Biochemical Studies on Carbohydrates.

CXXXIV. Mucosin.

First Report.

By

Tyuiti Isikawa.

(石川 忠一)

(From the Medico-chemical Institute, Tohoku University,
Sendai. Director: Prof. H. Masamune.)

(Received for publication, February 15, 1950)

Mucoitin (hyaluronic acid of Meyer) from umbilical cord was subjected to acid hydrolysis and amorphous mucosin given to esterification and acetylation. The final crystalline product proved to be heptacetyl-glucosamine-galacturonide-methylester (crystal water, $3\text{H}_2\text{O}$) by its behaviour toward Ehrlich reagent, analyses and molecular weight. It had the rotation of $[\alpha]_{\text{D}}^{10} = +14.8^\circ$ in chloroform and F.P. of 130°C .

EXPERIMENTAL.

Mucoitin.

Umbilical mucin was prepared according to Masamune and Yasuoka¹⁾ by precipitating the funis extract at pH 2.0. 8 g. of it were triturated in a mortar, dissolved in 500 cc. of water by the aid of the saturated $\text{Ba}(\text{OH})_2$ and shaken with 5~10 cc. of formalin for 3~4 days, maintaining the pH of the mixture at 7~9 by occasional addition of the baryta solution. The centrifuged supernatant liquid (turbid) was adjusted to pH 7.0 when necessary, and after distillation in vacuo to 30~40 cc., precipitated with 2 volumes of alcohol (95%). The deposit was dried, after washing with alcohol and ether, in a desiccator. 4 g. of substance were obtained on an average. Biuret positive. This crude product was next taken up in 250 cc. of water, removed from the insoluble part by repeated centrifugations and subjected again to the formalin treatment similar to above (shaken with formalin for 2 days). Some precipitate was centrifuged off. The supernatant liquid was not transparent and still gave the positive biuret reaction. Therefore it was shaken with 40~55 cc. of a chloroform-amylic alcohol mixture (2:1 by volume) for 5~6 hours at a pH of about 7.5 (brought to this pH by means of baryta) with following centrifugation. Another repetition of this operation gave a clear supernatant liquid that was biuret

negative. The liquid was dialyzed for 4~5 days and condensed by vacuum distillation to about 20 cc. and alcohol added. The precipitates were washed with alcohol and ether and dried. The product was non-colored powder. The mean crop 2 g. It gave strong positive reaction for hexuronic acid (Goldschmiedt) but the negative for ordinary sugar (Molisch).

Quantitative analytical results of one of the products are listed in Table I.

TABLE I.
Composition of a Preparation of Mucoitin.

Analysis	In per cent	In equivalents per equivalent weight
Nitrogen*	3.21	1.02
Glucosamine	39.96	1.00
Glucuronic acid		
By indole r.	42.68	
By orcinol r.	43.08	
Mean	42.88	0.99
Ash†	12.3	

* Micro Kjeldahl. † Micro analysis.

Muscoin.

3 g. of mucoitin were dissolved in 13 cc. of water and refluxed with 10 g. of oxalic acid at 100°C for 5 hours. The hydrolysate was cooled quickly and filtered. The bulk on the funnel was washed with several small portions of ice-cooled water and the filtrate and washings evaporated together on a boiling water-bath to a small volume (about 5 cc.). It was subsequently chilled, the precipitating oxalic acid separated off on a suction funnel and washed (5 cc. water used) and the filtrate and washing poured in drops into 150 cc. of absolute alcohol under agitation. The flocculent precipitates were centrifuged and washed with alcohol several times and with an alcohol-ether (1:1) mixture once and dried. About 1.5 g. of amorphous powder (crude mucosin oxalate) were given.

Free mucosin was separated by the aid of baryta:— 400 mg. of the above powder were taken up in 20 cc. of water, an insoluble part separated off and 158 mg. of crystalline barium hydroxide in 10 cc. of water added. Since baryta used was found insufficient, though calculated, to eliminate all oxalic acid, it was supplemented carefully and the centrifuged supernatant liquid (pH, 4.5) distilled in vacuo to dryness. The remainder was dissolved in about 10 cc. of water, filtered under suction and the filtrate again evaporated to dryness. Redissolution, filtration and re-evaporation was carried out more 3 times and finally to the condensate.

(about 3 cc.) obtained by stopping evaporation halfway were added 25 cc. of absolute alcohol. And the precipitates were separated by centrifugation and washed with alcohol and ether. The yield of this portion was 150 mg. (Fr. I). From its mother liquid a second portion was thrown down by addition of ether (Fr. II). This weighed 100 mg. when dried. The former was reprecipitated from water (2 cc.) with alcohol (13 cc.) and after washing with alcohol and drying (crop, 80 mg.) subjected to analysis that gave the figures in Table II. The neutral equivalent was calculated at 381 on an ash-free basis (calc.: 355). The measurement here was processed as follows. 12.99 mg. of the substance were dissolved

TABLE II.
Composition of Mucosin as Percentages.

Analysis	Found		Calc. on ash-free basis
	Actual	On ash-free basis	
Nitrogen*	3.37	3.83	3.94
Amino nitrogen†	3.33	3.78	3.94
Glucosamine	43.01	48.88	50.42
Glucuronic acid			
By indole r.	50.17		
By orcinol r.	51.31		
Mean	50.74	57.67	54.64
Reduction as glucose of ferricyanide‡	45.86	52.11	50.70
Ash§	12.01		

* Micro Kjeldahl. † Micro Van Slyke. ‡ Analyzed according to Masamune²³ on the principle of Fujita and Iwatake. § Micro analysis.

in 2 cc. of water and titrated with 0.01 n. NaOH after addition of 1 cc. of twice diluted and neutralized formalin.²⁴ The alkali required was 3.901 cc. in the main run and 0.022 cc. in the control. In the test for hexosamine, the positive Elson-Morgan reaction was given but not the indirect Osaki-Turumi reaction. Molisch test resulted in negative.

Mucosinmethylester Hydrochloride.

0.4 g. lots of mucosin (prepared as Fr. I above) completely dried were dissolved in 2 cc. of absolute methanol involving 5% HCl and stood as stoppered at 20°C for 3 days. To the mixture were then added 10 cc. of absolute methanol and 20~30 cc. of dried ether and the flocculent precipitates centrifuged, washed with ether and placed in a desiccator. Yield, 0.32 g. Analyses of a preparation are embodied in Table III. Chloride test was positive.

TABLE III.

Composition of a Preparation of Mucosinmethylester Hydrochloride as Percentages.

Analysis	Found		Calc. on ash-free basis
	Actual	On ash-free basis	
Nitrogen*	2.81	3.20	3.46
Glucosamine	36.99	42.13	44.20
Glucuronic acid			
By indole r.	44.06		
By orcinol r.	44.91		
Mean	44.49	50.67	47.90
Acidity as hydrochloric acid†	7.39	8.42	9.0
Ash‡	12.2		

* Micro Kjeldahl. † Formol titration. ‡ Micro analysis.

Attempts were made to obtain a crystalline product. For this purpose crude mucosin oxalate (s. above) was immediately esterified and to the mixture after the reaction was added ether cautiously to faint turbidity. On standing crystals in needle form deposited but too small a yield did not allow analysis.

Heptacetylmucosin Methylester.

1 g. of amorphous mucosinmethylester-hydrochloride was put into a flask which was cooled in ice and a mixture of 6.7 g. pyridine and 5 g. acetic anhydride, similarly chilled, was added. The vessel was stood as stoppered for about 17 hours, with frequent shaking until complete dissolution of the powder. The reaction mixture was then poured into 30 cc. of ice-water under violent stirring and occurring amorphous precipitates and the mother liquid were separated by centrifugation.

a) The precipitates were taken up in a smallest possible quantity of absolute alcohol by boiling under reflux and the hot solution quickly filtered to eliminate the insoluble part. When cold grayish irregular crystals came out. Yield here was 250 mg. and F.P. 125°C. They were recrystallized in a like manner again and again to constant melting point of 130°C. The amount of the final product (non-colored needles) was 200 mg.—Prod. I.

b) The centrifuged supernatant liquid was distilled in vacuo (<40°C) and the residual dried as perfectly as possible in a desiccator for re-acetylation. The crystals first obtained (from alcohol) melted at 120°C. After recrystallizations the substance (needles)—Prod. II—melted constant at 130°C and no depression was shown by the mixed melting point test with Prod. I. Yield 50 mg.

The products (air dry) were united for examining the physical and chemical properties.

The substance dissolved in acetone, chloroform, ethylacetate, glacial acetic acid and pyridine with ease but in methanol, ethanol and benzene with difficulty. It was insoluble in ether, petroleum ether and cold water. It gave naphthoresorcinol (Neuberg-Saneyoshi), α -naphthol (Goldschmiedt) and aniline acetate tests for hexuronic acid and the indirect Osaki-Turumi test for acetylhexosamine. Hot Fehling and Tollens (cold) solutions were reduced. No ash was left on burning.

Crystal water.

6.040 mg. substance lost 0.469 mg. of water at 105°C under reduced pressure.
 $C_{12}H_{13}NO_{10}(CH_3CO)_7(OCH_3)+3H_2O$. Found 7.76%; calc. 7.53%.

Nitrogen (micro Kjeldahl).

13.20 mg. substance, 1.79 cc. 0.01 n. acid neutralized.

$C_{27}H_{43}NO_{21}$. Found 1.97%; calc. 1.95%.

Glucosamine (Hamasato and Akakura).

18.37 mg. substance was taken for analysis. 2 cc. of the hydrolysate, neutralized, filled up to 25 cc. and filtered, contained 0.443 mg. glucosamine as hydrochloride. Found 25.02%; calc. 24.97%.

Glucuronic acid.

17.60 mg. substance was dissolved in 10 cc. of chloroform and 3 cc. and 2.5 cc. portions respectively were pipetted into test-tubes for indole and orcinol reaction with following drying. Then 1 cc. water was added for the colorimetric technique to each of the residues. Found 26.99% (by the indole reaction 26.70%, by the orcinol reaction 26.98%); calc. 27.06%.

CH_3CO (Suzuki's modification of Friedrich, Rapoport and Sternberg method).

10.620 mg. substance, 10.25 cc. of 0.01 n. thiosulfate solution used (4.4075 mg. CH_3CO). Found 41.50%; calc. 41.98%.

CH_3O .

3.626 mg. substance, 3.10 cc. 0.01 n. Na_2SO_3 used. Found 4.42%; cal. 4.32%.

Molecular weight from iodine value (Bergmann and Machemer⁵).

9.26 mg. substance, 3.249 mg. iodine used (iodine value 27.65). Found 723; calc. 717.

Optical rotation in chloroform.

$$[\alpha]_D^{10} = \frac{+0.45 \times 100}{3.042 \times 1} = +14.78^\circ \text{ (no mutarotation).}$$

The substance contained neither NH_2-N (micro Van Slyke) nor free acid group (by titration with alkali). For determination of NH_2-N 20.44 mg. substance was dissolved in 10 cc. of water by slight heating in a stoppered flask and 2 cc. of the solution subjected to analysis after cooling, whereby no crystals precipitated out within some time. Alkaline titration was made on 10.21 mg. substance in a mixture of 6 cc. water + 6 cc. absolute alcohol. Alkali employed was 0.1 n.

The costs of this work were defrayed by a grant given through the Grant Committee for Scientific Researches from the Education Department.

H. Masamune.

References and Notes.

- 1) Masamune and Yasuoka, Tohoku J. Exp. Med., 1947, **49**, 181.
 - 2) Masamune and Tanabe, J. Bioch., 1933, **18**, 259.
 - 3) Cf. a paper on chondrosin by Kobayasi (J. Bioch., 1944, **36**, 399).
 - 4) Analyzed by Mr. Yosizawa according to Vieböck and Brecher.
 - 5) Bergmann and Machemer, Ber. dtsh. chem. Ges., 1930, **63**, 1922.
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