Biochemical Studies on Carbohydrates.

LXIX. Preparation of Pure Chondroitinsulfuric Acid.

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Not a few approvals have accumulated until now for Levene's assertion that the monosaccharide components of chondroitinsulfuric acid are chondrosamine and glucuronic acid, but the final conclusion will be only reached when isolation in perfectly pure state of this polysaccharide is succeeded. α -Naphthol does not react on chondrosamine by the Molisch process, while it does on glucuronic acid, producing a green dyestuff, and hence chondroitinsulfuric acid ought to show green coloration by this reaction. On the contrary, however, violet coloration is produced by all its products obtained by the methods of predecessors as was already pointed out by Hisamura from this institute (1938, i). It is due most probably to contamination by the galactose-acetylchondrosamine complex as either detached from or involved in chondromucoid (cf. Hisamura, 1938, ii). The method of Meyer and Smyth, consisting in extraction at neutral reaction, is considered to give a mixture of the complex acid and chondromucoid, because the substance, which was prepared by Hisamura (1938, i) according to them, not only showed the Molisch test for ordinary sugar, but also involved nitrogen in excess over the corresponding to chondrosamine. In all the other methods strong alkali (sodium or potassium hydroxide) are used and therefore the products are contaminated with the mentioned galactose-involving polysaccharide or with this in addition to small fragments of protein moiety of the mucoid.

With the aim in view, we have adapted formaldehyde to cartilage powder at pH 7.5–8.0 in order to destroy the amino groups which are

acting as cations in salt formation of the protein with chondroitinsulfuric acid. It was also intended by this device to change the protein into an insoluble form without any distinct cleavage. The extract was precipitated with alcohol, and the deposit was removed from the galactose-acetylchondrosamine complex, which had come over in combination with protein, with phosphotungstic acid and kaolin. The deposit by three volumes of glacial acetic acid was nearly free from ordinary sugar and nitrogenous impurities. It was further submitted to electro-dialysis with following fractionation by means of glacial acetic acid and sodium acetate. The solution of the product mixed with α -naphthol gave a plain green zone at the junction with sulfuric acid. Analytical figures also accorded perfectly with Levene's conception of chondroitinsulfuric acid, as an acid sodium salt, in so far as the composition is concerned. Noteworthy in particular were that the intensities of colour developed by the orcinol and indole reactions coincided well as glucuronic acid with the computation from the furfural yield and further that the nitrogen content corresponded to the equivalent of chondrosamine. The substance gave transitory pink colour with dimethylaminobenzaldehyde, after preliminary heating with alkali, and reduced iodine (Cajori method), though to a slight extent. These reactions were given by all of our preparations.

On the structure of chondroitinsulfuric acid Levene retracted recently his original claim (in 1913 Levene and La Forge put forward the dimeric nature of this complex acid) and "suggested that chondroitinsulfuric acid may have a chain structure similar to that of other polysaccharides, each unit being a chondroitinsulfuric acid containing the four components."¹⁾ Levene adduced no experimental evidence for it, and moreover Fürth and Bruno supported Levene's old view again, standing on its molecular weight which was measured by the Northrop diffusion method.²⁾ But the new opinion appears more plausible from the remarkable colloidal nature of this carbohydrate and, as well, the recent mode of viewing the structure of polysaccharides. We asserted in a preceding communication (Masamune, Hiyama and Kobayasi), that chondrosin is a glucuronidochondro-

1) A description by Tipson and Stiller according to a private communication from Levene (Textbook of biochemistry, Philadelphia, 1935, 105).

2) They used evidently impure preparations from the view point of Molisch reaction as already discussed and accordingly the correctness of the result is highly dependent on the indication, which was chosen for determining the concentration of chondroitinsulfuric acid in the outer liquid of the diffusion cell, but no information was made in this respect.

samine and, in the present work, the positive reaction with dimethylaminobenzaldehyde has disclosed that an acetylchondrosamine rest holding free carbonyl lies at one end of chondroitin molecule. Chondroitin might be then expressed such as:

C Acetylchondrosamine

G Glucuronic acid

* denotes the component holding free carbonyl

In the diagram, x amounted to 21, when calculation from iodine use was made under the assumption that the aldehyde equivalent of C* is equal to that of free acetylchondrosamine. Because of a small quantity reduced of this oxidizing agent, the above value of x is regarded to show only an approximate number. Probably, chondroitin is made up of 20-25 molecules of chondrosin.

EXPERIMENTS AND CALCULATION.

Preparation of chondroitinsulfuric acid.

A typical example:

Nasal cartilage of whale freed from extraneous matter and perichondrium, was scraped and ground through a meat chopper, stood for several days with 95% alcohol and dried in the apparatus of Faust at room temperature. 110 gms. of thus dried material were suspended in 500 cc. of water and, under agitation, 5% sodium carbonate solution up to pH 8.0 and 40 cc. of formalin (sp. gr., 1.09) were added. After having been diluted with more 500 cc. of water, the mixture (pH, 7.8) was shaken for two days (room temp., 13-15°C), pH being maintained at 7.5-8.0 by frequent supply of the Na₂CO₃ solution. The mixture (pH at this stage, 7.8) was filtered on a cotton gauze and the liquid was finally squeezed out. The turbid solution thus obtained was acidified with 30% acetic acid (40 cc.) to pH 4.6. As turbidity increased thereupon, the solution was clarified by repeated suction through paper pulp. The filtrate (950 cc.) was precipitated by means of 3 volumes of 95% alcohol and 8 gms. of sodium chloride and dried in a desiccator after collecting on a suction filter. The amount of this substance was 7.2 gms. In similar manner, extraction was carried out again from the tissue residue above. This time, the yield was 0.7 gm. These were combined, taken up in 790 cc. of water and to the transparent greenish solution were added 10% hydrochloric acid, until its concentration in the solution reached 1%, and 2 cc. of 20% phosphotungstic acid. Only

a small amount of fine precipitates was given and more of either hydrochloric acid or phosphotungstic acid caused no increase of them. The solution together with the suspension was mixed thoroughly with 250 gms. of purified kaolin and filtered. 30 gms. of barium acetate were dissolved in the clear filtrate (700 cc.) for the purpose of separating chondroitinsulfuric acid as barium salt and the turbidity occurring thereby was removed again by treatment with 100 gms. of kaolin after dilution with 700 cc. of water. The liquid was precipitated with 3 volumes of glacial acetic acid¹⁾, and the supernatant solution after subsidence of the deposit was decanted off. The deposit was then collected by centrifuging, washed twice with dilute acetic acid (3 parts of glacial acetic acid + 1 part of water), once with glacial acetic acid, 8 times with 95% alcohol (pH of the last washing, 6.0) and twice with ether and dried finally in a vacuum desiccator. In the product ordinary sugar was hardly detectable (Molisch test). Since tests for Ca as well as Ba were positive and the watery solution was quite acid, chondroitinsulfuric acid is assumed to have been isolated as an acid salt involving barium, calcium and in addition probably sodium. It was changed into acid sodium salt with simultaneous purification as follows:

The whole substance, taken up in 150 cc. of water, was subjected to electrodialysis (Pauli's apparatus; 200 volts). The process was continued as long as 8 hours, after the shift of pH of water²⁾ in the outer compartments had stopped. Too much increase of the solution in volume was avoided by occasional vacuum distillation during the course. After dialysis, 4 volumes of glacial acetic acid did not give rise to precipitation from the solution (330 cc.), but on addition of sodium acetate (1.5 gms.) gellatinous half-transparent deposit appeared which was collected by centrifuging and washed three times with dilute acetic acid (4 parts of glacial acetic acid + 1 part of water) and a little sodium acetate, then 5 times with alcohol³⁾ and dried, after replacing ether for alcohol, in a vacuum desiccator. The product was purified by fractioning twice as described above with glacial acetic acid and finally precipitating with 10 volumes of 95% alcohol from water. Yield, 900 mgms.

1) With more 3 volumes of glacial acetic acid were about 1.8 gms of a substance deposited. This, however, was discarded, because the Molisch test indicated the presence of ordinary sugar, though in a trace, in it.

2) Renewed many times.

3) pH was raised to 5.6.

Properties of the acid sodium salt of chondroitinsulfuric acid.

Biuret reaction was not given. α -Naphthol gave by the Molisch procedure a plain green zone without any adjacent red layer which is formed when a trace of ordinary sugar is co-present. Hot Ehrlich's reagent of dimethylaminobenzaldehyde gave obvious red colour, though transitory and pale¹⁾, when it was heated preliminarily with alkali. This test was conducted by following a modification of Blix's direction (Osaki and Turumi, unpublished).

$$[\alpha]_D^{25} \text{ (in water)} = -0.158 \times 3.01862 / 0.02057 \times 1.00110 = -23.15^\circ$$

Quantitative analysis.

Furfural production and iodine reduction were measured by the Youngburg and the Cajori method respectively and chondrosamine was determined according to Hamasato and Akakura. The remaining analyses were made by the procedures often used in the investigations of this series. The figures of the typical product whose preparation processes were cited above are listed in table I. Other products analyzed similarly.

TABLE I.

Analysis	%	Expressed in equivalents per equivalent weight
N	2.67	1.01
Chondrosamine	33.54	1.00
Glucuronic acid		
By the furfural method ²⁾	36.04	0.99
" orcinol " " ³⁾	38.02	1.05
" indole " " ³⁾	38.46	1.04
Acid-hydrolytic S	6.12	1.02
Acetyl	7.73	
Ash	19.59	
Reducing force as glucose from iodine reduction	1.74	

Calculation of the molecular weight.

The acid sodium salt is formulated from the analytical figures⁴⁾ in table I as $(C_{14}H_{21\frac{1}{2}}NO_{15}SNa_{1\frac{1}{2}})_n - (H_2O)_{n-1}$, which may be expressed

- 1) 3 mgms. of the substance gave the positive reaction, while 2 mgms. could not.
- 2) By the Youngburg method the yield of furfural from glucuronic acid amounted to 40.44% (19.96% of the substance) in averages of the theoretical value.
- 3) Glucuronic acid was taken for preparing the standard solution of colorimetry.
- 4) Sodium was calculated out from the quantity of ash as sodium sulfate.

more simply as $(C_{14}H_{19\frac{1}{2}}NO_{15}SNa_{1\frac{1}{2}})_n$ when n is a large number.

86.7 mgms. of the substance reduced 0.168 cc. of 0.1 N iodine solution. Therefore, if the iodine use of the acetylchondrosamine rest, which holds free carbonyl in the chondroitin molecule, is assumed equal to that of acetylchondrosamine (s. below),

$$0.0867 : x = \frac{0.168}{1.12} : 20000 \quad (x = \text{gram molecule})$$

$$\text{m.w.} = 11560$$

Namely, n approximates 23 in the above formula. .

Preparation of acetylchondrosamine and its properties.

100 mgms. of chondrosamine hydrochloride were shaken with 77.3 mgms. (1 mol) of silveracetate in 2 cc. of absolute methanol, after addition of 0.1 cc. (2 mols) of acetic anhydride. After 9 hours, silverchloride was separated off on a filter, and the filtrate was placed in a desiccator containing P_2O_5 . Crystals came out gradually on the vessel wall over the liquid surface. From the mother liquid a much less amount of crystals was obtained by diluting with absolute alcohol, precipitating with ether and petroleum ether and standing. The substance together amounted to 40 mgms. 400 mgms. of the crude product were prepared by ten runs, because starting with more of the material decreased markedly the crop. Recrystallization was made twice from methanol by means of ether. The yield was 350 mgms. Crystalline form, thin needle (colorless); m.p., 154°C (uncor.). Anhydrous substance¹⁾ analyzed:

$C_8H_{15}O_6N$ Calc. 6.33% N, 19.46% CH_3CO , 0.0% $\text{NH}_2\text{-N}$.
Found. 6.61% N, 18.63% CH_3CO , 0.0% $\text{NH}_2\text{-N}$.

Iodine use under Cajori's conditions corresponded to the aldehyde equivalent of 1.12 (8.72 mgms. of the anhydrous substance reduced 0.88 cc. of 0.1 N. iodine solution).

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1) Dried at $100\text{--}110^\circ\text{C}$ in vacuo.

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