the filtrate liberated the 4-hydroxy-3-iodoquinoline. The latter was removed by filtration, dried and treated with 3 volumes of phosphorus oxychloride to yield the 4,5- and 4,7-dichloro-3-iodoquinolines according to the procedure originally used in their preparation.[1] By this method was obtained 0.06 g. of 4,5-dichloro-3-iodoquinoline (m.p. 109–110.5° after recrystallization from a mixture of Skellysolve A and B followed by recrystallization from methanol) from XVII and 0.35 g. of 4,7-dichloro-3-iodoquinoline (m.p. 111–112° after recrystallization from Skellysolve B) from XVIII. Mixed melting points with authentic samples were not depressed in either case.

Proof of Structure. Preparation of 3-Aminoquinoline from 4-Chloro-3-nitroquinoline.—Three grams of 4-chloro-3-nitroquinoline were reduced in methanol in the presence of Raney nickel at room temperature. After the hydrogen uptake had indicated the complete reduction of the nitro group, 2 g. of solid potassium hydroxide and a little fresh catalyst were added and the reduction continued at 50°. After filtering off the catalyst, the methanol was removed by distillation and the residue taken up in dilute hydrochloric acid, filtered with charcoal, the filtrate treated with excess sodium hydroxide solution and the aqueous solution extracted four times with ether. The ether solution, after drying with Drierite and filtering with charcoal, was evaporated to give a pale yellow oil which solidified on standing. This was crystallized from 200 ml. of Skellysolve B to give 0.5 g. of small white needles; m.p. 83.5–84.5°. The acetyl derivative, prepared according to the procedure of Mills and Watson[12] melted at 168–168.5°. These workers report this derivative as melting at 166–167°.

3-Amino-7-chloroquinoline.—A solution of 4,7-dichloro-3-nitroquinoline in methanol was reduced catalytically in the presence of Raney nickel as described in the preceding paragraph. The crude product was recrystallized from Skellysolve C followed by recrystallization from alcohol and water to give long silky white needles, m.p., 143–143.5°.

Anal. Calcd. for $C_9H_7ClN_2$: Cl, 19.85. Found: Cl, 19.74.

4-Chloro-6-nitroquinoline.—The nitration procedure was adapted from that used by Halcrow and Kermack[9] for the preparation of 6-nitro-4-hydroxyquinaldine.

A mixture of 10 ml. of concentrated sulfuric and 10 ml. of concentrated nitric acids was dropped into a solution of 20 g. of 4-hydroxyquinoline in 100 ml. of concentrated sulfuric acid maintaining the temperature at 0–5°. After addition was complete the mixture was allowed to warm to room temperature and stand for two hours. The solution was poured into 500 ml. of ice and water, allowed to stand overnight and the separated solid filtered off, washed with water and dried to give 18.3 g. (70%) of material which was largely 4-hydroxy-6-nitroquinoline. The product may be recrystallized from thirty volumes of glacial acetic acid but this purification is unnecessary if the product is to be chlorinated.

Sixteen grams of the above crude product was heated with 30 ml. of phosphorus oxychloride until solution occurred (5–10 minutes), the mixture poured into ice-water, warmed briefly to 40° and filtered with charcoal. The clear yellow solution was treated with sodium hydroxide solution, the free base taken up in ether, the latter solution dried over Drierite and the solvent distilled off to give 10.5 g. (60%) of pale yellow solid, m.p. 140–142°. Recrystallization from methanol gave long fluffy yellow tinted needles, m.p. 144–145°.[11]

Nitration of 4-hydroxyquinoline according to the procedure of Gouley, Moersch and Mosher[10] followed by treatment with phosphorus oxychloride and purification of the product gave the same compound, melting at 144–145°.

6-Aminoquinoline.—Three grams of 4-chloro-6-nitroquinoline, twice recrystallized from methanol, was reduced stepwise by the procedure described above for the preparation of 3-aminoquinoline. The residue, after evaporation of the methanol, was taken up in dilute hydrochloric acid, the solution adjusted with ammonium hydroxide until just basic to litmus and filtered with charcoal. The filtrate was made strongly basic with sodium hydroxide solution and the alkaline solution extracted three times with ether. Evaporation of the dried ether extracts gave 0.93 g. of a pale yellow solid, m.p. 115–116°. Recrystallization from Skellysolve C gave large white leaflets, m.p. 116–116.5°.[13] The acetyl derivative melted at 138.5–139°.[17]

6-Amino-4-chloroquinoline.—Three grams of purified 4-chloro-6-nitroquinoline was reduced catalytically to give 1.95 g. of crude material, m.p. 184–186°. Recrystallization from 70 ml. of methanol gave pale yellow needles, m.p. 186–187°.[16]

Anal. Calcd. for $C_9H_7ClN_2$: Cl, 19.85; N, 15.69. Found: Cl, 19.60; N (Kjeldahl), 15.30.

RENSSELAER, N. Y. RECEIVED NOVEMBER 22, 1950

[CONTRIBUTIONS FROM THE DEPARTMENT OF MEDICINE, COLUMBIA UNIVERSITY COLLEGE OF PHYSICIANS AND SURGEONS, AND THE EDWARD DANIELS FAULKNER ARTHRITIS CLINIC OF THE PRESBYTERIAN HOSPITAL]

Analysis of the Products Formed on Hydrolysis of Hyaluronic Acid by Testicular Hyaluronidase[1,2]

BY MAURICE M. RAPPORT, KARL MEYER AND ALFRED LINKER

A scheme for the analysis of enzymic hydrolysates of hyaluronic acid has been devised based upon adsorption on charcoal (Darco G-60) followed by fractional elution with 100-ml. volumes of a succession of solvents: water, 5% ethanol, 15% ethanol, 0.1% pyridine, 0.5, 1.0, 1.5, 2.0, and finally 5.0% pyridine. The effluent is collected in 10-ml. fractions which are analyzed for reducing sugar, uronic acid and acetylglucosamine equivalents. Analysis of the data so obtained shows the chromatographic technique employed to be capable of separating fractions of different molecular size but similar chemical composition. Although the technique does not yield definitive separations, its application to hydrolysates obtained after various time intervals of enzymic action has led to the following points being confirmed or established with regard to hyaluronic acid structure and the mechanism of *testicular hyaluronidase* action: (1) Hyaluronic acid is composed of a uniform structure of alternating acetylglucosamine and glucuronic acid residues. There is no evidence of the presence of a core of polyacetylhexosaminides or polyuronides. (2) The enzyme attacks the substrate close to the center of the polysaccharide chains breaking only the glucosidic bond formed by the oxygen on carbon-1 of the acetylglucosamine residue. (3) The enzymic hydrolysis does not proceed down to the liberation of monosaccharides.

Introduction

Hyaluronic acid is a very high molecular weight polysaccharide acid of widespread occurrence. It has been isolated from vitreous humor, synovial fluid, umbilical cord, skin, tumor fluids and hemolytic streptococci in the mucoid phase. It is a substance of considerable importance in such diverse problems as bacterial invasiveness, permeability of ground substance and the so-called "spreading reaction," fertilization, the etiology of rheumatic disease, and related fields in physiology and medicine.[3]

The detailed structure of hyaluronic acid is not known, although analyses of carefully purified preparations show that it is composed of equimolar

(1) Supported by grants from the Helen Hay Whitney and Josiah Macy, Jr., Foundations.

(2) Presented at the 118th Meeting of the American Chemical Society, Chicago, Illinois, September, 1950.

(3) *Ann. N. Y. Acad. Sci.*, **52**, 943 (1950).

parts of glucuronic acid, glucosamine and acetic acid,[4] the latter two components apparently combined into an N-acetylglucosamine residue.[5] From acid hydrolysates, glucosamine has been isolated as the hydrochloride in better than 70% yield, while the glucuronic acid has been identified by isolation in very good yield of saccharic acid as the acid potassium salt after nitric acid oxidation of the polysaccharide.[4] The composition suggests a structure for hyaluronic acid composed of alternating N-acetylglucosamine and glucuronic acid residues and evidence obtained by enzymic hydrolysis supports this view.[6] This concept of hyaluronic acid structure has been challenged recently by Kaye and Stacey[7] who suggest that hyaluronic acid may contain a chitin-like "core" of N-acetylaminosugar units attached to a complicated polyglucuronic acid structure.

Enzymes which hydrolyze hyaluronic acid also have a widespread occurrence,[8] the most readily available sources at present being mammalian testes and bacteria such as pneumococcus, streptococcus, staphylococcus and clostridium.

This paper is concerned with an analysis of the products of the action of a purified hyaluronidase from bull testis on one of our purest preparations of sodium hyaluronate derived from human umbilical cord. Products obtained by enzymic hydrolysis of hyaluronic acid have been studied in several laboratories.[8,9,10] However, the purity of the enzymes and, more important, the substrates employed by these workers is for comparative purposes difficult to assess, while the lack of a quantitative treatment precludes conclusive interpretations of their data. In the study reported here, some of these objections are avoided by (1) employing a purified enzyme preparation which is commercially available and therefore readily accessible, (2) employing an analyzed preparation of sodium hyaluronate, and (3) examining the total hydrolysate rather than a fraction. This latter approach in particular helps to define the limits of ambiguity which result from the presence of impurities in the starting materials.

The fractionation scheme is an extension of the method of Whistler and Durso[11] for the separation of sugars on charcoal. The absence of suitable reference compounds in this field made it mandatory to collect fractions sufficiently large so that analyses on isolated material could be performed. The chromatographic method does not yield definitive separations, but its application to hydrolysates obtained at various time intervals of the enzyme–substrate reaction combined with the quantitative examination of the properties of the fractions has yielded considerable information both on the mechanism of action of testicular hyaluronidase and on the structure of hyaluronic acid.

(4) K. Meyer and J. W. Palmer, *J. Biol. Chem.*, **114**, 689 (1936).
(5) K. Meyer, *Cold Spring Harbor Symposia on Quant. Biol.*, **VI**, 91 (1938).
(6) K. Meyer, *Physiol. Rev.*, **27**, 335 (1947).
(7) M. A. G. Kaye and M. Stacey, *Biochem. J.*, **46**, xiii (1950).
(8) L. Hahn, *Arkiv Kemi, Mineral, Geol.*, **19A**, 1 (1945).
(9) H. J. Rogers, *Biochem. J.*, **40**, 782 (1946).
(10) L. Hahn, *Biochem. Z.*, **318**, 123, 138 (1947).
(11) R. L. Whistler and D. F. Durso, THIS JOURNAL, **72**, 677 (1950).

Experimental

Materials.—The enzyme employed was a preparation of testicular hyaluronidase assaying 800 turbidity reducing units per mg. by our method.[6,12] The sodium hyaluronate was prepared from human umbilical cords by methods previously described.[4,13] An analysis of this material is presented in Table I.

TABLE I

ANALYSIS OF SODIUM HYALURONATE EMPLOYED AS STARTING MATERIAL[a]

Component	Per cent.		Moles per atom N
Nitrogen (Kjeldahl)[b]	3.15	3.16	
Hexosamine	39.7	39.8	0.98
Uronic acid (CO_2)	44.1		1.01
Sulfate (acid hydrolyzable)	2.5		0.12

$[\alpha]^{25}D$ −74° (*c*, 0.25% in H_2O)

[a] Dried over phosphorus pentoxide *in vacuo* at room temperature. [b] N calcd.: 3.40 (corrected for 12% sulfated polysaccharide).

Preparation and Fractionation of Enzymic Hydrolysates.—One gram of sodium hyaluronate was incubated with 25 mg. of enzyme in 100 ml. of 0.1 *M* sodium acetate buffer containing sodium chloride (0.15 *M*), final *p*H 5.0, at 37°, under toluene. At intervals of 10 min., 30 min., 1 hour, 2 hours, 4 hours and 24 hours, 10-ml. aliquots were withdrawn and placed on a well-washed column prepared with 2 g. of Darco G-60 and 1 g. of Celite-503, measuring 18 mm. in diameter and 35 mm. in length. Successive 100-ml. portions of water followed by 5% ethanol, 15% ethanol, 0.1% pyridine, 0.5% pyridine, 1.0, 1.5, 2.0 and finally 5.0% pyridine were then put through the column. The effluent was collected in 10-ml. fractions, and the course of the elution was followed by measurement of the reducing sugar and uronic acid content of each tube. On many fractions analyses for acetylglucosamine equivalent were performed. Where sufficient material was present in a fraction, the material was isolated by evaporation of the solvent under reduced pressure and analyzed on the basis of dry weight for hexosamine and uronic acid CO_2, in addition to colorimetric uronic acid, acetylglucosamine equivalent and reducing sugar.

Analytical Methods.—Analyses for the nitrogen, hexosamine, uronic acid CO_2 and hydrolyzable sulfate content of the polysaccharide were carried out as previously reported.[12] Reducing power was determined by a modification of the Hagedorn–Jensen method.[14] Uronic acid in small quantities was determined colorimetrically by the method of Dische.[15] Uronic acid contents of samples measured by both colorimetric and decarboxylation methods were in excellent agreement (within 5%).

Acetylglucosamine equivalents were determined by a new reaction for this grouping. It was found that N-acetylglucosamine reacts with acetylacetone to form a product producing a red color with *p*-dimethylaminobenzaldehyde under conditions almost identical with those employed for the determination of glucosamine. No color is produced in the absence of the acetylacetone. The color produced per mole of acetylglucosamine is about one-third of that formed by glucosamine. The sole modification in the procedure was the time employed for color development with the *p*-DAB, which was increased from 30 to 40 min. Synthetic N-acetyl-D-glucosamine was used as the standard. The possible interference of residues containing a free amino group was excluded by the sensitive ninhydrin spot test on paper, which showed that there was less than 1 μg. of glucosamine equivalent in 200 μg. of substance for several of the isolated fractions.

Results and Discussion

Since the limits within which the present work may be considered conclusive are dependent on the

(12) K. Meyer and M. M. Rapport, *Arch. Biochem.*, **27**, 287 (1950).
(13) K. Meyer, "Advances in Protein Chemistry," Vol. II, Academic Press, Inc., New York, N. Y., 1945, p. 253.
(14) M. M. Rapport, K. Meyer and A. Linker, *J. Biol. Chem.*, **186**, 615 (1950).
(15) Z. Dische, *ibid.*, **167**, 189 (1947).

purity of the hyaluronic acid and the quantitative aspect of the analysis of the hydrolysate, we believe some discussion of these factors is necessary.

The physical properties of hyaluronic acid are not sufficiently well-defined to establish its degree of purity. Such an estimate can at present only be obtained from analysis of its constituents. There are four important criteria. The first is the nitrogen content. When this value is higher than the calculated value (3.49), protein contamination is indicated. Factors contributing to low values are the degree of hydration and the presence of inorganic salts and other nitrogen-free impurities such as glycogen. The second, and far more significant, criterion is the molar ratio of hexosamine to nitrogen. Deviations of this ratio from 1 reveal the presence of nitrogenous impurities such as protein, a contaminant difficult to remove from hyaluronic acid preparations. The third criterion, the molar ratio of uronic acid to hexosamine, in general serves the same purpose as the second, but is included here because so much of the experimental work is based on the uronic acid analyses, inasmuch as they are carried out routinely much more readily than hexosamine analyses. The fourth criterion, the acid hydrolyzable sulfate content, is very important because the main polysaccharide contaminate encountered, other than glycogen, is a chondroitin sulfate, of composition similar to hyaluronate but containing a residue of ester sulfate. This substance is exceptionally difficult to remove from hyaluronic acid derived from umbilical cord. It can be seen (Table I) that the sodium hyaluronate employed in this study meets the first three criteria extremely well, but the fourth shows that the preparation still contains about 12% of a sulfated polysaccharide as a contaminant.[16] This quantity serves to define the limits within which conclusive interpretations of structure may be made based upon analysis of the products of hydrolysis.

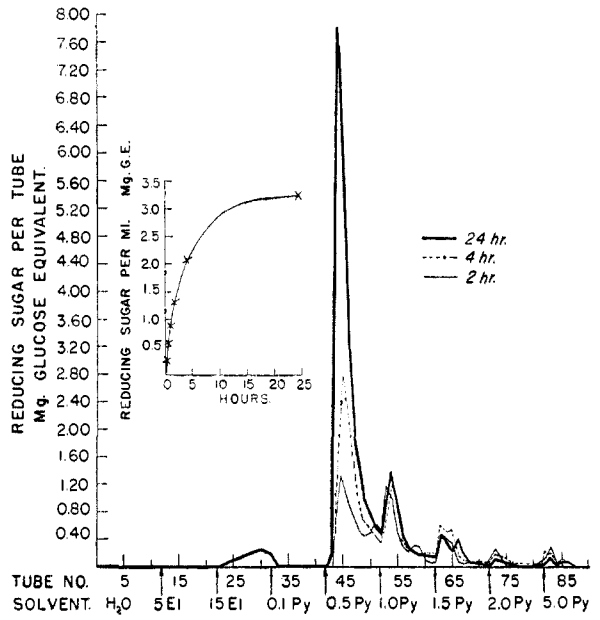

Fig. 1.—Reducing sugar content of tube plotted against tube number for 2-, 4- and 24-hour hydrolysates. Insert shows course of hydrolysis determined directly on incubation mixture.

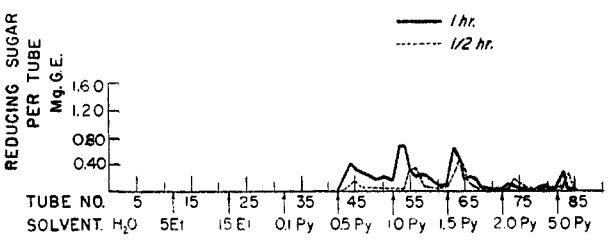

Fig. 2.—Reducing sugar content of tube plotted against tube number for one-half and 1-hour hydrolysates.

With regard to the quantitative aspect of the analysis of the hydrolysate, the examination of hydrolysis products may be considered quantitative since it was found that where uronic acid determinations were performed on all fractions collected from the chromatogram of a given hydrolysate, for example, with those representing the 2-, 4- and 24-hour hydrolysis intervals, 98% recoveries of the initial uronic acid were obtained.

The results obtained by plotting the reducing sugar content of the tube against the tube number are shown in Fig. 1 for the 2-, 4- and 24-hour hydrolysates and in Fig. 2 for the one-half and 1-hour hydrolysates. The 10-minute hydrolysate yielded reducing sugar values which were too small to be reliable. The insert in Fig. 1 shows the course of the hydrolysis as determined directly on aliquots of the incubated solution. It can be seen that the reaction is essentially complete in 24 hours. In one hour it has progressed about 25%.

From the results shown in these figures, four important observations may be made. The first is that no monosaccharides have been liberated even after 24 hours, since these would have at least begun to appear in the water and 5% ethanol eluates if they were present. This follows from the work of Whistler and Durso[11] and from an experiment carried out with a solution of 5 mg. of sodium glucuronate and 5 mg. of N-acetylglucosamine in acetate buffer carried out under conditions identical with those described. In this experiment, 50% of the glucuronic acid appeared in the water, 30% more was obtained in the 5% ethanol and an additional 10% in the 15% ethanol. All of the N-acetylglucosamine appeared in the first two 5% ethanol tubes. This observation of the absence of monosaccharide formation confirms the results of the adsorption analysis study reported by Hahn.[10]

The second observation is that although the reducing sugar content increases with time in almost all of the fractions, the relative quantity eluted with the lower pyridine concentrations is greater with more extensive hydrolysis. This indicates that the smaller hydrolytic breakdown products are less strongly adsorbed.

The third observation is that the final hydrolysis products are still somewhat heterogeneous and that the enzymic reaction apparently ceases before it is complete.

(16) It should be noted that a *sulfur* content of only 1% (sulfate, 3%) represents 15% of the sulfated polysaccharide.

The fourth observation is that at the end of the first hour, when more than 25% of the total reaction has been completed, no single fraction predominates. Unless large differences exist in the susceptibility of substrates of different chain lengths (hyaluronic acid appears to be polydisperse), this seems to be rather substantial evidence for the conclusion that the enzyme attack on the substrate occurs close to the center of the polysaccharide chains rather than at terminal periods. This identical conclusion had been arrived at previously from entirely independent considerations.[14]

Since the total uronic acid content (unlike the reducing power) remains constant during the enzymic process and is a direct measure of the weight of material, a clearer picture of the changes occurring from one time interval to another is obtained by plotting this quantity against the tube number. Such a plot is shown in Fig. 3 for the 4- and 24-hour hydrolysates. All the solvent fractions following the 0.5% pyridine can be seen to contain more material with the 4-hour sample, in essential agreement with the previous curves. The quantity of material eluted by the 15% ethanol is too small (less than 3% of the total) to merit consideration.

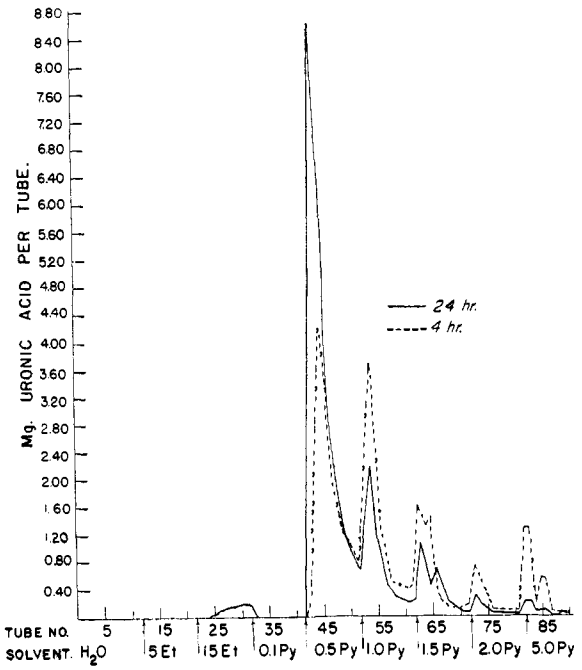

Fig. 3.—Uronic acid content of tube plotted against tube number for 4- and 24-hour hydrolysates.

These curves are irregular and interpretation of their detailed pattern would at this time be difficult, and perhaps misleading. For this reason the areas under the curves obtained *with each solvent* have been integrated and are presented in Fig. 4. In this figure the solvent fractions can be compared with each other for a particular hydrolysate and with the corresponding fraction from a different hydrolysate. Also, the different hydrolysates are more readily compared with each other. Two quantities are presented for each solvent fraction. The solid bar indicates the

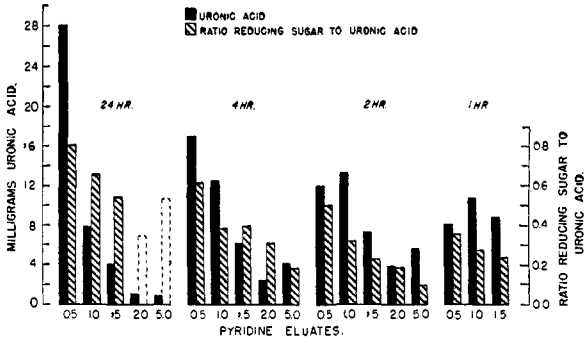

Fig. 4.—Uronic acid content and reducing sugar to uronic acid ratio for combined fractions eluted from column with single solvent. Reducing sugar to uronic acid ratio for 2.0 and 5.0% pyridine eluates for 24-hour hydrolysate shown dotted because reducing sugar equivalents were too small to obtain accurate values.

quantity of uronic acid and is a measure of the total weight of material, while the cross-hatch bar represents the ratio of reducing sugar equivalents to uronic acid. This ratio is a function of the molecular weight, and, as presented, the shorter the cross-hatch bar, the smaller the ratio, and hence the larger the molecular weight. The exact degree of dependence of the ratio on the molecular weight can of course not be known in the absence of information on the reducing power of the separate molecular species, but there is no reason to believe that the deviation from direct proportionality is appreciable. Two predominant trends are apparent when the analyses are examined in this way. The first confirms the observation already made that as the hydrolysis proceeds, the substances formed move into those fractions which are eluted with lower pyridine concentrations. In the 24-hour hydrolysate, 67% of the total material is in the 0.5% pyridine fraction with 19% in the 1.0% pyridine and 10% in the 1.5% pyridine, compared with 40, 30 and 15%, respectively, for the 4-hour hydrolysate, and 29, 32, and 17% for the 2-hour hydrolysate. Second, with increasing concentrations of pyridine, the average molecular weight of the fractions eluted is seen to increase. If we assume a uniform chemical composition for hyaluronic acid, it follows that the chromatographic technique is achieving some fractionation of the hydrolysis products dependent on molecular weights. That the resolution is not definitive can be seen by comparing the reducing sugar to uronic acid ratios for the same solvent fraction obtained with different hydrolysates. For example, these values are 0.80, 0.61, 0.50 and 0.35 for the 0.5% pyridine eluates of the 24-, 4-, 2- and 1-hour hydrolysates, respectively. This would seem to indicate that the efficiency of resolution of the adsorption system increases with decrease in the average molecular weight of the polymers with which it is loaded. The failure to obtain clear-cut separations has so far prevented us from obtaining one of the most important pieces of information which we originally hoped to be forthcoming from this study, namely, the value of this reducing sugar to uronic acid ratio for the ultimate product of testicular enzyme action. This in-

formation is the *sine qua non* for any rational interpretation of reaction rates and enzyme activities based upon measurements of reducing power. The course of the reaction suggested that this end-product would be found in the 0.5% pyridine fraction of the 24-hour hydrolysate which is represented by the very sharp peak. So far only amorphous products have been isolated from this fraction. The reducing sugar to uronic acid ratios measured directly on the individual tubes constituting this peak have the values 0.92, 0.91, 0.91, 0.77, 0.83, 0.77 and 0.78 for tubes 44 to 50, respectively, indicating that the value representing the end-product was close to 0.92. However, the appearance in subsequent experiments of fractions with higher values (see Fig. 5) leads us to suspect that the sharp peak may still contain several species, and this fraction is being subjected to further study.

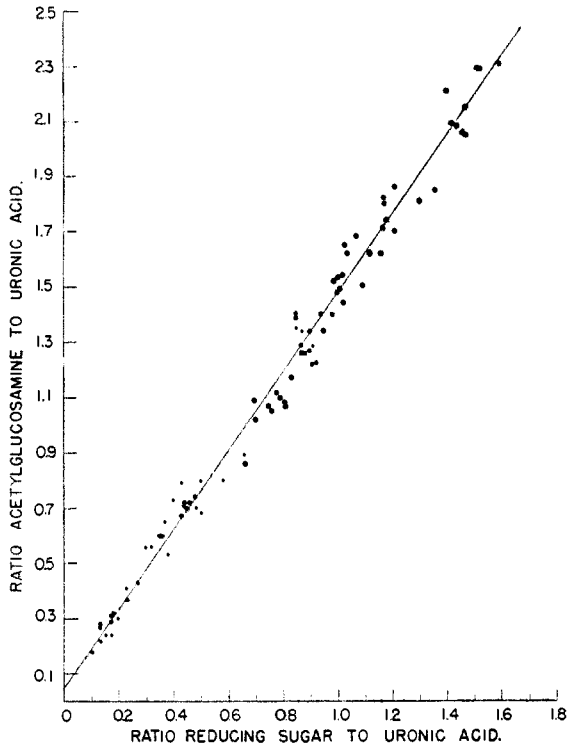

Fig. 5.—Scattergram showing the ratio of acetylglucosamine [equivalent] to uronic acid plotted as a function of molecular weight (*i.e.*, the ratio of reducing sugar [equivalent] to uronic acid) for 86 fractions obtained from the columns.

In every case where isolated material was examined, regardless of the hydrolysate from which it was derived, or the solvent employed for elution, or the apparent molecular weight, the same composition was found, namely, equimolar proportions of glucosamine and uronic acid. This is reasonably conclusive, within the limits set by this study of the homogeneity of hyaluronic acid and indicates the structure to be one of alternating residues of acetylglucosamine and glucuronic acid. There is no indication from any of these fractions of the existence of a "core" or a prosthetic group as suggested by Kaye and Stacey.[7]

The final study of the chromatographic fractions which we wish to report is that based on the analysis for acetylglucosamine equivalent. The most significant treatment of the data obtained is that shown in Fig. 5 in which the ratio of the values for acetylglucosamine and uronic acid, respectively, is plotted against the ratio of the values for reducing sugar and uronic acid, the latter ratio being a function of the molecular weight. The data are plotted in this way in order to emphasize the fact that the different fractions have different number average molecular weights. It can be seen that the results of these three analyses performed on 86 fractions ranging over at least a tenfold difference in average molecular weight fall on a straight line. The slope represents the ratio of acetylglucosamine (equivalent) to reducing sugar (as glucose), and its constancy shows that there is only *one* grouping which is responsible for *both* reactions, that is, that the opening of the *same type* of glucosidic bond, the one liberating the aldehydic function of the acetylglucosamine residue, is responsible for *all* the increase in reducing power. The liberation of a reducing group of a different type would produce a substance with distinctly different chemical properties. It might be expected that such a substance would appear at least somewhat concentrated in one of the fractions. The locus of a point representing the analyses of such a fraction would appear well below the line in the scattergram. No such observation was made. The enzyme preparation employed in this study has thus opened only one of the (presumably) two possible types of glucosidic bonds present in the substrate. This enzyme preparation is readily available and interpretations of results obtained with its use can be considerably simplified in view of the demonstration of this specificity of action with respect to hyaluronic acid.

The ratio of acetylglucosamine equivalent to glucose equivalent, the value of which is 1.42 by the methods employed, is a fundamental property of any product of the testis enzyme action irrespective of molecular size.

Similar studies are in progress with chondroitin sulfuric acid and hyaluronidases of bacterial origin.

Acknowledgments.—It is a pleasure to acknowledge the very considerable assistance of Miss Doris Anne Hall and Miss Jean McLean in this work. We also wish to express our gratitude to Dr. Joseph Seifter of the Wyeth Institute for the enzyme preparation employed in this study.

NEW YORK, N. Y. RECEIVED OCTOBER 21, 1950