

On the Structure of Hyaluronic Acid

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In a case of mesothelioma about 20 g of pure hyaluronic acid were isolated from the highly viscous pleural and peritoneal fluid¹. Advantage was taken of the relatively great amount of hyaluronic acid thus obtained to attempt to elucidate the structure of this substance, using the same principles as those applied by Meyer *et al.*² in their investigation of the structure of chondroitin sulfuric acid. When the present work had proceeded for some time, investigations on the same subject were communicated, first by Jeanloz³, in a preliminary note, and somewhat later, briefly, by K. H. Meyer and Fellig⁴.

Based on reasons which, owing to the rather short form of the communication, are somewhat difficult to judge, Jeanloz suggests that the acetylglucosamine and glucuronic acid residues of the hyaluronic acid are mutually joined by 1,3-linkages. According to Meyer and Fellig periodic acid at 0° C and pH 4.7 attack only the end groups of the hyaluronic acid molecule. The same was found to be true also with the fully methylated and subsequently methanolysed substance. Assuming the hyaluronic acid to be a linear polymer, composed of alternating residues of acetylglucosamine and glucuronic acid, both being present in the pyranose form, the results mentioned would indicate that the basic units of the chain are bound by glucuronido-4-glucosamine and glucosaminido-3-glucuronic acid linkages. Recently Kaye and Stacey⁵ reported the isolation of a dimethyl ether of methyl glucopyruronide methyl ester from the methylated product of a somewhat degraded hyaluronic acid, thus giving definite evidence of the pyranose form of glucuronic acid in that substance. On the basis of their analyses of the methylated hydrolysis products of hyaluronic acid they reject the idea that the hyaluronic acid consists of linearly linked disaccharidic units and suggest that the N-acetyl amino sugar groups form a chitin-like "core" to which is attached a complicated polyglucuronic acid structure. (The report is a short abstract of a paper read to the Biochemical Society in London.)

ISOLATION, COMPOSITION AND MOLECULAR WEIGHT OF THE HYALURONIC ACID INVESTIGATED

The isolation of the preparation used has earlier been described in detail ¹. The proteins of the tumor fluid were degraded by papain and mostly removed by dialysis. Protein residues were removed by the Sevag technique. The native tumor fluid was very viscous. As not infrequently happens with the procedure employed, the viscosity of the hyaluronic acid solution decreased markedly during the isolation, the pure product showing a relative viscosity of only 1.76 at +25° and pH 7.0 in 0.05 *M* phosphate and 0.05 *M* NaCl.

The reduction power as measured according to the method of Willstaetter and Schudel as modified by Linderström-Lang and Holter ⁶ indicated a mean molecular weight of about 28 000.

The analysis of the pure product gave the following results.

	%	Calculated for the Na-salt of hyaluronic acid %
Glucosamine	45.1	44.6
Glucuronic acid	47.5	48.4
Acetyl	9.8	10.7
Nitrogen	3.39	3.49
Sulfuric acid	0	0
Optical rotation (end value)	— 77.9° ± 1°	

Glucosamine was determined with the method of Elson and Morgan as modified by Blix ⁷, glucuronic acid according to Burkhart, Baur and Link ⁸ and acetyl according to Friedrich and Rapoport ⁹. — The values obtained showed the product to be very pure. A negative ninhydrin test indicated the absence of proteins and protein split products.

THE STRUCTURE OF HYALURONIC ACID

According to the chemical analyses the hyaluronic acid is composed of an equal number of acetylglucosamine and glucuronic acid residues. Studies of double refraction of flow and viscosity (Blix and Snellman ¹⁰) of the native substance have shown that it has a long chain structure. As a working hypothesis it therefore may be reasonably assumed that the molecule of the hyaluronic acid is composed of glucosidically linked repeating units of acetylglucosamine and glucuronic acid. Of the monosaccharides the glucuronic acid is with

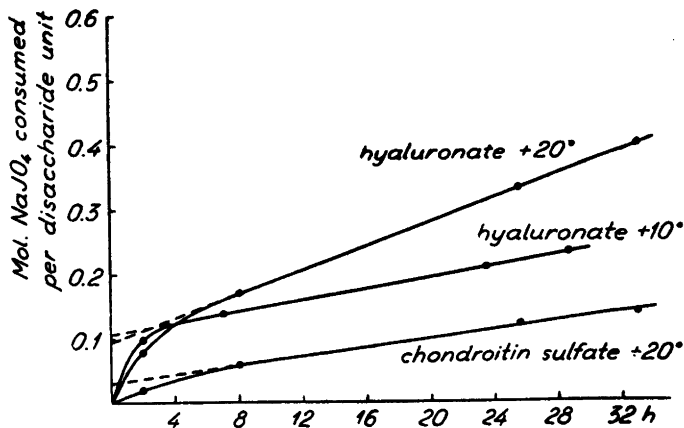


Fig. 1. Oxidation of unmethylated substances.

of structure I or II. However, the consumption of periodic acid is probably greater than can be due to the oxidation of the end groups of a straight chain composed of 70 disaccharide units. Two explanations may be suggested: 1. In addition to the main chain the molecule may have a few short side chains with nonreducing end groups. 2. In addition to a great number of units of structure I or II, the molecular chain may contain a small number of units with some other kind of linkages, for example 1,6 linkages.*)

2. Oxidation of the methylated and methanolysed substance

Several experiments were conducted of which one is given in Fig. 2. The consumption of periodic acid was found to be about 0.5 molecule per disaccharide unit. This result is most simply interpreted by the assumption that the hyaluronic acid molecule is composed of an equal number of disaccharide units of structure I and II. In agreement with this assumption one half or somewhat less of one molecule of ammonia per disaccharide unit was found to be liberated on the oxidation.

* As the determination of the molecular weight with the aid of the reduction power can be regarded only as a rather approximate method, the moderate excess oxidation found might possibly at least partly be due to the use of a too high value of the molecular weight.

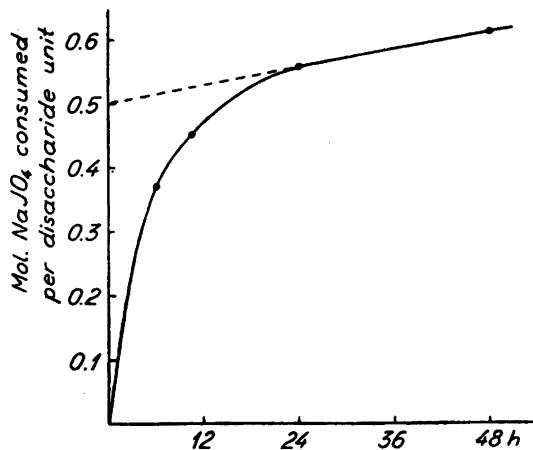


Fig. 2. Oxidation of methylated and methanolysed hyaluronic acid.

3. Oxidation of the methylated and hydrolysed substance

The results of the experiments given above might, to some extent, be checked by experiments on the methylated and hydrolysed product. With structure I or II two molecules of periodic acid should be consumed per disaccharide unit, whereas with most of the other structures three molecules should be consumed. Ammonia should be liberated in both cases. Actually, the hydrolysis showed a biphasic course. At $+55^{\circ}$ — $+60^{\circ}$ in 4 N HCl reducing values (obtained by the method of Linderström-Lang and Holter), corresponding to one reducing group per disaccharide, were reached in about 12 h, whereafter the reduction power increased only very slowly, reaching a constant value in 5—6 days. The reducing value arrived at after that time amounted to only about 80 % of that calculated for 2 reducing groups per dimer, the deficit most probably being due to secondary reactions involving the C₁ group of the glucuronic acid. After hydrolysis for 8 days the oxygen consumption found was about 2.0 atoms per dimer (Fig. 3) and the oxidation liberated about 0.8 molecule ammonia on the same basis.

Considering especially the secondary reaction in which the liberated ammonia is likely to be involved, these results agree fairly well with those of the preceding experiments and thus give some further support of the conclusions drawn

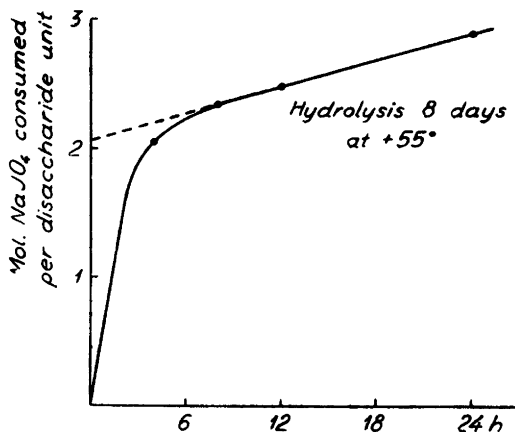


Fig. 3. Oxidation of methylated and hydrolysed hyaluronic acid.

from them. (The possibility of using the determination of the formed formic acid for further evidence was tried, but owing to a technical misfortune the results received are unreliable).

COMMENT

The observations made are consistent with the idea that the molecule of the hyaluronic acid investigated is in the main constituted of linearly linked dimers of N-acetyl-glucosamine and hyaluronic acid and indicate that the glucosamine residue is glucosidically linked to the 3rd carbon atom of the glucuronic acid residue. The results further make it probable that one half of the glucuronic acid residues are linked to the 3rd and the other half to the 4th C-atom of the glucosamine part. If this is true, these two kinds of linkages may either be present alternately in the same molecular chain or, less probably, may belong to two different kinds of molecules, present in equal amounts. The studies on hyaluronic acid from navel cord by Meyer and Fellig indicated presence exclusively of glucuronido-4-glucosamine linkages. Possibly the structure may vary in hyaluronic acid of different origin. It should also be emphasized that in the present work, contrary to Meyer's findings, a consumption of periodic acid of the native product was found which could not be ascribed purely to the end groups of a straight chain with glucuronido-4-(or -3-)-glucosamine and glucosaminido-3-glucuronic acid linkages, provided that

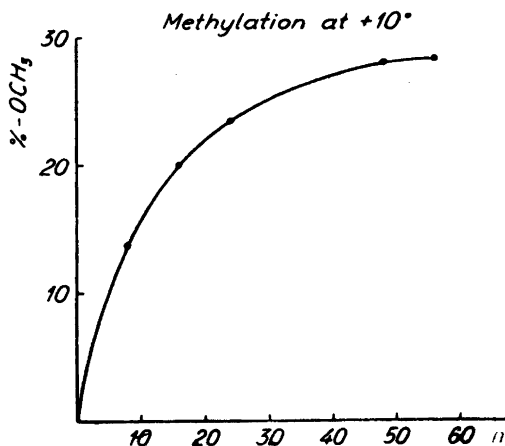


Fig. 4. Methylation of hyaluronic acid.

the molecular weight deduced from the reducing power is approximately correct. The oxidizability of the native substance was in the present work found to be the same for three different preparations of hyaluronic acid, two of which were isolated from navel cord, and was distinctly lower for a specimen of chondroitin sulfuric acid tried. This result agrees with that of Jorpes, Werner and Åberg¹¹, who in this regard found a distinct difference between the two substances.

The oxidizability of the native substance might indicate the presence of a few short side chains per molecule but may also be otherwise explained. Many unsuccessful attempts to get X-ray diffraction pictures of hyaluronic acid might perhaps be taken as an additional evidence of an irregular structure of the substance. Experiments with periodic acid can give valuable guidance in studies of the structure of hyaluronic acid and related substance, but they obviously do not suffice for a definite settlement of the structural problems of these compounds. However, the application of the classical methods for structural studies within the carbohydrate domain, seem to be connected with unusual difficulties in the case of hyaluronic acid and related substances.

EXPERIMENTAL

Methylation

Meyer *et al.* methylated chondroitin sulfate with the aid of dimethyl sulfate and obtained a fully methylated product on operating at +5° or +20°, whereas the methylation became incomplete at +40° and +60°, probably due to disturbing secondary reac-

tions. In a preliminary experiment on chondroitin sulfate, the methylation did not become quite complete at $+20^{\circ}$. The methylation of the hyaluronic acid was therefore carried out at $+10^{\circ}$. A quantity of 8 g was dissolved in 160 ml 1 % NaOH. Using an apparatus similar to that of Meyer, Wertheim and Bernfeld¹² and following the directions given by Meyer, Odier and Siegrist², 40 ml of dimethyl sulfate and 51.2 ml 30 % NaOH were added during 8 h. The solution was then precipitated by addition of 3.5 liters of alcohol, the mixture centrifuged, washed with alcohol and ether and dried. Of this product 0.3 g were dissolved in water and dialysed against 0.5 % sodium acetate until free from sulfate. The solution was then precipitated with alcohol, washed with alcohol and ether and dried *in vacuo*, at first at room temperature, and then at $+70^{\circ}$ over P_2O_5 . Some moisture was very tenaciously held by the methylated products and constant weight was usually attained only after drying for several days at the elevated temperature. The preparation thus obtained was used for the methoxyl determination, carried out according to Vieböck and Brecher¹³. The main portion was again dissolved in 1 % NaOH (200 ml) and methylated in the same way as before. After 6 methylations 3.5 g of a methylated, quite white product were obtained, which contained 28.1 % methoxyl; calculated for the Na-salt of the tetramethylated disaccharide unit, 27.1 %. For the precipitation of the highly methylated products ether had to be added. The course of the methylation is shown in Fig. 4.

Periodate oxidation

The oxidations were performed principally in the same way as given by Meyer, Odier and Siegrist².

A suitable quantity of the substance to be oxidized was dissolved in a small amount of water. In most instances 12 ml 2 *M* acetate buffer of pH 4.3 and 6 ml *M* sodium periodate were then added and the solution filled up to 60 ml with water. After standing, as a rule at $+18^{\circ}$ – $+20^{\circ}$ for suitable lengths of time, 10 ml portions were taken for titration. 2 ml of a 7.5 % KI solution and 4 ml 2 % HCl were added and the solution then titrated with 0.1 *M* $Na_2S_2O_3$, using a 5 ml burette allowing a reading of 0.01 ml.

The liberated ammonia was distilled after addition of magnesia.

Methanolysis and hydrolysis

Meyer, Odier and Siegrist² used 7.3 % HCl and reflux for 44 h. On boiling methanol containing 7.3 % HCl with reflux at ordinary pressure the greater part of the HCl escapes in a short time. In the present work the same results were obtained when the substance was boiled under reflux for 40 h in 7 % HCl as when this procedure was repeated once with the solution evaporated to dryness *in vacuo* after the first treatment. The outcome was also the same on heating with 7 % HCl in a closed tube for 6 or 12 h. (The formation of risky amounts of methyl chloride forbids the use of longer heating times in closed tubes.) In the experiment given in Fig. 2 the substance was boiled under reflux twice with (initially) 7 % methanolic HCl for 40 h.

The hydrolyses were carried out in closed glass bulbs at $+55^{\circ}$ or $+60^{\circ}$ in 4 *N* HCl. Small losses of fluid through inevitable evaporation were replaced by addition of water before samples were taken for analyses.

SUMMARY

Hyaluronic acid obtained from the tumor fluid in a case of mesothelioma was submitted to structural analysis with the aid of periodate oxidation of the native and the methylated product. The results found are consistent with the view that the molecule of the hyaluronic acid is in the main constituted of linearly linked dimers of N-acetylglucosamine and hyaluronic acid, and indicate that in this case the glucosamine residues were glucosidically linked to the 3rd carbon atom of the glucuronic acid residue. They further made probable the interpretation that half of the glucuronic acid residues were linked to the 3rd and the other half to the 4th C-atom of the glucosamine part.

Added in proof: The full report of the work of Meyer and Fellig⁴ has now been published in *Helv. Chim. Acta* **34** (1951) 939 and that of Kaye and Stacey⁵ in *Biochem. J.* **48** (1951) 249.

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