Note

A kinetic study of the esterification and glycosylation of D-galacturonic acid and its α-D-(1→4)-linked di- and tri-saccharide analogs with methanol*

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Jansen and Jang[1] demonstrated that the rate of esterification of D-galacturonic acid with methanol was about 25 times greater than that of glycoside formation at 25°, whereas at 0° the differential in rates was 55 times. They also demonstrated that pectic acid, under the same reaction conditions, undergoes esterification at a much lower rate. The latter observation could be explained on the basis of lack of solubility of the pectic acid. However, they also showed that alginic acid under the same conditions was esterified much more readily.

Since the methyl esters of oligosaccharides of D-galacturonic acid were required for studies with pectic enzymes, and because of the above observations, an investigation of the esterification and glycosylation of D-galacturonic acid (monomer) and its α-D-(1→4)-linked disaccharide (dimer), and trisaccharide (trimer) analogs was undertaken, and is reported here.

EXPERIMENTAL

General. — The preparation and characterization of the oligosaccharides of D-galacturonic acid has been reported[2]. The Dowex-50 resin (H^+ form, 100–200 mesh) was washed with abs. methanol, air-dried and then stored in a vacuum desiccator over calcium chloride. The exchange capacity, as determined by titration with sodium hydroxide, was 3.69 mequiv. per g. The 2,2-dimethoxypropane was obtained from Eastman Organic Chemicals, Rochester, New York. Abs. methanol (J. T. Baker Chemical Co., Phillipsburg, N. J.) was used directly without further treatment.

Preparation of reaction mixtures. — In all experiments the final concentration of substrate was 1.1% (w/v). The substrate was dissolved in methanol and sufficient M methanolic hydrogen chloride was added (1/10 dilution) to give a final concentration of 0.1 M. The M methanolic hydrogen chloride was always prepared on the day of use.

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In the case of the reaction catalyzed by Dowex-50, a sufficient amount of resin was added to give a final concentration of 0.1 mequiv. per ml (0.282 g/10 ml). A magnetic stirrer was used to provide proper mixing of the resin. All reaction-mixtures were incubated in a water-cooled water bath at 20±0.5° or in a melting ice-bath (0°).

Analysis of reaction rates. — The rate of esterification was determined by removing 1-ml samples at suitable time-intervals. The reaction was stopped by adding the sample to distilled water (in the case of the H^+-catalyzed reactions) or by removing the Dowex-50 resin by vacuum filtration through a sintered-glass filter and washing it with water. Samples were titrated to pH 7.0 with an automatic Fisher Titrimeter by using 10 mM sodium hydroxide. Carbon dioxide-free air was bubbled through the solution during titration. The hypoiodite technique[3] was used to measure the extent of glycosylation. Prior to analysis, the acetone formed from the 2,2-dimethoxypropane was removed by passing a stream of air over the sample for 45–60 min. The results are plotted as % of carboxyl or aldehyde groups remaining *vs.* time.

*Isolation of a monomethyl ester of 4-*O*-(α-*D*-galactosyluronic acid)-*D*-galacturonic acid.* — The dimer (5 g) was dissolved in 455 ml of methanol in a 500-ml flask. Dowex-50 resin (H^+, 12.8 g) was added and the reaction was allowed to proceed, with magnetic stirring, for 75 min at 20°. The resin was removed by filtration and washed with water. The methanol was removed by concentration of the filtrate on a rotary evaporator under vacuum at 30°. The sample was added to a column (3.5 × 38 cm) of Dowex-1 (100–200 mesh) in the formate form. The elution schedule was as follows: 1100 ml of water; 2 liters of linear gradient from 0.1M to 0.3M of formate (pH 4.7); and 500 ml each of 0.4, 0.5, and 1.0M sodium formate. Fractions of ~40 ml were collected. The monomethyl ester of the dimer (fractions 29–44) was passed over Dowex-50 (H^+) and freeze dried (yield 1.4 g). Since paper chromatography indicated the presence of a small proportion of free dimer, the sample was rechromatographed on a second column (2.4 × 44 cm) of Dowex-1 (formate). The yield of monomethyl ester of the dimer after treatment with Dowex-50 (H^+) and freeze drying was 0.82 g; $-CO_2Me/-CO_2H = 0.93$.

RESULTS AND DISCUSSION

The relative rates of esterification of monomer, dimer, and trimer with 0.1M hydrogen chloride are shown in Fig. 1. As observed by Jansen and Jang[1], the esterification reaction of the monomer was first-order. However, the dimer and trimer showed an initial, curvilinear rate, with a secondary rate that appeared to be first-order. Essentially the same observations can be made for the reactions catalyzed by Dowex-50 (Fig. 2) and for the reactions conducted at 0° (Fig. 3). Hamann *et al.*[4] stated that only in the later stages of esterification "are conditions achieved which yield sensible results". However, they were referring to reaction conditions where only the reactants (alcohol and acid) are present in approximately equivalent amounts. Furthermore, the data of Flory[5,6] indicate that the observed rates are lower in the initial stages. This behavior is exactly opposite to our observations with the uronic

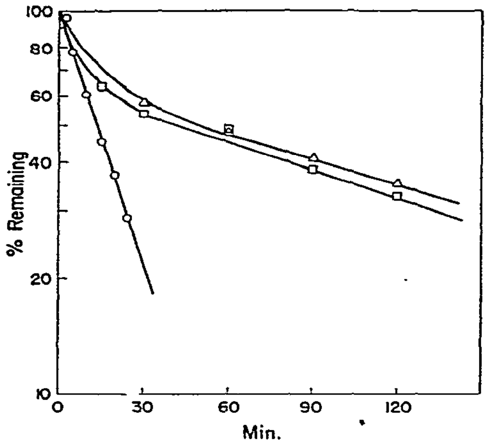

Fig. 1. Esterification of monomer (○), dimer (□), trimer (△) in 0.1M methanolic hydrogen chloride at 20°.

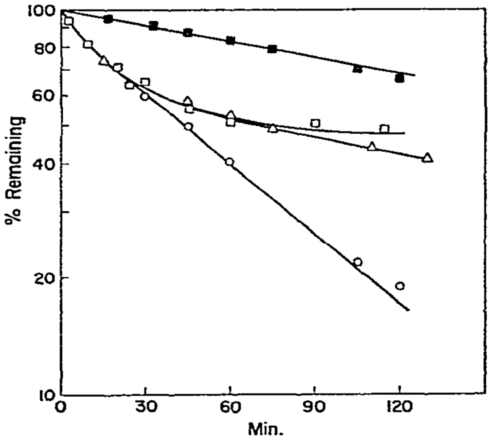

Fig. 2. Esterification of monomer (○), dimer (□), trimer (△), and monomethyl ester of dimer (■) with Dowex-50 (H^+) at 20°.

acids. Our reaction conditions differ in that methanol was used in large excess, so that the uronic acids were in a dilute solution. Because of the large excess of methanol, a first-order reaction would be expected, since only the concentration of uronic acid is limiting. This is, indeed, the case with D-galacturonic acid and the monomethyl ester of the dimer. In addition, a dicarboxylic acid (tartaric acid) gave first-order kinetics under the same conditions when Dowex-50 (H^+) was used as catalyst.

Further evidence to support the contention that esterification of the second carboxyl group in the dimer occurs at a lower rate is obtained from studies of the rate of esterification of the monomethyl ester of the dimer (Fig. 2) with Dowex-50. First-order kinetics are observed for the beginning of the reaction. The slope is similar to that observed for the trimer. No initial, rapid rate is observed. This evidence

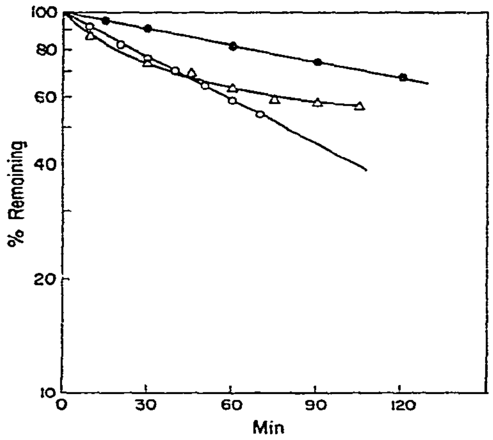

Fig. 3. Esterification of monomer (○) and trimer (△) in 0.1M methanolic' hydrogen chloride, and monomer (●) with Dowex-50 (H^+) at 0°.

would, therefore, support the conclusion that esterification of the first carboxyl group occurs at a more rapid rate than that of the remaining carboxyl groups.

From these data it can be concluded that, in the case of the dimer and trimer, since the initial rate of esterification is comparable to that of the monomer, either one of the carboxyl groups is esterified more readily or else once one of the carboxyl groups is esterified, this ester somehow hinders esterification of the carboxyl groups remaining. Although no direct evidence can be cited, the most likely explanation is that one of the carboxyl groups is more susceptible to esterification. Solubility is not a factor, and steric effects of an esterified carboxyl group that would influence the adjacent carboxyl group seems improbable because of the spatial relationships involved. From a study of structural models, if one of the carboxyl groups is more readily esterified, the most likely one is that located on the non-reducing and of the molecule.

Table I summarizes the rate constants determined for the first-order reactions. Dowex-50 (H^+) at the same concentration as hydrogen chloride resulted in lower rates of esterification. In addition, the rate constant for the monomethyl ester of the dimer is less than 25% of that observed for the monomer. Essentially identical rates of glycosylation of D-galacturonic acid were obtained with the two catalysts. With the trimer, Dowex-50 yielded a lower rate than with hydrogen chloride as the catalyst.

It is apparent that substitution at C-4 of the galacturonic acid interferes with the rate, since the constants for the dimer and trimer were approximately 5% of that obtained for the monomer. Since mutarotation constants for disaccharides are similar to those observed for monosaccharides and it is presumed that ring opening is required for this reaction to occur, it follows that substitution at C-4 of D-galacturonic acid would not be expected to interfere with ring opening. Therefore, this substituent must exert its effect in another manner. Schmidt and Neukom[7,8] have shown with D-galacturonic acid that, after formation of the ester, the first products formed are the methyl α- and β-furanosides. The pyranoside is formed at a much lower rate, presumably

TABLE I

RATE CONSTANTS FOR REACTION OF D-GALACTURONIC ACID AND ITS α-D-(1→4)-LINKED OLIGOMERS WITH METHANOL

Substrate	*Catalyst*	*Temp. (°C)*	*$10^6 \times k$ (sec^{-1})*
Esterification			
Monomer	H^+	20	830
Monomer	H^+	0	140
Monomer	Dowex-50	20	240
Monomer	Dowex-50	0	56
Monomethyl ester of dimer	Dowex-50	20	56
Glycosylation			
Monomer	H^+	20	50
Monomer	Dowex-50	20	56
Dimer	H^+	20	3.1
Trimer	H^+	20	2.6
Trimer	Dowex-50	20	2.1

from the furanoside. Since, in the dimer and trimer, the furanose ring cannot be formed because of substitution at C-4, it would seem logical to conclude that this in turn influences glycosylation. However, the same mutarotational information cited here tends to refute this hypothesis.

It is possible that formation of the methyl glycosides of substituted uronic acids occurs without opening of the ring. If so, the rate would be predictably lower, because of restriction of conformational changes. Thus, glycosylation of the dimer or higher oligomers could involve either an A-1 heterolytic cleavage or a nucleophilic displacement of a protonated, anomeric hydroxyl-group. Under the conditions used here, first-order kinetics are observed from the beginning, regardless of the nature of the substrate (Fig. 4).

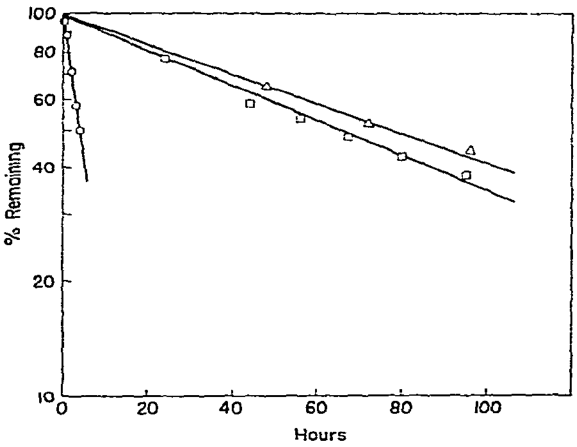

Fig. 4. Glycosylation of monomer (○), dimer (□), and trimer (△) in 0.1M methanolic hydrogen chloride at 20°.

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