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PROFESSOR W. J. WHELAN

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Enzymic Explorations of the Structures of Starch and Glycogen

THE FOURTH CIBA MEDAL LECTURE

BY W. J. WHELAN*

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With many a winding bout of linked sweetness, long drawn out (*Milton*, 1631)

I am not aware that Milton had starch and glycogen in mind when he composed the couplet with which I introduce this Lecture. Its uncanny aptness in describing the constitution of these polysaccharides was noted by Sir Norman Haworth, the head of the Department of Chemistry at the University of Birmingham, where I received my training as an organic chemist. I had won a Brewers' Society Scholarship to study biochemistry at Birmingham, under R. H. Hopkins, but on arrival was informed that wartime regulations would prevent me from taking a degree in Hopkin's Department. I was advised to take a degree in chemistry and maybe the war would be over before my studies were complete, when I could switch to biochemistry. This was in 1942, and I graduated in chemistry before the war's end, continuing as a Ph.D student in that Department, working on an antisubmarine device. My plans to receive at least some biochemical instruction were thwarted by Haworth's forbidding me to attend Hopkin's lectures, which I nevertheless did, *sub rosa*. Haworth's hostility to biochemists, typical of the organic chemist of that period, was, however, tempered by the excellent biochemistry in progress in his own Department, under the direction of Stanley Peat, with whom I had the good fortune to be associated for over 10 years, as a Ph.D. student and later as a member of his staff at the Department of Chemistry in the University College of North Wales, Bangor. But it was two actions of Haworth himself that rekindled the fires of biochemistry within me, specifically enzymology. The wartime shortage of laboratory assistants was combated by Haworth by steering some of us into the research laboratories, at the expense of our formal laboratory classes. I spent 9 undergraduate months with Peat's group engaged in the enzymic synthesis of α-glucose 1-phosphate. Then, 3 years later, when my antisubmarine research had led me to study the photo-oxidation of starch fractions (Whelan & Peat, 1949), I wanted to characterize the oxidative changes by enzymic methods. This phase coincided with a visit by Haworth to the United States, whence he returned with a sample of crystalline sweet-potato β-amylase, given to him by A. K. Balls (Balls, Walden & Thompson, 1948). With a fine disregard for enzyme fragility, Haworth had carried the sample around in his waistcoat pocket. We looked at it under the microscope. The beautiful prisms were dismissed by Haworth as ammonium sulphate. I was too intrigued to be sceptical and proceeded to use it on the starch fractions, amylose and amylopectin, with the totally unexpected result that amylose was only incompletely (70%) converted into maltose (Peat, Whelan & Pirt, 1949; Peat, Pirt & Whelan, 1952*a*,*b*). Amorphous β-amylase had given quantitative yields of maltose, leading to the belief that amylose was a linear homogeneous molecule. It was clearly not.

This experiment brought home the realization that, if one wished to explore the structures of starch and glycogen, the best possible tools would be the natural agents of catabolism. Instead of trying to devise chemical methods of analysis, which would be at best semiquantitative, and certainly lacking in specificity, one should use the ready-to-hand, totally specific and exquisitely sensitive natural agents of degradation. There would also be the added bonus that one would at the same time learn something about the natural catabolism of these polysaccharides.

The problems inherent in such an approach were several. Could the enzymes be purified? The example of crystalline versus amorphous β-amylase was illustrative of the dangers of incorrect conclusions being derived from the use of an impure enzyme preparation. [It was shown later that it was an α-amylase contaminant of amphorous β-amylase that was assisting the latter to achieve complete conversion of amylose (Cunningham, Manners, Wright & Fleming, 1960).] In this regard one could only proceed on the basis that to be forewarned was to be forearmed. A second problem was that to attack the highly branched polysaccharides, amylopectin and glycogen, there was needed an enzyme to split the (1→6)-branch points. None was then available. We only had enzymes that would split

* Address: Department of Biochemistry, University of Miami School of Medicine, P.O. Box 875, Biscayne Annex, Miami, Fla. 33152, U.S.A.

the (1→4)-bonds, i.e. α-amylase, β-amylase and phosphorylase.

Even more serious was our lack of knowledge of the specificity of these carbohydrases. We could not characterize enzymes of unknown specificity by using substrates (starch and glycogen) of unknown structure.

The solutions to the second and third problems came very quickly, assisted by good fortune. First, we discovered in beans an enzyme that would debranch amylopectin, i.e. R-enzyme (Hobson, Whelan & Peat, 1951). We were led to the discovery by the fact that debranching is accompanied by a marked increase in intensity of iodine stain. Secondly, paper chromatography of carbohydrates had just arrived, opening a whole new world by permitting a visual inspection of the course of enzyme reactions, and then Whistler & Durso (1950) put into practical terms a method of fractionating oligosaccharides in quantity on columns of charcoal, a development of a method originally described by Weibull & Tiselius (1945). We seized on this and in the same year had isolated pure specimens of maltotriose and higher homologues (Bailey, Whelan & Peat, 1950). Only one more stroke of fortune was needed, that these oligosaccharides, of known structure, would be substrates for our enzymes. For the most part this was true, and we were able to characterize the action patterns of potato phosphorylase and sweet-potato β-amylase (Whelan, Bailey & Roberts, 1953; Whelan & Bailey, 1954; Whelan & Roberts, 1954), salivary α-amylase (Whelan & Roberts, 1953) and the potato transglycosylase, D-enzyme (Peat, Whelan & Rees, 1956*a*). α-Amylase action on amylopectin and glycogen was also characterized by our ability, with the Whistler & Durso (1950) method, to separate the branched oligosaccharides that the enzyme formed (Whelan & Roberts, 1952; Whelan, 1960). Some of these oligosaccharides proved to be substrates for R-enzyme; the action of the latter enzyme was therefore also characterized (Whelan, 1953). The chain lengthening by phosphorylase of maltodextrin primers, with α-glucose 1-phosphate as donor substrate, was found to proceed by a simultaneous elongation of all primer molecules (Whelan & Bailey, 1954). In this way we could synthesize polymers of known average chain length, $\overline{CL}$, for use as characterizing substrates for enzymes that would not act on the small maltodextrins, for example the amylopectin-synthesizing enzyme, Q-enzyme (Peat, Whelan & Bailey, 1953*a*).

Structural studies with enzymes

R-Enzyme era. The primary goal of the studies recounted above had been that of developing a methodology for the exploration of the arrangement

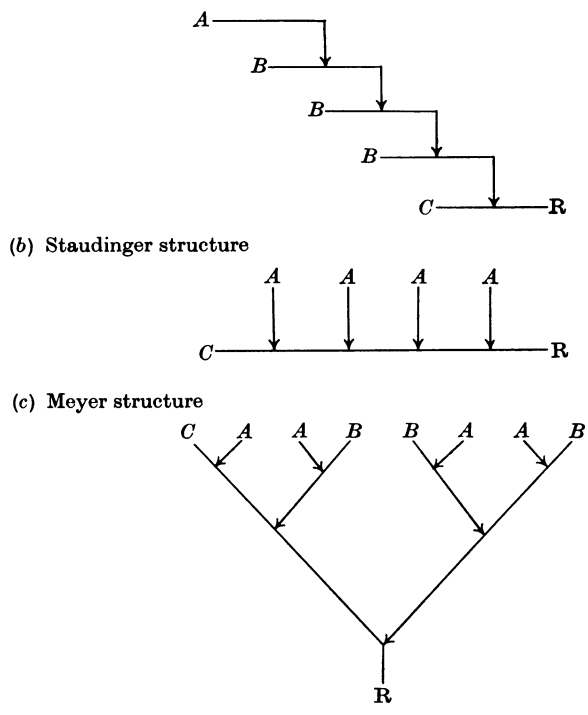


Fig. 1. Three representations of amylopectin and glycogen as proposed by Haworth *et al.* (1937), Staudinger & Husemann (1937) and Meyer and co-workers (Meyer & Bernfeld, 1940; Meyer & Fuld, 1941). The *A*, *B* and *C* chains (——) of (1→4)-bonded α-glucose units are defined in the text; ↓, (1→6)-bond; R, reducing end group.

of the unit chains in amylopectin and glycogen, and the stage was now set. As working models we had the three structures proposed by Haworth, Hirst & Isherwood (1937), Staudinger & Husemann (1937) and Meyer and co-workers (Meyer & Bernfeld, 1940; Meyer & Fuld, 1941) (Fig. 1). It is worth pointing out here some of the problems inherent in molecules of this type. These are homopolysaccharides, and the uniformity of the building units means that the only recognizable features that can be probed are the chain termini. There is no reason to expect that every molecule is identical in arrangement of chains or in molecular weight. If there were only one type of glycosidic linkage, the (1→4)-bond, the molecules would be linear and the problem would simply be one of determining average molecular weight and degree of polydispersity. It is the presence of the second, and minor, (1→6)-bond, 1 in about 24 for amylopectin and 1 in about 12 for glycogen, that gives rise to an infinity of possible arrangements, of which the formulae in Fig. 1 are only three expressions. It is the relative absence of

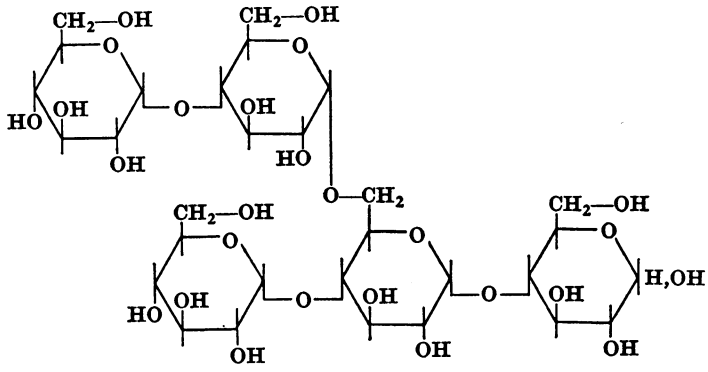


Fig. 2. Structure of a pentasaccharide formed from amylopectin by *Bacillus subtilis* α-amylase (Hughes *et al.* 1963).

recognition points in the molecules, coupled with the virtual certainty of inter- and intra-molecular variation in individual unit chain length, in positioning of branch points and in molecular weight, that makes the structural determination inherently more formidable than that of heteropolymers such as heteropolysaccharides, proteins or nucleic acids.

Even if the homopolymer is relatively simple, its structural determination by purely chemical methods may be impossible. Fig. 2 depicts a pentasaccharide formed from amylopectin by *Bacillus subtilis* α-amylase. It contains every known chemical feature of amylopectin and glycogen, i.e. chains of (1→4)-linked α-D-glucopyranose units joined through a (1→6)-branch point, a single unsubstituted reducing end group and a multiplicity of non-reducing ends. It proved impossible to arrive at a unique representation for this molecule by chemical methods, namely methylation analysis, periodate oxidation and fragmentation analysis. Yet analysis by enzymic methods (Hughes, Smith & Whelan, 1963) was so rapid and unequivocal as to be almost slightly sinful for one brought up as an organic chemist. If enzymes were essential for this simple pentasaccharide, was there any question that they would also be indispensable for the polysaccharides? This last point was further emphasized by the sterile and rather acrimonious correspondence that Meyer, Gürtler & Bernfeld (1947) and Haworth (1947) had been conducting on the relative merits of their formulae, sterile because their chemical methodology could not resolve the problem. It was hoped that the new enzyme methodology would do so.

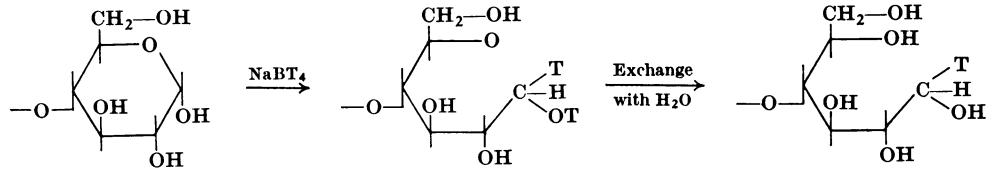
We were initially able only to look at amylopectin, since R-enzyme does not attack glycogen (Peat, Whelan, Hobson & Thomas, 1954), of which more later. The strategy we adopted then was one that has proved consistently successful in later work. First, one had to define the differences between the three formulae, and this was done by delineating three chain types (*A*, *B* and *C* in Fig. 1). The *A* chain is linked to the macromolecule only at one point, by the involvement of its reducing group in a branch link. The *B* chain is likewise linked, but is also substituted one or more times at a primary hydroxyl group by *A* or *B* chains. The *C* chain, of which there can only be one, whatever the structure, carries a free reducing group and is substituted only at primary hydroxyl groups. On this basis one sees that the Haworth and Staudinger molecules represent extremes, consisting almost wholly, in terms of numbers of chains, of one chain type. The Meyer molecule, in its most symmetrical, regularly rebranched, form, consists of equal numbers of *A* and *B* chains. But how to distinguish *A* from *B*? We could not expect that *A* and *B* chains could be distinguished on the basis of length. In any case, even if we branched amylopectin to set the chains free, we had no means of fractionating them. We had to convert one or other of the chain types into something of uniform length that could be recognized, separated and assayed. This was accomplished by prior treatment of the amylopectin with β-amylase to give the so-called β-limit dextrin.

β-Amylase is an exo-acting enzyme that splits alternate (1→4)-bonds in amylaceous polysaccharides to yield β-maltose. It cannot split (1→6)-bonds and therefore acts only on the outer chains of amylopectin. Since the *A* chains are wholly outer they must be trimmed by β-amylase to a definite length, even though in the native molecule they may vary in length. We had to expect that the *A*-chain stubs would be of two lengths, depending on whether the original chain had an even or odd number of glucose units. And so it proved to be. The β-limit dextrin was treated with R-enzyme and the products were fractionated on charcoal. There were found maltose and maltotriose; the next higher homologue was maltohexaose. The di- and tri-saccharides were clearly the stubs of the original *A* chains; the maltohexaose and higher saccharides were *B*

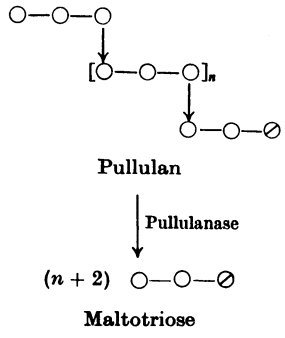
chains. The combined weight of maltose and maltotriose (12.8%) was close to the amount expected from the regularly rebranched Meyer structure (10.4%), in which there are equal numbers of *A* and *B* chains (Fig. 1). The amounts of maltose and maltotriose that would be formed from the Haworth and Staudinger molecules were vastly different. Clearly, of the three alternatives, the Meyer formula was the most appropriate (Peat, Whelan & Thomas, 1952, 1956*b*). Simultaneously, using a different type of debranching enzyme, the mammalian amylo-1,6-glucosidase, Larner, Illingworth, Cori & Cori (1952) applied a similar strategy to glycogen, using phosphorylase as the exo-enzyme, and concluded that the Meyer structure held for that polysaccharide also.

Pullulanase era. At this point our first impetus had run its course. The next step would be to determine what were the actual lengths of the unit chains in amylopectin and glycogen, and whether the chains really were arranged as Meyer had predicted. All that had, in effect, been established was a parity between *A* and *B* chains. There are many alternative ways of arranging them (French, 1964). We needed several things. The first was a method of fractionating the unit chains, once set free by debranching. Charcoal chromatography could only fractionate oligosaccharides. We had to wait the advent of molecular sieves to solve this problem. Secondly, we would have to characterize the chains by measuring $\overline{CL}$. Conventional chemical assays were too tedious. This problem was solved in two ways. A copper reagent (Nelson, 1944) is stoicheiometrically reduced by the maltodextrins and higher homologues (Robyt & Whelan, 1965) and is sufficiently sensitive for use up to $\overline{CL}$ 40–50. The second method has been to render practical the use of sodium boro[^{3}H]hydride to label the reducing chain end (Scheme 1; G. N. Richards & W. J. Whelan, unpublished work). A comparison of the amount of label introduced, compared with the total amount of polysaccharide, which may be determined by a sensitive and specific enzyme assay (Lee & Whelan, 1966), permits $\overline{CL}$ to be calculated. The idea of ^{3}H-labelling is by no means new (De Wulf, Lejeune & Hers, 1965), but its application has been plagued by a non-volatile ^{3}H-labelled impurity that contaminates commercially available sodium boro[^{3}H]hydride. We have succeeded in eliminating this material by the device of transferring the reduced polymer to a small square of filter paper, and washing this in acidified ethanol before counting its radioactivity. The impurity, but not the polymer, dissolves in the ethanol. The sensitivity of the method is such that values of $\overline{CL}$ up to several thousand can be measured.

Vital to our needs also was a better debranching enzyme, better in the sense that it would more completely debranch amylopectin and would have some action, at least, on glycogen. R-Enzyme debranches amylopectin β-dextrin efficiently, but amylopectin only incompletely (Hobson *et al.* 1951). The mammalian glycogen-debranching enzyme, amylo-1,6-glucosidase, discovered by Cori & Larner (1951), does not separate amylopectin and glycogen into their unit chains (see below). Our problem here was partly resolved by our good fortune in learning from Professor K. Wallenfels that he had discovered an extracellular enzyme from *Aerobacter areogenes* that converted a yeast (*Pullularia pullulans*) α-glucan, containing (1→6)-bonds, into maltotriose. Bender & Wallenfels (1961) were thereby able to describe the structure shown in Scheme 2 for pullulan. The significance for me was that this enzyme, pullulanase, was probably an R-enzyme, and this proved to be the case (Whelan, 1964; Abdullah *et al.* 1966; Drummond, Smith & Whelan, 1970). Though it had only a limited action on glycogen (indeed we now believe that some glycogens are not even attacked; Mercier & Whelan, 1970), it did attack glycogen β-limit and phosphorylase (ϕ)-limit dextrins. The use of pullulanase in determining glycogen and amylopectin structure was first described at a CIBA Foundation Symposium in 1963 (Abdullah, Taylor & Whelan, 1964), and within 3 years various of the Symposium participants had shown the great value of this enzyme in structural analysis and characterization



Scheme 1. Labelling of a reducing end group with NaB^3H_4 ($NaBT_4$), as used in the determination of $\overline{CL}$ of the unit chains of glycogen and amylopectin set free by a debranching enzyme and fractionated on a molecular sieve.



Scheme 2. Pullulan and its hydrolysis to maltotriose by pullulanase. ○, α-D-Glucopyranose; ∅, reducing end group; —, (1→4)-bond; ↓, (1→6)-bond.

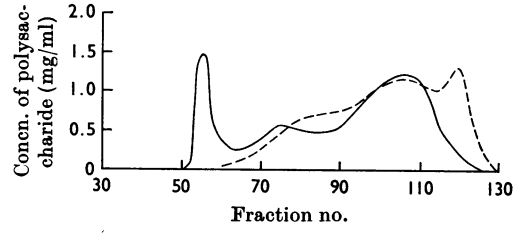


Fig. 3. Waxy-maize amylopectin (——) and its β-limit dextrin (----) after being debranched with pullulanase and fractionated on Sephadex G-50. [Redrawn from Lee *et al.* (1968).]

of enzyme action pattern (Brown, Illingworth & Kornfeld, 1965; Verhue & Hers, 1966; Bathgate & Manners, 1966; Abdullah & French, 1966, 1970).

The ability of pullulanase to attack both amylopectin and glycogen β-limit dextrins was exploited in a method for determining $\overline{CL}$ of these polysaccharides. By including β-amylase in a digest of pullulanase and polysaccharide, the β-limit dextrin is formed, debranched and continually regenerated, leading to an eventual qualitative conversion into maltose and glucose. The monosaccharide arises from the statistical 50% of chains containing an odd number of glucose units. Its selective enzyme determination permits $\overline{CL}$ to be calculated and offers a very convenient micro method for determining this parameter (Lee & Whelan, 1966).

The use of pullulanase on amylopectin and its β-limit dextrin, followed by fractionation on Sephadex G-50 and $\overline{CL}$ determination, permitted us to see for the first time the profile of unit chains in these molecules (Fig. 3; Lee, Mercier & Whelan, 1968). The results did not confirm the Meyer formula (Fig. 1). There were seen for amylopectin three populations of polysaccharide. That of highest molecular weight, not seen with the β-limit dextrin, is undebranched material. Penetrating the gel were two populations of chains having $\overline{CL}$ 20 and >50 at the peaks. A Meyer molecule, if translated into this kind of 'fingerprint' would give a continuous spectrum ranging from short (*A* chains, $\overline{CL}$ 12) to long chains, the latter being *B* chains of $\overline{CL}$ 36. Unlike the *A* chains, which might be expected to be distributed symmetrically in length around the average, the *B* chains would be at their shortest and most abundant at a length somewhat in excess of the $\overline{CL}$ for the *A* chains and would dwindle in number, while increasing in length (Fig. 1). If the molecular weight of amylopectin is measured in millions (Geddes, Greenwood & Mackenzie, 1965) the longest *B* chains would have $\overline{CL} > 100$, but there is little evidence of this in the totally debranched β-limit dextrin (Fig. 3). The dissymmetry in the unit-chain profile may mean, not only that the Meyer arrangement of chains is incorrect, but that we are seeing something that Meyer could not provide for in drawing his model, namely the effects on structure of the specificities of the enzymes of amylopectin synthesis. Clearly there are preferences for creating chains of particular lengths rather than any length.

Isoamylase era. At the time that this Lecture was delivered in December 1969 I described the action of pullulanase on glycogen, or rather its lack of action, and its limited action on the β-limit dextrin, which results in the release of maltose and maltotriose (the *A* chains) and other maltodextrins up to malto-octaose (C. Mercier & W. J. Whelan, unpublished work). I recounted these and other facts and argued that they were not compatible with a Meyer formulation for glycogen. I could not, however, offer an alternative structure. Rather than repeat this negative statement I prefer to mention briefly a discovery that was made shortly afterwards in the Miami laboratories. Yokobayashi, Misaki & Harada (1969, 1970) had described a *Pseudomonas* enzyme, isoamylase, that was unique in totally debranching glycogen and amylopectin. Being unable to acquire any of the Japanese enzyme, we instituted our own search for isoamylase and discovered it in a commercially available protein concentrate from *Cytophaga* (Gunja-Smith, Marshall, Smith & Whelan, 1970*a*). It seems identical in its action with the *Pseudomonas* enzyme, including its inability to remove *A* chains 2 units in length (Fig. 8). We have made use of this property by converting all the *A* chains of amylopectin and glycogen into maltosyl residues by the successive actions of muscle phosphorylase and β-amylase (Walker & Whelan, 1960). On successive treatments of this φ,β-limit dextrin with *Cytophaga* isoamylase and β-amylase, glycogen yields 44%

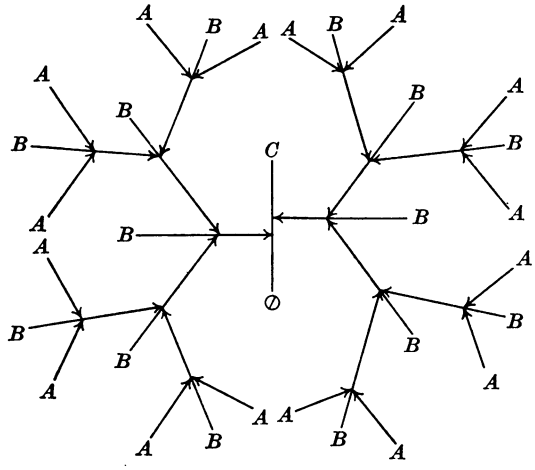


Fig. 4. Revision of the Meyer structure (Meyer and Bernfeld, 1940; Meyer & Fuld, 1941) for amylopectin and glycogen proposed by Gunja-Smith *et al.* (1970*b*). Symbols are as in Fig. 1; ∅, reducing end group. [Reproduced from Gunja-Smith *et al.* (1970*b*) by permission from *FEBS Letters*.]

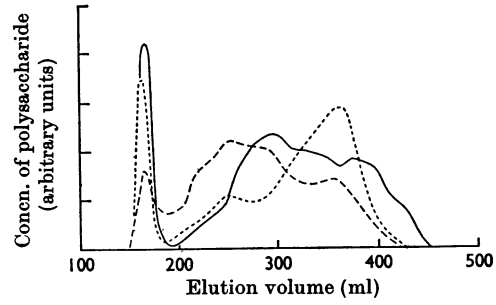
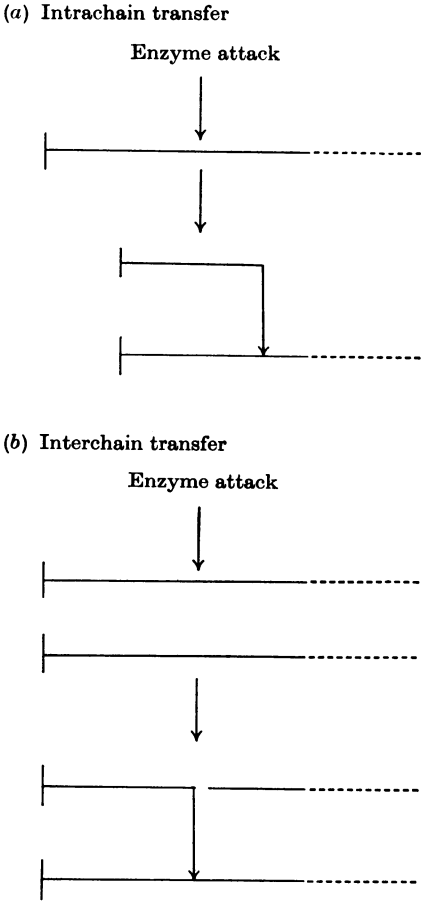


Fig. 5. Comparison of the length distribution of unit chains in synthetic and natural amylopectins. Waxy-maize amylopectin (·····), amylopectin synthesized from amylose by Q-enzyme (----) and amylopectin synthesized with phosphorylase, Q-enzyme and α-glucose 1-phosphate (Barker *et al.* 1950) (——) were debranched with pullulanase and fractionated on Sephadex G-50 (Drummond, 1970; Drummond, Smith & Whelan 1971).

maltose and amylopectin yields 29% maltose. Reference to Fig. 1 shows that in the Meyer molecule every *B* chain carries an *A* chain. A Meyer ϕ,β-limit dextrin after being debranched would still be a β-limit dextrin because the maltosyl *A* chains, terminating the *B* chains, would not be split away by isoamylase. Therefore a new arrangement of *A* and *B* chains in both glycogen and amylopectin is necessary to account for these results. This we have proposed (Fig. 4; Gunja-Smith, Marshall, Mercier, Smith & Whelan, 1970*b*). In this structure only half of the *B* chains carry *A* chains.

With the discovery of this debranching enzyme the millenium in terms of methodology for glycogen and amylopectin structure determination seems to have been reached, and we may expect rapidly to clothe the schematic representation in Fig. 4 with further details. One might add also that the use of this enzyme has provided what is the most convenient of any $\overline{CL}$ determination method yet described for branched polysaccharides (Gunja-Smith *et al.* 1970*a*). One simply measures the copper-reducing power of the totally debranched polysaccharide.

Biosynthesis of amylopectin. The dictum of the chemist carrying out structure determination is: 'analyse, then synthesize'. And so it should be for the biochemist. The biosynthesis of amylopectin-like material was in fact achieved over 20 years ago (Barker, Bourne, Peat & Wilkinson, 1950) by either of two routes. Branching enzyme, Q-enzyme, converts amylose into 'amylopectin', or the latter can be formed by incubating Q-enzyme, phosphorylase and α-glucose 1-phosphate. The enzymes are from potato. The products closely resemble natural amylopectin in terms of $\overline{CL}$, iodine stain and degree of β-amylolysis, and the claim to have synthesized amylopectin is legitimate. We have wondered, however, whether the resemblance of one or both of these synthetic materials to the natural article would persist when they were examined by the much more searching method of looking at their unit-chain profiles, after they had been debranched with pullulanase. It was not to be expected that both synthetic polymers would give identical profiles. Indeed it was hoped that, if they were different, one would be similar to the natural polymer, thereby indicating by which route amylopectin is formed *in vivo*. In the event it proved that the 'fingerprints' of both synthetic polysaccharides were very significantly different from that of natural amylopectin (Fig. 5), indicating that we do not yet know how amylopectin is synthesized. The obvious next step is to synthesize amylopectin with starch synthetase as the chain-extending enzyme, acting on UDP-glucose and/or ADP-glucose in conjunction with Q-enzyme. These findings have their parallel in the work of Leloir and his colleagues (Parodi, Mordoh, Krisman & Leloir, 1969), who have compared the physical properties of natural glycogen with those of glycogens synthesized with synthetase and phosphorylase. Their finding that it was 'synthetase' glycogen that more nearly resembled the natural article confirmed, if confirmation was needed, that phosphorylase has no major involvement in glycogenesis. The belief that plant phosphorylase does have a role in the synthesis of starch is, however, still



Scheme 3. Two types of chain transfer that can occur during the synthesis of amylopectin by Q-enzyme. Symbols are as in Fig. 1. The experiment in Fig. 6 proves that interchain transfer can occur.

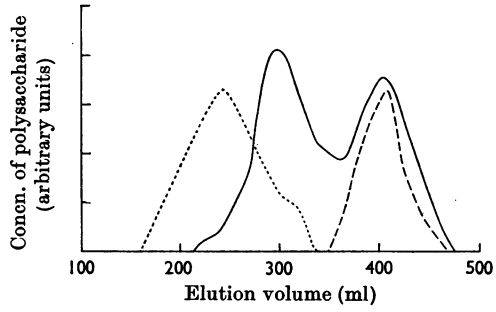


Fig. 6. Fractionation of synthetic amylopectins on Sephadex G-200. Potato Q-enzyme was allowed to act on amylose of $\overline{CL}$ 260 (·····) and $\overline{CL}$ 48 (----) and on a mixture of the two (——). The products were then fractionated (Drummond, 1970 Drummond *et al.* 1971).

current (e.g. Badenhuizen, 1969). Perhaps the results in Fig. 5 may undermine this belief.

The further pursuit of structural studies of starch and glycogen has now reached the point where the action patterns of the enzymes of biosynthesis must be explored in order to account for observations such as the dissymmetry of the unit-chain profile of amylopectin (Fig. 3). Much remains to be discovered about the branching enzymes in terms of their ability to act on chains of different lengths. One problem is whether the transglycosylation branching process proceeds by an interchain or an intrachain mechanism. (Scheme 3; Smith, Taylor & Whelan, 1968; Manners, 1968). We have partly resolved the question for potato Q-enzyme in the following manner (Drummond, 1970; Drummond *et al.* 1971). Amyloses of $\overline{CL}$ 260 and 48 were incubated with Q-enzyme separately and together, and the products frac tionated on Sephadex G-200. The 'amylopectins' formed from the separate amyloses were quite distinct in molecular profile (Fig. 6). If intrachain transfer were the exclusive mode of branch formation the same two profiles would have been seen in the experiment where Q-enzyme acted on the mixture. The result actually seen was of a change in profile such that the average weight of the heavier of the two amylopectins had decreased. This is indicative of interaction between the longer and the shorter amylose chains, i.e. interchain transfer is occurring. The possibility of intrachain transfer also occurring is, of course, not eliminated.

Structural anomaly in glycogen and amylopectin. We have developed new methodology for the determination of enzyme action pattern, polymer chain length and the detection of trace amounts of endo-enzyme in an exo-enzyme preparation, this beginning with the simple device of oxidizing a small proportion (5%) of the monosaccharide units with periodate. A similar principle was used some years ago by Nelson, Scaletti, Smith & Kirkwood (1963) to distinguish between exo- and endo-β-(1→3)glucanases on the basis of the ability of periodate to oxidize only the chain ends of a (1→3)-linked β-glucan, thereby blocking exo-β-glucanase action. This technique is not, however, limited to (1→3)-glucans oxidized specifically at the chain ends. A random oxidation of only a small proportion of the monomer units of a polysaccharide will render most of the molecule immune to an exo-enzyme. In this way we were able to confirm our finding (Drummond, Smith, Whelan & Han Tai, 1969) that pullulanase acts on pullulan in endo fashion (Smith, Drummond, Marshall & Whelan, 1970). We also argued that the method could be used for $\overline{CL}$ determination, since the degree of

limitation of attack by an exo-enzyme on a polymer in which a given number of monomer units has been oxidized will increase as CL increases. This is illustrated in Fig. 7 for glycogen, amylopectin and amylose, amyloglucosidase being used as the exo-enzyme. It was while conducting these experiments that we discovered that the first sample of amyloglucosidase we used, from *Aspergillus niger*, was contaminated with an endo-enzyme, probably α-amylase. This was discovered because the enzyme preparation continued to attack oxidized amylose. Accordingly we used a crystalline amyloglucosidase from *Rhizopus niveus*, which proved to be free from α-amylase. We then encountered an unexpected finding that put back the clock 20 years to the first use of crystalline β-amylase on amylose (see above). The *Aspergillus niger* enzyme preparation had been causing quantitative conversions of glycogens and amylopectins into glucose, providing a method for determining these polysaccharides (Lee & Whelan, 1966). The *Rhizopus niveus* enzyme gave conversions that in many cases were far from complete but that became so on addition of α-amylase (Table 1; Marshall & Whelan, 1970). Clearly the amylase-free glucosidase was encountering an obstacle to its progress into the macromolecules and, as with β-amylase (Peat *et al.* 1949), the results indicate the presence in glycogen and amylopectin of a new structural feature not hitherto suspected. This could be something quite novel, or it may be the result of a close packing of branch linkages. We know that the α-(1→6)-glucosidic bond is rapidly hydrolysed by amyloglucosidase only when the next bond in sequence is a (1→4)-bond (Abdullah, Fleming, Taylor & Whelan, 1963). If it happens that two (1→6)-bonds occur in sequence the first of these to be encountered by the enzyme may be hydrolysed only very slowly, accounting for the rest point at incomplete conversion of the polysaccharide. A small proportion of such structural features could block the further hydrolysis of large sections of a branched macromolecule. The solution to the problem will require the structural examination of the amyloglucosidase-limit dextrins.

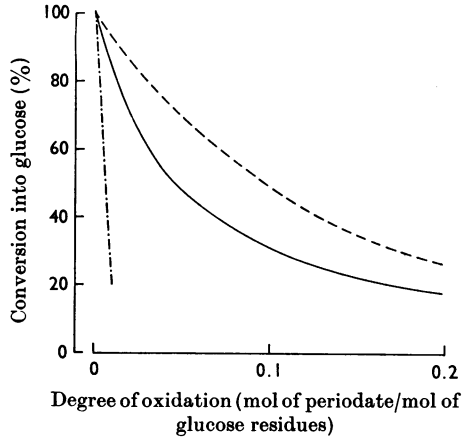


Fig. 7. Effect of limited periodate oxidation on the degree of hydrolysis of glycogen (----), amylopectin (——) and amylose (-·-·-) by *Rhizopus niveus* amyloglucosidase (Smith *et al.* 1970; J. J. Marshall, unpublished work). The polysaccharides were treated with $NaIO_4$ to achieve the degree of oxidation shown, and then hydrolysed with the enzyme.

It may be noted that the potential of the random oxidation procedure for determination of $\overline{CL}$ (Fig. 7), enzyme action pattern and enzyme purity is general to any class of polymer. All that is needed is a means of spattering the polymer with groups that will block exo-enzyme action.

Catabolism of starch and glycogen

I remarked earlier that the enzymic method of structural exploration has the added profitability

Table 1. *Incomplete hydrolysis of starch fractions and glycogens by* Rhizopus niveus *amyloglucosidase*

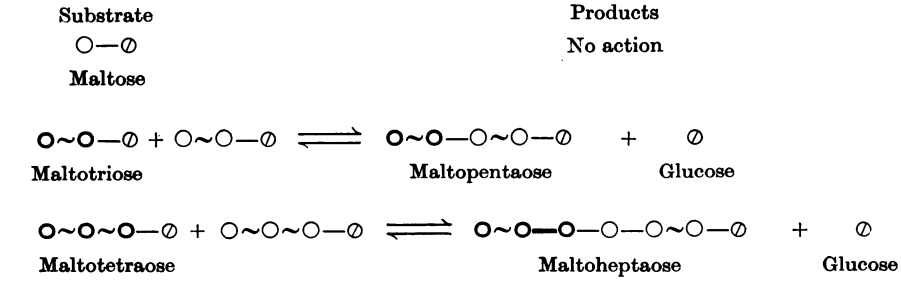
The data are taken from Marshall & Whelan (1970), where additional results will be found.

Substrate	Glucose released by enzymic hydrolysis (expressed as % of glucose released by acid)		
	Rhizopus niveus amyloglucosidase	*Rhizopus niveus* amyloglucosidase + α-amylase	*Aspergillus niger* amyloglucosidase
Potato amylose	90.1	101.0	97.0
Waxy-maize starch	97.6	103.0	100.0
Floridean starch	77.8	97.5	90.8
Cat liver glycogen	82.2	98.8	93.1
Human muscle glycogen	87.8	99.4	96.3
Skate liver glycogen	89.3	99.3	95.3

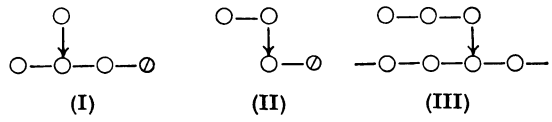
of giving an insight into the *modus operandi* of the enzymes *in vivo*. Thus the discovery of R-enzyme (Hobson *et al.* 1951) and D-enzyme (Peat, Whelan & Rees, 1953*b*) allowed me to postulate how starch is catabolized in the potato (Whelan, 1958). Phosphorylase was the likely main agent of catabolism, on account of its abundance relative to other enzymes, such as α-amylase, that hydrolyse the α-(1→4)-glucosidic bonds. Phosphorylase would, however, require assistance in completely catabolizing amylopectin, since it is an exo-enzyme, unable to split the (1→6)-branch bonds. R-Enzyme was clearly the agent for the latter role. But something was still missing. Phosphorylase is unable to shorten maltodextrin chains to fewer than four glucose units (Whelan & Bailey, 1954), so that the combined actions of phosphorylase and R-enzyme on starch would yield α-glucose 1-phosphate plus a residue of maltotetraose from each chain. Since amylopectin has $\overline{CL}$ 24, one-sixth of the molecule would be tied up in this way. How could the maltotetraose be metabolized? Hydrolysis was unlikely because starch hydrolases are present in potato only in trace amounts. The realization then came that a role had been found for the aforementioned transglycosylase, D-enzyme, discovered by W. R. Rees in the Bangor laboratories (Peat *et al.* 1953*b*, 1956*a*). (The 'D' represented disproportionation.) Thus maltotriose was reversibly converted into maltopentaose and glucose by transfer of an α-maltosyl residue from one molecule of maltotriose to another, with the resynthesis of a (1→4)-bond (Scheme 4). We had been puzzled why D-enzyme has the ability to cleave some (1→4)-bonds and not others, according to their position in the maltodextrin chain. It was established that the portion transferred was that containing the non-reducing end group (Walker & Whelan, 1957), and that the enzyme could not split the (1→4)-linkage at the non-reducing end or that penultimate to the reducing end (Scheme 4; Peat *et al.* 1956*a*; Jones & Whelan, 1969). The significance of this limitation on the linkages that can be broken becomes evident when one considers what it means for maltotetraose. Only one of the three (1→4)-bonds can be split and only one reaction is possible, the formation of maltoheptaose and glucose (Scheme 4). The result is that three glucose residues (in the maltoheptaose) now become available to phosphorylase. Therefore the coupled system, phosphorylase and D-enzyme, will convert maltotetraose into 3 mol.prop. of α-glucose 1-phosphate and 1 mol.prop. of glucose, and, with the addition of R-enzyme, amylopectin of $\overline{CL}$ n would yield $(n-1)$ mol.prop. of α-glucose 1-phosphate and 1 mol.prop. of glucose. The subsequent fate of these two products is obvious.

In the case of glycogen catabolism, Cori & Larner (1951) had described the formation of the same two products and in the same ratio. Indeed, a measurement of this ratio was proposed as a means of measuring $\overline{CL}$ (Illingworth, Larner & Cori, 1952). There were two differences from the plant system, namely that the debranching enzyme was of different specificity and, apparently, only phosphorylase and the debranching enzyme were required for complete catabolism. There was no involvement of a transglycosylase. The debranching enzyme, amylo-1,6-glucosidase, from muscle or liver, was, like R-enzyme, specific for the hydrolysis of α-(1→6)-glucosidic linkages, but with the difference that, where R-enzyme can only hydrolyse non-terminal branch linkages, amylo-1,6-glucosidase hydrolyses only terminal branch points (Fig. 8).

Cori & Larner (1951) had proposed that phosphorylase attenuates an *A* chain (Fig. 1) to a single (1→6)-linked glucose residue, which is then hydrolysed by amylo-1,6-glucosidase. By analogy with potato phosphorylase, we would have expected the *A* chain to be shortened only to four residues. We decided to look at the structure of the muscle



Scheme 4. Behaviour of potato D-enzyme towards maltose, maltotriose and maltotetraose. Symbols are as in Scheme 2, with ~ indicating a non-transferable (1→4)-bond and **bold** circles those glucose units undergoing transfer.



(I) (II) (III)

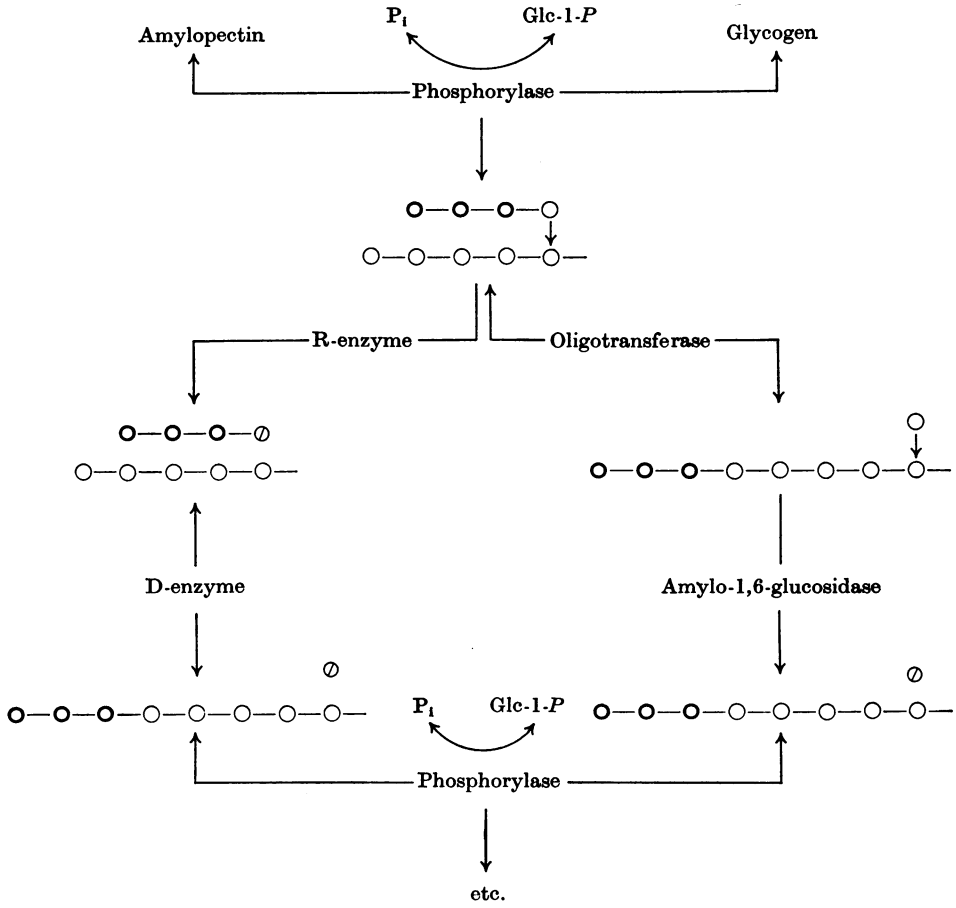
Fig. 8. Differences in specificity between the three types of debranching enzyme. Symbols are as in Scheme 2. Animal and yeast amylo-1,6-glucosidase will act only on structure (I). The pentasaccharide shown is the smallest substrate for the muscle enzyme (Brown *et al.* 1965). R-Enzyme (pullulanase) will act on structures (II) and (III), but not (I). The tetrasaccharide (II) is the smallest substrate for *Aerobacter aerogenes* pullulanase (Abdullah *et al.* 1966). *Cytophaga* isoamylase will not remove a maltosyl side chain from ϕ,β-limit dextrin, but will hydrolyse a maltotriosyl side chain (Gunja-Smith *et al.* 1970*a*,*b*; Yokobayashi *et al.* 1969, 1970). The smallest substrate is not known, nor have substrates with single-unit side chains (I) been tested.

phosphorylase(ϕ)-limit dextrin of amylopectin and found that the *A* chains were indeed four units long (Walker & Whelan, 1960). At that time we could not examine glycogen ϕ-limit dextrin, not having a glycogen-debranching enzyme, but when pullulanase became available we were able to demonstrate the presence of the same four-unit *A* chains in the glycogen dextrin (Abdullah *et al.* 1964). It was clear that the amylo-1,6-glucosidase preparations were likely to contain a D-enzyme-like transferase, and this prediction (Walker & Whelan, 1960) was confirmed (Brown & Illingworth, 1962; Abdullah & Whelan, 1963; Brown, Illingworth & Cori, 1963). The similarity to D-enzyme of the 'oligo-1,4 → 1,4-glucantransferase' was heightened by the finding that it preferred to transfer glucose units in groups of three (Brown & Illingworth, 1962), just as D-enzyme does when acting on maltotetraose (Scheme 4). This means that in a single step the transferase converts the ϕ-limit dextrin into the substrate for amylo-1,6-glucosidase (Scheme 5).

There is therefore a very close parallel between the catabolic routes for glycogen and amylopectin (Scheme 5), and one may speculate whether they share a common ancestry along the evolutionary pathway (Lee, Smith & Whelan, 1970). The only major difference between the two processes resides in the specificities of the debranching enzymes (Fig. 8). Attention is drawn to the fact that amylo-1,6-glucosidase, unlike R-enzyme, cannot act on the native polysaccharide: it requires the co-operation of the transferase. Applying hindsight, it could be said to be logical when Brown & Illingworth (1964) and Brown & Brown (1966*a*) observed that muscle amylo-1,6-glucosidase and transferase cannot be separated. The same has now been discovered for the similar system in yeast (Lee, Carter, Nielsen & Fischer, 1970). This seems to be a simple example of a multienzyme system (Taylor & Whelan, 1968).

A recent finding in our laboratory, and concerning this system, indicates that the reversal of a hydrolytic process, usually considered physiologically insignificant, may well be significant. Our observations stemmed from the paradox presented by type IV liver glycogen-storage disease, characterized by the presence in the liver of a 'glycogen' closely resembling amylopectin in $\overline{CL}$, iodine stain and degree of β-amylolysis. Yet the branching enzyme seems to be totally absent. Should the patient then not be synthesizing a linear amylose? We knew that the patients have a normal complement of the debranching enzyme system and, further, that the incorporation of [^{14}C]glucose into glycogen by amylo-1,6-glucosidase, though slight, since it is a reversal of the hydrolytic function, is a good assay for this enzyme (Hers, Verhue & Van Hoof, 1967). Is it possible that a reversal of the sequence of actions of amylo-1,6-glucosidase and transferase could occur (Scheme 5) so that new, synthetase-extendable, chains could be formed? We tested this possibility, by using phosphorylase as the chain extender, with α-glucose 1-phosphate as substrate, and used the branching-enzyme assay method of Brown & Brown (1966*b*) in which no primer is added, but branching enzyme, acting on the traces of primer always present, creates phosphorylase-extendable chains with ever increasing rapidity as the weight of polysaccharide builds up. We found that if, instead of branching enzyme, one adds the amylo-1,6-glucosidase–transferase complex and glucose, the system responds as if branching enzyme had been added (Fig. 9; Huijing, Lee, Carter & Whelan, 1970). Therefore one can account for the formation of a branched glucan by the type IV patient, the lower proportion of branch points reflecting the relative inefficiency of the 'branching' system. Considering, however, that the proportion of branch points is 50% of that in normal glycogen, the branching system is not so inefficient and the synthetic potential of a hydrolase is emphasized. We may note in passing that the resemblance of type IV glycogen to amylopectin proved to be more apparent than real when the glycogen was 'finger-printed' by debranching and fractionation on a molecular sieve (Mercier & Whelan, 1970). Just as the synthetic amylopectins had failed to yield a natural amylopectin 'fingerprint' (Fig. 5; see above), so also did the type IV glycogen.

The possibility that the hydrolytic carbohydrases play a biosynthetic role in normal metabolism deserves serious consideration, for example, in the context of the origin of primer for glycogen and starch synthesis. Synthetase and phosphorylase both require primer, even though this may, for



Scheme 5. Comparative enzymology of the debranching of amylopectin in plants and of glycogen in animals. Symbols are as in Scheme 2. In the plant system debranching (R-enzyme) precedes transglycosylase (D-enzyme) action. In the animal system the corresponding enzymes act in the reverse sequence. Each unit chain of the macromolecule, having $\overline{CL}$ n, is eventually converted into $(n-1)$ molecules of α-glucose 1-phosphate (Glc-1-P) and 1 molecule of glucose. The three **bold** glucose units are those repositioned by the transglycosylases.

glycogen synthetase, be a molecule as simple as maltose (Goldemberg, 1962; Leloir, 1964). But no mammalian or plant system is known that can synthesize maltose. It seems to have been a fixed idea that such synthesis would have to be via a glucose phosphate (e.g. Illingworth, Brown & Cori, 1962*a*,*b*). At the same time we have puzzled at the role of the small amounts of α-amylase and α-glucosidase ubiquitous in plant and animal tissues (Abdullah *et al.* 1964; Smith *et al.* 1968). To be sure, one of the mammalian α-glucosidases, that in the lysosome, has an assigned function, that of degrading glycogen (Hers & Van Hoof, 1968). But what of the non-lysosomal enzymes? A report by Hehre, Okado & Genghof (1969) prompts me to suggest an explanation of primer synthesis that provides a role for the hydrolases. Hehre *et al.* (1969) reported that α-amylase and β-amylase will dimerize the same anomeric forms of maltose that they liberate from starch, i.e. α-maltose and β-maltose respectively. They also dispelled the previous illusion that these reversion reactions are slow; they are just as rapid as is hydrolysis. Now α-glucosidase yields the whole spectrum of α-glucose disaccharides by reversion (Peat, Whelan & Hinson, 1955; Lukomskaya, 1962). The maltose so synthesized can then yield maltotetraose with α-amylase (Hehre *et al.* 1969). Once primer is made and elongated by synthetase, it can be fragmented by α-amylase into additional primer molecules. An alternative in multiplying primer, once formed, is by glycosyl transfer to glucose with a transferase, like D-enzyme (Whelan, 1958). So perhaps we have been handling the primer-synthesizing system without recognizing

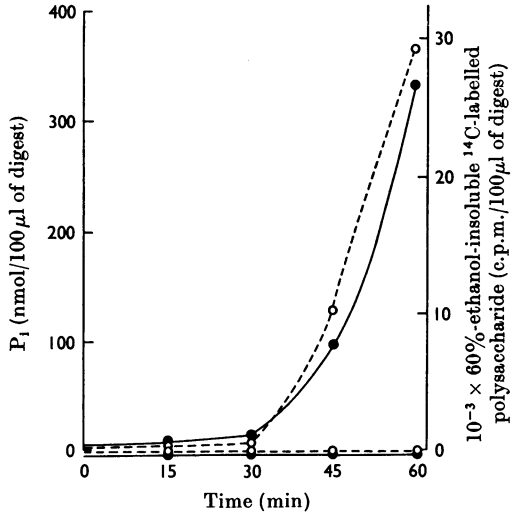


Fig. 9. Evidence that the rabbit muscle glycogen-debranching enzyme system can behave as branching enzyme in the assay system for the latter described by Brown & Brown (1966*b*). The ascending curves represent the complete system of rabbit muscle phosphorylase, amylo-1,6-glucosidase–transferase, α-glucose 1-phosphate and [^{14}C]glucose, and show release of P_i (----) and formation of 60%-ethanol-insoluble ^{14}C-labelled polysaccharide (——). When glucose or the debranching enzyme system was omitted no reaction occurred (horizontal curves). [Reproduced from Huijing *et al.* (1970) by permission from *FEBS Letters*.]

it for what it is. Yet another consideration in connexion with reversion concerns the above-mentioned ability of the α-glucosidases to synthesize a mixture of glucose disaccharides rather than a single product. This means that in any system where glucose and α-glucosidase came into contact, serum, liver, muscle, kidney etc., we must expect to encounter sugars such as αα-trehalose, kojibiose and nigerose. Their detection need not spark off speculation that some new metabolic pathway, and an importance of the sugar, have been discovered. It is in this context that I view reports such as that incubation of glucose with kidney-cortex slices gave rise to 0.4% αα-trehalose (Sacktor & Berger, 1969).

In conclusion

I used to have the feeling that glycogen and starch were played out as objects for further research, and indeed for several years after our first forays most of my energies were directed towards other aspects of carbohydrate chemistry and biochemistry. This was partly because deceptively satisfying cycles of metabolism had been worked out, partly because there were far more virgin territories to explore and partly because existing methodology had run its course. The picture began to change when Leloir & Cardini (1957) upset the metabolic cycles with the discovery of glycogen synthetase, and the new methodology of fractionating macromolecules, both enzymes and substrates, came along. I do not see another rest period developing. Rather is the knowledge of glycogen and starch metabolism expanding exponentially, and one is so impressed by the many 'firsts' scored in this field that the future, judged in terms of the past, may well offer major generalizations arising out of this area of metabolism. One may think of such facts as starch being the first polymer for which a helical structure was proposed, glycogen the first polymer to be synthesized *in vitro*, the first such synthesis for which primer-dependence was observed, for which phosphorylation and dephosphorylation of enzymes, as regulatory processes, was described, the first polysaccharide to be synthesized from a nucleoside diphosphate sugar, the first system in which the biological role of cyclic AMP (adenosine 3′:5′-cyclic monophosphate) was discovered, or in which protein kinase was found to be the onward messenger of cyclic AMP. This has been, and is, a most rewarding and exciting field.

As for the future, my own predilections are towards studying the same aspects covered in this Lecture, the scrutiny of the individual enzymes of metabolism and regulation and the integration of their activities. Almost all the liver enzymes remain unpurified, even unidentified; the situation in muscle is hardly better. Yet here is a polymer, glycogen, that constitutes 20% of the dry weight of liver and 3% of the whole man. To understand the actions of some of these enzymes will require model substrates more sophisticated than anything yet to hand. We need polysaccharides of precisely defined structure as well as the oligosaccharides already available. A beginning in this direction has been made in collaboration with Professor R. U. Lemieux. A 2,3-disubstituted amylose ester (Bines & Whelan, 1960) is condensed with 3,4,6-tri-*O*-acetyl-1-chloro-2-nitroso-α-D-glucopyranose, when an α-glycosidic linkage is formed. The resulting 2-oximo derivative is then converted into the 2-oxo compound, which is reduced stereospecifically with sodium borohydride to the *gluco* configuration. By the use of ^{3}H-labelled borohydride in this step, the 6-bonded α-glucosyl residue can be tagged and its subsequent fate when the amylose is used as an enzyme substrate can be followed. What has been synthesized then is a Staudinger-type molecule (Fig. 1), and the performance of the same reaction with maltosyl or larger maltodextrins will yield even more useful model substrates, corresponding to β-amylase

(maltosyl, maltotriosyl)- or phosphorylase (maltotetraosyl)-limit dextrins. The last-named compound should be capable of side-chain elongation by phosphorylase or synthetase. This range of polymers, when used as substrates, may be expected to shed further light on enzyme action patterns, and their physical and chemical properties may assist in elucidating the many unsolved problems of glycogen and starch structure.

I received the CIBA Medal with the greatest possible pleasure and a sense of flattery in being elevated to the company of the distinguished predecessors who have earned this award. In accepting it I remembered my debt to my teachers, especially Stanley Peat. The day that began with my learning of the award was greatly saddened by the news that Peat had died that day. I remembered also Walter Morgan, who had invited me to the Lister Institute, and the very many pupils of my own, whose work the Society was also acknowledging, the financial benevolence of the Agricultural Research Council, the Medical Research Council, the Science Research Council and the U.S. Department of Agriculture, and my wife, for her tolerance and encouragement of a biochemist's obsessions. I was especially pleased to receive the award at the 500th Meeting of the Society, and to recall another anniversary. Friedrich Miescher, whose portrait forms the obverse of the CIBA Medal, discovered DNA in 1869, in Hoppe-Seyler's laboratory in Tübingen. It seemed appropriate for a carbohydrate biochemist to be chosen for the award in the centenary year of this discovery, since I have always regarded DNA as a substituted polysaccharide, another example of a 'winding bout of linked sweetness'. And it was appropriate also to recall the advice given to Miescher by his uncle Wilhelm His, a most enlightened Professor of Anatomy. This was to study not anatomy, but rather the chemical composition of tissues, 'because the ultimate problems of tissue development would be solved on the basis of chemistry' (Mirsky, 1968).

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