Biochemical Studies on Carbohydrates.

LXXXIII. Mucoids of Sclera.

Second Paper.

By

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In the first paper Satoh¹ suggested the presence of mucoitin and chondroitinsulfuric acid together in "scleramucoid." He obtained the preparations by extracting bull sclera with a lime water with following precipitation by means of HCl, and made the inference above on the basis that the equivalent ratios of hydrolytic sulfur to amino sugar or glucuronic acid in them were 1:2.

It is conceivable that there exist in this tissue two glycoproteins, of which one involves mucoitin and the other chondroitinsulfuric acid. So we undertook in the present work to separately isolate these possible mucoids. After attempts was chosen a means to extract at first the fresh tissue with a saline solution and second the residue with a lime water. Precipitates were obtained from each extract by acidulation of the latter with HCl (scleramucoid " α " and " β "). The mucoids, which had been purified till free from phosphorus, analyzed differently from each other and the equivalent proportion of hydrolytic sulfur to glucuronic acid was approximately 3:2 in the α mucoid and 1:1 in the β .

These ratios differ from the corresponding ratio in Satoh's scleramucoid, and it is now unnecessary to accept the simultaneous presence of ititin and itinsulfuric acid in this tissue. Further analytical data seen in Table I imply that besides itinsulfuric acid an acetylhexosamine-mannose complex as sulfuric ester and a similar carbohydrate uncombined with sulfuric acid are involved in the α and the β mucoid respectively. Treatment of the mucoids with formaldehyde according to Masamune and Osaki² separated chondroitinsulfuric acid from both of them. The residues were subjected to the procedure of Sasaki³ that cleaves off the methylene groups.

In the β mucoid, the product, which was obtained thereby, analyzed as demonstrating that the part, constituting with chondroitinsulfuric acid the whole molecule of the mother mucoid, had been perfectly recovered, but the one from the α mucoid was not similar in this respect, its polysaccharide diverging, in percentage in the substance as well as in quantitative proportion between its monosaccharide constituents (mannose and acetylhexosamine), from what had been expected. In the make-up of the α mucoid partake probably two proteins, which have each a different polysaccharide, in addition to chondroitinsulfuric acid, and one of them was lost⁴ during the course of separation.

The chemical nature of hexosamine in the mannose-containing polysaccharides could not be established owing to the shortage of material, but it is plausibly assumed as glucosamine, because Levene and López-Suárez⁵ have already isolated glucosamine besides chondrosamine from a probable polysaccharide-mixture from sclera.

EXPERIMENTAL.

Preparation of Scleramucoids α and β

From ox eyeballs brought soon from a slaughter-house was separated sclera by removing all neighbouring tissues: lens, retina, choroid, vitreous humor, tendon, conjunctiva, blood vessels, cornea and optic nerve. Its parts (0.5 and 0.3 cm. wide respectively) surrounding cornea and the optic nerve were also dissected off. Then it was cut into smallest possible pieces with scissors.

A typical example to prepare the crude mucoids was carried out as follows:

(a) *Crude scleramucoid α* . 329 gms. of the tissue (from 100 eyeballs) obtained as above were washed with many (usually six) 1.5 l. portions of water (thymol was added as 20% alcoholic solution), until the washing was not stained anymore. After this preparatory treatment, the tissue was extracted repeatedly at room temperature by shaking with 5 times the weight of 0.85 % NaCl solution mixed with thymol for 48 hours. The extracts (pH 7.0) (faintly turbid and brownish) were acidified with hydrochloric acid to pH 5.6, where maximum precipitation occurred. (From the fourth extract nothing came out on acidification.) The deposits were washed together twice with a dilute hydrochloric acid solution at pH 5.6, with changes of 50% alcohol so long as the chloride test was given, twice with 95% alcohol and finally twice with ether and dried in a desiccator. The substance was then suspended, when dry, in 700 cc. of water and dissolved by the aid of 1% Na_2CO_3 solution. pH of the brownish solution obtained was

9.0. The insoluble part, which reached only a small amount, was centrifuged off and 0.2% hydrochloric acid was added to pH 5.6. The flocculi coming out were dissolved and precipitated once again in a like way, washed and dried as before (crude scleramuroid α). The yield of the greyish powder obtained was 1 gm.

(b) *Crude scleramuroid β* . The residue of the tissue above was next subjected to extraction with half-saturated lime water. The manner was similar. Into the fourth extract came no acid-precipitable substance over. The precipitates, which appeared on acidifying the extracts to pH 5.6, were combined, washed exhaustively with dilute hydrochloric acid at the same pH, and when the washing (the 6th washing) showed no biuret reaction, treated with alcohol (twice) and ether (twice) and dried in a desiccator. Dissolution in water (700 cc.) by the use of the lime water and precipitation of the powder obtained were made twice. Washed and dried. The brownish product weighed 0.5 gm.

Purification.

Since both of the above products contained phosphorus, they were purified by means of baryta water.

The procedure will be given in detail taking an example on the α mucoid. 1 gm. of the crude product was kneaded in water, water increased up to 100 cc. and saturated baryta water dropped in. Most of the substance went into solution at pH 9.0 (2.5 cc. of the baryta water were required). The insoluble part was removed by centrifugation and the supernatant liquid, which was turbid, was clarified by filtration with suction. To the yellowish filtrate were added 2 drops of 2 n. HCl (pH was shifted to 2.6) and the deposit was centrifuged and washed 3 times with a dilute hydrochloric acid (2 drops of 2 n. HCl in 40 cc. of water), whereby all barium could be eliminated. It was further washed with alcohol and ether, twice each, and dried. The white powder that was obtained amounted to 407 mgms. P was absent.

In the case of the β mucoid, the solution (pH, 9.2) prepared by means of $\text{Ba}(\text{OH})_2$ was brought to pH 4.5, where precipitation occurred at maximum. A dilute hydrochloric acid at this pH was employed for washing. The yield of the product (white powder) was in averages 0.43 gm. for 1.0 gm. of the crude substance. It was free from phosphorus.

Properties of the Mucoids.

1. Qualitative tests.

The mucoids behaved both similarly towards colour and precipitation tests for protein.

(a) The colour tests.

Reaction	Scleramu- coid α	Scleramu- coid β	Reaction	Scleramu- coid α	Scleramu- coid β
biuret	+	+	vanilline		
Millon	+(weak)	+(weak)	(for tryptophane) ...	+	+
xanthoprotein	+	+	Adamkiewitz	$\mp$	$\mp$
Sakaguchi	+	+	Ehrlich <i>p</i> -dimethyl- aminobenzaldehyde		
Ehrlich diazo	+	+	(for tryptophane) ...	+(weak)	+(weak)
Hopkins and Cole ...	—	—	Liebermann	+(„)	+(„)
reduced sulfur	$\pm$	$\pm$			

(b) The precipitation tests.

Solutions of the mucoids at pH 7.0 (prepared by the aid of NaOH) were adapted.

The mucoids were precipitated markedly by tannic, picric, hydrochloric (soluble in excess of this acid) and acetic acid and lead and mercuric acetate, but only faintly by lead subacetate, HgCl_2 and CuSO_4 and by saturation with $(\text{NH}_4)_2\text{SO}_4$, and were not at all by ferrocyanic and nitric (concentrated) acid and by saturation with NaCl , Na_2SO_4 and MgSO_4 . They came out on addition of sulfosalicylic and trichloroacetic acid but dissolved again in the excess of these acids, as when HCl was used.

When the solutions acidified with acetic acid were boiled with NaCl , precipitation became more remarkable.

2. Quantitative analysis.

The following methods were employed for quantitative analysis.

N: micro-Kjeldahl method. Hexosamine: Hamasato and Akakura method. Hexose: the second procedure of Masamune and Tanabe method. Glucuronic acid: the indole and orcinol method of Ōsaki⁶ and the furfural method of Tanabe. Acid-hydrolytic sulfur: gravimetrically under the conditions chosen by M. Suzuki. Acetyl: M. Suzuki method. Ash: micro analysis.

Figures obtained are embodied in Table I.

3. Optical rotatory power.

(a) Scleramuroid α .

19.4 mgms. of Prep. I were suspended in 2 cc. of water and dissolved by addition of 0.01 n. NaOH in drops. pH of the transparent solution obtained was 8.2.

$$[\alpha]_{\text{D}}^{11} = \frac{-0.36 \times 3.122}{0.0194 \times 1.001 \times 1} = -57.9^\circ$$

TABLE I.

Analysis	Scleramuroid α						Scleramuroid β					
	In per cent			In equivalents per equivalent weight			In per cent			In equivalents per equivalent weight		
	Prep. I	Prep. II	Prep. III*	Prep. I	Prep. II	Prep. III	Prep. I	Prep. II	Prep. III	Prep. I	Prep. II	Prep. III
Nitrogen	12.40	12.28	12.12				12.08	12.20	12.04			
Hexosamine	11.21	10.81	11.01	2.00	2.00	2.00	10.74	10.70	11.06	1.00	1.00	1.00
Acetyl	2.61			1.94			2.48	2.43		0.96	0.94	
Hexose as mannose												
Indole r.	5.38	5.33	5.35						2.54			
Orcinol r.	5.24	5.07	5.20						2.45			
Diphenylamine I r. ...	5.08	5.07	5.20						2.41			
Average	5.23	5.16	5.25	0.93	0.95	0.95			2.46			0.22
Glucuronic acid												
Osaki method:												
Indole r.		6.21							10.55			
Orcinol r.		6.31							10.46			
Average		6.26			1.07				10.51			0.88
Furfural method: ...	6.85	6.24	6.26	1.12	1.07	1.05			10.18	0.88	0.82	0.84
Acid-hydrolysable S ...	1.40	1.54	1.54	1.40	1.58	1.56	1.71	1.68	1.69	0.89	0.88	0.85
Ash			1.02				0.70		1.55			

* Electrolyzed (200 volts). For this purpose 550 mgms. of a specimen obtained similarly as Preps. I and II were dissolved in 60 cc. of water by the use of NaOH. At the end of the dialysis, the substance came out of the solution. After washing with alcohol and ether and drying, the white product weighed 400 mgms.

(b) Scleramuroid β .

1.76 mgms. of Prep. I were used. The solution was prepared in a similar manner as above. Its pH was 8.2.

$$[\alpha]_D^{10} = \frac{-0.387 \times 3.204}{0.0176 \times 1.004 \times 1} = -70.1^\circ$$

Separation of the Scleramuroids into Chondroitinsulfuric Acid and the Remaining Part or Parts.

1. Scleramuroid α .

7 gms. of the mucoid were subjected⁷ to the procedure for preparation of chondroitinsulfuric acid of Masamune and Osaki². 280 mgms. of acid sodium salt of chondroitinsulfuric acid were yielded. It was readily soluble in water and gave a plain green colour by the Molisch reagents and the positive naphthoresorcinol reaction for hexuronic acid (2 mgms. of the substance were used). Hydrolytic sulfur was present (2.4 mgms. were hydrolyzed with 4cc. of 1 n. HCl and filtered and to the filtrate was added 5% BaCl₂ solution in drops). Faint red colour was developed from it by *p*-dimethylaminobenzaldehyde, only when its alkaline solution had been preliminarily heated (Osaki and Turumi procedure⁸; the substance taken was 4 mgms.).

The quantitative composition and optical rotatory power embodied in Table II demonstrate it to be chondroitinsulfuric acid (acid sodium salt), though not perfectly pure (comp. with the corresponding figures of the preparation of Masamune and Osaki² and that of Osaki and Hasegawa⁹).

TABLE II.

	In per cent	In equivalents per equivalent weight
Nitrogen... ..	2.74	1.02
Glucuronic acid		
Indole r.	36.97	
Orcinol r.	37.39	
Average	37.18	1.00
Ash	24.63	
$[\alpha]_D^{10}$ (in water)	$-24.6^\circ \left(= \frac{-0.149 \times 3.019}{0.0183 \times 1.0006 \times 1} \right)$	

From the residue¹⁰ in the above stage, 200 mgms. were heated for 30 minutes with 0.05 n. HCl in boiling water according to Sasaki³. Most of the substance dissolved thereby. After centrifugation, the supernatant slightly colored liquid was brought to pH 5.6 with sodium

carbonate and precipitated with 5 volumes of 95% alcohol. The substance, reprecipitated from water (sodium carbonate was employed for dissolution; pH of the solution, 9.0) and washed with alcohol and ether, weighed 180 mgms. Hydrolytic sulfur was detected. The quantitative analysis and the optical measurement (the solution for the optical measurement was prepared by the aid of 0.01 n. NaOH; pH of the solution was 8.0) gave the values as follow:

Nitrogen	14.82%	Average	3.21%
Ordinary sugar as mannose:		Hexosamine	0.71 "
Indole r.	3.36 "	NH ₂ -N	1.25 "
Orcinol r.	3.10 "	Ash	2.06 "
Diphenylamine I r.	3.16 "		

$$[\alpha]_D^8 = \frac{-0.646 \times 3.0411}{0.0265 \times 1.0005 \times 1} = -74.1^\circ$$

The figures of the monosaccharide-constituents diverge from those anticipated from the composition of the α mucoid as already mentioned.

2. Scleramucoid β .

The processes applied were the same as those in 1. 300 mgms. of chondroitinsulfuric acid (acid sodium salt) and 3.8 gms. of the remaining part were recovered from 5 gms. of the β mucoid.

The preparation of chondroitinsulfuric acid was similar to the one from the α mucoid in not only qualitative but also quantitative (Table III) respects.

The moiety other than chondroitinsulfuric acid analyzed as follows:

Nitrogen	15.52%
Hexosamine	2.18%
Ordinary sugar as man- nose	
Indole r.	2.77%
Orcinol r.	2.77%
Diphenylamine I r.	
	<u>2.83%</u>
Average	2.79%

TABLE III.

	In per cent	In equivalents per equivalent weight
Nitrogen...	2.78	1.03
Glucuronic acid		
Indole r. ...	37.30	
Orcinol r. ...	37.34	
Average ...	37.32	1.00
Ash ...	19.08	
$[\alpha]_D^{10}$ (in water) ...	$-23.4^\circ \left(= \frac{-0.152 \times 3.0198}{0.0196 \times 1.0001 \times 1} \right)$	

$[\alpha]_D^6$ of its solution at pH 8.0 was $-34.7^\circ \left(= \frac{-0.199 \times 3.5165}{0.0201 \times 1.0020 \times 1} \right)$ and the solubility resembled that of the above protein from the α mucoid.

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Bibliography and Notes.

- 1 Satoh, J. Bioch., 1940, **31**, 351.
- 2 Masamune and Osaki, Tohoku J. Exp. Med., 1943, **45**, 121.
- 3 Sasaki, *ibid.*, 1945, **48**, 307.
- 4 It may have remained, for instance, in the mother liquid when the alkaline solution of the mucoid was precipitated with acid after the formaldehyde treatment.
- 5 Levene and López-Suárez, J. Biol. Chem., 1918, **36**, 105.
- 6 Osaki, Tohoku J. Exp. Med., this volume, p. 15.
- 7 The suspensions in the successive formaldehyde treatments were acidified, as in the case of tendomucoid β (Sasaki³), without separation in advance of the product insoluble in alkali.
- 8 Osaki and Turumi, Tokoku J. Exp. Med., this volume, p. 11.
- 9 Osaki and Hasegawa, *ibid.*, 1943, **45**, 176.
- 10 It amounted to 3.5 gms.