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Inter-residue Lactones Formed by Treatment of Periodate-oxidised Polysaccharides with Aqueous Bromine

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When amylose was oxidised with periodate until 64 % of the glucose residues had been converted into "dialdehyde" units, and then treated at 20° with aqueous bromine buffered at pH 4.6, a product was isolated in which 60 % of the aldehyde groups had been converted into free carboxylic acid, and 40 % into lactones.

The remaining 36 % of intact glucose residues in the product were resistant to oxidation by periodate, but after hydrolysis of the lactones with mild alkali, they became freely oxidisable. After hydrolysis with alkali, treatment with aqueous acid or bromine did not regenerate the lactones. Sodium alginate and a xylan also contained lactones after partial oxidation with periodate and treatment with aqueous bromine.

The lactones must have arisen by direct oxidation, in their closed-ring form, of hemiacetals formed between the aldehyde groups of periodate-oxidised sugar residues and the secondary hydroxyl groups of intact sugar residues in the same molecular chain.

In connection with studies [1–5] of the hemiacetals that occur in polysaccharides after partial oxidation with periodate, a better method was needed to identify the aldehyde and hydroxyl groups involved. It was thought that, if the hemiacetals could be converted directly into lactones by oxidation with aqueous bromine, the required information might be provided by circular-dichroism spectroscopy of the products. A study of conditions was therefore undertaken, and the present paper describes results that appear promising.

EXPERIMENTAL

Materials. The preparation of 64 % periodate-oxidised amylose has been described elsewhere,[3] as also has the preparation of 44 % periodate-oxidised sodium alginate.[1] The sample of xylan was prepared from the red seaweed, *Rhodymenia palmata*,[6] by Dr. Arne Jensen, and kindly donated by him. It was water-soluble, of high intrinsic viscosity, and contained 78 % of 1,4-linked xylose residues, as indicated by its periodate-oxidation

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limit.[5] A portion of the xylan was oxidised in 25 mM periodate to the extent of 40 %, essentially as described elsewhere for a sample of maize-cob xylan.[2]

Kinetics of oxidation of 64 % periodate-oxidised amylose with aqueous bromine. A 0.5 M sodium acetate-acetic acid buffer of pH 5.0 was almost saturated with bromine at room temperature. A portion (10 ml) was added to a mixture of aqueous potassium iodide (30 % w/v; 10 ml) and 2 N hydrochloric acid (2 ml), and titrated with 0.1 M sodium thiosulphate. A bromine content of 0.176 g atom l^{-1} was thus found. A volume (150 ml) of this solution, and a solution of 64 % periodate oxidised amylose (0.5 g) in water (100 ml) were brought separately to 20°, and then mixed. At intervals, portions (10 ml) were withdrawn and titrated as just described. To correct for bromine lost by evaporation, a parallel experiment was done on 150 ml of the bromine-containing buffer, diluted to 250 ml with water. The pH of both the test and control solutions remained constant at 4.6 throughout.

Isolation of 64 % periodate-oxidised amylose after complete oxidation with bromine. A portion (1 g) of the 64 % periodate-oxidised amylose was oxidised for 48 h under the analytical conditions just described. Residual bromine was removed by addition of an excess of formic acid, and the solution was dialysed against water. It was concentrated in the rotary evaporator to 100 ml, and dialysed for 6 h against 0.01 N hydrochloric acid at 4°, with hourly replacement of the dialysate. It was then dialysed against water, and freeze-dried. The product (yield, 850 mg) had an equivalent weight, determined by titration with 0.02 N sodium hydroxide to a faint pink end-point with phenolphthalein, of 215. Another sample of the same product was isolated, as its sodium salt, by dialysis, first against 0.1 M sodium acetate, and then against water. Its equivalent weight, determined by titration with cetylpyridinium chloride,[7] was 283.

Kinetics of saponification of the lactones in the amylose derivative. A portion (100 mg) of the product (free acid), in water (20 ml), was titrated with 0.02 N sodium hydroxide in the presence of phenolphthalein (0.1 %; 2 ml) to a faint pink end-point. This solution, at 20°, was mixed with 0.02 N sodium hydroxide (50 ml), and then quickly diluted to 100 ml with water, and kept at 20°. As a control, phenolphthalein (0.1 % w/v; 2 ml), in water (20 ml), was titrated to a faint pink end-point, after which 0.02 N sodium hydroxide (50 ml) was added, followed by water to give 100 ml. At intervals, portions (10 ml) were withdrawn and back-titrated with 0.01 N oxalic acid.

Analytical oxidation of the saponified amylose derivative with periodate. A portion (100 mg) of the unsaponified amylose derivative (sodium salt), in water (50 ml), was mixed with 0.02 N sodium hydroxide (50 ml) and kept at 20° for 5 h. Acetic acid (1.0 N; 4.0 ml) was then added, followed by water (90 ml), and the solution was cooled to 2° in an ice-bath. Its pH was 4. Sodium metaperiodate (0.25 M; 4.0 ml) was then added, and the solution was quickly diluted to 200 ml with water. It was kept in the dark at 2°, and at intervals, portions (10 ml) were pipetted into an ice-cold mixture of 0.5 M sodium phosphate buffer (pH 7.0; 20 ml) and aqueous potassium iodide (30 % w/v; 3 ml), and the liberated iodine was titrated with 0.01 M sodium thiosulphate. A control solution, containing everything but the substrate, was also prepared and titrated.

Analytical oxidation of the unsaponified amylose derivative with periodate. To a mixture of 0.02 N sodium hydroxide (50 ml) and 1.0 N acetic acid (4.0 ml) was added a solution of the unsaponified amylose derivative (sodium salt) (100 mg) in water. The remainder of the procedure was as described above.

Preparative oxidation of the saponified and unsaponified amylose derivative, and analysis of the products for glucose. The conditions of oxidation were the same as in the analytical procedures, except that 2.5 times the quantities were used. After oxidation for 6 h, ethanediol (10 ml) was added to each reaction mixture. The solutions were then evaporated to 100 ml, dialysed thoroughly against water, and freeze-dried. A portion (10 mg) of each product was heated for 5.5 h in a sealed ampoule with 0.5 N hydrochloric acid (2.5 ml). The solutions were then neutralised with 0.2 M sodium acetate (3 ml) and 0.5 N sodium hydroxide (2.5 ml). A blank was also prepared, and, as a control, a sample of the original amylose derivative containing the lactones, but not subsequently treated with periodate, was treated similarly. Triplicate portions (0.06 ml) of each solution, and of an external glucose standard, were measured with an Agla micrometer syringe, and analysed for glucose by the glucose-oxidase procedure.[8]

Position of the equilibrium between the lactones and the corresponding free acids. A portion (100 mg) of the amylose derivative, in water (20 ml), was mixed with phenolphthalein

(0.1 %; 2 ml) and 0.02 N sodium hydroxide (50 ml), and kept 1 h at 20°. Oxalic acid (0.1 N; 30 ml) was then added, and the solution was diluted to 100 ml with water, and kept at 20°. Portions (10 ml) were withdrawn at intervals and titrated with 0.02 N sodium hydroxide. The titres were constant over a period of 50 h. In another experiment, the amylose derivative (100 mg) was kept at 20° in 0.033 N oxalic acid (100 ml; pH 1.5), and the change in the acidity of the system was followed by titrating portions (10 ml) with 0.02 N sodium hydroxide. The acidity increased, indicating hydrolysis of the lactones with a time of half change of approximately 48 h.

Oxidation of 40 % periodate-oxidised xylan with aqueous bromine. The kinetics were followed in the same way as, and were closely similar to, those of the periodate-oxidised amylose. After oxidation for 8 h, a sample was isolated as its sodium salt. It had an equivalent weight, determined by titration with cetylpyridinium chloride,[7] of 223. After saponification, this decreased to 207.5 (theory, assuming complete oxidation, requires 203).

Oxidation of 44 % periodate-oxidised alginate with aqueous bromine. An amount of bromine corresponding to the oxidation of one-half of the aldehyde groups was consumed very rapidly, after which further consumption was very slow. The half-oxidised material was isolated as its sodium salt, and its equivalent weight, determined by titration with cetylpyridinium chloride,[7] was 227. The kinetics of saponification were studied in the same way as for the amylose derivative, but the reaction mixture was made 2 M with respect to sodium chloride, to minimise the Donnan effect. The consumption of alkali became constant after 20 h, and indicated a final equivalent weight of 153 (theory, assuming 50 % oxidation, requires 167).

RESULTS

In the first series of experiments, the partially periodate-oxidised polysaccharides were treated with unbuffered, aqueous bromine. Under these conditions, there was an initial, rapid consumption of bromine, but the reaction then became very slow, as the pH dropped to about 1.5 because of the formation of hydrogen bromide. Isolation and examination of the partly oxidised materials revealed the presence of lactones as well as free carboxylic acid, but as the materials were successively given further treatments with bromine water in an attempt to complete the oxidation, the proportion of lactones decreased. With the partially periodate-oxidised amylose and xylan, products were finally obtained which contained no remaining aldehydic functions, as judged by a lack of reactivity with aqueous phenylhydrazine hydrochloride, but they also contained no lactones.

The kinetics of the oxidation with bromine were next studied under buffered conditions. A pH range of 4 – 5 was chosen, in the expectation that the rate of hydrolysis of the lactones would be minimal in this region. Fig. 1 shows the consumption of bromine by the 64 % periodate-oxidised amylose at pH 4.6. After oxidation for 48 h under these conditions, a product was obtained whose equivalent weight depended upon the conditions of isolation. When the free acid was isolated, after dialysis in the refrigerator against hydrochloric acid at pH 2, it had an equivalent weight of 215, as determined by direct titration with alkali. The corresponding theoretical value, assuming that all the carboxyl functions were present as free acid, was 142. This implies that about one-third of the carboxyl functions were present as lactones.

When the oxidised amylose derivative was isolated after dialysis against sodium acetate at pH 6, its sodium salt had an equivalent weight, determined by titration with cetylpyridinium chloride,[7] of 283. This compares with a

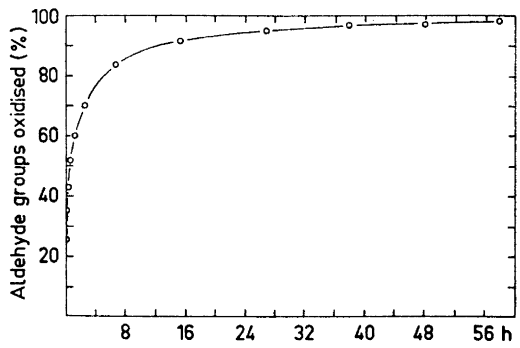

Fig. 1. Oxidation of 64 % periodate-oxidised amylose in aqueous bromine at pH 4.6 and 20°. The initial concentrations of bromine and aldehydic groups (free and combined) were 94 and 27 mequiv. l^{-1}, respectively.

theoretical figure for the sodium salt of 164, and implies the presence of 42 % of lactones. This figure is probably the more accurate of the two, since some hydrolysis of lactones would be expected to occur during isolation of the free acid.

Fig. 2 illustrates the saponification of the amylose derivative at pH 12 and 20°, and shows how the equivalent weight, calculated on a basis of the free acid, decreased to approximately the theoretical value of 142. Further experimentation as described in the experimental section showed that acidification of the reaction mixture did not cause the lactones to re-form. On the contrary, acid merely hydrolysed the lactones in the unsaponified material, as was already indicated by the oxidations carried out in unbuffered bromine water. No attempt was made to measure the exact position of the equilibrium between the free-acid and lactone forms in aqueous solution, but the results are sufficient to indicate that this must lie very far on the side of the free acid. Freeze-drying of the fully hydrolysed material in its free-acid form did not

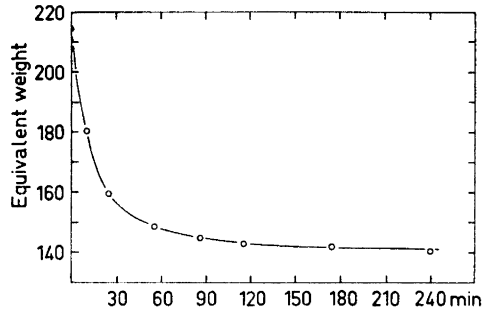

Fig. 2. Saponification of the lactones in 64 % periodate-oxidised amylose after complete oxidation with aqueous bromine. The initial concentration of sodium hydroxide was 0.01 N, and it was present in a molar excess of 4:1. The temperature was 20°.

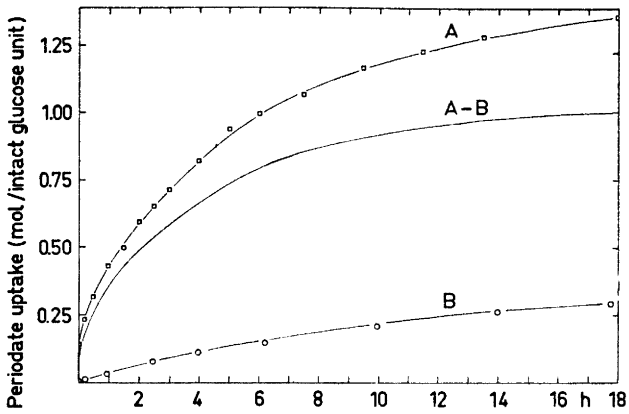

Fig. 3. Oxidation at 2° in 5 mM sodium metaperiodate of 64 % periodate-oxidised amylose after complete oxidation with aqueous bromine. Curve A: after saponification for 5 h under the conditions shown in Fig. 2. Curve B: without prior saponification. The pH of the reaction mixture was 4 in both experiments.

regenerate any lactones. It is possible that lactones would have been formed by heating the material in a vacuum, but this was not investigated.

Similar oxidations and assays of the free acid and lactones in the products were carried out on 40 % periodate-oxidised xylan and 44 % periodate-oxidised sodium alginate, as described in the experimental section. The xylan derivative was readily oxidised to completion, but the product contained only about 10 % of its carboxyl groups as lactones. The alginate consumed only about half the theoretical amount of bromine, without any decrease in equivalent weight at all; the aldehyde groups concerned were converted quantitatively into lactones.

An experiment was next carried out to determine whether any of the lactones in the amylose derivative involved the secondary hydroxyl groups of the intact glucose residues remaining in the material. Fig. 3 shows the consumption of periodate by the saponified and unsaponified material. Both consumed periodate, but when the curve for the unsaponified material was subtracted from that for the saponified material as shown, a limiting difference in consumption corresponding almost exactly to the Malapradian oxidation of the glucose residues in the latter material was indicated.

The consumption of periodate in excess of 1 mol per intact glucose residue in the saponified material must have been due to non-Malapradian oxidation, and it seemed likely that the parallel consumption by the unsaponified material was also due to this alone. To confirm this, the two substrates were isolated after oxidation for 6 h, and analysed for intact glucose residues by acid-hydrolysis and application of the glucose-oxidase method of analysis. Whereas the unsaponified material still contained 28 % of glucose residues after the treatment with periodate, the saponified material contained only 4 %.

DISCUSSION

The present investigation was prompted by the classical work of Isbell and his associates,[9–12] who first showed that glucose, and other aldohexoses and aldopentoses, are directly oxidised by aqueous bromine in their cyclic, hemiacetal forms, to give δ-lactones as the initial products. The delta-lactones formed in this kinetically-controlled reaction are unstable, and they spontaneously undergo hydrolysis in water to give mainly the free aldonic acid. In unbuffered solutions, this reaction is autocatalytic.[10] If the free aldonic acids are kept in aqueous solution for a relatively long time, they spontaneously and partially re-lactonise, but the lactones formed in this way are the thermodynamically more stable γ-lactones.[9,10] It was therefore impossible for the δ-lactones to have been formed as secondary products from the free acids, and the pyranose-ring structure of the parent sugars in solution was thereby established.

Apart from the possible presence of D-erythrofuranose rings in the periodate-oxidised amylose,[13] all the hemiacetals in the materials examined in the present work were expected to be derivatives of dioxan.[1–5] The lactones derived from them were therefore expected to resemble the aldonic δ-lactones in properties, even though some differences in stability could be expected to arise from the presence of two hetero-atoms in the rings, and the presence of any inter-residue lactones as parts of fused-ring systems.

Initial attempts to carry out the oxidation with unbuffered bromine quickly confirmed that the lactones were unstable in acid, and could not have been formed as secondary products from previously oxidised aldehyde groups. By carrying out the oxidation at pH 4.6, hydrolysis of the lactones was kept to a minimum, and any lactonisation of previously formed carboxyl groups would have been virtually precluded. It was also hoped that any trans-lactonisation (intramolecular trans-esterification) would be kept to a minimum at this pH, but the possibility of this reaction unfortunately cannot be excluded.

The yield of a particular lactone is unfortunately no indication of the relative amount of its parent hemiacetal in the starting-material. In the case of the xylan derivative, for example, it was known from earlier work [2] that the position of the equilibrium between the aldehydic and hemiacetal forms lay about 85 % on the side of the latter, and yet only 10 % of them were recovered as lactones. In contrast, half the hemiacetals in the periodate-oxidised alginate were quantitatively converted into lactones, while the remainder were very resistant to oxidation.

There are clearly two reasons for this. The first is that every lactone is subject to spontaneous hydrolysis from the moment of its formation, and the rate of hydrolysis will depend upon its stereochemistry.[10,14] The second is that the yield of a lactone is determined not only by the position of the equilibrium between its parent hemiacetal and the corresponding open-chain aldehydic form, but also by the relative rates of oxidation of these. The rate of oxidation of a cyclic hemiacetal is known [11,12,15] to be highly dependent upon the stereochemistry of the ring.

It may not always be useful to attempt complete oxidation with bromine,

as was done in the present work, because during the period of time required for this, extensive hydrolysis of the less-stable lactones could occur. Fig. 1 shows that half the aldehyde groups in the periodate-oxidised amylose were oxidised in a few minutes, while it took many hours to complete the last 10 % of the oxidation. It should be noted that this kinetic behaviour is certainly due in part to the fact [16,17] that the tribromide ion, Br_3^-, does not oxidise hemiacetals.

In conclusion, there is a strong element of unpredictability in the use of bromine-oxidation to study this kind of hemiacetal, but possibilities exist for overcoming the problems encountered, and the results appear to be generally encouraging.

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