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The Isolation of Chondrosamine from Gangliosides and from Submaxillary Mucin *

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According to Klenk [1], the gangliosides are composed of fatty acids, sphingosine, hexoses (in the main galactose), and a hydroxyamino acid, *i.e.* neuraminic acid ("Neuraminsäure"), with the probable composition $C_{10}H_{19}NO_9$. The structure of the latter is unknown. In 1938 one of us [2] noted an agreement in certain qualitative reactions between gangliosides and a mucopolysaccharide present in submaxillary mucin, and therefore submitted hydrolysate of gangliosides to hexosamine determination according to the colorimetric method of Elson and Morgan. Such analyses showed a hexosamine content of about 9 % in pure gangliosides. In view of this result, Klenk [3] tested neuraminic acid for hexosamine, but found no reducing substance in the hydrolysate of this substance. In 1948 Brante [4] applied paper partition chromatography in studies on water-soluble split products of brain lipids. He obtained from ganglioside hydrolysates a very distinct spot, which reduced *m*-phenylendiamine and gave a positive reaction with the Elson-Morgan reagent and with ninhydrin. With different solvents, this spot moved at practically the same rate as chondrosamine in chondroitin sulphuric acid hydrolysates, a rate which differed slightly from that of glucosamine in identical conditions. These findings thus strongly supported the supposition that the ganglioside contains hexosamine, and also suggested that the hexosamine present was chondrosamine. We felt, however, that the question could only be definitely answered by isolation of the substance.

Owing to its greater solubility, chondrosamine hydrochloride does not crystallize from water or water methanol mixtures as readily as glucosamine hydrochloride. When preparing chondrosamine hydrochloride from chondroi-

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tin sulphuric acid by Levene's [5] method we at first had some difficulty in obtaining a crystalline product. If, however, the hydrolysate is evaporated *in vacuo* to a sticky syrup and then dissolved in methanol, and this process repeated a few times, there appears on resolution a crystalline residue which increases on standing. From the mother liquors more crystalline material can obtained by the same treatment. Probably, the removing of the last traces of water by the repeated evaporations with methanol favoured the crystallization. This experience was acted on in the subsequent work. As will be described in detail in the experimental part, we succeeded in isolating from the ganglioside a hexosamine which, by its optical rotation and X-ray diffraction pattern, was identified as chondrosamine. Nothing indicated the coexistence of glucosamine in the analysed material.

The finding that the ganglioside contains chondrosamine prompted us to investigate whether the main carbohydrate of the submaxillary mucin does so, too. — In 1936 one of us [6] showed this mucin to contain two different carbohydrate groups. The main carbohydrate was isolated in small amounts in crystalline form. It was thought to be of disaccharide nature, and to consist of an acetylated hexosamine and a polyhydroxy acid, which was not a hexuronic acid. This view was based on the results of elementary analyses and acetyl determinations, as also on the reducing power and acidity of the substance, and on certain colour reactions, in the first place the Ehrlich reaction with *p*-dimethyl-aminobenzaldehyde. Hexosamine was not isolated from the substance, however. Unlike ordinary N-acetylhexosamine compounds, the main carbohydrate of the submaxillary mucin gives a strong purple reaction on heating with the Ehrlich reagent, even when not previously boiled in alkaline solution. It is further characterized by a very marked humin formation on treatment with acids in the heat and, as has been found later on, by giving a purple colour when tested with Bial's reaction, and by giving off CO_2 on heating with 12 % HCl in the same way as hexuronic acids. Besides this substance, for which we propose the provisional name *sialic acid*, the mucin contains small amounts of carbohydrate of the dihexose-hexosamine type, as well as of the bloodgroup antigen type. If the mucin is purified by frequent reprecipitation from acid solution by diluting with water, the content of the two neutral carbohydrates is reduced to traces, whereas the sialic acid content increases to about 25—30 % of the mucin. A good yield of hexosamine isolated from such a mucin preparation could not be derived from the neutral carbohydrate components. In fact, from 2 g of such a mucin preparation we succeeded in isolating somewhat more than 0.2 g almost pure chondrosamine hydrochloride. — However, this chondrosamine did not derive from the sialic acid. This is shown in the following observations. Boiling the mucin in water

for 1—2 hrs partially decomposes it. If the material thus obtained is evaporated *in vacuo* to dryness and the residue extracted with methanol, part of it dissolves. Fractionation of the methanol solution with increasing amounts of ether yield products which contain increasing amounts of sialic acid (as determined quantitatively with the help of the Bial reaction or of the direct Ehrlich reaction) and decreasing quantities of hexosamine (as determined according to Elson and Morgan after hydrolysis with 2 *N* HCl for 2—12 hrs).

The gangliosides give the reactions characteristic of sialic acid, *i.e.* direct Ehrlich reaction, purple Bial reaction, and strong humin formation and liberation of CO_2 on heating with mineral acid. It may therefore be assumed that the gangliosides contains this (or some closely related) substance. As to the relation between the sialic acid and the neuraminic acid, it should only be pointed out that the isolation procedure for the neuraminic acid includes heating with methanolic HCl and a prolonged heating with baryta, by which certain groups, *e.g.* acetyl groups, might have been split off. The neuraminic acid has so far been isolated only in the form of its non-reducing methoxyl compound. Also this substance gives a strong purple Bial reaction and the direct reaction with the Ehrlich reagent.

Taken altogether, the above observations indicate that the sialic acid both in the submaxillary mucin and in the gangliosides is accompanied by, and probably bound with an easily split linkage to the chondrosamine. As regards the composition of the acid, it is clear that if it contains hexosamine, of which there is at present no direct evidence, this must be in such a combination that it is not liberated on treatment with mineral acids in the heat.

As sialic acid occurs not only in submaxillary mucin and in the gangliosides but also in several other glycoproteins, *e.g.* those of the blood serum, the elucidation of the structure of the sialic acid should be an important task. Attempts to this end are at present in progress in this Institute, as is work on the questions of the relation of the sialic acid to the acid glycoprotein in serum demonstrated by Winzler *et al.*[7], and to the influenza-virus haemagglutination inhibitors.

EXPERIMENTAL

Preparation of ganglioside. In the main the directions given by Klenk [1] were followed. However, the substance was in addition dissolved in water and thoroughly dialysed, in order to remove low-molecular organic and inorganic impurities. The preparation used contained only 0.04 % P. The hexosamine content (determined according to the Elson-Morgan method as modified by Blix [8]) was about 8 %, and the content of neuraminic acid, as determined according to Klenk with the aid of the Bial reaction, about 20 %. The substance gave a clear solution in water.

Isolation of chondrosamine hydrochloride from ganglioside. 1.5 g was heated under reflux with 30 ml 5 *N* HCl for 12 hrs. The humin formed was filtered off and the filtrate

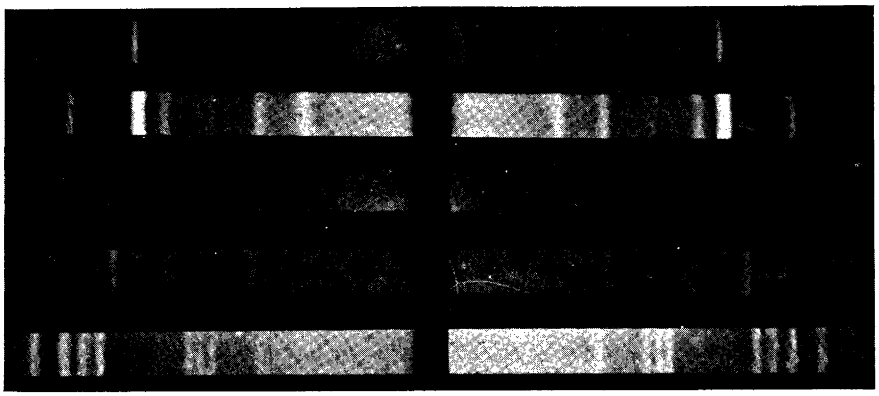

Fig. 1. X-ray powder diagrams of α-chondrosamine HCl (1), hexosamine HCl from ganglioside (2), β-chondrosamine HCl (3), hexosamine HCl from submaxillary mucin (4), α-glucosamine HCl (5).

was shaken twice with two vol. petroleum ether. 1.5 g blood coal was then added to the aqueous phase. The mixture was boiled for a few minutes, filtered and evaporated to a syrup. The syrup was redissolved in warm methanol, and insoluble residues (which contained very little hexosamine) filtered off and discarded. As repeated evaporations from methanol solution yielded no crystalline deposit, the material was heated to 100° with 1 *N* HCl for 24 hrs. The hydrolysate was then treated exactly as described above. After the treatment with blood coal, the solution was evaporated to a syrup. This was taken up in 5 ml methanol, and the solution once more evaporated. After standing for some days, a crystalline deposit appeared, which did not dissolve on addition of methanol. The crystals were washed repeatedly with methanol. On evaporation of the mother liquor, more crystalline material was formed and treated as above. Further repeated evaporations of methanol solution of the material gave more crystalline substance. All crystalline material was collected, and dissolved in about ½ ml distilled water. Methanol was added until crystallization started, and the mixture kept for some days at + 4°. The crystalline deposit was then filtered off, washed and dried. Hexosamine determination according to Elson and Morgan give 84.0 % (calc. 83.1 %). N = 6.32 % (calc. 6.50 %). Optical rotation $[\alpha]_D^{20}$: + 88° (end value). To judge from the N value and the rotation value, the substance still contained some impurities. The X-ray diffraction picture of a once more recrystallized specimen (Fig. 1) showed complete agreement with that of an authentic specimen of α-chondrosamine hydrochloride.

Preparation of submaxillary mucin. The substance was prepared according to the old method of O. Hammarsten, as given by one of us [6]. Some details which are of practical importance in the preparation but which have not been pointed out earlier, should, however, be given here. In order to obtain a coherent mucin clot on dilution of the acid mucin solution with water, it is essential that the submaxillary glands should not be finely minced before the extraction. The gland should only be divided into pieces of about the size of a hazel nut, or somewhat larger. When the glands are minced, the coextracted globulins will prevent the mucin precipitating as a clot, and thereby render it difficult or impossible to isolate. A closer observation of the extraction procedure has shown that

the mucin is in fact, not extracted in the true sense, but flows slowly out into the water from all greater and smaller ducts. If the "extraction" is carried out at + 2°— + 4°, mucin clots may be obtained from the water "extracts" on 5—6 subsequent days. The preparation here investigated was reprecipitated 6 times from acid solution by diluting with water. The dry substance contained 16 % hexosamine and 9.9 % N.

Isolation of chondrosamine hydrochloride from submaxillary mucin. 2 g of the mucin was heated under reflux on the boiling water bath with 30 ml 5 *N* HCl for 14 hrs. To the solution were then added 100 ml methanol and 1.5 g blood coal. The mixture was boiled for some minutes, and filtered. The filtrate was evaporated to a syrup, the latter warmed with 10 ml methanol, and undissolved material filtered off. The filtrate was again evaporated, taken up in 5 ml methanol, and filtered. After a new evaporation, the residue was heated for 18 hrs to + 100° under reflux with 1 *N* HCl. The treatment of the hydrolysate described above was then repeated, the residue taken up in methanol, and the solution thereafter evaporated three times more. Finally, the residue was taken up in 50 ml methanol, and ether was added until a precipitate began to appear. On standing in an open dish for four days, a crystalline deposit was formed. This was filtered off and washed. Repeated evaporations and dissolvings in methanol enabled us to obtain additional crystalline material from the mother liquor. The crystals were collected, dissolved in very little water, and crystallized by adding methanol. Of the fairly pure substance somewhat more than 0.2 g was obtained, which is about 2/3 of the hexosamine present in the mucin taken. Hexosamine determination according to Elson and Morgan gave 84.3 %. N = 6.28 (Dumas). $[\alpha]_D^{20}$: + 93° (end value). The X-ray diffraction pattern was that typical for β-chondrosamine hydrochloride (Fig. 1).

SUMMARY

The isolation and identification of chondrosamine from submaxillary mucin and from gangliosides is described. In these substances, the chondrosamine accompanies and seems to be loosely bound to a probably disaccharidic, structurally still unknown, substance, which is provisionally called *sialic acid*. Some characteristics of the latter are given, and its relation to the so-called neuraminic acid, isolated by Klenk from gangliosides, is shortly discussed.

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