Biochemical Studies on Carbohydrates

CLVIII. On Karl Meyer's Mucoitinsulfuric and Hyaluronosulfuric Acid

First Paper

By

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(Received for publication, May 23, 1952)

With his coworkers Karl Meyer separated in 1937¹⁾ a polysaccharide, containing glucosamine, an uronic and sulfuric acid, by alkaline hydrolysis of commercial gastric mucin, and later on in 1940²⁾ a polysaccharide of resembling composition from cornea, which diverged from the former in optical rotation and digestibility by the pneumococcus enzyme. He²⁾ believed the latter polysaccharide to be a monosulfate of "hyaluronic acid," on the glucosamine nature of the hexosamine, its hydrolysableness by the pneumococcus enzyme and optical rotation which "becomes quite similar to that of hyaluronic acid, when calculated on the basis of an ash and sulfate free compound," but did not establish the nature of the uronic acid. Also regarding the former polysaccharide, which he designated "mucoitinsulfuric acid," the uronic acid in it was not strictly identified, whereas glucosamine was separated as hydrochloride.

Lately Wolfrom *et al.*³⁾ isolated D-glucosaccharic acid after oxidative hydrolysis of the mucoitinsulfate of Meyer. However, only 48 mg. of the saccharic acid were yielded from 1 g. of the material. By their preparation procedure of the acid polysaccharide, an itin and an itinsulfuric acid, if simultaneously existent in one and the same tissue, are separated together and, on the other hand, pig gastric mucosa contains mucoitin (Karl Meyer's hyaluronic acid), as already mentioned by Takahasi and Yasuoka.⁴⁾ Therefore the saccharic acid they obtained may have originated in glucuronic acid constituting mucoitin as a contaminant of the preparation.

Thus we undertook re-examination of those polyuronic acids, particularly with concern about their uronic acids. The substances were at first prepared by following Meyer as strictly as possible and further purified by the aid of glacial acetic acid until products were given, that were

Molisch-negative, exhibiting mere vivid Goldschmiedt reaction with α -naphthol and conc. H_2SO_4 . Analyses indicated that either of the polyuronic acids was an itinsulfuric acid. The depths of coloration by indole and orcinol of the cornea polysaccharide coincided with each other as expressed as glucuronic acid, but, contrariwise, those of the gastric polysaccharide agreed as expressed as galacturonic acid rather than as glucuronic acid. The developing colours by those reagents from mannuronic acid is characterized with green fluorescence, which was not noticed in the reactions of the preparations. They rotated D-light almost similar, $[\alpha]_D$'s being approximately -25° . In short, one of the claims of Meyer appeared to be acceptable as real that the cornea polysaccharide is monosulfuric acid ester of hyaluronic acid, but the other polysaccharide to be in need of a scrutiny. Our preparation of the cornea substance diverged from Meyer's in the magnitude of negative optical rotation, which, however, the writer thinks, can not be an evidence against the identity of the carbohydrate skeleton of this polysaccharide and hyaluronic acid.

Thanks to a new devise of Masamune and Maki,⁵⁾ not only hexosamines but also hexuronic acids have become sharp-separable between themselves by paper partition chromatography. So, the writer hydrolyzed the stomach substance under various conditions to subject to that chromatography. Diagrams were obtained with spots of glucosamine and galacturonic and glucuronic acid besides that corresponding to the position common to the basic disaccharide units of hexosamine-containing polyuronic acids, which are generally difficult to completely hydrolyze without decomposition. The spot of glucuronic acid was comparatively weak and looked due to mucoitin separated together with the itinsulfuric acid in view. Moreover, the writer prepared the basic disaccharide of the polysaccharide and desaminized it. The hydrolysate of this desamination product gave a spot of galacturonic acid but none of glucuronic acid. Most probably the mucoitin that was contained in the polysaccharide preparation was so little that mucosin neither amounted beyond a trace nor was present at all in the unit disaccharide preparation.

Needless to say, Levene applied the name mucoitinsulfuric acid to acid polysaccharides composed of acetylglucosamine, glucuronic acid and sulfuric acid in the molecular proportions of 1:1:1, irrespective of their structure. Therefore, the itinsulfuric acid in the gastric mucin, whose uronic acid component is galacturonic acid, does not conform to his terminology of mucoitinsulfuric acid, whereas the one in cornea does. The writer proposes to call the former "gastroitinsulfuric acid" and the latter mucoitinsulfuric acid. That "hyaluronic acid" is deemed to possess a structure same as the carbohydrate skeleton of the cornea sub-

stance gives a favourable argument for the denomination of "mucoitin" we applied to it.

EXPERIMENTAL

The itinsulfuric acids in problem were prepared according to Meyer and others^{1,2)} with some unavoidable modifications and purified.

I. Cornea Itinsulfuric Acid

164 fresh ox corneas were cut into smallest possible pieces with scissors. The total weight amounted to 87 g. They were mixed with 500 cc. of 50% urea, alkalized to pH 9.0 with NaOH (1 *N*) and stood for 3 weeks at room temperature, with occasional agitation and adjustment to the original alkalinity. Centrifuged. The centrifugate was again extracted for 1 week with 250 cc. of the urea solution and centrifuged. The supernatant fluids were combined, diluted four times and acidified to pH 3.8 with acetic acid (20%), whereby precipitation occurred. The precipitate centrifuged was washed with four 20 cc. portions of water, taken up in 120 cc. of 10% CaCl₂ and after alkalinizing to pH 7.4, shaken. The turbid solution given was then shaken with 32 cc. of a chloroform-amylic alcohol (3:1 by volume) mixture for 15 hours on an apparatus. The centrifuged upper layer (translucent) was again treated with 16 cc. of the same organic mixture for 10 hours. Centrifugation gave this time a transparent solution. This was precipitated with 2 volumes of abs. alcohol and a little anhydrous sodium acetate, washed well with abs. alcohol and thereafter dissolved in 20 cc. of water by the aid of 0.3 cc. of 1/5 *N* NaOH. The solution (pH 7.0) was mixed with an equal volume of 2 *N* acetic acid and vigorously shaken with 2 g. of purified kaolin for 1 hour and the centrifugate stirred up with 5 cc. of 1 *N* acetic acid and again centrifuged. The successive supernatant fluids (opalescent) were further filtered to eliminate floating particles, and 2 volumes of abs. alcohol and a little abs. alcohol saturated with anhydrous sodium acetate added. Then the deposit was washed thoroughly with abs. alcohol and taken up in 3 cc. of water, and the insoluble part centrifuged and washed with 2 cc. of water. The solution and washing were precipitated with 20 cc. of glacial acetic acid and a little anhydrous sodium acetate, washed with abs. alcohol repeatedly to free from acetic acid as far as possible and with acetone and dried in a desiccator. 120 mg. of a white powder that corresponded to Meyer's preparations were thus yielded. Since the product here gave the positive Molisch reaction besides the positive Goldschmiedt, purification was processed as follows.

It was dissolved in 10 cc. of water, a small insoluble part rejected by centrifugation and the clear supernatant mixed with 30 cc. of glacial

acetic acid. On addition of anhydrous sodium acetate to the opalescent solution, an abundant flocculent precipitate occurred. This was further on reprecipitated in a similar manner more than ten times. The substance took more and more glassy appearance (translucent jellylike form) and showed less and less positive and finally negative Molisch reaction. Thereupon it was washed with alcohol several times to remove acetic acid completely and dried. Furthermore it was liberated from an insoluble contaminant after taking up in 3 cc. of water, precipitated and washed with alcohol and dried. Crop, 59 mg. Goldschmiedt strongly positive, biuret negative.

For the measurement below the substance was dried to constant at 100–110°C under reduced pressure.

Analysis. See Table I.

TABLE I
Composition of the Cornea Polysaccharide

Analysis	In per cent	In equivalents per equivalent weight
N	3.0	1.1
Glucosamine*	33.2	1.0
Hexuronic acid†		
i) as glucuronic acid‡		
Indole reaction	34.2	
Orcinol r.§	34.7	
Mean	34.5	1.0
ii) as galacturonic acid//		
Indole r.	18.6	
Orcinol r.§	40.3	
Acetyl¶	10.5	1.3
Hydrolysable sulfur**	7.0	1.2
Iodine use as glucose††	1.8	
Ash‡‡	16.8	

* Hamasato & Akakura method. † The orange colours developed by mannuronic acid with the orcinol and indole reagents are accompanied with green fluorescence which impairs the colorimetry against the standard prepared with glucose. That fluorescence did not occur here. ‡ Cf. Hisamura⁶⁾ and Masamune & Hisamura.⁷⁾ § Cf. Ozaki.⁸⁾ // Cf. Masamune, Suzuki & Kondoh.⁹⁾ ¶ Suzuki's modification of Friedrich, Rapoport & Sternberg method.¹⁰⁾ ** Suzuki method.¹⁰⁾ †† Macleod & Robison method. ‡‡ Pregl method without the use of H₂SO₄.

$$\text{Optical rotation in water. } [\alpha]_D^{13} = \frac{-0.35 \times 100}{1.38 \times 1} = -25.4^\circ.$$

II. The Itinsulfuric Acid in Pig Stomach Mucosa

Preparation procedure

Since the commercial "gastric mucin" was inaccessible, it was first

prepared by the method briefly described by Meyer *et al.*¹⁾:—Gastric mucosa was torn off together with the mucus from 15 fresh pig stomachs and masticated (total weight 6120 g.), then it was kneaded with 200 cc. of water and enough 10% HCl to shift the pH to 2.0, and after addition of 40 cc. toluene and 40 cc. chloroform, placed in a thermostat at 37°C. Additional hydrochloric acid was added from time to time to maintain the mixture at the incipient acidity. After 10 days the digestate (a translucent yellow-brown fluid) was separated from the non-digestible residue and 4 volumes of alcohol added. And the white gummous precipitate was centrifuged, washed well with alcohol and dried in a desiccator over CaCl_2 . 105 g. of a non-sticky powder were obtained.

The material thus obtained was agitated in 1 l. of 1 N NaOH and stood for 2 days at room temperature (15–19°C). Then, the brown solution containing insoluble particles was shaken with 20 cc. of toluene and centrifuged, whereby the mixture separated into three layers. The middle transparent layer was transferred to a beaker and 2 volumes of alcohol added. The slimy precipitate was washed with alcohol several times and a sticky clump was given, which was dissolved in 50 cc. of water, precipitated with 10 volumes of glacial acetic acid and washed with alcohol exhaustively and with acetone. 9.5 g. was the brown heavy powder given here. This was dissolved in 500 cc. of water and neutralized with 10% NaOH (4.2 cc. used), and followingly 1 N HCl (56.02 cc.) added up to 0.1 N. Vigorously shaken with 50 g. of kaolin and filtered. The bulk on the funnel was shaken with 50 cc. of water and again filtered. And the filtrates were precipitated with 4 volumes of alcohol and after washing with alcohol twice, extracted with two 30 cc. portions and one 10 cc. portion of water. The combined solutions (70 cc.) were precipitated with 350 cc. (5 volumes) of alcohol and anhydrous sodium acetate, washed many times with alcohol and dried. The product (a yellowish powder) at this stage, which corresponded to Meyer's preparations, amounted to 4.8 g. Almost biuret-negative but strongly Molisch-positive. Neuberg & Saneyoshi test for hexuronic acid resulted in positive.

Purification. It was dissolved in 500 cc. of water and 10 g. of heated charcoal were added, while hot (70–80°C), under agitation. The mixture was further stirred up for 30 minutes and filtered with suction. The almost colorless filtrate was distilled in vacuo to 30 cc. 150 cc. of alcohol were added and the precipitate was centrifuged, washed with alcohol and dried. The substance diminished to 2.6 g. and its Molisch reaction became less strong. Repeating treatments with glacial acetic acid were next effected:—

1) It was taken up in 100 cc. of water and 400 cc. of glacial acetic acid were added in small portions. The precipitate was subjected to a

similar process again and again, using later NaCl, in addition, to complete the precipitation (Without NaCl-addition the centrifuged supernatant remained turbid). The precipitate after more than 20 repetitions was washed with many changes of alcohol and dried. 1.2 g. of a white powder, showing only faint Molisch reaction, were given.

2) This was freed from some insoluble after shaking with 50 cc. of water and 4 volumes of glacial acetic acid and some NaCl added. The centrifugate was likewise reprecipitated from water scores of times. The consecutive precipitates changed more and more glassy, but a trace of a Molisch-giving impurity could not be eliminated. The final precipitate was again washed with alcohol and dried.

3) The product in 2) (a white powder, 900 mg.) was dissolved in 50 cc. of water (A minute insoluble part was not discarded), the pH moved to 7.4 with 5% Na_2CO_3 and after addition of 1 cc. of formalin, the Na_2CO_3 solution added again to re-elevate the pH to 7.4. The mixture was stood for 1 hour at room temperature and precipitated with 5 volumes of alcohol with subsequent washing with alcohol. The powder dried was again taken up in 50 cc. of water, the insoluble part rejected by centrifugation and 4 volumes of glacial acetic acid added. The dissolution and precipitation were further carried on many times with addition, when necessary, of NaCl besides glacial acetic acid. At last the substance in water showed no turbidity at all on addition of glacial acetic acid alone but came out in flakes on adding NaCl in addition. Scores of reprecipitations were again conducted. Thereafter, the transparent jellylike deposit was washed with abs. alcohol exhaustively and after drying once, carried down from a small volume of water with abs. alcohol and a little NaCl. The present deposit was also washed with abs. alcohol repeatedly and dried—**Prep. I**, a white powder weighing 520 mg.

In another similar run started with 40 g. mucin (from 2460 g. fresh mucosa), 120 mg. of **Prep. II** were produced (The intermediate product corresponding to Meyer's preparations amounted to 2.0 g.).

Chemical and physical properties

Qualitative tests. Preps. I and II resembled each other in chemical qualitative respects. Biuret and Molisch negative; Goldschmiedt, Neuberg & Saneyoshi and aniline acetate test distinctly positive. The indirect Osaki & Turumi reaction resulted in positive but not the direct.

Quantitative analysis. For the analysis, the preparations were dried to constant at 100–110°C under low pressure. The compositions are illustrated in Table II. To calculate out galacturonic acid from the furfural yields, the latter were multiplied with a factor 10.36, which was obtained by treating 1–4 mg. of barium galacturonate (Ba found 25.9%;

TABLE II
Composition of the Stomach Itinsulfuric Acid

Analysis	In per cent		In equivalents per equivalent weight	
	Prep. I	Prep. II	Prep. I	Prep. II
N	2.4	2.7	1.0	1.1
Glucosamine	31.6	32.7	1.0	1.0
Hexuronic acid				
i) as glucuronic acid				
Indole reaction	67.0	70.2		
Orcinol r.	34.4	36.8		
ii) as galacturonic acid				
Indole r.	34.4	36.8		
Orcinol r.	36.1	38.6		
Mean	35.3	37.7	1.0	1.1
Calc. fr. furfural yield*		39.2		1.1
Acetyl	8.8	10.0	1.1	1.3
Hydrolysable sulfur	5.5	5.7	0.97	0.97
Iodine use as glucose	1.9	2.0		
Ash	15.7	16.5		

All the analytical methods employed were the same as those in Table I, exclusive of the one to measure the furfural production. * Youngburg method¹¹; also see the text.

calc., 26.2%) according to Youngburg method.¹¹) Within those limits of amount taken, the galacturonate gave almost coinciding furfural yields per mg.

Optical rotation in water

$$\text{Prep. I, } [\alpha]_D^{20} = \frac{-0.35 \times 100}{1.56 \times 1} = -22.4^\circ.$$

$$\text{Prep. II, } [\alpha]_D^{20} = \frac{-0.56 \times 100}{1.80 \times 1} = -31.1^\circ.$$

Paper partition chromatography (The writer is indebted to Mr. M. Maki). Another batch of the stomach itinsulfuric acid was produced similar to above for the chromatographic investigation.

1) At first the preparation was hydrolyzed under various conditions with sulfuric acid, subjecting mucoitin (barium salt) and chondroitinsulfuric acid (acid sodium calcium salt) to a parallel run for comparison, which had been prepared after Isikawa¹²) and Jorpes respectively. The hydrolysates were removed from most of sulfuric acid with baryta, and evaporated in vacuo to a small volume for putting on paper strips. The volumes and concentrations of sulfuric acid used, temperatures and durations of time for hydrolysis, the volume of the condensates and else are tabulated as follows:

Method No.	Polysaccharide		Hydrolysis				Dilution of the hydrolysates with water to precip. out H ₂ SO ₄	Condensate	
	name*	amount taken (mg.)	agent: sulfuric acid		tempera- ture C	duration (hrs.)		vol. cc.	taken for irrig. cc.
			conc.	vol. cc.					
I	M C G }	100	1 N	10	100°	6	2 times	0.5	0.02
II	M C G }	"	5 N		37°	82	10 times		
III	M C G }		10 N				20 times		
IV	M C G }	"	5 vol. of H ₂ SO ₄ (sp. gr. 1.84)+ 2 vol. of H ₂ O	0.7	in an ice chest (0 ~6°)	96	to 100 cc.		

* M=mucoitin (acid barium salt), C=chondroitinsulfuric acid (acid sodium calcium salt), G=gastrositinsulfuric acid (acid sodium salt).

The solvent used for irrigation, amylalcohol-pyridine-water (7:7:6). Solvent fronts run 40 cm. Temperature 30°C. Duration of test 23 hours. Developers, aniline-phthalic acid of Partridge and 0.2% ninhydrin in butanol. In Fig. 1 (e-t) are diagrammatically reproduced the chromatograms obtained. All of the native polysaccharides were found to have not been completely hydrolyzed, showing a spot of the residual of respective disaccharide units. The different basic disaccharides had R_f values practically one and the same ($R_f=0.025$), which did not vary whether the disaccharides were in a salt form or free. Mucosin- and chondrosin-sulfate once separated were also run (1 and 2 in Fig. 1). The scission of the stomach itinsulfuric acid and mucoitin hardly reached beyond the disaccharide stage under the conditions of Method I, but, by the more drastic procedures (Methods II-IV) the monosaccharide components were liberated sufficiently to give distinct spots demonstrating the presence of galacturonic acid and glucosamine (aniline-phthalic acid as well as ninhydrin positive) and a small amount of glucuronic acid (weak spots) in the preparation of the stomach itinsulfuric acid, and glucuronic acid and glucosamine in mucoitin. Chondrosin, the basic disaccharide of chondroitinsulfuric acid, proved extremely resistant to hydrolysis under the conditions of all of those methods.

2) To ensure that the itinsulfuric acid in question contains galacturonic acid, its basic disaccharide ("gastrosin") was isolated by the procedure Levene had employed in obtaining chondrosin and further desaminized to make the uronide union less stable (Cf. former works¹⁴ on chondrosin and mucosin published from our Institute):—

1 g. of gastroitinsulfuric acid was heated with 3 cc. of 10% HCl and 0.47 g. of $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ on a boiling water-bath for 1 hour. The precipitate was filtered off and washed with 10 cc. of hot water. The filtrate and washing were freed from the excess barium by means of sulfuric acid and distilled in vacuo to a syrup. A colorless substance separated. To the still residue were added 3 cc. of methanol and the insoluble centrifuged off. The supernatant was precipitated with 10 volumes of ether, dissolved again in 2 cc. of methanol and reprecipitated with ether. 650 mg. of a dark brown powder were thus yielded—gastrosin hydrochloride (crude). Elson & Morgan and Neuberg & Saneyoshi intensively positive. The positive Molisch reaction was given, though not strong. Ferricyanide reduction of the hydrolysate (Hydrolysis: 1 *N* H_2SO_4 , 100°C, 4 hrs.) as glucose 41.2%, ash (Pregl method without H_2SO_4) 9.7%.

Desamination of the product was effected as was by Masamune, Maki and Hiyama¹⁵⁾ in preparing desaminochondrosin. The material taken was 600 mg. and 72 mg. of a white powder given—desaminogastrosin (crude). Elson & Morgan and Molisch test were faintly positive, indicating incomplete desamination. Neuberg & Saneyoshi strongly positive.

Gastrosin hydrochloride was applied immediately on a paper strip as a 1% solution (0.03 cc. was used), but desaminogastrosin was irrigated after hydrolysis by Method I (See 1)) as follows. 60 mg. of desaminogastrosin in 6 cc. of 1 *N* H_2SO_4 were heated at 100°C for 6 hours, and the hydrolysate was diluted twofold, freed from barium and condensed to 0.5 cc., of which 0.03 cc. was taken for the irrigation. As a control, crude desaminoglucosamine, prepared similar to above, was run after putting 0.015 cc. of its 1% solution (Fig. 1 (d)). The chromatograms are also shown in Fig. 1 (u, v and w). The gastrosin specimen gave only one spot at the same position as mucosin and chondrosin, but the hydrolysate of the crude desaminogastrosin four spots at positions corresponding to gastrosin (a faint spot), galacturonic acid, glucosamine (a faint spot) and desaminoglucosamine. Here no spot of glucuronic acid appeared. Probably because of the small mucoitin content of the gastroitinsulfuric acid preparation, the amount of mucosin produced was no more than a trace. Since the crude desaminoglucosamine still gave the positive Elson & Morgan reaction though weak, its chromatogram demonstrated a spot of glucosamine besides that of desaminoglucosamine itself (*R_f* 0.65). Similar was the case with the crude desaminogastrosin, namely, the spot of glucosamine came from the non-desaminized gastrosin in the preparation.

Taken altogether, gastroitinsulfuric acid contains galacturonic acid as its uronic acid component.

SUMMARY

1) Karl Meyer's hyaluronosulfuric acid of cornea and his mucoitin-sulfuric acid of "gastric mucin" were prepared and purified.

2) Analysis of the former itinsulfuric acid confirmed the result of the author regarding the composition, and moreover the figures obtained by the indole- and orcinol-method pointed to glucuronic acid as the uronic acid component in this polysaccharide.

3) The latter itinsulfuric acid was suspected not to contain glucuronic acid on rough identification by the analytical way, which rather suggested galacturonic acid. This polysaccharide and its basic disaccharide de-saminized were then investigated by chromatography, and the presence of galacturonic acid in them was established.

4) It was proposed to call the stomach itinsulfuric acid "gastroit-insulfuric acid" and to call the cornea substance mucoitinsulfuric acid.

The expenses of this work were defrayed by a grant which was given from the Education Department through the Grant Committee of the Science Council.

H. Masamune.

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