THE POLYSACCHARIDE OF THE VITREOUS HUMOR

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Since the work of Mörner (1) the existence of a mucoid in the vitreous humor has seemed well established. All subsequent workers used his method of preparation: the precipitation of the diluted native vitreous humor with dilute acetic acid. In his recent book on the nature of the vitreous body (2), Duke-Elder gives its concentration as 0.021 per cent, or about 30 per cent of the total protein present. The only analysis we were able to find on this mucoid is that of Mörner: N, 12.27 per cent; S, 1.19 per cent.

The stability of a typical mucoid, as, for example, egg mucoid, toward splitting into its polysaccharide and protein components is very remarkable. Thus Levene and Mori (3) state that the egg white must be hydrolyzed on the steam bath with 10 times its volume of 10 per cent barium hydroxide for 7 hours.

In an effort to prepare the supposed vitreous mucoid for other studies, we obtained, by very gentle methods, a free polysaccharide acid of high molecular weight, which is apparently in the vitreous humor in a salt-like combination. It appears to be a substance unique in higher animals, and may be best compared with some of the specific polysaccharides of bacteria.

EXPERIMENTAL

Our starting material was the acetone precipitate of fresh cattle vitreous humor. Lots of 100 eyes were brought from the abattoir packed in ice; the vitreous humor was immediately removed, strained through loose cotton gauze, and poured into 10 times its volume of cold acetone with vigorous stirring. After standing overnight in the ice box, it was filtered by suction, washed abun-

dantly with acetone and ether, dried *in vacuo* over P_2O_5, and powdered. The yield from 100 eyes was about 3.2 gm., containing about 7 per cent nitrogen, 11 per cent moisture, and 40 per cent ash (ashed with H_2SO_4). The pH of an aqueous suspension of this powder was greater than 10, while the original vitreous humor has a pH of about 7.8. This alkaline reaction cannot be explained by loss of CO_2. The supernatant acetone after evaporation was also alkaline. By a similar treatment, no carbonate was formed from bicarbonate. Other protein solutions, *e.g.* serum, become slightly more acid after acetone precipitation.

In the first experiments aqueous extracts of the acetone powder were precipitated by acidified alcohol. The powders thus obtained had 5 to 6 per cent nitrogen and 30 to 40 per cent reducing substances as glucose (Hagedorn-Jensen method (4) after 2 hours of hydrolysis in sealed tubes with 2 N H_2SO_4 in boiling water). Their solutions were not precipitated by dilute acetic acid, barium hydroxide, or neutral lead acetate, but were precipitated by basic lead acetate. The Molisch reaction was strongly positive. From the analytical figures and the reactions it was evident that the substance was not a mucoid, but a polysaccharide.

For obtaining the purified polysaccharide acid, the acetone powder from 100 eyes is extracted three times with 200 cc. portions of 90 per cent acetic acid. The residue is washed with alcohol until most of the acetic acid is removed, then suspended in water, and neutralized with N NaOH to facilitate centrifuging. This extraction with water is repeated on the centrifuged residue.

This residue consists of a fibrous mass, insoluble in all solvents except hot alkali, having a nitrogen content of 13.5 per cent (ash-free), and giving a strong Molisch reaction after hydrolysis. It is similar to collagen, and probably identical with the "residual protein" of Duke-Elder (2). Its yield is between 0.7 and 1.0 gm. per 100 eyes.

The combined aqueous extracts from above are poured into 6 times their volume of alcohol to which a few cc. of glacial acetic acid are added. After standing cold overnight, the mixture is centrifuged, taken up in a small volume of water, and poured into 15 times the volume of glacial acetic acid. The stringy material stands overnight in the ice box, and is washed abundantly with alcohol, acetone, and ether, powdered, and dried *in vacuo* over

P_2O_5. The yield from 100 eyes is about 0.73 gm.; *i.e.*, 30 per cent of the organic material. It contains a varying amount of inorganic material (2 to 10 per cent), mostly $CaSO_4$, most of which can be removed by dissolving in 0.2 N HCl and reprecipitating in glacial acetic acid.

By a similar procedure no polysaccharide was obtained from egg white.

In Table I are given some of the data on the preparations of this acid for which we propose, for convenience, the name "hyaluronic acid," from hyaloid (vitreous) + uronic acid.

TABLE I

Analysis of Preparations of Hyaluronic Acid

Preparation No.	Per cent nitrogen	Reducing substance as per cent glucose*		Equivalent weight	Per cent ash	Remarks
		(*a*)	(*b*)			
4-A	4.77	49.4	58.9	460†	4.04	
20-A	5.16	49.0	61.2	464	3.48	20.5% hexuronic acid
27-III	4.41	52.6	60.7	446	10.1	
30-I‡	3.84	51.0	59.4	453	1.01	20.5% acetyl

* (*a*) indicates values obtained after precipitation of the neutralized hydrolysate with $Zn(OH)_2$; (*b*) indicates values obtained directly on the neutralized hydrolysate.

† Electrometric titration value 507.

‡ Prepared from Preparation 27-III by reprecipitating from 0.2 N HCl in glacial acetic acid.

The free acid is very hygroscopic, but is not easily soluble in water. The salts are very soluble, forming highly viscous solutions. The following qualitative tests were positive: carbohydrate (Molisch), pentose (Bial), pentose or hexuronic acid (Tollens' phloroglucinol), hexuronic acid (Tollens' naphthoresorcinol), amino sugar (Elson and Morgan (5)); the following were negative: protein (biuret), galactose (mucic acid formation). The preparations contain no phosphorus, and those with a low ash content contain only traces of sulfur (shown to be $CaSO_4$).

Optical rotation in 2 per cent neutral solution in a 0.25 dm. tube, sodium light, was 0°; after hydrolysis, in 1.13 per cent solution, in a 1 dm. tube, $-0.07°$ at 30°, sodium light.

In one preparation (No. 20-A) we found 20.5 per cent uronic acid calculated as hexuronic acid (6) (0.1995 gm. gave 0.01774 gm. of CO_2). As a check on the method, 0.2072 gm. of pure glucuronic acid (for which we wish to thank Mr. L. L. Engel of the Department of Biological Chemistry) gave 0.0480 gm. of CO_2 compared with a theoretical of 0.0470 gm. In Preparation 30-I, acetyl estimations by a slight modification of the method of Kuhn and Roth (7) showed 20.5 per cent acetyl, indicating two acetyl groups per equivalent weight.

The quantitative amino sugar estimation (5) was unreliable, since the color from the glucosamine hydrochloride standard (violet-red) did not match well with the color produced by the hydrolysate (brown-red). With different standards we obtained values between 46 and 64 per cent of total nitrogen as amino sugar nitrogen, or 35 to 49 per cent of the total reducing substance as hexosamine.

The reducing sugar content before hydrolysis indicated one reducing group present for about fourteen after hydrolysis. The hydrolysate yielded a mixture of phenylosazones which we have not as yet been able to separate.

On electrotitration with the glass electrode in a current of hydrogen to exclude CO_2 we obtained an apparent equivalent weight of 507 (18.56 mg. required 3.31 cc. of 0.01106 N NaOH), while the value by titration, with phenolphthalein as the indicator, was between 446 and 464. The electrotitration was made in a volume of about 80 cc., while the final volume in the colorimetric titration was about 4 cc. With the electrotitration data the apparent dissociation constant, calculated from the formula of Van Slyke (8), is 4.58×10^{-5} at 32°, and the acid is therefore about 2.5 times stronger than acetic acid. (We wish to thank Mr. F. Rosebury of the Department of Biological Chemistry for assistance with the electrotitration.)

It is evident that hyaluronic acid is not identical with what Levene and López-Suárez (9) considered a mucoitin sulfuric acid prepared from vitreous humor by alkaline treatment. Their material contained 3.6 per cent sulfur, while our material contains only traces as an impurity ($CaSO_4$).

One might suspect that the "mucoid" obtained by acidification of the fresh vitreous humor should be in our insoluble residue,

since the latter gives a strong Molisch reaction after hydrolysis. However, the known mucoids retain their solubility in water after treatment with acetone or similar agents.

When the 90 per cent acetic acid extract above is evaporated, the residue taken up in water, and made alkaline with ammonium or sodium hydroxide, a precipitate is obtained containing on an ash-free basis 17.1 per cent nitrogen. The yield from 100 eyes is about 0.25 gm. The material is soluble in dilute acids and is reprecipitated by alkalies. On dialysis in 0.1 N HCl it passes through the collodion membrane. It seems to be of the nature of a histone or a simpler base.

It is noteworthy that, according to Redslob (10), Abé found in vitreous humor two isoelectric points, one at pH 3.8, the other at pH 9.4. The latter would probably correspond to a complex containing the above basic substance.

One may speculate as to the possible connection between the polysaccharide acid and the problem of glaucoma. Redslob and Reiss (11) have demonstrated that the injection of alkali into the vitreous humor produces a long lasting rise in intraocular pressure, while the introduction of acid or neutral solutions causes only transient changes. They also state that the introduction of acid into a glaucomatous eye lowered the tension and relieved the symptoms. They report the production in the rabbit of a picture "with all clinical symptoms of glaucoma" by isotonic sodium hydroxide injection. There is a possibility of the spontaneous occurrence of such an alkaline reaction by the lactonization of the polysaccharide acid and the simultaneous liberation of the base which originally neutralized it.

SUMMARY

From the vitreous humor of cattle eyes a polysaccharide acid of high molecular weight has been obtained by methods avoiding strong hydrolytic agents. The acid has an apparent equivalent weight of about 450. As constituents there have been recognized a uronic acid, an amino sugar, and possibly a pentose. The dissociation constant has been determined as 4.58×10^{-5} at 32°. An attempt will be made to relate the acid to the pathogenesis of glaucoma.

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