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Genetic variation in the eel

II. Transferrins, haemoglobins and esterases in the eastern North Atlantic. Possible interpretations of phenotypic frequency differences.

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SUMMARY

Comparison, by electrophoretic methods, of eel samples from Iceland, Scotland and Germany confirmed previous reports of uniformity in haemoglobin types. When liver esterases were examined, significant differences in the frequencies of phenotypes in the 'fast' zone of the electropherogram were found between samples from Germany and samples from Iceland and Scotland. The frequencies of transferrin phenotypes in Icelandic and Scottish eels differed significantly from those of French and American eels.

It follows that regional differences in gene frequencies probably exist within the species *Anguilla anguilla*. The implications for the 'atlantic eel problem' are briefly discussed.

1. INTRODUCTION

Many papers have been published about the 'atlantic eel problem'. Detailed reference to them will be made in the Discussion. It may suffice here to say that one question at issue is whether in fact European eels spawn in the Sargasso Sea; and more specifically, whether they interbreed there with American eels.

The problem has been approached by searching for similarities or differences between American, European and other eels in characteristics such as haemoglobins and transferrin patterns. In the first paper of this series improvements in the resolution of haemoglobin types by electrophoresis were introduced, and it was also demonstrated that there is variability in esterase patterns (Pantelouris & Payne, 1968).

In this report we compare haemoglobins, some serum proteins and some liver esterases in samples collected in Iceland (Kollafjördur), Western Scotland (Loch

Long) and Germany (Elbe Hahnofersand; Elbe by Tinsdal; Helgoland; Stettiner Haff). The collections were made in June and July 1969. Most fish caught were yellow eels. Some brown and silver eels were also caught and were used for developmental comparisons.

2. MATERIALS AND METHODS

(i) *Samples*

Blood was collected by transecting the eels at the level of the heart about 30 min after an intramuscular injection of heparin (10000 i.u.). Plasma and red cells were separated by centrifugation. Where this was not possible and blood had to be frozen without centrifugation, haemolysis of course occurred. Such samples were still useful for the detection of haemoglobin type, despite difficulties arising from the presence of haptoglobins. It was not possible, however, to use these samples for the demonstration of transferrin phenotypes.

Liver samples were frozen. Later they were minced with scissors in an equal volume of distilled water and disintegrated by ultrasonic treatment with an MSE 60 W disintegrator and by further freezing and thawing. The homogenate was centrifuged at 5000 rev/min for 15 min and the supernatant was used for electrophoresis. No advantage was found in centrifuging at 20000 rev/min.

(ii) *Electrophoresis*

The technique of horizontal electrophoresis in gels of partially hydrolysed starch, developed by Smithies (1955), was used throughout this work. A description of the procedure applied has been given (Pantelouris, 1968). In all cases the discontinuous version of the technique was adopted.

Vessel buffer for haemoglobin: pH 8·6, 1·4 g diethylbarbituric acid and 7·7 g sodium 5,5-diethylbarbiturate dissolved in water and made up to 1000 ml.

Gel buffer for haemoglobin: pH 8·6, 4·85 g tris, 0·26 g EDTA and 0·74 g citric acid dissolved in water and made up to 1000 ml.

Vessel buffer for plasma proteins and esterases: pH 8.7, 18·6 g boric acid and 0·2 g sodium hydroxide dissolved in water and made up to 1000 ml.

Gel buffer pH 8·6 for esterases; 8·0 g tris, 1·5 g citric acid and 100 ml of borate vessel buffer made up to 1000 ml.

Gel buffer pH 7·6 for esterases: 812·5 ml of 0·05 M citric acid and 187·5 ml of 0·76 M tris. Diluted 1:10 before making gel.

Gel buffer for plasma proteins: pH 8·0 prepared as the pH 7·6 tris-citrate buffer with the addition of sufficient tris to raise the pH to 8·0.

(iii) *Staining of gells*

Haemoglobins were stained by the *o*-dianisidine, and esterases by the Fast Garnet methods. A 1% solution of nigrosine (Gurr) was used for the general staining of proteins. The position of transferrins on the starch gel was confirmed

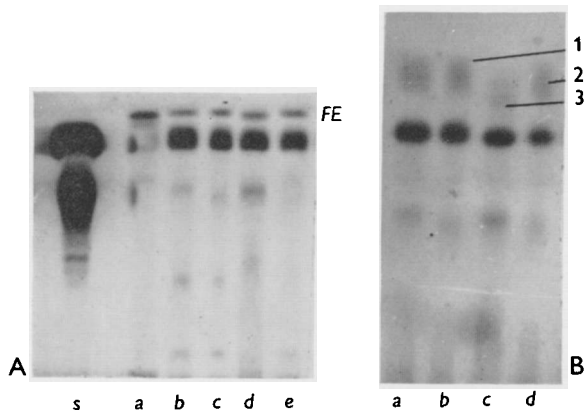


Fig. 1. Electropherogram of five samples (*a–e*) of washed red cell homogenates and, for comparison, of a sample of plasma, *s*. A, Tris-citrate buffer, pH 7·6. B, tris-citrate-borate buffer, pH 8·6. Note *FE*, a zone of esterases specific to red cells, which at the higher pH resolves in combinations in pairs of three fractions, 1–3. Substrate: 1-naphthyl acetate.

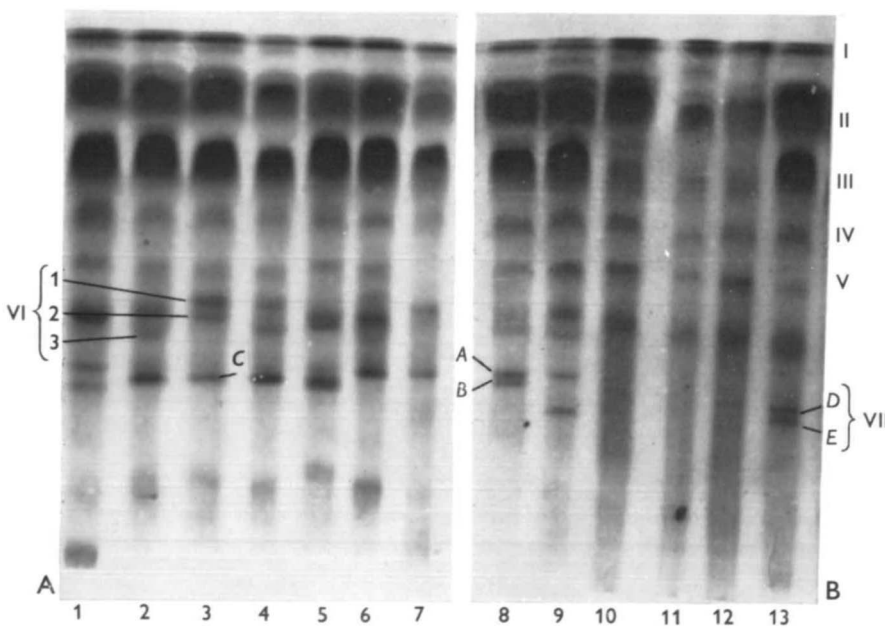
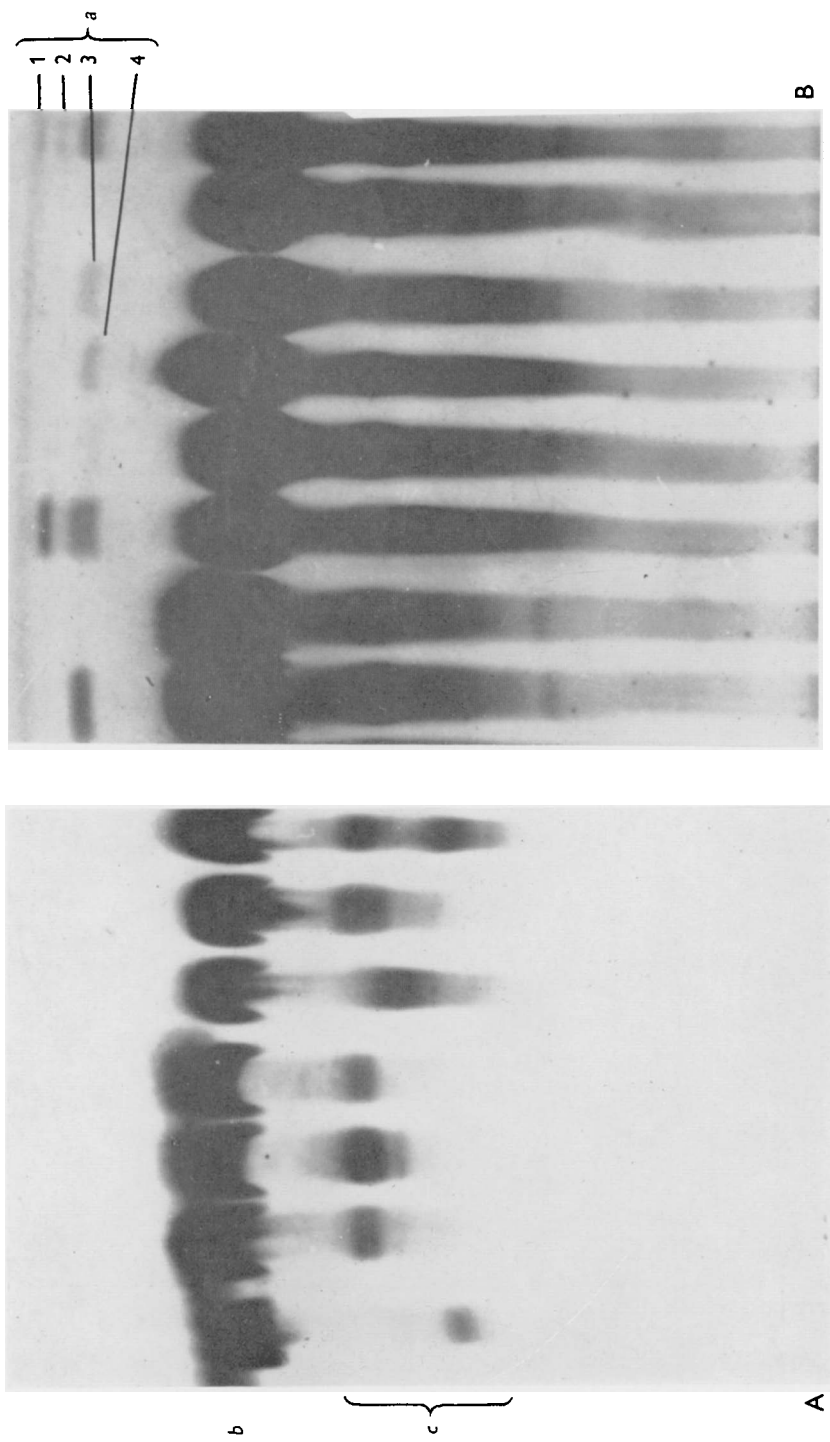


Fig. 2. Electropherogram of eel plasma stained with nigrosine. A, Specimens 1–7; B, specimens 8–13. Tris-citrate, pH 8·0. Note the phenotypic variation in all zones (I–VIII). Zone VI resolves into three and zone VII into five fractions.



Esterases of eel plasma. Substrate: 1-naphthyl acetate. A, Ontogenetic changes in zone *c*. 1, Young specimen 12 cm; 7, young green brown specimen 25 cm; 5, brown specimen 30 cm; 6, silver eel 32 cm; 2–4, silver eels 50–63 cm. B, Phenotypes of zone *a* (combinations of fractions 1–4) appearing if the gel slice is allowed to overstain.

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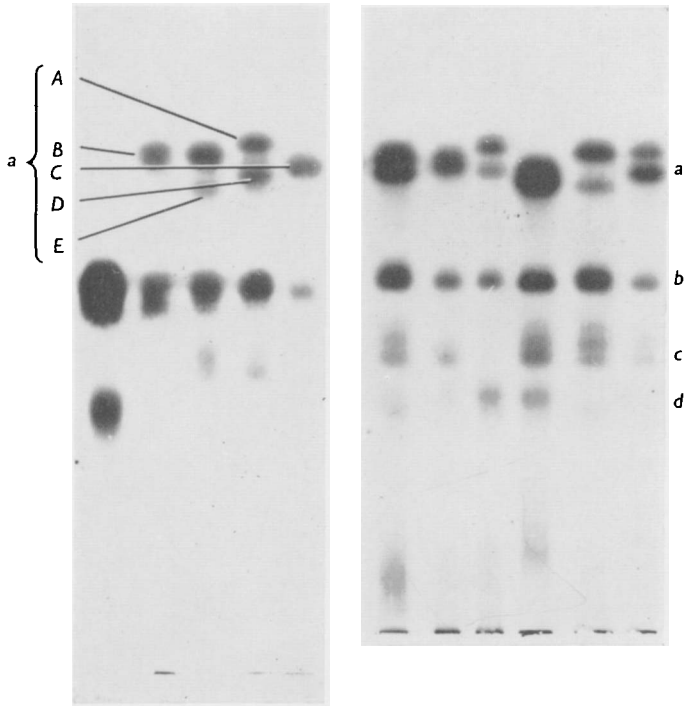

Fig. 1. Understained electropherogram of eel liver homogenates to show the main esterase zones. Tris-citrate-borate, pH 8·6. Substrate: 1-naphthyl acetate. Note fractions *A–E* in zone *a*. The first sample is plasma for comparison.

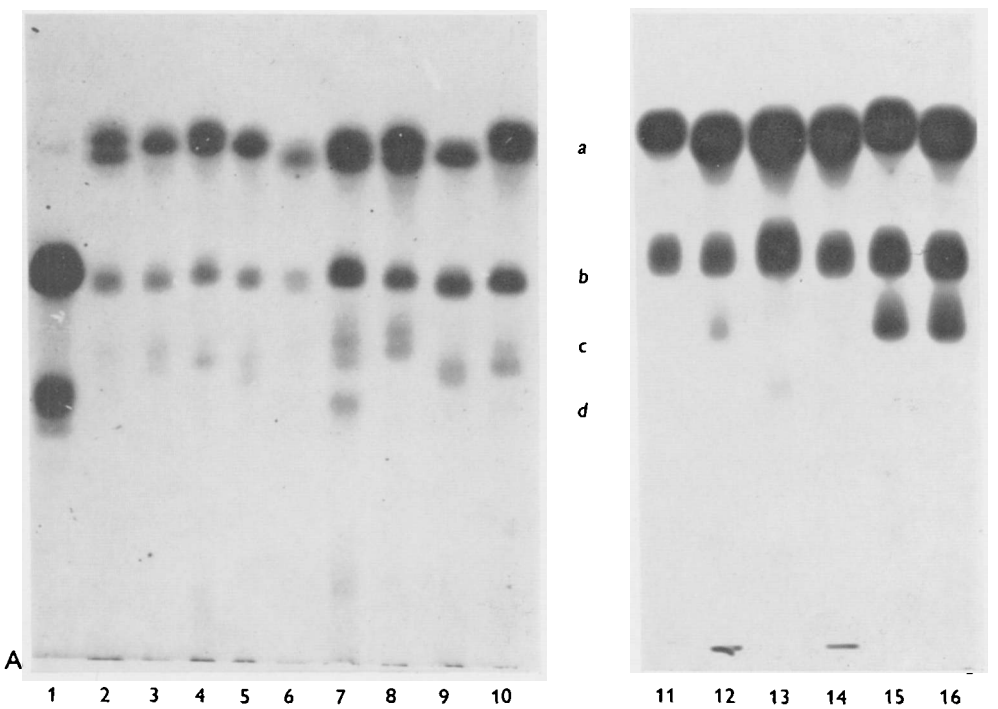

Fig. 2. Esterase electropherogram of liver homogenates obtained by the same method as fig. 1 but overstained (for 3 h against the normal 20 min) to emphasize details of zone *c*. A, Resolution of zone *c* into five weakly staining fractions. B, Silver eels (11–14) without zone *c*, which is strong in the brown eels, 15–16. Sample 1 is plasma.

by the Nitroso-R method of Mueller, Smithies & Irwin (1962). Details of all these staining procedures are given in Pantelouris & Arnason (1967).

3. RESULTS

(i) *Haemoglobins*

A total of 337 specimens from all the areas of collection listed in the Introduction were examined. All showed the four electrophoretic fractions described previously (Pantelouris & Payne, 1968).

(ii) *Red-cell esterases*

As Plate 1, fig. 1, shows, there is a zone of fast esterases (*FE*) specific to red cells. It appears as a single intensely staining band in pH 7·6 gels, but in pH 8·6 gels it is resolved into bands 1, 2 and 3. Only two of these are present in any one animal, but combinations other than 1–2 are extremely rare. In view of its weak staining, this zone was not used for our comparisons.

(iii) *Plasma proteins*

The resolution achieved is demonstrated in Plate 1, fig. 2. There is phenotypic variation in every zone of the electropherogram, but we focused our attention on the two zones marked VI and VII.

Zone VI comprises 1 or 2 out of 3 possible bands. The frequency of these combinations is given in Table 1. In zone VII there are four bands, of which two only are present in any given sample (Table 2).

(iv) *Plasma esterases*

Plate 2 illustrates the plasma esterase phenotypes. Although there is undoubtedly variation in zone *a*, it was not investigated because of the weak staining. In a general way, however, it was observed that bands 1 and 2 are very rare.

Zone *c* encompasses six bands. In pH 7·6 gels a developmental change can be detected: bands 5–6 are characteristic of the smallest and youngest eels, the serum of which has a high content of the blue-green pigment described by Kochiyama *et al.* (1966). Brown eels have usually bands 3–4, but in some cases 5–6 or 1–2 are also present. Silver eels show bands 1–2 exclusively. Neuraminidase treatment of the plasma in the manner already described (Pantelouris & Arnason, 1967) retards 1–2 and 3–4 to the positions 3–4 and 5–6 respectively. This suggests that the differences between developmental stages concern the number of sialic acid residues.

Having discovered that differences in zone *c* are ontogenetic, that zone *a* is rather difficult to demonstrate clearly and that *b* apparently requires further resolution, we felt that plasma esterases were not likely to provide, at this stage, reliable data for the purpose of this investigation.

(v) *Liver esterases*

The four main zones of the liver esterase pattern are shown in Plate 3, figs. 1 and 2. Five electrophoretic fractions are separated in zone *a*. The incidence of these and their combinations in pairs are set out in Table 4. We feel that these fractions are the most convenient for comparisons of populations. On the other hand, zone *b* is not well resolved, *d* stains weakly and *c* is subject to ontogenetic change. It is extremely weak in silver eels but stronger in earlier stages (see Plate 3, fig. 2).

Table 1. *Phenotype and allele frequencies, plasma proteins, zone VI*

(*a*) *Incidence of electrophoretic fractions*

	A	*B*	*C*	Totals
Iceland	22	18	7	47
Scotland	18	36	13	67
	40	54	20	114

$\chi^2 = 4{\cdot}83$ (2 D.F.)—not significant.

(*b*) *Incidence of phenotypes*

	A	*AB*	*AC*	*B*	*BC*	*C*	Totals
Iceland	8	9	5	7	2	0	31
Scotland	6	10	2	17	9	2	46
	14	19	7	24	11	2	77

$\chi^2 = 10{\cdot}66$ (5 D.F.)—not significant.

(*c*) *Frequencies of presumed alleles.*

From the totals under (*b*)... *A* 0·375 *B* 0·472 *C* 0·135

(*d*) *Hardy–Weinberg equilibrium of presumed genotypes*

	AA	*AB*	*AC*	*BB*	*BC*	*CC*	Totals
Observed	14	19	7	24	11	2	77
Expected	10·9	27·3	8·8	17·1	11·2	1·8	77
	24·9	46·3	15·8	41·1	22·2	3·8	114

$\chi^2 = 6{\cdot}50$ (5 D.F.)—not significant.

4. STATISTICAL COMPARISONS

(i) *Zone VI of plasma proteins*

There is no significant difference in the frequency with which bands *A*, *B* and *C* occur in our samples from Iceland and Scotland, nor in the frequency of their combinations in pairs (Table 1).

The three bands may be viewed as products of alleles, and the frequency of these can be calculated. Similarly, the expected numbers of presumed genotypes can be estimated on the usual assumptions of Hardy–Weinberg equilibrium. When this is done, no significant difference is found between observed and expected numbers (see Table 1).

(ii) *Transferrins*

The same reasoning and tests as for zone VI lead to the conclusion that there is no difference in the frequency of transferrin phenotypes between eels from Iceland and Scotland (Table 2).

Other authors, comparing samples from France and from America detected differences in the frequencies of transferrin phenotypes (Drilhon & Fine, 1969).

Table 2. *Phenotype and allele frequencies, plasma proteins, zone VII*

(*a*) *Incidence of electrophoretic fractions*

	A	*B*	*C*	*D*	Totals
Iceland	7	24	17	9	57
Scotland	7	14	31	6	58
	14	38	48	15	115

$\chi^2 = 7{\cdot}4$ (3 D.F.)—not significant.

(*b*) *Incidence of phenotypes*

	A	*AB*	*AC*	*AD*	*B*	*BC*	*BD*	*C*	*CD*	*D*	Totals
Iceland	0	4	1	2	10	6	4	7	3	0	37
Scotland	1	1	4	1	3	7	3	18	2	0	40
	1	5	5	3	13	13	7	25	5	0	77

$\chi^2 = 6{\cdot}61$ (8 D.F.)—not significant.

(*c*) *Frequencies of presumed alleles*

From the totals of phenotypes... *A* 0·097 *B* 0·331 *C* 0·474 *D* 0·097

(*d*) *Hardy–Weinberg equilibrium of presumed genotypes*

	AA	*AB*	*AC*	*AD*	*BB*	*BC*	*BD*	*CC*	*CD*	*DD*	Totals
Observed	1	5	5	3	13	13	7	25	5	0	77
Expected	0·7	4·9	7·1	1·4	8·5	24·2	4·9	17·3	7·2	0·7	77
	1·7	9·9	12·1	4·4	21·5	37·2	11·9	42·3	12·1	0·7	114

$\chi^2 = 15{\cdot}9$ (9 D.F.)—not significant.

Table 3. *Comparison of transferrin phenotype frequencies (data from Drilhon & Fine (1969) and this paper)*

	A	*AB*	*AC*	*AD*	*B*	*BC*	*BD*	*C*	*CD*	*E*
(*a*) Iceland–Scotland	1	5	5	3	13	13	7	25	5	0
(*b*) Atlantic France–Mediterranean	20	106	8	1	126	61	0	0	0	0
(*c*) America	0	10	0	0	79	14	0	1	0	0

χ^2: *a*–*b* = 210 (8 D.F.)—significant.

a–*c* = 80 (8 D.F.)—significant.

b–*c* = 58 (8 D.F.)—significant.

We have not yet examined American eels, but in Table 3 we have brought together our data and those of the above authors. We are pooling their data from Atlantic France with the data of Drilhon *et al.* (1966) from the Mediterranean, because there is in fact no significant difference between the two groups (χ^2 = 24·0 for 24 D.F.).

It emerges that there are significant differences between all three samples, i.e. from Iceland–Scotland, America and France–Mediterranean.

(iii) *Liver esterase zone* a

Samples from four locations are included in this comparison (Table 4), namely from Iceland, Scotland, Hahnofersand and Stettiner Haff. The frequency of bands *A*, *B*, *C*, *D* and *E* was again compared by the contingency chi-square test. The value obtained for χ^2 is highly significant at the 5% level (60·0 for 12 D.F.). There are, however, no significant differences in the incidence of the various combinations of the five fractions in pairs.

Table 4. *Phenotype frequencies, 'fast zone' of liver esterases*

(*a*) *Incidence of electrophoretic fractions*

	A	*B*	*C*	*D*	*E*	Totals
Iceland	23	45	30	17	13	128
Scotland	13	8	18	13	2	54
Hahnofersand	28	37	26	11	0	102
Stettiner Haff	22	49	9	1	1	82
	86	139	83	42	16	366

χ^2 = 60·0 (12 D.F.)—significant.

(*b*) *Incidence of phenotypes*

	A	*AB*	*AC*	*AD*	*AE*	*B*	*BC*	*BD*	*BE*	*C*	*CD*	*CE*	*D*	*DE*	Totals
Iceland	4	7	6	3	3	18	8	7	5	14	4	3	3	1	86
Scotland	2	1	5	5	0	4	0	3	0	11	0	2	5	0	38
Hahnofersand	13	8	5	2	0	22	4	3	0	13	4	0	2	0	76
Stettiner Haff	15	4	2	0	1	42	2	1	0	5	0	0	0	0	72
	34	20	18	10	4	86	14	14	5	43	8	5	10	1	272

χ^2 = 31·0 (39 D.F.)—not significant.

5. DISCUSSION

After 16 years or so of investigation at sea and in the laboratory, Schmidt (1922) developed his generally accepted theory of eel migration. All eels from European rivers migrate to a breeding region in the Sargasso Sea, whence eventually the next generation of larvae are carried towards the continent. The breeding area of American eels has its centre S.W. of the European (see also Vladykov, 1964). There are morphological differences between the two groups, summarized by Bruun (1963), mainly in the number of vertebrae and in dentition of the intermaxillary vomerine band. The two groups are considered separate species, *Anguilla rostrata* LeSueur (American) and *A. Anguilla* L. (European). In Schmidt's words, 'the areas embraced by the two species, however, are apparently not separated, but seem to overlap'.

The 'atlantic eel problem' was reopened by Tucker (1959, 1960), who believes that the mature European eels are physically incapable of swimming 7000 km to the Sargasso Sea to breed. He hypothesizes that they die on the way and that therefore it is only the American adults that provide the next generation of elvers for both sides of the Atlantic.

The arguments against Tucker's hypothesis have been presented by Deelder (1960) and, in more detail, by Bruun (1963). Also, Tesch (1967) established that eels transferred out to sea 'home' to their continental living grounds at a pace of 2 km/hr. This would be sufficient to take them to their spawning areas, starting from Europe in October, in time for the breeding season in March.

It is to be expected that a separation into two species would be reflected in at least some protein patterns. With this in mind, Sick, Westergaard & Frydenberg (1962) compared by electrophoresis the haemoglobins of eels from Europe, America and Japan. The specimens from the two continents were identical but differed from the Japanese sample. It was at the same time confirmed that the chromosome number for all three groups is $2n = 38$. A rare haemoglobin variant was subsequently discovered in American but not in European material (Sick *et al.* 1962).

Drilhon & Fine (1969) consider that the significant differences in the frequencies of transferrin phenotypes between American and European samples that they examined constitute a confirmation of the two-species theory.

Our data suggest a complex situation in that we demonstrated certain regional differences in transferrin and liver esterase phenotypes within European-caught eels. This raises for consideration a number of possibilities:

(1) On the one-species hypothesis, European eels may be the offspring of either (i) American eels (Tucker's hypothesis) or (ii) eels from both sides of the Atlantic.

(2) Under the two-species theory, European eels may constitute either (i) one panmictic population or (ii) a number of populations separated to a smaller or greater extent.

Regional differences such as we report here may reflect subdivision of populations of the European eel, but they may also be attributed to differential selection acting on one population of elvers as it fans out towards the Continent. As our next step, we are engaged in a systematic comparison of samples from a chain of stations around the Atlantic. It is hoped to establish whether differences in phenotype frequencies are widespread and whether or not they form continuous gradients or clines correlated with geographic factors.

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