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Note

Unusual properties of glycuronans [poly(glycosyluronic) compounds]

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Mucoid variants of *Pseudomonas aeruginosa* isolated from a variety of patients have been shown to produce slime polysaccharides closely related to the alginic acids[1,2]. These polymers have high molecular-weights and contain (1→4)-linked units of β-D-mannopyranosyluronic acid and α-L-gulopyranosyluronic acid[3].

Though the bacterial polysaccharides appear to be closely related to the alginic acids, they do show significant differences and some unusual properties. The Pseudomonas polymers are highly viscous, contain variable amounts of *O*-acetyl groups, do not precipitate at an acid pH, and, when having a high D-mannuronic acid content, they show a large loss of color in the orcinol reaction[4] for uronic acids (but not in the carbazole reaction[5]) after alkali treatment[1]. This loss in color value appears to be reversible by brief treatment with alginases[1]. In addition, the infrared spectra and the alginase-degradation products of seaweed alginic acids and bacterial-slime polysaccharides are different[3]. However, the major backbone structures appear to be the same, and polymers from both sources show a large variation in D-mannuronic to L-guluronic acid ratio. Therefore, the unusual properties of the slime polysaccharides that differentiate them from the alginates may be due to the presence of *O*-acetyl groups or to the particular block-like arrangement of uronic acids[6] in the polymers. This paper is concerned with an investigation of some of the chemical properties of these glycuronans.

EXPERIMENTAL

Analytical methods. — Uronic acid content was determined by the orcinol method[4] and a modified carbazole procedure[5] with D-mannuronic acid as the standard. *O*-Acetyl groups were determined by a colorimetric method[7] or by g.l.c.[8], and reducing sugar by a ferricyanide procedure[9] with D-glucose as the standard. The proportion of D-mannuronic to L-guluronic acid in the polysaccharides was determined by acid hydrolysis followed by paper chromatography and densitometry[3].

Materials. — Polysaccharides were isolated from the slime produced by strains of *Pseudomonas aeruginosa* as described previously[3]. Samples of alginic acids

differing in uronic acid composition were obtained from Dr. A. Haug. A lyase that degrades alginates and the slime polysaccharides of Pseudomonas was isolated from Alginomonas induced to grow on alginic acid[3].

Alkaline deacetylation. — For *O*-deacetylation, the polysaccharides were dissolved in distilled water (5 mg per ml), and to the resulting viscous solution 0.3M sodium hydroxide was added (0.5 ml per ml) with stirring. After one h at 25°, the solution was neutralized to pH 7.0 with 0.3M hydrochloride acid and dialyzed, and the material isolated as described[3].

Acetylation. — Deacetylated polysaccharides were treated with acetic anhydride and perchloric acid according to the method of Schweiger[10]. The extent of acetylation was measured by the i.r. spectrum and g.l.c.[8].

Alginase degradation. — A solution (10 mg per ml) of polysaccharides in 0.1M sodium acetate was incubated at pH 7.0 and 37° with 2 mg of alginate lyase per ml. Portions of the solution were withdrawn at time intervals and analyzed for reducing sugar and uronic acid.

RESULTS

Effects of alkali treatment. — Analyses of two Pseudomonas polysaccharides[3] and of two alginate samples differing in uronic acid composition are shown in Table I. A large drop in the proportion of uronic acid, as measured by the orcinol reaction, was observed after deacetylation of Pseudomonas Variant 2 polysaccharide (having a

TABLE I

ANALYSES OF NATIVE AND ALKALI TREATED GLYCURONANS

Polysaccharide	*ManA* (%)[a]	*Uronic acid* (%)		O-*Acetyl* (%)
		Orcinol	*Carbazole*	
Pseudomonas				
Variant 2 (native)	90	62	59	11.6
Variant 2 (alkali-treated)		20	70	0
Pseudomonas				
Variant 11 (native)	50	75	65	9.7
Variant 11 (alkali-treated)		67	60	0
Alginate I	88	30	93	0
Alginate L.h.	36	56	76	0

[a]The difference from 100% represents the proportion of L-guluronic acid.

high D-mannuronic acid content), whereas a low value was given by this reaction for Alginic acid Sample I (having a high D-mannuronic acid content). The analyses by the carbazole reaction, on the other hand, were not affected by alkali treatment or a high D-mannuronic acid content *per se*. Variant 11 showed only a slight decrease in

the orcinol value after deacetylation, and Alginic acid L.h. (having a low D-mannuronic acid content) showed a fairly high value in the orcinol reaction*.

Infrared spectra showed no significant differences, except those due to the loss of the acetyl group, between native and alkali-treated samples. Polysaccharide preparations obtained from several other variants of Pseudomonas[3] showed similar effects of deacetylation related to their uronic acid composition.

Alginase degradation. — Changes in orcinol value during degradation of alkali-treated polysaccharides by alginase had been noticed previously[1]. This reaction was, therefore, examined here further. As shown in Fig. 1, a rapid increase in the orcinol values can be observed during degradation of alkali-treated Variant 2 and Alginic acid I. Variant 11 showed no significant change, except for a final decrease in value (due to α-keto acid formation[11]). Alginic acid L.h. (not shown here) gave a pattern identical with that of Variant 11.

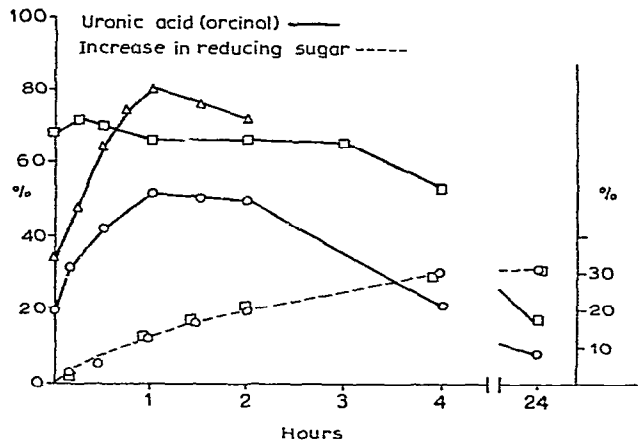

Fig. 1. Degradation of polysaccharides by alginase. Aliquots of the reaction mixture were analyzed for uronic acid content by the orcinol method and for increase in reducing sugar by a ferricyanide method: □, Pseudomonas polysaccharide Variant 11 (alkali treated); △, Alginate Sample I; ○, Pseudomonas polysaccharide Variant 2 (alkali treated). The right hand ordinate, reduced in scale, shows values after 24 h.

It should be noted that the increase in reducing sugar (a direct measure of the extent of degradation) proceeded at a rate much lower than that of the increase in orcinol color and was the same for Variants 2 and 11.

In order to check whether the effect of alginase degradation on the orcinol reaction was due to the degradation *per se* or to some unknown activity of the crude

*Alginic acids are generally prepared by an alkaline extraction procedure. However, a sample having a high D-mannuronic acid content, prepared by Dr. Haug without alkali, also showed a low orcinol-reaction. As can also be noted, total uronic acid content of all samples measured by either reaction are somewhat low. This is not due to the presence of other monosaccharides or impurities but to the nature of the color reactions used[2].

enzyme, samples of akali-treated, Variant 2 polysaccharide and Alginic acid I were hydrolyzed (24mM sulfuric acid). Aliquots of the hydrolyzate were removed at the time intervals shown in Fig. 2 and analyzed by the orcinol reaction. As can be seen, an increase in color values occurred with time, though not as rapidly as in the enzymic reaction (Fig. 1).

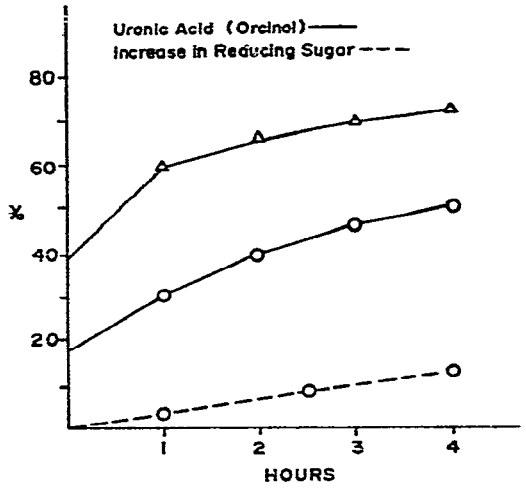

Fig. 2. Hydrolysis of polysaccharides by 25mM sulfuric acid. Aliquots of the reaction mixture were analyzed with the same methods as in the legend of Fig. 1: △, Alginate Sample I; ○, Pseudomonas polysaccharide Variant 2 (alkali treated).

The drastic lowering of the uronic acid content, as measured by the orcinol reagent (though not by the carbazole reagent), after alkali treatment of Variant 2 and related polysaccharides raised the question of whether alterations in the molecule other than removal of acetyl groups had occurred. The following experiments were performed to solve this.

Alkali treatment of alginase-degraded polysaccharides. — Variant 2 polysaccharide was treated with alkali, followed by a 1-h digestion with alginase. The alginase digest was neutralized, treated with sodium hydroxide to a concentration of 0.1M, and kept at room temperature for 2 h. Aliquots were removed, first after alginase digestion, and then after the second alkali treatment, and analyzed for uronic acid by the orcinol method. The increased value after alginase treatment (see Fig. 1) was not affected by the subsequent alkaline conditions, thus indicating that alkali *per se* did not produce changes in the polysaccharide.

Reacetylation of polysaccharides. — A Pseudomonas polysaccharide having a high D-mannuronic acid content[3] (Variant 16) and one containing 55% of L-guluronic acid[3] (Variant 17) were deacetylated with alkali and reacetylated with acetic anhydride. Analytical values are shown in Table II. As can be seen, reacetylation of Variant 16 polysaccharide restored the orcinol color-reaction to about its original

value. No significant effect of deacetylation or reacetylation on the orcinol reaction of Variant 17 (containing 55% of L-guluronic acid) was observed. When reacetylated Variant 16 was treated with alkali and deacetylated, the orcinol value decreased again significantly.

TABLE II

DEACETYLATION AND REACETYLATION OF PSEUDOMONAS POLYSACCHARIDES

Polysaccharide	*ManA* (%)[a]	*Uronic acid* (%)		*Acetyl* (%)
		Orcinol	*Carbazole*	
Variant 16	85	73	74	11.0
Variant 16 (deacetylated)		26	84	<0.5[b]
Variant 16 (reacetylated)		65	73	13.8
Variant 16 (reacetylated and deacetyled)		38	75	<0.5
Variant 17	45	79	87	8.5
Variant 17 (deacetylated)		66	84	<0.5[b]
Variant 17 (reacetylated)		70	69	16.4

[a]The difference from 100% represents the proportion of L-guluronic acid. [b]The i.r. spectrum showed a complete absence of acetyl peaks.

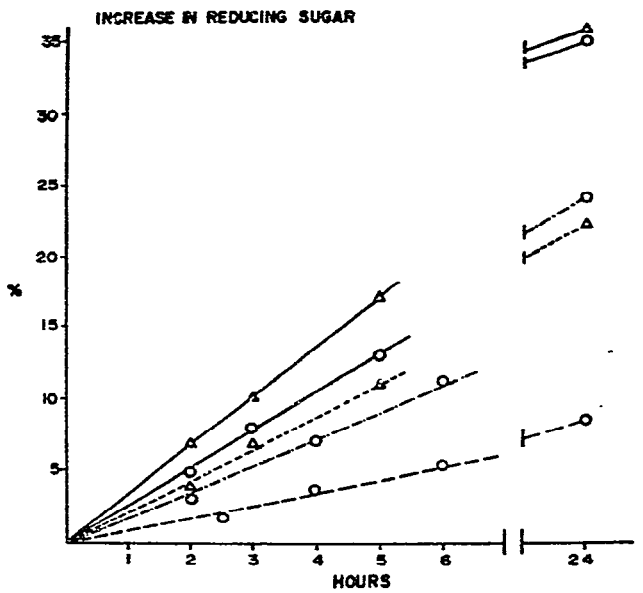

Fig. 3. Hydrolysis of polysaccharides by 0.1M hydrochloric acid. The increase in reducing sugar was measured at the time intervals shown by the ferricyanide method. △, Pseudomonas polysaccharide Variant 11; the solid line is the native polymer and the dashed line is the alkali-treated material; ○, Pseudomonas polysaccharide Variant 16: the solid line is the native polymer, the dashed line is the alkali treated, and the dotted, dashed line is the alkali-treated polysaccharide that has been re-acetylated.

Rate of hydrolysis. — In view of a recent publication[12] describing the effects of the presence of *O*-acetyl groups on the rate of hydrolysis of methyl glycosides of *N*-acetylneuraminic acid, the following experiments were performed: Polysaccharides from Variant 16 and 11 were hydrolyzed with 0.1M hydrochloric acid, before and after deacetylation, and after reacetylation for Variant 16. Aliquots of the hydrolyzates were removed at several time intervals and analyzed for reducing sugar (see Fig. 3). A significant difference in rate of hydrolysis and also in the final value for reducing sugar reached after 24 h of hydrolysis was observed in the sample having a high D-mannuronic acid content in the presence and absence of *O*-acetyl groups. The sample containing 50% of L-guluronic acid showed a much smaller effect of the *O*-acetyl groups on the rate of hydrolysis.

Determination of block composition. — The alginic acids have been shown[6] to be linear polymers in which the D-mannuronic and the L-guluronic acid residues are distributed in a blockwise fashion. Long segments of contiguous D-mannuronic acid residues and similar segments of L-guluronic acid residues are joined by segments containing alternating sequences of the same monomers. Samples of native and deacetylated polysaccharides were hydrolyzed with 0.3M hydrochloric acid for 2 h and the proportion of soluble to insoluble material, which indicates block arrangement, determined as described by Haug *et al.*[6] (see Table III). Large differences between native and deacetylated polysaccharides were observed. The native polysaccharide having a low acetyl content gave results similar to those of the alkali-deacetylated polymer having the same D-mannuronic acid content. The deacetylated glycuronans showed properties that are appropriately related to their block structures, like the alginic acids having similar uronic acid composition[6], whereas the native polymers (having a high acetyl content) behaved quite unexpectedly.

TABLE III

BLOCK COMPOSITION OF PSEUDOMONAS POLYSACCHARIDES[a]

Polysaccharide	*ManA* (%)[b]	*Acetyl* %	*Soluble*[c] %	*Insoluble*[c] %
M-98 (native)	>95	7.7	39	61
M-98 (deacetylated)		0	3	97
K8 B-3 (native)	70	10.4	88	12
K8 B-3 (deacetylated)		0	37	63
M-106 (native)	70	<0.5	31	69

[a]Differing in D-mannuronic acid and *O*-acetyl groups content. [b]The difference from 100% represents the proportion of L-guluronic acid. [c]After partial acid hydrolysis (see text).

DISCUSSION

Alginic acids and the Pseudomonas-slime polysaccharides related to them belong to a group of glycuronans having a composition showing considerable structural variation within the polymer family. Variation in the proportion of D-

mannuronic to L-guluronic acid and the arrangement of the uronic acids in homopolymeric blocks *vs.* alternating sequences[6] lead to significant differences in conformation and physical properties of the polysaccharides[13,14]. The presence of *O*-acetyl groups in the Pseudomonas polymers adds another parameter of potential variability.

We noticed previously[1] that the bacterial polysaccharides having a high D-mannuronic acid content showed a significantly lower uronic acid content, as measured by the orcinol reaction, after removal of the acetyl groups by alkali. Though the mechanism of color reactions in general is poorly understood, this large difference obviously had significance. It can also be observed (Table I) that the alginic acids themselves, when having a high D-mannuronic acid content, show quite low values in this particular color reaction, though they apparently do not contain *O*-acetyl groups. As the orcinol reaction is carried out in strong hydrochloric acid solution, the reversal of color loss by alginase degradation or weak acid hydrolysis was surprising. However, this and the lack of effect of further alkali treatment on these partially degraded polysaccharides indicated that the absence of acetyl groups *per se* was responsible for the low orcinol-reaction of the deacetylated or nonacetylated polymers. The restoration of the color value by reacetylation confirmed this hypothesis.

In general, glycosyluronic linkages are much more resistant to acid hydrolysis than glycosyl linkages, at least at low pH values[15]. The best hypothesis accounting for this difference is an inductive effect of the carboxyl group that results in a destabilization of the carbonium-ion intermediate[16]. On the other hand, the presence of a vicinal carboxyl group in glycosides of the sialic acids seems to contribute to their ease of hydrolysis. A recent paper describing the effects of *O*-deacetylation on the rate of hydrolysis of glycosides of *N*-acetylneuraminic acid[12] prompted us to investigate the effect of *O*-acetyl groups on the hydrolysis of the glycuronans.

As can be seen in Fig. 3, both rate and extent of hydrolysis in 0.1M hydrochloric acid were affected by the presence of *O*-acetyl groups in the polysaccharide having a high D-mannuronic acid content. The polymer containing 50% of L-guluronic acid was hydrolyzed readily and seemed less affected by the presence of *O*-acetyl groups. This polysaccharide probably contains a substantial portion of areas where the two uronic acid residues alternate; these areas are more readily hydrolyzed than homopolymeric blocks containing only one uronic acid[6]. We have never isolated any Pseudomonas polymers having a very high L-guluronic acid content, so we were not able to test this aspect further. We could not increase the L-guluronic acid content by growing the organisms in the presence of calcium ions, as reported by Haug[17] for Azotobacter.

The presence of *O*-acetyl groups appears to stabilize glycosyl linkages in hexoses[18] as well as in sialic acids[12] during acid hydrolysis. The opposite effect is noticed here for the glycuronans where glycosyl linkages are made labile. Since the carboxyl group stabilizes the linkages in glycosyluronic acids, but destabilizes them in sialic acids, as compared to hexoses, the same mechanism may be responsible for

both effects. It is of interest that chemical reacetylation, which is random, restores the orcinol color-reaction to about its original value (Table II) and increases the rate of hydrolysis to an intermediate value (Fig. 3).

As hydrolysis must precede chromophore formation, the unusual reactivity of glycuronans in the orcinol reaction can be accounted for by the differential rate and extent of hydrolysis due to acetyl content or the presence of significant amounts of L-guluronic acid (Fig. 3). Because of this, the otherwise excellent method of hydrolysis of Haug *et al.*[6] for the determination of block *vs.* alternating areas in alginic acids is not applicable to glycuronans containing *O*-acetyl groups. According to this method, the material solubilized by controlled acid hydrolysis represents alternating areas of the polymer, whereas the insoluble material is derived from homopolymeric blocks containing residues of only one uronic acid. As shown in Table III, large differences in results can be observed between the native and deacetylated polysaccharides. The compounds containing *O*-acetyl groups gave a high yield of the soluble fraction, which leads to an erroneous value for the extent of alternating areas in the polymer. It is obvious that polysaccharide M-98, which contains above 95% of D-mannuronic acid, cannot contain 40% of alternating sections. The value of 3% obtained after deacetylation is much more acceptable. Therefore, in order to apply the method of Haug *et al.*[6], alkaline deacetylation has to be performed first.

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