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THE ORIGIN OF THE PERIODATE-RESISTANT D-GLUCOSE RESIDUES IN STARCHES AND GLYCOGENS

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ABSTRACT

Under ordinary analytical conditions, the proportion of periodate-resistant D-glucose residues in starches and glycogens was consistently about one-third of the proportion of branching points. The resistant D-glucose residues became freely oxidisable after the limit-oxidised glucans had been reduced with sodium borohydride. The results can be explained by assuming that, when a D-glucose residue carrying a branch at position 6 is oxidised, the resulting two aldehyde groups both form 6-membered hemiacetal rings with the closest hydroxyl groups on neighbouring, unoxidised residues in the same, (1→4)-linked chain, whereas when the other D-glucose residues are oxidised, only one of the aldehyde groups shows a strong tendency to form a hemiacetal of this kind. It is suggested that, in the unbranched units, the other aldehyde group preferentially forms a hemiacetal with the primary hydroxyl group in the same unit.

INTRODUCTION

Formation of intramolecular hemiacetals between the aldehyde groups of oxidised sugar residues and hydroxyl groups on adjacent, unoxidised sugar residues occurs generally in the periodate oxidation of polysaccharidic chains[1]. The position of the equilibrium that is set up between these inter-residue hemiacetals and other states of combination of the aldehyde groups depends very much upon whether or not the oxidised sugar residues still contain other hydroxyl groups[1–5].

When they do not, as in glycuronans[1,2], for example, both the aldehyde groups of oxidised sugar residues show a strong tendency to form inter-residue hemiacetals, and the subsequent oxidation of adjacent, unoxidised units in the chains is strongly inhibited[1–3]. When they do, as in amylose[4,5], for example, only one of the aldehyde groups shows a strong tendency to form an inter-residue hemiacetal, while the other appears preferentially to form an intra-residue hemiacetal[6] with the primary hydroxyl group in the same unit[4,5].

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So far as the kinetics of periodate oxidation are concerned, the two classes of polysaccharide fall into fairly distinct groups. This is because, in the former, an unoxidised unit can be inhibited from oxidation by two oxidised neighbours, whereas in the latter, the inhibition is largely unidirectional along the chain[1–5]. Therefore, even supposing that the position of the equilibrium is the same in both cases, the inhibitory effect of two such equilibria is compounded in the former kind of polysaccharide. For example, if the equilibrium lies 90% on the side of the inter-residue hemiacetal, the rate of the slow, terminal phase of the oxidation[1] will be 1% of the initial rate in the first kind of polysaccharide, but 10% in the second kind. The starches and glycogens represent a group of polysaccharides in which the two situations can exist together in the same molecule, and the present paper examines the consequences of this.

The presence of a small proportion of periodate-resistant D-glucose residues in starches and glycogens has been recognised for many years[7–11]. Initially, this was interpreted as evidence for the presence of "anomalous" linkages in the chains [(1→3)-linkages in linear parts of the molecule, or branching through secondary hydroxyl groups[7–10]], but Manners and Mercer[11] subsequently found that, under forcing conditions (400mM periodate at 20° for several weeks), the "resistant" D-glucose residues could be oxidised. They therefore suggested formation of inter-residue hemiacetals as a more likely explanation, but envisaged the hemiacetals as macrocyclic structures, formed between different chains of the ramified glucan molecules[11]. In the light of later work[1], it now seems unlikely that stable structures of this kind would be formed, in competition with 6-membered hemiacetal and hemialdal structures.

In the work now described, an attempt has been made to measure accurately the proportion of D-glucose residues in amylose, starch, amylopectin, and glycogen that can meaningfully be described as "periodate resistant", when these materials are oxidised under typical, analytical conditions. The results of earlier estimates[7–11] were found to be too low, because correction had not been made for the destruction of D-glucose that occurs during acid hydrolysis of the reduced polyaldehydes. Such destruction is indeed slight when pure, unoxidised glucans are hydrolysed under the same conditions, but in the presence of the large excess of glycolaldehyde that is liberated from the reduced polyaldehydes by hydrolysis, it is very severe.

THEORY

The decision to describe any D-glucose residue as "periodate resistant" is clearly subjective, and the concept must be properly understood in kinetic terms. The problem here is to calculate the proportion of D-glucose residues that would be expected to undergo oxidation very much more slowly than any of the others. This is the fraction that, as a result of the stochastic nature of the attack on the chains, would ultimately exist with both adjacent residues in the oxidised state, and receive a high degree of inhibition from both of them.

All the D-glucose residues of interest would therefore have an oxidised,

branched D-glucose residue in one neighbouring position, and an oxidised, unbranched residue in the other. Moreover, since the inhibition provided by oxidised, unbranched residues is essentially unidirectional[4], only one of the two neighbours of a branched D-glucose residue would be potentially capable of becoming periodate resistant.

Given, therefore, that the proportion of potentially resistant D-glucose residues is the same as the proportion of branching points, the problem is to calculate what fraction of them actually become resistant. It is only possible here to indicate an approximate solution. The complexity of an exact analysis will be apparent from an earlier, Monto-Carlo treatment of the simpler case of amylose[5].

The simplifying assumption is that singly inhibited units are oxidised so much more slowly than uninhibited units that they can be regarded as being "resistant" to oxidation until all the uninhibited units have been oxidised. The whole oxidation is thus regarded as taking place in two discrete steps; an initial, fast one, generating chains in which every unoxidised unit is inhibited by at least one oxidised neighbour; and a second, slow step in which all singly inhibited units are oxidised, leaving, finally, only the doubly inhibited units that are of interest.

On the assumption of random attack, it is first recognised that, in half of the cases, the potentially resistant D-glucose residue will be oxidised before the branched D-glucose residue to which it is adjacent, and it will therefore be lost. In the other 50%

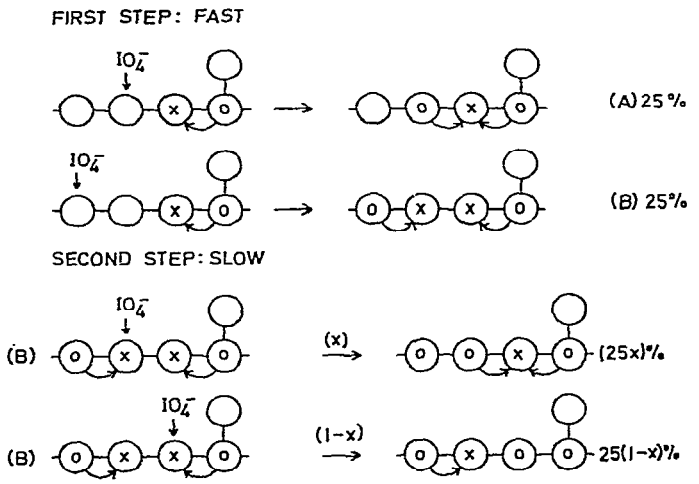

Fig. 1. Proposed mechanism for the formation of periodate-resistant D-glucose residues in starch and glycogen. Oxidised D-glucose residues are marked with an "O", the inter-residue hemiacetals formed by their aldehyde groups are shown as curved arrows, and unoxidised residues that are inhibited from oxidation by the hemiacetals are marked with an "X". The branched D-glucose residue on the right has been oxidised, and is able to inhibit the oxidation of the residue on its left. Unbranched residues, when oxidised, are only able to inhibit the oxidation of residues lying to their right. Residues for which oxidation is uninhibited by hemiacetal formation are oxidised in the initial, "fast" step, whereas residues for which oxidation is inhibited by a single hemiacetal are oxidised in the ensuing, "slow" step. Residues for which oxidation is inhibited by two hemiacetals are described as "periodate resistant".

of cases, two different, possible sequences of events can be recognised, as shown in Fig. 1.

In Fig. 1, it is assumed that unbranched units, when oxidised, inhibit the oxidation of units lying to their right. The potentially resistant D-glucose residue is shown, singly inhibited by the oxidised, branched residue on its right. On its left are the two preceding, unbranched D-glucose residues in the chain, and subsequent events depend upon which of them is attacked first.

When the unbranched unit that is adjacent to the potentially resistant unit is oxidised first (that is, in half of these cases, or one quarter of all cases), a "fully resistant" (that is, doubly inhibited) unit (A) is generated. When the next-nearest neighbour of the potentially resistant unit is oxidised first (that is, in half of these cases, or again, one quarter of all), sequences of type B are generated.

The subsequent fate of sequences of type B is determined by the relative reactivities of the two singly inhibited units in the second, slow step of the oxidation (Fig. 1). When the nearest neighbour of the potentially resistant unit is oxidised first, a "fully resistant" unit is generated, but when the potentially resistant unit itself is oxidised first, it is lost. The fraction (X) of sequences of type B that produces "fully resistant" D-glucose residues is not known. In the absence of this information, it is only possible to conclude that the proportion of periodate-resistant D-glucose residues will be at least 25%, and not more than 50%, of the proportion of branching points in the glucan.

It is now necessary to consider how this estimate should be affected, if the simplifying assumption is relaxed, and the possibility is admitted that singly inhibited units are susceptible to oxidation from the moment of their formation. This can be done by considering another extreme case, namely, that in which the singly inhibited units are oxidised at virtually the same rate as the uninhibited units, and only doubly inhibited units are regarded as "resistant".

In this model, it is necessary to consider only the triplet of D-glucose residues consisting of the potentially resistant residue and its two neighbours. This triplet can be attacked in six different, possible sequences, and in only two of these will the potentially resistant unit become "fully resistant", by virtue of being attacked last. Thus, the proportion of periodate-resistant D-glucose residues in this case will be one-third of the proportion of branching points.

These two special cases are sufficient to indicate that, even when the position of the equilibrium lies very far on the side of the inter-residue hemiacetals, the fraction of D-glucose residues that are oxidised most slowly will always have some value between 25 and 50% of the fraction of branching points, and that any decrease in the degree of inhibition will cause this value to tend towards 33%.

EXPERIMENTAL

Materials. — Amylose was supplied by Avebe A/G, Veendam, Holland. The same company provided a sample of a non-linear fraction, described as "amylo-

pectin G". This material, however, was much less highly branched than ordinary amylopectin, and it appeared to correspond fairly closely to the fraction of starch described by Whistler[12] as "intermediate fraction". A fraction described as "soluble starch", which appeared to consist largely of ordinary amylopectin, originated from Riedel de Haën, A/G, Hanover, Germany. Oyster glycogen was supplied by Koch–Light Laboratories, Ltd., Colnbrook, England. D-Glucose oxidase and peroxidase were supplied by Sigma Chemical Company, St. Louis, U.S.A. All other reagents were of Merck analytical grade.

Analytical methods. — The polysaccharides were not dried before use; their moisture contents were determined by drying to constant weight at 110°, and the weights of undried samples were corrected accordingly. Periodate-oxidised polysaccharides were isolated by freeze-drying, after which their moisture contents were negligible. The ash contents of the glucans were all below 1%. The ash content of each periodate-oxidised glucan was also measured, to check for complete removal of salts by dialysis, and was regarded as acceptable if less than 1%.

In samples of glucan that had been oxidised with periodate, reduced with borohydride, and oxidised again, erythrose was measured by the method of Dische[13]. In these experiments, a sample of amylose that had been fully oxidised with periodate, and then reduced with borohydride, was used to correct the results for the absorbance due to glycolaldehyde[13].

Iodine "blue values" were determined as described by McCready and Hassid[14].

Assay of unoxidised D-*glucose residues.* — Periodate-oxidised glucans were first reduced with borohydride[2,4] and then hydrolysed as follows. A sample (100 mg) of reduced polyaldehyde was dissolved in 0.5M hydrochloric acid (3 ml) at room temperature. A portion (2.5 ml) of the solution was then transferred to a glass ampoule of 5-ml capacity, which had previously been cleaned with chromic acid and dried. The sealed ampoule was heated for 5 h in a boiling water-bath, and then cooled and opened, and 0.2M sodium acetate (3 ml), followed by 0.5M sodium hydroxide (2.5 ml), were added to bring the pH close to 7.

A "blank" solution was prepared by heating a mixture of erythritol (60 mg) and glycolaldehyde (40 mg) with acid in the same way, and a D-glucose standard was prepared by similarly treating a mixture of erythritol (60 mg), glycolaldehyde (40 mg), and D-glucose (5.5 mg). Portions (0.06 ml), measured accurately with an Agla micrometer syringe, were then taken in triplicate from each solution, for the assay of D-glucose by the D-glucose-oxidase procedure of Richterich[15]. Comparison of the D-glucose control with an external D-glucose standard revealed that ~75% of the D-glucose was destroyed upon being heated in acid under the stated conditions.

Analytical oxidations. — Soluble starch or glycogen were oxidised at 20° in the dark, as 0.2% w/v solutions in 25mM sodium metaperiodate containing 10% (v/v) of propan-1-ol, which inhibited depolymerisation by acting as a radical scavenger[2]. For the assay of periodate uptake, samples (5 ml) of reaction mixture were pipetted into an ice-cold mixture of 0.5M sodium phosphate buffer (pH 7.0; 20 ml) and 30% (w/v) aqueous potassium iodide (2 ml), and the liberated iodine was titrated rapidly with

0.01M sodium thiosulphate, with starch as the indicator. Formic acid liberated during the oxidations was measured by pipetting samples (20 ml) of reaction mixture into ethane-1,2-diol (1.5 ml), waiting 10 min, and then titrating the mixture with 5mM sodium hydroxide, using Methyl Red as the indicator.

Amylose and amylopectin G were oxidised in the same way, but were first dissolved in a minimum of cold, M sodium hydroxide under nitrogen. The solutions were diluted with water, almost up to the final volume required, and then neutralised with an exactly equivalent amount of hydrochloric acid, immediately before addition of the oxidant.

For the re-oxidation of oxidised glucans after they had been reduced with borohydride, 0.2% (w/v) solutions in 5mM sodium metaperiodate, at 20° in the dark, were employed; 10-ml samples were removed for assay of periodate.

Preparative oxidations. — These were simply large-scale versions of the analytical oxidations. At chosen times, oxidation was stopped by addition of about a 10-fold excess of ethane-1,2-diol, and the solutions were concentrated on a rotary evaporator at 30° to ~10% of their original volume. They were then dialysed against distilled water, and concentrated again to remove water imbibed during dialysis. Sodium borohydride was then added to give a concentration of 2% (w/v), and after 12 h at room temperature, the solutions were brought to pH 6 with acetic acid, dialysed exhaustively against water, concentrated again, centrifuged for 1 h at 40,000*g*, and dried in the frozen state.

RESULTS

Confirmation of the existence of "periodate-resistant" D-glucose residues. — Glycogen was chosen for this experiment, and Fig. 2 shows that, under ordinary analytical conditions, the fraction of unoxidised D-glucose residues became virtually constant at ~2.5% after oxidation for 9 days. The consumption of periodate is also shown in Fig. 2, and it is seen that significant over-oxidation was occurring under the conditions of the experiment.

Characterisation of the glucans. — Attempts to measure the degree of branching by assay of the formic acid liberated from the non-reducing end-groups were complicated by the over-oxidation just noted, and the consequent liberation of excessive amounts of formic acid. On the assumption that the Malapradian oxidation and the over-oxidation were simultaneous reactions, the amount of formic acid arising from the former was estimated by extrapolation of the curve for the latter to zero time. The results for the four glucans are shown in Fig. 3. The degrees of branching measured in this way, and also the iodine "blue values", are recorded in Table I.

Correlation of the degree of branching of the glucans with their content of "periodate-resistant" D-glucose residues. — The samples of amylose, amylopectin G, and soluble starch were preparatively oxidised for 9 days, and the content of residual D-glucose was then measured. The results, together with that obtained earlier for glycogen, are included in Table I. A clear correlation between the fraction of residual

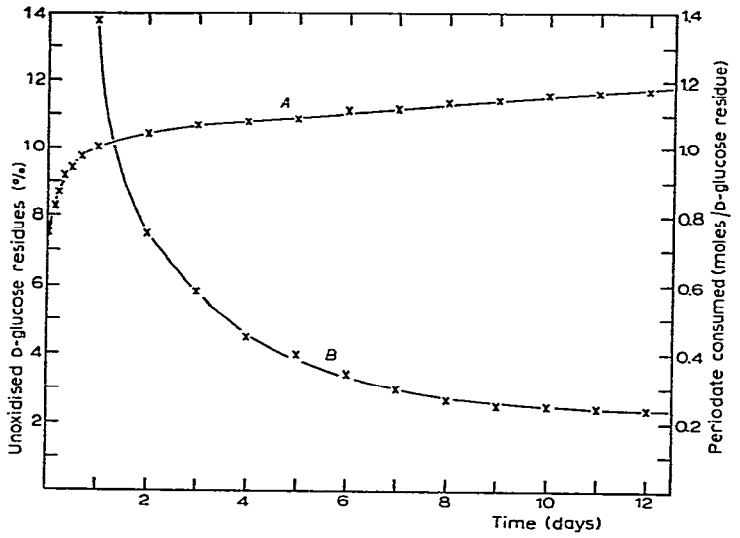

Fig. 2. Oxidation of glycogen in 25mM sodium metaperiodate at 20°. Curve *A*, moles of periodate consumed per D-glucose residue; *B*, percentage of unoxidised D-glucose residues.

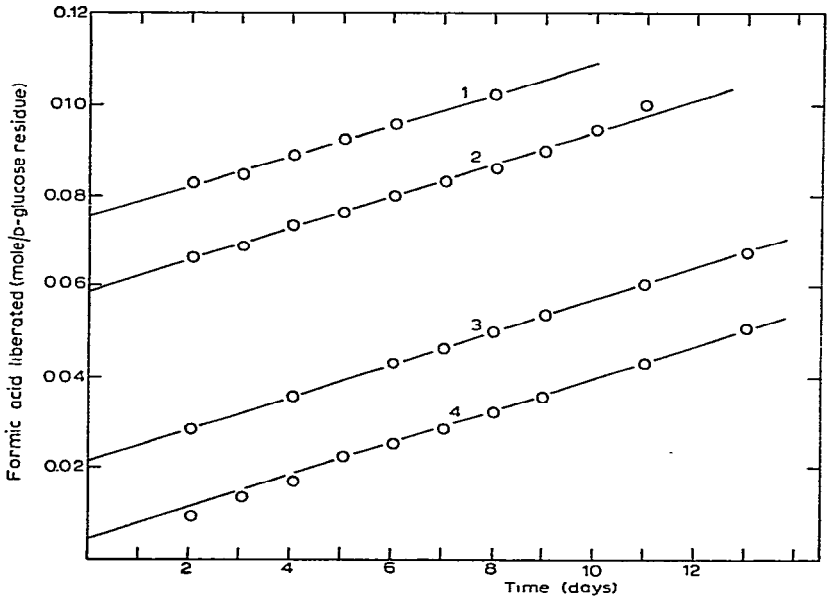

Fig. 3. Liberation of formic acid from various glucans during oxidation in 25mM sodium metaperiodate at 20°. Curve 1, glycogen; 2, soluble starch; 3, amylopectin G; 4, amylose.

D-glucose residues and the degree of branching of the glucan is indicated, the ratio of these two quantities being ~1:3.

TABLE I

IODINE BLUE-VALUES (BV), DEGREES OF BRANCHING (DB), AND PROPORTIONS OF PERIODATE-RESISTANT D-GLUCASE RESIDUES (PRGR) IN VARIOUS GLUCANS. THE DEGREE OF BRANCHING IS REGARDED AS THE FRACTION OF D-GLUCOSE RESIDUES EXISTING AS END GROUPS.

Glucan	*BV*	*DB*	*PRGR*	*DB/PRGR*
Amylose	1.28	0.005	0.0021	2.4
Amylopectin G	0.248	0.021	0.0072	2.9
Soluble starch	0.136	0.059	0.019	3.1
Glycogen	0.024	0.076	0.025	3.1

Effect of borohydride reduction upon the resistance of the residual D-*glucose residues to periodate.* — Accurate measurement of the amount of additional periodate consumed by the polyaldehydes after they had been reduced with borohydride was difficult, because of their small content of residual D-glucose. A definitive result was, however, obtained with a sample of glycogen that had been oxidised for 7 days. As indicated by the data in Fig. 2, this material had consumed 1.12 moles of periodate per D-glucose residue, and still contained 3.0% of residual D-glucose, a little more than the limiting content of 2.5%.

Fig. 4 shows the consumption of periodate by this material, both before and after reduction with borohydride. The reduced material consumed a total of 0.033 mol. of periodate, and showed no over-oxidation. The unreduced material showed over-oxidation as before, but extrapolation of the curve to zero time indicated that

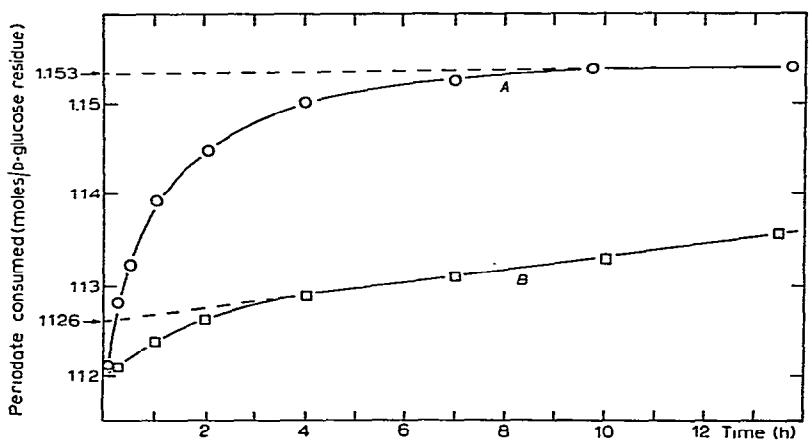

Fig. 4. Oxidation (in 5mM sodium metaperiodate at 20°) of a sample of glycogen that had been oxidised for 7 days under the conditions of Fig. 2. Curve *A*, after reduction with borohydride; *B*, before reduction with borohydride.

0.006 mol. of periodate had been consumed in the Malapradian manner*. It thus appeared that an additional 2.7% of D-glucose residues had been rendered susceptible to periodate as a result of reduction with borohydride.

With the sample of soluble starch, it was concluded that the additional periodate consumed after reduction with borohydride corresponded very roughly to the oxidation of the residual D-glucose residues, but with the amylopectin G, it was not possible to show that there was any additional consumption of periodate within the limits of experimental error.

All three branched fractions gave a definitive result when the reduced polyaldehydes, isolated after oxidation for 7 days, were re-oxidised for 4 h under the conditions of Fig. 4, isolated again, and analysed for D-glucose by the D-glucose-oxidase procedure. In every case, negligible quantities of D-glucose remained. Confirmatory evidence for this result was obtained by analysing the re-oxidised products for erythrose. Although the accuracy of this method was not high, because of the need to apply a fairly large correction for the reaction given by glycolaldehyde[13], the results clearly showed that the residual D-glucose residues had been replaced by an approximately equimolar amount of erythrose.

DISCUSSION

Fractions of starch and glycogen contain a well-defined proportion of D-glucose residues that are oxidised very slowly by periodate. Under ordinary, analytical conditions, their rate of oxidation is too low to be measurable on a practical time-scale. It is therefore meaningful to describe these units as "periodate resistant".

It appears from the work of Manners and Mercer[11] that the resistant units can be oxidised under forcing conditions, but, as these authors recognised, there is some doubt as to whether such oxidation occurs in the normal manner. The liberation of large proportions of formic acid under the conditions of the experiment showed that extensive over-oxidation was occurring, and it is therefore possible that the "resistant" residues were undergoing oxidation only after depolymerisation had exposed them as end groups.

Although a discussion of this problem of over-oxidation is outside the scope of this paper, it is relevant to mention that strong evidence has been obtained in this laboratory to show that, during the periodate oxidation of starch and glycogen, scission of internal linkages in the chains occurs by a free-radical mechanism similar to that reported earlier[2] for sodium alginate. This depolymerisation is inhibited, but not completely prevented, by the inclusion of propan-1-ol in the reaction mixture, which is thought to act as a radical scavenger[2].

If, however, it is accepted that the oxidation observed by Manners and Mercer[11] was taking place in the normal manner, it is possible from the reported times of half-

*The apparent rate of over-oxidation was higher than before. This, as well as the initial, rapid consumption of 0.006 mol. of periodate, was probably due to depolymerisation occurring during isolation of the unreduced polyaldehyde, and a consequent increase in the proportion of end groups.

change to estimate that the rate of oxidation, at 18–20°, was very roughly 0.02–0.04 l.mole^{-1}.h^{-1}. This may be compared with a figure of 280 l.mole^{-1}.h^{-1} for the initial rate of oxidation of amylose at 20°, and of 9l.mole^{-1}.h^{-1} for the final rate of oxidation of amylose at the same temperature[4].

The proportion of periodate-resistant D-glucose residues in starches and glycogens is undoubtedly higher than any of the earlier estimates have indicated. This is because of the hitherto unrecognised fact that extensive decomposition of D-glucose occurs when it is heated with dilute acid in the presence of a large excess of glycolaldehyde. The mechanism of this decomposition or destruction is not known, but it may be supposed that the D-glucose becomes irreversibly incorporated into the brown polymer that is formed when glycolaldehyde is heated in acid.

Under the conditions of acid hydrolysis used in the present work (0.5M hydrochloric acid at 100° for 5 h), such destruction occurred to the extent of 75%. Other workers have mostly used more-concentrated solutions of acid, but sulphuric acid has been preferred[7–11]. Another reason for believing that the present estimates are more accurate than any of the earlier ones is that the superior, D-glucose-oxidase method of assay was not available to the earlier workers.

The fraction of periodate-resistant D-glucose residues is directly proportional to the degree of branching of the glucan. The residues concerned must therefore be located near the branching points or near the non-reducing ends of the chains. However, if they were situated near the non-reducing ends of the chains, simple oligosaccharides of the maltose series would be expected to show anomalously low, periodate-oxidation limits, whereas it is well known that they do not. The resistant residues must therefore occur near the branching points of the chains.

The ratio of periodate-resistant residues to branching points is consistently ~1:3, and no explanation can be considered that does not take account of the statistical nature of the attack of periodate on the chains. A mechanism such as that discussed in the theoretical section is therefore strongly indicated.

Reduction of the polyaldehydes with borohydride permits the rapid and complete oxidation of the remaining D-glucose residues by periodate. Indeed, the rate of the reaction shown by curve *A* in Fig. 4 is very close to the initial rate of oxidation of amylose[4]. Conversion of the aldehyde groups into primary alcohol groups thus increases the rate of oxidation of the "resistant" D-glucose residues by a factor of ~10^4.

In the light of this and earlier[1–5] evidence, it is concluded that the periodate-resistant D-glucose residues in starches and glycogens arise because of the formation of 6-membered hemiacetal rings between the aldehyde groups of oxidised, 6-*O*-substituted D-glucose residues and the closest hydroxyl groups on adjacent, unoxidised residues in the same, (1→4)-linked chain. Such hemiacetals are no doubt formed with both adjacent residues, but, because of the essentially unidirectional inhibition provided by oxidised, unbranched D-glucose residues, only one of these adjacent residues is in a position to receive the extreme measure of inhibition that results from hemiacetal formation with both of its secondary hydroxyl groups.

In contrast with the present findings, Hamilton and Smith[10] reported that the periodate-resistant D-glucose residues in a sample of amylopectin did not become oxidisable after reduction of the polyaldehyde. This result is difficult to understand. It is possible that the sample of amylopectin studied really contained "anomalous" linkages as the authors suggested[10], but resistant residues of the type found here should still have been present in the polyaldehyde, and re-oxidation after reduction with borohydride should have led to a decrease in the proportion of intact D-glucose residues in the material. It is suggested that the explanation must be sought in the low accuracy of the analytical methods that were available to the earlier workers.

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