Biochemical Studies on Carbohydrates

CXCI. Esteric and Ethereal Links in the Molecule of Group Mucopolysaccharide from Pig Stomach Mucus

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(Received for publication, April 7, 1956)

In a preceding article,¹⁾ we pointed out the presence of N-glycosidic links between the carbohydrate and polypeptide moieties in the group mucopolysaccharide from pig stomach mucus. Regarding the union between the two moieties, a further study has been made.

At first, it was tried to prepare a substance containing amino acids as few as possible without considerable degradation of the carbohydrate moiety by successive boiling of the group mucopolysaccharide (MP I) with Dowex 1 and 50.²⁾ The substance given (MP II) was crystalline quite unexpectedly. It was devoid of the acetal links mentioned in the foregoing article,¹⁾ and contained serine and alanine alone as amino acids and only 3, 2, 5 and 1 molecules of glucosamine, galactosamine, galactose and L-fucose respectively as sugars for one unit molecule, which corresponds to the minimum molecular weight. Practically all the carboxyl groups in MP II were in bound state, but one carboxyl group became free per unit molecule on treatment of the substance with a mixture of equal volumes of water and 0.4 *M* sodium ethylate at room temperature, with simultaneous occurrence of a tetrasaccharide, which is designated "gastro-N-tetrasaccharide," and two new undialyzable polypeptide-polysaccharide complexes. The carboxyl which was unbound proved to belong to alanine by the old hydrazinolysis procedure of Akabori.³⁾ The tetrasaccharide was found to be composed of 1 molecule each of glucosamine and L-fucose and two molecules of galactose, of which one stands at the reducing terminal of the chain. One of the polypeptide-polysaccharide complexes contained 12 molecules of hexosamine and 9 molecules of galactose for a unit molecule corresponding to the minimum molecular weight of 4400 calculated from the iodine use and looked to be the unit fragment of MP II deprived of gastro-N-tetrasaccharide. The other polypeptide-polysaccharide complex was probably a condensation product of gastro-N-tetrasaccharide,

the polypeptide-polysaccharide complex above and resembling fission products.

On the other hand, when hydrolyzed with a strong acid and paper-chromatographed, MP II often gave a spot reacting with ninhydrin as well as with Partridge sugar reagent of an R_f value lower than that of the spot or spots of hexosamines. That spot might be ascribed to a compound stable to strong acids and composed of an amino acid or amino acids and a sugar or sugars. Therefore, trials were made to isolate it. In the successful attempt, a H_2SO_4 -hydrolysate was fractionated by means of ion exchangers and a chromatopack with halfway acetylation, and crystals in plates were obtained. It has been established to be an acetylglucosamine-galactoside, whose galactose residue is bound etherally with serine.

The findings above led us to conclude that 1: There must be at least two kinds of carbohydrate chain short ones and a very long one in MP II; 2: The main chain is linked with polypeptides by ether linkage and the polypeptides are connected each with a gastro-N-tetrasaccharide molecule by ester linkage in turn, as shown in Fig. 6; and 3: The galactose molecule standing at the reducing terminal of gastro-N-tetrasaccharide is combined through its C atom 1 with two N-acetylglucosamine molecules, one of which is linked to aspartic acid N-glycosidically, in MP I, as will be represented in Fig. 7.

EXPERIMENTAL

Preparation and properties of MP II

2 g. of MP I were taken up in 50 cc. of water. The solution was mixed with 6 g. of Dowex 1 (OH-form, 250–500 mesh) in a two-mouthed flask equipped with a stirrer and a return condensor (ground joint), and heated in an oil-bath at 110–120°C for 6 hours. The mixture was cooled in iced water, further stirred for 30 minutes and filtered through Toyo Roshi filter paper No. 5C. The filtrate was mixed with 6 g. of Dowex 50 (H-form) of 250–500 mesh, heated under agitation for 2 hours and after further agitation at 0°C for 1 hour, filtered. The filtrate here was decolorized with active charcoal⁴⁾ and dialyzed for 3 days against six 200 cc. portions of distilled water, then the inner fluid was distilled in vacuo to a very thick solution and added to with abs. ethanol. The syrup changed into fine, tetragonal platelets which exhibited birefringence in polarized light with refractive index of 1.499.⁵⁾ Fig. 1 shows their photograph, which was kindly taken by Dr. H. Haseyama in the Pathological Department.

The crystals were centrifuged, washed with ether and placed in a desiccator containing $CaCl_2$ (ordinary pressure)—MP II. Yield 0.4 g. This product was very soluble in water and pretty soluble in 70% ethanol. The indirect Osaki-Turumi test of it resulted in positive but the direct in negative. It contained approximately 3, 2, 5, 1, 1 and 1 molecules of glucosamine, galacto-

Fig. 1. Microphotograph (polarized light) of crystals of MP II.

TABLE I
Analyses of MP II

Analysis	In per cent	In equivalents per equivalent weight
Nitrogen*	5.1	
Glucosamine†	25.2	3.1
Galactosamine†	14.3	1.8
Total hexosamines	39.5	4.9
Galactose‡	39.1	4.8
L-Fucose§	5.6	0.8
Serine//	4.0	0.8
Alanine//	3.7	0.9
Ash¶	1.5	
Iodine use as glucose**	8.0	

* Micro Kjeldahl. † Masamune and Yosizawa.¹⁶⁾ ‡ Masamune and Sakamoto.¹⁷⁾ § Masamune and Sakamoto.¹⁸⁾ // Masamune and Hakomori.¹⁹⁾ ¶ Pregl method without use of H₂SO₄. ** Macleod and Robison²⁰⁾; The minimum molecular weight is calculated therefrom at 2250.

samine, galactose, L-fucose, serine and alanine respectively for a unit structure corresponding to the minimum molecular weight of 2250 calculated from the iodine use (Macleod and Robison) and was devoid of sialic acid (Table I). The group activities are shown in Table II.

A batch of MP II was prepared by repetition of the procedure above with new lots of MP I.

Absence of the acetal link in MP II

50 mg. of MP II were dissolved in 5 cc. of a 2% mercuric chloride solution, heated for 30 minutes at 60°C and dialyzed against several changes of distilled water. The dialysate was freed from mercuric chloride and concentrated in the manner described in the foregoing article¹⁾, and the syrupy residue was

TABLE II
Group Activities of MP II

The group potencies were measured on a glass plate as described by Masamune *et al.*²¹⁾ Anti-OS: an anti-OS saliva chicken serum; G: a normal anti-O goat serum. — no hemagglutination, $\pm$ faint hemaggl., + slight hemaggl., ++ marked hemaggl.

Antiserum	Red cells	Dilution of the substance (1:)							
		5×10^2	10^3	2×10^3	4×10^3	8×10^3	1.6×10^4	3.2×10^4	6.4×10^4
α	A	—	—	—	—	—	—	+	++
β	B	+	+	+	+	+	+	++	++
G	O	—	—	+	+	+	++	++	++
Anti-OS	O	—	—	$\pm$	+	+	++	++	++

paper-chromatographed. However, neither hexosamine nor acetylhexosamine nor galactose was detected. Furthermore, a 1 *N* HCl-hydrolysate of the residue gave no Elson-Morgan reaction.

Alkaline hydrolysis of MP II

1) Presence of ester links in MP II. 160 mg. of MP II were dissolved in 10 cc. of water and four 2.0 cc. portions of the solution were poured separately into 50 cc.-containing Erlenmeyer flasks with a glass stopper (ground joint). Into Flasks 1 and 2 were additionally pipetted 2.0 cc. of 0.4 *M* sodium ethylate in abs. ethanol and into Flasks 3 and 4 were additionally pipetted 2.0 cc. of CO₂-free water. The flasks were stoppered tightly by greasing and kept at room temperature (20–22°C) for 12 hours. Thereupon, 1.93 cc. of 0.2 *N* HCl, which neutralize 2.0 cc. of the 0.4 *M* sodium ethylate, were added to each of the contents in the former flasks and an equal volume of CO₂-free water to each of the contents of the latter flasks, and the acidities of the mixtures were measured by simple and formol titrations with 0.01 *N* NaOH. Indicator phenolphthalein.

Volumes of 0.01 *N* NaOH used in simple titration:

Flask 1 1.75 cc. (faintly red), 1.82 cc. (distinctly red)

Flask 3 0.22 cc. (faintly red), 0.25 cc. (distinctly red)

Diff.: 1.53 cc. 1.47 cc.

Volumes of 0.01 *N* NaOH used in formol titration:

Flask 2 1.78 cc. (faintly red), 1.92 cc. (distinctly red)

Flask 4 0.30 cc. (faintly red), 0.38 cc. (distinctly red)

Diff.: 1.48 cc. 1.54 cc.

There was no difference of alkali expenditure between formol and simple titrations. Hence the acidic group which was set free by the alkaline treatment is involved in ester linkage but not in peptide linkage in MP II. The volume of alkali required for neutralization of it corresponds approximately to one carboxyl group per unit molecule of MP II.

2) Fractionation of the hydrolysis products. 600 mg. of MP II were taken up in 30 cc. of water and after addition of an equal volume of 0.4 *M* sodium ethylate in abs. ethanol, stood at room temperature (20–22°C) for 12 hours. Then abs. ethanol was supplemented to 90% (v/v). The precipitate (Ppt. A) that occurred thereby was centrifuged, washed with 90% ethanol and set aside, and the supernatant was neutralized with 1 *N* alcoholic sulfuric acid and filtered. The filtrate and washing were distilled together in vacuo. The still-residue was dissolved in about 10 cc. of water, shaken with a mixture of one spoonful each of Amberlite IR 120 and IR 4B for 2 hours and filtered. The filtrate here was distilled in vacuo to dryness and dissolved in a few cc. of 90% methanol. A trace of the insoluble part was rejected after centrifugation, and the supernatant was precipitated with a large excess of dry ether, centrifuged and dried in a vacuum desiccator containing CaCl₂. Yield 125 mg.

It was paper-chromatographed by irrigation with a 5:3:2 mixture by volume of butanol, pyridine and water (Irrigation ascending; solvent front run 40 cm.; temperature of test 24°C) and found to be made up of at least three ingredients, giving three spots of R_f values of 0.7, 0.35 and 0.15. Therefore, the substance was fractionated by paper-chromatography on a preparative scale with the same mixed solvent, namely, 60 mg. of it were dissolved in 1 cc. of water and 0.1 cc. portions were sampled on 40×40 cm. paper squares along a horizontal line 2 cm. apart from one edge, followed by irrigation similar to above. Each band was eluted with water by capillary siphoning and the eluates (2 cc. each) of the corresponding bands were evaporated together in a small dish in vacuo over CaCl₂. Recovered were 35, 5 and 4.5 mg. of substances from the lower, middle and upper bands respectively. They will be referred to as Fr. I, II and III each to each. The results of assays will be tabulated in the following.

Assay	Fraction		
	I	II	III
Osaki-Turumi			
Indirect	—*	—	+
Direct	—	—	++†
Paper-chromatography after hydrolysis with 0.5 <i>N</i> HCl			
Glucosamine	+	+	+
Galactosamine	—	—	—
Galactose	+	+	?
L-Fucose	+	—	?
Unidentified spot‡	—	+	++
Serine	—	—	—
Alanine	—	—	—

* A yellow-red spot appeared. † An intensive color with absorption maxima at 540 and 570 m μ occurred promptly on heating.

‡ Indicator aniline hydrogen phthalate.

Fractions II and III were too small for further examinations. Fr. I analyzed hexosamine as glucosamine 25.8%, galactose 50.5%, L-fucose 22.0% and iodine

use as glucose 23.5%. The molecular weight is calculated at 766 from the iodine use. This tetrasaccharide is named "gastro-N-tetrasaccharide."

Ppt. A above was dissolved in 5 cc. of water, and after dialysis in a parchment paper bag against running water for 2 days, distilled in vacuo to dryness, followed by placing in a vacuum desiccator containing CaCl_2 . It weighed 360 mg. The substance was next extracted with 80, 70 and 60% ethanol in succession. The yields of fractions given (Fr. IV, V and VI) were 10.9, 229 and 99 mg. respectively. A few milligrams each of the fractions were subjected to paper electrophoresis in a 0.1 M KH_2PO_4 -0.05 M borax mixture of pH 8.6 (Kolthoff) by 0.9-1.2 mA current per cm. width at 500 V (Distance between electrode vessels 30 cm.) for 8 hours according to Kunkel and Tiselius.⁶⁾ The fractions IV, V and VI gave all a single, diffuse zone but at different distances 3, 3.5 and 2.0 cm. towards the anode from the start respectively. Based thereupon, the balances of Fr. V and VI were purified in the electrophoretic sedimentation apparatus of Masamune *et al.*⁷⁾ The solvent was the same mixture as above, current 0.8 mA (constant), temperature 20°C and duration of test 10 hours. In the case of Fr. V the fluid above cock *b* in tube *a* was drawn out and in the case of Fr. VI the fluid between diaphragm *e* and the upper aperture of the hole in cock *b* was drawn out. The fluids were each dialyzed in a parchment paper bag against running water, distilled in vacuo to a syrup, and after precipitating with abs. ethanol, dried in vacuo over H_2SO_4 . The yields were 90 and 42 mg. respectively. These substances will be referred to as Fr. V' and VI'. Fr. IV was so small that its purification was given up. The results of qualitative tests and quantitative analyses of Fr. V' and VI' are shown in Table III (a, b).

Fr. V' did not give the indirect-Osaki-Turumi reaction, and contained hexosamine and galactose in the molecular proportion of 4:3 but no L-fucose. Its minimum molecular weight calculated from the iodine use was twice as large as that of MP II. Judging from these facts, this fraction looks to have been a fragment whose multiple makes up MP II deprived of gastro-N-tetrasaccharide. Fr. VI' might be a condensation product of gastro-N-tetrasaccharide, Fr. V' and other fission products.

Reducing terminal of gastro-N-tetrasaccharide

20 mg. of the tetrasaccharide were dissolved in 3 cc. of water, and after addition of 3 cc. of 0.02 N iodine solution and 0.2 cc. of 5% Na_2CO_3 , kept at 20°C for 30 minutes. The reaction mixture was passed through an Amberlite IR 120 (H-form) column of 0.8 cm. diameter and 3 cm. height. The acidic effluent was distilled in vacuo at below 5°C. The still-residue was kneaded with portions of dry ether until a washing of pH 2-3 was given, taken up in 0.5 cc. of 0.5 N HCl and heated at 100°C for 2 hours. The hydrolysate was placed in a vacuum desiccator containing CaCl_2 and the evaporated residue was subjected to paper-electrophoresis in 0.05 M borax. Galactonic and glucosaminic acids were used for reference, and indication was effected according to Buchanan *et al.*⁸⁾ after spraying the paper with 1 N acetic acid and according to Partridge. Galactonic acid was detected (Fig. 2), revealing that one of the galactose molecules stands at the reducing terminal.

TABLE III
Qualitative Tests and Analyses of Non-dialyzable, High
Molecular Fractions Obtained after the Alkaline
Treatment of MP II

	Fr. V'	Fr. VI'		
a) Qualitative tests				
Molisch	++	++		
Osaki-Turumi				
Direct	—	—		
Indirect	—	+		
Indirect-Osaki-Turumi after treatment with acetic acid*		++		
Spots given by paper-chromatography				
Glucosamine	+	+		
Galactosamine	+	+		
Galactose	+	+		
L-Fucose	—	+		
Unidentified†	+	—		
Serine	+	+		
Alanine	+	+		
b) Analyses				
	In per cent	Number of molecules per unit molecule	In per cent	Number of molecules per unit molecule
Hexosamine‡	49.0	12.0	47.9	14.9
Galactose§	36.9	9.02	38.5	12.0
L-Fucose	0.0		5.3	1.5
Iodine use as glucose¶	4.09		3.2	

* The substances were heated with 1 *N* acetic acid for 1.5 hours. † Located between the spots of mannose and L-fucose (Indicator aniline hydrogen phthalate). ‡ Masamune and Yosizawa.¹⁶⁾ § Masamune and Sakamoto.¹⁷⁾ || Masamune and Sakamoto.¹⁸⁾ ¶ Macleod and Robison²⁰⁾; The minimum molecular weights of Fr. V' and VI' calculated therefrom amount to 4400 and 5600 respectively.

Acid-stability of the link binding the L-fucose molecule in gastro-N-tetrasaccharide

5 mg. of the tetrasaccharide were taken up in 0.5 cc. of 1 *N* CH₃COOH and heated in a small sealed tube at 100°C for 1.5 hours. The hydrolysate was placed in a vacuum desiccator containing solid caustic soda and anhydrous calcium chloride. The evaporated residue was evaporated again with water to dryness and the residue here was dissolved in 0.05 cc. of water. 0.02 cc. of the solution was paper-chromatographed, employing a mixture of butanol, pyridine and water in the proportions of 5:3:2 by volume as the irrigation

Fig. 2. Paper electrophoresis pattern ($1/5\times$) of the hydrolysate of the hypiodite-oxidation product of gastro-N-tetrasaccharide in 0.05 *M* borax; current 0.9–1.3 mA per cm. width at 500 V (The distance between the electrode vessels 35 cm.); duration of test 4 hrs. Indicated by Buchanan and others' method⁽⁸⁾.

a, a': reference run. *b*: the hydrolysate. 1: galactonic acid, 2: glucosaminic acid, 3: galactose.

(The chromatogram sprayed with Partridge reagent is omitted.)

solvent. A spot of galactose and a spot of a lower *R_f* value (probable trisaccharide) were given but no spot at the position of L-fucose. Namely, the L-fucose residue in gastro-N-tetrasaccharide is the one acid-stably linked but not the one acid-unstably linked of the L-fucose residues in MP I.⁽¹⁾

Hydrazinolysis of MP II and its undialyzable products by alkaline hydrolysis

Hydrazinolysis was processed on the principle of the old method of Akabori.⁽³⁾ 30 mg. of MP II, perfectly dried in an Abderhalden apparatus at 56°C over P_2O_5 , were dissolved in 1 cc. of freshly prepared anhydrous hydrazine and heated in an oil-bath at 120° for 8 hours under reflux. When cold, the hydrazinolysate was freed from excess hydrazine by repeated vacuum distillations with water until it smelled of hydrazine no more. The final still-residue was taken up in a few cc. of water, and after addition of 0.1 cc. of benzaldehyde drop by drop, vigorously shaken under cooling in iced water for 30 minutes. The mixture was adjusted to neutral with 0.1 *N* NaOH and shaken further on for 1 hour. The voluminous precipitate was centrifuged off and the supernatant was evaporated in vacuo over $CaCl_2$. The dry residue was dissolved in 0.1 cc. of water and 0.02 cc. portions of the solution were sampled on paper slivers for chromatography.

On the other hand, 60 mg. of MP II were hydrolyzed in a mixture of 8 cc. each of water and 0.4 *N* ethanolic sodium ethylate as above. Then the reaction mixture was dialyzed against several changes of water, and after distillation in vacuo to dryness, kneaded with 95% ethanol, followed by centrifugation. The centrifugate was dried in a vacuum desiccator over $CaCl_2$ at first and afterwards in an Abderhalden apparatus over P_2O_5 at 56°C. 43 mg. of a powder were given—MP II'. 30 mg. of MP II' were hydrazinolysed similar to above and sampled on the same slivers.

As shown in Fig. 3, the hydrazinolysate of MP II' gave a strong spot of alanine in contrast to that of MP II which gave only a faint spot of this amino acid. The alkaline hydrolysis under the above conditions does not split the peptide link as has been mentioned in the section "Presence of ester links in MP II", and moreover serine-OH participates in ether linkage but not in ester linkage as will be explained below. Therefore, the alanine-carboxyl must be

esterified with an OH-group of a sugar belonging to the molecule of gastro-N-tetrasaccharide, which is liberated from MP II by the alkaline treatment.

Fig. 3. Paper partition chromatograms (6/25 $\times$) of the hydrazinolysates of MP II and MP II', developed with 80% phenol (A dish containing 3% ammonia was placed in the cylinder) (A) and those (6/25 $\times$) developed with a mixture of butanol, acetic acid and water in the proportions of 4:1:2 by volume (B). Irrigation descending. Duration and temperature of test 24 hrs. and 25°C respectively. Indicator 0.2% ninhydrin in butanol. Slivers Toyo Roshi filter paper No. 2.

a : reference run. *b* : the hydrazinolysate of MP II'. *c* : the hydrazinolysate of MP II. 1 : glutamic acid, 2 : serine, 3 : threonine, 4 : alanine.

(In either of A and B, the spot of alanine in *b* was very strong contrary to the corresponding spot in *c*, which was scarcely recognizable. The very elongated diffuse spots in *b* as well as *c* of A and B are probably due to the hydrazides).

Preparation of N-acetylglucosamine-galactoside serine ether⁹⁾

3 g. of MP II were dissolved in 300 cc. of 1 *N* H₂SO₄ and hydrolyzed in a boiling water-bath for 6 hours. The solution became considerably dark colored. When cold, the hydrolysate was neutralized with the saturated baryta to pH 6.5. The humin precipitated together with BaSO₄. The centrifuged supernatant, which was almost colorless, was distilled in vacuo to about 100 cc. and passed through a 3.5 $\times$ 20 cm. column of Dowex 50 (H-form, 250–500 mesh). The column was followingly washed with about 500 cc. of distilled water until no more of Molisch- and ninhydrin-positive substances came out. Next, about 500 cc. of 0.5 *N* ammoniacal water were poured onto the resin gradually. The early portion of the effluent contained no ammonia and was discarded. The remaining portion, which smelled of ammonia and was alka-

line, was bubbled with carbon dioxide to nearly neutral and distilled in vacuo continuously as long as ammonium carbonate was expelled off from the solid still-residue. The substance left behind was exhausted with changes of abs. methanol. The solutions here were distilled together to dryness, dissolved in a mixture of 100 cc. of water and 30 cc. of methanol and transferred to a beaker, and 30 cc. of Dowex 1 (carbonate form) were added. The mixture was placed in ice-water, added to with 1.5 cc. of acetic anhydride under vigorous agitation with a stirrer, and after further agitation for 90 minutes, filtered. The filtrate was heated to boiling, cooled and distilled at 35–40°C in vacuo to about 50 cc., followed by passing through a 2×20 cm. column of Dowex 50 (H-form). After washing the column with 100 cc. of water, the part absorbed on the resin was eluted by displacement with 0.5 *N* NH₃ in the way described above, and the eluate was distilled under reduced pressure to dryness, dissolved in the smallest possible quantity of methanol and precipitated with dry ether. The deposit was centrifuged off and dried. The powder given amounted to 90 mg. Next, it was fractionated by means of a chromatopack of Porter¹⁰⁾ as follows. 200 mg. of a batch of powders obtained as above were dissolved in 4 cc. of methanol, and eighty 0.05 cc. portions were applied separately on squares of 7.5×30 cm. size of Toyo Roshi filter paper No. 2 along a horizontal line 2 cm. apart from the shorter edge. The paper squares were tightly packed together, fixed between two glass plates by the aid of Suzuki's tool¹¹⁾ and irrigated in an ascending way with solvent butanol-acetic acid-water (4:1:2 by volume). When the solvent front reached the top, the squares were dried by hanging separately in a well-ventilated chamber. They were again irrigated with solvent butanol-pyridine-water (5:3:2 by volume) in the same way. After drying, one of them was cut longitudinally into halves to spray one with ninhydrin and the other with aniline hydrogen phthalate. There appeared two bands, the lower reacting to the both reagents and the upper reacting only to Partridge indicator. The areas corresponding to the lower band were cut out from the squares, sheared into fine pieces, and after washing with ether, kneaded with water with subsequent filtration. The filtrate was distilled in vacuo to about 50 cc., shaken with Amberlite IRC 50 (H-form) for 30 minutes and again filtered. The filtrate here was distilled to perfect dryness and taken up in the smallest possible quantity of abs. methanol, and the insoluble part was rejected. The clear solution was precipitated with dry ether and dried in vacuo. A yellowish amorphous powder given amounted to 45 mg. It was dissolved in 2 cc. of water and treated with active charcoal, and the non-colored centrifuged supernatant was evaporated in vacuo over CaCl₂ to dryness. The residue was dissolved in 0.5 cc. of water, added to carefully with ethanol to persistent turbidity, and after clarifying by slight warming, stood in a refrigerator. A deposit in platelets was centrifuged off, washed with ethanol and placed in a desiccator containing CaCl₂ (ordinary pressure). 28 mg. was the yield. Fig. 4 shows the microphotograph of the crystals in polarized light which was kindly taken by Dr. N. Sasano in the Phthological Department.

Properties of the ether

It decomposed at 145°C with gas evolution. $[\alpha]_D^{25}$ (water) +53.2° (5

Fig. 4. Microphotograph (polarized light) of crystals of N-acetylglucosamine-galactoside serine ether.

minutes after dissolution). Analytical figures are embodied in Table IV. They agree with the calculated values for N-acetylglucosamine-galactoside serine ether containing one molecule of crystal water. The force of reducing iodine and the amount of alkali required for neutralization are also in accord

TABLE IV

Analyses of N-Acetylglucosaminegalactoside Serine Ether

Figures express percentages, excepting the case of formol titration where volumes (cc.) of 0.01 *N* NaOH used are shown.

Analysis	Found	Calcd. for $C_{17}H_{29}O_{13}N_2 \cdot H_2O$
Crystal water*	3.5	3.64
Nitrogen†	5.9	5.7
Galactose‡	36.6	36.9
Glucosamine§	37.9	36.6
Iodine use as glucose//	38.2	36.9
Formol titration		
5.3 mg. substance	1.15	1.09
7.9 mg. "	1.65	1.62

* Measured by heating the substance in vacuo over P_2O_5 at 118°C. † Micro Kjeldahl. ‡ Masamune and Sakamoto.¹⁷⁾ § Masamune and Yosizawa.¹⁶⁾ // Macleod and Robison.²⁰⁾

with theory. The substance gave strong ninhydrin reaction and moderate indirect-Osaki-Turumi reaction. Moreover, chromatography of its hydrolysate with 6 *N* HCl revealed that N-acetylglucosamine constitutes the reducing residue and the galactose residue is bound with serine, because the hydrolysis gave rise to abundant humin formation on one hand and on the other the hydrolysate gave spots of glucosamine and serine but no spot of galactose. The chromatography was carried out as follows. 5 mg. of the substance were hydrolyzed with 0.5 cc. of 6 *N* HCl by heating in a sealed tubelet at 100°C for

24 hours. The hydrolysate freed from the humin was evaporated at 40°C in a vacuum desiccator containing anhydrous calcium chloride and solid caustic soda to remove the hydrochloric acid. The evaporated residue was taken up in 0.5 cc. of water, and after rejecting the insoluble part, evaporated again in the desiccator. The residue here was dissolved in 0.3 cc. of water, and 0.02 cc. portions were sampled on different paper slivers. Each pair of slivers were irrigated with one solvent and one of them was sprayed with ninhydrin and the other with aniline hydrogen phthalate. The chromatograms are reproduced in Fig. 5. The spots of serine are exceedingly elongated. According to Narita,¹²⁾ this amino acid is very liable to decomposition and gives unknown products reacting with ninhydrin, when heated with a strong acid in the presence of sugars.

Fig. 5. Chromatograms reproduced diagrammatically (1/5 $\times$) of N-acetylglucosamine-galactoside serine ether and of its HCl-hydrolysate. Irrigation was effected in the ascending way with 80% collidine (**A**, **B**), 80% phenol (**C**) and butanol-pyridine-water (5:3:2 by volume) (**D**). Temperature of test 24°C. Indicators ninhydrin (**A**, **C**) and the aniline hydrogen phthalate reagent of Partridge (**B**, **D**).

a, *a'*, *a''*: reference run. *b*: the substance unhydrolyzed. *c*: the hydrolysate of the substance. 1: serine, 2: glucosamine, 3: galactose, 4: threonine, 5: alanine, 6: galactosamine, 7: one of the decomposition products of serine (s. the text).

(The chromatogram developed with 80% phenol and indicated with Partridge reagent and that developed with butanol-pyridine-water and indicated with ninhydrin are omitted.)

That the disaccharide is a galactoside but not an N-acetylglucosaminide has been again established by isolation of a galactose-serine ether as a DNP-derivative, which will be described in an article regarding systematic fractionation of a hydrolysate of MP II.⁹⁾

DISCUSSION

Judging from what have been described above, many molecules of gastro-N-tetrasaccharide and a polymer of Fr. V' constitute together the whole MP II. Fr. V' showed a lesser reduction power than MP II itself. Hence MP II must contain two kinds of carbohydrate chain gastro-N-tetrasaccharide present in every unit molecule of it and a very long chain, and the reduction of iodine by MP II is mostly ascribed to the reducing terminal (galactose) of the tetrasaccharide. It has been concluded that this tetrasaccharide is fastened by the alanine residue through an ester link. On the other hand, the acetylglucosamine-galactoside which is bound ethereally with serine is conceived to remain in Fr. V' because of the stableness against alkali of ether linkage. Suzuki¹³⁾ found that the same group mucopolysaccharide prepared like as MP I had molecular weight of 160,000–170,000 and axial ratio of 19/1. MP II is produced from MP I by splitting off of two hexosamine molecules and one molecule each of L-fucose and sialic acid per unit molecule besides aspartic acid and other amino acids preceding serine in the polypeptide chain. Of those sugar components bound in MP I, the L-fucose and sialic acid molecules are easily cleft off by the dilute acetic acid¹¹⁾ and easily decomposed by metaperiodate,¹⁴⁾ suggesting that they form side chains. And those two hexosamine molecules constitute together with gastro-N-tetrasaccharide a short carbohydrate chain which is connected probably at its terminals with the polypeptide chain in MP I, as will be described below. Therefore the trunk of the main carbohydrate chain in MP I remains as such in MP II. It probably has many alkali-unstable glycosidic links at definite intervals within the chain, as surmised from the unneglectable amount of iodine consumed by Fr. V'. These views led us to formulate a partial structure of MP II in Fig. 6.

Next, it was concluded in the foregoing article¹⁾ that the monosaccharide constituting the reducing residue in the unit structure of MP I is acetylglucosamine and that this and another acetylhexosamine molecule, which is N-glycosidically bound with the aspartic residue, are linked by acetal union to C atom 1 of an indirect-Osaki-Turumi negative sugar-residue. MP II does not possess the acetal link as well as aspartic acid and is two molecules less than MP I regarding hexosamine content per unit molecule. On the other hand, gastro-N-tetrasaccharide has galactose but no acetylhexosamine at its reducing end. Since no intermediate monosaccharides of the carbohydrate chains are cleft off, when MP I is degraded into MP II, it may be plausibly assumed that the galactose residue at the reducing terminal of gastro-N-tetrasaccharide is the hexose unit whose C₁ is linked with the two acetylhexosamine molecules by acetal union in MP I. In addition, the acetylhexosamine molecule N-glyco-

Fig. 6. Graphical representation of a partial structure of MP II. The monosaccharides within the gastro-N-tetrasaccharide residue are arbitrarily arranged, the galactose molecule at the reducing terminal excepted. Allocation of the ester link in gastro-N-tetrasaccharide is also arbitrary.

sidically bound with aspartic acid in MP I is acetylglucosamine because all the galactosamine molecules in MP I¹⁵⁾ are retained in MP II. Fig. 7 shows a combination of the partial structure of MP I discussed in the

Fig. 7. Graphical representation of a partial structure of MP I. X, Y, Z: amino acids which vary from one unit molecule to another. The monosaccharide components within gastro-N-tetrasaccharide are arbitrarily arranged excepting the galactose residue whose C atom 1 partakes in the acetal union. Allocation of the ester link in gastro-N-tetrasaccharide is also arbitrary.

foregoing article¹⁾ and that of MP II in Fig. 6.

SUMMARY

1. In continuation of the foregoing work, the group mucopolysac-

charide, prepared by alkaline scission of pig stomach mucus at room temperature, (MP I) was investigated with respect to the union between its polypeptide and carbohydrate moieties.

2. A partial degradation product of MP I, which was still group active, (MP II) was obtained in crystalline form. It contains three molecules of acetylglucosamine, two molecules of acetylgalactosamine, five molecules of galactose and one molecule each of L-fucose, serine and alanine per unit molecule and is devoid of sialic acid. Moreover, it lacks acetal link.

3. As products by alkaline hydrolysis of MP II, a tetrasaccharide composed of one molecule of acetylglucosamine, two molecules of galactose and one molecule of L-fucose ("gastro-N-tetrasaccharide") and two undialyzable high-molecular polypeptide-polysaccharide complexes were separated. In gastro-N-tetrasaccharide, one of the galactose molecules stands at the reducing terminal and the L-fucose molecule is bound in a manner stable against acetic acid. One of the polypeptide-polysaccharide complexes is most probably a fragment whose multiple makes up MP II deprived of the tetrasaccharide.

4. Ester linkage between the carboxyl of alanine and a hydroxyl in gastro-N-tetrasaccharide was suggested.

5. Crystalline N-acetylglucosamine-galactoside serine ether was isolated from a H_2SO_4 -hydrolysate of MP II.

6. From the findings above and those in other articles from this laboratory a partial structure of MP I and II are discussed.

Through the Grant Committee for Scientific Researches the Ministry of Education gave a grant in aid to us, which is gratefully acknowledged. H. Masamune.

References and Notes

- 1) Masamune, H., Hakomori & Masamune, O., *Tohoku J. Exp. Med.*, 1956, **64**, 281.
- 2) Hydrolysis with Dowex 50 was first attempted by Deuel, Senis, Angas-Weisz & Huber (*Helv. chim. acta*, 1951, **34**, 1849) on polysaccharides, and by Underwood & Deatherage (*Science*, 1952, **115**, 95) on casein and a coffee protein, and this method was applied by us to the urinary group glucidamins (Masamune, H., Hakomori, Masamune, O. and Takase⁷⁾). Furthermore, while our present work was being carried on, a paper in which ovomucoid was treated in a similar manner, was published by Dixon (*Bioch. J.*, 1955, **59**, No. 3, Proceedings xxi). However, the strongly basic resin Dowex 1 has never before been employed as a hydrolysis agent. By short heating with this resin, peptide links are split but not the ordinary O-glycosidic.
- 3) Akabori, Ohno & Narita, *Bull. Chem. Soc. Japan*, 1952, **25**, 215.
- 4) An occurring cloudiness by colloidal carbon was removed by passing the solution through a Celite layer.
- 5) We are indebted to Prof. K. Omori in the Department of Mineralogy, Petrology and Economic Geology, who measured this index.

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 - 9) The procedure has been modified with the aim of systematic fractionation of the hydrolysate of MP II, which will be published elsewhere.
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