Studies on Alginase

Part III. Constitution of a New Unsaturated Diuronide

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Chemical investigations were made on a new unsaturated crystalline diuronide isolated from alginase hydrolysate of alginic acid. This uronide has $[\alpha]_D^{22} - 8^\circ$ (in water), $E_{1\%}^{232m\mu} = 143$ and m.p. $135.5 \sim 136.5^\circ\text{C}$ (decomp.). The presence of an α,β -unsaturated carboxylic acid formulation is supported by the following evidences: (a) an ultraviolet absorption band at $232m\mu$, (b) infrared absorption bands at 1648 cm^{-1} due to double bond and at 1720 cm^{-1} due to conjugated carboxylic group, (c) the consumption of about 1 mole of bromine per mole of the compound, (d) the production of oxalic acid on oxidation with ozone, (e) the formation of a substance that shows absorption maximum at $550m\mu$ caused by the addition of thiobarbituric test. After hydrolysis, crystalline mannuronic lactone was obtained from the unsaturated diuronide. Occurrence of mannuronic moiety in the reducing unit was observed by paper chromatography of the hydrolysate of borohydride-reduced unsaturated compound. From these results it can be seen that the possible structure of this unsaturated diuronide is 4-O-(β -D-4,5-mannoseenpyranosyluronic acid)-D-mannuronic acid.

In recent years there has been increased interest in the formation of unsaturated intermediates during the course of enzymatic degradation of polyuronides, e.g., hyaluronic¹⁾, chondroitinsulfuric²⁻⁴⁾, pectic acids^{5,6)} and pectin⁷⁾. The experiments concerning the occurrence of the unsaturated intermediates have been based principally on spectrophotometric method. Some of these compounds have been isolated in chromatographically pure amorphous form¹⁻⁵⁾, but not in

pure crystalline form. The enzymes, which produce such unsaturated compounds, were all obtained from several microorganisms.

In the previous paper⁸⁾, isolation of a new crystalline unsaturated compound, obtained from abalone alginase digests of alginic acid, was reported. To the best of the writer's knowledge, no other example of the formation of an unsaturated intermediate during the course of animal enzymatic degradation of polyuronide has been observed. This uronide exhibited a strong absorption in the ultraviolet range with the maximum at $232m\mu$ and similar properties to those of known unsaturated uronides derived from other polyuronides. This unsaturated compound gave an empirical formula, $\text{C}_{12}\text{H}_{16}\text{O}_{12} \cdot \text{H}_2\text{O}$.

1) A. Linker, K. Meyer and P. Hoffman, *J. Biol. Chem.*, **219**, 13 (1956).

2) A. Linker, P. Hoffman, K. Meyer, P. Sampson and E.D. Korn, *ibid.*, **235**, 3061 (1960).

3) P. Hoffman, A. Linker, V. Lippman and K. Meyer, *ibid.*, **235**, 3066 (1960).

4) S. Suzuki, *ibid.*, **235**, 3580 (1960).

5) C.W. Nagel and R.H. Vaughn, *Arch. Biochem. Biophys.*, **94**, 328 (1961).

6) M.P. Starr and F. Moran, *Science*, **135**, 920 (1962).

7) P. Albersheim, H. Neukom and H. Deuel, *Helv. Chim. Acta*, **43**, 1422 (1960).

8) I. Tsujino and T. Saito, *This Journal*, **26**, 115 (1962); *Nature*, **192**, 970 (1961).

Investigations of the chemical character of this new unsaturated diuronide will be reported in this paper.

EXPERIMENTAL PROCEDURE

Analytical Procedure. Unless otherwise noted, analytical methods used for estimation of reducing power, color reactions and for paper partition chromatography are those which have been described previously. Lactones were detected by spraying with hydroxylamine-ferric chloride reagent⁹⁾, the borohydride-reduced product and the product of its hydrolysis were detected with periodate-benzidine reagent¹⁰⁾, and acidic compounds were detected with 0.04% bromophenol blue. The compounds absorbing ultraviolet light were visualized as dark shadow under ultraviolet lamp*. Bromine uptake was measured by the method of Linker et al.¹¹⁾ and of Suzuki¹²⁾

Spectroscopy. Ultraviolet absorption spectra were obtained in a Shimadzu spectrophotometer using 1 cm quartz cells. Infrared absorption spectra** were obtained with a KBr disk in a Hitachi infrared spectrometer with a NaCl prism.

Ozonation. In order to confirm the presence of an α,β -unsaturated carboxylic acid, ozonation was carried out as follows: unsaturated diuronide (500 mg) was dissolved in 60 ml of water, and ozone was bubbled through the solution at room temperature. After 1 hour the solution was evaporated in vacuo to 20 ml and heated on the water bath for 1 hour. Calcium acetate solution was added and the precipitate which formed was dried (30 mg). The precipitate was dissolved in dilute sulfuric acid and extracted with ether. After removal of ether, resulting crystals were recrystallized from water.

Reduction with Sodium Borohydride. The end unit was ascertained by reducing the unsaturated diuronide with sodium borohydride. Unsaturated diuronide (200 mg) was converted to sodium salt and dissolved in 8 ml borate buffer of pH 8.1, and 100 mg of sodium borohydride in 5 ml of water was added slowly with stirring. After 3 hours at room temperature, cations were removed with Amberlite IR-120 (H^+) and the residual borate was removed by distillation as methyl

ester. The syrupy product formed by this treatment reacted positively to test for lactone reagent.

Hydrolysis of the Unsaturated Diuronide. On hydrolysis with distilled water at 100°C, hydrolysates of the unsaturated diuronide showed identical ultraviolet absorption spectrum and paper chromatograms like those of hydrolysates with sulfuric acid. The hydrolysate showed a strong absorption peak at 246 $m\mu$ and a weak one at 286 $m\mu$. The change in light absorption at 245 $m\mu$ during the course of hydrolysis, as shown in Fig. 1, indicated that the hydrolysis was complete after 1 hour. From this result, the hydrolysis of the unsaturated diuronide was carried out by heating a water solution of the compound at 100~105°C for 1 hour.

FIG. 1. Increased Absorbancy at 245 $m\mu$ During the Course of Hydrolysis with Distilled Water at 100°C.

Materials. The unsaturated diuronide was prepared by the method of carbon column chromatography as described previously. D-Mannuronic acid was prepared by Spoehr's method¹¹⁾ and isolated as its lactone, m.p. 140~141°C. Anal. Found: C, 40.85; H, 4.42; Mol. Wt., 172. Calcd. for $C_6H_8O_6$: C, 40.91; H, 4.58%; Mol. Wt., 176. An infrared spectrum of this lactone showed a band at 1765 cm^{-1} due to lactone. On oxidation with nitric acid, the lactone gave mannosaccharo-dilactone, m.p. 186~188°C. D-Mannonic acid was prepared by the bromine oxidation of D-mannose and

9) M. Gee and R.M. McCready, *Anal. Chem.*, **29**, 257 (1957).

10) J.A. Cifonelli and F. Smith, *ibid.*, **26**, 1132 (1954).

* Toshiba's FI-3-S. type was used.

** The author wishes to thank Dr. Y. Matsuoka for these measurements.

11) H.A. Spoehr, *Arch. Biochem.*, **14**, 153 (1947).

precipitated as calcium salt. By treatment with cation exchanger, calcium mannorate was converted to its lactone which was evaporated to a syrup and used as standard for paper partition chromatography.

RESULTS

Properties and analytical data of the compound are given as follows: m.p. 135.5~136.5°C (decomp.), $[\alpha]_D^{22} - 8^\circ$ ($c=1.0$, in water), $E_{1\%}^{232m\mu}=143$, reducing sugar 0.58 mg per mg of the diuronide, carboxyl content 2.01 moles per mole of the diuronide, bromine uptake 0.97 mole per mole of the diuronide.

The bromine uptake was approximately 1 mole per mole of this compound, although hexauronic acids and also common saturated disaccharides did not consume bromine under the same conditions.

The bromine uptake and ultraviolet absorption at 232 $m\mu$, which disappeared on bromination or on palladium hydrogenation, suggest the presence of an α,β -unsaturated acid. After ozonolysis, the isolation of oxalic acid, m.p. 99.5~101°C (monohydrate) and

187.5~188°C (anhydrous), evidenced the presence of a double bond in the 4,5 position of the carboxylic acid moiety.

An infrared absorption spectrum of this compound, as shown in Fig. 2, showed a sharp band at 1648 cm^{-1} due to the double bond. The carboxyl band was seen at 1720 cm^{-1} (due to α,β -unsaturated carboxyl). After palladium hydrogenation, disappearance of the band at 1648 cm^{-1} and shift of the band at 1720 cm^{-1} to 1735 cm^{-1} , as seen in Fig. 2, supported the fact that one of the carboxylic group in this compound subsisted as an α,β -unsaturated formula.

2-Thiobarbituric acid reaction¹²⁾ of this unsaturated diuronide, exhibiting an absorption maximum at 550 $m\mu$ as seen in Fig. 3, demonstrated that the compound gives rise to β -formylpyruvate after periodate oxidation. The formation of β -formylpyruvate showed that an unsaturated moiety exists as β -deoxy α,β -unsaturated carboxylic acid.

On hydrolysis with distilled water, four

FIG. 2. Infrared Spectra of the Unsaturated Diuronide (below) and the Palladium Hydrogenated Diuronide (above).

12) A. Weissbach and J. Hurwitz, *J. Biol. Chem.*, **234**, 705 (1959).

FIG. 3. Absorption Spectrum of the Product Obtained by Heating the Periodate Reaction Mixture with 2-Thiobarbituric Acid.

spots were observed on paper chromatograms. Two spots of them showed the characteristics of mannuronic acid and of mannuronic lactone with color reactions, and the other two spots exhibited both ultraviolet absorptive and acidic characters. Fig. 4 indicates the procedure used for the isolation of the hydrolysis products of the unsaturated diuronide. The compound (370 mg) was dissolved in 3 ml of distilled water in a sealed glass tube and heated in an oven at 100~105°C for an hour. Slightly darkened hydrolysate was lyophilized and crystallized from ethanol; 57 mg of hexagonal thin plates (A) was obtained. The recrystallized material had a m.p. of 139.5~141°C and on oxidation with nitric acid gave mannosaccharodilactone, m.p. 182~184°C. The crystallized material was identified by paper chromatography^{13,14)} and by

infrared spectrophotometry as D-mannuronic lactone.

To collect the ultraviolet absorbing materials, the author extracted mother-liquors from the crystallization of mannuronic lactone with ether, evaporated and crystallized the residues (B) from water. The recrystallized material (C), identified paper chromatographically and ultraviolet spectrophotometrically as 2-furoic acid, had a m.p. 123~128°C, λ_{max} 246 mμ (in water), yield 34 mg.

Mother-liquors (D) from the crystallization of 2-furoic acid, exhibited absorption peaks at 246 mμ (strong) and at 286 mμ (weak), as seen in Fig. 5. Two spots, having both acidic and ultraviolet absorbing character, were observed on paper chromatograms of (D). The appropriate areas including the ultraviolet absorbing materials were cut and eluted with water. The absorption spectra of the water-eluted solution are shown in Fig. 5.

The faster moving spot (E) exhibited an absorption maximum at 246 mμ and coincided paper chromatographically and spectrophotometrically with authentic 2-furoic acid. The slower moving spot (F) exhibited an absorption maximum at 286 mμ and agreed with that of 5-formyl-2-furoic acid reported by Okamoto and Ozawa¹⁵⁾.

The hydrolysates of the palladium hydrogenated diuronide, showed spots corresponding to mannuronic acid and mannuronic lactone, but negligible increase in ultraviolet absorption at 246 mμ.

The borohydride-reduced diuronide exhibited reducing power of 7% as compared with that of nontreated unsaturated compound, moved as almost one component on paper chromatograms and behaved as lactone on paper chromatograms. The ultraviolet absorption spectra of the borohydride-reduced compound and of its hydrolysate were iden-

13) F.G. Fischer and H. Dörfel, *Z. physiol. Chem.*, **301**, 224 (1955).

14) H. Masamune and M. Satake, *Tohoku J. Expt. Med.*, **69**, 167 (1959).

15) K. Okamoto and J. Ozawa, *Nogaku Kenkyu*, **48**, 39 (1960).

FIG. 4. The Method of Separation of the Products of Hydrolysis of the Unsaturated Diuronide.

tical with that of non-reduced diuronide and of its hydrolysate respectively. The hydrolysate of borohydride-reduced diuronide showed 3 spots corresponding to mannuronic lactone, 2-furoic acid and 5-formyl-2-furoic acid on paper chromatograms, respectively.

DISCUSSION

It has been generally accepted that alginic acid is a polymer made up of uronic acid units united by β -1,4 linkages¹⁶⁾. Recently, Jayme and Kringstad¹⁷⁾ isolated a crystalline D-mannuronic acid disaccharide, 4-O-(β -D-

16) R.L. Whistler and C.L. Smart, "Polysaccharide Chemistry", Academic Press (New York), 1953, p. 339.

17) G. Jayme and K. Kringstad, *Chem. Ber.*, **93**, 2263 (1960).

FIG. 5. Absorption Spectra of the Water-eluted Solution Obtained from Chromatogram of (D).

E: Faster moving spot (2-furoic acid).

F: Slower moving spot (5-formyl-2-furoic acid).

FIG. 6. Summary of Reactions of the Unsaturated Diuronide.

mannopyranosyluronic acid) -D-mannopyranosyluronic acid, from oxalic acid hydrolysate

of alginic acid.

From the results described in the present paper it can be seen that the structure of this unsaturated diuronide is possibly 4-O-(β -D- $\Delta^{4,5}$ -mannoseenpyranosyluronic acid)-D-mannuronic acid.

The reactions of this new unsaturated diuronide, described in the present study, are summarized in Fig. 6.

On hydrolysis, the formation of 2-furoic acid and of 5-formyl-2-furoic acid from unsaturated moiety of the diuronide was proved as compared with the hydrolysis of the palladium hydrogenated diuronide. Analogous degradative reaction has been observed during the course of hydrolysis of the intermediate compound isolated from *Erwinia* digestion of pectin¹⁵.

Crystalline D-mannuronic lactone has previously been isolated from acid hydrolysates

of alginic acid by a number of workers and reported that this lactone existed in two cry-

stalline forms, a lower-melting isomer^{11,18-21}) and a higher-melting isomer²²⁻²⁵). Recently in addition to the well established mannuronic acid, the presence of a second uronic acid, L-guluronic acid, was found in alginic acid by several workers²³⁻²⁸). Although the uronic moiety in this unsaturated diuronide was identified as D-mannuronic acid in the present investigation, it is very interesting that D-mannuronic acid and L-guluronic acid might yield an identical unsaturated compound wherein the configurational difference

is eliminated by the introduction of unsaturation between C-4 and C-5.

Recently, Yoshikawa²⁸) and Preiss et al.²⁹) also reported that alginic acid was degraded to a series of unsaturated oligouronides by the action of bacterial alginase. From the present and other workers' results, it may be one of the routine pathways that alginic acid is cleaved dehydratively by the action of both animal and bacterial alginase to produce a series of oligouronides containing $\Delta^{4,5}$ -uronic acid moiety.

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