Biochemical Studies on Carbohydrates

CLXII. High-molecular Components of Pig Kidney-cortex, particularly a Carbohydrate or Carbohydrates Containing Hexosamine

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Pig kidney-cortices were extracted with a saline solution after dehydration with alcohol in one case and in the other immediately. And, by fractionation of the solutions two biuret negative carbohydrate-preparations were obtained which proved inhomogeneous after electrophoresis, but analyzed similar to each other. In either of them, the nitrogen content amounted more than twice the calculated from the hexosamine content, suggesting a complex or complexes of a polypeptide and a polysaccharide. Whether the preparations were mixtures of differently aggregate molecule of one carbohydrate or those of different carbohydrates is not decided for the present. The details regarding the preparation procedure and the properties of the products will be described below.

EXPERIMENTAL

Preparation Procedure

1. 50 pig kidneys were taken. The cortices (4.8 kg.) separated mechanically were ground in a masticator, stood as covered under 4.5 volumes of denat. alcohol containing 3.5% methanol for 5 days and then dried in a Faust apparatus. 125 kg. of the material thus obtained were boiled with 5 and 3 volumes of abs. alcohol in sequence for 2 hours each. The residue was dried as above. 0.97 kg. was the amount. It was divided into three equal portions for successive treatments as below: The divisions were mixed with 10 volumes of a 10% saline solution and the mixtures shaken for 3 days (Toluene was used as a preservative). The extraction was repeated three times, followed by centrifugation until none came out anymore. The supernatant fluids were treated separately again. The description below concerns the first centrifuged supernatant in the case of the first division of the defatted material. It (a somewhat turbid, light brown supernatant, 2.7 l.) was acidified with 10% HCl to

a pH of 1.5 and centrifuged.

The voluminous centrifugate was washed with a 10% NaCl solution, which had been acidified to pH 1.5 with HCl, and, then, after vigorous agitation in 10% NaCl, neutralized with NaOH (10%). The insoluble part was rejected and the solution was precipitated with 5 volumes of abs. alcohol. The deposit was washed with abs. alcohol and dried—**Fr. I** (1.3 g.). Molisch +; biuret +; Neuberg-Saneyoshi (for hexuronic acid) +(weak); Goldschmiedt (for hexuronic acid) $\pm$. P ++; purine +; Bial-Mejbaum (for pentose) +; Dische (for desoxypentose) +(weak). Thus this Fr. proved to be a mixture of proteins, nucleic acids (mostly a pentosenucleic acid) and a small amount of a hexuronic acid-containing polysaccharide (polyuronic acid). From its watery solution, freed from the insoluble part, the nucleic acids were carried down with barium acetate and baryta, and the centrifuged supernatant was removed from barium with H_2SO_4 , dialyzed and condensed, followed by addition of alcohol. The deposit here showed only weak Molisch reaction contrary to strong biuret reaction. It was further divided into the precipitable and unprecipitable by adjusting its solution to pH 4.2 with HCl. But these subdivisions were not more than faintly Molisch-positive. Thus glucidamins appeared absent among the proteins in Fr. I.

The clear, light brown supernatant was dialyzed for 3 days and some precipitate occurring thereby was discarded. The clear mother fluid was distilled in vacuo to about 30 cc., freed from some precipitate that had come out during the condensation, and 2.5 cc. of 20% barium acetate were added in small portions, until no more precipitation. The voluminous flocculent deposit was centrifuged off.

The centrifugate was washed with 1.6% barium acetate and suspended in a little water and after changing into sodium salts by agitation with portions of a sodium sulfate solution, filtered. And the filtrate was dialyzed and condensed and alcohol added. The precipitate, washed with alcohol and dried, (a white powder) weighed 0.7 g.—**Fr. II**. Molisch +; biuret +(weak); Neuberg-Saneyoshi, Goldschmiedt and Dische —; P ++; purine +; Bial-Mejbaum +. Hence it consisted chiefly of a pentosenucleic acid

To the supernatant was added the saturated $\text{Ba}(\text{OH})_2$ to pH 8.0.

An occurring white flocculent precipitate was also transformed into sodium salts similar to above. 0.75 g. of a white powder was given—**Fr. III**. Molisch and biuret +(weak); P ++; purine +; Bial-Mejbaum +; Dische +. From these consequences of tests, the fraction is regarded to have contained pentose- and desoxypentose-nucleic acid as the main components.

The mother fluid was neutralized with 2% HCl and dialyzed for 4 days until no more barium ions were detected. After centrifuging off of some deposit that had occurred during the dialysis, the solution was distilled in vacuo to a syrup, precipitated with 5 volumes of abs. alcohol

and washed with the alcohol repeatedly, adding sodium acetate when requisite. 1.2 g. of a slightly brown powder were given.—**Fr. IV (Cr. Ch.)**.

Frs. IV's from the whole defatted cortex material amounted to 4.8 g. They were combined and assayed. Molisch ++; biuret ++; Bial-Mejbaum ++; Neuberg-Saneyoshi, Goldschmiedt, Dische and tests for P and purine —. The balance of the batch was taken up in 75 cc. of water (pH of the solution, 5.6), saturated with ammoniumsulfate (a voluminous precipitate occurred) and after standing overnight, filled up to 200 cc. with the saturated $(\text{NH}_4)_2\text{SO}_4$. Centrifuged. The supernatant fluid was freed from suspended particles by filtration.

The centrifugate was dissolved in a small quantity of water, and reprecipitated by saturation with $(\text{NH}_4)_2\text{SO}_4$. After further addition of the saturated $(\text{NH}_4)_2\text{SO}_4$ up to 100 cc., the mixture was centrifuged. And the centrifugate was dissolved in 600 cc. of water (A brown, slightly turbid solution was given, whose pH was 6.0) and dialyzed for 5 days when NH_4 - and SO_4 -ions were not detected. A small precipitate during the dialysis was rejected, and the solution distilled in vacuo to about 40 cc., followed by addition of 10 volumes of alcohol and a little anhydrous sodium acetate. The deposit was washed with alcohol and dried. A light brown powder given was 3.1 g.—**Fr. V**. Molisch +(weak); Bial-Mejbaum ±; biuret ++; Neuberg-Saneyoshi, Goldschmiedt, Dische and test for P —. The present fraction thus looked to be a mixture of water-soluble, carbohydrate-poor proteins. It was further treated by the procedures of Kerr and Seraidarian¹¹ and Sevag. But the divisions also showed only weak Molisch reaction, which suggested no glucidamins.

The filtrate was diluted fourfold with water and dialyzed against running water for 5 days. Since it proved to still contain SO_4 -ions, it was condensed to about half the volume by vacuum distillation and dialyzed again. The sulfate-free solution was concentrated as above to about 30 cc. Then the slightly turbid, light yellow solution of pH 6.0 was subjected to centrifugation and the clear supernatant was precipitated with abs. alcohol containing some sodium acetate, washed with alcohol and dried in vacuo over CaCl_2 . A white powder given weighed 0.83 g. Molisch ++; biuret ++, Bial-Mejbaum +.

0.82 g. of the product was then dissolved in 20 cc. of water and 10% lead acetate was added carefully to just carry down all the precipitable (2.5 cc. of the salt solution were required). Stood for 5 hours and centrifuged.

The precipitate liberated from lead by the aid of H_2SO_4 and H_2S —**Fr. VI**—showed only slightly positive Molisch but strong biuret reaction. The presence of glucidamins was again disproved.

The supernatant, which was somewhat turbid, became clear on re-centrifugation after standing overnight in an ice chest. It was then bubbled with H_2S and the black sediment filtered off. The filtrate was vacuum-distilled to a syrupy solution, precipitated with 5 volumes of abs. alcohol and after washing with the alcohol, dried. 0.36 g. was the white powder yielded. Molisch $\#$; biuret $\pm$; Bial-Mejbaum $+$.

Finally the balance (0.35 g.) of the product was kneaded with 1 cc. of water and to the viscid solution were added 11.25 cc. of glacial acetic acid (b.p. 117–118°C, 98%). The flocculent sticky precipitate was centrifuged and washed with 90% acetic acid twice and further with ether five times and dried—**The Acetic Acid-Insoluble I (Ch. I)**. Crop, 180 mg. To the light yellow supernatant were added three volumes of ether and the centrifugate also washed with ether thoroughly and dried—**The Acetic Acid-Soluble I (Ch. I')**. Crop, 140 mg. Either of these fractions was a white powder. The Acetic Acid-Insoluble I: Molisch $\#$, Bial-Mejbaum $+$, biuret, Neuberg-Saneyoshi, Goldschmiedt, Dische, Osaki-Turumi (the direct as well as the indirect), ninhydrin and Masamune *et al.* $HgCl_2$ test^{1a)} —, hydrolysable sulfur, phosphorus and purine —. The Acetic Acid-Soluble I did not differ in test-tube tests from above, but that it gave weak biuret reaction.

2. In this case a fresh tissue material was immediately treated with physiological saline and the solutions fractioned as above with a few modifications.

The cortex of 5 pig kidneys, weighing 0.6 kg. in total, were masticated and shaken with 3 l. of a 0.85% saline solution for 12 hours, using toluene as a preservative. Centrifuged. (The centrifugate was subjected to further extractions.) To the supernatant fluid, which was turbid and red-brown colored, was added 10% HCl in small portions to pH 4.5. (The voluminous flocculent precipitate was separated off for preparing the pentosenucleic acid which will be reported elsewhere.²⁾). The nearly clear mother fluid was dialyzed for 2 days. A pretty large amount of a precipitate came out thereby. This was rejected. The fluid was distilled in vacuo to about 200 cc., whereby again precipitation occurred. After removing of the deposit, it was further concentrated to syrupy consistency, precipitated with 5 volumes of abs. alcohol and washed with the alcohol, followed by drying in vacuo over $CaCl_2$. A brown powder given weighed 5.0 g. and gave the strong biuret reaction. It was also Molisch positive but Neuberg-Saneyoshi and Goldschmiedt negative. Phosphorus was detected. After the second extraction of the same material, 0.5 g. of a similar substance was given. 5 more runs were carried out, employing 2.89 kg. of fresh cortices in all, and 35.1 g. in total of the products were obtained. They were combined together—**Fr. A**.

5.5 g. of Fr. A were taken up in 50 cc. of water. The insoluble part was filtered off. To the filtrate were added 4.2 cc. of 20% barium acetate until no more precipitation occurred, then to the centrifuged supernatant was added the saturated baryta to pH 8.0. The centrifuged supernatant was neutralized with HCl, dialyzed for 4 days against running water to eliminate SO_4 -ions and after rejecting some sediment, distilled in vacuo. The remaining thick solution was precipitated and washed with abs. alcohol and dried. A brownish powder given amounted to 2.1 g. It was perfectly free from phosphorus. Molisch and biuret positive. Dische negative. From the whole Fr. A were yielded 11.1 g. of the present fraction—**Fr. B**—by repetition of the operations. To remove proteins, fractionation by means of $(\text{NH}_4)_2\text{SO}_4$ was processed as follows:—

11.1 g. of Fr. B were dissolved in 200 cc. of water (A little insoluble part was centrifuged off; pH of the solution, 6.2), and $(\text{NH}_4)_2\text{SO}_4$ added till saturation. The voluminous deposit was filtered off after standing overnight and the clear yellowish filtrate was diluted three times and dialyzed for 5 days. SO_4 -ions did not entirely disappear, and so after condensation to about 150 cc., the solution was further dialyzed for 2 days when it was perfectly free from SO_4 - and NH_4 -ions. After condensation to about 10 cc., it was precipitated with 10 volumes of abs. alcohol and some sodium acetate. The precipitate was washed with abs. alcohol and dried as above. 0.70 g. of a white powder was given. Molisch and Bial-Mejbaum positive. Biuret-giving impurities were still detected, though little—**Fr. C**.

0.69 g. of Fr. C was taken up in 20 cc. of water. A somewhat turbid light yellow solution of pH 6.0 was given. 2.8 cc. of a 10% lead acetate solution was added in small portions until no more precipitation. Stood overnight and centrifuged. The clear supernatant fluid was bubbled with H_2S gas. The black precipitate was filtered off and the filtrate (pH 5.8, 30 cc.) was diluted up to 200 cc. and electrodialyzed in a Pauli apparatus through cellophane membranes³⁾ against changes of water until outer fluids renewed did not undergo any pH shift. Some deposit, that had appeared, was centrifuged off and the supernatant was distilled in vacuo to about 5 cc., precipitated with 10 volumes of abs. alcohol containing some anhydrous sodium acetate. The deposit here was washed with plain abs. alcohol and dried. A white powder weighing 0.23 g. was given—**Fr. D**. Molisch strongly positive; Bial-Mejbaum moderately positive; biuret faintly positive.

The balance (0.22 g.) of this fraction was kneaded with 1 cc. of water. To the solution formed (pH 6.2) were added 11.25 cc. of glacial acetic acid (b.p. 117–118°C, 98%) under shaking. The sticky flocculent precipitate was centrifuged, washed twice with 90% acetic acid and thorough-

ly with ether and dried. 95 mg. of a white powder were obtained—**The Acetic Acid-Insoluble II (Ch. II)**. To the supernatant fluid were added 3 volumes of ether and the deposit hereby was also washed repeatedly with ether and dried. 103 mg. was this division (a white powder)—**The Acetic Acid-Soluble II (Ch. II')**. The former division was Molisch and Bial-Mejbaum positive. Biuret, Neuberg-Saneyoshi, Goldschmiedt, Dische, Osaki-Turumi (the direct as well as the indirect), ninhydrin and Masamune *et al.* (a HgCl_2 -test^{1a)}) all negative. Phosphorus, purine and hydrolysable sulfur were not detected. The latter division resembled the former qualitatively, but was faintly biuret-positive.

Properties of the Products

The Acetic Acid-Insoluble I (Ch. I) and II (Ch. II) and the Acetic Acid-Soluble II (Ch. II') were examined. The Acetic Acid-Soluble I (biuret-positive, N 10.9%, hexosamines 6.9%, ash 7.9%) was scarcely examined because it was contaminated with much of protein.

Solubilities

They dissolved perfectly in water; 10% trichloroacetic acid, 90% phenol and the saturated ammonium sulfate, and were not precipitated from water by cupric chloride, lead acetate, barium acetate and baryta. In 90% acetic acid the last one was soluble but the first two were not.

Analyses

The figures obtained are embodied in Table I.

TABLE I
Composition of the Carbohydrate Preparations (Chs. I, II and II')

Analysis	In per cent			In equivalents per equivalent weight		
	Ch. I	Ch. II	Ch. II'	Ch. I	Ch. II	Ch. II'
N*	4.3	3.9	6.1	7.9	6.8	11.8
Hexosamines	20.8†	21.4‡	18.9‡	3.0	2.9	2.8
Galactose§	21.5	23.7	18.9	3.1	3.2	2.8
Mannose§	7.0	7.4	6.7	1.0	1.0	1.0
Pentose as xylose//	6.8	7.1	6.2	1.2	1.1	1.1
L-Fucose¶	8.3	9.6	7.3	1.3	1.4	1.2
Ash**	4.7	2.5	4.8			

* micro Kjeldahl, † Hamasato-Akakura method, ‡ Blix method⁶⁾, § Masamune-Ogawa method⁷⁾ // Masamune-Ogawa method⁸⁾, ¶ Masamune-Ogawa method⁹⁾, ** micro Pregl method without use of H_2SO_4 .

Optical rotations

The Acetic Acid-Insoluble

$$\text{I: } [\alpha]_{\text{D}}^{25} = \frac{-0.08 \times 1}{0.0098 \times 1} = -8.1^{\circ}$$

$$\text{II: } [\alpha]_{\text{D}}^{16} = \frac{-0.13 \times 1}{0.0093 \times 1} = -13.9^{\circ}$$

The Acetic Acid-Soluble

$$\text{III: } [\alpha]_{\text{D}}^{15.5} = \frac{-0.23 \times 1}{0.0104 \times 1} = -22.1^{\circ}$$

Anti-hemoagglutinative potencies

By our usual procedure (Cf. Oh-Uti⁴¹) the forces of inhibiting agglutination of A, B and O erythrocytes by normal α and β serum and eel serum respectively were estimated. The results are shown in Table II.

TABLE II

Occurrence of Agglutination of A, B and O Erythrocytes by Human B- and A-serum and Eel Serum respectively after Treatment of the Latter with the Preparations (I, II, I' and II')

The B, A and eel serum were so diluted as to have titre 16. $\pm$: faint aggl., +: slight aggl., ++: marked aggl., ##: strong aggl.

Preparation (Ch.)	Group of red cells	Dilution of preparations (1 :)				
		2×10^2	4×10^2	8×10^2	1.6×10^3	3.2×10^3
I	A	++	++	##	##	##
	B	++	++	##	##	##
	O	++	++	++	##	##
II	A	++	++	++	##	##
	B	$\pm$	+	+	##	##
	O	++	++	++	++	##
I'	A	++	++	##	##	##
	B	++	++	##	##	##
	O	++	++	++	++	##
II'	A	##	##	##	##	##
	B	$\pm$	+	++	++	##
	O	++	++	++	++	##

Paper partition chromatography of the sugar components

The procedure of Masamune and Yosizawa⁵¹ was adapted.

The Acetic Acid-Insoluble I (Ch. I). 40 mg. of the preparation were heated for 5.5 hours with 4 cc. of 1 N H_2SO_4 at 100°C under reflux. The hydrolysate was diluted twice and nearly freed from H_2SO_4 with baryta (Crystals and the saturated solution were added to a pH of 4.6) and condensed to 0.5 cc., of which 0.02 cc. portions were sampled.

The Acetic Acid-Insoluble II (Ch. II). 29 mg. of this preparation were hydrolyzed with 3 cc. of the sulfuric acid solution and after freeing from most of sulfuric acid, condensed to 0.4 cc., and 0.03 cc. portions were

Fig. 1. (A): the chromatograms of Ch. I; (B): the chromatograms of Ch. II. The *n*-butanol-pyridine-water (5:3:2) chromatograms to the left and the *n*-butylacetate-acetic acid-ethanol-water (3:2:1:1) chromatograms to the right in both (A) and (B). Temperature of test: (A), 23–26°C; (B), 20–22°C. Duration of test, 24 hrs. Indicator, aniline hydrogen phthalate of Partridge.

a and b (a 1 *N* H₂SO₄-hydrolysate of tragacanth): reference runs. c: Ch. I in (A); Ch. II in (B). 1, 1': chondrosamine sulfate; 2, 2': glucosamine sulfate; 3: galactose; 4: mannose; 5: xylose; 6: L-fucose; 7: rhamnose.

sampled.

Figs. 1A and 1B are diagrammatic reproductions of the chromatograms demonstrating glucosamine, chondrosamine, galactose, mannose, L-fucose and probable xylose as the sugar components.

Electrophoresis

Chs. I and II were subjected to electrophoresis in a Tiselius apparatus.

Fig. 2. (A) An electrophoretic pattern of a 1% solution of Ch. I in phosphate buffer, pH 7.8, $I=0.29$; 14°C ; 10 ma; exposure 60 minutes; mobility: the first moving boundary $\frac{1.5 \times 0.0117 \times 0.3}{0.01 \times 3600} = 14.6$

$\text{cm}^2 \cdot \text{volt}^{-1} \cdot \text{sec}^{-1} \cdot 10^5$, the second moving $\frac{0.9 \times 0.0117 \times 0.3}{0.01 \times 3600} = 8.7 \text{ cm}^2 \cdot \text{volt}^{-1} \cdot \text{sec}^{-1} \cdot 10^5$, the third moving $\frac{0.2 \times 0.0117 \times 0.3}{0.01 \times 3600} = 1.9 \text{ cm}^2 \cdot \text{volt}^{-1} \cdot \text{sec}^{-1} \cdot 10^5$. (B)

A pattern of a 1% solution of Ch. II in carbonate buffer, pH 8.9, $I=0.105$; 3°C ; 10 ma; exposure 30 minutes; mobility: the first moving boundary $\frac{1.0 \times 0.00401 \times 0.3}{0.01 \times 1800} = 6.6 \text{ cm}^2 \cdot \text{volt}^{-1} \cdot \text{sec}^{-1} \cdot 10^5$, the second moving $\frac{0.15 \times 0.00401 \times 0.3}{0.01 \times 1800} = 1.0 \text{ cm}^2 \cdot \text{volt}^{-1} \cdot \text{sec}^{-1} \cdot 10^5$. Ascending limbs to the left.

The patterns (Figs. 2A and 2B) show three boundaries in the former preparation and two in the latter. The first moving, low boundary of Ch. I appears to represent a contaminating peptide, because Ch. II which contained less nitrogen than Ch. I perfectly or nearly lacked it. The second and third moving components of Ch. I most probably correspond to the first and second moving of Ch. II respectively.

COMMENTS AND DISCUSSION

1. Of the fractions of the tissue extract obtained by the aid of 10% NaCl (the first procedure), Fr. I only showed faintly positive Neuberg-Saneyoshi and Goldschmiedt reaction for hexuronic acid, and its subdivisions gave the negative reactions. Hence, pig kidney cortex looks to be very scanty of polyuronic acids.

2. Glucidamins are also believed to be scarce, if present, as were revealed by the experiments on Frs. I, V and VI.

3. The quantitative ratio of the two components represented by the second and third electrophoretic boundaries of Ch. I was the inverse of the corresponding ratio of the corresponding components of Ch. II. Nevertheless, regarding the kinds of sugar component as well as the mutual equivalent proportions of these components in respective substances, the two preparations were quite similar to each other, suggesting that the two electrophoretically different components of either of Chs. I and II were only differently aggregated forms of one and the same carbohydrate

(polypeptide-polysaccharide complex).

SUMMARY

Saline extracts of pig kidney cortices were fractioned and carbohydrate preparations obtained were studied analytically, optically, electrophoretically and serologically. The intermediate products in the course of preparation were also examined which indicated that the tissue is scanty of polyuronic acids and devoid of glucidamins.

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