beta-ELIMINATION IN URONIC ACIDS: EVIDENCE FOR AN ElcB MECHANISM

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ABSTRACT

Methyl esters of methyl pyranosides of 2,3,4-tri-*O*-methyl-α-D-mannuronic acid (**3**), -α-D-glucuronic acid (**2**), and -β-D-galacturonic acid (**1**) undergo rapid *beta*-elimination of the 4-methoxyl group in 0.50, 0.20, and 0.02M sodium methoxide in methanol at room temperature to give 4,5-unsaturated esters *via cis*, *cis* and *trans* elimination, respectively. The ease of *cis* elimination in methyl (methyl 2,3,4-tri-*O*-methyl-α-D-glucopyranosid)uronate (**2**) and methyl (methyl 2,3,4-tri-*O*-methyl-α-D-mannopyranosid)uronate (**3**) to form the 4,5-unsaturated glycoside is explained by ring flexibility which allows a change in conformation. Only the methyl (methyl 2,3,4-tri-*O*-methyl-α-D-mannopyranosid)uronate (**3**) yields a 2,3:4,5-di-unsaturated ester (**7**) (*trans* elimination), and this occurs in 0.50M methoxide, in a much slower reaction. The favoring of *trans* elimination over the *cis* elimination that would have been needed in the other two compounds in order to form the second double bond is explained by the rigidity of the half-chair conformation of the 4,5-unsaturated esters. It is suggested that both eliminations proceed *via* an ElcB mechanism. The results of treatment of hydroxypropyl alginate with potassium hydroxide confirmed that its depolymerization is much slower, and more dependent on the concentration of alkali, than that of pectin.

INTRODUCTION

Ordinary, internal glycosidic linkages of a polysaccharide are considered to be alkali-resistant in the absence of oxygen, except under extreme conditions of alkalinity and temperature. However, it has been known for some time that anaerobic, alkaline de-esterification of pectin (galacturonoglycan methyl ester) to produce pectate (pectic acid, galacturonoglycan, polygalacturonate) results in extensive depolymerization that is complete when de-esterification is complete[1–6].

Kenner[7], Whistler and BeMiller[8], and Neukom and Deuel[9] explained the observed depolymerization as a *beta*-elimination. According to this mechanism, the carbonyl group of the methyl ester, through its electron-withdrawing power, makes the *alpha* proton (H-5) sufficiently acidic to be removed by alkali[7]. Hence, pectin, which has a true ester carbonyl group and whose glycosyl units are C-4 substituted,

is depolymerized in dilute alkaline solutions. Pectate, which has a free carboxyl group, is quite stable under the same conditions[10], for the carboxylate anion has much less electron-withdrawing capacity, because of charge dispersal[10,11].

Therefore, depolymerization and de-esterification are competitive reactions and, because esters of α,β-unsaturated acids are slowly hydrolyzed[12], they should reach completion simultaneously. Vollmert[5] verified this experimentally when he found, from osmotic-pressure measurements on solutions of the nitrate derivative of pectin, that depolymerization in alkali ceases when de-esterification is complete. Launer and Tomimatsu[13] reported that, at 25°, all reactions of pectin in a buffer of pH 10.2 are complete in 30 min and average about one glycosidic bond cleavage for each 80 ester groups hydrolyzed. Skola and Fak[14] studied the reaction of pectin with ammonia in the presence of calcium salts at temperatures ranging from 3.5° to 24°, and also found that degradation is a reaction concurrent with de-esterification, and that the ratio between ester hydrolysis and glycosidic cleavage is constant at a given temperature.

Pectin is also unstable in neutral solutions, being degraded in a temperature-dependent reaction[10,15–18]. Again, the rapid decrease in viscosity and increase in reducing end-groups is indicative of splitting of glycosidic bonds[18]. When held in a buffer (pH 6.8) for 1 h at 95°, citrus pectin containing 70% of esterified carboxyl groups had 6.3% of the original glycosidic bonds broken, and a decrease in the ester content to 27%; the product was determined to be a 4,5-unsaturated compound[10]. In an investigation of a model compound, Heim and Neukom[19] showed that the methyl glycoside of dimethyl di-D-galactopyranuronate is readily cleaved by cold sodium methoxide to yield methyl D-galacturonate and methyl (methyl α-D-*xylo*-hex-4-enopyranosid)uronate.

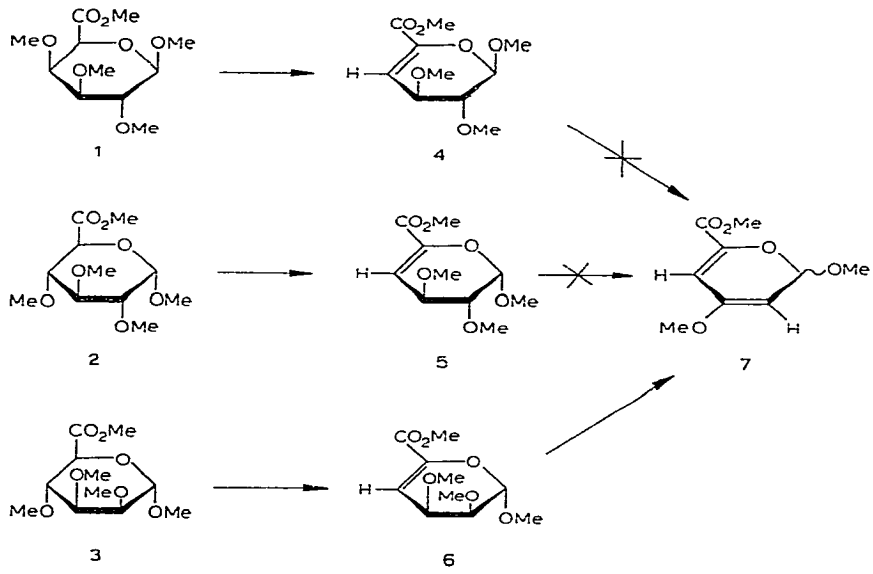
Alginates (mannuronoglycans) behave similarly, that is, their esters are depolymerized, whereas their salts are not[20]. However, the reaction is slower, and is more pH-dependent[21]. It has been suggested that this behavior is due to the fact that *beta*-elimination of an alginate would be a *cis* (*a,e*) elimination, whereas that of pectin would be a *trans* (*a,a*) elimination when each is in the $^4C_1$ (D) conformation[10,22].

However, Huang[23] studied the effect of 0.1M potassium hydroxide at 50° on methyl (methyl 4-*O*-methyl-D-glucopyranosid)uronate (which has the same configuration at C-4 as has D-mannuronic acid and, hence, undergoes *cis beta*-alkoxy elimination and ester hydrolysis), and found that both reactions occur very rapidly, with the production of 4 to 6 carboxylate anions for each double bond (α,β-unsaturated ester).

It was the purpose of the present investigation to examine *beta*-alkoxy elimination reactions in carbohydrate systems, in order to determine (*a*) the relative rates of *cis* and *trans* eliminations and (*b*) whether these differences can be used to explain the different behaviors of pectin and alginates. Methyl esters of methyl pyranosides of 2,3,4-tri-*O*-methyl-β-D-galacturonic acid (**1**), -α-D-glucuronic acid (**2**), and -α-D-mannuronic acid (**3**) were chosen. In each, elimination of the 4-methoxyl group could occur, perhaps followed by the removal of H-3, activated by the vinylic system, and elimination of the 2-methoxyl group. Eliminations in these compounds would be

trans-cis, cis-cis, and cis-trans respectively. In order to overcome competitive de-esterification, sodium methoxide in methanol was chosen as the base.

The depolymerization of alginates was also further examined, in order to substantiate that the depolymerization of alginic esters is slower, and more dependent on the concentration of alkali, than that of pectin.



EXPERIMENTAL

Chromatography. — Paper-chromatographic separations were performed at room temperature, by the ascending method, on Whatman No. 4 paper with 6:4:3 (v/v) butyl alcohol–pyridine–water. The components were detected with a silver nitrate–sodium hydroxide spray[24].

Thin-layer chromatographic separations were achieved on plates of Silica Gel H (thickness, 0.25 mm), dried for 2 h at room temperature and then activated for 1 h at 130°, and developed in the following solvent systems: solvent *A*, 4:1 (v/v) petroleum ether (b.p. 30–60°)–acetone; solvent *B*, 9:6:3:1 (v/v) butyl alcohol–acetic acid–ether–water. Components were located by spraying with a 50% solution of sulfuric acid in ethanol and heating the plate for 15 min at 130° (oven) and, on preparative plates, by u.v. irradiation.

*Oxidation of methyl 2,3,4-tri-*O*-methyl-*D*-hexopyranosides.* — *A. Methyl (methyl 2,3,4-tri-*O*-methyl-α-*D*-mannopyranosid)uronate* (3). Methyl α-D-mannopyranoside was prepared from D-mannose by treating it with 2% methanolic hydrogen chloride[25]; m.p. 188–190° (lit.[25] m.p. 190–191°). Methyl 6-*O*-trityl-α-D-mannopyranoside was prepared from methyl α-D-mannopyranoside by treating it with chlorotriphenylmethane for 5.5 h at 100–105° (Ref. 26). Complete methylation, followed by detrityla-

tion, oxidation with alkaline permanganate, and esterification[27], yielded **3**; $[\alpha]_D^{30}$ +45° (*c* 0.94, chloroform) [lit.[27] $[\alpha]_D^{18}$ +45° (*c* 1.0, chloroform)]. This preparation contained a single component (t.l.c.).

*B. Methyl (methyl 2,3,4-tri-*O*-methyl-α-*D*-glucopyranosid)uronate* (**2**). Methyl 6-*O*-trityl-α-D-glucopyranoside was prepared from commercial methyl α-D-glucopyranoside by reaction with chlorotriphenylmethane for 5.5 h at 100–105°. Complete methylation, followed by detritylation, oxidation with alkaline permanganate, and esterification, yielded **2**; $[\alpha]_D^{30}$ +90° (*c* 1.0, water) [lit.[28] $[\alpha]_D^{18}$ +87° (*c* 1.0, water)]. This preparation contained several components, as determined by t.l.c.

Direct oxidation of methyl D-*hexopyranosides with potassium ferrate* (K_2FeO_4). — *A. Methyl (methyl 2,3,4-tri-*O*-methyl-α-*D*-glucopyranosid)uronate* (**2**). Methyl α-D-*gluco*-hexodialdo-1,5-pyranoside[29] was oxidized with sodium hypoiodite[30] to methyl α-D-glucopyranosiduronic acid, and the reaction mixture was passed through a column of Amberlite IR-120 (H^+) cation-exchange resin. The pH of the eluate was adjusted to 7.5–8.0 with barium hydroxide solution, and the resulting solution was concentrated to about 100 ml and filtered; ethanol (2–3 volumes) was added to the filtrate to precipitate barium (methyl α-D-glucopyranosid)uronate, which was collected by filtration, washed well with 75% ethanol, and redissolved in hot water; then the precipitation and washing were repated. Finally, the precipitate was dissolved in hot water, the solution was treated with Amberlite IR-120 (H^+) cation-exchange resin to remove barium ions, and filtered, and the filtrate was evaporated to a pale-yellow syrup of methyl α-D-glucopyranosiduronic acid; $[\alpha]_D^{30}$ +140° (*c* 1.0, water) [lit.[31] $[\alpha]_D$ +129° (*c* 1.0, water)].

Methyl α-D-glucopyranosiduronic acid was methylated according to the method of Anderson and Cree[32]. The methylated compound (**2**) was purified by preparative, thin-layer chromatography with solvent *A*: $[\alpha]_D^{30}$ +153° (*c* 1.0, water) [lit.[28] $[\alpha]_D^{18}$ +87° (*c* 1.0, water)]. This preparation contained a single component as determined by t.l.c.

*B. Methyl (methyl 2,3,4-tri-*O*-methyl-β-*D*-galactopyranosid)uronate* (**1**). Methyl β-D-galactopyranoside was prepared by treating D-galactose with 2% methanolic hydrogen chloride[25,33]. Methyl β-D-galactopyranosiduronic acid was prepared from methyl β-D-galactopyranoside by successive ferrate and hypoiodite oxidations[29,30]. Methylation to afford the title compound was effected according to the method of Anderson and Cree[32]: $[\alpha]_D^{30}$ −8.0° (*c* 0.33, methanol) [lit.[34] $[\alpha]_D^{18}$ −20° (*c* 1.2, methanol)].

*C. Methyl (methyl 2,3,4-tri-*O*-methyl-α-*D*-mannopyranosid)uronate* (**3**). The title compound was prepared by ferrate[29] and hypoiodite[30] oxidations of methyl α-D-mannopyranoside, followed by methylation; $[\alpha]_D^{30}$ +53° (*c* 1.0, chloroform) [lit.[27] $[\alpha]_D^{18}$ +45° (*c* 1.0, chloroform)].

Reaction with base. — All reactions of the foregoing compounds were performed in a cuvet at 30 ±0.5° with base of known concentration, and absorbances were measured from 320 to 200 nm with a Beckman-DB spectrophotometer. Before reaction, the esters were dissolved in reagent-grade methanol, and the absorption spectra

of these solutions were recorded and examined. Each had an absorption maximum at 209 nm, and all solutions were adjusted to the same absorbance at 209 nm by dilution with methanol.

To 1.5 ml of each of these ester solutions in a cuvet was added 1.5 ml of the base (known strength), and the absorbance was quickly scanned from 320 to 200 nm. After the reaction was complete, the reaction mixture was made neutral with Amberlite IR-120 (H^+) cation-exchange resin, the suspension filtered, and the filtrate evaporated under diminished pressure.

Depolymerization of alginates. — All degradations were conducted with 1%, oxygen-free, aqueous solutions of polysaccharide. Hydroxypropyl alginate* was treated with 0.1M potassium hydroxide at 25° and at 50°. Also, hydroxypropyl alginate was placed in a solution of potassium hydroxide (pH 8.1) at 50°.

Sodium alginate (13.8% moisture) was treated with 0.1M potassium hydroxide at 50°.

Determination of uptake of bromine[35]. — Aliquots (50 ml) of a solution of the material under study were placed in 250-ml bottles, and neutralized to the Methyl Orange end-point with 0.1M hydrobromic acid. Phosphate buffer (2M; pH 6.8; 10 ml) was added to each, and the solutions were cooled to ~5° in a refrigerator; the final pH was 6.7–6.8. Then the pressure in the bottles was diminished with a water aspirator, 0.1M Br_3^- reagent (20 ml) was added without introduction of air, and the bottles were shaken in the dark for 1 h at 15°. Potassium iodide (15%; 10 ml) and 0.5M sulfuric acid (10 ml) were added, the vacuum was released, and the contents were titrated with 0.05M sodium thiosulfate, with starch as the indicator. Blank determinations were conducted on 0.1M potassium hydroxide.

Determination of ester hydrolysis. — Aliquots (10 ml) of a solution of the material under study were placed in bottles containing 0.05M sulfuric acid (10 ml). The solutions were back-titrated with 0.1M potassium hydroxide to the phenolphthalein end-point.

RESULTS AND DISCUSSION

A new approach to the synthesis of methylated glycosiduronic esters has been used, *viz.*, oxidation to the uronic acid followed by permethylation, as follows. Methyl D-glycopyranoside $\xrightarrow{\text{potassium ferrate}[29]}$ methyl D-hexodialdo-1,5-pyranoside $\xrightarrow{NaOI}$ methyl D-glycopyranosiduronic acid $\xrightarrow[\text{methyl iodide, DMF}]{\text{sodium hydride}}$ methyl (methyl 2,3,4-tri-*O*-methyl-D-glycopyranosid)uronate.

Before this sequence was tried, published methods[27,28] were used for preparing the permethylated uronic acids; however, a number of problems were encountered, primarily involving methylation of tritylated glycosides and extraction of methylated compounds with water after detritylation. Complete methylation of methyl 6-*O*-trityl-

*Kelcolloid O, an 80% propylene glycol ester (15.9% moisture) of alginic acid, from the Kelco Co., New York, N. Y.

β-D-galactopyranoside could not be accomplished, possibly because of steric hindrance by the bulky 6-*O*-trityl group to the entering methyl groups. The optical rotations of the compounds that we prepared by the published procedures agreed with literature values, but, in some cases, t.l.c. revealed that the products were actually mixtures of several compounds. The new method gives chromatographically pure products.

Sodium methoxide in methanol was used as the base in the *beta*-elimination studies in order to exclude the competitive reaction of de-esterification. Elimination of a *beta*-alkoxyl group from methylated glycopyranosiduronates can occur when the proton at C-5 is removed by the base. The remaining pair of electrons shift to form a double bond between C-4 and C-5, with elimination of a methoxide anion from C-4.

It may be assumed that three steps are involved when methyl (methyl 4-*O*-methyl-β-D-glucopyranosid)uronate (**2**) is the substrate: (*a*) removal of the proton on C-5, to form a carbanion, (*b*) conformational "inversion", and (*c*) elimination (*i.e.*, an ElcB mechanism), and also that **1** and **3** react by the same mechanism. Inversion after anion formation is possible in the monomeric model compound, which has considerable ring flexibility and can readily undergo change of conformation, and this could lead to overall *cis* elimination.

To determine whether elimination in a *trans* system is favored over that in a *cis* system, the formation of double bonds in the products from the methyl esters of methyl pyranosides of 2,3,4-tri-*O*-methyl-β-D-galacturonic acid (**1**), -α-D-glucuronic acid (**2**), and -α-D-mannuronic acid (**3**) was studied with various concentrations of base at 30 ±0.5°. It was found that the formation of products having 4,5-unsaturation, which causes absorption at ~220 nm, from methyl esters **1**, **2**, and **3** is dependent upon the concentration of the base. The formation of the 4,5-unsaturated products was very rapid with 0.50, 0.20, and 0.02M sodium methoxide in methanol. Absolute rate-constants could not be determined at any of these base concentrations, because of the speed of reaction. There was no measurable formation of unsaturation with lower concentrations of the base (for example, 2mM).

The formation, with time, of a second double-bond (2,3-unsaturation), which causes absorption at ~232 nm, was found for the D-mannuronic acid ester (**3**) treated with 0.50M base (see Table I). As the amount of the 2,3-unsaturated compound **7** increased, the peak became broader, and λ_{max} shifted from 228 to 232 nm. Esters **1** and **2**, so treated, showed no increase in absorbance at 232 nm (which would have been indicative of conjugated double bonds). Hence, for this second elimination, there is a real difference in the ease of *trans* and *cis* elimination.

After neutralization, and evaporation under diminished pressure, of the reaction mixture from methyl (methyl 2,3,4-tri-*O*-methyl-α-D-mannopyranosid)uronate (**3**), t.l.c. revealed two new components. One of these (having R_F 0.76) is probably the compound possessing a single double bond (4,5-unsaturation), and the second (R_F 0.68), the compound with two double bonds (2,3:4,5-di-unsaturation); the starting compound (**3**) has R_F 0.80. The formation of 2,3-unsaturation to give the pyran derivative (**7**) occurs because of the acidity of the proton on C-3 and the favored

TABLE I

REACTION OF METHYL ESTERS OF METHYL 2,3,4-TRI-*O*-METHYL-β-D-GALACTOPYRANOSIDURONIC ACID (**1**), -α-D-GLUCOPYRANOSIDURONIC ACID (**2**), AND -α-D-MANNOPYRANOSIDURONIC ACID (**3**) WITH 0.50M SODIUM METHOXIDE IN METHANOL[a]

Time (min)	*Compound* **1** A_{228}	*Compound* **2** A_{228}	*Compound* **3** A_{232}
0	0.38	0.27	0.35
5	—	—	0.38
15	0.415	0.27	0.42
30	0.43	0.27	0.48
45	—	—	0.55
60	0.47	0.28	0.63
75	—	—	0.71
95	—	—	0.79
120	—	—	0.85
180	—	—	1.05
270	—	—	1.10

[a]The reaction mixture contained 1.5 ml of ester solution and 1.5 ml of 0.50M sodium methoxide in methanol at 30 ±0.5°.

trans-elimination. A greater concentration of base was needed in order to effect this elimination. Elimination resulting in 2,3-unsaturation takes place in a compound (**6**) having a fairly rigid ring in which no conformational "inversion" is possible as the 4,5 double-bond fixes it in a half-chair conformation.

Whereas no evidence was adduced for the formation of 2,3-unsaturation (*cis*-elimination) in the products from **1** and **2**, there is spectral evidence for the formation of a product having a second double-bond (2,3-unsaturation) from the D-mannuronic ester **3** (*trans*-elimination). Owing to the relative rigidity of the ring of the starting compound (the 4,5-unsaturated ester **6**), a distinction between the E2 and ElcB mechanisms is not possible. However, because of the similarity to the first elimination (*i.e.*, presence of a poor leaving-group and of conditions conducive to carbanion stabilization), it is likely that this elimination, too, proceeds *via* an ElcB mechanism.

Taken together, these data indicate that the rigidity of the ring determines whether or not *trans*-elimination is favored over *cis*-elimination, and that it is favored only when the ring is relatively inflexible. These considerations, therefore, apparently constitute further evidence for the ElcB mechanism, for which direct evidence has recently been obtained by Crosby and Stirling[35].

To compare the depolymerization of alginic esters with that of pectin[1–6], hydroxypropyl alginate was treated with 0.1M potassium hydroxide at 50°. After 60 h, 171 mmoles of bromine were consumed per esterified unit, and 26% of the ester groups were hydrolyzed (see Table II). Therefore, 1.5 ester groups were hydrolyzed per glycosidic bond broken. When the depolymerization was conducted at 25°, 0.05 mole of bromine was consumed per esterified unit after 3 h, and this value remained constant thereafter; after 5 h, 2% of the ester groups had been hydrolyzed

and this value, too, then remained constant. Therefore, 1 ester group was hydrolyzed per 2.5 glycosidic bonds broken.

TABLE II

DEGRADATION[a] OF KELCOLLOID O IN 0.1M POTASSIUM HYDROXIDE AT 50°

Time (h)	*Δ ml of 0.1M KOH*	*Δ ml of 0.05M* $S_2O_3^{2-}$
0	0	0
2	0.14	0.80
5	0.135	1.31
12	0.295	1.92
18	0.41	2.85
24	0.66	4.44
36	0.74	4.56
42	0.87	5.10
48	0.97	5.40
60	1.01	5.81

[a]The reaction mixture consisted of 1% of Kelcolloid O in oxygen-free, 0.1M potassium hydroxide.

When hydroxypropyl alginate was dissolved in a solution of potassium hydroxide (pH 8.1) at 50°, little color had been produced after 32 h, and there was little change in viscosity; little glycosidic-bond cleavage or ester hydrolysis had taken place. The pH after 78 h was 5.1 (see Table III). On treatment of sodium alginate

TABLE III

DEGRADATION[a] OF KELCOLLOID O AT 50° (INITIAL pH 8.1)

Time (h)	*Δ ml of 0.1M KOH*	*Δ ml of 0.05M* $S_2O_3^{2-}$
0	0	0
6	0.033	0.01
12	0.05	0.04
24	0.033	0.03
30	0.047	0.43
36	0.067	0.48
48	0.087	0.19
54	0.083	0.39
60	0.067	0.35
72	0.087	0.43
78	0.107	0.51

[a]The reaction mixture consisted of 1% of Kelcolloid O in an oxygen-free, solution of potassium hydroxide (pH 8.1).

with 0.1M potassium hydroxide at 50°, 100 mmoles of bromine were consumed per uronic acid unit after 60 h; this is equivalent to cleavage of 10% of the glycosidic bonds (see Table IV).

TABLE IV

DEGRADATION OF SODIUM ALGINATE[a] IN 0.1M POTASSIUM HYDROXIDE AT 50°

Time (h)	*Δ ml of 0.1M KOH*	*Δ ml of 0.05M* $S_2O_3^{2-}$
0	0	0
6	0.10	0.45
12	0	0.90
24	0.29	2.06
30	0.37	2.40
36	0.41	3.05
48	0.29	3.93
54	0.30	4.59
60	0.32	4.95

[a]The reaction mixture consisted of 1% of sodium alginate in oxygen-free, 0.1M potassium hydroxide.

Thus, there is a facile *beta*-elimination reaction in polymers of (1→4)-linked D-galactopyranosiduronic acid esters where ring rigidity dictates a *trans* (*a,a*) elimination, and a much slower, more base-dependent, *beta*-elimination in polymers consisting of D-mannopyranosiduronic acid esters linked (1→4) where ring rigidity dictates a *cis* (*a,e*) elimination, as shown in this work and that of others[20,21].

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