hepatomegaly, moderate hypoglycaemia and mild acidosis.

Figure 7.

[see Schmidt, 1964]

A slight defect in glucose-6-phosphatase activity has been shown to accompany Type III glycogenosis (Hers, 1959) but a complete absence of this enzyme is the surest indication of von Gierke's disease.

In Type II glycogenosis, the symptoms are first noticed within a few months of birth. The patient is exceedingly weak, unable to hold up his head, and lies in a completely flaccid position. Survival beyond infancy is rare, particularly when the heart is greatly enlarged. The pathological manifestations preceding death appear to be due to progressive, generalised deposition of glycogen resulting in a weakness of the respiratory muscles rather than to a specific enzymic deficiency, although a causal connection due to a lack of the enzyme acid maltase (Hers, 1963) has been suggested. Diagnosis can be made, in addition to observations on the physical condition of the patient, (1) by muscle biopsy, demonstrating a high concentration (<u>ca</u>. 10%) of a glycogen of normal structure; (2) fasting blood-sugar levels are normal, and a galactose or glucose tolerance test elicits a normal response; (3) responses to administration of adrenaline and glucagon are within normal limits; (4) blood lipid and lactate levels are normal; (5) acidosis and ketosis are absent; (6) absence of the enzyme acid maltase in biopsy specimens.

In Type III glycogenosis, the clinical symptoms are generally those /

those of hepatomegalic glycogenosis, but less marked e.g. the fasting hypoglycaemia, acidosis and ketonuria are less severe. Accordingly, a diagnosis can be made on the following grounds - (1) by experimental verification of the main enzyme defect, i.e. absence of amylo-1,6-glucosidase, and (2) by chemical characterisation of the deposited glycogen proving the presence of the shorter outer chains; (3) infusion of galactose or fructose elicits a normal response; (4) administration of glucagon or adrenaline is also followed by a fairly normal or normal hyperglycaemic response; (5) Sidbury (1961) has shown that the erythrocytes of patients with limit dextrinosis contain elevated levels of glycogen of abnormal structure, and this provides an exceedingly convenient method for diagnostic purposes.

Hers (1959) has suggested that cases of limit dextrinosis may be further subdivided according to whether the enzyme defect occurs in both liver and muscle tissue (limit dextrinosis A), and when it is only apparent in the liver (limit dextrinosis B). These types may be distinguished sometimes by the different amounts of glycogen deposited in the muscle and the liver.

Amylo-1,6-glucosidase is most conveniently assayed by measurement of the incorporation of C^{14}-glucose into glycogen (Hers, 1959). This method has also been adapted to leucocytes by Williams <u>et al</u> (1963).

Type III and Type I glycogenosis may be associated in the same /

same family. Illingworth et al (1961) report on a pair of siblings, one of whom exhibited a diminished glucose-6-phosphatase activity, and the other symptoms of a Type III glycogenosis. A similar instance (Manners, 1954, and Calderbank et al, 1960) has also been reported. Two siblings, one of whom had Type I and the other Type III glycogenosis (Eberlein et al, 1962) were both shown to have an abnormally high erythrocyte glycogen level.

Type IV glycogenosis, or Andersen's disease has appeared twice in the literature (Andersen, 1956, and Sidbury et al, 1962). The isolated glycogen resembled amylopectin and stained blue with iodine. No specific enzyme defect has been demonstrated, though a deficiency of branching enzyme would seem plausible (Illingworth & Cori, 1952). The liver was enlarged and cirrhosed. In both cases, the hyperglycaemic response to glucagon and adrenaline was reduced, and the glucose tolerance near normal; the disease was fatal after 17 months (Andersen's case) and after 42 months (Sidbury's case). Structurally abnormal glycogen was isolated from the heart, muscle and erythrocytes as well as from the liver. In Andersen's case the material was only sparingly soluble in water, which led Recant (1955) to suggest that by "precipitating", the "glycogen" became inaccessible to phosphorolytic attack.

Type V glycogenosis, or McArdle's disease was first described /

described by McArdle (1951) for a male adult who had suffered from muscular cramping after exercise. In his original paper, McArdle showed the absence of a rise of venous lactate on exercise, and a normal response to administration of adrenaline. Schmidt and Mahler (1959) and also Mommaerts et al (1959) demonstrated that this type of glycogenosis is due to a lack of the enzyme phosphorylase in skeletal muscle. A biopsy carried out on McArdle's original patient confirmed the absence of muscle phosphorylase activity (Mellick et al, 1962). The course of the disease is progressive (Schmidt & Mahler, 1959) Mommaerts et al, 1961); children complain of easy fatigability, and later whilst patients are able to walk on the level, they find stairs difficult. After forty years actual wasting of muscle may take place, although the prognosis for this type of disease appears to be good (Schmidt & Hammeker, 1961). The concentration of glycogen is moderately high (4%) and is of normal structure. Diagnosis is therefore made (1) because of a lack of muscle phosphorylase, and (2) no rise in venous lactate during exercise; (3) administration of fructose or glucose (Pearson & Rimer, 1959; Mellick et al, 1962) can increase a patient's tolerance for work; the utilisation by muscle of fructose as an energy source was a further observation. (4) Similarly glucagon (Schmidt & Mahler, 1959) or adrenaline (McArdle, 1951) can increase a patient's work capacity.

The enzyme phosphorylase is conveniently assayed by the method /

method of Hers (1959) - an adaptation of the Cori method (1943). The incorporation of glucose from glucose-1-phosphate into glycogen is measured by the release of inorganic phosphate that occurs in the presence of AMP (see preceding section).

Type VI glycogenosis, or Hers' disease is not a very clearly defined condition. Patients growth is generally retarded during the first year of life, and the liver is enlarged. A subnormal hyperglycemic response to glucagon or adrenaline is observed (Hers, 1959), but the Schwartz test is followed by a normal rise in blood glucose. A low liver phosphorylase activity has been shown to be accompanied by a corresponding defect in the leucocytes (Hulsmann <u>et al</u>,(1961);Williams & Field,(1961)). However, it has been found (Hers, 1961) that hepatomegalic glycogenosis can occur among siblings not all of whom have any obvious enzyme defect, and whose muscle glycogen concentration is not abnormal. Hers (1964a) concludes that this type of glycogenosis is probably the most frequently encountered, and points out that the distinction between normal and low phosphorylase activities is not very clear cut, especially since the child may be suffering from another form of glycogen storage disease, or something else altogether.

Tarui (1965) reports on 3 cases of a new type of glycogenosis, in which all three patients complained of easy fatigability. The enzyme defect was identified as an almost complete lack of muscle /

muscle phosphofructokinase. A moderate deposition of glycogen was apparent, and of especial interest, an accumulation of hexose monophosphate. The phosphofructokinase activity of hemolysates was partially reduced. The offspring (siblings of both sexes) resulted from a consanguinous mating - a feature common to ca. 10% of cases of glycogen storage disease (Hers, 1965).

In the experimental section that follows, a number of different techniques have been used in an attempt to classify seven different biopsy samples.

EXPERIMENTAL - METHODS.

(1) The Preparation of Homogenates for Enzyme Assays.

When a biopsy specimen is taken it must be deep-frozen at once to preserve the *in vivo* levels of enzymic activity, and also to maintain metabolically active non-enzymic constituents in an undegraded form. One method of doing this is to place the specimen in a sealed container, which is then covered with powdered Cardice. This method ensures that minimum losses due to evaporation occur, an important point, since enzymic activities are often expressed in terms of fresh tissue weight. This was the method generally adopted for the present work. Whenever possible the assays were carried out within 48 hours of the operation. When the operation took place away from Edinburgh the biopsy specimen was placed on a train to Edinburgh the same day.

The following method was adopted for the preparation of the homogenates. The deep-frozen liver sample was placed on a cold microscope slide, and a slice (50-150mg) removed with a scalpel. The slice was weighed in a tared, ice-cold glass homogeniser tube and cold water added (2.0 ml per 100 mg tissue). The tissue was briefly homogenised, i.e. for 15 sec. followed by a 5 sec. pause, and for a further 15 sec., and the homogenate filtered through two thicknesses of muslin into a small test-tube. 0.2 ml portions of the filtered homogenate were /

were used for each glucose-6-phosphatase assay, and 0.5 ml of a 5-fold dilution for the phosphorylase assay. The remainder of the biopsy specimen was kept deep-frozen for glycogen extraction.

(2) The Extraction of Glycogen from Biopsy Specimens.

Two main points have to be borne in mind during any extraction procedure. First, the material being extracted must be as little affected by the method used as possible. - ideally not at all, and second, the extracted material must be in a state suitable for analysis. Several ways of extracting glycogen have been used by different authors including homogenisation in hot water (Greenwood and Manners, 1957), cold water (Lazarow, 1942; Orrell and Bueding, 1958) maceration in cold trichloroacetic acid (Stetten <u>et</u> <u>al</u>, 1956) and alkali extraction (Pfluger, 1910). Detergents and 8M urea have also been used. All the methods described above, except the cold water extraction, in themselves facilitate the removal of contaminating protein. The subject has been extensively reviewed by Orrell <u>et</u> <u>al</u>, 1964. Where water is the extractant, an organic solvent is often employed for deproteinisation, for example chloroform and octyl alcohol (Sevag, 1934).

In order to obtain the maximum amount of material with which to work, extraction with hot concentrated alkali was preferred /

preferred for all the biopsy specimens. Although limited alkaline degradation does undoubtedly occur, ~~(Stetten, 1963~~) together with a decrease in molecular size (Orrell, 1964), these factors do not interfere materially with the determination of β-amylolysis limits, α-amylolysis limits or iodine staining power in half-saturated ammonium sulphate solution. Attempts to use trichloroacetic acid and dimethylsulphoxide did not produce a quantitative yield of glycogen. The alkaline extraction was carried out by placing 1-3 ml 40% potassium hydroxide in a small tapered centrifuge tube, and heating it in a boiling water-bath. Frozen tissue (100-600mg) was dropped into the hot alkali, and heating continued for a further 30 to 45 minutes. The contents of the tube were occasionally stirred with a glass rod until dissolution. A loosely fitting glass-stopper (from a reagent bottle) was used to close the mouth of the tube. After half an hour, a layer of fat usually separated out on top of the glycogen-alkali solution and was removed with a pipette.

After cooling the tube in crushed ice, 2-3 volumes of ethanol were added to precipitate the polysaccharide (Kjolberg Ph.D. Thesis, 1962). The precipitate was centrifuged and then taken up in 2-3 ml of water. At this stage, the polysaccharide material was estimated by the phenol sulphuric method. A further five re-precipitations were carried out in /

in the same tube using decreasing volumes of water and ethanol. For the last few precipitations, one small crystal of ammonium acetate was added. The final white solution was frozen and freeze-dried if more than 10 mg were present.

(3) The Extraction and Estimation of Glycogen from Erythrocytes.

Red blood cells from human blood, (10 ml saline washed and centrifuged), were treated with an equal volume of 20% trichloroacetic acid (TCA) at 0-5° and stirred vigorously to disrupt the cells (Sidbury, 1961). The TCA-complex was centrifuged, and the supernatant dialysed against distilled water for 48 hr. A suitable portion of the dialysate was estimated by the phenol sulphuric method for polysaccharide content, and the concentration expressed in terms of g glycogen per 1 g. haemoglobin (the haemoglobin figure was determined by the clinician providing the blood).

(4) The Estimation of Inorganic Phosphate.

The method used was a modification of that described by Allen (1940).

Reagents: Perchloric acid (60%)

Ammonium molybdate (0.5% in 0.05 N ammonium hydroxide)

Amidol reagent: amidol (0.5 g.) sodium bisulphite in water (50 ml) filtered through a glass-wool plug. The reagent kept for ca. 2 weeks in a dark bottle placed in a refrigerator before discolouring.

Method.

A portion of the solution to be tested (1 ml) containing not more than 1 μM of inorganic phosphate was pipetted into a test tube (1 in. dia.), to which was added perchloric acid (0.5 ml), amidol reagent (0.5 ml) followed by ammonium molybdate solution (5.0 ml). The well-mixed solution was allowed to stand (15 min) and then the optical density was read in 1 cm cells on a Unicam SP 600 spectrophotometer. Standard inorganic phosphate solution was prepared from A.R. potassium dihydrogen phosphate.

At room-temperature, maximum colour development was reached in five minutes and thereafter was stable. An optical density reading of 0.575 was equivalent to 1 μM P_i under the conditions described above.

(5) The Assay of Glucose-6-phosphatase.

Essentially the method described by Cori and Cori (1952) was used.

Conditions:-

Substrate:	0.01 M glucose-6-phosphate dipotassium salt (hydrated).
Enzyme:	liver-tissue homogenate (see p. 141): concentration 100 mg fresh-weight per 2 ml water.
Digest:	Substrate (0.5 ml) Citrate buffer (0.3 ml, 0.1 M, pH 6.8) Homogenate (0.2 ml).

Assay: the digest was incubated for 60 min., at 30° in stoppered centrifuge-tubes suspended in a stirred, thermostatically controlled water-bath. The reaction was stopped by the addition /

addition of ice-cold 10% TCA (1 ml). The tubes were placed in crushed-ice for 5 min., and then centrifuged. A portion (1 ml) of the supernatant was examined for inorganic phosphate by the modified Allen procedure. Assays were carried out in triplicate, and a blank containing no substrate was simultaneously incubated with the assay digests. A phosphate standard was included.

(6) The Phosphorylase Assay.

Conditions (Hers, 1959; Haynes, 1963):-

Substrate:	0.1 M glucose-1-phosphate 2% rabbit liver glycogen 0.003 M AMP 0.2 M NaF adjusted to pH 6.1.
Enzyme:	5x dilution of the glucose-6-phosphatase homogenate.
Digest:	Substrate (0.5 ml) Enzyme (0.5 ml) Citrate buffer (0.5 ml, 0.1M, pH 6.1).

Assay: The digest was incubated in stoppered centrifuge-tubes for 30 min., at 37°. The reaction was stopped by the addition of 20% TCA (0.5 ml). The centrifuge tubes were placed in crushed-ice for 5 min., and then centrifuged. Portions (1 ml or less) of the supernatant were then analysed by the modified Allen procedure for inorganic phosphate. The assays were carried out in triplicate, with an enzyme blank.

(7) /

(7) The Units of Activity.

The unit used by Hers was adopted, i.e. the phosphate release was expressed as μM P_i released per minute per gramme of tissue. This is a unit of convenience rather than one of any great theoretical justification, for it assumes (a) that the weight of frozen tissue in a stoppered tube cannot alter, and (b) it is not always very easy to ensure that the homogenate was prepared from 100% liver-tissue.

(8) Iodine-staining in Half-saturated Ammonium Sulphate Solution.

Standard iodine solution (2.0 ml)
Hydrochloric acid (0.15 ml, 6N)
Saturated ammonium sulphate solution (10 ml)
Glycogen solution (5.0 ml containing ca. 1 mg).
Made up to a final volume of 20 ml.

The wavelength of maximum absorption was read on the P.E. 137 recording spectrophotometer, using 2 cm cells. The reading thus obtained was used to estimate the average chain-length of the glycogen (Archibald *et al*, 1961), using the equation $\overline{CL} = 16 + 0.114\ (\lambda\,\mathrm{max} - 500)$.

(9) The Phenol Sulphuric Method and Polysaccharide Concentration.

The standard phenol sulphuric method (Dubois *et al* 1956) was used directly to estimate glycogen (Montgomery, 1957), and it was found to give an absorption value 1.11 times greater than an equal weight of glucose.

(10) Small-scale Enzyme Digests.
(Kjolberg, Ph.D. Thesis, 1962)

β-amylolysis Limits: Typically, polysaccharide solution (10 ml containing ca. 2.0 mg) was taken and incubated with β-amylase (1.0 mg in acetate buffer, 5.0 ml, pH 4.6, 0.2 M) for 24 hr at 37°. Portions (1.0 ml) of the digest were then estimated by the modified Park and Johnson method.

α-amylolysis Limits: The same polysaccharide solution was used, (10 ml) and α-amylase (calibrated enzyme, see Kjolberg & Manners, 1962) in citrate buffer (5.0 ml, containing 1 ml sodium chloride 0.5%, enzyme, 1 mg, and citrate 0.1 M, pH 7.0) was added, and the digest incubated for 24 hr at 37°. Portions (1.0 ml or less) were then estimated by the Park and Johnson method.

Concentration of the Polysaccharide Solutions: this was determined by the phenol sulphuric method (see 9 above), on 0.2 ml portions.

(11) The Determination of Chain-length ($\overline{CL}$) by α-amylolysis of Glycogens.

The method described by Kjolberg and Manners (1962) was used. The formula $P_{1,6} = 23.4 - 0.20\ (P_M)$ was used in conjunction with the enzyme prepared by Dr. Kjolberg, where $P_{1,6,}$ the percentage of 1,6-linkages $= \frac{100}{\overline{CL}}$

(12) /

(12) The Estimation of Reducing Sugars: Park & Johnson (1949).

Reagents (as reference) viz:

Potassium ferricyanide 0.5 g/l ... (1)

Sodium Carbonate 5.3 g and
Potassium Cyanide 0.65 g/l ... (2)

Ferric ammonium sulphate 1.5 g and
Duponol 1.0 g/l ... (3)

The Duponol was recrystallised several times, as Kjolberg, (1962, Ph.D. Thesis) found that the commercial reagent gave a high reagent blank.

Method (modified).

Sugar solution (1 ml)
Mixed reagents 1 and 2 (5 ml)
Water (4.0 ml)

The mixed solutions were heated for 15 min. on a boiling water bath in stoppered tubes.

Portions (1.0 ml) were added to reagent 3 (5.0 ml) and the optical density read after 15 min. at 690 m in 1 cm cells. Triplicate readings were taken, and a reagent blank and a control were included.

EXPERIMENTAL - RESULTS.

A Comparison of Three Methods of Glycogen Extraction.

(1) Dimethylsulphoxide Extraction: Approximately 1.0 g of frozen human liver tissue was placed in dimethylsulphoxide (10 ml), and homogenised with an Ultra-Turrax mixer for 15 sec., followed by a pause and then again for 15 sec. The tube containing the tissue:dimethylsulphoxide mixture was immersed in an ice:salt mixture. The extracted glycogen was precipitated by the addition of methanol, centrifuged and taken up in water, and re-precipitated a further three times with ethanol, using three volumes of alcohol for each precipitation. A final aqueous solution of glycogen was freeze-dried, and then taken up in water (20 ml). Suitable aliquots were estimated by the phenol sulphuric method, and showed that the glycogen extracted by dimethylsulphoxide represented only 0.01% of the fresh-tissue weight. (Whistler & BeMiller, 1963).

(2) Silver-sand Dimethylsulphoxide Extraction: Approximately 0.4 g of frozen human liver tissue was ground with silver sand and dimethylsulphoxide (10 ml) in a mortar placed in crushed ice using a pestle. The resulting mixture was centrifuged and re-extracted a further two times in the same way. After centrifugation, the polysaccharide was precipitated from the supernatant by the addition of methanol (3 vol.), followed by three further re-precipitations with ethanol, as described for the /

the dimethylsulphoxide extraction procedure. The final precipitate was taken up in water, and estimated for glycogen by the phenol sulphuric procedure. The yield of glycogen was 0.08%.

(3) Potassium hydroxide (40%) Extraction: Approximately 0.5 g of frozen human liver tissue was placed in 40% potassium hydroxide (2ml) and heated in a boiling water bath for 30 min. The cold alkaline solution was cooled, diluted twice, and glycogen precipitated by the addition of ethanol (3 vol). The precipitate was centrifuged, taken up in dimethylsulphoxide, re-precipitated, and then taken up in water. Suitable portions of this solution were estimated by the phenol sulphuric method. The yield of glycogen was 0.3%.

The technique last described was therefore adopted for the extraction of biopsy samples, and the estimation of glycogen content.

Method	Yield (%)
1	0.01
2	0.08
3	0.30

(4) The Stability of Glucose-6-phosphatase in Tissue Samples.

One sample of human liver tissue was stored at room temperature (18°) and another in the cold room (0-5°). At 24 hr intervals portions of the tissue were excised and estimated /

estimated for glucose-6-phosphatase activity by the procedure described on p. 145. The results expressed as optical density readings, obtained for each assay are tabulated below.

Time (hr)	Reading	Blank	Activity	Percent.
		Cold Room		
0	0.332	0.035	0.297	100
24	0.360	0.067	0.293	99
48	0.184	0.049	0.135	45
96	0.248	0.132	0.113	38
		Room Temperature		
0	0.332	0.035	0.297	100
24	0.112	0.097	0.015	5
48	0.113	0.125	0.008	3
96	0.175	0.154	0.021	7 ø

ø the sample had by now begun to putrefy.

Thus at room temperature, over 90% of the activity is lost within the first 24 hr. It is also of interest to note the increasing blank values: a large blank reading could therefore be taken as a measure of the lack of care with which a biopsy sample had been handled.

(5) Effect of Storage-Conditions on the Activity of Glucose-6-phosphatase.

The release of inorganic phosphate during the estimation of glucose-6-phosphatase was measured at 0.5, 1.0, 2.0 and 4 hr, using a freshly prepared homogenate. A similar experiment, using /

using an homogenate that had been frozen and stored overnight, was also carried out. Optical density readings from which blanks have been subtracted are presented below.

Time (hr)	fresh homogenate O.D. 720	stored homogenate O.D. 720
0.5	0.032	-
1.0	0.042	0.039
2.0	0.059	0.058
4.0	0.051	

The phosphorylase assay was also examined by determining the inorganic phosphate release at 0.5 and at 1.0 hr., when the optical density readings were respectively 0.089 and 0.084.

These results are shown graphically on the next page.

Ricketts (1963) has reported a non-linear release of inorganic phosphate during the glucose-6-phosphate assay under the conditions of Hers which could be rectified by the addition of isotonic sucrose to the medium. In the present work the original conditions were used for purposes of comparison.

(4) THE EFFECT OF STORAGE CONDITIONS ON GLUCOSE-6-PHOSPHATASE ACTIVITY .

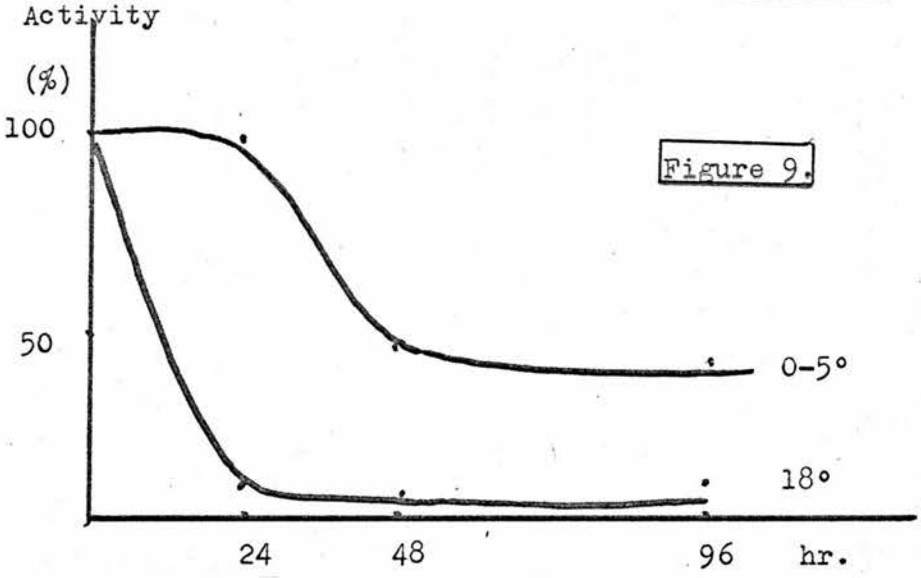


Figure 9.

(5) THE ENZYMIC HYDROLYSIS OF GLUCOSE-6-PHOSPHATE: TIME-CURVE.

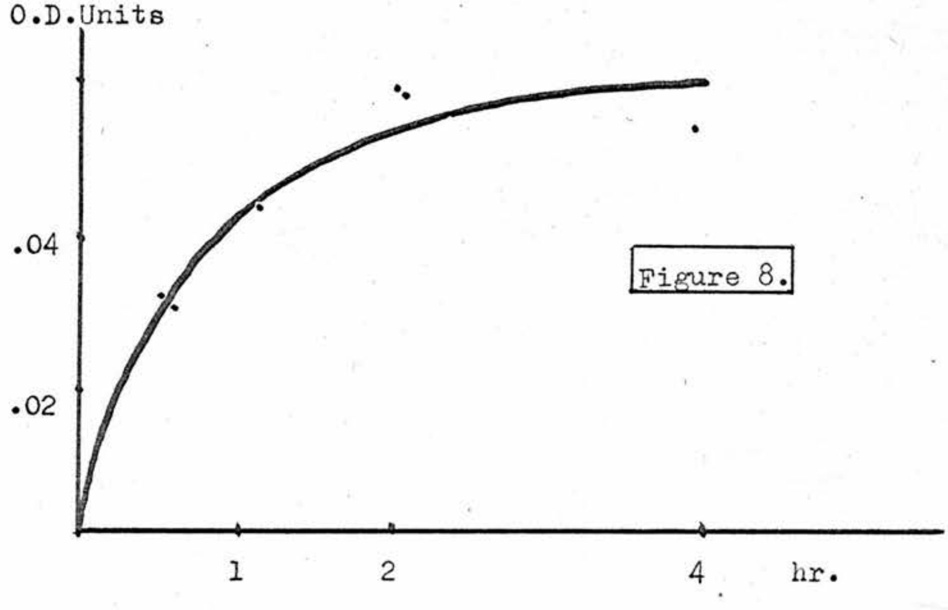


Figure 8.

ANALYSIS OF HUMAN TISSUES.

(6) Control Liver Autopsy Specimen (Edinburgh).

This intact liver, kindly provided by the Sick Childrens' Hospital through Dr. D. Bain gave the following results on analysis.

Glycogen Content	0.3%	Liver Autopsy
Glucose-6-phosphatase*	1.71	
Phosphorylase*	10.3	

* the enzyme units adopted in this and all subsequent enzyme assays are μM P_i released per min. per g. tissue (see p.147).

(7) Case I (D.S.)

This patient (male, aet. 6) had an ordinary glucose tolerance curve, and showed a transient hyperglycaemic response to the administration of adrenaline and glucagon. The fasting blood sugar was generally low. There was no relevant family history.

Results (I) Clinical:

Glucose tolerance, fasting blood sugar level:	59mg/100ml
Maximum rise, after 60 min. to:	132mg/100ml
Glucagon test, fasting blood sugar level:	30 mg/100ml
Maximum rise, after 90 min. to:	138mg/100ml
Adrenaline test, fasting blood sugar level:	25mg/100ml
Maximum rise, after 30 min. to:	30mg/100ml

(The results of the clinical tests, and the liver biopsy specimen were kindly provided by Dr. J. Lorber of the Children's Hospital, Sheffield).

Results /

Results (II) Biochemical:

Glycogen Content	9.1%	Liver Biopsy
Glucose-6-phosphatase activity	0.73	
Phosphorylase activity	11.4	
Glycogen Analysis:		
β-amylolysis limit	49%	
α-amylolysis limit	82%	
Average Chain-Length		
(a) via P_M	14.3	
(b) via Iodine-Stain	12.5	

(2) Case II (M.A.)

This patient (male, aet. 4) had a normal glucose tolerance curve, but showed very little hyperglycaemic response to the administration of glucagon. The child was slightly stunted. The father and mother were first cousins, and the 3rd and 4th children are apparently normal. The second child died from an unknown cause at the age of 7 months. The initial diagnosis of hepatomegaly was made in Cairo when M.A. was 1 year old.

Results (I) Clinical:

Glucose tolerance, fasting blood sugar level:	35mg/100ml
Maximum rise, after 30 min. to:	130mg/100ml
Glucagon test, fasting blood sugar level:	30mg/100ml
Maximum rise after 60 min. to:	None.

(The clinical results, and the liver, muscle and erythrocyte biopsy specimens were kindly provided by Dr. E. Thompson from the Royal Free Hospital, London).

Results (II) Biochemical:

Glycogen Content	17.1%	Liver Biopsy
Glucose-6-phosphatase	1.04	
Phosphorylase	17.0	
Glycogen Analysis:		
β-amylolysis limit	35%	
α-amylolysis limit	76%	
Average Chain-Length		
(a) via P_M	12.2	
(b) via Iodine-Stain	13.0	
Glycogen Content	3.7	Muscle Biopsy
β-amylolysis limit	40%	
Erythrocyte Glycogen Content	1410	R.B.C. Glycogen

(9) Case III (E.A.)

This patient (female, aet. 6) was admitted to hospital at the age of two with a grossly enlarged liver. The glucose tolerance test produced a slow rise and fall in the blood sugar level, and administration of adrenaline was not followed by a positive hyperglycaemic response; in fact a drop in the blood sugar level was observed. The patient, an only child, had no relevant family history, and was anaemic.

Results (I) Clinical:

Glucose tolerance test, fasting blood sugar level	43mg/100ml
Maximum rise, after 90 min. to:	161mg/100ml
Glucagon test, fasting blood sugar level	94mg/100ml
Maximum rise, after 30 min. to:	144mg/100ml

(The clinical results, and the liver biopsy and R.B.C. specimens were kindly provided by Dr. P.M.G. Broughton of St. John's Hospital, Chelmsford).

Results (II) Biochemical:

Glycogen Content	7.8%	**Liver Biopsy**
Glucose-6-phosphatase activity	0.01	
Phospharylase activity	20.2	
β-amylolysis limit of Glycogen	51%	
Erythrocyte Glycogen Content	585	**R.B.C. Glycogen**

(10) Case IV (J.A.)

This patient (female, aet 3) had an abnormally low fasting blood sugar level, and the glucose, glucagon and adrenaline functional tests all produced abnormal results.

Results (I) Clinical:

Glucose tolerance, fasting blood sugar level	35mg/100ml
Maximum rise, after 90 min. to:	79mg/100ml
Glucagon test, fasting blood sugar level	35mg/100ml
Maximum rise, after 90 min. to:	48mg/100ml
Adrenaline test, fasting blood sugar level	15mg/100ml
Maximum rise, after 106 min. to:	38mg/100ml

(The results of the clinical tests, and liver biopsy specimen were kindly provided by Dr. P.M.G. Broughton of St.John's Hospital, Chelmsford).

Results (II) Biochemical:

Glycogen Content	10.9%	**Liver Biopsy**
Glucose-6-phosphatase activity	0.63	
Phosphorylase activity	19.6	
Glycogen Analysis:		
β-amylolysis limit	35%	
α-amylolysis limit	79%	
Average Chain-Length (a) via P_H	13.2	
(b) via Iodine-Stain	12.5	

(11) Case V (G.H.)

This patient (male, aet. 11) was shown to have an atypical glucose tolerance curve, and there was no hyperglycaemic response to adrenaline administration. The liver was greatly enlarged, and the patient's height was much below normal. There are two siblings, nine and four years older than the patient, both apparently normal. The patient was seen in hospital at the age of 7 months.

Results - Clinical:

Glucose tolerance test: fasting blood sugar 70-80mg/100ml
Lag curve, followed by a slow rise and fall.

Adrenaline test: no response observed.

(The results of the clinical tests and the liver biopsy specimen were kindly provided by Dr. P.M.G. Broughton of St.John's Hospital, Chelmsford).

Results - Biochemical:

Glycogen Content	10.3%	Liver Biopsy
Glucose-6-phosphatase activity	0.01	
Phosphorylase activity	11.2	
β-amylolysis limit	43%	

(12) Case VI (M.D.)

This patient (female, aet. 29) had an abnormal glucose tolerance curve: very flat and with a minimal rise. Generally she was described by her physician as evidencing a fairly widespread systemic disorder. Both the patient and her mother showed an abnormal E.C.G. There was a slight possibility /

possibility that the patient was suffering from some form of glycogenosis, and so a muscle biopsy was taken.

(The clinical details, and the muscle biopsy and R.B.C. specimen were provided by Dr. J.A. Friend of the Western General Hospital, Edinburgh).

<u>Biochemical Results</u>:

<u>Glycogen Analysis</u>:

Glycogen Content	0.6%	<u>Muscle Biopsy</u>
β-amylolysis limit	52%	
α-amylolysis limit	76%	
Average Chain-Length (a) via P_M	12.2	
(b) via Iodine Stain	12.5	
Erythrocyte Glycogen Content	214	<u>R.B.C. Glycogen</u>

<u>(13) Case VII (S.S.)</u>

This patient (male, aet. 34) was suffering from some undefined disorder: one of the possibilities was considered to be some form of glycogen storage disease, since no hyperglycaemic response could be detected after glucose administration.

(The clinical results and the needle biopsy and R.B.C. specimen were kindly provided by Dr. J. McManus, of the Western General Hospital, Edinburgh).

<u>Biochemical Results</u>.

Glycogen Content	4.9%	<u>Liver Needle Biopsy</u>
Glucose-6-phosphatase activity	2.28	
Phosphorylase activity	15.6	
Erythrocyte Glycogen Content	144	<u>R.B.C. Glycogen</u>

(14) Erythrocyte Glycogen Contents.

Cell Source	Glycogen Content (μg/g Hb)	β-amylolysis Limit (%)
Normal	49.234,115,135	54 (pooled)
Myocardial Infarction	203,136	27
Diabetic	123	38
S.S. (Edinburgh)	144	-
M.D. (Edinburgh)	214	-

DISCUSSION.

A number of liver biopsy specimens taken from patients suspected of having glycogen storage disease have been examined (a) in order to establish the nature of any possible enzyme defects, and (b) to extract and determine the glycogen content of the liver. In addition, when there was sufficient material, the molecular structure of the extracted glycogen was partially analysed. Two muscle biopsy specimens were also examined, together with a needle biopsy taken from the liver of a patient who was shown, on biochemical analysis, not to be suffering from glycogen storage disease. The erythrocyte glycogen from a number of non-glycogenosis patients has been examined, and also that from two of the glycogen storage disease patients.

Case I (D.S.)

The results of the glucose-6-phosphatase and phosphorylase assays showed both enzymes were present in reduced amounts. Whilst the glycogen structure was normal (β-amylolysis limit 49%, chain-length 13-14), the glycogen content of the liver biopsy specimen was exceedingly high (9.1%). The glucose tolerance curve was normal, though the maximum sugar level attained was low; injection of adrenaline or glucagon elicited marked hyperglycaemic responses. Accordingly, Type I glycogenosis is ruled out because of the normal glucose-6-phosphatase activity, and also Type IV on account of the normal glycogen /

glycogen structure. Types II and V are eliminated on clinical grounds. Accordingly this is a case of either Type III or Type VI glycogenosis, although in the former a glycogen of low β-amylolysis limit is usually found. The latter is often characterised by a diminished phosphorylase activity, and a high liver glycogen content. The low fasting blood sugar level is in accordance with either type of glycogenosis. However, a low level of amylo-1,6-glucosidase was shown to be present (by Dr. J.R. Stark) and so D.S. is classified as a mild case of Type VI glycogenosis.

<u>Case II (M.A.)</u>

The enzyme assays showed near normal activity for phosphorylase and glucose-6-phosphatase in the liver biopsy specimen obtained from this patient. The average chain-length was not abnormally low, and the β-amylolysis limit (35%) was slightly reduced from normal. The erythrocyte glycogen content of 1410 g/g.Hb was much elevated, and suggests a Type III glycogenosis. This diagnosis is in accord with the other data available; for example, the feeble hyperglycaemic response to the injection of glucagon, and the low fasting blood sugar level. Glycogenosis Types I,II,IV, and V are therefore ruled out, and as a high RBC glycogen level has not been reported for Type VI, it seems probable that Case II is an example of Type III glycogenosis. A muscle biopsy specimen contained 3.7% glycogen, with a β-amylolysis limit of 40%. Thus the enzyme /

enzyme defect would appear not to be restricted to the liver, and accordingly a diagnosis of Limit Dextrinosis A is plausible.

Case III (E.A.)

The virtually complete absence of glucose-6-phosphatase indicated that this patient suffered from Type I glycogenosis. The phosphorylase activity and glycogen structure were normal. The glucose tolerance curve, and the failure of adrenaline to produce a rise in the level of blood sugar are in accord with this diagnosis. The hyperglycaemia induced by the injection of glucagon is of interest since the results of adrenaline and glucagon tests have previously been found not to agree (Hers, 1964). The high erythrocyte glycogen content (585 g/g.Hb) is unusual, since Type III glycogenosis is usually associated with such elevated levels. Case III is identified as a Type I glycogenosis.

Case IV (J.A.)

The glucose-6-phosphatase activity was low, and phosphorylase normal. The glycogen content of the liver was high (10.9%) and the β-amylolysis limit of the extracted glycogen low. The average chain-length of 13 was normal. The fasting blood sugar levels were low, and the glucose tolerance curve abnormally flat. There was very little glycaemic response to the injection of either adrenaline or glucagon. Accordingly the picture presented is that of a Type /

Type III glycogenosis, although Type VI cannot conclusively be ruled out.

Case V (G.M.)

As in case III, there was a complete absence of glucose-6-phosphatase activity, indicating a clearly defined Type I glycogenosis. The glycogen content was high (10.3%) and of normal structure. A flat glucose tolerance curve and a lack of response to adrenaline injection confirm the biochemical diagnosis of Type I glycogenosis.

Case VI (M.D.)

A muscle biopsy specimen obtained from this patient contained ca. 0.6% glycogen of normal structure. The erythrocyte glycogen content was normal. It would therefore seem unlikely that M.D. suffered from any form of glycogen storage disease, although the determination of glycogen content and structure should not, without supporting evidence from enzyme assays, be considered sufficient either to classify or rule out a case of glycogen storage disease. The contrary clinical evidence, viz: a flat glucose tolerance curve, could well have some other cause since the metabolism of this patient was generally disturbed.

Case VII (S.S.)

As in the previous case, the lack of response to the glucose tolerance test suggested a disturbed carbohydrate metabolism /

metabolism; this was complicated by the presence of jaundice. An erythrocyte glycogen content proved to be within normal limits, and enzyme assays of phosphorylase and glucose-6-phosphatase yielded normal results. The glycogen content of the needle biopsy sample obtained from this patient was high, though not abnormal for liver tissue. On this evidence it is apparent that any form of glycogenosis can be ruled out.

Erythrocyte Glycogen Contents.

The glycogen content of four normal specimens of red blood cells varied between 49 and 243 μg/g.Hb. The β-amylolysis limit of 54% determined on the pooled glycogen compares with normal liver glycogen values. The significance of the low values obtained from diabetic or myocardial infarction patients is not obvious. However, if all the other glycogen contents are taken as normal, the average value obtained is 150, and the mean deviation from this value is 43; thus a normal range of values is 150 ± 43 μg/g.Hb.

Summary of the Biochemical Results from Biopsy Specimens.

Case Number	I	II	III	IV	V	VI	VII
Liver Biopsies.							
Glucose-6-Phosphatase*	0.73	1.04	0.01	0.63	0.01	-	2.28
Phosphorylase*	11.3	17.1	20.2	19.6	11.2	-	15.6
Glycogen Content (%)	9.1	17.0	7.8	10.9	10.3	-	4.9
β-Amylolysis Limit (%)	49	35	51	35	43	-	-
α-Amylolysis Limit (%)	82	76	-	79	-	-	-
Average Chain-Length (a)	14.3	12.2	-	13.2	-	-	-
(b)	12.5	13.0	-	12.5	-	-	-
Erythrocyte Glycogen Content (%)	-	1410	585	-	-	214	144

Muscle Biopsies.

Case Number	II	VI
Glycogen Content (%)	3.7	0.6
β-Amylolysis Limit(%)	40	52
α-Amylolysis Limit (%)	-	76
Average Chain-Length (a)	-	12.2
(b)	-	12.5

* Hers Units.

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APPENDIX I.

1. MANNERS & ROWE (1964) Chem.& Ind. p. 1835.
2. MANNERS, MERCER & ROWE (1965) J. chem. Soc. p. 2150

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Conversion of Amylopectin into a Glycogen-type Polysaccharide by a Plant Enzyme

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In a previous Communication,[1] we described the conversion of amylopectin into a glycogen-type polysaccharide by yeast branching enzyme. This reaction, which increased the proportion of α-1,6-glucosidic inter-chain linkages in amylopectin from 4·5 to 7·4% (and hence, decreased the average chain length from 22·0 to 13·5), differentiated the yeast enzyme from potato[2] and other plant branching enzymes (Q-enzymes) which have no action on amylopectin.[3] In contrast to most higher plants which contain a two-component starch, sweet corn (*Zea mays*) appears to be unique in synthesising both starch and a glycogen-type polysaccharide (phytoglycogen).[4] It was therefore of interest to examine the branching enzyme of sweet corn.

In preliminary experiments, sweet corn flour was extracted with citrate buffer (pH 6·0) and the extract fractionated with ammonium sulphate. The protein precipitated between 32 and 55% ammonium sulphate was collected, and shown to be substantially free of phosphorylase and α-amylase impurities. On incubation with amylopectin, there was a rapid decrease in iodine-staining power at the $\lambda_{\text{max.}}$, 550 mμ and a change in $\lambda_{\text{max.}}$ to lower wavelengths; various phytoglycogen samples have $\lambda_{\text{max.}}$ in the range 430–480 mμ.[5] This change was accompanied by a small decrease (4%) in the β-amylolysis limit of the amylopectin. With amylopectin β-dextrin, there was a small increase (4%) in β-amylolysis limit and a small decrease (5%) in iodine-staining power at 535 mμ. These results suggest that the sweet corn preparation, like yeast branching enzyme,[1,6] can introduce additional branch points into both amylopectin and amylopectin β-dextrin.

A sweet corn preparation was fractionated by continuous electrophoresis using a Beckman-Spinco model CP apparatus. Fractions were assayed for α-amylase and branching enzyme by measurement of the change in iodine-staining power on incubation with glycogen and amylopectin respectively. Those fractions containing branching enzyme, but not α-amylase, were combined and a portion incubated with amylopectin. Enzyme action caused a decrease in α- and β-amylolysis limits (see Table) which indicated that further branching had taken place. (Analytical data for phytoglycogen isolated from the same sample of sweet corn are also given.) Proof that these branch points were α-1,6-glucosidic was obtained by treating maize amylose (β-amylolysis limit, 101%) with the sweet corn enzyme, and then with isoamylase (a specific α-1,6-glucosidase which hydrolyses the 1,6-inter-chain linkages in amylopectin and glycogen).[8] The β-amylolysis limit of the "branched"-amylose was 77% before, and 98% after isoamylolysis, showing that the inter-chain linkages had been hydrolysed. Isoamylase also caused a marked increase in the β-amylolysis limit of the synthetic polysaccharide.

Table

A comparison of the properties of amylopectin–glycogen type polysaccharides

	Amylopectin	*Synthetic polysaccharide*	*Phytoglycogen*
α-Amylolysis limit, PM (%)	90	84	87
Proportion of 1,6-linkages, $P_{1,6}$ (%)*	5·4	6·6	6·0
Average chain length (glucose residues)	19†	15	16–17‡
β-Amylolysis limit (%)	59	54	53
Exterior chain length	14	10–11	11
β-Amylolysis limit after isoamylolysis (%)	80	85	—

*Calculated from the equation: $P_{1,6}=23{\cdot}4-0{\cdot}20$ (PM), ref. 7.

†Average chain length 22 by periodate oxidation.

‡Average chain length 15 by periodate oxidation.

The sweet corn branching enzyme activity was optimum at *ca.* pH 6·0, was completely inhibited by 5×10^{-5}M mercuric chloride, and partly inhibited by 1% ammonium molybdate solution; these inhibitors have less effect on yeast branching enzyme.[1]

We conclude that extracts of sweet corn contain a branching enzyme, which, unlike potato Q-enzyme, can introduce further α-1,6-inter-chain linkages into amylopectin. During the completion of this work, Dr. C. E. Cardini kindly informed us that his colleagues Drs. N. Lavintman and C. R. Krisman have also obtained evidence for the presence of an atypical branching enzyme in sweet corn.*

Certain of the electrophoresis fractions caused an increase in the iodine-staining power of glycogen and in the β-amylolysis limit of phytoglycogen; this indicates the presence of a "debranching" enzyme (*cf.* isoamylase[8]) in sweet corn.

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α-1,4-GLUCOSANS. PART XIX. THE ACTION OF ACID ON MALTOSE AND STARCH-TYPE POLYSACCHARIDES

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384. *α-1,4-Glucosans. Part XIX.*[1] *The Action of Acid on Maltose and Starch-type Polysaccharides*

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When dilute solutions (0·4—1·0%) of maltose are heated with mineral acid, significant quantities of isomaltose and nigerose are produced; the yields are greater than that due to acid-reversion from D-glucose. The isolation of minute quantities of nigerose from partial acid hydrolysates of starch-type polysaccharides cannot, in the absence of other supporting evidence, be regarded as proof of the presence of α-1,3-glucosidic linkages.

ALTHOUGH it is now generally accepted that amylopectin is composed of chains of α-1,4-linked glucose residues interlinked by 4—5% of α-1,6-glucosidic interchain linkages,[2] the presence of a small proportion of α-1,3-glucosidic linkages has also been suggested. This was originally based on the isolation of *ca.* 0·13% of nigerose from a partial acid hydrolysate of waxy maize starch, under conditions in which its formation by " reversion " from glucose was negligible.[3] Partial acid hydrolysates of beef liver glycogen [4] and the algal glucan Floridean starch [5] also contain small quantities of nigerose (Table). However, nigerose has been prepared by the action of acid on a mixture of maltose and glucose,[6] whilst commercial starch dextrins are prepared by roasting starches in the presence of acidic reagents, which causes fragmentation and the formation of 1,3-glucosidic linkages by transglucosidation.[7] It therefore seemed possible that despite the difference in these experimental conditions, and those in the Table, limited acid-catalysed transglucosidation of maltose could account for the observed minute yields of nigerose. We now report evidence for the correctness of this view, a preliminary account of which has been published.[8]

The presence of nigerose in partial-acid hydrolysates of starch-type polysaccharides

		Yield of nigerose		
Wt. of polysaccharide (g.)	Conditions of hydrolysis	(mg.)	(%)	Ref.
Waxy maize starch, 130	0·4% in 0·1N-HCl at 100° for 8 hr.	170 *	0·13	3
Beef liver glycogen, 92	0·4% in 0·1N-HCl at 100° for 5·8 hr.	1 *	0·001	4
Floridean starch, 12·5	1% in 0·33N-H_2SO_4 at 100° for 130 min.	35	0·28	5

* Isolated as twice this weight of octa-acetate.

Since a reference specimen of nigerose was required, and the fact that the sample prepared by Pazur and Budovich [6] differed markedly in specific rotation (+87°) from other literature values (+135 to +137°),[9] we have repeated the preparation of nigerose by the action of acid on maltose and glucose. A mixture of glucose (12 g.) and maltose (24 g.) was heated at 100° in 0·1N-sulphuric acid (90 ml.) for 5 hr., cooled, neutralised, and the product fractionated by charcoal–Celite column chromatography. The products were glucose (26·1 g.), isomaltose (0·19 g.), maltose (3·15 g.), and a mixture of nigerose and other sugars (1·98 g.). Part of this mixture was fractionated on a second column, as the borate complexes,[10] to give glucose, α,α-trehalose, kojibiose, maltose, and nigerose. The yield of the last, which had $[\alpha]_D$ +134°, in agreement with other literature values, corresponded to the presence of 0·388 g. in the original mixture, an amount similar to that (0·4 g.) recorded by Pazur and Budovich.[6]

The action of hot acid on a dilute solution of maltose was then examined, and in view of our previous interest in Floridean starch,[11] the conditions of Peat and his co-workers,[5] with a final concentration of 1% carbohydrate, were used. Paper chromatography showed the presence of five sugars with R_G 1·0, 0·70, 0·55, 0·37, and 0·28, corresponding to glucose, maltose, isomaltose, panose, and an oligosaccharide. The maltose spot was elongated and showed the probable presence of an additional sugar of slightly higher R_G.

The mixture of sugars was neutralised, concentrated, and fractionated on a charcoal–Celite column. The products were glucose (8·48 g.), and mixtures of glucose and 1,6-anhydroglucose (0·05 g.), of disaccharides (2·50 g.), and of oligosaccharides (0·23 g.). One portion of the disaccharide mixture was chromatographed, as borate complexes, on charcoal. This gave traces of glucose and panose, isomaltose (65 mg.), and a mixture of nigerose and maltose which on preparative paper chromatography gave 12·5 mg. of a reducing sugar with the R_G of nigerose, the same electrophoretic mobility as laminaribiose, and whose acetate had m. p. 147° undepressed on admixture with β-nigerose octa-*O*-acetate kindly supply by Dr. J. R. Turvey. A second portion of the mixed disaccharides (0·65 g.) was fractionated by preparative paper chromatography, to give a further 9·7 mg. of nigerose, $[\alpha]_D$ +142° (in water). A third portion of the disaccharide mixture (0·56 g.) was analysed by quantitative paper chromatography, the various disaccharides being eluted with water, and estimated using a phenol–sulphuric acid reagent.[12] The apparent composition of the original disaccharide mixture was: maltose, 2344 ± 12 mg.; isomaltose, 72 ± 6 mg.; nigerose, 87 ± 6 mg. It is clear that substantial losses of nigerose had occurred during the various attempts to obtain a chromatographically pure specimen.

The oligosaccharides had the R_G values of maltotriose, panose, and isomaltotriose (0·44, 0·32, and 0·21, respectively). The last has been isolated from partial acid hydrolysates of glycogen, and assumed to be structurally significant.[4] This would mean that, in parts of the molecule, at least two branch points were linked together. This is not very likely in terms of the mode of action of branching enzyme,[13] and it is possible that this trisaccharide is also formed by transglucosidation, either from maltose (as above), or from isomaltose.

The fact that approximately similar yields of nigerose and isomaltose are formed from maltose was confirmed in small-scale experiments in which maltose (0·50 g.) was heated in 1% solution with dilute sulphuric acid, and the products analysed by quantitative paper chromatography. In one experiment, the relative yields of maltose, isomaltose, and nigerose were 392, 23, and 26 μg., respectively.

The effect of hot dilute hydrochloric acid on 0·4% solutions of maltose (cf. ref. 3) was also studied. In all experiments, paper chromatographic evidence for the formation of both isomaltose and nigerose was obtained; the yields were similar to that formed from 1% maltose by hot dilute sulphuric acid. During an 8 hr. heating period, isomaltose could be detected after only 1 hr.

We conclude that significant amounts of nigerose are formed by the action of hot dilute mineral acid on dilute (0·4—1·0%) solutions of maltose. Since these conditions are similar to those used for the linkage analysis of amylopectin-type polysaccharides (Table), the presence of nigerose cannot be regarded as uniquivocal evidence for the presence of α-1,3-glucosidic linkages. Other evidence for the presence of these linkages in amylopectin [14] was based on periodate oxidation studies, but was recently [1] shown to be inconclusive. Moreover, the fact that intestinal extracts hydrolyse nigerose does not, despite a suggestion to the contrary,[3] offer further support for the presence of 1,3-linkages in starch.

In the case of Floridean starch, the evidence for α-1,3-linkages is based on (a) the presence in partial acid hydrolysates of nigerose, and also of trisaccharides containing α-1,3-linkages, (b) the release of nigerose following enzymic degradation,[15] a process which does not, in this instance, involve transglucosidation reactions. The latter observation thus provides stronger evidence for the presence of α-1,3-linkages, and will be considered in detail in a later Communication.

Our conclusions differ from those of Wolfrom and his co-workers,[16] who have also considered the possibility that the nigerose was an artifact formed by the action of acid on maltose and glucose. A mixture of these sugars (114 and 30 g., respectively) was heated in 37 l. of 0·08N-hydrochloric acid for 10 hr. After separation of glucose by column chromatography, paper chromatography of the oligosaccharide fraction showed the presence of maltose, with smaller amounts of glucose, 1,6-anhydroglucose, " a significant amount of (1→6)-linked disaccharide, and a trace of nigerose." However, on acetylation of this mixed fraction, nigerose octa-acetate could not be isolated, and it was therefore concluded that the previous isolation of this derivative (*e.g.*, 2 mg. from 92 g. of glycogen [4]) was structurally significant. It must be noted that these workers have disregarded their own paper chromatographic evidence; moreover, neither the acetylation of sugars nor the chromatographic separation of sugar acetates is a quantitative procedure.

The absence of β-linked disaccharides in our experiments proves that isomaltose and nigerose are formed not by acid-reversion from glucose (which gives various sugars including gentiobiose and cellobiose [17]) but by a relatively rapid acid-catalysed transglucosidation reaction. This occurs with retention of configuration, and is not confined to maltose. For example, when methyl α-D-glucoside is heated with dilute acid, isomaltose is formed (cf. ref. 18), whilst, with methyl β-D-glucoside, gentiobiose is the product. When isomaltose in 0·4% solution was heated with acid, maltose and a higher oligosaccharide could be detected.

Additional evidence for the formation of oligosaccharides from maltose was obtained by hydrolysing amylopectin in the presence of [^{14}C]glucose, and comparing the radioautogram with that of (a) maltose and (b) glucose, each of which was heated in dilute acid solution with [^{14}C]glucose. The formation of radioactive oligosaccharides was much greater from amylopectin and maltose than from glucose. This confirms the view [17b] that, in the linkage analysis of polysaccharides, the action of acid on the constituent disaccharide(s) rather than the component monosaccharide(s) should be used for control purposes.

EXPERIMENTAL

Methods.—(a) *Paper chromatography.* Ascending and descending chromatograms were developed using *A*, ethyl acetate–pyridine–water (10 : 4 : 3, v/v); *B*, butan-1-ol–pyridine–water (6 : 4 : 3, v/v); *C*, butan-1-ol–ethanol–water (40 : 11 : 19, v/v); *D*, butan-1-ol–acetic acid–methanol–water (517 : 100 : 219 : 166 v/v) [19] as solvents, together with aniline phthalate, and silver nitrate spray reagents.

(b) *Electrophoresis.* Separation of oligosaccharides on Whatman No. 1 paper was effected using an apparatus similar to that of Foster,[20] at 750 v and 10 mA. Borate [20] or germanate [21] buffers (pH 10·7) were used.

(c) *Column chromatography.* Charcoal–Celite columns were prepared from Ultrasorb 120—240 charcoal [22] and Celite 545, and oligosaccharides eluted with increasing concentrations of aqueous ethanol. In some experiments, borate complexes of oligosaccharides were eluted [10]; column fractions were then acidified to pH 3 and extracted with isopentyl alcohol for 24 hr. to remove borate ions.

(d) *Estimation of reducing sugars.* Glucose and disaccharides were determined by the method of Park and Johnson,[23] or by the phenol–sulphuric acid reagent of Dubois and his co-workers.[12] For disaccharides, a calibration curve against maltose was used.

Materials.—Glucose and maltose were commercial samples which had been purified by three recrystallisations. The glucose was chromatographically pure. The maltose sample contained a trace of maltotriose (identified by α-amylolysis to maltose and glucose), but no other disaccharides. Maltose was chromatographed on Whatman 3MM paper, and the leading and trailing edges of the band of sugar separated and rechromatographed in solvent *C* for 7 days. On spraying with both aniline oxalate and silver nitrate reagents, only maltose was present. For the experiments involving quantitative paper chromatography, maltose which had been freed from maltotriose by charcoal–Celite chromatography was used.

Authentic samples of isomaltose, isomaltotriose, and panose were available from previous studies.[24]

Action of Acid on a Mixture of Maltose and Glucose.—A mixture of glucose (12 g.) and maltose (24 g.) was dissolved in 0·1N-sulphuric acid (90 ml.) and heated at 100° for 5 hr. The solution was cooled, neutralised (barium carbonate), centrifuged, and the precipitate washed three times with water. The supernatant solution and washings were combined and concentrated;

paper chromatography showed the presence of at least five sugars. The concentrate was applied to a charcoal–Celite column (100 × 6 cm.) which was eluted with water (5 l.) and then 10% ethanol (25 l.). The aqueous eluate did not contain carbohydrate; the ethanol eluate was collected in 500 ml. fractions, each of which was concentrated to *ca.* 1 ml. before chromatography in solvent *B* (two ascents on Whatman 3MM paper).

Fraction O (column fractions 11—19). This material crystallised, yield 26·1 g. The sugar had $[\alpha]_D$ +52° in water, the R_G value of glucose in solvents *A* and *B*, and the derived acetate had m. p. 130—131°. D-Glucose had $[\alpha]_D$ +52·6°, and the penta-acetate had m. p. 131°.

Fraction A (column fractions 20—24). Chromatography in solvent *B* showed the presence of one major and two trace components, with R_G values of isomaltose, glucose, and maltose, respectively. The isomaltose was purified by preparative paper chromatography (yield 195·2 mg.). It had $[\alpha]_D$ +122·8° in water, and the derived acetate had m. p. 142° (cf. authentic values of $[\alpha]_D$ +122° and m. p. 143°, respectively).

Fraction B (column fractions 25—32). This material crystallised (yield 3·15 g.) but contained a mixture of glucose and maltose. A portion was fractionated by preparative paper chromatography to give glucose (34 mg.) and maltose (303 mg.) which had $[\alpha]_D$ +132·9° and gave an acetate with m. p. 158° (cf. authentic values of $[\alpha]_D$ +136° and m. p. 158—159°). Fraction B thus contained 315 mg. of glucose and 2·835 g. of maltose.

Fraction C (column fractions 33—50). Concentration to dryness gave 1·98 g. of material which, by chromatography in solvent *B*, contained three sugars with R_G values similar to those reported by Pazur and Budovich[6] for nigerose, maltose, and isomaltose. Chromatography with solvents *A* and *B*, using both descending and multiple-ascent techniques, failed to give an adequate resolution for quantitative separation.

A portion of fraction C (170 mg.) was applied to a charcoal–Celite column (60 × 5 cm.) previously washed with water and then with borate buffer pH 8·7,[10] and the column eluted with borate buffer (6 l.) and an ethanol gradient of 0—20%. Fractions (50 ml.) were collected, and their carbohydrate content estimated on a 1-ml. aliquot using the phenol–sulphuric acid reagent.[12] Various fractions were combined, concentrated, freed from borate, evaporated to dryness, and redissolved in known volumes of water for measurement of optical rotation and disaccharide content.

Fraction C/A (column fractions 8—23) (yield 1·2 mg.). The sugar had the R_G value of glucose, and $[\alpha]_D$ +50° in water.

Fraction C/B (column fractions 24—27) did not contain carbohydrate.

Fraction C/C (column fractions 28—35) (yield 5·4 mg.) had $[\alpha]_D$ +186° in water and did not react with Fehling's solution, aniline oxalate, 3,5-dinitrosalicylic acid, or triphenyltetrazolium chloride.[25] This suggests a 1,1-linked disaccharide; α,α-trehalose has $[\alpha]_D$ +178°.

Fraction C/D (column fractions 36—43) (yield 9·4 mg.) had $[\alpha]_D$ +136° in water, did not react with Fehling's solution, aniline oxalate, and triphenyltetrazolium chloride, but reduced 3,5-dinitrosalicylic acid. These reactions are characteristic of 1,2-linked disaccharides; kojibiose has $[\alpha]_D$ +133 to +140°.[26]

Fraction C/E (column fractions 44—59) (yield 33·3 mg.) had $[\alpha]_D$ +134° in water. The R_G and M_G value in both borate and germanate buffer were the same as that of nigerose isolated from a partial acid hydrolysate of isolichenin.[27] The derived β-acetate (prepared by the sodium acetate–acetic anhydride method) had m. p. 148° (lit.,[9] $[\alpha]_D$ +134 to +139°, m. p. 147—153°).

Fraction C/F (column fractions 60—90) (yield 97·5 mg.) had $[\alpha]_D$ +135° in water, and had the R_G and M_G values of maltose. The derived β-acetate had m. p. 159° (lit., $[\alpha]_D$ +136°, m. p. 159°).

From the above results, the composition of fraction C was calculated to be: glucose, 14 mg.; α,α-trehalose, 63 mg.; kojibiose, 110 mg.; nigerose, 388 mg.; maltose 1136 mg.

The ratio of isomaltose : maltose : nigerose in the reaction products was measured in a separate experiment in which glucose (2 g.) and maltose (4 g.) were heated in 0·1N-sulphuric acid (15 ml.). After neutralisation, the combined solutions were diluted to 50 ml. with distilled water, and aliquots (10 μl.) chromatographed on Whatman No. 1 paper using solvent *C* (which was not known to us during the previous experiment) for 8 days. Appropriate areas of the chromatograms were eluted, and the disaccharide content measured by Park and Johnson's method.[23] The relative yields were: isomaltose, 14 μg; maltose, 129 μg; nigerose 13 μg; the last is equivalent to the formation of 390 mg. nigerose in the large scale experiment.

Action of Acid on Maltose.—(a) 1% *Maltose and sulphuric acid.* Maltose (12·5 g.) was dissolved in 0·33N-sulphuric acid (1250 ml.), preheated to 100°, and heated for 125 min. at 100°. The cooled solution was neutralised (barium carbonate), centrifuged, and the precipitate washings and supernatant solution were combined. Paper chromatography (solvent *B*, two ascents) showed the presence of five sugars with the R_G values of glucose, maltose, isomaltose, panose, and an oligosaccharide. The maltose spot was elongated, suggesting the probable presence of an additional sugar of slightly higher R_G value. The solution was concentrated to 50 ml. and applied to a charcoal–Celite column (45 × 7 cm.) which was eluted with 12 l. of aqueous ethanol (1—20%, as a linear gradient). Fractions (50 ml.) were collected, the carbohydrate content determined on 1-ml. aliquots, and after concentration, appropriate fractions combined after paper-chromatographic analysis (solvent *B*, two ascents).

Fraction	Column fraction	Wt. (g.)	Probable identity
A	4—12	0·001	—
B	13—69	8·390	Glucose
C	70—82	0·085	Glucose
D	83—103	0·048	Glucose, 1,6-anhydroglucose
E	104—163	2·454	Maltose, isomaltose, nigerose *
F	164—173	0·049	Maltose, isomaltose, nigerose, panose *
G	174—193	0·113	Maltose, maltotriose, panose *
H	194—230	0·117	Maltose, maltotriose, isomaltotriose *

* Traces of glucose were also present.

Fraction B had $[\alpha]_D$ +53° in water, and the R_G value of glucose in solvents *A* and *C*, and the acetate had m. p. 128° (cf. $[\alpha]_D$ +52·6° and m. p. 131° for glucose).

Fraction C also had the R_G value of glucose.

Fraction D was a mixture of glucose and 1,6-anhydroglucose. The relative yields of these sugars, by quantitative paper chromatography, were 18·7 and 26·2 mg., respectively.

Fractions E and F were combined, and one half was applied to a charcoal–Celite column (30 × 5 cm.) previously washed with borate buffer, and eluted with 10 l. of buffer with a linear gradient of 0—20% of ethanol. Fractions (*ca.* 40 ml.) were collected, analysed, and combined as appropriate, as follows.

Fraction EF/A (column fractions 15—22) was non-carbohydrate.

Fraction EF/B (column fractions 36—46) was a trace of glucose.

Fraction EF/C (column fractions 47—65) (yield 64·7 mg.) had the R_G value of isomaltose in solvents *A* and *C*, and $[\alpha]_D$ +117° in water (cf. +122° for isomaltose).

Fraction EF/D (column fractions 66—220) was a mixture of maltose and nigerose (chromatography in solvents *A* and *C*). These chromatograms were slightly streaked, and attempts to remove inorganic ions by treatment with ethanol caused the loss of some disaccharides. The mixture was fractionated by preparative paper chromatography (Whatman 3MM paper, solvent *C*, 4 days), to give 12·5 mg. of a disaccharide which had the R_G value of nigerose, an M_G value similar to that of laminaribiose, and formed a crystalline acetate with m. p. 147° undepressed on admixture with authentic nigerose β-octa-*O*-acetate.

Fraction EF/E (column fractions 221—269) contained a trace of panose (paper chromatography).

A second portion of fraction EF (650 mg.) was fractionated by preparative paper chromatography to give 9·7 mg. of a disaccharide with the R_G and M_G values of nigerose, and $[\alpha]_D$ +142° in water (lit., +134 to +139°). From a third portion of fraction EF (150 mg.), maltose was isolated by preparative paper chromatography, and identified by R_G and M_G values, and $[\alpha]_D$ +140° (lit., +136°).

Fractions G and H were combined, and examined by paper chromatography in solvent *B*. Three sugars with the R_G values of maltotriose, panose, and isomaltotriose were present.

(b) *Ratio of disaccharides formed from maltose.* The remainder of fraction EF (560 mg.) was dissolved in water (25 ml.), and 5 μl. portions chromatographed on sheets using solvent *C* for 9 days. Appropriate areas of the sheets were eluted and analysed by the phenol–sulphuric acid method. The calculated yields in EF were: isomaltose, 72 ± 6 mg.; maltose, 2344 ± 12 mg.; and nigerose, 87 ± 6 mg.

The similarity in the yields of isomaltose and nigerose was confirmed in a second experiment in which maltose (0·50 g.) was heated in 0·33N-sulphuric acid (50 ml.) for 130 min., cooled, neutralised (barium carbonate), and the solution and washings were adjusted to 10 ml.

Portions were chromatographed as above for 7 days, and appropriate areas estimated by Park and Johnson's method.[23] The relative yields were: isomaltose, 23 μg.; maltose, 392 μg.; nigerose, 26 μg.

(c) 0·4% *Maltose and hydrochloric acid*. Maltose (500 mg.) was heated in 0·4% solution in 0·1N-hydrochloric acid for 8 hr., cooled, and neutralised by passage through a column of Duolite A-4 resin.[3] The solution was adjusted to 50 ml., and portions (0·2 ml.) chromatographed against authentic nigerose using solvent *D* for 4 days. Nigerose was detected, and amounted to *ca.* 2% of the total carbohydrate. Nigerose was also formed in similar experiments in which (a) the hydrochloric acid was neutralised with silver carbonate, and (b) 1% maltose was heated in 0·33N-sulphuric acid.

Considerable difficulty arose in these and the previous experiments in effecting complete separation of maltose and nigerose. In some experiments the following bands were eluted: isomaltose, maltose plus nigerose, and glucose; the mixed fraction was then rechromatographed in solvent *D* for 4 days. Even this technique did not always give chromatographically pure nigerose; hence, the quoted figures for paper-chromatographic analyses are not considered to be strictly quantitative.

When 0·4% solutions of maltose were heated in 0·1N-hydrochloric acid, isomaltose was not present after 30 min. but could readily be detected after 1 hr.

Action of Acid on other Glucosides.—(*a*) Isomaltose (70 mg.) was heated in 0·4% solution in 0·1N-hydrochloric acid for 4 hr., cooled, neutralised (Duolite A-4), concentrated, and chromatographed. In addition to glucose and isomaltose, small quantities of sugars with R_G values of a di- and tri-saccharide were present. The latter were eluated from a sheet of paper, and tentatively identified as maltose and isomaltotriose, by borate electrophoresis and R_G value, respectively.

(*b*) Methyl α-D-glucoside (500 mg.) was heated in 2N-hydrochloric acid (25 ml.) at 70° for 2 hr., cooled, neutralised (De-Acidite FF resin column), and concentrated to *ca.* 3 ml. Part of the solution was chromatographed on Whatman 3MM paper, and guide strips showed the presence of isomaltose or gentiobiose. This sugar was eluted (yield 4 mg.); it had the same M_G value as isomaltose, and was not hydrolysed by an almond β-glucosidase preparation. When methyl β-D-glucoside was heated similarly, the product was hydrolysed by the enzyme preparation (whose specificity had been tested against known substrates), and is therefore identified as gentiobiose.

Acid-transglucosidation Experiments using [^{14}C]*Glucose.*—Generally labelled [^{14}C]glucose (0·1 mc, 0·37 mg. in 0·5 ml. of water, supplied by the Radiochemical Centre, Amersham) was used. Solutions were chromatographed on Whatman No. 1 paper, air-dried, and placed on sheets of Ilford Fast Industrial-G *X*-ray film, which were developed after a suitable period. Exposure for up to 8 days failed to reveal any radioactive oligosaccharides in the sample of glucose.

Maltose (10 mg.), [^{14}C]glucose solution (0·1 ml.), in 0·33N-sulphuric acid (0·9 ml.) were heated for 130 min., cooled, neutralised (barium carbonate), and the solution and washings were concentrated to 0·1 ml. Radioautography showed the presence of radioactive sugars with the R_G values of 1,6-anhydroglucose, isomaltose, and an elongated maltose spot. The intensities of the radioactive disaccharides were much greater than that of a similar solution prepared from 10 mg. of glucose. When potato amylopectin (9 mg.) was hydrolysed under these conditions, radioactive disaccharides with the R_G values of maltose, isomaltose, and nigerose were formed in amounts similar to that from maltose. (In all the radioautograms, 5 μl. portions of solutions were used.)

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