Molisch-positive Mucopolysaccharides of Gastric Cancers as Compared with the Corresponding Components of Gastric Mucosae

First Report

Fractionation Procedure of Gastric Cancer and Gastric Mucosa*

By

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Several papers¹⁾⁻⁴⁾ have been already published from this Medico-chemical Institute regarding the group mucopolysaccharides of gastric cancers and of gastric mucosae to establish how the cancerous group substances deviate in composition from the corresponding non-cancerous. However, the methods of isolating high-molecular components from animal tissues and of analyses of them have been recently amazingly improved, necessitating a reinvestigation. Fortunately, large amounts of the same materials as above have become accessible to us by favour of a grant from the Ministry of Education and friendly support of many hospitals. Therefore, a thoroughgoing chemical study has been undertaken anew to carry out on as many mucopolysaccharides as obtainable from those tissues, including those which are group active, with respect not only to their composition but also to their structure.

The present account deals with a great part of the procedure for separating the tissue components in view in a pure state.

EXPERIMENTAL

Materials

Gastric cancers and mucosae of non-cancerous stomachs, which had been covered separately according to blood group A, B or O under

* 214th report of Masamune and co-workers' "Biochemical Studies on Carbohydrates." A part was read before the XVIth General Meeting of the Japanese Cancer Association, Osaka, October 30-31, 1957.

TABLE
Isolation of

I

MPSs I-IV

4–5 volumes of ethanol for various durations from 1/2 month to 2 years, were squeezed, masticated, and after drying in a Faust apparatus, pulverized. The six different materials were further dried in a desiccator (CaCl_2).

Isolation Procedure of the Mucopolysaccharides

The case started with the dry group A cancer material will be described as an example. The principle is illustrated in Table I.

First stage. 180 g. of the dry material was shaken with 2 l. of water for 2 days at room temperature (20°C), avoiding foam formation with 5 drops of octyl alcohol. The insoluble residue was further shaken with four 1.3 l. portions of water in succession, each extraction taking 1 day. The first yellowish turbid extract of pH 7.0—**Extract 1**—which amounted to 1.4 l. was distilled in vacuo to 300 cc. and dialyzed against 1.5 l. of water for 12 hours. The yellowish, transparent dialysate was distilled under reduced pressure to a syrup and placed in a vacuum-desiccator (CaCl_2). 4.5 g. of a hygroscopic yellow powder was given—**Dialysate 1**. The inner fluid in the dialysis bag (500 cc.) was acidified to pH 4.5 with 20% acetic acid. A white flocculent precipitate that occurred was centrifuged off, washed with water adjusted to pH 4.5, ethanol and acetone in turn and dried. 1.6 g. of a gray fibrous substance was given—**Ppt CH_3COOH** . The mother fluid (yellowish turbid) and watery washing were combined, filtered through a cellulose pad with suction, neutralized to pH 6.0 with NaOH and distilled in vacuo to a syrup. It was precipitated with 200 cc. of ethanol, washed with ethanol and acetone and dried. 6.5 g. of a yellowish powder was given—**Supern CH_3COOH** .

The successive tissue extracts were treated similar to above with yields of the fractions following:

No. of extract	Yield (g.).		
	Dialysate 1	Ppt CH_3COOH	Supern CH_3COOH
1	4.51	1.55	6.46
2	2.25	1.70	1.92
3	0.73	1.10	0.84
4	0.55	0.89	0.30
5	0.30	0.85	0.28
Total	8.34	6.09	9.80

Second stage. 9.0 g. of Supern CH_3COOH was dissolved in 90 cc. of water and dialyzed for 24 hours against 1.5 l. and 1.2 l. portions of water in succession, and the yellow transparent dialysates were concentrated together by distilling in vacuo to dryness to give 3.5 g. of a hygroscopic yellow substance—**Dialysate 2**. The almost clear inner fluid was distilled under reduced pressure to about 200 cc., saturated with 150 g. of ammonium

sulfate and acidulated to pH 5.6 with 3% sulfuric acid. The flocculent deposit was centrifuged off after standing for 3 hours at room temperature (15°C) and reprecipitated twice from decreasing volumes of water. Then it was taken up in 120 cc. of water, dialyzed against running water for 3 days and distilled in vacuo to syrupy consistency, and 150 cc. of ethanol containing some anhydrous sodium acetate was added. The precipitate here was separated, washed with ethanol and ether and desiccated. 2.94 g. of a grayish powder with yellow hue was given—**Ppt**_{(NH₄)₂SO₄}. The filtrates saturated with (NH₄)₂SO₄ (yellowish transparent) were united, diluted with 2 volumes of water, and after 2 days' dialysis against running water, condensed to about 1/10 the volume, followed by dialysis for the same duration of time. The fluid was then distilled to a syrup, precipitated, washed and dried as when **Ppt**_{(NH₄)₂SO₄} above was separated. 0.93 g. of a white powder was given—**Supern**_{(NH₄)₂SO₄}.

Third stage. 0.93 g. of **Supern**_{(NH₄)₂SO₄} was dissolved in such a volume of 0.2 M acetate buffer of pH 4.6 (I 0.2) as gives a 2% solution. To the yellow, almost clear solution was added 99% ethanol drop by drop. Turbidity began to occur at the ethanol concentration of 35% (v/v) and turned flocculent at that of 60%. The adhering precipitate was centrifuged off after standing for 1 hour. It was reprecipitated from 10 cc. of the same buffer in a similar manner, washed with ethanol and ether and dried. A white powder given—**Ppt**_{buffer-35~60}—weighed 440 mg. The supernatant fluids containing 60% ethanol were combined, and after further addition of ethanol to 90%, stood for 2 hours. The centrifugate, which was not adhering at all, was washed and dried as above. 440 mg. was the white powder given here—**Ppt**_{buffer-60~90}. The centrifuged supernatant was distilled in vacuo to a syrup, dehydrated with excess ethanol, washed and dried as above. Yielded was 10 mg. of a substance—**Super-n**_{buffer-90}.

The fractional precipitations above were processed at 0°C. Operations at 15°C entail the same result.

Fourth stage. a) 435 mg. of **Ppt**_{buffer-35~60} was taken up in 50 volumes of 1% Ba(AcO)₂. To the faintly opalescent solution cooled on ice was added ethanol in drops. Turbidity began to increase at the ethanol concentration of 10% and changed flocculent at that of 30%. Ethanol was added additionally to 35%, the mixture was stood for 1 hour in ice-water, and the non-sticky precipitate was centrifuged off and washed with three 6 cc. portions of a solution containing as much Ba(AcO)₂ and ethanol as the mother fluid did—**Ppt**_{Ba(AcO)₂-0~35}. The centrifuged supernatant and washing were combined, and the ethanol concentration was raised to 60% similar to above, whereby a sticky precipitate occurred. After standing for 2 hours under continuous cooling, the deposit was centrifuged off,

washed with ethanol and dried. It was next dissolved in 30 cc. of water, and the clear solution of pH 7.0 was agitated with 10 g. of Amberlite IR 120 (H-form) and the same weight of Amberlite IRA 410 (OH-form) in turn. The treatment with the resins was repeated four times, until the solution showed a constant pH value of 5.2. The resins were washed with changes of water, and the solution and washings were distilled together in vacuo to a syrup, precipitated with a mixture of 80 cc. of ethanol and 0.5 cc. of ethanol saturated with anhydrous sodium acetate, washed with ethanol and ether and dried. 243 mg. (0.15% of the dry starting tissue material) of a white powder—**MPS (Mucopolysaccharide) I**—was given. It proved homogeneous electrophoretically as well as ultracentrifugally. Molisch positive, biuret slightly positive; N 4.7%, hexosamine 30.6%, ash 1.6%, $[\alpha]_D^{25}(\text{water}) = -37.5^\circ$; group A- and O-active, toxohormone active. The centrifuged supernatant containing barium acetate and 60% ethanol was distilled in vacuo, and the thick condensate was diluted with water, treated with the resins and so on as above with yield of only 14 mg.

—**Supern**_{Ba(AcO)₂-60}.

b) Since **Ppt**_{buffer-60~90} above was found to be almost devoid of group potency, it was united with the corresponding fractions of the cancer materials from group A and O persons. 820 mg. of the mixed substances was taken up in 25 cc. of 1% Ba(AcO)₂, the insoluble part (176 mg.)—**Ins'**_{Ba(AcO)₂}—was centrifuged off, washed with 10 cc. and 5 cc. portions of 1% Ba(AcO)₂, and the supernatant and washings were cooled and fractionally precipitated with ethanol as above. The precipitate at the ethanol concentration range from 0 to 40%, that at the ethanol concentration range from 40 to 60% and that at the ethanol concentration range from 60–90% were separated, washed with ethanol and ether and dried. The subdivisions thus obtained will be referred to as **Ppt'**_{Ba(AcO)₂-0~40}, **Ppt'**_{Ba(AcO)₂-40~60} and **Ppt'**_{Ba(AcO)₂-60~90} respectively. The mother fluid of the last precipitate was freed from barium acetate, concentrated, precipitated and dried also as above—**Supern'**_{Ba(AcO)₂-90}—with a yield of 70 mg. **Ppt'**_{Ba(AcO)₂-60~90} was liberated from barium acetate by the aid of Amberlite IR 120 and IRA 410 in the manner also in a), and 185 mg. (0.06% of the starting dry tissue material) of a white powder was recovered—**MPS II**. It gave a single boundary after electrophoresis in an acid and an alkaline buffer. Molisch positive, biuret slightly positive; N 5.3%, hexosamine 27.0%, ash 2.1%, $[\alpha]_D^{25}(\text{water}) = -19.5^\circ$.

Fifth stage. 2.5 g. of **Ppt**_{(NH₄)₂SO₄} in the second stage was taken up in 100 cc. of 1.9% Ba(AcO)₂, the insoluble part (70 mg.)—**Ins''**_{Ba(AcO)₂}—was centrifuged off and washed with the barium acetate solution, and the supernatant and washings were combined (125 cc. altogether, pH 7.0, yellowish-brown opalescent), chilled on ice and fractionally precipitated

with ethanol, separating the precipitates at the ethanol concentration ranges of 0–20% (v/v), of 20–40% and of 40–60%. Those precipitates were washed with ethanol and acetone and dried—**Ppt''**_{Ba(AcO)₂-0~20}, **Ppt''**_{Ba(AcO)₂-20~40} and **Ppt''**_{Ba(AcO)₂-40~60}. The respective yields were 107, 1,752 and 505 mg.

The mother fluid of **Ppt''**_{Ba(AcO)₂-40~60} was distilled in vacuo to about 50 cc., passed through 13 × 2 cm. columns of Amberlite IR 120 in H-form and of Amberlite IR 45 in OH-form in turn at a flow rate of 5 cc. per minute. The columns were followingly washed with 500 cc. of water. The effluent and washing united (pH 6.8) were distilled in vacuo to a syrup, precipitated with ethanol containing some anhydrous sodium acetate, washed with ethanol and dried—**Supern''**_{Ba(AcO)₂-60}. It was a yellowish powder. Yield 305 mg.

Sixth stage. a), i) 1.55 g. of **Ppt''**_{Ba(AcO)₂-20~40} (Fifth stage) was kneaded with a small quantity of water, water was supplemented up to 40 cc., and 10% NaOH was added in drops to the suspension under stirring. The yellowish brown turbid solution of pH 8.0 was neutralized with 3% H₂SO₄ to pH 7.0 and made up to 50 cc. with water, and 25 cc. of 15% Na₂SO₄ was added. This mixture was stood at 20°C for 12 hours, and the small precipitate (BaSO₄) was rejected after centrifugation. The total Na₂SO₄ concentration in the solution was increased to 20% by addition of a calculated amount of anhydrous sodium sulfate to the solution at 30°C, which was continuously maintained at the same temperature. The deposit was centrifuged off, dissolved in 30 cc. of water, and after 3 days' dialysis against running water, concentrated to a very thick solution. The condensate was precipitated with ethanol, washed with ethanol and acetone and dried as above—**Ppt**_{5-20% Na₂SO₄}. It was found perfectly insoluble. The centrifuged supernatant containing 20% Na₂SO₄ was diluted to 150 cc. and warmed to 33°C, and the Na₂SO₄ concentration was raised to 30% with anhydrous sodium sulfate, whereby a flocculent precipitate occurred. The mixture was stood for 1 hour at the same temperature and centrifuged. The centrifugate was reprecipitated four times from 100 cc. of water at 33°C by addition of Na₂SO₄ to 30%. Finally it was taken up in 150 cc. of water, dialyzed and passed through 2 × 23 cm. columns of Amberlite IR 120 and of Amberlite IR 45. The effluent and washing were concentrated, precipitated with acetone with subsequent washing with acetone and dried. The yellowish brown powder given amounted to 0.78 g.—**Ppt**_{20-30% Na₂SO₄}. The mother fluids containing 30% Na₂SO₄ were diluted with 3 volumes of water, dialyzed for 2 days, condensed to 1/10 the volume and again dialyzed for the same duration of time. The solution was then distilled to a syrup, precipitated with ethanol containing some anhydrous sodium acetate, washed with ethanol and acetone and dried.

445 mg. (0.4% of the starting dry tissue material) of a yellowish powder was given—**Supern_{30%} Na₂SO₄**.

ii) 0.75 g. of Ppt_{20-30%Na₂SO₄} was taken up in 50 cc. of water. The yellowish turbid solution of pH 4.8 was centrifuged (59,400×g; 30 min.) in a preparative ultracentrifuge (Type 40P, Hitachi). The centrifugate was washed with 20 cc. of water twice (**Ins_{H₂O}**), and the supernatant (yellowish, almost transparent) and washings were freeze-dried. 300 mg. (0.25% of the starting dry tissue material) of a fluffy powder was given—**MPS III**. Only one component was demonstrable after electrophoresis in an acid and an alkaline buffer. Molisch positive, biuret positive; N 7.3%, hexosamine 21.5%, ash 1.6%, $[\alpha]_D^{25}(0.01\ N\ NaOH) = -18.6^\circ$; toxohormone active.

b), i) 500 mg. of Ppt''_{Ba(AcO)₂-40~60} (Fifth stage) was dissolved in 60 cc. of water, the yellowish, translucent solution of pH 6.8 was acidified with 5% HCl to pH 2.4 and the tiny precipitate (40 mg.) was centrifuged off. The clear supernatant was neutralized to pH 7.0 with 10% NaOH and freed from inorganic salts by the aid of 1×25 cm. columns of Amberlite IR 120 and of Amberlite IR 45. The effluent and washing united (pH 6.0) were condensed, precipitated with ethanol containing some anhydrous sodium acetate, washed with ethanol and acetone and dried. The powder given which weighed 380 mg. was dissolved in 15 cc. of 90% (v/v) phenol, and ethanol was added to 10%. The flocculent precipitate occurring was centrifuged off after 1 hour, washed with ethanol and acetone. 166 mg. of a white powder was given—**Ppt_{phenol-0~10}**. The centrifuged supernatant was fractionated by increasing ethanol to 20% at first, and after separating the deposit, to 60%. The successive precipitates here (**Ppt_{phenol-10~20}** and **Ppt_{phenol-20~60}**) were washed with ethanol and acetone and dried. Their yields were 76 and 117 mg. respectively. The mother fluid of Ppt_{phenol-20~60} gave almost no precipitate on addition of excess ethanol and ether.

ii) 160 mg. of Ppt_{phenol-0~10} in i) was dissolved in 8.0 cc. of 0.2 M acetate buffer of pH 4.6 (I 0.2) and chilled on ice. The solution was fractionated with ethanol. The precipitate at the ethanol concentration range of 45 to 60% was centrifuged off, washed with ethanol and ether and dried. The amount of the white powder given was 125 mg., that is, 0.09% of the starting dry tissue material—**MPS IV**. It was homogeneous electrophoretically and ultracentrifugally. Molisch positive, biuret slightly positive; N 4.8%, hexosamine 30.8%, ash 1.5%, $[\alpha]_D^{25}(\text{water}) = -18.8^\circ$; group A- and O-active, toxohormone active. No precipitate occurred at the ethanol concentration range of 0–40%. The precipitate at 40–45% ethanol amounted no more than 8 mg. The precipitate at 60–90% ethanol containing a comparatively large amount of MPS IV was 23 mg.,

and only a trace of substance remained in the mother fluid.

Sometimes the fractionation in *b*, *ii*) may be omitted.

Remarks

Of the fractions left without further fractionation in the above, $\text{Ppt}_{\text{Ba}(\text{AcO})_2-0\sim 35}$, $\text{Supern}_{\text{Ba}(\text{AcO})_2-60}$, $\text{Ins}'_{\text{Ba}(\text{AcO})_2}$, $\text{Ppt}'_{\text{Ba}(\text{AcO})_2-0\sim 40}$, $\text{Ins}''_{\text{Ba}(\text{AcO})_2}$, $\text{Ppt}''_{\text{Ba}(\text{AcO})_2-0\sim 20}$, $\text{Ppt}_{5-20\% \text{Na}_2\text{SO}_4}$ and $\text{Ins}_{\text{H}_2\text{O}}$ were discarded, because the first six gave markedly positive Goldschmiedt, Neuberg-Saneyoshi, biuret and phosphorus test besides the positive Molisch so that they looked to be mixtures of hexuronic acid-containing mucopolysaccharides, nucleic acids and proteins, and the last two were insoluble in water. $\text{Supern}_{\text{buffer}-90}$, $\text{Ppt}'_{\text{Ba}(\text{AcO})_2-40\sim 60}$, $\text{Supern}'_{\text{Ba}(\text{AcO})_2-90}$, $\text{Supern}''_{\text{Ba}(\text{AcO})_2-60}$, $\text{Supern}_{30\% \text{Na}_2\text{SO}_4}$, $\text{Ppt}_{\text{phenol}-10\sim 20}$ and $\text{Ppt}_{\text{phenol}-20\sim 60}$ were too small in yield and hence their fractionation was postponed until sufficient quantities of them are obtained from later materials. Fractionation of Dialysate 1 and 2 and $\text{Ppt}_{\text{CH}_3\text{COOH}}$ will be dealt with in a forthcoming paper, since they proved to be scanty of mucopolysaccharides or mucoproteins.

MPS III of non-cancerous gastric mucosa somewhat differs from that of cancer tissue in precipitability and is not isolable in the manner described above. The procedure of preparing it will be reported in another article. Furthermore, the cancerous group B and O tissue materials and non-cancerous group A and O materials have been subjected until now to the 1st–4th stages only of the fractionation. Fractions $\text{Ppt}_{(\text{NH}_4)_2\text{SO}_4}$ given of them were too small in quantity and are set aside to combine with the corresponding fractions after other runs with new batches of tissue materials.

The yields of the intermediate fractions and final products hitherto separated are embodied in Table II.

SUMMARY

1. A procedure of fractioning gastric cancer and non-cancerous gastric mucosa is described.

2. Two kinds of group A mucopolysaccharide and two kinds of group inactive mucopolysaccharide have been isolated in a pure state from a cancer material (group A), and one kind each of the corresponding group active mucopolysaccharides and group inactive mucopolysaccharides from a non-cancerous group B mucosa material.

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TABLE II

Yields of Intermediate Fractions and Final Products in g.

The figures in parentheses express fractions in per cent of the respective starting dry tissue materials.

Stage	Fraction	Stomach cancer			Non-canc. gastric mucosa		
		A	B	O	A	B	O
First	Starting dry tissue material	180	75	125	280	515	375
	Dialysate 1	8.3	2.7	2.9	10.1	10.4	8.9
	PptCH ₃ COOH	6.09	1.0	4.2	3.8	9.9	5.4
	SupernCH ₃ COOH	9.80 (5.4)	2.31 (3.1)	4.75 (3.9)	7.66 (2.7)	10.16 (2.0)	7.50 (2.0)
Second	Starting SupernCH ₃ COOH	9.00	2.10	4.50	7.00	9.00	7.00
	Dialysate 2	3.50	0.27	1.33	2.20	3.45	2.40
	Ppt(NH ₄) ₂ SO ₄	2.94 (1.8)	0.93 (1.4)	1.34 (1.1)	2.05 (0.8)	2.55 (0.6)	1.06 (0.3)
	Supern(NH ₄) ₂ SO ₄	0.93 (0.6)	0.33 (0.5)	0.59 (0.5)	0.76 (0.3)	1.49 (0.3)	0.85 (0.3)
Third	Starting Supern(NH ₄) ₂ SO ₄	0.93	0.33	0.59	0.75	1.46	0.85
	Pptbuffer-35~60	0.440 (0.3)	0.170 (0.3)	0.253 (0.2)	0.405 (0.2)	0.940 (0.2)	0.425 (0.1)
	Pptbuffer-60~90	0.440 (0.3)	0.133 (0.2)	0.288 (0.2)	0.225 (0.1)	0.325 (0.1)	0.364 (0.1)
	Supernbuffer-90	0.010	0.008	0.024	0.020	0.047	0.022
Fourth, a)	Starting Pptbuffer-35~60	0.435	0.170	0.245	0.400	0.930	0.425
	PptBa(AcO) ₂ -0~35	0.172	0.060	0.104	0.108	0.120	0.148
	MPS I	0.243 (0.15)	0.097 (0.14)	0.110 (0.10)	0.270 (0.11)	0.700 (0.16)	0.265 (0.08)
	SupernBa(AcO) ₂ -60	0.014	0.01	0.006	0.014	0.048	0.009
Fourth, b)	Starting Pptbuffer-60~90	0.415	0.125	0.280	0.220	0.320	0.350
	Ins'Ba(AcO) ₂	0.820			0.890		
	Ppt'Ba(AcO) ₂ -0~40	0.176			0.95		
	Ppt'Ba(AcO) ₂ -40~60	0.057			0.105		
	Supern'Ba(AcO) ₂ -90	0.285			0.146		
		0.070			0.050		
	MPS II	0.185 (0.06)			0.347 (0.03)		
Fifth	Starting Ppt(NH ₄) ₂ SO ₄	2.50			2.50		
	Ins''Ba(AcO) ₂	0.070			0.300		
	Ppt''Ba(AcO) ₂ -0~20	0.107			0.077		
	Ppt''Ba(AcO) ₂ -20~40	1.752 (1.2)			1.280 (0.3)		
	Ppt''Ba(AcO) ₂ -40~60	0.505 (0.4)			0.640 (0.1)		
	Supern''Ba(AcO) ₂ -60	0.305 (0.2)			0.332 (0.1)		

TABLE II—*Concluded*

Stage	Fraction	Stomach cancer			Non-canc. gastric mucosa		
		A	B	O	A	B	O
Sixth, a, i)	Starting Ppt''Ba(AcO) ₂ -20~40	1.550				1.200	
	Ppt5-20% Na ₂ SO ₄	0.215				0.200	
	Ppt20-30% Na ₂ SO ₄	0.780 (0.6)				0.408 (0.1)	
	Supern30% Na ₂ SO ₄	0.445 (0.4)				0.452 (0.1)	
Sixth, a, ii)	Starting Ppt20-30% Na ₂ SO ₄	0.750				0.400	
	InsH ₂ O	0.420				0.203	
	MPS III	0.300 (0.25)				0.190 (crude) (0.05)	
Sixth, b, i)	Starting Ppt''Ba(AcO) ₂ -40~60	0.500				0.620	
	Pptphenol-0~10	0.166 (0.12)				0.193 (MPS IV) (0.04)	
	Pptphenol-10~20	0.076				0.078	
	Pptphenol-20~60	0.117				0.120	
Sixth, b, ii)	Starting Pptphenol-0~10	0.160					
	MPS IV	0.125 (0.09)					

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