Biochemical Studies on Carbohydrates.

CV. Structural Study of A-specific Carbohydrate from the Mucin of Pig Stomach-mucus.

First Paper.

By

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From the complex carbohydrate in the heading acetolysis, deacetylation and following fractionation by means of organic solvents gave an acetylglucosamine galactoside which makes the unit structure of the mother substance. The chemical and physical properties of this disaccharide will be seen in the experimental part.

EXPERIMENTAL.

Preparation of the Group Specific Polysaccharide Employed as a Material.

The principles of Kobayasi-Tiba¹⁾ and Satoh²⁾ methods were combined.

First stage. 85 gm. of pig stomach mucus were stood with 170 cc. of 1.5 n. NaOH solution at room temperature (10–17°C) for one day and to the digestate was added glacial acetic acid to pH 5.0 under cooling. Centrifuged. The supernatant fluid and the washing (a dilute acetic acid at a similar pH was used) of the centrifugate were united, dialyzed (cellophane membrane) against running water until neutral (three days were required), and filtered through a folded paper to remove some material which had separated. It was then evaporated under reduced pressure to 8 cc., 85 cc. of 94% alcohol added, and the precipitates washed with two 8 cc. portions of 96% alcohol and dried in a desiccator over H₂SO₄. The obtained powder weighed 2.1 gm.

Second stage. The substance was at first made into a thick dough with a little water, and after increasing water to 35 cc., the insoluble-part was centrifuged off. The supernatant liquid was treated further as the

alkaline digestate of the mucus:— a) Acidified, centrifuged repeatedly, and when made water-clear, subjected to dialysis; b) then filtered, condensed and precipitated by the use of alcohol. The precipitates were washed with alcohol and dried. Crop, 1.1 gm.

Third stage. The product was agitated thoroughly with about 20 cc. of glycol, and to the transparent supernatant liquid after prolonged centrifugation were added 10 volumes of glacial acetic acid (96%). The deposit was centrifuged and washed with four 5 cc. portions of 95% alcohol and dried. Crop, 0.81 gm.

Fourth stage. This substance was dissolved in 40 cc. of water and shaken with 4 cc. of 12.5% CuSO_4 solution and 8 cc. of 10% NaOH solution. The centrifugate was washed with 8 cc. of 2% NaOH solution four times and dissolved in 4 cc. of 10% HCl solution, followingly the solution was precipitated with 60 cc. of 95% alcohol and the precipitates washed repeatedly with 95% alcohol, till free from HCl, and dried. The processes were repeated once more, when no biuret reaction was given. Crop, 0.61 gm.

Fifth stage. Electrodialysis. 0.61 gm. was adapted as 1% solution to electrodialysis (Pauli apparatus, collodione membrane, 200 volts). On the fourth day, the new changes of water in the outer compartments showed no pH shift. The precipitates, which appeared in the meanwhile, were centrifuged off and the clear supernatant liquid distilled under reduced pressure to about 3 cc. The substance thrown down with 40 cc. of 95% alcohol was washed twice with 10 cc. of 95% alcohol and dried in a desiccator over H_2SO_4 . A powder weighing 0.51 gm. was yielded.

Acetolysis.

The procedure of Bergmann and others³⁾ was applied:—

3 gm. of the carbohydrate prepared as above and perfectly dried were taken in an Erlenmeyer flask and placed in a freezing mixture. To it was added a mixture⁴⁾ of 15 cc. acetic anhydride and 2.1 cc. sulfuric acid (99.5%), cooled as well, in small portions and the stoppered vessel (ground joint) was kept in a dark room (15–20°C) for two days with occasional shaking (the substance turned yellow), and subsequently in a thermostat at 52–53°C. In 10 hours about 2/3 of the substance dissolved, giving a dark red solution. The suspension was cooled and centrifuged to again subject the undissolved part to the same treatment. The supernatant liquid was poured into 90 cc. of ice-cooled water containing 15 gm. of crystalline sodium acetate under agitation, whereupon red-brown, oily deposit came out, and sodium carbonate at first and afterwards sodium bicarbonate added to neutral. The oil changed thick-pasty by the

neutralization, but without separating it off, the mixture was shaken with chloroform repeatedly (with one 50 cc. portion and two 30 cc. portions). On the other hand, the part of the material, which was re-acetolyzed, gave a complete solution. This was also diluted, neutralized and extracted with chloroform. The chloroform extracts from the both solutions were united altogether and after washing with 100 cc. of water twice, dried by means of CaCl_2 (5 gm.) in a dark room. CaCl_2 was filtered off on a creased filter and washed with chloroform, then the filtrate and washings combined were condensed at a low temperature (in vacuo, $<30^\circ\text{C}$). The syrupy residue was taken up in 5 cc. of methanol. Since a few flocculent precipitates came out of the solution on standing in an ice chest, these were separated off on a Hirsch funnel and washed with methanol (3 cc., three times), and the filtrate and washings were evaporated together similar to before. The remainder dried in a vacuum desiccator was 3.0 gm. and proved clearly soluble in methanol (a red solution was given).

Deacetylation of the Cleavage Products and Following Fractionation.

The whole of the product above was deacetylated by shaking with 135 cc. of 1/5 n. KOH solution for 4 hours (room temperature, 15°C). The red solution obtained was neutralized with 1 n. H_2SO_4 solution (2.5 cc. were used) and distilled in vacuo ($<38^\circ\text{C}$) to a syrup. This was again evaporated twice with 50 cc. of methanol and the completely dried remainder was boiled three times with 50 cc. of methanol under reflux. The extracts were filtered hot and concentrated⁵⁾ as above ($<35^\circ\text{C}$) to a dark red syrup and treated with boiling absolute alcohol (two 30 cc. and one 10 cc. portions of the alcohol were used). Flocculi separated from the extract, when cold, and increased on raising the volume of the solvent. Therefore cold absolute alcohol (100 cc. in total) was supplemented, until no more precipitation occurred. The centrifuged supernatant liquid was distilled (under reduced pressure, $<40^\circ\text{C}$) to dryness, and the amorphous residue was taken up in 100 cc. of cold methanol (the substance dissolved well), decolorized with activated charcoal (2 gm. were employed) and after distilling off the solvent, extracted with cold absolute alcohol repeatedly. When evaporated and dried, the extract left a substance which weighed even 3.2 gm. and largely consisted of ash. 20 cc. of butanol dissolved it almost entirely but precipitation happened successively by further addition in portions of butanol up to 200 cc. The precipitates were collected by centrifugation and dissolved in 25 cc. of 70% ethanol. A small part undissolved was separated off on a Büchner funnel and washed with 70% alcohol (two 10 cc. portions) and the filtrate and washings were evaporated together under reduced pressure ($<40^\circ\text{C}$) to a syrup (red-

coloured). It⁷⁾ was then dissolved in 10 cc. of 70% ethanol and 50 cc. of absolute ethanol added. Some precipitates coming out on addition of 20 cc. ether were discarded and the clear mother liquid was precipitated most possibly with ether (500 cc.). The flocculent precipitates here were collected by centrifugation and dried up (in vacuo over H_2SO_4)—550 mgm.

In order to eliminate ash, which was largely potassium acetate, the following method was used. The substance was dissolved in 10 cc. of water, and after cautious addition of 10% H_2SO_4 to pH 1.2, extracted with ether continuously overnight. It was then neutralized with the saturated $\text{Ba}(\text{OH})_2$, distilled ($<35^\circ\text{C}$), without antecedent removal of BaSO_4 -precipitates, nearly to dryness and extracted with hot absolute methanol (four 30 cc. portions). Condensation and drying of the extracts gave 490 mgm. of yellowish white very hygroscopic powder. This was the disaccharide in view as will be demonstrated by analytical figures.

Physical and Chemical Properties of the Starting Polysaccharide and the Product.

For the purpose of analytical and other quantitative determinations the substances were dried constant in vacuo over P_2O_5 at room temperature.

1) The group-specific carbohydrate for acetolysis.

Biuret and ninhydrin reactions and the tests for methylpentose (Rosen-thaler's and Oshima-Tollens' methods) were negative. Molisch, intensively positive. Quantitative figures given were: N (micro Kjeldahl), 4.12%; acetyl (a modification of Friedrich, Rapoport and Sternberg's method by Suzuki), 10.77%; glucosamine (Hamamoto and Akakura method), 44.52%; galactose, 45.16% (the indole reaction of Dische, 45.16%; the orcinol reaction of Tillmans and Philippi simplified by Ozaki, 45.16%); ash (micro analysis), 0.03%. It exhibited anti-isoagglutinative reaction to A erythrocytes at 1: 10^6 dilution.

2) The final product (acetylglucosamine-galactoside).

A red dye developed by *p*-dimethylaminobenzaldehyde, when the substance had been heated in advance with alkali (Osaki and Turumi procedure), and strong Molisch reaction was also given. It had not the serological potency above at a dilution of 1:100. Decomposition point, $175\text{--}185^\circ$ (s. below). Molecular weight (Rast method), 386.65 (calc., 383.35). Rotation of light in water: it had the initial $[\text{D}]_D^{25}$ of $+66.38^\circ$ (3 minutes after dissolution) and equilibrium rotation of $+57.81^\circ$ (after 12 hours from dissolution). N (micro Dumas), 3.61% (calc., 3.65%); acetyl, 11.21% (calc., 11.23%); galactose, 47.13% by the indole method,

47.03% by the orcinol method, in averages 47.08% (calc., 47.0%); iodine use as glucose (Macleod and Robison), 49.54% (calc., 47.00%); ash, 0.1%. It tasted sweet.

Details will be appended regarding the molecular weight, optical rotatory power and melting point :—

Molecular weight. 0.557 mgm. of the substance was mixed with 4.943 mgm. of camphor. The depression of melting point was 11.6 degrees. $M. w. = \frac{1000 \times 0.557 \times 40}{4.943 \times 11.6} = 386.65.$

Optical rotation. 22.40 mgm. of the substance were dissolved in water at 4°C and made up accurately with water to 1 cc.

Duration of time after dissolution (min.)	α (degree)	$[D]_a^4 = \frac{\alpha \times 100}{l \times 2.240}$ (degree)
3	+1.487	+66.38
5	+1.437	+64.15
10	+1.397	+62.37
15	+1.379	+61.56
20	+1.367	+61.03
25	+1.357	+60.58
30	+1.351	+60.31
40	+1.339	+59.78
50	+1.333	+59.51
60	+1.331	+59.42
60 × 2	+1.320	+58.93
60 × 3	+1.313	+58.62
60 × 12	+1.295	+57.81
60 × 24	+1.295	+57.81

Melting point. It began to swell at some 98°C to contract again at 135°C and upwards, and melted very slowly at 175–185°C under decomposition.

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Bibliography and Notes.

- 1) Tiba, Tohoku J. Exp. Med., 1949, **50**, 281.
- 2) Satoh, Tohoku J. Exp. Med., in press.
- 3) Bergmann, Zervas and Silberkweit, Ber. deutsch. chem. Ges., 1931, **64**, 2436.
- 4) Acetic anhydride and sulfuric acid were both cooled before they were mixed together.
- 5) On standing in air the filtrates became turbid but were immediately subjected to distillation.
- 6) They were insoluble in absolute alcohol.
- 7) Insoluble in butanol.