ON CHONDROITIN SULPHURIC ACID.

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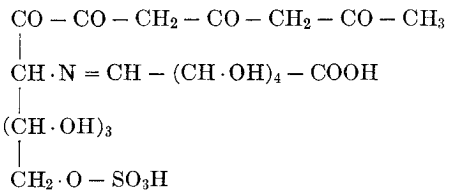
Chondroitin sulphuric acid has been the subject of repeated investigations, yet the information furnished by them added little to that advanced by the work of Schmiedeberg.[1] There is scarcely a new fact brought out by recent investigators which remained unchallenged and undisputed in the light of subsequent investigations.

According to the conception of Schmiedeberg the nucleus of chondroitin sulphuric acid is chondrosin. This, in its acetylated form (chondroitin) combines with sulphuric acid to yield chondroitin sulphuric acid.



The investigation here presented deals primarily with chondrosin. All writers are in accord in the view that chondrosin is composed of two substances in some way or other related to carbohydrates. According to Schmiedeberg the components are glucosamine and glucuronic acid. Orgler and Neuberg[2] contradicted Schmiedeberg on both points, claiming the components to be aminotetrahydroxycaproic acid and a hexose of undetermined configuration. S. Fränkel[3] further modified the view of the two preceding writers in that he interpreted the nature of the nitrogenous body as that

[1] Schmiedeberg: *Arch. f. exp. Path. u. Pharm.*, xxviii, p. 358, 1891.

[2] Orgler and Neuberg: *Zeitschr. f. physiol. Chem.*, xxxvii, p. 407, 1903.

[3] S. Fränkel: *Ann. d. Chem.*, cccli, p. 344, 1907.

of an aminoglucuronic acid, the other component supposedly having the structure of a very labile hexose with an undetermined configuration. Still later Kondo,[4] working in Hofmeister's laboratory, reached the conclusion that one of the components was xylose. Finally reference has to be made to the observation of Mandel and Neuberg[5] that chondroitin sulphuric acid gave a test with naphtoresorcin characteristic of glucuronic acid.

It must be added here that the only crystalline substances for which absolute purity was claimed by the writer were the salts of tetrahydroxyaminocaproic acid described by Orgler and Neuberg.

It is seen from this brief review that there exists an absolute lack of agreement on the nature of the components of chondrosin. No greater is the harmony of various writers on the mode of union of the two components. According to the view of Schmiedeberg, the carbonyl group of the glucuronic acid is attached to the amino group of the glucosamine, hence giving the following expression to the molecule:

$$\begin{array}{l} CH_2\,(OH)\,(CH\cdot OH)_3\,CH-CHO \\ \qquad\qquad\qquad\qquad\qquad\quad | \\ COOH\cdot(CHOH)_4\,CH=N \end{array}$$

Orgler and Neuberg criticized severely the conception of Schmiedeberg as inconsistent with the properties of the substance, one of the reasons being the observation of Schmiedeberg that the reducing power of a chondrosin solution is not diminished after oxidation with nitric acid; and also on the ground of the great resistance of chondrosin against the hydrolytic action of mineral acid. Orgler and Neuberg, however, do not advance a definite view on the mode of the union of the two components. Also S. Fränkel and Kondo furnish little information in that direction. The latter however argues against the presence of a free carboxyl group in the chondrosin molecule

In course of the present investigations, which are as yet not completed, definite information was obtained of the nature of one of the components, and also of the condition of some of the characteristic groups of both components.

The difficulty of getting hold of the components is conditioned

[4] Kondo: *Biochem. Zeitschr.*, xxvi, p. 116, 1910.

[5] Mandel and Neuberg: *Biochem. Zeitschr.*, xiii, p. 148, 1908.

by the fact that chondrosin displays a great resistance towards usual hydrolytic agents, so that it was not possible to bring about hydrolysis of the molecule without simultaneously affecting the integrity of the components. This peculiarity of chondrosin was known to Schmiedeberg.

In course of the present investigation a method was found which brought about a cleavage of the chondrosin molecule permitting the isolation of at least one component. The method consisted in the use of sodium amalgam. The details of the method are described in the experimental part. The substance isolated was the usual glucuronic acid.

The substance was identified by the phenyl and parabromphenylhydrazine derivatives, by the fact that it yielded saccharic acid on oxidation with bromine, as well as by oxidation with nitric acid. The phenylhydrazine derivative had all the properties and the composition of the phenylhydrazid of the osazone. The substance was found to be identical with the one obtained under similar conditions from glucuron and first described by Thierfelder.

The *p*-bromphenylhydrazine derivative had the composition of the substance described by Guido Goldschmiedt and Ernest Zerner.[6] Under the same conditions we were able to obtain the identical body from pure glucuron. In accord with Goldschmiedt and Zerner we were unable to obtain the substance described by Neuberg. However, the formation of a derivative with only one molecule of hydrazine to one molecule of glucuron is *a priori* not impossible. We made no special effort in obtaining it for the reason that Goldschmiedt and Zerner's substance formed very readily from both pure glucuron and from the hydrolytic products of chondrosin.

Regarding the mode of the union between the glucuronic acid and the second component it was established first that the glucuronic acid is not bound to the amino group of the second component for the reason that the presence of an unsubstituted amino group in the chondrosin was demonstrated by the nitrous acid process.

Further, it was also made obvious that the carbonyl group of the glucuronic acid does not take part in the linking of the two components. The reasons are the following. On oxidation of chondro-

[6] Goldschmiedt and Zerner: *Ber. d. deutsch. chem. Gesellsch.*, xlvi, p. 113, 1913.

sin with nitric acid a product is obtained which on distillation with hydrochloric acid gives rise to a minimal quantity of furfurol, while the original substance yields a quantity required by a complex composed of one molecule of glucuronic acid and one of a carbohydrate of approximately equal molecular weight. The same oxidation product does not contain free saccharic acid, which could be identified as the acid potassium salt. However, the salt is readily obtained as soon as the oxidation product is hydrolyzed with alkali. Hence, the original oxidation product contains saccharic acid in conjugated form. This in its turn carries convincing evidence that the carbonyl group of the glucuronic acid is not the place of the union between the two components.

Whether or not the carboxyl group of the glucuronic acid is free or serves for linking the two components cannot be stated with certainty at present. Decision has to be postponed until the chemical nature of the second constituent is established. Chondrosin apparently contains only one free carboxyl group, and if the second component contains none then the conclusion will be obvious, that the carboxyl group of the glucuronic acid is present in the chondrosin molecule in a free state.

The fact that chondrosin contains both the carboxyl and the amino groups in a free state, while chondroitin sulphuric acid does not possess reducing properties, and does not react with nitrous acid to form nitrogen gas, indicates that both groups are combined in the more complex molecule with other radicals.

EXPERIMENTAL.

Preparation of chondroitin sulphuric acid barium salt.

Nasal septums of cattle were freed from bone and other extraneous material and ground through a meat chopper. Portions of 5 kgm. each were allowed to stand for two days with 10 liters of 2 per cent potassium hydrate solution. The extract was strained through a cloth and the residue again subjected to the same treatment with 5 liters of potassium hydrate solution and finally washed once with water. The united extracts were acidified with acetic acid and concentrated on the steam bath with an excess of barium carbonate to about half of their volume. The clear liquid was then poured off and the residue thrown on a folded filter and allowed

to drain off. This filtrate was united with the decanted liquid and the whole acidified with acetic acid and evaporated as before with barium carbonate to about 2 liters.

The separated protein and barium carbonate were removed by centrifugalization and the clear yellow liquid dropped into eight times its volume of glacial acetic acid and kept agitated by a turbine. The acid potassium salt thus obtained was filtered by suction, washed with glacial acetic acid and finally with alcohol and ether.

Two hundred grams of this product, which gave a slight biuret test, were dissolved in 10 liters of water and while the solution was kept stirred with a turbine, a solution of basic lead acetate was dropped in until complete precipitation had taken place. The lead salt, after having been washed several times by grinding in a mortar with water and filtering with suction, was suspended in 5 liters of water and with the addition of 100 grams of barium acetate and 50 cc. of acetic acid decomposed by long treatment with hydrogen sulphide with constant stirring. After standing for twelve hours the lead sulphide was filtered off and the slightly turbid solution of the barium salt precipitated by the addition of about one-third of its volume of 95 per cent alcohol. After filtering and washing with 50 per cent alcohol, then with 95 per cent, and finally with absolute alcohol and ether, the product after drying was a pure white powder, showing no trace of biuret.

This product is a mixture of the barium salts of chondroitin and chondroitin sulphuric acid. It showed no reduction of Fehling's solution and in the apparatus of Van Slyke no amino nitrogen.

0.5070 gram substance gave 7.75 cc. $\frac{N}{10}$ NH_3.
0.4650 gram substance gave 7.40 cc. $\frac{N}{10}$ NH_3.
0.4166 gram substance gave 0.1125 gram $BaSO_4$.

	Calculated for $C_{18}H_{27}NSO_{17}$:	Found:
N	2.01 per cent.	(1) 2.14 per cent.
		(2) 2.23 per cent.
S	4.60 per cent.	3.72 per cent.

Preparation of chondrosin.

Fifty grams of the barium salt of chondroitin sulphuric acid were dissolved in 150 cc. of equal parts of concentrated hydrochloric acid

and water, and heated for an hour on the water bath. Barium sulphate begins to separate at once, and after one hour the solution, which is only slightly colored, shows its maximum reduction of Fehling's solution, and all the nitrogen is present as amino nitrogen. The filtered solution was evaporated in vacuum to a very thick syrup and this was taken up in about 40 cc. of hot water and poured into 500 cc. of absolute alcohol. Partial precipitation of the chondrosin hydrochloric acid salt as a nearly colorless flocculent precipitate takes place. After standing over night, two volumes of absolute ether were added and the precipitate filtered with suction and thoroughly washed with absolute ether. For a final purification the product thus obtained is dissolved in about its own weight of water and precipitated and washed again as above described. It is a quite colorless powder, which when properly washed is not hygroscopic. The yield of the first product, dried over calcium chloride for two days in vacuum, was 27 grams.

0.1966 gram substance dried to constant weight at 100° gave 12.7 cc. amino N at 21°, 764 mm. N = 3.67 per cent.

0.3319 gram chondroitin sulphuric acid barium salt gave 5.8 cc. $\frac{N}{10}$ NH_3. N = 2.44 per cent.

0.5044 gram substance hydrolyzed for one hour with one part HCl and one part H_2O gave 18.3 cc. amino N at 16°, 760 mm. N = 2.11 per cent.

Cleavage of chondrosin with sodium amalgam.

Twelve grams of chondrosin hydrochloride in 100 cc. of water were allowed to stand with 100 grams of 2.5 per cent sodium amalgam. After about twenty minutes at ordinary temperature the solution takes on a bright yellow color and at the same time an evolution of ammonia begins. The solution is then neutralized with sulphuric acid and 100 grams of sodium amalgam are again added, the temperature always being kept at about 25°. After about one hour the solution is again acidified with sulphuric acid and allowed to stand over night, after the addition of a third 100 grams of amalgam. The solution is then separated from the mercury and filtered from the sodium sulphate with the addition of some animal charcoal.

Preparation of phenylhydrazine compound.

The solution obtained by the above treatment was diluted to about 200 cc. and after the addition of 15 grams of phenylhydrazine in 50 per cent acetic acid allowed to stand on the water bath. After about 20–30 minutes a dark tarry material has separated together with a small amount of solid material. At this point the solution is quickly filtered with suction on a hot funnel into a hot flask and the filtrate allowed to stand for two to three hours on the water bath. After this time the solution is filled with long yellow needles to which very little of the light-colored oil adheres. The crystals were filtered and washed with warm water and then with cold absolute alcohol until no more oil drops could be discerned under the microscope. When dried in vacuum the product melts with decomposition at about 115°. Attempts to recrystallize did not effect a purification and therefore the first product was used for the analysis.

0.1188 gram substance gave 0.2484 gram CO_2; 0.0634 gram H_2O.
0.1278 gram substance gave 19.2 cc. N, 17°, 758 mm.

	Calculated for $C_{24}H_{26}N_6O_4 \cdot 1 \cdot 5H_2O$:	Found:
C	58.93 per cent.	58.80 per cent.
H	5.93 per cent.	6.12 per cent.
N	17.17 per cent.	17.33 per cent.

0.0599 gram substance in 5 cc. pyridine alcohol mixture rotated in a 0.5 dm. tube with D-light – 0.32°.

Phenylhydrazine compound from glucuron.[7]

One gram glucuron was warmed on the water bath for two hours with a little more than the required amount of normal sodium hydrate. The solution was neutralized with acetic acid, and 4 grams of phenylhydrazine in 50 per cent acetic acid and 4 grams of sodium acetate were added. After a short time crystallization of the phenylhydrazine compound in long yellow needles began and after three hours their amount had reached 1.6 grams. The material was purified by washing with cold alcohol and ether. It decomposed at about 115°.

[7] Thierfelder: *Zeitschr. f. physiol. Chem.*, xi, p. 395, 1887.

0.0598 gram substance in 5 cc. pyridine alcohol mixture rotated in a 0.5 dm. tube with D-light – 0.322°.

By prolonged heating in vacuum at 100° the substance loses weight but before becoming constant decomposition sets in, while at lower temperatures no loss of weight was observed.

Parabromphenylhydrazine compound from chondrosin.

Twenty grams of chondrosin hydrochloride were treated in the usual way with sodium amalgam, and the resulting solution, after acidifying with acetic acid, heated on the water bath with 4 grams of parabromphenylhydrazine hydrochloride. After about one hour the solution was filtered from the separated tarry material and allowed to stand three hours longer on the water bath. The impure phenylhydrazine compound obtained was washed with alcohol until the impurities had been removed and then with ether. The substance may be recrystallized by dissolving in as little as possible of a mixture of one part of 50 per cent acetic acid and one part alcohol and then precipitating by the addition of two parts of hot water.

0.0568 gram of the substance in 5 cc. pyridine alcohol mixture rotated with D-light in a 0.5 dm. tube – 0.8°.

0.0614 gram twice recrystallized under the same conditions rotated – 0.75°.

0.1454 gram substance gave 12.5 cc. N, 22°, 762 mm.

0.1126 gram substance gave 0.0118 gram AgBr.

	Calculated:	Found:
Br	28.95 per cent.	27.09 per cent.
N	10.15 per cent.	9.72 per cent.

Parabromphenylhydrazine compound from glucuron.

One gram of glucuron in 100 cc. of water was heated on the water bath with 2.5 grams of parabromphenylhydrazine hydrochloric acid salt, which had been purified by twice recrystallizing from dilute hydrochloric acid and washing with ether, and 2.5 grams of sodium acetate. After about one hour 0.3 gram of a yellow crystalline substance had separated. The mother liquor filtered from the first crystallization gave upon further heating 0.2 gram more of the same substance. After recrystallization from 50 per cent acetic acid and alcohol it had the following composition.

0.1436 gram substance gave 13 cc. N at 22°, 758 mm.
0.1268 gram substance gave 0.0824 gram AgBr.
0.1338 gram substance gave 0.0124 gram Na_2SO_4.

	Calculated for $Br_2C_{18}H_{17}O_5N_4Na$	Calculated for $(C_{12}H_{17}N_2O_7Br)$:	Found:
Br	28.95 per cent.	20.97	27.65 per cent.
Na	4.17 per cent.		3.01 per cent.
N	10.15 per cent.	7.21	10.20 per cent.

0.0653 gram substance in 5 cc. pyridine alcohol mixture rotated in a 0.5 dm. tube with D-light − 0.90°.

Nitric acid oxidation of the products of hydrolysis of chondrosin.

Twenty-five grams of chondrosin hydrochloride were treated with sodium amalgam in exactly the same manner as described in the previous experiment. The solution, after having been freed from inorganic salts by precipitation with alcohol, was evaporated to a syrup. This syrup was quickly evaporated in a flat dish with nitric acid composed of one part of nitric acid, specific gravity of 1.42, and one part of water. The residue was then evaporated several times with water and finally taken up in 15 cc. of water and neutralized with potassium hydrate. Upon addition of glacial acetic acid the crystallization of the acid potassium saccharate began after a short time. After two days the yield amounted to 1.1 grams. For analysis it was recrystallized from water.

0.1253 gram substance gave 0.0427 gram K_2SO_4.

	Calculated:	Found:
K	15.72 per cent.	15.32 per cent.

Nitric acid oxidation of chondrosin and subsequent hydrolysis.

Ten grams of chondrosin hydrochloride were evaporated in a flat dish on a water bath with 10 cc. of nitric acid and 10 cc. of water. The residue was dissolved in 10 cc. of water and 5 cc. of nitric acid and again evaporated to dryness. The final residue was then dissolved in 10 cc. of water and the solution divided into two parts of 7 and 3 cc. each and neutralized in the cold with potassium hydrate. The larger portion, after addition of 2 cc. of 50 per cent potassium hydrate, was allowed to stand for two hours on the water bath and then acidified with acetic acid. After several

hours the acid potassium saccharate began to separate. The yield amounted to 0.5 gram after two days. From the smaller portion, after addition of acetic acid, only a trace of the same substance separated after long standing.

0.1276 gram substance gave 0.0440 gram K_2SO_4.

	Calculated:	Found:
K	15.72 per cent.	15.46 per cent.

Brom oxidation of the products of hydrolysis of chondrosin.

A solution of 25 grams of chondrosin hydrochloride was treated in the usual way with 2.5 per cent sodium amalgam. The solution was acidified with hydrochloric acid and allowed to stand for five days at ordinary temperature with an excess of bromine. It was then concentrated in vacuum to about 100 cc. and the principal amount of the salt separated by pouring the substance into hot absolute alcohol. The alcoholic solution was concentrated in vacuum to a syrup, taken up in water, and the halogen determined in an aliquot part. The requisite amount of lead acetate was then added to the remainder of the solution and the lead chloride and bromide removed by filtration. The excess of lead was then removed by hydrogen sulphide and the solution evaporated in vacuum to about 30 cc. It was then neutralized with potassium hydrate and after the addition of 10 cc. of glacial acetic acid allowed to stand for two days in the refrigerator. The separated crystals were filtered on suction and the product recrystallized from water. After drying it amounted to 1.6 grams.

0.1209 gram substance gave 0.0419 gram K_2SO_4.
0.1210 gram substance gave 0.0466 gram H_2O; 0.1242 gram CO_2.

	Calculated for $C_6H_9O_8K$:	Found:
H	3.65 per cent.	4.28 per cent.
C	27.90 per cent.[8]	27.98 per cent.
K	15.72 per cent.	15.55 per cent.

Furfurol from chondrosin after oxidation with nitric acid.

0.4219 gram of chondrosin (calculated from the nitrogen content) was evaporated to dryness with 5 cc. concentrated nitric acid and

[8] Considering that one atom of carbon is contained in the ash as K_2CO_3.

5 cc. of water. After repeated evaporation with water the solution of the residue was distilled in the usual way with hydrochloric acid of specific gravity 1.06, until no more furfurol was given off. Upon addition of 0.1 gram of phloroglucin 0.0076 gram of phloroglucoside was obtained, corresponding to 0.0218 gram of glucuronic acid, or about one-tenth of the amount present in chondrosin.

Desamido chondrosin.

Three grams of chondrosin hydrochloride in 50 cc. of water were treated with the calculated amount of silver nitrite (1.1 grams). After standing for several hours at ordinary temperature the reaction mixture was warmed on the water bath with occasional shaking. After the solution had been allowed to stand over night at ordinary temperature it was again warmed on the water bath for about two hours, after addition of 0.3 gram of silver nitrate and about 5 cc. of diluted hydrochloric acid. The excess of silver was then removed with a slight excess of hydrochloric acid and the solution evaporated in vacuum to a syrup which was taken up in very little water and poured into dry acetone. The gummy precipitate hardened quickly and was then ground with more dry acetone and washed with ether. The product was then a white amorphous powder resembling chondrosin in all its physical properties and its power to reduce Fehling's solution and gave the same amount of furfurol.

0.3710 gram substance dried at 100° in vacuum gave 0.0575 gram phloroglucoside corresponding to 0.1725 gram glucuronic acid.