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Serum Transferrins in Cod

By

Dag Møller

Institute of Marine Research, Bergen.

Introduction.

The presence of an ironbinding protein in human serum was found by Holmberg and Laurell (1945). This substance binds ferrous ions in such a way that the iron do not react with $\alpha\alpha'$-dipyridyl.

Inherited variation of these proteins, the transferrins, has been established by electrophoresis primarily used by Tiselius (1937), modified and improved by Smithies (1955) using the method of starch-gel electrophoresis, and by autoradiography with ferrous-59, described by Giblett, Hickman and Smithies (1959), in numerous mammalian species (see Schmid, 1961, Cooper and Sharman, 1964, for references), in chicken (Ogden et al., 1962) and pigeons (Mueller, 1961, Mueller, Smithies and Irwin, 1962). Transferrins have also been found in sera of amphibians and reptiles (Dessauer and Fox, 1964).

The transferrin polymorphism studied in different species has its origin in a genetic system with two or more co-dominant autosomal alleles each controlling one or more molecular types. In the macaque, Macaca mulatta, ten molecular forms each controlled by one gene, and 24 phenotypes have been detected (Goodman et al., 1965). The bovine transferrin types are commonly controlled by three alleles and each produces three bands (Ashton, 1958). The last observation can be explained by the paired random association of two types of units with a genetic base (Hines, Ludwick and Rausch, 1965).

Fish proteins have been studied by Hamoir (1955), who states that the protein composition of fish can be compared with that of the highest classes of vertebrates. This comparative study of plasma or serum proteins by electrophoresis shows a very different picture, and the variations observed have apparent no common characters within the fish class. Sanders (1964) examined the electrophoretic patterns in sera of three trout species. In two species he found characteristic protein fractions for the particular species. The rainbow trout, however, exhibited either 6 or 7 fractions. The amount of protein differed both inter- and intraspecificly, but he suggested that the amount present varied with age, sex, species and diet. Khailov (1962) studied the protein variation in sera from cod, plaice and haddock in the autumn. He found distinct differencies between the species and individual variations of the fractions with time.

A single transferrin band was demonstrated in jack (Caranx sexfasciatus) by Blumberg (1960) who studied biochemical polymorphisms in animals. Creyssel et al. (1964) found the existence of a polymorphism of transferrins in carp (Cyprinus carpio) by starch-gel electrophoresis. As the individuals studied were too few, the authors could not with sufficient certainty decide the heredity.

The present paper describes transferrin patterns in cod (Gadus morhus) and the genetic basis for the observed differencies. The investigation was carried out in an attempt to identify individual genetic characters in cod. Such characters, the haemoglobin polymorphism, have previously been demonstrated by Sick (1961) using agar electrophoresis of freshly prepared oxyhaemoglobin.

The gene frequencies of the different transferrin alleles will later be used as parameters in connection with the gene frequencies of the haemoglobin alleles (Sick, 1961), the frequencies of different otolithtypes (Rollefsen, 1933) and bloodtypes (Møller, 1965, in preparation) in studies of cod populations.

Material and methods.

The samples used in the present study are selected from a material collected in 1964-1965 for population studies. Part of this material were unfortunately destroyed before the sera could be treated in the laboratory. Sera used are from panmixed populations and from different geographic areas with different frequency of the characters studied from one sample to another. As indicators for panmixing, haemoglobin types (Sick, 1961), otolith types (Rollefsen, 1933) and bloodtypes (Møller, 1965, in preparation) were examined. Table 1 give a survey of the material containing six samples with sera from 682 individuals, listed in a geographical order, starting with the sample from Hordaland in the south and ending with the sample collected 27 nautic miles SW of Bjørnøya in the north. In the material cods of different sex and age are represented, the majority being 4 and 5 years old.

Blood was obtained by puncture of bulbus arteriosus on live cod. After clotting overnight at 2-4 C the samples were centrifuged and the sera pipetted off, then examined or stored at -25 C for later shipment to Bergen.

Preserving the samples was a major problem at far-away fishing grounds onboard fishing boats lacking sufficient capacity for freezing. The samples can not be stored in a regfrigator for more than five days. Frozen samples gave usable results even after two to three months in the freezer, but fresh sera gave a better result than freshly frozen samples.

The electrophoretic technique was a modification of Giri's method (1956a, b), made by Sick (1961 and personal communication). The most effective buffer system to enhance individual differencies among the protein types were 30.25 g/l Tris(hydroxymethyl)-aminomethane, 3 g/l Titriplex II and 2.3 g/l boric acid. The gel was made by mixing 2% starch (BDH) and 0.8% agar ("Ionagar" No. 2, Oxoid) in the buffer and heating for half an hour at 96 C in a waterbath while gently stirring during the warming. Prepared microscope slides with a 2 mm thick layer of gel were stored for a quarter of an hour in a regfrigator before use.

Each electrophoretic run lasted for two hours applying voltages between 65 and 70 V across the slide giving a total current of 35 to 45 mA in the apparatus taking six slides in each run. The slides were stained with Amido Black 10B after fixation and drying of the gel.

Cases where the determination of a pattern have been difficult to establish, which happen with poorly preserved samples, the serum have been tested in a second run. This run often gave a good result, provided that particular care was taken to keep the slide temperature low, since fish proteins are more readily destroyd than e.g. mammalian proteins.

In order to identify the transferrin bands Giblett's et al.'s (1959) method were used with some modifications. Approximately 0.1 ml of ferrous (Fe^{59}) citrate of specific activity 100 μC/ml were added to 0.2 ml selected sera. After half an hour in waterbath at 2 C the sera were subjected to starch/agar-gel electrophoresis.

The total numbers of the different patterns observed in each sample have been compared with totals expected according to the Hardy-Weinberg law. The statistical significance between observed and expected proportions was tested by a chi-square test.

Results.

In fig. 1 ten different patterns found by starch/agar-gel electrophoresis of cod sera are shown. The figure is made up of eight slides from different runs with two samples of serum on each slide. In all cases the transport of substances was towards the anode. All the patterns have a fast moving component (albumin) which frequently reached the filter paper, the connection between gel and buffer during the run. Slower moving components of uncertain nature are

seen as one or two faint bands. The patterns which are studied in this paper consist of five components called A, B, C_1, C and D, A being the fastest moving component. The bands are represented in the serum phenotypes: A, or called here AA, AB, AC, AD, BB, BC, BD, CC, CD and C_1C. The band D alone has also been found. As the bands only appear alone or two together, eleven out of fifteen possible combinations have been found.

Fig. 2 show four slides with the four patterns AC, CC, BC and CD comprared with autoradiographs of the same slides. The iron-59 is bound to the bands and some radioactivity to the fast moving albumin component.

A genetic hypothesis involving the five co-dominant alleles Tf^A, Tf^B, Tf^{C1}, Tf^C and Tf^D may be adopted to explain the transferrin variation observed, giving the homozygotes Tf^ATf^A, Tf^BTf^B, $Tf^{C1}Tf^{C1}$, Tf^CTf^C and Tf^DTf^D which are responsible for the phenotypes AA, BB, C_1C_1, CC and DD, and giving the hetrozygotes Tf^ATf^B, Tf^ATf^{C1}, Tf^ATf^C, Tf^ATf^D, Tf^BTf^{C1}, Tf^BTf^C, Tf^BTf^D, $Tf^{C1}Tf^C$, $Tf^{C1}Tf^D$ and Tf^CTf^D for the phenotypes AB, AC_1, AC, AD, BC_1, BC, BD, C_1C, C_1D and CD. The distributions of the different transferrin patterns in sera from six samples are presented in Table 2. The column "Not rep.", is the expected totals of the not-represented types AC_1, BC_1, C_1C_1 and C_1D. All the observed data are in accordance with the expected Hardy-Weinberg distribution. The chi-square is calculated for each sample giving a probability between 0,98 and 0,95 of all the samples together. The gene Tf^C dominates the material particularly in samples from southern Norway, and the gene frequencies of Tf^A (q^A) seem to increase northward, while the Tf^B allele fluctuate between 0.1000 and 0.1735. Tf^{C1} and Tf^D are rare in the areas investigated.

Discussion.

This investigation was carried out in order to identify individual genetic characters in blood of cod for later use in population studies. Eleven different transferrin patterns have been demonstrated to be present in codfish serum. The totals of the different patterns were compared with totals expected according to the Hardy-Weinberg distribution giving a probability between 0.98 and 0.95 by chi-square tests, implying that these transferrins are controlled by co-dominant alleles and not being affected by age or environmental factors.

Further evidence in favour of this statement is the fact that the transferrin pattern does not seem to change during a cod's life, as is the case with the haemoglobins in salmon (Kock, Bergstrøm and Evans, 1964). The bands are represented in every age-group from 3 to 16, in male and female, in immature as well as mature cods. The material consists of individuals of different sex and age and the transferrin distribution still fit Hardy-Weinberg law. Khailov (1962) found variations of the amounts of different fractions with time insera of cod using colourometry by elusion of the different fractions. In this study, however, it has not been detected any visible variations of the amounts of transferrin according to the season of the year.

The selection of samples was made so as to be sure that the material was from panmixed populations. The material presented here was taken in the autumn of 1964, and only one of six sample is omitted in this report. But the collection from other areas at other times has given other distributions of transferrin patterns, partly with deviations from Hardy-Weinberg law, than stated in this report. The reason for this is very likely because these populations were not panmixed as determined by analysis of haemoglobins, otoliths and bloodtypes.

The transferrins are well separated and the patterns of different runs can easily be compared with each other with the starch/agargel electrophoresis used. The determination of the pattern do not offer any difficulties in fresh sera, and the technique of examining is quick and simple in treating high numbers of sera.

The determination of the phenotype have been difficult to establish with poorly preserved samples. If a satisfactory result was not obtained after a second, perhaps a third, run of a serum, the serum was rejected. Since all the sera in a sample have been given the same treatment, they have also all

either been used or discarded. In other words, from the different localities listed in this report, all sera have been used, in other cases they have all been rejected. Thus, no methodical selection of the material collected has taken place. It has not been possible to test the rare C_1 protein autoradiographically. In this report, however, the band is interpreted as a very rare ferrous-binding protein because of the appearance of the bands; both the position and the strength are in accordance with the others. Together with A, B or D this band could perhaps be difficult to distinguish from AC, BC or CD. Since C_1 is rare, however, this is a minor problem and a higher frequency of C_1 would primarily give a higher number of the pattern C_1C.

The transferrins have been identified earlier in fish by Blumberg (1960) and Creyssel et al. (1964). The transferrin patterns studied in this report, however, cannot be compared in details with those of their report because these authors all used different techniques. The positions and intensity of a band depend upon the electrophoretic conditions used. The transferrin patterns in carp (Creyssel et al., 1964) seems to a great extent to be like the patterns in cod, but genetic studies confirming the patterns in carp have not been undertaken as far as is known. On the other hand, the inheritance of transferrins in cod as an polyallele system without dominance non-affected by age or environment is in strict accordance with what is found in higher vertebrates (Beckman, 1962, Ogden et al., 1962, Schmid, 1961).

The transferrin patterns in cod, which in any case can be separated in four different molecular types labeled TfA, TfB, TfC and TfD (according to Cohen and Shreffler, 1961) represent four co-dominant alleles Tf^A, Tf^B, Tf^C and Tf^D. A fifth molecular type called TfC_1 represent perhaps a fifth allele Tf^{C_1}. The frequencies of the four alleles will be used as genetic parameters in connection with the gene frequencies of the haemoglobin alleles (Sick, 1961), the frequencies of the otolithtypes (Rollefsen, 1933) and the frequencies of the bloodtypes (Møller, 1965, in preparation) in taxonomic studies of cod populations.

Summary.

Sera from 682 cods from six different localities along the Norwegian coast and in the Barents Sea have been investigated by starch/agar-gel electrophoresis. Eleven different patterns have been found which consist of five molecular types. Four of them have been shown to be ironbinding proteins by autoradiography. The different distributions of the transferrin patterns observed were in agreement with totals expected according to the Hardy-Weinberg formula, implying that the bands have their origin in five co-dominant alleles.

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Table 1.

Material

Sample no	Date of sampling	Localities	No. in sample	Gear
XVII64	18.12.64	Hordaland, coastwards	100	Trap-net
XVI64	4.12.64	Smøla, coastwards	100	"
XV64	1.12.64	Helgeland, coastwards	98	"
I64	26.10.64	Nordskot, Vestfjorden	153	"
VIII64	1.11.64	Varangerfjorden	83	shrimp-trawl
XII64	19.11.64	Bjørnøya, N73°55'E18°15'	148	trawl
		Total	682	

Table 2.

Distribution of transferrin types and gene frequencies in cod.

Sample no		Fenotypes AA	AB	AC	AD	BB	BC	BD	CC	CD	DD	CC_1	Not rep.	q^A	B	q^C	q^D	q^{C_1}
XVII64	obs.			3		2	13	3	74	2		3		0.015	0.100	0.845	0.025	0.015
	exp.	0.02	0.30	2.54	0.08	1.00	16.90	0.50	71.40	4.23	0.06	2.54	0.45					
XVI64	obs.		1	7		1	23	1	63	3		1		0.040	0.135	0.800	0.020	0.005
	exp.	0.16	1.08	6.40	0.16	1.82	21.60	0.54	64.00	3.20	0.04	0.80	0.20					
XV64	obs.		4	14		2	26		48	2		2		0.092	0.174	0.714	0.010	0.010
	exp.	0.83	3.12	12.86	0.18	2.95	24.29	0.35	50.00	1.43	0.01	1.43	0.56					
I64	obs.		3	13		4	31		98	4				0.052	0.137	0.797	0.013	
	exp.	0.42	2.20	12.76	0.21	2.88	33.49	0.55	97.28	3.19	0.03							
VIII64	obs.	2	1	12		2	15		49	2				0.102	0.121	0.765	0.012	
	exp.	0.87	2.05	13.01	0.20	1.20	15.31	0.24	48.59	1.53	0.01							
XII64	obs.	5	5	29	2	6	27	3	63	7	1			0.155	0.159	0.639	0.047	
	exp.	3.57	7.30	29.37	2.18	3.73	30.01	2.22	60.34	8.94	0.33							

Sample no	Chi-square	D.f.	Probability
XVII64	1.13	1	$0.3 > p > 0.2$
XVI64	0.30	2	$0.9 > p > 0.8$
XV64	0.37	2	$0.9 > p > 0.8$
I64	0.44	2	$0.9 > p > 0.8$
VIII64	0.22	2	$0.9 > p > 0.8$
XII64	1.79	3	$0.7 > p > 0.5$
Total	4.25	12	$0.98 > p > 0.95$

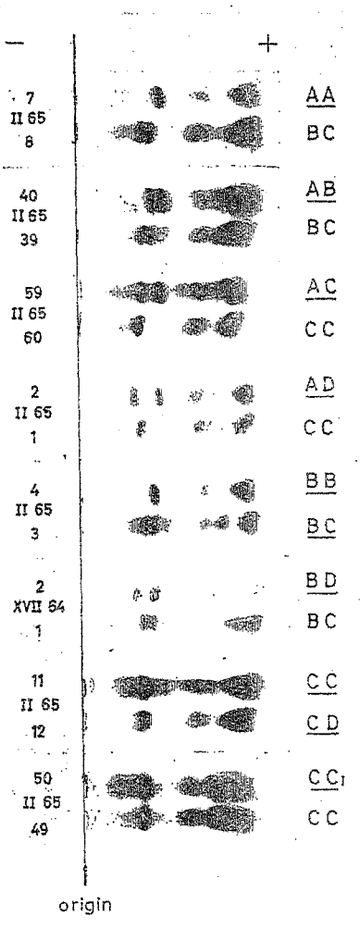

Fig. 1. Starch/agar-gel electropherograms of cod sera. 'Origin' indicates the site of sera application, numbers to the left state the number of fish in the sample and the sample number and letters to the right the type of pattern.

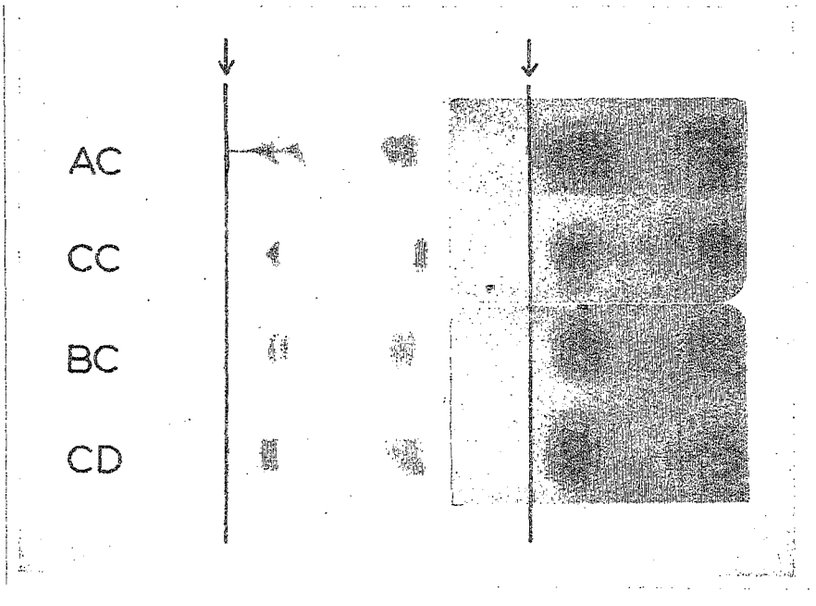

Fig. 2. Starch/agar-gel electrophoresis of transferrins in cod sera on the left localized from the autoradiographs on the right. Arrows indicate the site of serum application and letters the type of pattern.