Biochemical Studies on Carbohydrates.

CXXXVIII. A Search for the Unknown Nitrogenous Determinant Group of Masamune in the Group Carbohydrate from the Mucus-mucin of Pig Stomach.

By

Yasuaki Matuda and Eitaroh Itoh.

(松田 順 皓)

(伊 藤 榮 太 郎)

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

(Received for publication, June 1, 1950)

Masamune¹⁾ claimed that group (ABO-system) carbohydrates possess two kinds of determinant group, one a structure formed by either glucosamine or chondrosamine together with either galactose or galacturonic acid or D-fucose and the other an unknown nitrogenous structure or constituent. As to the components of group lipoids one²⁾ of the writers has fractionated the hydrolysate of a preparation of the group lipid from A human liver and isolated choline and arginine besides glucosamine, galactose and others. Whether or not choline and arginine were real constituents of that lipid is not certain at present, but it will be worthy of experimentation for the scrutiny of the second determinant group to decide if preparations of group carbohydrates involve phosphotungstic acid-precipitable components particularly choline and allied quaternary methylamine derivatives such as neurine and glycollbetaine. In the present work the writers picked out Group A carbohydrate from the mucus of pig stomach as a material and searched after the compounds in question among the split products. The hydrolysis was exerted in two steps, first with 1 n. H_2SO_4 and next with 6 n. HCl . Choline and neurine which are cast down from alcohol by platinum chloride, glycollbetaine whose hydrochloride is insoluble in alcohol and which is precipitated from water with gold chloride, histidine and lysine were not found, whereas arginine was isolated as flavianate. Arginine, however, was yielded in too small an amount to be accepted as a constituent of that carbohydrate.

EXPERIMENTAL.

The group carbohydrate was prepared immediately from the mucus by our usual method (Cf. f. i. Yosizawa³⁾) Electrodialysis was not effected. The copper alkali procedure was carried out as described by Matuda.⁴⁾

The product analyzed: nitrogen 4.3%, glucosamine 40.1%, galactose 41.6%, ash 2.1%. The inhibition of isoagglutination of A erythrocytes was exhibited at its dilution of 1:16⁶. Negative biuret despite of positive Sakaguchi test for arginine. Tollens ammoniacal silver solution was reduced but very slowly. Positive reduced sulfur test and Acree-Rosenheim test for tryptophane were not given.

1) *Primary Hydrolysis of the Group Substance and Fractionation with Phosphotungstic Acid.* 5 g. of the material were taken up in 250 cc. of 1 n. H_2SO_4 and refluxed for 5 hours at 100°C. When cold, the hydrolysate was filtered and to the filtrate was added 20% phosphotungstic acid in portions until no more precipitation occurred. After standing for 5 hours the deposit was centrifuged and washed with 1 n. H_2SO_4 containing 2.5% phosphotungstic acid. It was then suspended in 30 cc. water and freed from phosphotungstic and sulfuric acid with the saturated $\text{Ba}(\text{OH})_2$. The solution obtained had 41.5 mg. nitrogen.

2) *Fractionation at Acid Reaction of the Phosphotungstic Acid-precipitable Substance with Silver Sulfate.* The final solution above was distilled under diminished pressure to 25 cc. and after centrifuging off of some appearing deposit, dilute H_2SO_4 and fine powdered Ag_2SO_4 added whereby precipitates were given. The centrifuged supernatant liquid and the centrifugate suspended in water were separately freed from silver and sulfuric acid by the aid of H_2S and the saturated $\text{Ba}(\text{OH})_2$, and the fluid containing the substance, that had been precipitated with Ag_2SO_4 , was further condensed to a small volume (about 1 cc.). The condensate (neutral and somewhat turbid) gave neither murexide reaction with halogen nor diazo reaction with NaOH , whereas its dried up residue showed the positive Lassaigne reaction of nitrogen, suggesting the presence of guanine. The other fluid containing the fraction unprecipitated by Ag_2SO_4 were assayed qualitatively with following results: ninhydrin+, biuret+, arginine (Sakaguchi)+, histidine (Totani, Hanke-Koessler)—?, tyrosine (Totani, Hanke-Koessler)—?.

3) *Subdivision with Alcohol of the Fraction Unprecipitated by Silver Sulfate.* The balance of the fluid concerned was acidified to pH 1.0 with HCl and distilled in vacuo to a small volume. This was subjected repeatedly to vacuum distillation with water or with alcohol and when excess HCl was eliminated, the residue was dissolved in 2 cc. of water and 20 cc. of absolute alcohol added, whereupon brownish-colored precipitates came out. The centrifuged supernatant fluid was distilled to dryness and absolute alcohol added. A part of the substance did not dissolve. Centrifugation and vacuum distillation of the supernatant liquid and addition of absolute alcohol to the remainder was repeated until all the alcohol-insoluble part was separated. The final alcoholic solution gave no precipitates with

alcoholic platinum chloride solution (2%) which indicated that no choline nor neurine was present, because the hydrochlorides of these quaternary amines are soluble in absolute alcohol while that of glycocollbetaine is not. The balance of the solution was evaporated similar to above and the residual taken up in 10 cc. of water. A small insoluble part was centrifuged and set aside. Precipitation tests on the clear solution: It gave 1) precipitates with phosphotungstic acid (A part of the solution was acidified with H_2SO_4 and the precipitant added), and also 2) some precipitates with picric acid as dissolved in water and with flavianic acid as dissolved in water (The presence of a small amount of arginine was suspected) but 3) no precipitation with watery platinum chloride solution (2%) (Neurine was scarce, if present).

4) *Extensive Hydrolysis of the Balance of the Fraction Examined in 3) with Following Fractionation with Phosphotungstic Acid.* The alcohol-insoluble portions, the water-insoluble substance and the watery solution above were combined and distilled in vacuo to dryness. The residual was hydrolysed with 20 cc. of 6 n. HCl by heating for 16 hours in a boiling water-bath (under return condenser). The hydrolysate was diluted six times with water and filtered and the filtrate (biuret-negative) precipitated with phosphotungstic acid with following removal of the precipitant from the precipitates in a similar manner as in 1). The solution obtained was made up to 100 cc. for determination of nitrogen. 30.8 mg. N was present. Then 1 cc. of 10% HCl was added and the mixture distilled at a low temperature to about 2 cc. Addition of 10 cc. of alcohol gave rise to mere turbidity but no precipitation. The turbid fluid was distilled as such to dryness. Excess HCl was eliminated by repeated vacuum distillation of the residue with water and with alcohol, discarding a very small amount of water-insoluble substance in the course of the operations. And the final evaporated residue was taken up in 4 cc. of water. The solution here (pH, 5.0) was found non-precipitable by watery gold chloride solution (2%) (glycocollbetaine —). A few drops of the solution were taken for that test. Again the balance of the solution was distilled. The dried-up remainder was taken up in 20 cc. of absolute alcohol. The insoluble part centrifuged and then the supernatant liquid distilled to dryness. From the remainder the alcohol-insoluble part was further separated in a similar manner and this time the solution was condensed to about 5 cc. A few drops of the solution were put to the test with the alcoholic platinum chloride solution but no precipitation happened (choline —, neurine —).

5) *Fractionation with Silver Nitrate at Alkaline Reaction of the Substance Precipitated with Phosphotungstic Acid in 4).* The remaining portion of the alcoholic solution was distilled and the residual thereby and the substance undissolved in alcohol were dissolved together in 15 cc. water. The solu-

tion was acidified with HNO_3 and sufficient 20% AgNO_3 added for the most possible precipitation. The deposit was centrifuged⁵⁾, and to the supernatant liquid was further added the AgNO_3 solution in small portions until a drop of the mixture gave brown precipitates on bringing together with a drop of the saturated $\text{Ba}(\text{OH})_2$, and then at first neutralized with the saturated $\text{Ba}(\text{OH})_2$ and afterwards saturated with powdered baryta. The dark precipitates were centrifuged and washed with changes of dilute $\text{Ba}(\text{OH})_2$ with centrifugation. The supernatant fluids were soon freed from barium and silver with H_2SO_4 and H_2S , subsequently distilled in vacuo to about 5 cc., and after addition of an equal volume of 10% H_2SO_4 , precipitated with 20% phosphotungstic acid. The precipitates obtained (very few) were suspended in water and liberated from phosphotungstic acid with a mixture of amylalcohol and ether (1:1) and from H_2SO_4 with a just sufficient quantity of the saturated $\text{Ba}(\text{OH})_2$ and the resultant clear solution was condensed to about 0.5 cc. and diluted with 2 cc. of absolute alcohol. Addition of saturated alcoholic picric acid solution caused no precipitation (lysine —): The mixture was diluted with 20 cc. of water, acidified with HCl and shaken with ether. And the picric acid-free solution was distilled in vacuo to dryness and to the little rest were added 2 cc. of absolute alcohol. However, all the substance dissolved thereby, which proved the absence of glycocollbetaine. Really precipitation test on drops of the solution with alcoholic platinum chloride solution resulted in negative. Further, the balance of the solution was distilled to dryness and the remainder dissolved in 1 cc. of water (All the substance dissolved). This solution, too, gave no precipitate with watery gold chloride solution (2%).

6) *Isolation of Arginine.* To the dark Ag-precipitates in the preceding section was added dilute H_2SO_4 in excess and the centrifuged supernatant liquid bubbled with H_2S . Again centrifuged. Ag_2S (the centrifugate) was washed with boiling water and filtered. Washing was repeated. The supernatant liquid and washings were boiled together to expel off H_2S . (Dialysis test for histidine was negative at this stage). The excess H_2SO_4 was removed with an equivalent quantity of the saturated $\text{Ba}(\text{OH})_2$. And the solution was distilled in vacuo to about 1 cc., 5 cc. of 5% flavianic acid (a watery sol.) added and the mixture stood for 2 days. Yellow crystalline deposits (octahedrons) were collected (on a suction funnel) and dried at 105°C — 24.2 mg. The product was recrystallized from a hot very dilute solution of flavianic acid and dried as above — 20.2 mg. N (Friedrich), 17.12% (calculated for arginine flavianate, 17.21%). It began charring at 258°C and decomposed at 268°C .

7) *Examination of the Filtrate from the Crude Arginine Flavianate.* This filtrate was freed from flavianic acid by addition of the saturated $\text{Ba}(\text{OH})_2$.

After quantitative removal of excess barium with H_2SO_4 , the solution was distilled to about 5 cc., when its pH lay at 4.5. The condensate gave no positive ninhydrin reaction.

CONCLUSION.

Group A carbohydrate from the mucus-mucin of pig stomach contains neither choline nor neurine nor glycocollbetaine nor histidine nor lysine. From the hydrolysis products of this group substance arginine was isolated as flavianate but the yield was too small for a constituent.

We express gratitude to the Education Department which gave a grant through the Grant Committee of the Science Council in aid to us.

H. Masamune.

References and Notes.

- 1) Masamune, Tokyo Iji Shinshi, 1949, **66**, 269. Cf. also Matuda (2)).
- 2) Matuda, Tohoku J. Exp. Med., 1951, **53**, 373.
- 3) Yosizawa, *ibid.*, 1949, **51**, 51.
- 4) Matuda, *ibid.*, this volume, p. 1.
- 5) The centrifugate was suspended in water, and after removing Ag with H_2S , the solution was distilled but nothing left.

A Method to Take a Kymogram of the Heart along the Perimeter of the Transsection.*

A Preliminary Report.

By

Shinji Takahashi and **Tatsuyo Shinozaki.**

(高橋 信次)

(篠崎 達世)

(From the Department of Radiology, Hirosaki University
School of Medicine. Hirosaki)

(Received for publication, March 28, 1951)

An X-ray tube, a rotation table, a lead slit and a film cassette are arranged in this order for the operation.

The film moves just behind the stationary slit which is 1 mm wide,

* Read at the 3rd Meeting of the Tohoku Radiological Society. Oct. 15, 1948. Sendai.