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On the Chemical Composition of some Mucous Substances of Fish

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The mucous substances of some fishes have been studied. The predominant component of the external surface mucus seems to be a simple protein, in the case of myxinoids a protein which precipitates in fibres. Together with the simple protein there are also small, varying amounts of nucleic acid and glycoproteins. The glycoproteins contain hexosamine, galactose, fucose, and sialic acid, and thus seem to be of the same types as are present in mucous secretions of other vertebrates.

In addition to ribose (nucleic acid) and in some cases small amounts of fucose, and/or glucose, the fish-roe preparations contained hexosamine, galactose, mannose, and sialic acid as monosaccharide constituents. The carbohydrate moiety probably forms the substantial part of the jelly coat of the roe.

The epithelial mucus of mammals has a fairly uniform composition, its main components being two glycoproteins[1]. One of these has a prosthetic group containing acetylated hexosamine, galactose, and fucose; the other contains acetylated hexosamine and sialic acid. As a rule they occur together, and are usually inseparable by ordinary physico-chemical procedures. The chemical differences found in mucus specimens from different organs, species, or individuals may be differences in proportion between the two types of prosthetic groupings, differences in the relative content of some monosaccharide constituent such as fucose or galactosamine, or slight variations in the acyl groups substituted to the sialic acid molecule. Up to now no exception from this pattern has been reported.

Investigations on frog spawn have shown the mucous, gelatinous coating of the spawn to contain the same main carbohydrate constituents, *viz.* hexosamine, galactose, fucose, and probably, sialic acid [2,3].

Preliminary analyses of mucosal scrapings from birds (pheasants and geese) have shown the same main carbohydrate components (Werner, unpublished data).

Thus the same types of glycoproteins seem to be prevalent in the epithelial mucus of mammals, birds, and amphibians. We therefore decided to investigate some mucous substances of the lower vertebrates, fishes, and cyclostomes.

MATERIAL

The external mucous coatings of plaice (*Pleuronectes platessa*), cod (*Gadus callarias*), whiting (*Gadus merlangus*), eel (*Anguilla vulgaris*), pike (*Esox lucius*), ray (*Raja sp.*), and hagfish (*Myxine glutinosa*) were collected, care being taken to avoid contamination from both external and internal sources (roe, milt, *etc*,). The preparations were dehydrated in ethanol, acetone, and ether, and subsequently analysed.

Samples of roe from perch (*Perca fluviatilis*), pike-perch (*Lucioperca sandra*), cod (*Gadus callarias*), burbot (*Lota vulgaris*), whitefish (*Coregonus lavaretus*), and Baltic herring (*Clupea harengus*), and of milt from burbot (*Lota vulgaris*) were freed from membranes, washed with aqueous (50 %) ethanol, dehydrated by treatment with ethanol, acetone, and ether, and analysed.

Some other samples of roe from pike-perch, burbot, and whitefish, and of milt from burbot were instead lyophilized, and subsequently analysed. These preparations, however, gave slightly turbid solutions in the orcinol-sulphuric-acid reaction, probably owing to the presence of some lipid constituents, and they were therefore not used for quantitative analysis.

ANALYTICAL METHODS

Nitrogen was determined by the Kjeldahl method [4].

Hexosamine was assayed according to Blix's modification of the Morgan-Elson method [5]. Before analysis the samples were hydrolysed for 14 and 18 h with 2 N hydrochloric acid at 100°C. The two hydrolysis-times gave nearly identical hexosamine values. The readings were made at 530 mμ in a Beckman B spectrophotometer.

Hexose, *pentose*, and *methylpentose* were determined by the orcinol-sulphuric-acid method in Vasseur's modification [6]. After a heating time of 20 min the absorbancies were measured at 495 mμ, where the sugars present have roughly the same extinction coefficients. As chromatography revealed galactose to be the predominant sugar in most preparations this sugar was chosen as standard.

The *methylpentose* content was determined separately by Dische's method [7]. A heating time of 10 min was used. A standard with 10 μg of fucose was run simultaneously with the unknown samples. The readings were made 4 to 6 h after the addition of cysteine, because at this time the absorbancy depending on the fucose content has reached a maximum and nearly constant value [8,9].

Most of the preparations were found to contain material interfering with the determination. When cysteine was added, a colour with maximum absorption at about 395 mμ developed instantly, and then gradually disappeared in 2 to 3 h. The absorption due to fucose seemed to develop, independently of the interfering reaction, in the course of 4 to 6 h. Thus when very little or no fucose was present there was only a fading of the initial colour after addition of cysteine; but when the fucose content was higher, the absorption first decreased and later increased. When the fucose content exceeded about 1 % there was no fading, the colour intensity rising from the beginning. Thus the influence of the interfering material was probably eliminated by not making the readings until at least 4 h after the addition of cysteine.

The absorptions developing during the heating with sulphuric acid were read off before the addition of cysteine, and subtracted from the values obtained 4 to 6 h after cysteine had been added.

The methylpentose values found are reported as fucose, because this sugar was the only methylpentose revealed by chromatography.

Sialic acid was assayed by the quantitative Ehrlich and Bial reactions described previously [10]. The results obtained with the two methods tallied very well, and it is thus probable that the preparations contain a sialic acid. This is further supported by the fact that the absorption spectra in all but two cases (ray and hagfish) showed absorption maxima at the same wavelengths as crystalline sialic acid.

For qualitative demonstration of *hexuronic acid* most of the preparations were examined by the naphthoresorcinol method of Fishman *et al.*[11] No hexuronic acid could be traced by this method.

Table 1. Percentage composition of the dried external mucus of some fishes. The figures are corrected for ash and moisture content.

Species	N	Carbohydrate (total)	Hexosamine	Hexose + Pentose + Methylpentose	Fucose	Sialic acid	P
Teleostei							
Pleuronectes platessa	13.5	8.5	3.0	5.0	2.1	0.5	
Gadus callarias	15.2	4.0	0.6	2.6	0.1	0.8	1.0
Gadus merlangus	13.8	4.8	0.9	3.1	0.1	0.8	
Anguilla vulgaris	14.1	3.7	1.0	1.3	[0.1][1]	1.4	
Esox lucius	13.8	8.3	3.5	3.2	0.4	1.6	
Elasmobranchii							
Raja sp.	12.7	14.4	4.2	10.2	1.4	<0.2	
Cyclostomi							
Myxine glutinosa	13.6	3.3	1.8	1.5	[0.1][2]	<0.2	0.2

[1] No fucose spot could be detected on the chromatogram.
[2] A fucose spot could not with certainty be detected on the chromatogram.

All analytical figures given are means of at least two separate determinations, and are corrected for ash and moisture content.

Chromatography. The carbohydrate composition was studied by one-dimensional chromatography on Munktell OB filter-paper. Lutidine-*n*-amyl alcohol [12] proved to be the most suitable solvent. The papers were sprayed with the aniline hydrogen phthalate reagent of Partridge [13]. The preparations were hydrolysed with 2 N sulphuric acid at 100°C for one hour. $Ba(OH)_2$ was added to pH 6, and, after centrifuging, the clear supernatant was taken for analysis. A hydrolysis time of one hour was found suitable. Mannose was the sugar most slowly split from the protein. After about one hour it had reached a concentration high enough to be demonstrable by chromatography. A ribose spot was visible on all chromatograms after hydrolysis for one hour, but after a hydrolysis time of 2 h no ribose could be traced by paper chromatography.

The amino acids were assayed by two-dimensional paper chromatography, using ascending phenol-ammonia-cupron in one direction and descending pyridine—amyl-

Table 2. Percentage composition of dried roe and milt from some fishes. The figures are corrected for ash and moisture content.

Material	Carbohydrate (total)	Hexosamine	Hexose + Pentose + Methylpentose	Fucose	Sialic acid	P
Roe from						
Perca fluviatilis	12.5	1.8	8.6	[0.3] [1]	2.1	
Lucioperca sandra	8.5	2.0	3.9	0.0	2.6	
Gadus callarias	3.3	0.9	1.9	0.0	0.5	1.1
Lota vulgaris	4.8	1.3	2.2	0.0	1.3	1.0
Coregonus lavaretus	7.9	1.6	3.9	0.3	2.4	
Clupea harengus	5.1	1.3	3.0	0.2	0.8	
Milt from						
Lota vulgaris	4.0	0.4	3.2	0.0	0.4	3.3

[1] No fucose spot could be detected on the chromatogram.

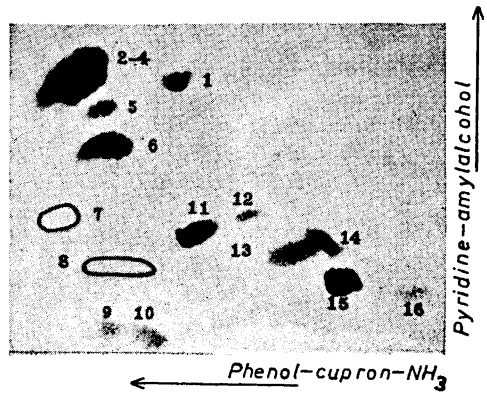

Fig. 1. Two-dimensional chromatogram showing the amino acid pattern of the external mucus of eel. Solvents, phenol-cupron-ammonia/pyridine-amyl alcohol.

1. Tyrosine
2. Phenylalanine
3. *Iso*-leucine
4. Leucine
5. Methionine
6. Valine
7. Proline
8. Histidine
9. Arginine
10. Lysine
11. Alanine
12. Threonine
13. Glycine
14. Serine
15. Glutamic acid
16. Aspartic acid

alcohol in the other. The technique described by de Verdier and Ågren was employed [14]. This procedure allows good separation of all ordinary amino acids except phenylalanine, leucine, and isoleucine.

RESULTS

In the dried state the preparations of external mucus were light white-greyish powder, except the one from the hagfish. The hagfish mucus maintained throughout the drying and grinding procedure (several different methods were used) a distinct fibrous structure and was, in the dry state, similar to

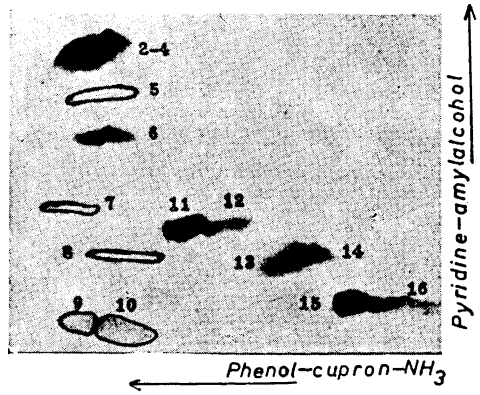

Fig. 2. Two-dimensional chromatogram showing the amino acid pattern of the external mucus of ray. Solvents and designation as in Fig. 1.

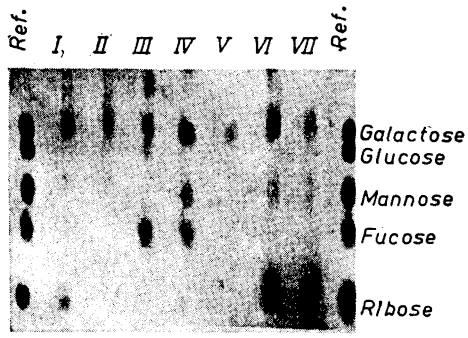

Fig. 3. Chromatogram showing sugar components of the external mucous coatings of some fishes. I, cod; II, eel; III, plaice; IV, ray; V, hagfish; VI, pike; VII, whiting. Solvent, lutidine-*n*-amyl alcohol.

short-clipped cotton wool. The dried roe and milt preparations were yellowish powders.

The analytical figures are given in Tables 1 and 2.

Amino acid chromatography of the mucous coatings revealed in most preparations the presence of all the common amino acids. A characteristic chromatogram is shown in Fig. 1. In the mucus of the ray no tyrosine could be demonstrated (Fig. 2).

The sugar chromatograms are shown in Fig. 3 and 4. A rough quantitative evaluation based on the size and intensity of the spots is given (Tables 3 and 4). This evaluation holds only for the individual chromatograms, and should not be used for mutual comparison of different chromatograms.

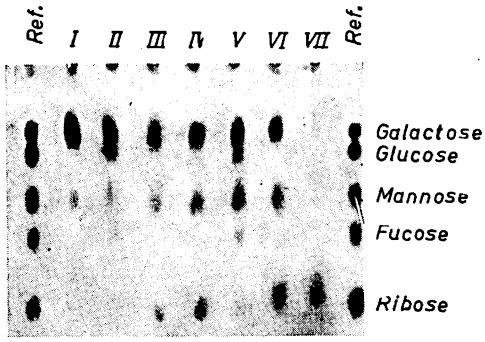

Fig. 4. Chromatogram showing sugar components of roe from I, perch; II, Baltic herring; III, cod; IV, pike-perch; V, whitefish; VI, burbot; and VII, milt from burbot. Solvent, lutidine-*n*-amyl alcohol.

DISCUSSION

The presence of carbohydrate in some piscine mucins has been observed earlier. In a study of the surface slime of the Pacific hagfish (*Polistotrema stouti*), Ferry [15] found carbohydrate contents of 1.4 and 2.9 % (expressed as glucose) in two different preparations. From loach (*Misgurnus anguilli caudatus*) Turumi and Saito [16] obtained a mucous product said to contain 17.9 % glucosamine, 9.8 % galactose, and sialic acid.

Hammarsten [17] examined roe from perch and found that the yolk protein was not a glycoprotein, whereas the egg-jelly gave, after acid hydrolysis, large quantities of a reducing substance, the nature of which he did not further investigate. Young and Inman [18] found 1.0 % glucosamine in the protein of the egg-casings of the salmon. A few per cent of reducing substance has also been reported in roe from *Barbus fluviatus* [19], pike [19], king salmon [20], trout [21], herring [22-24], and salmon [25].

All our preparations of external fish mucus contained carbohydrate. The carbohydrate content is comparatively low, and the quantitative variation between different species is small, except that the ray mucus contains about twice as much as that of other fishes.

The main constituent of the mucus seems to be a simple protein component containing very little or no carbohydrate. In all species investigated the amino-acid pattern of the protein seemed to be mainly the same except in the ray, the mucus protein of which does not contain tyrosine and thus also differs in this respect.

Besides this simple protein component, the mucus also contains variable amounts of one or more glycoproteins. The presence of hexosamine, galactose, fucose, and sialic acid indicates the same types of glycoproteins as are present in mucous secretions of higher vertebrates, *viz.* fucomucin and sialomucin [1]. It is interesting to note that sialic acid is present in the mucus of all ordinary fishes, but not in the mucus of the ray and the hagfish.

In addition to the sugars mentioned, all the preparations except perhaps that of the hagfish, contained ribose and mannose, and one of them also glucose. The ribose probably derives from nucleic acid. The presence of nucleic acids in the mucus is not unexpected, as it is known that many of the epidermal cells discharge their entire contents directly onto the surface. Mannose is

Table 3. Relative sugar composition (hexoses + pentoses + methylpentoses) of the external mucous coatings of some fishes, as shown by chromatography after acid hydrolysis.

Material	Galactose	Glucose	Mannose	Fucose	Ribose
External mucus from					
Pleuronectes platessa	++	+	(+)	+++	+
Gadus callarias	+		(+)	(+)	++
Gadus merlangus	+		(+)	(+)	++
Anguilla vulgaris	+		(+)		+
Esox lucius	++		+	(+)	+
Raja sp.	+++		++	++	+
Myxine glutinosa	+		?	?	(+)

Table 4. Relative sugar composition (hexoses + pentoses + methylpentoses) of the roe and the milt of some fishes, as shown by chromatography after acid hydrolysis.

Material	Galactose	Glucose	Mannose	Fucose	Ribose
Roe from					
Perca fluviatilis	++++		+		+
Lucioperca sandra	+++	+	++		++
Gadus callarias	+++		+		+++
Lota vulgaris	+++		++	(+)	++
Coregonus lavaretus	+++	++	++	+	+
Clupea harengus	+++	++	+	+	++
Milt from					
Lota vulgaris	+				++

a well-known component in many serum and tissue glycoproteins, and is often found in small amounts in mucous secretions of mammals. The mannose might thus be explained as belonging to some tissue protein present. The possibility of mannose belonging to some of the mucous glycoproteins mentioned above cannot of course be excluded. The glucose probably derives from glycogen, which has been shown to be present in the epidermal cells [26].

The view that the mucus is a mixture of one major, simple protein component and smaller amounts of one or more glycoproteins is supported by histological observations. Firstly, there are in most fishes several different types of "glands", specific glandular structures such as the large mucous glands of the myxinoids, the simple pouch-like epidermal organs, and the monocellular "glands" of the goblet-type, indicating a complex nature of the secretion [27]. Secondly, the mucus and some of the epidermal cells, especially the goblet cells, take various degrees of staining with fuchsin after pretreatment with per-iodic acid. They also show metachromasia with toluidine blue in the same way as do many glycoproteins [26, 28].

The finding of fucose- and sialic acid containing glycoproteins in secretion from external mucous epithelium is notable. Glycoproteins of this type so far known have all been of entodermal or closely related origin. Here we find tissues of obviously ectodermal origin producing the same types of substance.

The same monosaccharides as in the external mucus are also found in hydrolysates of fish roe. It is reasonable to assume here, too, that the ribose derives from nucleic acid. Galactose, mannose, hexosamine, and sialic acid are invariable constituents, and are probably due to the presence of glycoproteins. Some of the preparations also contained glucose and/or fucose. The origin of the glucose is obscure. The method of preparation employed (treatment of the pulverized roe with aqueous ethanol) is certain to remove free glucose, which is known to occur in fish eggs [29]. Glycogen has not been found in undeveloped fish eggs [21, 25]. Fucose may indicate the presence of small quantities of mucus of the usual epithelial mucus type.

The largest carbohydrate content is found in those roe specimens that have the thickest mucous layers, *viz.* whitefish and perch, and the smallest content in those with very thin layers, *e.g.* cod and burbot. Considering that

the mucous layer only contributes a minor part of the total dry weight of the roe, and assuming that the carbohydrates are mainly derived from this, it is clear that the relative carbohydrate content of this mucous layer must be high and similar to that of frog-spawn mucin [2].

Ribose was the predominant sugar in the milt sample examined.

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