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The Influence of pH on the Rate of Hydrolysis of Acidic Polysaccharides

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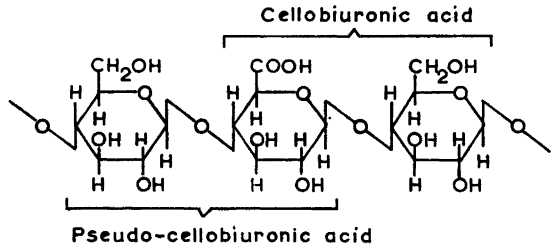
The rate of hydrolysis was determined for a number of poly- and oligosaccharides as a function of the pH. Neutral carbohydrates were hydrolysed at a rate nearly proportional to the acid concentration. At low pH-values, carbohydrates containing sulphate or carboxyl groups were hydrolysed at a lower rate while the opposite was the case at low acid concentrations.

Poly- and oligouronides showed a particularly high rate of hydrolysis at pH-values above 2, and the results indicate that the rate depends on the degree of dissociation of the carboxyl group.

The higher rate of hydrolysis of charged polysaccharides at low acid concentrations is supposed to be due to a higher proton concentration near the negatively charged polysaccharide chain than in the bulk of the solution. An intramolecular catalysis by the undissociated carboxyl groups is proposed as the mechanism responsible for the specific effect obtained with 1,4-linked uronic acids.

Acid hydrolysis is a widely used tool for studying the chemical composition and structure of polysaccharides. Partial hydrolysis of polysaccharides, followed by isolation and characterization of di- and oligosaccharides, is fundamental in structural studies. For polysaccharides composed of different sugar residues particularly good yields may be obtained if the rates of hydrolysis of the different glycosidic linkages in the polysaccharide are sufficiently different. The isolation of aldobiuronic acids from hemicelluloses may serve as an example of this technique.

Kinetic studies of the rate and mechanism of the hydrolysis of glycosides and disaccharides are, therefore, of considerable interest also in polysaccharide chemistry. The mechanism now generally accepted (for references see Timell [1]) involves three steps: I) A protonation of the glycosidic oxygen to give the conjugate acid; this step is rapid and the acid will exist in its equilibrium concentration. II) A unimolecular heterolysis of the conjugate acid with the formation of a non-reducing end-group and a carbonium-oxonium ion: this step is slow and rate determining. III) A rapid addition of water to the carbonium-oxonium ion with the formation of a reducing end-group and a



proton. This mechanism has been used to explain the different rate of hydrolysis of the different types of glycosides and disaccharides. The acid resistance of the glycosidic linkage in an aldobiuronic acid is supposed to be caused by an inductive effect of the carboxyl group, lowering the concentration of conjugate acid (step I) and decreasing the rate of electron transfer in step II. It was proposed by Marchessault and Rånby [2] that this inductive effect increases the rate of hydrolysis of the glycosidic linkage from a neutral sugar residue to the uronic acid (pseudo-aldobiuronic acid). Such an effect could explain the existence of "weak linkages" in commercial cellulose samples containing small amounts of uronic acid residues.

This was investigated by Johansson, Lindberg and Theander [3] who synthetized cellobiuronic acid and pseudo-cellobiuronic acid and determined the rate of hydrolysis in 1 M sulphuric acid. Under these conditions the same rate of hydrolysis was obtained for cellobiose and pseudo-cellobiuronic acid, while cellobiuronic acid was hydrolysed at a significantly lower rate.

Most kinetic studies have been carried out at high acid concentration where the carboxyl groups of the polysaccharide are not dissociated. The effect of the carboxyl groups in ionized form seems not to have been investigated. The effect of the highly dissociated sulphate half-ester groups has been investigated by Clancy and Turvey [4] who found that the presence of a sulphate half-ester group in the 3- or 6-position decreased the rate of hydrolysis of methylglycosides. The authors explained this as a steric effect of the sulphate group resulting in a decreased tendency of the carbonium-oxonium ion for existing in its most stable half-chair conformation. Another effect of sulphate half-ester groups in polysaccharides is that the polysaccharide, in its acid form, may be hydrolysed by its own acid strength. This autohydrolysis technique was utilized by Painter [5] to produce sulphated oligosaccharides from furcellaran.

Many investigations have been carried out concerning the stability of polysaccharides under neutral or slightly acidic conditions, usually by viscosity measurements. The intrinsic viscosity has, in most cases, not been determined, and it is therefore difficult to compare the rate of hydrolysis of different polysaccharides. The same also applies to comparison with data obtained under strongly acidic conditions where end-group estimations are used for determining the hydrolysis rates.

The object of this investigation was to determine the rate of hydrolysis of alginate and some model substances over a wide range of pH-values.

EXPERIMENTAL

Materials. The alginate was prepared from *Laminaria digitata*, harvested at Tarva 29/8, as previously described.[6] The ratio between mannuronic and guluronic acid residues, M/G, was 1.6 determined according to Haug and Larsen,[7] and the intrinsic viscosity [8] was 18 (100 ml/g).

An acid soluble oligouronide was prepared from the above alginate by partial hydrolysis at 100°C in 1 M oxalic acid.[9] The soluble fraction removed after 1 h of hydrolysis had a number average degree of polymerization of 13 and a M/G-ratio of 1.8. An oligouronide preparation with a lower degree of polymerization was prepared by partial hydrolysis for 10 h in 1 M oxalic acid. The solution was neutralized by adding calcium carbonate, the precipitate removed by filtration, and the solution evaporated to a carbohydrate concentration of approximately 1 %. Ethanol was added to a concentration of 30 % (v/v) and the brown precipitate formed was removed by filtration and discarded. More ethanol was added to a final concentration of 64 %. The precipitate was washed with ethanol and ether, and dried. The number average degree of polymerization was 2 and the M/G-ratio was 2.5.

The methylcellulose was a commercial sample from Mo och Domsjö AB, Sweden. The dextran and dextran derivatives were prepared by Pharmacia, Sweden. The dextran and the dextran sulphate were commercial samples, while the carboxymethyldextran was kindly supplied on request. The number of acidic groups per sugar residues was 1.6 for the dextran sulphate and 1.0 for the carboxymethyldextran.

The cellobiose and the pectic acid were commercial samples from Fluka AG. The cellobiuronic acid and the pseudo-cellobiuronic acid were prepared by Lindberg *et al.*[3]

All the disaccharides were reduced with three times their weight of potassium borohydride overnight at room temperature, and gave zero reducing power at the start of the hydrolysis.

Determination of hydrolysis rates. In all the kinetic experiments 0.05 M citrate buffer was used for pH-values of 2 and higher. Hydrochloric acid was used below pH 2. The acid strengths given in the figures are the amount of hydrochloric acid in excess of that required to liberate the acidic groups of the carbohydrate. The degradation of alginate and methylcellulose was carried out by keeping the solutions in a thermostated bath at 50.1°C. 1 % solutions of methylcellulose and 0.3 % solutions of alginate were used, and samples were removed at intervals for viscosity determinations. Sodium chloride was added to the solutions to 0.1 N, and the viscosity determined in an Ubbelohde No. 2 viscometer. At pH-values below 3, the alginate was not soluble, and a different technique was employed. One part of alginate was suspended in 100 parts of buffer in a large test tube. After a suitable degradation time, the test tube was removed from the water bath, and the contents poured through a glass-sinter filter. The insoluble alginic acid was washed with cold water, suspended in water, dissolved by addition of alkali, and the solution diluted to an alginate concentration of 0.3 %. Sodium chloride was added to 0.1 N before the viscosity measurements. The filtrates did not in any experiments contain significant amounts of carbohydrate (phenol-sulphuric acid reaction). The intrinsic viscosity of the alginate was determined by means of empirical curves [8] relating the viscosity at 0.3 % concentration to the intrinsic viscosity. The intrinsic viscosity of the methylcellulose was found by determining the viscosity at three different concentrations and extrapolating to zero concentration.

Two different techniques were used for determining the hydrolysis rates at 100°C. The two oligouronide samples, the dextran, the dextran sulphate, the pectic acid, and the carboxymethylcellulose, were all boiled under reflux in 0.3 % solutions. To minimize the effect of evaporation on the concentration of carbohydrate, the volume of the solutions was kept large (200 ml at the beginning and not less than 100 ml after the removal of all samples). The carboxymethyldextran and samples of reduced cellobiose (4-*O*-β-D-glucopyranosyl-D-glucitol), cellobiuronic acid (4-*O*-β-D-glucopyranuronosyl-D-glucitol) and pseudocellobiuronic acid (3-*O*-β-D-glucopyranosyl-L-gulonic acid) were heated in sealed glass tubes placed in boiling water. The concentration of carbohydrate in the tubes was 0.05 %. Tubes were removed at intervals. All the degradations at 100°C were followed by determining the reducing power of the solutions according to Nelson.[10]

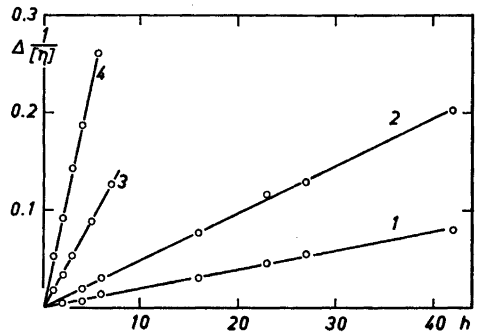


Fig. 1. Homogeneous and heterogeneous hydrolysis of alginate at 50.1°C. 1 = Citrate-HCl, pH 4.15; 2 = Citrate-HCl, pH 3.63; 3 = HCl, 0.123 N; 4 = HCl, 1.23 N.

RESULTS

For a first order random cleavage of the glycosidic linkages in a homopolysaccharide, the inverse of the number average degree of polymerization plotted against time gives straight lines with a slope equal to the rate constant. The inverse of the intrinsic viscosity plotted in the same way also gives straight lines if the intrinsic viscosity is directly proportional to the number average degree of polymerization during the degradation. [2] The results of four degradation experiments with alginate where the degradation was observed by the viscosity decrease are plotted in this way in Fig. 1. Curves 1 and 2 show results obtained at pH-values where the alginate is soluble, while curves 3 and 4 show results of experiments at low pH where the alginate is insoluble. Both the heterogeneous and the homogeneous reactions gave straight lines. The slope of the curves are proportional to the rate constants and are in this work used as such.

The rate of degradation of alginate was determined at different pH-values, and the results, plotted as $\Delta(1/[\eta])$ per hour against acidity, are given in Fig. 2. The acidity is expressed as the pH for pH-values higher than 2 and

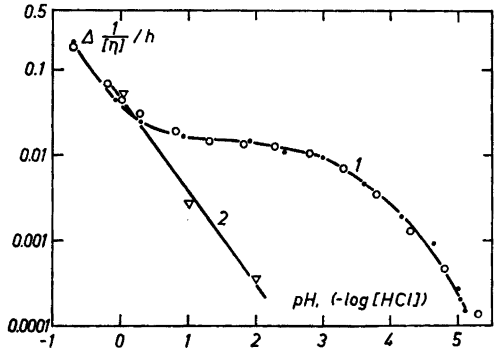


Fig. 2. Hydrolysis of alginate and methylcellulose at 50.1°C.
1 ● = Experimental points for alginate.
1 ○ = Values calculated from eqn. (2) with $k_{HA}' = 0.035$ and $k_{A^-}' = 28$;
2 ▽ = Experimental points for methylcellulose.

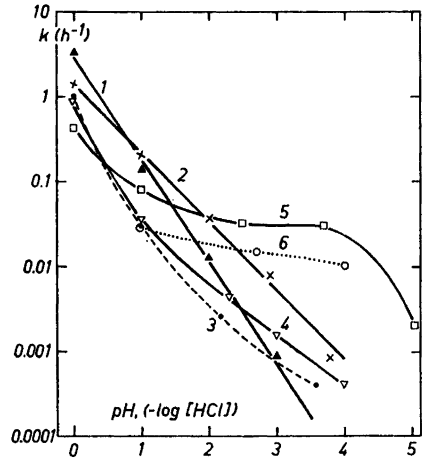


Fig. 3. Hydrolysis at 100°C. 1 = Dextran; 2 = Dextran sulphate; 3 = Carboxymethyldextran; 4 = Carboxymethylcellulose; 5 = Oligouronide, DP = 13; 6 = Pectic acid.

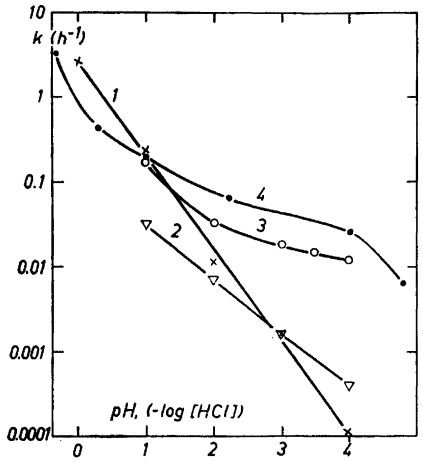


Fig. 4. Hydrolysis at 100°C. 1 = 4-*O*-β-D-glucopyranosyl-D-glucitol; 2 = 4-*O*-β-D-glucopyranuronosyl-D-glucitol; 3 = 3-*O*-β-D-glucopyranosyl-L-gulonic acid; 4 = Oligouronide with DP = 2, reduced with potassium borohydride.

as the negative logarithm of the hydrochloric acid concentrations at higher acidities. A similar experiment was carried out with methylcellulose. Since in this case the curves of $\Delta(1/[\eta])$ against time were slightly curved, the initial slope is taken as proportional to the rate constant. The results are plotted in Fig. 2, and demonstrate a marked difference in the pH-dependence of the rate constants of the two polysaccharides. The rate constant of the degradation of methylcellulose is nearly proportional to the acidity, while the degradation rate of alginate is only slightly dependent on the pH in the region between pH 1 and 3.

Alginate is a poly-electrolyte, and attempts were made to establish if the unusual pH-dependence of the degradation rate was a common feature of acidic polysaccharides. The hydrolysis rate was determined for dextran, dextran sulphate, carboxymethyldextran, carboxymethylcellulose, and pectic acid at different pH-values. An oligouronide, soluble over the whole pH range, was also included. The rate of hydrolysis was determined by measuring the reducing power at intervals during the degradation, which in this case was carried out at 100°. Plotted as a 1st order reaction, straight lines were obtained in all cases for the first part of the degradation (20—30 % bonds broken). The rate constants, taken as the slope of the straight lines, are plotted as a function of the acidity in Fig. 3. The rate constant of the neutral polysaccharide dextran is nearly proportional to the acid concentration, as was the case for methylcellulose. The curve representing the degradation of the dextran sulphate is also a straight line, but with a slope significantly lower than that of dextran. The two carboxymethyl derivatives gave curves with a slope decreasing with increasing pH. The degradation rate of the carboxymethyl

derivatives decreases, however, much more with increasing pH than does the degradation rate of the two uronic acid-containing compounds; pectic acid and the oligouronide.

The results indicate that the unusual pH dependence of the degradation rate of alginate is connected with the uronic acid residues. Further information was obtained by comparing the degradation rates of some low molecular compounds. Cellobiose, cellobiuronic acid and pseudo-cellobiuronic acid were reduced with borohydride and hydrolysed at different pH-values at 100°. A degradation product of alginate, with a number average degree of polymerization of 2, was also reduced and hydrolysed. The samples were reduced in order to avoid degradation from the reducing end during hydrolysis, and in order to obtain more reliable analytical results. The results are given in Fig. 4. The reduced cellobiose (4-*O*-β-D-glycopyranosyl-D-glucitol) was hydrolysed with a rate nearly proportional to the acid concentration. The reduced pseudo-cellobiuronic acid (3-*O*-β-D-glucopyranosyl-L-gulonic acid) and the reduced oligouronide mixture were hydrolysed with a rate showing the same unusual pH dependence as observed for the polyuronides. The curve representing the degradation of the reduced aldobiuronic acid (4-*O*-β-D-glucopyranuronosyl-D-glucitol) was nearly a straight line with a slope lower than that of the neutral cellobiose derivative. The relatively rapid degradation of polyuronides at pH-values above 2 thus seems to be connected with the glycosidic linkage to the C_4 in the uronic acid residue.

DISCUSSION

According to the mechanism of the hydrolysis of the glycosidic linkages described in the introduction, the rate constants should be proportional to the proton activity. The logarithm of the rate constants (log k) plotted against the acidity function of Hammett, H_0, should therefore give straight lines with a slope of unity. A linear relationship between log k and H_0 has been found for methylglycosides.[1,11] The slopes of the curves did, however, deviate from unity and were found to be different both for different acids[1] and for different alkylglycosides.[12] The pH, or the negative logarithm of the hydrochloric acid concentration, only deviates significantly from the Hammett function at high acid concentrations, and also plots of the type given in Figs. 2, 3, and 4 should, according to the described mechanism, give nearly straight lines with a slope close to unity.

The curves for the neutral carbohydrates, methylcellulose (Fig. 2), dextran (Fig. 3), and reduced cellobiose (Fig. 4) were all found to be straight lines with a slope slightly higher than unity, and the results are thus in good agreement with the accepted mechanism and with the results observed for the alkylglycosides. The curve obtained for the dextran sulphate is also a straight line, but with a slope considerably lower than unity. The hydrolysis rate of dextran sulphate is lower than that of dextran at low pH-values, in agreement with experiments with methyl glycosides of sulphated sugars,[4] but the sulphated derivative is hydrolysed at a higher rate than the neutral polysaccharide at higher pH-values. Owing to the negative charge of the polysaccharide, the proton concentration near the chain must be considerably higher than

that in the bulk of the solution when the proton concentration is low, and this effect may explain the higher rate of hydrolysis of the charged polymer at pH-values above 1. The non-linearity of the curves for the carboxymethyldextran and carboxymethylcellulose, may be explained in the same way, taking into account the dissociation of the carboxyl groups in the pH region investigated.

The experiments with the carbohydrates containing uronic acid residues, alginate (Fig. 2), pectic acid and the two oligouronide preparations (Figs. 3 and 4), indicate that a different effect occurs in the hydrolysis of these substances. The hydrolysis curve of alginate in Fig. 2 may be used for a further evaluation of this effect. Formally we can associate one rate constant (k_{AH}) with the proton-catalysed hydrolysis of the undissociated uronic acid residues (AH) and another (k_{A^-}) with the dissociated form (A^-). In addition we must take into account the possible catalysing activity (characterized by a rate constant k_s) of the undissociated carboxyl group. The total rate constant k may, therefore, be described by an equation containing three terms

$$k = k_{HA}\,[HA]\,[H^+] + k_{A^-}\,[A^-]\,[H^+] + k_s\,[HA] \qquad (1)$$

For an acid where the dissociation follows the usual formula

$$\frac{[H^+]\ [A^-]}{[HA]} = K_a$$

the two last terms of eqn. (1) are proportional and the equation may be written as

$$k = k_{HA}[HA]\,[H^+] + k_{A^-}'\,[A^-]\,[H^+] = k_{HA}\,[HA]\,[H^+] + k_{A^-}'K_a\,[HA] \qquad (2)$$

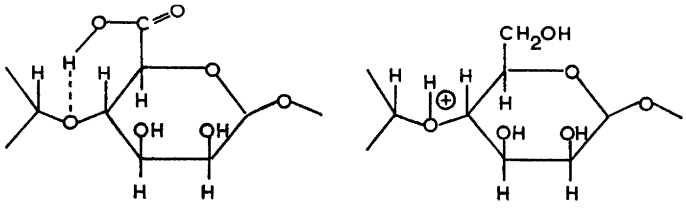
where $k_{A^-}' = k_{A^-} + k_s/K_a$.

The dissociation curves of alginate [13,14] show that the apparent dissociation constant, as usual for polyacids, varies with the degree of dissociation, the ionic strength and the polyacid concentration. The uronic acid composition of the alginate also influences the apparent dissociation constant. When the ionic strength is as high as 0.1, however, the apparent dissociation constant is almost independent of the degree of dissociation, and for an alginate sample with a ratio between mannuronic and guluronic acid residues of 1.6, a dissociation constant of $10^{-3.42}$ should be applicable at room temperature. The degradation of alginate (Fig. 2) was carried out at 50°C and a dissociation constant of $10^{-3.3}$ seems a good approximation in our case. Using this dissociation constant, eqn. (2) was found to give a good fit with the experimental result when values of 0.035 and 28 were chosen for k_{HA} and k_{A^-}', respectively, (Fig. 2, open circles).

The results given in Fig. 2 may be explained either as a much higher rate of degradation of the charged than the uncharged polysaccharide ($k_{A^-}'/k_{HA} \approx 800$), or as an intramolecular catalysis of the undissociated carboxyl groups. As demonstrated by eqn. (2), the shape of the curve correlating the degradation rate and the acidity can not be used to distinguish between these two possibilities. Marchessault and Rånby [2] discussed the

possibility of intramolecular catalysis by carboxyl groups as the cause of weak linkages in cellulose. Capon [15] investigated the hydrolysis of *o*-carboxyphenyl-β-D-glucoside and found the correlation between the rate of hydrolysis and acidity to be of the type given in eqn. (2). Based on some additional evidence, Capon favoured an intramolecular acid catalysis.

In this work we have found a very marked difference between the hydrolysis of polysaccharides containing uronic acid residues, and polysaccharides with carboxymethyl groups. We have also found that the effect of the carboxyl group on the hydrolysis of reduced, acidic disaccharides (Fig. 4) depends very much on whether the carboxyl group belongs to the originally reducing sugar unit or to the non-reducing residue. We consider these observations, which demonstrate the importance of the location of the carboxyl group relative to the glycosidic linkage, to be in favour of the hypothesis of an intramolecular acid catalysis of the type shown in the formula (A).



For comparison, the conjugate acid supposed to be an intermediate in the ordinary acid hydrolysis is also shown (B). It should be emphasized that the spatial arrangement of the carboxyl group in the reduced disaccharide is slightly different (owing to the open-chain structure) from the arrangement in the polymer.

We should finally like to point out some practical consequences of our findings. Polyuronides have generally been regarded as being more stable to acid hydrolysis than neutral polysaccharides. This is the case only at low pH-values (pH$<$1), where the acid hydrolysis usually is carried out. At higher pH-values polyuronides are hydrolysed at a rate much higher than neutral polysaccharides. It is possible that this may be utilized in structural work. Neutral polysaccharides containing small amounts of uronic acid residues, *e.g.* commercial samples of cellulose, should, according to our results, be expected to contain a small amount of linkages which are broken at a much higher rate than the rest of the linkages at low acid concentrations (pH$>$2). Such weak linkages in cellulose are, as pointed out by Grassie,[16] most frequently observed under mild acid conditions.

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