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UNBOUND SIALIC ACIDS IN FISH EGGS

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SUMMARY

Relatively large quantities of sialic acids occur in an unbound form in trout eggs.

Both N-glycolyl and N-acetylneuraminic acid are found within one cell, the trout egg.

Preliminary studies on the permeability of the egg to free sialic acids are reported.

INTRODUCTION

Sialic acids[1–3] are found in mammals[4,5], birds, amphibians[5], fish[6,7], bacteria[8], and in the eggs of the sea urchin[9]. Several forms of sialic acids (N-glycolyl, N-acetyl, N,O-diacetylneuraminic acid) have been described in the tissues of different animal species. Virtually all of the sialic acids found in tissues are bound either in large protein-carbohydrate complexes or in oligosaccharides such as neuramin-lactose of the mammary gland[10,11].

In this paper we wish to report some unusual characteristics of the sialic acids of trout eggs. Not only is much of the sialic acid free, but two forms of sialic acid are found in one egg.

MATERIALS AND METHODS

Eggs of the eastern brook trout (*Salzelinus fontinalis*) and rainbow trout (*Salmo gairdneri*) were kindly supplied by Dr. K. E. Wolf, Eastern Fish Disease Laboratory, United States Fish and Wildlife Service, Leetown, West Virginia. The eggs were removed from the female, placed in Tyrode's solution and transported in a container containing crushed ice. Experiments commenced within 20 h of removal from the female and continued for a further 48 h. Eggs were stored in Tyrode's solution at 4°. The eggs of shad (*Alosa sapidissima*) and perch (*Perca flavescens*) were removed from fish obtained from commercial fishermen within 2 h of the time of catching.

Determinations of sialic acids reported in this paper were carried out by the thiobarbituric acid assay[12]. The orcinol assay for sialic acid[13] was used in some of the permeability studies. The thiobarbituric acid assay was carried out on hydrolyzed (80°, 1 h, 0.1 *N* H_2SO_4) (see ref. 14, 15) and unhydrolyzed preparations of egg homogenates. Since the thiobarbituric acid assay measures only unbound sialic acids the two determinations gave the total, free and bound sialic acid content of the eggs. For standards, N-acetylneuraminic acid isolated from blood proteins and N-glycolylneuraminic acid from pig submaxillary gland mucin, were used[16]. The molecular weight of N-acetylneuraminic acid, 309, is used for all calculations.

Ascending chromatography was carried out on Whatman No. 1 paper for 20 h in two solvents which were capable of separating N-acetyl from N-glycolylneuraminic acid. Solvent 1 is *n*-butanol–*n*-propanol–0.1 *N* HCl, (1:2:1) (see ref. 17), Solvent 2, ethanol–water–concentrated ammonia, (80:20:1). Two other solvents were also used: Solvent 3, *sec.*-butanol–acetic acid–water, (4:1:5) and Solvent 4, ethyl acetate–pyridine–water, (1:1:2).

Material isolated from trout eggs was chromatographed alone and also mixed with N-acetylneuraminic acid and N-glycolylneuraminic acid. Sialic acids were located on paper chromatograms by spraying with modified reagents of the thiobarbituric acid assay[18].

RESULTS AND DISCUSSION

It can be seen in Table I that the sialic acid contents of trout, shad and perch eggs are high and that approximately half of the sialic acids of brook and rainbow trout eggs are free. Sialic acids account for over 4% of the dry weight of shad eggs. However,

TABLE I

SIALIC ACID CONTENT OF FISH EGGS

Approximately 1 ml of eggs was added to a graduated tube containing 5 ml of 0.1 *M* KCl solution. The eggs were homogenized in a glass homogenizer and aliquots were used for determination of free and total sialic acid content. Aliquots were also dried *in vacuo* over P_2O_5 for 48 h for dry weight and nitrogen analysis. Determinations were made on 5 samples of brook trout eggs, 3 samples of rainbow trout eggs, and one sample each of shad and perch eggs.

Source	*Free mg/g dry egg*	*Bound* mg/g dry egg*	*Total mg/g dry egg*	*% Free*	*% Nitrogen in dried egg***
Brook trout	2.44 (1.86–2.97)	2.74	5.18 (4.55–5.86)	47.2	11.11
Rainbow trout	8.01 (7.08–8.78)	6.99	15.0 (14.06–16.6)	53.5	10.79
Shad	0.35	43.50	44.10	0.8	11.94
Perch	0.48	21.12	21.60	2.2	8.55

* Calculated by difference.
** Determined by the micro Kjeldahl method.

very little of the sialic acid of shad and perch is free. The free sialic acid of the trout egg was dialysable and could not be precipitated by a solution of 5 % phosphotungstic acid in 2 N HCl. Each brook trout egg, with a volume of 0.07 ml, contains a total of 70 μg of sialic acid of which 33 μg is free. In contrast to the large amounts of sialic acid in eggs, the semen of shad and trout contains little or no sialic acids.

In order to see whether sialic acids in eggs were bound in small dialysable oligosaccharides such as neuramin-lactose[10,11], extracts of trout eggs were dialyzed and the dialysis fluid hydrolyzed (0.1 N H_2SO_4, 80° for 1 h) and assayed. There was no difference in the values of the sialic acid content of hydrolyzed and unhydrolyzed dialysis fluid as determined by the thiobarbituric acid method. This indicates the absence of small, dialysable sialic acid-containing oligosaccharides.

Characterization of sialic acids

The sialic acids from trout egg resembled authentic sialic acids in that they were adsorbed by Norit A and could be eluted with 50 % aqueous methanol[19]. They also behaved similarly on columns of Dowex-1 formate and Dowex-1 acetate. The absorption spectra in the direct Ehrlich, orcinol, diphenylamine[13], resorcinol[20], and thiobarbituric acid reactions were virtually the same. Neuraminidase, purified from the culture fluid of *Clostridium perfringens*, was capable of freeing the bound sialic acid of trout eggs.

Sialic acids were isolated by chromatographic methods[15,16,21] from hydrolyzed brown trout egg homogenates (total sialic acids), from unhydrolyzed egg homogenates (free sialic acids) and from hydrolyzed egg protein (bound sialic acids). Egg protein was obtained by precipitation, by addition of water to eggs which had been homogenized in physiological saline. In one preparation approximately half of the total sialic acids were found to co-chromatograph in solvents 1 and 2 with N-glycolylneuraminic acid and half with N-acetylneuraminic acid (Table II). The infrared spectrum of this material was identical with that of an equimolar mixture of N-acetyl and N-glycolylneuraminic acid (KBr pellet).

TABLE II

R_F VALUES OF SIALIC ACID FROM TROUT EGGS

Solvent		*Sialic acid from trout ova*		*N-Acetylneuraminic acid*	*N-Glycolylneuraminic acid*
		Free	*Bound**		
1. *n*-butanol *n*-propanol 0.1 N HCl	1 2 1	0.35 0.45	0.35 0.45	0.45	0.35
2. Ethanol Water Ammonia	8 2 1	0.20 0.30	0.20 0.30	0.30	0.20
3. *Sec*-butanol Acetic acid Water	4 1 5	0.78	0.76	0.75	0.76
4. Ethyl acetate Pyridine Water	1 1 2	0.69	0.71	0.71	0.71

* After hydrolysis of egg protein.

Both types of sialic acids were found free and bound and as estimated on paper chromatograms there was great variation from one batch of eggs to another in the relative amount of N-glycolyl to N-acetylneuraminic acid in the free and bound compartments. In one sample no N-acetylneuraminic acid was found in the free form. However, the total amount of sialic acid in several batches of trout eggs was found to be constant.

Permeability studies

Preliminary studies have been carried out on the changes in the concentration of free sialic acid upon fertilization of trout eggs. When trout sperm are added to trout eggs suspended in distilled water at room temperature (23°) there is a marked outpouring of free sialic acid (Table III). However a similar, but variable response has been found upon the addition of boiled sperm, magnesium or calcium ions to eggs suspended in distilled water. Antagonism between calcium and magnesium ions has been observed. Temperature has a marked effect upon the system. Loss of sialic acid does not take place at 0°, but at 37° there is great loss of sialic acid from eggs suspended in distilled water even without additions. It should be emphasized that the system is very sensitive, and is erratic in its response. Whether the loss of sialic acid by eggs has any relevance to the process of fertilization is not known.

TABLE III

RELEASE OF SIALIC ACIDS FROM TROUT EGGS

Each tube contained 4 brown trout eggs in 1 ml of suspending medium which contained the additions listed below. Each egg contains approximately 70 μg of sialic acid of which 33 μg is free. After incubation for 40 min at 23°, 0.75 ml of the medium was removed, 0.75 ml 5% phosphotungstic acid in 2 *N* HCl was added to the aliquot and this was centrifuged. The orcinol reaction[13] was carried out on 1.0 ml of the supernatant solution. Comparable experiments have been carried out in which 0.3 ml of the suspending medium was assayed by the thiobarbituric acid assay without phosphotungstic acid treatment. Concentrations of cations in Expt. B are optimal.

Additions	*μg sialic acid lost per egg*
A. None	2.2
Trout semen	25.2
Trout semen (heated 100°, 15 min)	25.0
Trout semen (no eggs)	0.3
B. None	0.7
$CaCl_2$ (0.1 μmole)	15.0
$MgCl_2$ (1.5 μmoles)	18.0
$CaCl_2$ (0.1 μmole) + $MgCl_2$ (1.5 μmoles)	1.2

DISCUSSION

Free sialic acids have not been described in nature except for small amounts in human cerebrospinal fluid[22]. The amount of free sialic acids in the trout egg is far greater and is a large fraction of the total sialic acid of the cell. Free sialic acid has also been found in hog gastric tissue. However the relatively acidic condition at this site is probably sufficient to cause postmortem release of sialic acid[23]. Sialic acid is known to be bound by acid labile bonds.

The presence of a sialidase (neuraminidase) in trout eggs might account for the occurrence of free sialic acids but none has been detected. Neuraminic acid aldolase[24] also appears to be absent from trout eggs. In this regard there is only 12 % as much free N-acetyl hexosamine as free sialic acid on a molar basis in trout eggs. If neuraminic acid aldolase was present one would expect to find these proportions reversed, for the equilibrium of the aldolase reaction favors degradation of sialic acids.

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