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The Role of Intramolecular Autocatalysis in the Acid Hydrolysis of Polysaccharides Containing 1,4-Linked Hexuronic Acid

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At pH values from 2 to 4, oligomannuronic acids (OM) were hydrolysed faster at 100° than oligoguluronic acids (OG), the ratio (k_{OM}/k_{OG}) of the rates being maximal at about 4.3 near pH 2.8. Oligouronides containing both monomers (OGM) were hydrolysed at intermediate rates, and to a good approximation the initial rates could be expressed in terms of k_{OM}, k_{OG}, and the monomeric composition (M/G ratio) of the oligouronides.

After partial hydrolysis of OGM at pH 2.8, 80 % of the isolated diuronides contained both monomers in the same molecule, and of these, 90 % had mannuronic acid as the non-reducing terminal unit.

The results support the view that the high rate of hydrolysis of 1,4-linked polyuronides in weakly acidic media is due largely to intramolecular catalysis of glycosidic cleavage by the carboxyl groups in the respective aglycone units.

The rates of hydrolysis of alginic and pectic acids depend only slightly upon hydrogen-ion activity in the pH range 2—4, and are much higher than those of neutral polysaccharides under the same conditions.[1] Similar behaviour is shown by 3-*O*-β-D-glucopyranosyl-L-gulonic acid, whereas with 4-*O*-β-D-glucuronopyranosyl-D-glucitol, dependence of the rate upon pH is much more pronounced.[1] It was therefore suggested [1] that the behaviour of the polyuronides is due largely to intramolecular catalysis [2–4] of the hydrolysis of glycosidic linkages by the carboxyl groups in the respective aglycone units.

It was nevertheless desirable to seek more definitive evidence for the role of intramolecular catalysis in the hydrolysis of long polyuronide chains. This has now been obtained by making use of the block-copolymeric structure of alginate, which consists of long sequences of contiguous mannuronic acid residues, similar groups of guluronic acid residues, and a third kind of sequence,

characterised by a high proportion of alternating mannuronic and guluronic acid residues.[5]

Oligouronides representative of the three kinds of block were split out by partial acid hydrolysis, and conditions were found under which the oligomannuronic acids were hydrolysed 4.3 times faster than the oligoguluronic acids. Hydrolysis of the predominantly alternating material under the same conditions then led to the observation that the mannuronic acid residues were exposed as non-reducing terminal groups much faster than were the guluronic acid residues. A preliminary report of some of the results has been published previously.[6]

EXPERIMENTAL

Preparation of oligouronides. Partial hydrolysis of alginate from *Laminaria digitata* was carried out in 0.3 N hydrochloric acid as described previously, and the fractions A, B, and C, all having number-average degrees of polymerisation (P_n) of 20 ± 1, and containing 65 %, 91 %, and 8 % of mannuronic acid, respectively, were isolated as their sodium salts.[5] Fraction A, which was rich in alternating sequences,[5] was soluble in acid, and was used directly for kinetic measurements; it is hereinafter referred to as OGM. Fractions B and C were insoluble at the lower pH values, and had to be degraded further to increase their solubility in acid.

A solution (1 % w/v in water) of fraction B was acidified to pH 2.9 with sulphuric acid, heated 1 h at 100°, and then cooled. The pH was adjusted to 2.0, and, after standing overnight, the precipitate was removed in the centrifuge. The soluble material (OM; yield, 75 % w/w) then had $P_n=5$; the neutralised solution was stored at 0°, and used directly for the kinetic measurements.

Fraction C (1 % w/v in 0.05 M citrate buffer, pH 3.75) was heated 16 h at 100°. After removal of the material insoluble at pH 2.0, the acid-soluble product (OG; yield, 75 %; $P_n=3.5$) was stored in neutral solution at 0°.

Analytical methods. The total carbohydrate in the stock solutions of OM and OG was determined by the phenol method,[7] with mannuronic and guluronic acid, respectively, as the standards. Number-average degrees of polymerisation (P_n) and degrees of scission (α, $=1/P_n$) were measured as reducing power according to Nelson;[8] the two monomers gave the same molar extinction, and mannuronic acid was used throughout as the standard. In hydrolysis with 2 N hydrochloric acid, the high concentration of sodium chloride obtained after neutralisation of the samples affected the reproducibility of the Nelson estimations, and in this case, measurement of the total carbohydrate [7] before and after reduction with potassium borohydride [9] was used to determine the degrees of scission.

Kinetic measurements. Values of pH higher than 2 were achieved with citric acid-sodium citrate buffers (0.05 M), and the others with hydrochloric acid. Values of pH of 2 and above were measured with a Radiometer-4 pH-meter. The reaction mixtures contained 0.2 % w/v of carbohydrate, and were boiled under reflux in a slow stream of nitrogen; their volume was kept large compared with the total volume removed for test, to minimise errors due to loss of water by evaporation. Reaction was initiated by rapid addition of pre-heated carbohydrate solution to the boiling buffer; measured samples were withdrawn and rapidly cooled immediately after starting, and thereafter at intervals. Samples of low pH were neutralised with sodium hydroxide, and the others with sodium acetate (to pH 6); they were then diluted appropriately with water prior to analysis.

Isolation and examination of diuronides from OGM. The pH of a solution (1 % w/v) of OGM in water was adjusted to 2.8 with N sulphuric acid. The solution was then boiled under nitrogen for 23 h, after which the carbohydrate had $P_n=2$. The product was fractionated on a column of Sephadex G 25 as described earlier.[5] The total diuronide fraction (yield, 19 % w/w of OGM) was divided into homopolymeric and heteropolymeric subfractions [5] by electrophoresis [10] on well-washed filter paper. The two bands, after location by spraying guide-strips, were eluted and estimated for total carbohydrate by

the phenol [7] and orcinol [11] methods; a blank pherogram was used to correct the results for polysaccharidic material extracted from the paper.

A portion (58 mg) of the heteropolymeric subfraction, in water (10 ml) was reduced (20 h) with sodium borohydride (70 mg) and then acidified with acetic acid. After removal of sodium ions with Dowex-50 (H^+ form) resin, and of boric acid by distillation with added methanol, the product was hydrolysed 5 h at 100° with N sulphuric acid. The ratio of mannuronic to guluronic acid (M/G ratio) in the hydrolysate was determined in the usual way.[12]

RESULTS

The acid-hydrolysis of polysaccharides containing hexuronic acids is complicated by significant losses of uronic acid due to acid-catalysed dehydration, with or without decarboxylation, with formation of furfural, reductic acid, 5-formyl-2-furoic acid,[13–16] and, ultimately, brown polymers of the type referred to as "humins". However, it is reasonably clear that the reactions leading to glycosidic cleavage and decomposition are, at least mainly, consecutive rather than simultaneous. Thus, whereas the decarboxylation of galacturonic acid obeyed first-order kinetics, that of acid-soluble oligouronides derived from pectic acid did not, the reaction curve showing a pronounced induction period.[16]

In the present work, marked induction periods were noted at all pH values examined in the formation from the oligouronides of material absorbing in the ultraviolet (260—280 mμ), whereas only a relatively insignificant induction period was observed in some cases when the monouronic acids were examined under the same conditions. This is illustrated in Fig. 1, which compares the behaviour of mannuronic acid and polymannuronic acid (fraction B) at pH 2.8 and 100°, as shown by the changes in reducing power and absorbance at 260 mμ. The initial rates of increase in reducing power were therefore regarded as representing the cleavage of glycuronosidic linkages.

When OM and OG were hydrolysed at six different acidities, and the results plotted according to first-order kinetics [ln(1—α) against time], straight-

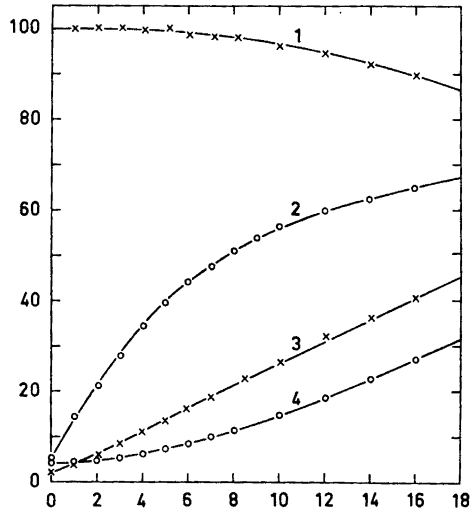

Fig. 1. Changes in reducing power (curves 1 and 2) and absorbance at 260 mμ (curves 3 and 4) of mannuronic acid (×) and polymannuronic acid (○) at pH 2.8 and 100°. The ordinates represent either reducing power as a percentage of the theoretical maximum or absorbance at 260 mμ, expressed arbitrarily as millimoles of ascorbic acid.

line relationships were obtained for degrees of scission (α) up to at least 0.4. The rate-constants calculated from them (k_{OM} and k_{OG}) are shown in Table 1, together with the respective values [17] of the Hammet H_0 function.

Table 1. Rate constants (h^{-1}) for acid-hydrolysis of oligomannuronic (OM) and oligoguluronic (OG) acids at different acidities.

Acid strength	H_0	k_{OM}	k_{OG}	k_{OM}/k_{OG}
2.0 N	−0.69	2.4	3.4	0.71
1.0 N	−0.20	0.80	0.80	1.0
0.1 N	0.98	0.13	0.090	1.5
pH 2.0	2.0	0.076	0.030	2.6
pH 2.8	2.8	0.073	0.021	3.5
pH 3.6	3.6	0.035	0.015	2.3

The variable ratio (k_{OM}/k_{OG}) between the two rate-constants suggested that hydrolysis was proceeding by more than one mechanism. It has been shown [1] that the pH-dependence of the rate of hydrolysis of whole alginate is described by the following equation:

$$k = k_{HA}\ (1 - x)[H^+] + k_I\ (1 - x) \qquad (1)$$

where k is the observed, net rate constant, x is the degree of dissociation of the carboxyl groups, k_{HA} is the rate-constant for proton-catalysed hydrolysis of the undissociated polyuronide, and k_I is the rate-constant for that part of the hydrolysis which is considered to be brought about by intramolecular catalysis.

The shape of the curve given by eqn. (1) is determined by k_{HA}, k_I and the dissociation constant (K_a) of the uronic acid. By choosing appropriate values for these three constants, it was possible to match theoretical curves with the experimental data given in Table 1, and the two sets of values giving the

Table 2. Rate constants (h^{-1}) and dissociation constants used for calculating the solid-line curves in Fig. 2.

	Oligomannuronic acid	Oligoguluronic acid
k_{HA}	0.45	0.60
k_I	0.08	0.02
pK_a	3.5	3.8

closest possible fit with the results for OM and OG, respectively, are shown in Table 2. The two dissociation constants determined in this way are slightly higher than those determined earlier for monomeric mannuronic and guluronic acids (pK_a=3.38 and 3.65, respectively) at room temperature.[18] In Fig. 2, the experimental points taken from Table 1 are plotted, while the two solid-line curves are those of the following equations, obtained by substitution of the figures in Table 2 into eqn. (1):

$$k_{OM} = (0.45\ [H^+] + 0.08)(1 - x)$$
$$k_{OG} = (0.60\ [H^+] + 0.02)(1 - x)$$

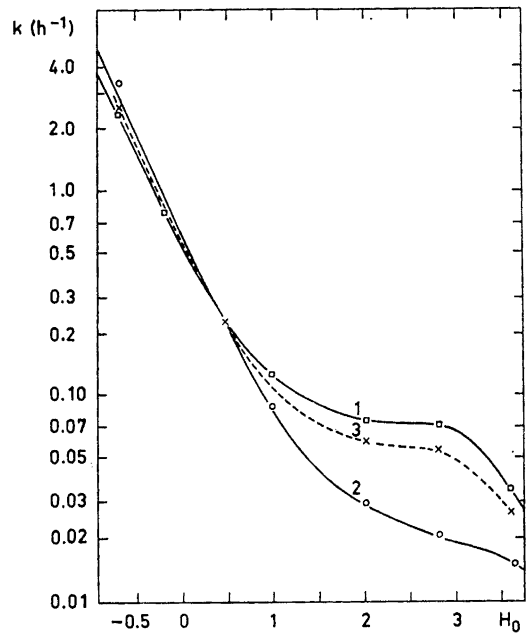

Fig. 2. pH-Dependence of the rates of hydrolysis of oligomannuronic acids (OM), curve 1; oligoguluronic acids (OG), curve 2; and oligouronides of mixed composition (OGM), curve 3.

These equations permit calculation of that part of the total rate which is due to proton-catalysed hydrolysis of the undissociated polyuronide, and this, expressed as a percentage of the total rate of hydrolysis, is shown in Table 3 for five different acidities.

Table 3. The contribution of proton-catalysed hydrolysis of undissociated uronic acid as a percentage of the rate of hydrolysis at different acidities.

Acid strength	2 N	0.3 N	pH 2.0	pH 2.8	pH 3.6
H_0	−0.69	+0.45	2.0	2.8	3.6
Oligomannuronic acid (OM)	97	67	6.0	1.5	0.0
Oligoguluronic acid (OG)	99	92	33	6.0	1.2

When OGM was hydrolysed at five different acidities, and the results were plotted according to first-order kinetics, linear relationships were obtained only at the lower pH values. At the higher pH values, curved relationships were obtained, indicating that more than one rate-constant was needed to describe the reaction, and in these cases the initial rates were measured from the slopes of the tangents at zero time. The possible error in the measurement of these slopes was inevitably large, about ±10 %. The initial rates are

plotted in Fig. 2 (broken-line curve), and are seen to be intermediate between those for OM and OG throughout the range of pH studied.

Although neither OM or OG was completely homopolymeric, it is reasonable as a first approximation to regard k_{OM} and k_{OG} as the rate-constants for the corresponding pure oligouronides. If the rate of hydrolysis of OGM is determined only by its monomeric composition (M/G ratio), and is independent of the distribution of the two monomers along the chain, the initial rate will be given by:

$$k = k_M \, M + k_G \, G \qquad (2)$$

where k_M and k_G are the two rate-constants, and M and G the mole fractions of the mannuronic and guluronic acid residues, respectively. From the monomeric composition of OGM (63 % mannuronic acid), the theoretical, initial rates of hydrolysis were calculated on the assumption that $k_M = k_{OM}$ and that $k_G = k_{OG}$. The results are given in Table 4, and show good agreement between the calculated and observed initial rates.

Table 4. Comparison of calculated and observed initial rates of hydrolysis for preparation A.

Acid strength	2 N	0.3 N	pH 2	pH 2.8	pH 3.6
H_0	−0.69	0.45	2	2.8	3.6
k, observed	2.6	0.24	0.060 [b]	0.055 [b]	0.025 [b]
k, calculated	2.8	0.24 [a]	0.059	0.053	0.028

[a] Interpolated value from Fig. 2.
[b] Possible error, about ±10 %.

Since OM was prepared by degradation of fraction B at pH 2.9, it was expected that its relative content of intact linkages to guluronic acid residues had thereby been enhanced. To obtain an indication of the error due to this, intact fraction B was hydrolysed at pH 2.8, at which it was soluble. The measured rate was now 0.080 h^{-1}, compared with 0.073 h^{-1} for OM.

It was expected that the error in the value of k_{OG}, due to the presence of mannuronic-acid residues, would be smaller, since the undesired linkages to mannuronic acid would have been selectively cleaved during the preparation of OG from fraction C by hydrolysis at high pH. Therefore a second approximation for k_M could be obtained from k_{OG}, eqn. (2), and the quantitative composition of fraction B:

$$0.08 = k_M \cdot (0.91) + (0.021)(0.09)$$

This gives $k_M = 0.086$ h^{-1}, and hence, a value for the ratio $k_M/k_G = 4.3$. Recalculation of the initial rate for OGM, using eqn. (2), gives $k = 0.062$ h^{-1}, compared with 0.055 h^{-1} obtained experimentally. This difference is within the limits of error involved in measuring the slopes of the initial tangents, for hydrolysis of OGM at the higher pH values.

When OGM was hydrolysed at pH 2.8 to a degree of scission of 0.5, and the diuronide fraction was isolated by chromatography on Sephadex G-25,

it was found that 80 % of the diuronides contained both mannuronic and guluronic acid residues in the same molecule, and of these, 90 % had mannuronic acid as the non-reducing residue.

DISCUSSION

At acid-concentrations of 0.5 N and above, OM, OG, and OGM were all hydrolysed at similar rates, and the degradation was approximately random, although not completely so. The marked divergence in the rates of hydrolysis of OM and OG above pH 1 must, in the light of the present results, be attributed mainly to an influence of the configuration at C-5 of a uronic acid residue upon the rate of cleavage of the glycosidic linkage attached to C-4 of the same residue. If the influence were mainly upon the rate of cleavage of the glycosidic linkage formed at C-1 of the same residue, then the mannuronic acid residues would have been more rapidly exposed as reducing, rather than as non-reducing end-groups. That this influence becomes important only when the carboxyl groups are partly dissociated suggests at first sight that a dissociated carboxyl group assists proton-catalysed hydrolysis of the glycosidic linkage attached to C-4 of the same residue. If this is the case, it is difficult to explain the importance of the stereochemistry of the aglycone otherwise than on the assumption that the carboxylate anion participates directly in the reaction. Two possible ways in which it may do this are discussed by Capon [3] in connection with the hydrolysis of *o*-carboxyphenyl β-D-glucopyranoside.

On the other hand, since $[A^-][H^+]=K_a[HA]$, the kinetics are equally explicable on the assumption that catalysis is brought about by the undissociated carboxyl group acting directly as the proton donor.[1,3] Capon and Smith [4] have shown that this is indeed the mechanism of hydrolysis of 2-methoxymethoxybenzoic acid in the pH range 3.1—5.5, and it is therefore to be favoured as a likely explanation for the present results.

Although it is clear that intramolecular catalysis of the hydrolysis of glycosidic linkages by either the dissociated, or more probably the undissociated, carboxyl groups in the respective aglycones plays an important role in the degradation of alginate in weakly acidic solutions, the present work does not prove that it is the only mechanism responsible for the high rate of hydrolysis under these conditions.

Capon and Ghosh [19] have shown that, whereas 2-naphthyl β-D-glucuronopyranoside is hydrolysed 45 times more slowly than the corresponding glucoside in N hydrochloric acid, it is hydrolysed 35 times faster at pH 4.8. This effect was attributed to a much higher rate of proton-catalysed hydrolysis of the dissociated glucuronide than of the undissociated form. In this laboratory, it was found that, when glucitol is the aglycone, the glucuronoside is hydrolysed only about 4 times faster than the glucoside at pH 4, whereas 3-*O*-β-D-glucopyranosyl-L-gulonic acid is hydrolysed 100 times faster.[1] It therefore appears that the magnitude of the effect discussed by Capon and Ghosh [19] depends upon the nature of the aglycone, and that when the possibility for intramolecular catalysis by the aglycone exists, the rate is greatly enhanced, and the intramolecular mechanism is likely to become the dominant one.

Recently, Roy and Timell [20] studied the pH-dependence of the hydrolysis of 2-*O*-(4-*O*-methyl-α-D-glucuronopyranosyl)-D-xylose and 4-*O*-β-D-glucopyranosyl-D-glucuronic acid with results that are consistent, in part, with these conclusions. However, in contrast with all the results obtained so far in this laboratory, both viscosimetrically and by reducing end-group determinations,[1,21] the rate of hydrolysis of the last-named compound was reported to increase about 3-fold as the pH increased from 3 to 6, and was interpreted as evidence for intramolecular catalysis by the dissociated carboxyl group. This explanation, however, is untenable, since eqn. (1) does not permit of such an increase in rate with increasing pH. The β-alkoxy carbonyl elimination, which was shown by Deuel and co-workers [22] to be responsible for the degradation of methyl pectate near neutral pH, is also unlikely to account for the results of Roy and Timell,[20] since the same workers [22] showed that unesterified pectate is stable under these condiditions. Significant degradation of unesterified alginate by this elimination reaction occurs only at high pH (Ref. 23). Roy and Timell reported that their preparation of 4-*O*-β-D-glucopyranosyl-D-glucuronic acid decomposed in weakly acidic solutions, and it seems unlikely that their results can be interpreted in terms of acid hydrolysis only. Considerable difficulty with the same compound was previously experienced in this laboratory,[1] and it was necessary to work with the corresponding alditol in order to obtain reliable kinetic data.

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