Biochemical Studies on Carbohydrates

CL. On Chondrosin and Chondroitinsulfuric Acid

By

Hajime Masamune, Zensaku Yosizawa and Masahiro Maki

(正宗 一)

(吉澤 善作)

(眞木 正博)

(From the Medico-chemical Institute, Tohoku University, Sendai)

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Kurt H. Meyer *et al.*¹⁾ assigned to chondroitinsulfuric acid the following formula:

Namely, regarding the structure of its basic disaccharide (chondrosin) they considered that glucuronic acid takes the pyranose form and glucosidically links with C_{III} of chondrosamine contrary to Kobayasi²⁾ from our Institute, who had put forward chondrosin to be chondrosamine-6-glucuronide on the basis that oxidation of it with mercuric oxide gives formylglucuronide. In order to decide the mode of union in question we subjected N-acetylchondrosinethylester to periodic acid oxidation and found 1 mole of formaldehyde is produced consuming 4 moles of periodic acid, which presented the formula below of chondrosin, in accord with the view of Kurt H. Meyer *et al.* in so far as the constitution of that basic

Chondrosin

disaccharide concerns. As a control experiment N-acetylchondrosamine and N-acetyl- α -methylchondrosaminide were examined as to the periodic acid consumptions under similar conditions. The results were the expected, that is, 3 moles and 1 mole respectively of periodic acid were used per mole.

In the next, chondroitinsulfuric acid (mol. w. 22300) and its two products (I, II) (mol. w.: I 12200, II 4100) obtained by partial degradation at the glucosidic and sulfuric ester linkages were investigated. All of these materials showed the positive indirect-Osaki & Turumi reaction distinctly and on the other hand, analysis gave the equivalent ratios of the aminosugar and hexuronic acid of 1:1. Therefore, acetylchondrosamine stood at the reducing end of the respective molecules and consequently glucuronic acid at the non-reducing. In the reaction with periodic acid, chondroitinsulfuric acid required 3 moles of the oxidizing agent per mole while the cleavage products 4 moles, which led us to the following conclusions: 1) in any of the substances, the inner part of the chain did not react at all; 2) the sulfate group linked with one of the terminal hexose units, reducing and non-reducing, of the cleavage products was cleft off during the preparation with acid (6 N HCl), for, when not, no more than 3 moles of periodic acid should be needed; 3) chondrosamine combines glucosidically C_{III} of glucuronic acid belonging to the adjacent acetylchondrosin, or, otherwise, intermediate glucuronic acid molecules would consume periodic acid if free or when freed, by the strong mineral acid, from the sulfate rest; 4) intermediate chondrosamine molecules are a pyranose, because, whether the sulfate rest is present or not, acetylchondrosamine bound at C_I and C_{III} is not oxidized at all in the pyranose form whereas the furanose would be, reducing 1 mole of the oxidizing agent if it is free from the sulfate rest or when the latter has been split off.

Nevertheless, from the above findings the sulfate groups can not be allocated to a definite position. Those rests can naturally combine any free position in the inner part of the chain of chondrosins without effect upon the HIO₄ consumption, since the mentioned part of the chain even in the state unattached by the rests is inert to the reagent, and moreover, chondroitinsulfuric acid can use 3 moles of the reagent when any one of the positions C_{II} and C_{IV} of glucuronic acid and C_{IV} and C_{VI} of acetylchondrosamine is occupied by the rests. Kurt H. Meyer and co-workers found that completely methylated chondroitinsulfuric acid had used 3 moles of HIO₄ per period after hydrolysis. Our acquaintance is through C. A. so that we don't know the details of their experiments. However, the conditions they preferred for the oxidation appear different from ours, because such a hydrolysate ought to require by our procedure only 2 moles of the reagent if the sulfate groups lie at C_{VI} of acetylchondrosamine

as they claimed. Be that as it may, where the sulfate groupings are situated is as yet an unsettled problem to us, and we content ourselves for the present with expression of chondroitin in the diagram:

n amounts to 45-50. That the β -form is assigned to chondrosaminide is arbitrary. Cf. also Masamune & Osaki³⁾, Kobayasi⁴⁾, and Nagaoka.⁵⁾

That Kobayasi obtained formylglucuronic acid after oxidation of chondrosin might be explained if one concedes the attack of mercuric oxide on the chondrosamine molecule from both sides of C_{III}.

EXPERIMENTAL

Materials

- 1) *Ethylester of N-acetylchondrosin* prepared according to Masamune Hiyama & Kobayasi.⁶⁾
- 2) *N-Acetylchondrosamine*.
- 3) *N-Acetyl- α -methylchondrosaminide*.

These two were prepared as reported by Masamune, Maki & Hiyama.⁷⁾

4) *Acid Na-salt of chondroitinsulfuric acid*. 2 g. of acid Na,Ca-salt of chondroitinsulfuric acid (Jorpes) were subjected, as dissolved in 100 cc. of water, to electro dialysis (200 volts) against changes of water, until no more shift of pH of the fluids in the outer compartments occurred, which took 2 days, and after rejecting some precipitates, the solution was neutralized with NaOH and concentrated to about 8 cc. The transparent yellowish condensate was precipitated with 10 volumes of abs. alcohol 1/4-saturated with sodium acetate. The centrifugate was washed with 20 cc. of abs. alcohol three times (The final washing was somewhat turbid) and thoroughly dried in a desiccator. 1.3 g. of a white powder were given. N (micro Kjeldahl) 2.63%, chondrosamine 32.57%, acetyl 7.60%, glucuronic acid 37.94% (indole method: 38.30%; orcinol method: 37.58%), hydrolysable sulfur 5.80%, (equivalent ratios: 1.03:1.0:0.97:1.07:1.0). Ash (Na₂SO₄) 21.82%. Iodine value 1.75. *The molecular weight calculated from the iodine value, 24000 (22300 for free acid). Optical rotation in water:

$$[\alpha]_D^{20} = \frac{-0.88 \times 100}{3.33 \times 1} = -26.4^\circ.$$

5) *Cleavage Product I of chondroitinsulfuric acid.* 20 cc. of a solution containing 2 g. of the material above and an equal volume of 10 *N* HCl were mixed and stood at room temperature (18–22°C) for 1 day, and then 10 volumes of abs. alcohol and 5 volumes of ether were added. The occurring purplish deposits were centrifuged, washed with two 40 cc. portions of abs. alcohol and dried in a vacuum desiccator over H₂SO₄. The powder amounting to 1.4 g., that was obtained, was next taken up in 60 cc. of water (A clear purplish solution formed) and electrodialed as above for 3 days followed by rejecting some precipitates and distilling in vacuo to about 5 cc., to which abs. alcohol and ether were added similar to above. The deposits were dried in vacuo over H₂SO₄ at first and afterwards over P₂O₅. 1.15 g. of a greyish powder were yielded. N (micro Kjeldahl) 3.60%, chondrosamine 44.96%, acetyl 10.32%, glucuronic acid 49.0% (indole method: 49.57%; orcinol method: 48.43%), hydrolysable sulfur 3.11%, (equivalent ratios: 1.03 : 1.0 : 0.96 : 1.01 : 0.39). Ash 0.0%. Iodine value 3.45. The molecular weight calculated from the iodine value, 12200. $[\alpha]_D^{20} = \frac{-0.35 \times 100}{1.44 \times 1} = -24.3^\circ$ (in water).

6) *Cleavage Product II of chondroitinsulfuric acid.* 2 g. of chondroitin-sulfate (4) were dissolved in 40 cc. of water and added to with 60 cc. of 10 *N* HCl. The mixture was allowed to stand for 3 days at room temperature (18–20°C) and precipitated with 9 volumes of abs. alcohol and 4 volumes of ether. The deposits were centrifuged, washed with two 30 cc. portions of abs. alcohol and dried over H₂SO₄. 1.2 g. was the powder obtained. This was subjected to electro dialysis after dissolving in 80 cc. of water (a clear purplish solution) for 3 days and some appearing precipitates centrifuged off. The supernatant fluid was condensed and precipitated and the precipitates desiccated, as above. A greyish powder weighing 0.8 g. was the product. N (micro Kjeldahl) 3.63%, chondrosamine 45.40%, acetyl 10.27%, glucuronic acid 50.14% (indole method: 50.67%; orcinol method: 49.60%), hydrolysable sulfur 0.84%, (equivalent ratios: 1.02 : 1.0 : 0.94 : 1.02 : 0.10). Ash 0.0%. Iodine value 10.17. The molecular weight calculated from the iodine value, 4100. $[\alpha]_D^{20} = \frac{-0.28 \times 100}{1.38 \times 1} = -20.3^\circ$ (in water).

Methods of Oxidation and Separation of Formaldehyde

The methods described by Yosizawa⁸⁾ and by Masamune and Yosizawa⁹⁾ were adapted.

N-Acetylchondrosinethylester, *N*-acetylchondrosamine and *N*-acetyl- α -methyl-

chondrosaminide. 5 cc. of 0.1 *M* solution of a substance and 10 cc. of a HIO_4 solution were mixed and let react at 20°C until the change of optical rotation stopped. Thereupon, 3 cc. portions were pipetted out to measure the excess of HIO_4 and the amount of formaldehyde produced.

Acid-Na-chondroitinsulfate and the cleavage products I and II. 50 cc. of 3.01% solution of the first substance, 10 cc. of 4.33% solution of the second and 10 cc. of 4.13% solution of the third were maintained at 20°C together with 10 cc., 20 cc. and 20 cc. respectively of a variously concentrated HIO_4 solution. And immediately after the cessation of the change of optical rotation 12, 6 and 3 cc. respectively were taken out of the mixtures for determination of the excess HIO_4 .

Results

See Table I.

TABLE I

Requirements of Periodic Acid by N-Acetylchondrosinethylester, N-Acetylchondrosamine, N-Acetyl- α -methylchondrosaminide and Chondroitinsulfuric Acid (Acid Sodium Salt) and Its Cleavage Products, and Productions of Formaldehyde from the First Three

	N-Acetyl- chondros- inethyl- ester	N-Acetyl- chondros- amine	N-Acetyl- α -methyl- chondros- aminide	Chondroitinsulfuric acid		
				Acid Na-salt	Cl. Prod. I	Cl. Prod. II
Requirements of HIO_4						
Molarity of HIO_4 sol. added	0.2634	0.2634	0.1317	0.0436	0.0218	0.0436
Time (min.) till cessation of the change of opt. ro- tation	6×60	21	35	45	60	70
Moles of the excess HIO_4 in total	$0.123 \times \frac{5}{1000}$	$0.214 \times \frac{5}{1000}$	$0.151 \times \frac{5}{1000}$	$0.046 \times \frac{5}{1000}$	$0.058 \times \frac{5}{1000}$	$0.046 \times \frac{5}{1000}$
Mols of HIO_4 required per mole	4.04	3.13	1.12	3.29	4.11	4.09
Yields of formaldehyde						
Mg. substance in 3 cc. of the oxidation mixtures	44.3	22.1	24.4			
Mg. formaldehyde-dime- don compound (ppt.)	28.2	28.7	0.0			
F.P. of ppts. (cor.) $^{\circ}\text{C}$	189-190	189-190				
HCHO recovered, % theor.	96.6	98.3	0.0			

SUMMARY

N-Acetylchondrosinethylester, and a chondroitinsulfate and its partial

cleavage products were allowed to react with HIO_4 and the requirements of the oxidizing agent per mole of them and also the production of formaldehyde from the first one were estimated. The findings thereby and else suggested that 1) chondrosin is chondrosamine-3- β -glucuronide, and 2) chondroitin is expressed by Fig. 3 with acetylchondrosamine at the reducing end of the chain and glucuronic acid at the non-reducing.

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H. M.

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