On Proteins of Gastric Cancer

I. Preparation of Protein Fractions and Some of their Properties, Physical and Chemical*

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A large number of gastric cancers were employed and fractioned, by combining the principles described in foregoing articles^{1), 1a), 1b), 2)} from this Institute. The present account deals chiefly with separation of the protein fractions and some properties of the products. They are not precluded to have been mixtures of proteins, which contained a minute quantity of carbohydrate or were made up wholly of amino acids, and the group carbohydrate (an amino acid-polysaccharide complex), which had not been perfectly removed due to its tenacious adsorption. But here glucidamins^{2a)} are tentatively assumed in place of the mentioned carbohydrate. The corresponding preparations were also prepared from gastric mucosa and examined.

EXPERIMENTS AND COMMENTS

Preparation Procedure

Gastric cancer

Fresh human gastric cancers excised were mechanically freed from adjacent tissue and necrotic matter, and after cutting into small pieces, kept under 5 volumes of alcohol for a duration of time varying from 3 days to 1 year and 8 months. The materials thus obtained were put together according to the blood group of the patients (A and O) and after squeezing and mincing, dried in a Faust apparatus. The number of cancers and the weight of fresh and dried materials were the following.

Blood group	Fresh cancers		Total weight of the dried materials (g.)
	number	total weight (g.)	
A	30	632	98.5
O	30	667	101.0

* The 6th report of Masamune and coworkers' "On Proteins and Amino Acids."

The batches of the dried materials will be referred to as Batches A and O.

1) Batch A

Preliminary fractionation

It was exhausted with boiling alcohol (5 and 3 volumes of abs. alcohol were employed) (Regarding the details, cf. Masukawa¹⁾). The solutions were united (720 cc.) and set aside for isolation of the blood group lipoid (cf. Masamune *et al.*³⁾).

The residue amounting to 88.5 g. was subjected to extraction with changes of water (1000, 900, 900, 800, 800 and 700 cc.). The remainder here, washed with alcohol and dried, weighed 71 g. To the solutions (light-yellow and turbid) were added as much acetic acid (30%) as to raise the pH to 4.5, whereupon marked precipitation occurred, and a little NaCl. The centrifuged supernatant fluids of the earlier solutions were turbid, while those of the later ones were nearly transparent. The centrifugates were washed once with a little water and then three times with abs. alcohol and dried. The successive grayish coarse powders amounted to 0.45, 0.51, 0.86, 0.62, 0.62 and 0.68 g. (in total 3.74 g.—**Ppt. I**).

The supernatant fluids were dialyzed against running water for 24 hours, and distilled in vacuo to about 50 cc., whereby they became much more cloudy. Centrifuged. The centrifugates were washed with water and abs. alcohol and dried. The powders yielded from the 1st to 3rd condensates were 0.23, 0.28 and 0.15 g. respectively (in total 0.66 g.—**Ppt. II**). The centrifugate of the 4th to 6th were very small. The fluids were further condensed to a syrup and precipitated with 5 volumes of abs. alcohol and a little NaCl, followed by repeated washing of the precipitates with abs. alcohol. The amount of the substances dried (light-yellow powders) were 4.43, 0.94, 0.71, 0.56, 0.17 and 0.22 g. (in total 7.03 g.). These were united and agitated with many changes of water (20–40 cc. portions) containing a little NaCl. The insoluble part was washed with abs. alcohol (with addition of a little anhydrous sodium acetate) several times and dried. It amounted to 0.54 g.—**Ins. I**.

The solutions (all slightly turbid) combined were adjusted to pH 9.5 (with Na_2CO_3) and proteins that precipitated were rejected. Following acidulation (with acetic acid) caused extreme turbidity, but gave almost no centrifugate (r. p. m. 3,000). It was then distilled in vacuo to about 10 cc. and 6 volumes of abs. alcohol and a little anhydrous sodium acetate were added. The precipitate was fractioned, after washing with abs. alcohol and drying (6.02 g.), into glacial acetic acid-soluble and -insoluble parts (The substance was kneaded with a little water (2.5 cc.) and an abundant quantity (500 cc.) of 98% acetic acid was added. A little

anhydrous sodium acetate was also used besides). And the insoluble was centrifuged, washed with glacial acetic acid and thoroughly with abs. alcohol and dried—3.61 g. This (a white powder, **Cr. Ch.**) was furnished for separation of the group carbohydrate whose description will be given elsewhere. From the solution—**Fl. I**—were isolated the glacial acetic acid-soluble proteins as below.

Purification

a) Glacial acetic acid-insoluble proteins

a) The proteins precipitable from water by acid

Ppts. I-II and Ins. I (4.9 g. altogether) were agitated together in 100 cc. of water and after addition of the saturated $\text{Ba}(\text{OH})_2$ to pH 8.5, stood overnight. To the mixture was again added the baryta solution to pH 9.3, and the abundant precipitate was centrifuged off.⁴⁾ The supernatant was acidified to pH 2.0 by means of 3% HCl, and after addition of a little NaCl, subjected to centrifugations (one single centrifugation gave only an extremely turbid supernatant fluid). And the final translucent fluid was dialyzed, concentrated by vacuum distillation to a syrup and mixed with abs. alcohol and a little anhydrous sodium acetate. The deposit was washed with abs. alcohol again and again and dried. A white powder given weighed 1.58 g.—**Ppt. III**. This was combined with Ppts. IV and V below for separation of the fraction which is insoluble in glacial acetic acid but does not precipitate at pH 2.0 from water by acid.

The centrifugate above (the precipitate at pH 2.0) was washed likewise with abs. alcohol and dried. The grayish powder given (0.81 g.) contained phosphorus. It was fractioned by means of glacial acetic acid:—The substance was kneaded with a few drops of water and 3% NaOH. The volume of 98% acetic acid added was 40 cc. The glacial acetic acid-insoluble part, thoroughly washed with the glacial acetic acid and with abs. alcohol (A little anhydrous sodium acetate was added) and dried, amounted to 0.77 g. On it was the sequence of fractionations from the treatment with baryta to that with glacial acetic acid was repeated twice more.⁵⁾ A grayish powder amounting to 0.11 g. was yielded—**Prep. I**. Phosphorus was scarcely detected. The fractions recovered from the supernatant fluids of pH 2.0 here will be referred to as **Ppts. IV and V**. They weighed 0.19 and 0.07 g. respectively.

β) The proteins non-precipitable from water by acid

Ppts. III-V were united. This batch (1.84 g.), which also contained phosphorus though little, was taken up in 40 cc. of water and 3% HCl was added to pH 2.0. The precipitate was centrifuged off and washed with three 25 cc. portions of a dilute HCl of pH 2.0, each time followed

by centrifugation.⁶⁾ The supernatant fluids (All but the last were turbid) were dialyzed together for 1 day and distilled in vacuo to about 15 cc., then, the condensate was acidified again to pH 2.0 with HCl and precipitated with 20 volumes of a mixture of abs. alcohol and ether (1:1 by volume). The precipitate was washed with the alcohol-ether mixture above and dried. 0.68 g. of a white powder was given here. Another run of removing the part insoluble at pH 2.0 was carried out in a similar manner.⁷⁾ The product (a light colorless powder)—**Prep. II**—was free from phosphorus. Crop, 0.51 g.

b) Glacial acetic acid-soluble proteins

To Fl. I were added about 3 volumes of ether. The precipitate, washed with abs. alcohol exhaustively and dried, was fractioned by means of glacial acetic acid over again. Water was not used here at all. The quantity of glacial acetic acid (98%) added was 80 cc. and a little anhydrous sodium acetate was employed besides. After centrifugation, the supernatant fluid (light brown, transparent) and the washings (one 20 cc. and two 10 cc. portions of the glacial acetic acid were used) of the centrifugate⁸⁾ were united, precipitated with 4 volumes of ether and centrifuged. By washing the present centrifugate with abs. alcohol containing a little anhydrous sodium acetate and drying, a brownish coarse powder weighing 0.69 g. was given.

By following the procedure in a foregoing account,¹⁾ it was liberated from nucleic acid and basic and acidic proteins precipitable at slight alkalinity and acidity respectively. Namely, the pH of its solution was shifted hither and thither twice with baryta and HCl, followed by removal of barium, concentration of the solution after neutralization with NaOH, and precipitation with alcohol. When thus no contaminants separable in this manner remained anymore, the substance was carried down from the clear solution by means of alcohol—0.54 g. This was light yellow granules and phosphorus-free. Subsequent electro dialysis reduced the quantity of substance to 0.26 g. Finally it was treated over again with glacial acetic acid similar to above, because it was found still partially insoluble in this solvent.⁹⁾ The product which was a light-yellow coarse powder—**Prep. III**—amounted to 0.1 g. and was both Molisch- and biuret-positive.

2) Batch O

Fractionation of this batch (101 g.) and purification of its protein fractions were carried out in a manner quite similar to above. The volume of the solutions obtained by extraction with boiling alcohol were 790 cc. altogether. Be the products corresponding to those above represented with the same symbols, Ppts. I and II and Ins. I weighed 3.04,

0.43 and 0.21 g. respectively (in total 3.68 g.) and Ppts. III to V 1.16, 0.28 and 0.09 g. respectively (in total 1.52 g.). Cr. Ch. 3.24 g. Of the purified fractions, which were insoluble in glacial acetic acid, the one precipitable by acid (Prep. I) was 0.15 g. and the one non-precipitable by acid (Prep. II) 0.32 g., and the glacial acetic acid-soluble (Prep. III) 0.18 g. These fractions resembled the corresponding fractions of Batch A in appearance as well as the result of biuret and phosphorus test.

Normal human gastric mucosa

The mucosa was teared from the whole or a part of a human stomach and after freeing from mucus, cut into small pieces, which were preserved in 5 volumes of alcohol for a various duration of time from 13 days to 17 months. The materials thus obtained from stomachs were also divided according to the blood group of the persons, squeezed and dried—Batches A and O. Details are tabulated as below:

Blood group	Fresh materials		Total weight of the dreed materials (g.)
	number	weight (g.)	
A	19	1,254	224
O	14	927	174

The fractionation procedure was strictly same as above.

1) Batch A

Batch A amounted to 224 g. After treatment with boiling abs. alcohol, it weighed 208 g. The light brown alcoholic solutions amounted to 1800 cc. in all. The fractions corresponding to Ppts. I and II and Ins. I above were 4.81, 0.61 and 1.13 g. respectively (in total, 6.55 g.) and those corresponding to Ppts. III to V 1.60, 0.21 and 0.04 g. (in total, 1.85 g.). The crude fraction (Cr. Ch.) set aside for separating the group carbohydrate was 1.39 g. The purified proteins, insoluble in glacial acetic acid and precipitable from water by acid, 0.11 g.; those, insoluble in glacial acetic acid and unprecipitable from water by acid, 0.35 g.; the glacial acetic acid-soluble, 0.11 g.

2) Batch O

Batch O, 174 g. The hot-alcoholic solutions, 1260 cc. The crude fractions of the proteins which were insoluble in glacial acetic acid, 0.75, 0.75 and 0.45 g. respectively (in total 1.95 g.). The material containing the group carbohydrate (Cr. Ch.), 0.68 g. The fractions of glacial acetic acid-insoluble proteins could not be thoroughly purified due to shortage of material. The purified glacial acetic acid-soluble amounted only to 57 mg.

The fractions, crude and purified, from the both batches of the mucosa had appearance like the corresponding fractions from gastric cancers and also gave same consequences in qualitative tests.

Physical and Chemical Properties of the Products

The corresponding protein fractions from the different sources resembled one another in solubility, precipitability, test-tube assays and quantitative analyses.

1) Solubility and precipitability

Prep.-I's dissolved in water on alkalization and half saturation with $(\text{NH}_4)_2\text{SO}_4$ gave rise to remarkable precipitation.

Prep.-II's dissolved also in water, forming a clear solution, on faint alkalization. Trichloroacetic acid and $(\text{NH}_4)_2\text{SO}_4$ (even at half saturation) gave rise to a precipitate from its solution, but picric acid could not. The solution became turbid on acidification (turbidity maxima at pH's 4.0 and 2.4), but no precipitation occurred.

Prep.-III's. Their solutions behaved similar to Prep.-II's towards trichloroacetic acid and $(\text{NH}_4)_2\text{SO}_4$.

2) Chemical qualitative tests

Prep.-I's. Biuret-, Millon-, Sakaguchi-, reduced sulfur-, Hopkins-Cole- and Molisch-test positive. The positive Goldschmiedt and Neuberg-Saneyoshi reaction were given, although aniline acetate test resulted only in faint positive. Rosenthaler weak. The indirect Osaki-Turumi reaction was shown but not the direct.

Prep.-II's. The biuret test and the assays for amino acids resulted similar to above. Molisch and the reactions of hexuronic acid and methyl-pentose occurred more intensively than in Prep.-I's. The direct Osaki-Turumi reaction was given, though faint, besides the positive indirect reaction.

Prep.-III's. The tests resulted similar as in Prep.-I's but that more vivid Rosenthaler was given.

3) Quantitative composition

Analysis was made by our usual procedures. The figures obtained are embodied in Tables I-III, which demonstrate that the corresponding fractions from gastric cancers and the mucosa were generally similar in so far as the rough composition concerns. Prep.-III's from gastric mucosa were richer in carbohydrate than those from gastric cancers, but such a divergency as was found may happen between mixture preparations

of this kind even if separated from parts of one and the same batch of a tissue pulp.

4) Optical rotation

As is indicated in Tables II and III, Prep.-II's and Prep.-III's of cancer

TABLE I

Composition of Prep.-I's (the protein fraction of gastric cancer and gastric mucosa insoluble in glacial acetic acid and precipitable from water by acid)

Analysis	The protein fraction		
	of gastric cancer		of normal gastric mucosa from Group A persons
	from Group A persons	from Group O persons	
N*	12.3	13.0	14.2
Hexosamines†	7.9	6.1	5.4
Non-amino sugars as galactose			
Orcinol reaction	8.8	8.7	8.1
Indole r.	15.2	16.5	16.8
Diphenylamine r. I.	14.7	12.3	10.8
Ash	11.1	11.7	12.6

* Micro Kjeldahl.

† Hamasato-Akakura method

TABLE II

Composition and Optical Rotation of Prep.-II's (the protein fraction of gastric cancer and normal gastric mucosa insoluble in glacial acetic acid and unprecipitable from water by acid)

Analysis and rotation	The protein fraction		
	of gastric cancer		of normal gastric mucosa from Group A persons
	from Group A persons	from Group O persons	
Composition (Figures express percentages.)			
N	9.5	9.7	10.3
Hexosamines	20.0	21.2	20.8
Non-amino sugars as galactose			
Orcinol reaction	20.1	21.0	18.0
Indole r.	27.1	28.6	27.8
Diphenylamine r. I.	35.2	36.2	25.1
Ash	4.2	3.9	2.7
Optical rotation			
$[\alpha]_D$	-26.8° (11°C)	-25.1° (11°C)	-41.0° (11°C)

TABLE III

Composition and Optical Rotation of Prep.-III's (the protein fraction, soluble in glacial acetic acid, of gastric cancer and normal gastric mucosa)

Analysis and rotation	The protein fraction			
	of gastric cancer		of normal gastric mucosa	
	from Group A persons	from Group O persons	from Group A persons	from Group O persons
Composition (Figures express percentages; those in parentheses are the calculated on an ash-free basis)				
N	10.8 (13.1)	10.7 (12.8)	9.7 (11.4)	10.5 (11.8)
Hexosamines	10.6 (12.9)	10.6 (12.7)	15.9 (18.6)	18.2 (20.4)
Non-amino sugars as galactose Orcinol reaction	14.8 (18.0)	13.8 (16.5)	18.4 (21.5)	21.0 (23.5)
Indole r.	20.0 (24.3)	20.0 (24.0)	22.8 (26.7)	29.3 (32.8)
Diphenylamine r. I.	22.4 (27.2)	25.7 (30.8)	27.5 (32.2)	30.2 (33.9)
Ash	17.6	16.6	14.6	10.7
Optical rotation				
$[\alpha]_D$	-50.9° (11°C)	-51.8° (12°C)	-40.6° (12.5°C)	-55.8° (13°C)

had rotations almost equal between the Groups A and O within each pair of the preparations. Moreover the rotations of the latter preparations (the Prep.-III's) lay between those of the corresponding mucosa preparations.¹⁰⁾ Prep. II could not be prepared from the gastric mucosa of Group O persons, but the rotation of that from gastric mucosa of Group A persons suggests that a similar relation also holds within the cancer and mucosa Prep.-II's. These facts remind one of Oh-Uti's finding^{1b)} on the rotations of the Group A and Group O carbohydrates of gastric cancer and gastric mucosa.

SUMMARY

1. Three protein-fractions (the fraction which is insoluble in glacial acetic acid and precipitates from water at pH 2.0 (I), the one insoluble in glacial acetic acid and non-precipitable at any pH from water (II), and the glacial acetic acid-soluble (III)) were separated from a gastric cancer of persons belonging to Group A and a gastric cancer of Group

O persons. Also the corresponding preparations were prepared from human gastric mucosa.

2. The solubilities, qualitative reactions and quantitative analyses resembled each other between the corresponding fractions of the gastric cancer and mucosa.

The rotation of a pair of preparations from cancers of persons belonging to Group A and from those of persons belonging to Group O were coincident with each other and lay between the rotations of the corresponding preparations from gastric mucosa.

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References and Notes

- 1) Masukawa, Tohoku J. Exp. Med., 1951, **54**, 227; Matuda, *ibid.*, 1951, **53**, 333.
 - 1a) Satoh, *ibid.*, 1949, **51**, 275.
 - 1b) Oh-Uti, K., *ibid.*, 1949, **51**, 297.
 - 2) Aikawa, *ibid.*, 1949, **51**, 367.
 - 2a) Masamune, *ibid.*, 1949, **50**, 107; Masamune, Yosizawa & Maki, *ibid.*, 1951, **53**, 237 (footnote).
 - 3) Masamune, Siozima & Masukawa, *ibid.*, 1951, **54**, 327.
 - 4) The centrifugate was washed with a dilute baryta solution of pH 9.3 and then with abs. alcohol (A little anhydrous sodium acetate was employed besides) and dried—Ppt. A. It amounted to 2.1 g.
 - 5) The portions precipitated by baryta—Ppts. A' and A''—weighed 0.3 g. and 70 mg., when they were dried after washing with a dilute baryta of pH 9.0 and alcohol.
 - 6) The centrifugate was then washed well with alcohol and dried—Ppt. B. This weighed 0.8 g.
 - 7) The centrifugate here, washed and dried, amounted to 155 mg.—Ppt. B'.
 - 8) This was further washed with abs. alcohol several times and dried—Ppt. C. It amounted to 0.35 g.
 - 9) The insoluble part, centrifuged, was washed once with glacial acetic acid and many times with abs. alcohol and dried. A yellowish powder weighing 86 mg. was given—Ppt. C'.
 - 10) Judging from the analyses, Prep.-III's from the mucosa and those from the cancers don't seem to strictly correspond to each other regarding the quantitative ratios of the proteins composing the respective substances, and hence their rotations must be compared mutually with some reservation.
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