[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE OHIO STATE UNIVERSITY]

Heparin—Hydrolytic Characteristics

BY M. L. WOLFROM AND J. V. KARABINOS[1]

In a previous communication,[2] the analytical data for the crystalline barium acid heparinate of Charles and Scott[3] were rather thoroughly reinvestigated. Some indications of minimum molecular size were also reported. From a degradative standpoint, the crystalline barium acid salt was hydrolyzed to D-glucosamine, isolated in high yield, thus confirming the previous work of Jorpes and Bergström on the amorphous sodium salt.

It was suggested in the previous communication[2] that a constituent other than D-glucosamine and uronic acid might be present. A search for another sugar component has now been made with a negative result. The uronic acid still eludes identification, but there is no reasonable doubt that it is present.

In order to proceed intelligently with the study of the degradation of the heparin molecule, a knowledge of its hydrolytic characteristics was desirable. For this purpose, a hydrochloric acid concentration of 4 *N*, a temperature of 98° and a heparin salt concentration of 1.5% or under, were employed. These conditions were chosen because it was known[2] that they were sufficient to liberate completely the sulfate and hexosamine, without destruction of the latter. The variations of the analyses with time are diagrammed in Fig. 1.

Curves A and B (Fig. 1) for the glucosamine liberation and uronic acid destruction roughly parallel each other, with the glucosamine being liberated initially at a rate faster than the uronic acid is destroyed. This would seem to point toward the possibility that under these hydrolytic conditions, the uronic acid is destroyed about as rapidly as it is liberated. It is also conceivable that decarboxylation of the uronic acid could proceed while the uronic acid was still in glycosidic combination. Curve C shows that the sulfate is hydrolyzed rapidly. The anticoagulant activity (Curve D) dropped quickly, the first sample (at fifteen minutes) analyzed being inactive. The activity may have been lost before this point. In curve E, the reducing sugar (Hagedorn–Jensen) levels off after ten hours at 21% (as glucosamine) after the destruction of all of the hexuronic acid initially present. This serves as a check on the glucosamine assay of Curve A, which reaches a maximum of 23% at the same time. The maximum in the rotation value (Curve F) roughly corresponds to the maximum in the reducing sugar curve. It is to be noted that an increase in reducing value does not necessarily indicate an hydrolysis to a monosaccharide unit, since chain rupture produces a reducing group as the terminal unit of a new chain. Attempts to isolate a uronic acid by interrupting the reaction at the point of maximum reduction were without success.

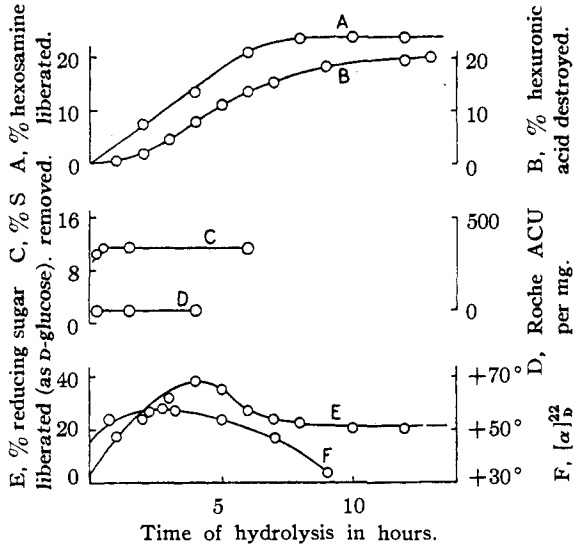

Fig. 1.—Hydrolysis of barium acid heparinate (*c* 0.25 g. per 100 cc. soln.) with 4 *N* hydrochloric acid at 98°; *c* 1.5 in B and F; *c* 0.8 in A; purified sodium heparinate used in A and F, but % calcd. to barium acid salt basis.

With the general characteristics of the heparin molecule when undergoing complete hydrolysis thus established, it is planned to study further the hydrolytic degradation of this substance under conditions short of complete hydrolysis.

Experimental

Qualitative Tests for Other Sugars.—Acid-hydrolyzed crystalline barium acid heparinate gave a negative Seliwanoff[4] test for ketoses and a negative aniline test[5] for pentoses (in the presence of uronic acids). The absence of 6-desoxyhexoses is shown by the negative CH_3-C assay[2] (Kuhn–Roth[6] chromic acid oxidation) previously reported. An amount of 2.0 g. of crystalline barium acid heparinate, purified through the benzidine salt, was hydrolyzed for eighteen hours under reflux with 100 cc. of 0.5 *N* sulfuric acid, whereupon the sulfate ion was removed with barium hydroxide, the resultant solution neutralized with barium carbonate, concentrated under reduced pressure to a thin sirup and precipitated by pouring into an excess of ethanol. The precipitate was removed by filtration, washed with 80% ethanol, the combined filtrate and washings concentrated to dryness under reduced pressure and the residue extracted with warm absolute methanol. A precipitate that formed on the addition of ether to this methanolic extract was removed by centrifugation and the centrifu-

(1) Hoffmann-La Roche Fellow of The Ohio State University Research Foundation.

(2) M. L. Wolfrom, D. I. Weisblat, J. V. Karabinos, W. H. McNeely and J. McLean, THIS JOURNAL, **65**, 2077 (1943); *Science*, **97**, 450 (1943).

(3) A. F Charles and D. A. Scott, *Biochem. J.*, **30**, 1927 (1936).

(4) T. Seliwanoff, *Ber.*, **20**, 181 (1887).

(5) W. E. Militzer, *J. Chem. Education*, **18**, 25 (1941).

(6) R. Kuhn and H. Roth, *Ber.*, **66**, 1274 (1933).

gate was concentrated to a sirup under reduced pressure. This sirup (67 mg.) was treated with 1 cc. of concentrated hydrochloric acid (d. 1.19) and some insoluble material removed by filtration. The filtrate was stirred at 0° for one hour with 1 cc. of ethyl mercaptan, whereupon the solution was neutralized with concentrated ammonium hydroxide, concentrated to dryness under reduced pressure and the dried residue was acetylated for twenty-four hours with acetic anhydride and pyridine (2:1). The acetylation mixture was poured into water, the water extracted with chloroform and the extract washed with 5% hydrochloric acid to remove the pyridine, then with aqueous sodium bicarbonate and water. On solvent removal from the dried chloroform solution there was obtained 10 mg. of a sirupy product that could not be brought to crystallization.

Hydrolytic Characteristics.—Crystalline barium acid heparinate (*c*, 0.25 g. per 100 cc. soln.) was hydrolyzed under reflux with 4 *N* hydrochloric acid and the rate of hydrolysis was followed in several ways. The data obtained are diagrammed in Fig. 1. The hexosamine liberation data (Curve A) have been reported previously[2] but are included for comparative purposes. They were obtained on the sodium salt (*c*, 0.8) purified through the crystalline barium acid salt. The hexuronic acid destruction (Curve B) was followed by placing the solution (*c*, 1.5) in the uronic acid assay apparatus of Burkhart, Baur and Link[7] and weighing the carbon dioxide evolved at the various time intervals. The sulfate liberated (Curve C) was determined in a separate portion to which an excess of barium acetate had been added. The anticoagulant activity (Curve D) was determined essentially according to the procedure of Foster[8] on portions of the solution that had been carefully neutralized with 2 *N* sodium carbonate and maintained at 0° until the activity was determined. The initial activity of the material used was 500 Roche ACU per mg. The reducing sugar liberated (Curve E) was determined on neutralized (by 2 *N* sodium carbonate) portions according to the method of Hagedorn and Jensen.[9] The polarimetric data (Curve F) were obtained on the neutral sodium salt, purified through the crystalline barium acid salt, employing a concentration of 1.5 g. per 100 cc. of solution.

Attempts to isolate a uronic acid by interrupting the reaction at the point of maximum reduction were without success.

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Summary

1. The general behavior of the heparin molecule on undergoing complete acid hydrolysis has been ascertained by following the time rate of change in rotation, reducing value, free sulfate, D-glucosamine, anticoagulant activity and uronic acid destruction.

2. No other sugar derivatives than D-glucosamine and a uronic acid (not isolated) were found on hydrolysis.

(7) B. Burkhart, L. Baur and K. P. Link, *J. Biol. Chem.*, **104**, 171 (1934).

(8) R. H. K. Foster, *J. Lab. Clin. Med.*, **27**, 820 (1942).

(9) H. C. Hagedorn and B. N. Jensen, *Biochem. Z.*, **135**, 46 (1923).

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF OREGON]

The Conductance of Non-aqueous Solutions of Magnesium and Calcium Perchlorates[1]

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The conductance data reported in the present paper were obtained in the course of the general investigation on organic solutions of metallic salts, particularly of the alkaline earth perchlorates, which is being carried out in this Laboratory. The conductances of solutions of magnesium perchlorate in *n*-propyl and isopropyl alcohols were recently reported by Van Rysselberghe and Hunt.[2] We are now presenting the conductances of the same salt in acetone, methyl alcohol and nitromethane. Since the only data on conductances of organic solutions of alkaline earth perchlorates in the literature preceding our work are the conductances of dilute solutions of barium perchlorate in acetone, measured by Walden, Ulich and Busch,[3] we have turned our attention to calcium perchlorate, and are, therefore, able to compare the conductances of the perchlorates of three alkaline earth ions, magnesium, calcium, and barium in the same solvent, acetone.

(1) Based in part upon a thesis submitted by Robert M. Fristrom in partial fulfillment of the requirements for the M.A. degree at the University of Oregon, August, 1944.

(2) P. Van Rysselberghe and G. J. Hunt, THIS JOURNAL, **66**, 1488 (1944).

(3) P. Walden, H. Ulich and G. Busch, *Z. physik. Chem.*, **123**, 429 (1926). Data available in W. A. Roth and K. Scheel, Landolt-Börnstein, "Physikalisch-chemische Tabellen," Erster Ergänzungsband, 1927, p. 636.

Experimental

Salts.—Most of the magnesium perchlorate used was prepared and dehydrated according to the methods previously described.[2] Some portions of this salt and all of the calcium perchlorate were dehydrated in a vacuum oven of the Abderhalden type using the vapor of boiling nitrobenzene as a source of heat. The calcium perchlorate was prepared from c. p. calcium oxide and perchloric acid. The purity of both perchlorates was checked by analysis.

Solvents.—High-grade solvents were used: Merck C. P. acetone had, without additional purification, the correct boiling point and a conductivity in close agreement with the value given by Scudder[4]; J. T. Baker C. P. methyl alcohol was refluxed over calcium oxide and fractionated, the final product having, again, a conductivity in close agreement with Scudder's value[4]; Eastman Kodak Co. white label nitromethane was treated with sodium amalgam and calcium oxide, then fractionated, the final product having the correct boiling point but a conductivity somewhat higher than Scudder's value.[4] Corrections for solvent conductivity were made whenever necessary.

Conductivity Bridge, Conductivity Cells, Temperature Control, Preparation of Solutions.—The equipment used was the same as that previously described.[2] Conductiv-

(4) H. Scudder, "The Electrical Conductivity and Ionization Constants of Organic Compounds," D. Van Nostrand Co., New York, N. Y., 1914.