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Neuraminic acid. - VI. Acetylneuraminic acids in lamprey liver and eggs, and in eggs from two teleostei species *

by

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The lampreys, cyclostomata which possess a very simple organization, are considered as one of the most primitive vertebrates. Some of their biochemical aspects, such as the nature of their haemoglobin molecule (5, 16), collagen (15), and fibrinogen (8), have been studied recently. Referring to acylneuraminic acids (or sialic acids), EYLAR *et al* (9) have indicated that they exist in the blood cells of the sea lamprey (*Petromyzon marinus*) and mentioned that both NGNA ** and NANA ** exist in its erythrocytes. WARREN (22) has found sialic acid in the skin of this species, and SEAL (17) has determinated a relatively high concentration of it in its serum.

The eventual differences or identity between the nature of the sialic acids extracted from the eggs and liver of this primitive cyclostomata and those from the eggs of two more evolved fish species, belonging to the class of the teleostei (*Scomber scombrus* and *Cyclopterus lumpus*), have been studied in this paper. The acylneuraminic acids from the liver of the sea lamprey have also been characterized.

Material and Methods

Materials

Livers from 43 sea lampreys, which come up the rivers of Galicia (N. W. Spain) during the Spring months, were stored in 96 % ethanol at 0-4° during several days. The eggs proceeding of the

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** Abbreviations:
NGNA = N-glycolylneuraminic acid;
NANA = N-acetylneuraminic acid.

same lot (24 animals) were also stored in the same manner. The *Scomber* eggs were used immediately after they were removed from the fresh animals. And the *Cyclopterus* eggs, 80 g, come from a commercial product, named in Germany «Deutscher caviar» (Christensen-Hamburg 4) conserved without chemical preservative.

Preparative methods

For the separation and purification of the acylneuraminic acids from the four materials assayed, we have mainly followed a procedure similar to KLENK-UHLENBRUCK's method (12), as described in a previous publication (10).

The livers, homogenized with ethanol in a mixer, were boiled in the ethanol; the supernatant liquid was discarded, a new quantity of ethanol was added and all was reboiled; the precipitate was washed five times with 0.01 N H_2SO_4 at 0-4° until the pH became 2.5; then it was defatted with 1 li of ethylic ether at 0-4°, twice; the dry product was hydrolyzed five times (80°, 1 h, 0.03 N H_2SO_4), neutralized with $Ba(OH)_2$, passed through the two exchange (connected) columns (Dowes 50 W × 4, 20-50 mesh, and Lewatit MIH, acetate form) and then eluted from the anionic column with formic acid; the fractions rich in sialic acids were eluted and gave a great acute peak, followed by another little peak; after lyophilisation, 263 mg of product were obtained.

The lamprey eggs accompanying tissues were removed and the eggs homogenized, boiled, reboiled and washed as in the above-metioned procedure; after the sixth washing (pH = 2.6), the first two hydrolyzates were put together (the third, giving a non-typical colour with resorcinol, was discarded); the 48.9 mg of acylneuraminic acid (expressed as NANA) calculated in the said hydrolyzates become 34.5 mg after elution.

The *Scomber* eggs, treated in the same manner, gave 36.3 mg of acylneuraminic acids corresponding to two batches of them. Finally, the *Cyclopterus* eggs, after the third washing (pH = 2.8), were hydrolized and the three hydrolyzates put together, totalizing a theoretical concentration of 83 mg; the crystallization of the resulting powder (127 mg) gave crystals in needle form.

Analytical methods

Paper chromatography of the sialic acids was made on Schleicher & Schüll 2043 b paper according to our previous publication (6), using the following solvents: (I), *n*-butanol-pyridine-water (6 : 4 : 3, v/v) (3); (II), *n*-butanol-acetic acid-water (4 : 1 : 5, v/v) (3); and (III), *n*-butanol-*n*-propanol-0.1 N HCl (1 : 2 : 1, v/v) (19). The sprays used were: The Ehrlich (2), (6) (direct), Bial (4) and thiobarbituric reagents (1, 20); for the detection of the O-acetyl group the Hestrin (3) reagent was employed.

The infrared spectrogram of the preparations, run in compressed BrK, were obtained in a Perkin-Elmer apparatus, model I.R.8.*

The quantitative determination of the sialic acids (expressed as NANA) was made by the resorcinol (18)-butyl acetate (13) and/or by the thiobarbituric methods (1, 20).

Results

The characteristics of the crystals of the last material assayed and the behaviour of all the products on columns of Dowes and Lewatit, as well as the absorption spectra in the resorcinol-butyl acetate and thiobarbituric reactions and in the infrared (see fig. 1) were the typical of acylneuraminic acids and virtually identical to those of the NANA standard.

Furthermore, as it can be appreciated on the chromatogram I (fig. 2), the four materials produce one spot corresponding to NANA and another one, quicker (of fairly great intensity when the solventi

time of development is 48, 56 and 72 hours. Finally, with the solvent III the NANA spot is the biggest and only one weak and quicker spot appears. The same result is obtained at 40 hours of de-

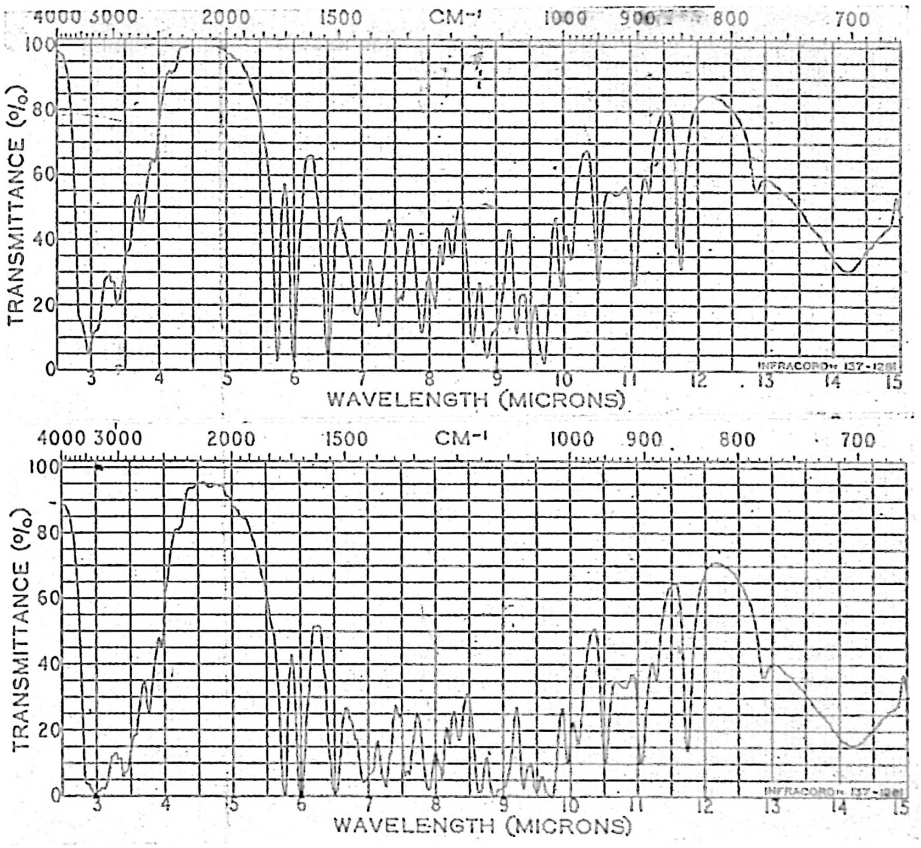

FIG. 1. Infrared patterns of sialic acids from *Cyclopterus lumpus* eggs *(above)*, and standard of N-acetylneuraminic acid *(below)*.

is used) due to the pluriacetylneuramin-derivatives (see below). Identical results were obtained in all the chromatograms developped during 36, 42 and 52 hours, often accompanied by a third, weak and quicker spot. On the chromatogram II the spot corresponding to NANA is clearly the biggest, followed sometimes by another weak spot and by a third spot in the case of the *Cyclopterus* eggs. The same spots are obtained when the velopment. All the chromatograms referred have been revealed with Ehrlich (direct) spray.

Another chromatogram with the same four problem-products was developped during 56 hours with the solvent II and then cut into several straps; each one was revealed with Bial's and thiobarbituric spray, which possess, like the former, both great specificity and sensibility in the conditions employed. The

results were identical to those obtained with the Ehrlich spray.

Finally, the existence of O-acetyl was proved with the Hestrin reagent in form of weak spots, when bigger quantities of substance were put on a paper that was developped with the solvent I during 48 hours; these spots appeared precisely at the height of the diacetylneuraminderivatives.

We have not found NGNA in the four materials employed, but mainly NANA, and a small concentration of N-acetyl-O-acetylneuraminderivatives, the last being probably different of the 7-O-acetyl type (1, 14, 20).

The possibility of finfing free acylneuraminic acids in the above materials was investigated in the following manner:

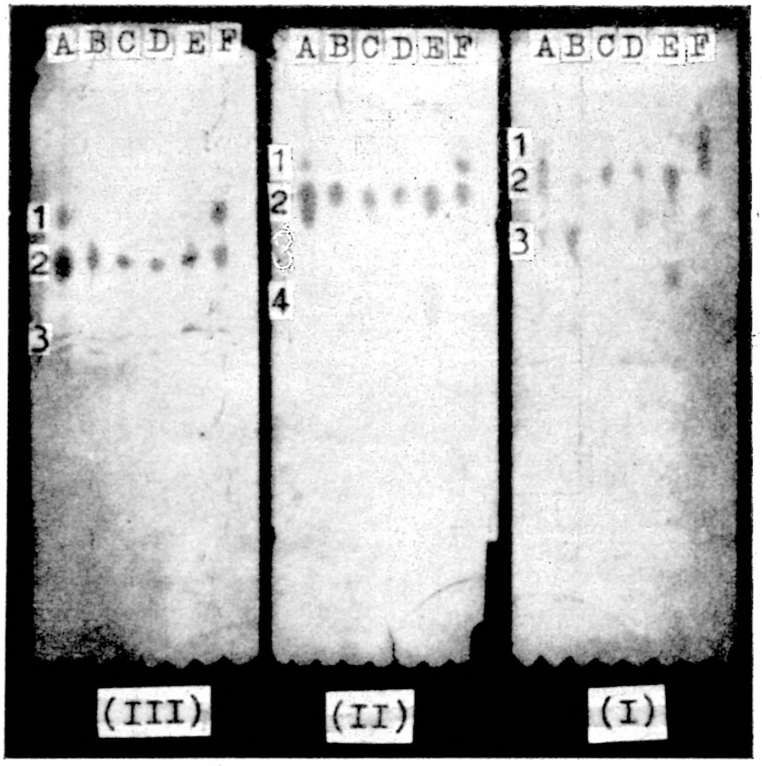

FIG. 2. Chromatography on paper Schleicher & Schüll 2043 b, descending free, Ehrlich's spray. (I) solvent series I, 50 hours. (II), solvent series II, 56 hours. (III), solvent series III, 30 hours. A = Standard (sialic acids from goat colostrum) (7). B = Problem: sialic acids from lamprey liver. C = Problem: sialic acids from lamprey eggs. D = Problem: sialic acids from *Scomber scombrus* eggs. E = Problem: sialic acids from *Cyclopterus lumpus* eggs. F = Standard (sialic acids from goat milk) (7). 1 = NGNA. 2 = NANA. A1 = F1 = NGNA Standard. A2 = F2 = NANA Standard. B2, C2, D2, E2 = problems.

a) 67 ml ethanol in which 16 lamprey eggs batches have been stored were concentrated *in vacuo*. *b*) A small quantity of the same eggs (before boiling) was homogenized with ethanol and filtrated. *c*) Another small quantity of eggs was extracted with distilled water. The dry product proceeding from *a*) and the residue and concentrated filtrate from *b*)

and *c*) were tested by the thiobarbituric assay; the results were negative. On the other hand, the first sulfuric washing liquid from the *Cyclopterus* eggs gave a sialic acid-positive reaction with the resorcinol test, but none of the other washing liquids did; it was concentrated *in vacuo* and the residue assayed by paper chromatography (solvent III); some weak spots appeared with the Ehrlich spray. Also an appropiate aliquot of the ethanol in which the eggs were stored was concentrated and analyzed in the same manner; the result was confirmatively positive. It can be deduced that the acetylneuraminic acids, which are the sialic acids found in the four materials investigated, are chiefly bound, but also free at small concentration in the *Cyclopterus* eggs.

The concentration in total acylneuraminic acids from each material is as follows: the biggest corresponds to the *Cyclopterus* eggs; it is followed by the *Scomber* and lamprey eggs; the lamprey liver contains besides a great quantity of lipids and carbohydrates which make the isolation and characterization of the sialic acids more difficult.

Discussion

If these results are compared to those obtained by WARREN (21) in the eggs from other teleostei species, it can be appreciated that they are similar in what concerns the nature and state of the sialic acids from shad and perch eggs, but not in what refers to the brook trout and the rainbow trout eggs sialic acids.

In spite of the fact that the sea lamprey is a primitive vertebrate, it can be deduced that it contains (in its liver and eggs) the same acylneuraminic acids (acetylneuraminic, precisely) as the other two more evolved fish species assayed, at least in what refers to their eggs.

On the other hand, WARREN (22) thinks that the sialic acids appear relatively late in the evolution processus and that they are of the glycolyl type in the case of the invertebrates; besides it is well known that the glycolyl substituted type is accompanied by the acetylneuraminderivatives in mammals, birds, etc.

The nature of the sialic acids from the eggs and liver of the sea lamprey differs from the result obtained by EYLAR and colb. (9) in the blood cells of the same species. From our work we can deduce that all the sialic acids found in the four materials assayed are acetylneuraminderivatives.

Summary

The acylneuraminic acids (or sialic acids) from the eggs and liver of the cyclostomata *Petromyzon marinus* (sea lamprey), a primitive vertebrate, have been isolated and characterized. In view of establishing a comparison, the acylneuraminic acids from the eggs of two other more evolved fish species (*Scomber scombrus* and *Cyclopterus lumpus*), belonging to the class of the teleostei, have been studied in the same manner. The results are very similar: In all four analyzed materials we have found chiefly the N-acetylneuraminic acid (NANA), accompanied by a small concentration of pluriacetylneuraminic acids, but not the N-glycolylneuraminic acid (NGNA). These acids appeared only bound in the first three materials, and also free in a very weak concentration in the case of the fourth product.

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