Alginic Acid Metabolism in Bacteria

I. ENZYMATIC FORMATION OF UNSATURATED OLIGOSACCHARIDES AND 4-DEOXY-L-ERYTHRO-5-HEXOSEULOSE URONIC ACID

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Alginic acid, a high molecular weight linear polymer obtained from seaweed, is widely used as an added product to food due to its property of forming highly viscous solutions. Originally described as a polymer of D-mannuronic acid, it has now been demonstrated, by the chemical studies of Fischer and Dörfel (1), to be a β-1-4-linked polymer of D-mannuronic and L-guluronic acid. The ratio of the two uronic acids vary widely in preparations of alginic acid derived from various species of seaweed. The alginic acid of Macrocystis pyrifera, the species examined in the present study, has been reported to have a L-guluronic acid content of 20 to 40% (2). At the present time, little is known of the sequence of the two uronic acids in the polymer.

In an attempt to delineate the enzymatic mechanism of alginic acid metabolism, as a prelude to studies on sequence determination, an organism capable of growth on alginic acid as sole carbon source was isolated. The results of metabolite studies with this organism have been reported briefly (3). According to the reaction sequence illustrated in Fig. 1, alginic acid is cleaved by an enzyme fraction referred to as alginase into a series of oligosaccharides containing an unsaturated uronic acid on the non-reducing end of the oligosaccharide chain. These unsaturated oligosaccharides are further degraded with the ultimate production of the monosaccharide, 4-deoxy-L-erythro-5-hexoseuloseluronie acid.

The present paper is concerned with the purification and properties of alginase with further documentation on the identity of the reaction products.

The following paper (4) describes the purification of a TPNH-linked dehydrogenase, from alginate-induced Pseudomonas, capable of converting the alginase end product, 4-deoxy-L-erythrose-5-hexoseuloseluronie acid, to 2-keto-3-deoxy-D-gluconate.

EXPERIMENTAL PROCEDURE

Various chemicals used in this study were generously made available to us as follows: D-Mannuronic acid by Dr. H. Isbell of the National Bureau of Standards, L-guluronolactone by Dr. F. G. Fischer, the α-and β-metalsaccharinic acids by Dr. H. Fletcher of this Institute, and algic acid by the Kelco Company, Los Angeles, California. Chondroitin sulfates A and B and hyaluronic acid were isolated. The results of metabolic studies with the undegraded alginic acid to brief heating in alkali. Both the monosaccharide end product and the intermediate reducing end of the oligosaccharides give negligible orcinol reaction after alkali treatment.

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I. Alginic Acid Metabolism

II. Thiobarbituric Acid Assay—This method is based upon the formation of compounds giving rise to $\beta\text{-formylypyruvate}$, whereas intact alginic acid does not so react. Thus, aliquots of the above described incubation mixture were assayed with thiobarbituric acid according to the modification of Weissbach and Hurwitz (8) whereby 0.01 $\mu$mole of $\beta$-formylypyruvate gave an optical density reading of 0.290 at 549 $\mu$m. A unit is defined as that amount of enzyme required to yield 1 $\mu$mole of $\beta$-formylypyruvate under the stated incubation conditions.

III. Ultraviolet Absorption Assay—This method is based upon the marked absorption peak at 230 $\mu$m of the unsaturated uronic acid portion of the intermediate oligosaccharides. Neither intact alginic acid nor the final monosaccharide end product is measured in this assay. The same reaction mixture described in the previous two methods was used except that the concentration of sodium alginate was halved, i.e. 2.55 $\mu$moles added (as mannuronic acid). The reaction was initiated by the addition of an appropriate aliquot of the enzyme solution to a 1-cm cuvette and the absorption determined at 230 $\mu$m in a Beckman DU spectrophotometer. Readings, taken at 1 and 5 minutes after addition of the enzyme, were compared with a reaction blank in which the alginate was omitted. In this assay a unit is defined as that amount of enzyme required to cause an optical density increase of 1.0 at 230 $\mu$m in 4 minutes at room temperature. A similar assay has been reported by Nakada et al. (9) for measuring bacterial mucopolysaccharides.

Enzyme Purification

Crude Extract—The frozen bacterial paste was suspended in 4 volumes of 0.1 M potassium phosphate buffer, pH 7.5, containing 0.01 M glutathione, and disrupted by sonic vibration for 20 minutes in a 10-kc Raytheon oscillator. The broken cell mixture was centrifuged at 10,000 r.p.m. for 15 minutes and the supernatant solution was used as the starting material for purification of the enzyme. All ensuing operations were carried out at 0-3°C.

Streptomycin Precipitation—Sixteen milliliters of a 5% (weight per volume) streptomycin sulfate solution were added slowly, with continuous stirring, to 80 ml of the crude extract. After standing for 10 minutes, the suspension was centrifuged at 15,000 r.p.m. for 10 minutes and the supernatant retained (93 ml).

Ammonium Sulfate Fractionation—To 90 ml of the above fraction an equal volume of cold, saturated ammonium sulfate was added. After 10 minutes, the mixture was centrifuged as above, and the precipitate discarded. The supernatant was brought to 95% saturation by addition of solid ammonium sulfate and allowed to stand for 20 minutes before centrifugation. The resultant precipitate was dissolved in 20 ml of 0.03 M Tris buffer, pH 8.5, and dialyzed overnight against 500 ml of the same buffer (26 ml).

DEAE-Cellulose—Twenty five milliliters of the above solution were added to a DEAE-cellulose column (2 X 12.5 cm) which had been equilibrated with 0.03 M Tris buffer, pH 8.5. Upon washing of the column with the same buffer solution, the enzyme appeared in the first two resin bed volumes. These were collected separately and designated DEAE-cellulose Fractions I and II (20 and 23 ml, respectively).

Table I summarizes the data on the partial purification of the alginate-degrading enzyme. The ratio of activity in the thiobarbituric acid assay with respect to protein concentration.

buffer, pH 7.5, 30 $\mu$mules, and enzyme in a total volume of 1.0 ml, was incubated for 8 minutes at 37°C. The reaction was stopped by heating in a boiling water bath for 30 seconds and a 0.05 ml aliquot pipetted into 1.45 ml of 0.1 N NaOH. The latter tube was heated to 100°C for 5 minutes, cooled, and assayed by the Dische modification of the orcinol reaction (7). A heated enzyme control was run with every assay. A unit is defined as that amount of enzyme required to cause a decrease of 20 units of optical density at 665 rnp in the total reaction mixture under the above conditions. The calorimetric assay was performed on a Beckman DU spectrophotometer with cuvettes with a 1-cm light path.

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Table I summarizes the data on the partial purification of the alginate-degrading enzyme. The ratio of activity in the thio-
barbituric and ultraviolet assay remained constant with respect to each other during purification but changed appreciably in relation to the orcinol assay. Since each assay reflects different parameters of the same over-all reaction, the significance of this observation is not immediately clear. However, on the basis of preliminary experiments, it would seem reasonable to anticipate that more than one enzymatic activity is involved in the degradation of alginic acid.

Properties of Alginase

Stability—After 3 months of storage at -15°, during which time the enzyme was repeatedly frozen and thawed, the activity decreased approximately 10% after 6 months, a decrease of 30% was noted.

pH Optimum—As measured by all three assays, alginase activity exhibited maximal velocity in the pH range of 7.0 to 8.0 (Fig. 3).

Salt Activation—The striking dependence of enzymatic activity upon salt concentration is illustrated in Fig. 4. Maximal activation was obtained at 0.05 M KCl; higher concentrations were inhibitory. A similar pattern of behavior was observed when assayed by the orcinol or thiobarbituric acid methods.

Specificity—Under conditions similar to those used in the alginic acid assay, hyaluronic acid, chondroitin sulfate A, B, and C, and polygalacturonate were inactive in the thiobarbituric acid or orcinol assays. Furthermore, no inhibitory effect of these compounds upon the enzymatic degradation of alginic acid was observed. Similarly, when the free uronic acids, D-mannuronic and L-guluronate, were incubated with the enzyme, no detectable reaction occurred (thiobarbituric acid assay) and the uronic acids were recovered quantitatively. The inability of the enzyme to metabolize these compounds is in accord with the concept that the free uronic acids are not involved as intermediates in the formation of 4-deoxy-5-ketouronic acid.

Isolation and Identification of Reaction Products

The products found upon incubation of the partially purified enzyme with sodium alginate for 90 minutes at room temperature were examined by paper chromatography in Solvent A. All of the components shown in Fig. 5 exhibited a strongly positive reaction when sprayed with the thiobarbituric acid reagent (6) or treated with AgNO₃ (5). Corresponding areas of ultraviolet light absorption were observed in all cases with the exception of the fastest moving compound. Prolongation of the incubation time to 24 hours failed to produce a qualitative change in the chromatogram, although a distinct increase in the staining intensity of the leading spot was apparent. On the basis of the relative rates of migration, as well as the differential response to thiobarbituric acid and ultraviolet absorption, it was presumed that the most rapidly moving component represented a monosaccharide end product, whereas the slower moving components indicated the presence of a series of unsaturated oligosaccharides.

To accumulate an amount of the major reaction products sufficient for isolation and identification, a large-scale incubation was prepared. The reaction mixture, containing 2 mmoles of

| TABLE I Purification of alginase from Pseudomonas extracts |
|-----------------|-----------------|-----------------|-----------------|
| Fraction        | Total units     | Protein  mg/ml  | Ratio of activities orcinol to thiobarbituric acid to ultraviolet |
| Crude extract   | 2,340.4,890.5,500 | 13.0          | 2.8:1:2:1:2.3 |
| Streptomycin supernatant | 2,320.4,920.5,540 | 11.3          | 2.7:1:2:1:2.4 |
| Ammonium sulfate fraction | 1,100.1,700.1,800 | 5.1           | 8.6:1:1:5:1.6 |
| DEAE-cellulose Fractions: |             |               |               |
| I               | 504            | 693           | 800           | 0.31:78 | 1:1.4:1:6 |
| II              | 408            | 570           | 600           | 0.35:49 | 1:1.4:1:7 |

*Orcinol assay.

Fig. 3. Alginase assays as a function of pH. The assay procedures are described under "Methods." The buffer used; however, in the pH 6 to 7 range was 0.02 M potassium phosphate instead of Tris-HCl.

Fig. 4. Effect of salt concentration on alginase action. The ultraviolet assay procedure is described in "Methods" except that the various salts were substituted for KCl as indicated.
were chromatographed in Solvent A.

The formic acid in Peak I was removed by ether extraction in a continuous flow liquid-liquid extractor as described previously (10). Peaks II and III were treated similarly after pretreatment with Dowex 50 (H+). All three fractions were concentrated by lyophilization.

Identification of Peaks I and II—When chromatographed in Solvents A and B, both peaks revealed a single thiobarbiturate-reacting component after periodate oxidation, which were indistinguishable from each other in both solvent systems (Table II). The thiobarbiturate chromogen of both peaks yielded identical spectra, closely paralleling that of known β-formylpyruvate, and indicated a recovery of 1,200 μmoles of product in Peak I and 200 μmoles in Peak II.

Twenty microliters of the reaction mixture described in the text were obtained. A total of 92% of the thiobarbiturate-reacting material was recovered in the three peaks.

Fig. 5. Paper chromatography of the alginase reaction mixture. Twenty microliters of the reaction mixture described in the text were chromatographed in Solvent A.

sodium alginate, 11 mmoles of Tris buffer (pH 7.5), and 4.5 mg of enzyme (DEAE-cellulose Fraction I) in a total volume of 75 ml, was incubated for 24 hours at 37°. During this time, 1,630 μmoles of alginate (80%) was converted to thiobarbiturate-reacting products. The reaction mixture was diluted to 150 ml and placed on a Dowex 1-formate column (10% cross-linked, 4-cm diameter × 14 cm). The column was washed with 680 ml of water and elution begun with 0.15 N formic acid. Fractions (90 ml) were collected and assayed colorimetrically by the thio-
barbituric acid procedure. The elution pattern is illustrated in Fig. 6. After collection of the major portion of the reaction product in Peak I (1.5 liters), the 0.15 N formic acid eluent was replaced by a linear gradient system containing 2 liters of water in the mixing chamber and 2 liters of 2.0 N ammonium formate in the reservoir. Two additional peaks were subsequently ob-
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Subjection of both compounds to periodate oxidation failed to produce significant amounts of formaldehyde as determined by chromotropic acid test (11). However, when the compounds were reduced with NaBH₄ before periodate oxidation, a stoichiometric recovery of formaldehyde was found (Table II). Further supporting evidence for the presence of an intact aldehyde group in the molecule was provided by the ultraviolet spectrum of the semicarbazone derivatives (12) which exhibited a sharp maximum at 233 nm (Fig. 7).

Both compounds gave a positive reaction when treated with o-phenylenediamine (13), thus inferring the presence of an α-keto acid. Similarly, both compounds were shown to be decarboxylated readily in the presence of ceric sulfate (14) with the liberation of a stoichiometric yield of CO₂ (Table II).

Determination of periodate uptake, assayed spectrophotometrically (15), indicated a value of 1.93 moles of periodate consumed per mole of compound. The theoretical amount expected from a 4-deoxy-5-ketohexuronic acid is 2 moles per mole of compound oxidized.

In an attempt to demonstrate the orientation of the hydroxyl groups on carbon atoms 2 and 3, the compounds from both Peak I and II were converted to the corresponding α- and β-metasaccharinic acids by reduction with NaBH₄ (Fig. 8). They were identified by their cochromatography in Solvents A and B with authentic crystalline standards. The reduction was shown to be quantitative by two assay methods: (a) determination of formaldehyde released by periodate oxidation, and (b) measurement of lactone formation with FeCl₃-hydroxylamine after a brief heating at 100° in 1 N HCl (16). The α- and β-metasaccharinic acids were then converted to 2-deoxyribose in 10 to 12% yields by the Ruff degradation (17); a control microdegradation with authentic metasaccharinic acid resulted in a 15% yield.

The identification of the resulting products as 2-deoxyribose was based upon the following data: both products reacted to give the characteristic spectrum of authentic 2-deoxyribose in the cysteine-H₂SO₄ (18) and diphenylamine (19) assay procedures. Repetition of the thiobarbituric acid assay no longer gave a maximum at 549 nm but resulted in spectra identical to that shown by malonaldehyde with peak absorption at 534 nm.

### Table II

<table>
<thead>
<tr>
<th>Fraction</th>
<th>β-Formylpyruvate after periodate oxidation</th>
<th>Formaldehyde before reduction</th>
<th>Formaldehyde after reduction</th>
<th>Ceric sulfate decarboxylation</th>
<th>Migration</th>
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<tr>
<td></td>
<td>µmoles/ml</td>
<td>µmoles/ml</td>
<td>µmoles/ml</td>
<td>cm/3/hr</td>
<td>cm/5/hr</td>
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<tr>
<td>Peak I</td>
<td>19.3</td>
<td>0.8</td>
<td>16.8</td>
<td>20.8</td>
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<tr>
<td>Peak II</td>
<td>14.5</td>
<td>0.2</td>
<td>14.7</td>
<td>15.5</td>
<td>26</td>
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</table>

**Fig. 7.** Absorption spectra of the deoxyketouronic acid semicarbazone at a concentration of 4.4 × 10⁻⁵ M based on the thiobarbituric acid assay (7).

**Fig. 8.** Chemical conversion of deoxyketouronic acid semicarbazone to 2-deoxyribose.
Fig. 9. Rate of malonaldehyde production from 2-deoxypentoses. Malonaldehyde assayed by the thiobarbituric acid reaction. For each time point, 0.019 μmole of deoxypentose reaction product and 0.020 μmole of authentic deoxyribose and deoxyxylose based on the diphenylamine assay (18) were used.

Fig. 10. Ultraviolet absorption spectra of the unsaturated oligosaccharide fraction (Peak III) at a concentration of 1.15 × 10^-4 M.

Both compounds cochromatographed with 2-deoxyribose in Solvents A, B, C, and D, the last two of which clearly resolved 2-deoxyribose and 2-deoxyxylose. Optical rotation of the degradation product from Peak I was demonstrated to have [α]_D of -40° which approximates the reported value of -50° for 2-deoxy-d-ribose (21) and establishes the former as the expected d isomer. The difference in the [α]_D obtained from the reported value is attributed to the small amount of material available for measurement; insufficient material derived from the Peak II compound was available for a specific rotation determination.

On the basis of the above data, the compounds isolated in Peaks I and II appear to be identical and have been identified as 4-deoxy-L-erythro-5-hexoseulose uronic acid (Fig. 1). It is not apparent why this compound appears as two separate peaks upon Dowex-formate chromatography. However, in addition to the chemical evidence cited above, enzymatic similarity has also been demonstrated that both compounds are converted to 2-keto-3-deoxygluconic acid by a purified TPNH-linked dehydrogenase isolated from the same organism (4).

Characterization of Peak III—In contradistinction to the two fractions described above, Peak III exhibited a marked absorption in the ultraviolet range with a sharp maximum at 235 mμ (Fig. 10).

When subjected to paper chromatography in Solvent A, Peak III was resolved into four separate components which were eluted from the paper. The data, compiled in Table III, indicate that each component possessed an extinction coefficient of 8,500 at 235 mμ and consumed 1 mole of bromine per mole of P-formylpyruvate released by periodate oxidation. Comparison of the relative amounts of saturated to unsaturated uronic acid, based upon the orcinol to thiobarbiturate assay values, revealed a ratio which, in each case, approximated a characteristic integral number. On the basis of this evidence, together with the behavior toward column and paper chromatography, it is suggested that these compounds represent the tri-, tetra-, hexa-, and nonasaccharides. Further analysis of these unsaturated oligosaccharides is in progress.

The observation that the unsaturated oligosaccharide gives

### Table III

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Thiobarbituric acid assay</th>
<th>Occinol</th>
<th>Br₂ uptake*</th>
<th>cm/45 hr</th>
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<tr>
<td>Peak III</td>
<td>μmoles</td>
<td>μmoles</td>
<td>μmoles</td>
<td>cm/45 hr</td>
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<tr>
<td>1</td>
<td>17.2</td>
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<td>8,500</td>
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<td>2</td>
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<td>4</td>
<td>0.06</td>
<td>4.7</td>
<td>0.89</td>
<td>8,600</td>
</tr>
</tbody>
</table>

* Bromine uptake was measured by the method of Suzuki (22); values are micromoles per μmole of thiobarbituric acid.
† Absorption from the paper was too high to permit an accurate estimation of the extinction coefficient of Fraction I.

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6 Supporting evidence for the assignment of the tri- and tetrasaccharide structure to Fractions I and II, respectively (Table III), is based upon the sensitivity of the free reducing group of the terminal uronic acid to bromine oxidation and the apparent lack of reactivity of the unsaturated uronic moiety toward the orcinol reagent. Treatment of Fraction I with aqueous bromine at room temperature under conditions which completely destroyed the free uronic acid control resulted in a 50% loss of orcinol-reacting material, whereas the orcinol titer of Fraction II was decreased 33%. Since the nonreducing uronides are stable to mild oxidation, a decrease of 50 and 33% is in agreement with the theoretical values for unsaturated tri- and tetrasaccharides, respectively.

In addition, enzymatic degradation of Fractions I and II, by the crude extract, resulted in formation of close to the theoretical yield of 3 and 4 μmoles of deoxyketouronic acid, respectively.
rise to β-formylpyruvate after periodate oxidation provides suggestive evidence for the location of the unsaturated bond at carbon atoms 4 and 5. From the studies of Waravdekar and Saslaw (20), it was shown that the release of malonaldehyde from galactal was more rapid than from 2-deoxyribose. No apparent reason was mentioned for this different rate. However, as is illustrated in Fig. 11, galactal may be considered as the enol form of a 2-deoxy sugar just as the unsaturated uronide may be regarded as the enol form of a 2-keto-3-deoxycarboxylic acid. By analogy with galactal, β-formylpyruvate formation from the unsaturated oligosaccharide should be and is faster than from the corresponding 4-deoxy-5-ketouronic acid (Figs. 12 and 13).

**DISCUSSION**

The pattern of alginic acid utilization described in this study appears to be closely analogous to the bacterial degradation of mucopolysaccharides, as reported by Linker, Meyer, and Hoffman (23) and Linker et al. (24). These workers isolated and identified a disaccharide composed of a Δ-4,5-uronide and N-acetylhexosamine which was subsequently shown to be enzymatically hydrolyzed to yield 4-deoxy-l-threo-5-hexoseulose uronic acid, the 2-epimer of the compound reported here.

A tentative mechanism proposed for the enzymatic degradation of alginic acid is shown in Fig. 1. The polymer is pictured as being cleaved by an “endoalginase,” resulting in the formation of a series of oligosaccharides containing an unsaturated uronic acid residue on the nonreducing end of the chain. The unsaturated oligosaccharides, in turn, are further fragmented with the eventual formation of 4-deoxy-l-erythro-hexoseulose uronic acid.

Provisional evidence in accord with the intermediate role of the unsaturated oligosaccharides is the finding of a series of slow moving, ultraviolet light-absorbing products after brief incubation with minimal amounts of enzyme. Upon longer incubation, these products diminish in intensity and are accompanied by the appearance of the fast moving monomer. Under comparable conditions, D-mannuronic and L-guluronic acid are not metabolized as determined by the orcinol and thiobarbituric acid assays.

**SUMMARY**

An alginic acid degrading enzyme, alginase, has been partially purified from cell-free extracts of an adapted pseudomonad and a study of its properties undertaken. The metabolism of alginic acid has been shown to involve the intermediate formation...
of a series of unsaturated oligosaccharides and the resultant accumulation of a monosaccharide end product which has been isolated and characterized as 4-deoxy-L-erythro-5-hexoseulose uronic acid.

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Alginic Acid Metabolism in Bacteria: I. ENZYMATIC FORMATION OF UNSATURATED OLIGOSACCHARIDES AND 4-DEOXY-1-ERYTHRO-5-HEXOSEULOSE URONIC ACID

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