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POLYMORPHISM OF SERUM TRANSFERRIN IN COD

By

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INTRODUCTION

An ironbinding protein in human serum was found by HOLMBERG and LAURELL (1945). This protein binds ferrous ions in such a way that the iron does not react with *aa'*-dipyridyl, and is now usually called transferrin (HOLMBERG and LAURELL 1947, GIBLETT, HICKMAN and SMITHIES 1959).

Inherited variations in this protein has been revealed by the use of electrophoretic techniques (TISELIUS 1937, SMITHIES 1955) in numerous mammalian species (SCHMID 1961, COOPER and SHARMAN 1964), in chicken (OGDEN, MORTON, GILMOUR and MCDERMID 1962) and in pigeon (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 in species studied has its origin in a genetic system with two or more codominant autosomal alleles, each controlling one or more bands in the electrophoretic pattern. In the macaque, *Macaca mulatta*, ten molecular forms, each controlled by one gene, and 24 phenotypes have been detected (GOODMAN, KULKARNI, POULIK and REKLYS 1965). The transferrin types in Norwegian reindeer are controlled by eight alleles and each allele produces two bands (BRÆND 1964).

Fish proteins have been studied by HAMOIR (1955), who states that the main characteristics of fish protein are satisfactorily defined, and that a comparison of the highest and lowest classes of vertebrates is possible from the point of view of protein composition. The comparative study of the electrophoretic pattern of plasma or serum shows, however, that the lowest vertebrates apparently do not have common characters *inter se* or with the mammalian ones (HAMOIR 1955). 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 differences between the species and individual variations of the fractions with time.

A single transferrin band was demostrated in jack (*Caranx sexfasciatus*) by BLUMBERG (1960). CREYSSEL, SILBERZAHN, RICARD and MANUEL (1964) found polymorphism of transferrins in carp (*Cyprinus carpio*) by starch gel electrophoresis, and FINE, DRIHLON, AMOUCH and BOFFA (1964) detected the presence of several transferrin types in eel (*Anguilla anguilla*) by paper electrophoresis. However, too few carp and eel individuals were studied to decide the heredity with sufficient certainty.

The present paper describes transferrin patterns in cod (*Gadus morhua*) and discusses the genetic basis for the observed differences. The investigation was carried out to identify individual genetic characters for studies of cod populations. Haemoglobin polymorphism has been demostrated in cod, using agar electrophoresis of freshly prepared oxyhaemoglobin (SICK 1961).

MATERIAL AND METHODS

The samples used in the present study are selected from a material collected in 1964 and 1965 for population studies. Unfortunately, part of this material was destroyed before the sera could be analyzed in the laboratory. The samples 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) and otolith types (ROLLEFSEN 1933) were used. Table 1 gives a survey of the material containing six samples, with sera from 682 individuals, listed in a geographical order, starting with the

Table 1. Date, locality, and gear used, of the collected samples.

Date of sampling	Locality	Gear	Number of fishes in sample
18 Dec. 64	Hordaland, coastwards	Trap-net	100
4 Dec. 64	Smøla, coastwards	—»—	100
1 Dec. 64	Helgeland, coastwards	—»—	98
26 Oct. 64	Nordskot, Vestfjorden	—»—	153
1 Nov. 64	Varangerfjorden	Shrimp-trawl	83
19 Nov. 64	Bear Island N73°55'E18°15'	Trawl	148

sample from Hordaland in the south and ending with the sample collected 27 nautical miles SW of Bear Island in the north. Cod of different sex and ega are represented in the material, the majority being 4 to 5 years old.

Blood was obtained by puncture of *bulbus arteriosus* in live cod. After clotting overnight at 2—4°C the samples were centrifuged and the sera pipetted off. The material collected near Bergen was examined fresh, whereas the sera from the northernmost localities were shipped to the laboratory in frozen state (—25°C). Without sufficient capacity for freezing, preservation of the samples from distant fishing grounds is a major problem. In the present investigation cod sera could not be stored in a refrigerator for more than five days without destruction of the transferrin molecules. However, frozen sera gave reliable results even after two or three months, although fresh sera gave a better result.

The electrophoretic technique was a combination of Giri's method (1956 a, b), modified by SICK (1961 and personal communication). The most effective buffer system to enhance individual differences among the protein types was 30.25 g/l Tris (Tris(hydroxymethyl)-aminomethane), 3 g/l EDTA (Ethylenediaminetetraàcetic acid), and 2.3 g/l boric acid (AARONSON and GRØNWAL 1957). 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 stirring gently. Microscope slides covered by 2 ml of gel were kept in a refrigerator for a quarter of an hour before use.

Each electrophoretic run lasted for two hours. Voltages of 65 to 70 V between the ends of the filterpapers were applied, giving currents of 6 to 7 mA for each slide. Six slides, each with two specimens, were placed in the apparatus in each run. After fixation the proteins were stained with Amido Black 10B and the gel dried.

In cases where the transferrin patterns have been difficult to determine, usually poorly preserved samples, the sera were tested in a second run. This run often gave a good result, provided that particular care was taken to keep the temperature of the gel low.

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

The numbers of the different patterns observed in each of the six samples have been compared with numbers expected according to the Hardy-Weinberg law of genotype distributions in large random mating populations. The differences between observed and expected distributions were tested by applying a chi-square test.

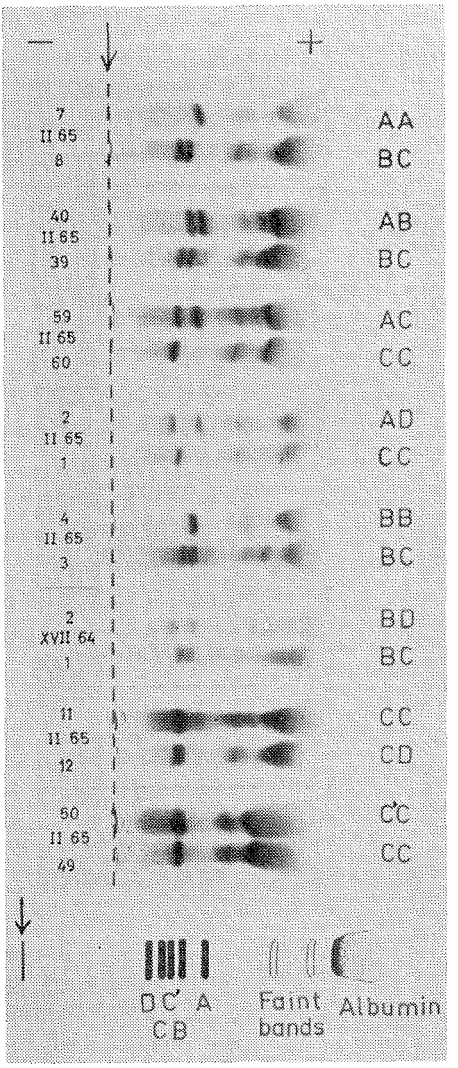

Fig. 1. Starch/agar gel electrophoretograms of cod sera. The arrow indicates the site of sera application, numbers to the left state the number of fish in the sample and the samp'e number, and letters to the right the type of pattern. An enlarged schematic drawing of all components is shown at the bottom of the figure.

RESULTS

Fig. 1 shows ten different patterns made up of eight slides from different runs. An enlarged schematic drawing of all the components is seen at the bottom of Fig. 1. In all cases the proteins move towards the anode. All patterns have a fast moving component (albumin) which frequently reaches the filter paper. Slower moving components of uncertain nature

are seen as one or two faint bands next to the albumin. The components which are studied in this paper are five distinct bands called A, B, C', C, and D; A being the fastest moving component. The bands appear alone or two together, and thus fifteen combinations are possible. Of these eleven have been found. The bands are represented in the serum phenotypes: AA, AB, AC, AD, BB, BC, BD, CC, CD, and C'C. The band D alone has also been found. The mobility for the five components are clearly different, the C' band, however, being only slightly faster than the C band under the electrophoretic conditions used in this investigation. The distance between the stained bands A and B is only slightly longer than the distance between B and C, while this distance is essentially longer than the distance between the bands C and D.

The intensity of the bands vary, and consequently the content of transferrin in cod sera differs from one individual to another. Generally the one band patterns are stronger than the two band patterns. However, visible variations in the amount of protein according to season of the year have not been detected.

The present material contains specimens of different sex and age, and the components A, B, C, and D are represented in every agegroup from 3 to 16, in males and females, and in immature as well as mature cod.

Fig. 2 shows four slides with the three patterns AC, BC and CD compared with autoradiograms of the same slides. The Fe^{59} is bound to the bands described above, and variable quantities of radioactivity appear also in the albumin component except in one case. No radioactivity is bound to the faint bands of uncertain nature.

A hypothesis involving five codominant alleles Tf^A, Tf^B, $Tf^{C'}$, Tf^C, and Tf^D may be adopted to explain the transferrin variation observed. This hypothesis gives the homozygotes Tf^ATf^A, Tf^BTf^B, $Tf^{C'}Tf^{C'}$, Tf^CTf^C, and Tf^DTf^D which are assumed to be responsible for the phenotypes AA, BB, C'C', CC, and DD, giving the hetrozygotes Tf^ATf^B, $Tf^ATf^{C'}$, Tf^ATf^C, Tf^ATf^D, $Tf^BTf^{C'}$, Tf^BTf^C, Tf^BTf^D, $Tf^{C'}Tf^C$, $Tf^{C'}Tf^D$, and Tf^CTf^D for the phenotypes AB, AC', AC, AD, BC', BC, BD, C'C, C'D, and CD. The distributions of the different transferrin patterns from six samples are presented in Table 2 together with the expected distributions according to the Hardy-Weinberg law. The column «Not rep.», contains expected totals of the notrepresented types AC', BC', C'C' and C'D.

The chi-square value for the observed distributions and the expected Hardy-Weinberg distributions in samples from Helgeland, Vestfjorden, and Bear Island, gives a probability between 0.7 and 0.5 when d.f. $=3$. According to the test it is therefore no significant deviation between the observed and expected distributions in these samples. The

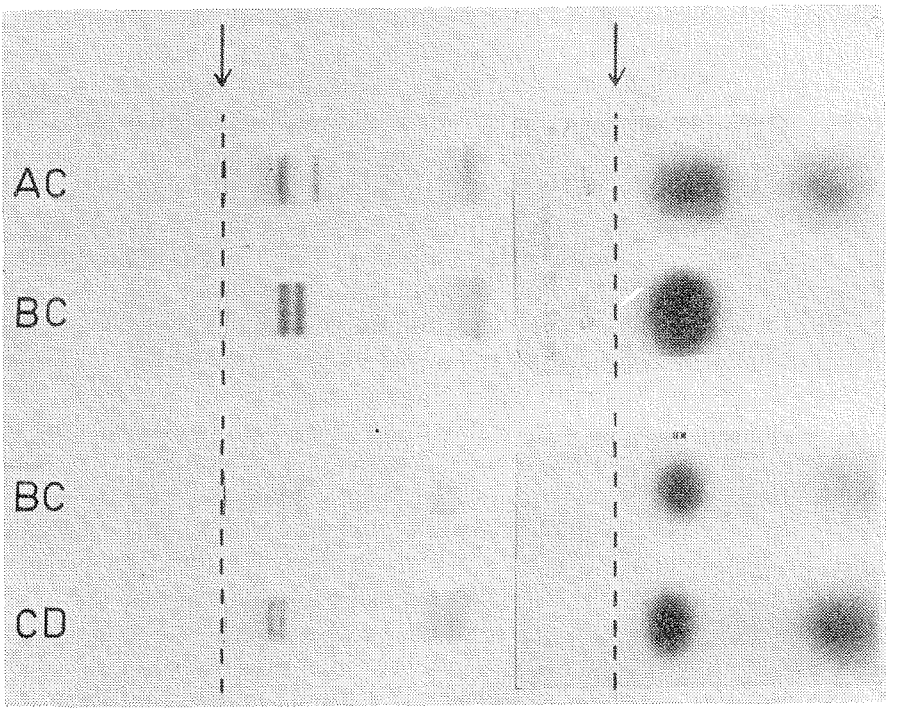

Fig. 2. Starch/agar gel electrophoretograms of transferrin in cod sera (left) localized from autoradiograms (right). Arrows show the site of serum application, and letters indicate the type of pattern.

samples from Hordaland, Smøla and Varangerfjord are omitted because of the low number in the classes of the expected distributions.

The gene Tf^C dominates the material, particulary in samples from southern Norway, and the frequencies of Tf^A (q^A) seem to increase northward, while the frequencies of the Tf^B allele fluctuate between 0.1000 and 0.1735. $Tf^{C'}$ and Tf^D are rare in the areas investigated.

DISCUSSION

The present investigation has demonstrated that cod transferrins are controlled by codominant alleles.

The transferrin patterns consist of one or two strong bands and was not found to vary with the age of the fish. This is in contrast to the variation of the haemoglobin patterns in salmon (KOCH, BERGSTRØM and EVANS 1964), which was found to be ontogenetic. The amount of the cod transferrins may vary with time, though it has not been possible to detect any visible variation by the method used in this study. KHAILOV (1962) found variations in the amounts of different fractions during

Table 2. Distribution of transferrin phenotypes observed in samples of cod from different areas compared with the expected distribution of genotypes according to the Hardy-Weinberg law.

Sample		Transferrin patterns											Not rep.	Observed gene frequencies				
		AA	AB	AC	AD	BB	BC	BD	CC	CD	DD	C'C		A q	B q	C q	D q	C' q
Hordaland	obs.			3		2	13	3	74	2		3		.015	.100	.845	.025	.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					
Smøla	obs.		1	7		1	23	1	63	3		1		.040	.135	.800	.020	.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					
Helgeland	obs.		4	14		2	26		48	2		2		.092	.174	.714	.010	.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					
Vestfjorden ...	obs.		3	13		4	31		98	4				.052	.137	.797	.013	
	exp.	0.42	2.20	12.76	0.21	2.88	33.49	0.55	97.28	3.19	0.03							
Varangerfj. ...	obs.	2	1	12		2	15		49	2				.102	.121	.765	.012	
	exp.	0.87	2.05	13.01	0.20	1.20	15.31	0.24	48.59	1.53	0.01							
Bear Island	obs.	5	5	29	2	6	27	3	63	7	1			.155	.159	.639	.047	
	exp.	3.57	7.30	29.37	2.18	3.73	30.01	2.22	60.34	8.94	0.33							

the autumn in sera of cod, but he used colourometry to evaluate the different fractions.

The samples were selected to ascertain that the material was from panmixed populations. Samples with distributions of haemoglobin types not in accordance with Hardy-Weinberg law were not used, nor samples with mixed types of otoliths. The material presented was collected during the autumn of 1964, and only one of seven samples has been omitted from the report.

The transferrins are well separated and the patterns of different runs can easily be compared with each other when using this starch/agar gel electrophoresis. The determination of the pattern is not difficult for fresh sera, and the technique is quick and simple, and large samples can be treated.

The determination of the phenotypes has been difficult for poorly preserved samples. If a satisfactory result was not obtained after a second, or perhaps a third run of a serum, the specimen was rejected. Since all sera in a sample were preserved in the same way, they also were either used or discarded together. Methodical selection of the material therefore has been avoided.

It has not been possible to test the rare C' protein autoradiographically. In this report, however, the band C' is interpreted as a very rare ferrous-binding protein because of the appearance of the band: both the position and the strength are comparable to characteristics of the other transferrins. Together with A, B, or D, this band could be difficult to distinguish from AC, BC, or CD. Since C' is rare, however, this is a minor problem, and a higher frequency of C' would primarily give a higher number of the pattern C'C.

Transferrins have earlier been identified in jack, carp, and eel, respectively, by BLUMBERG (1960), CREYSSEL *et al.* (1964), and FINE *et al.* (1964). The transferrin patterns studied in this report, however, cannot be compared in detail with those, because different techniques have been used. The position and intensity of a band depend upon the electrophoretic conditions used. The transferrin patterns in carp (CREYSSEL *et al.* 1964) and in eel (FINE *et al.* 1964) seem to a great extent to be like the patterns in cod, but genetic studies confirming the patterns in carp and eel have apparently not been undertaken. On the other hand, the inheritance of transferrins in cod as a polyallele system without dominance is in strict accordance with results from studies of higher vertebrates (BECKMAN 1962, OGDEN *et al.* 1962).

The variable ironbinding capasity of the albumin component can not be explained by the results in this study. However, the ironbinding cod albumin will be investigated.

The transferrin patterns in cod, which in any case can be separated into four different molecular types named TfA, TfB, TfC and TfD (according to COHEN AND SHREFFLER 1961) represent four codominant alleles Tf^A, Tf^B, Tf^C, and Tf^D. A fifth molecular type called TfC' may represent a fifth allele $Tf^{C'}$.

SUMMARY

Sera from 682 cod from six different localities along the Norwegian coast and in the Barents Sea have been investigated by starch/agar gel electrophoresis. Eleven different patterns composed by five molecular types have been found. By autoradiography it has been demonstrated that four of them are ironbinding proteins. The distributions of the observed transferrin patterns were in agreement with distributions expected according to the Hardy-Weinberg formula, implying that the bands have their origin in five codominant alleles.

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