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Genetic variation in the eel

I. The detection of haemoglobin and esterase polymorphisms

By E. M. PANTELOURIS and R. H. PAYNE
Biology Department, University of Strathclyde, Glasgow, C. 1

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INTRODUCTION

The accepted theory on the life-history and migrations of the European freshwater eel, *Anguilla anguilla* L., is based on the classical work and ideas of Schmidt (1913, 1915, 1922), whose theory was that the maturing eels migrate to spawn in the Sargasso sea, their larvae finding their way back to the eastern Atlantic coast as the result of transportation by the Gulf Stream. A similar theory was developed for the American eel but these animals were placed in a separate species, *A. rostrata* Le Soeur, mainly on differences in the number of vertebrae, a morphological character of doubtful taxonomic significance.

Renewed attention has been focused on the problem by Tucker (1959; see also Deelder, 1960), who considers Schmidt's theory unproven and in fact very unlikely. Tucker suggests that it is more likely that the adult eels leaving Europe perish before they reach the spawning grounds and the European stocks are replenished by larvae produced by the American population, which has a much shorter distance to travel to the spawning area.

Tucker's theory contains two unproven hypotheses: that the adult European eels perish before breeding and that all North Atlantic eels belong to one and the same species. The fate of adult European eels is a problem for marine biologists, but it has been pointed out by Sick *et al.* (1962, 1967) that the existence of genetic differences between *A. anguilla* and *A. rostrata* would be incompatible with Tucker's assumption that they are derived from the same breeding population. Sick *et al.* (1962, 1967) have found that American and European eels possess the same two haemoglobins, although one of these haemoglobins exists in two electrophoretically distinct forms, the rarer of which was found only in American eels. However, they have shown that the haemoglobins of Japanese eels differ from the Atlantic populations in both number and electrophoretic mobility.

2. MATERIALS AND METHODS

(i) *Samples*

The eels used in this investigation were collected from rivers at the following locations within the British Isles: near Dublin, at Toomebridge (Northern Ireland), and in North Wales. All the animals were 12–40 cm. in length and all at the silver eel stage.

The animals were injected intramuscularly with 0·5 to 1·0 ml (depending upon size) of 1 % heparin in 0·85 % saline and bled 10 min later by decapitation just in front of the pectoral fins so as to cut across the conus arteriosus. Blood was allowed to flow into small tubes containing a few crystals of heparin.

The serum was removed by centrifugation at 4000 rev/min for 5 min and the compacted erythrocytes were washed three times with two volumes of ice-cold CO-saturated 0·85 % saline. The washed erythrocytes were treated with CO and lysed by the addition of an equal volume of cold CO-saturated tris–EDTA–citric acid gel buffer. The samples were stored deep-frozen under an atmosphere of carbon monoxide.

Tissue samples were deep-frozen and later homogenized, followed by ultrasonic treatment for 2 min with a 100 W M.S.E. ultrasonic disintegrator. Before use the homogenates were centrifuged for 20 min at 4000 rev/min in a refrigerated centrifuge, and the supernatants were used as samples.

(ii) *Electrophoresis*

Horizontal starch gel electrophoresis was used as described already (Pantelouris & Arnason, 1966). Agar gels (1 % 'Oxoid Agar No. 3') were also used as specified.

For haemoglobins the buffer system was as follows:

Gel buffer, pH 8·6, 0·485 % tris, 0·026 % EDTA, 0·074 % citric acid.

Vessel buffer, pH 8·6, 0·14 % diethylbarbituric acid, 0·77 % sodium 5,5-diethylbarbiturate.

The buffer system for esterases was:

Gel buffer, pH 8·6, 0·27 % tris, 0·05 % citric acid plus 10 % of the vessel buffer.

Vessel buffer, pH 8·6, 1·18 % boric acid, 0·12 % sodium hydroxide.

Before electrophoresis the haemoglobin samples were diluted 1:2 with gel buffer and centrifuged over carbon tetrachloride at 4000 rev/min for 60 min in a refrigerated centrifuge at 5 °C. Samples were applied to the gels on Whatman No. 3 filter-paper sample-holders.

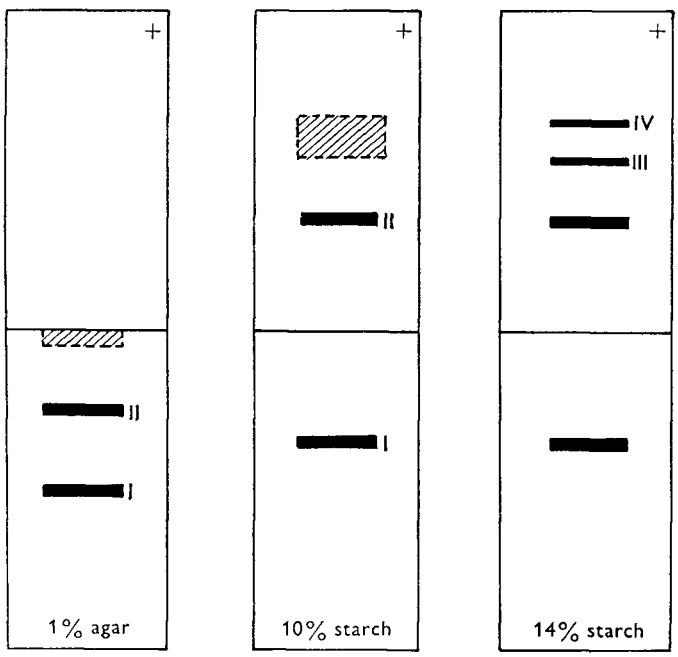
The haemoglobins were applied to the gels untreated (carboxyhaemoglobin), following vigorous aeration in the light (oxyhaemoglobin) or after oxidation with potassium ferricyanide (methaemoglobin). In addition samples were subjected to electrophoresis in gels containing 0·75 % 2-mercaptoethanol. This is a reducing agent which converts haemoglobin polymers to monomers and methaemoglobins to haemoglobins (Morton, 1966).

(iii) *Staining of gels*

The haemoglobins were stained with 0·25 % *o*-dianisidine in pH 4·6 acetate buffer, or with a 1 % solution of nigrosine in 50 % acidified methanol. The *o*-dianisidine stain was prepared by dissolving 0·25 g *o*-dianisidine in the minimum of acetone and diluting to 100 ml with 0·1 M acetate buffer. Before use, 1·0 ml hydrogen peroxide solution (20 vol.) was added to the staining solution. In practice this stain was found superior to *o*-dianisidine stains prepared by dissolving the

reagent in 1:1 mixture of ethanol and phosphate buffer, pH 5·0. Gels stained in *o*-dianisidine were washed for 2 h in warm 1:250 'Teepol' solution and stored overnight in 50% methanol before photography. Gels stained with nigrosine were washed repeatedly in acidified 50% methanol. In some instances, haemoglobins were eluted from gels with a measured volume of gel buffer and estimated quantitatively in a 'Unicam S.P. 800' spectrophotometer.

Detection of esterases on the starch gels were accomplished by the Fast Garnet technique described previously (Pantelouris & Arnason, 1966), employing the following esters as substrates: 1-napthtyl acetate, 2-naphthyl acetate, naphthyl butyrate, naphthyl stearate, and 1-naphthol acetate (Sigma).



Text-fig. 1. Diagram to show the resolution of eel haemoglobins in different gels: 1% agar, 10% starch and 14% starch. The weak diffuse region corresponding to Hb III and Hb IV moves slowly towards the cathode in the agar gel but becomes a fast anodal band when starch is used. However, a 14% starch gel is required to separate these two minor haemoglobins into discrete bands. The same buffer system was used with all gels.

3. RESULTS

(i) *Haemoglobins*

Electrophoresis of oxyhaemoglobin and carboxyhaemoglobin samples in agar gels permitted the resolution of two haemoglobin fractions which migrated towards the cathode. In addition a very slow, weak and diffusely staining region was observed close to the origin. This diffuse band moved towards the cathode but could not be resolved further with the agar gel system (Text-fig. 1). The same samples were run on the 10% starch gels. One haemoglobin fraction (Hb I)

moved towards the cathode while the other (Hb II) migrated anodally. The diffuse region moved quickly towards the anode in front of Hb II. No improvement in resolution was obtained. However, when the starch concentration was increased to 14% the diffuse band was immediately resolved into two distinct minor bands (Hb III and Hb IV). The haemoglobins of all ninety-seven animals examined could be resolved into these four fractions.

It was decided to test whether Hb III and Hb IV were polymers of Hb I and/or Hb II, or methaemoglobin derivatives of these haemoglobins. Electrophoresis in gels containing 2-mercaptoethanol did not cause the disappearance or alter the mobility of any of the four bands. In the case of samples treated with potassium ferricyanide it was also possible to resolve four fractions, although electrophoretic mobilities were greatly changed. The methaemoglobin derivative of HbI increased slightly in mobility towards the cathode, while the anodal mobility of met-Hb II was less than that of oxy-Hb II. Met-Hb III also migrates more slowly than oxy-Hb III but slightly faster than oxy-Hb II. It would appear that Hb III is quite resistant to oxidation as conversion to methaemoglobin was not quantitative. Hb IV is also retarded in mobility by treatment with potassium ferricyanide, the conversion being complete in this case. These observations are illustrated in Plate 1, fig. 1. The electrophoretic mobilities of eel oxyhaemoglobins and carboxy-haemoglobins are apparently identical.

Quantitative estimations of Hb I and Hb II have demonstrated that these haemoglobins are not present in equal amounts. The ratio Hb I/Hb II measured spectrophotometrically at 416 mμ is in the region of 0·4.

(ii) *Esterases*

Serum esterase zymograms show eight bands as numbered in Plate 1, fig. 2. The first two (1, 2) are not usually seen, unless the starch gel is left in the reaction medium overnight; and unless 1-naphthyl acetate is the substrate. Individuals differ in that they may possess one or the other or both of these bands.

The strong band 3 stains with all substrates used, whilst 4 reacts with all substrates other than naphthyl stearate and naphthol acetate.

The four slower bands 5–8 appeared with all our substrates. With some substrates, the intensity of the reaction makes them coalesce into a single broad zone, but naphthol acetate resolves them well. Not all samples possess all four bands, and the following phenotypes were encountered: 5 + 6 + 7, 5 + 6 + 8, 5 + 6, 6 and 7 (Plate 1, fig. 2).

A survey was made of extracts from homogenized tissues or organs. As expected, organs differ from each other in their esterase patterns. The most constant feature is the fraction numbered 3 in serum zymograms: it is present, and is in fact the strongest band, in a whole series of organs: liver, spleen, pancreas, muscle, gonads. There are individual variations suggestive of genetically determined polymorphism in all these organs, for example, the liver (Plate 2).

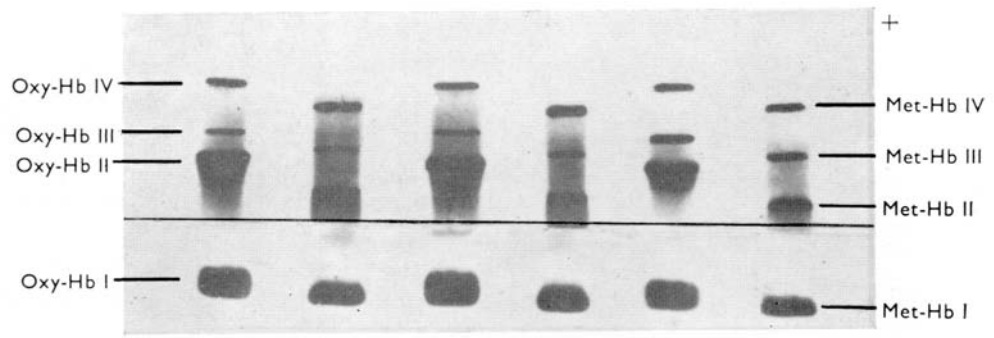


Fig. 1. Starch gel electrophoresis of the haemoglobins of the common European eel demonstrating the relative electrophoretic mobilities of the four haemoglobins in their oxyhaemoglobin and methaemoglobin forms. Electrophoresis for 1 hr. at 200 V., pH 8·6, stained with nigrosine.

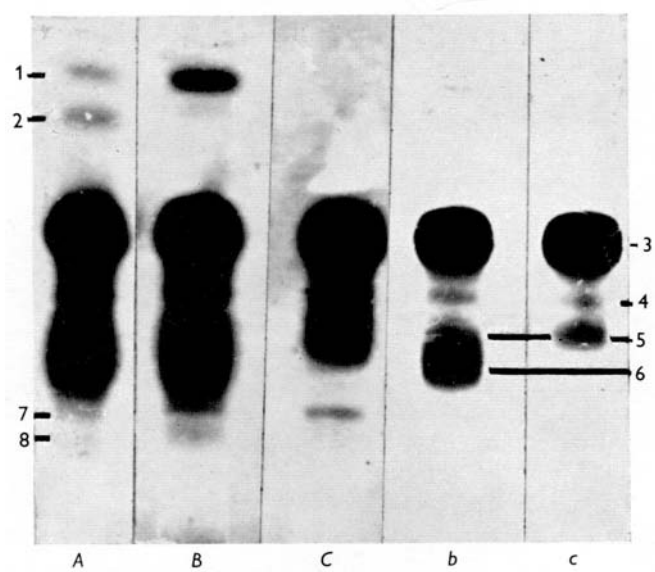


Fig. 2. Variation in esterase zymograms of three eel sera; *A*, *B* and *C*. *b* and *c* are earlier photographs of the last two, taken before the intense staining reaction resulted in fusion of the strongest bands. 1 to 8 are the esterase bands demonstrated, but not all samples possess them all. For example, *A* has 1 and 2 but *B* has only 1. Also *B* has 5 and 6 whilst *C* exhibits only 5.

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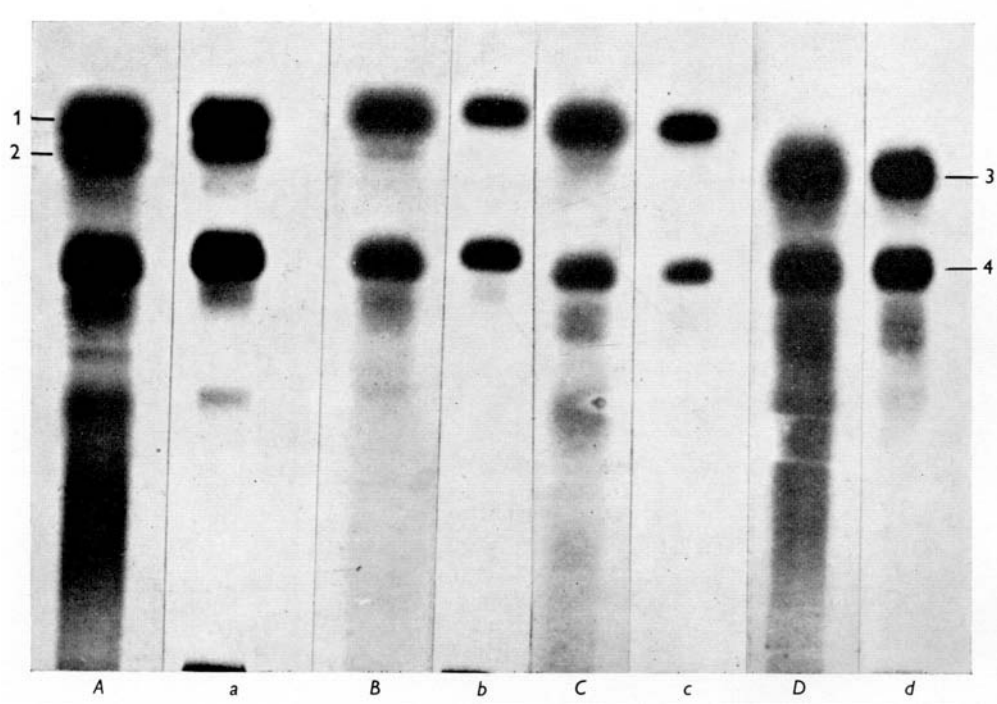

Esterase zymograms of eel liver extracts. *A* and *a* are photographs of the same zymogram taken at the end of the staining reaction and at its beginning, hence the weaker fractions are only seen in the former. The same applies to *B* and *b*, *C* and *c* and *D* and *d*. Whilst all four individuals have band 4, some have both 1 and 2, others have only 1. This suggests that 1 and 2 could be governed by a pair of alleles.

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4. DISCUSSION

The main point at issue in the eel controversy is whether eels found in rivers on both sides of the Atlantic belong to the same species and are derived from a common breeding population, or whether they represent two distinct populations which do not interbreed.

Sick *et al.* (1962, 1967) have pointed out an approach to this problem: if it can be shown that the eels of the European and American Atlantic rivers differ genetically with regard to qualitative characters, they cannot be derived from the same population. We believe that this is a useful basis for research but it cannot be asserted absolutely. European and American samples may show genetic differences even if derived from a common breeding population. Such a situation could arise if selection pressure against certain genotypes was unequal on fish migrating in the two directions. It is reasonable to suggest that different selective pressures may apply during a 3-year migration of 3000 miles to Europe and a much shorter migration to North America. Sick *et al.* (1967) have shown that a haemoglobin variant is found in American samples at low frequency but is absent from European material; this may equally well be evidence of selective pressure or of derivation from separate breeding populations.

Despite these difficulties of interpretation, it is clearly necessary to collect the evidence for genetic differences or similarities between eels caught in America and in Europe. Obviously attention should be focused on qualitative rather than metric characters, for the latter are more prone to environmental influences. One would wish to find a number of particular mutations in the one group only, or large differences in phenotype frequencies incompatible with random breeding, to suggest that the populations are quite distinct. On the other hand, similar phenotype frequencies or a gradual decrease in phenotypic variation with distance from the origin would suggest one breeding population. Whether European adults contribute to this breeding population is a different matter.

It is clear from the findings of Sick *et al.* (1962) that eels from both sides of the Atlantic have much in common with respect to haemoglobin types, while Japanese samples are distinctly different.

Our results show that there are in fact four and not two haemoglobins in *Anguilla anguilla* L., our Hb I and Hb II corresponding to the haemoglobins described by Sick *et al.* These workers failed to discover Hb III and Hb IV because resolution with agar gel is not as good as with starch gel electrophoresis, and because they did not use, as we did, concentrated samples and also 14 % starch gel instead of the usual 10 %.

The minor haemoglobins are probably not polymers of Hb I and Hb II, as they appear with equal clarity when 2-mercaptoethanol is incorporated into the gels. However, it was found that 2-mercaptoethanol inhibits the peroxidase activity of haemoglobin and gels must be stained with nigrosine.

It is also true that Hb III and Hb IV are not methaemoglobin derivatives of Hb I and Hb II as (1) they appear in the same position when 2-mercaptoethanol is

incorporated into the gels and (2) the methaemoglobin derivatives of the four fractions have been prepared and their electrophoretic mobilities characterized.

The serum proteins of the eel have been included in an electrophoretic survey of fish sera by Drilhon (1953, 1959). In a similar way, Augustinsson (1959) examined the esterases of several species by column electrophoresis and found the eel serum to be the richest source of enzymes hydrolysing aromatic esters and triglycerides. Neither of these authors recorded individual differences.

Our results show the existence of such individual differences in two regions of the serum esterase zymogram: the 1–2 and the 5–8 regions. The differences are such as to suggest genetic polymorphism. It can be surmised that 1 and 2 are allelomorphs at one locus. In the slow region, it appears likely that 5–6 (and 7–8) are allelomorphs. The electrophoretic patterns of tissue extracts indicate that here also there is a wealth of individual variation which should be studied further so as to decide whether or not it represents genetic polymorphism. No direct proof can be expected so long as it is not possible to set up controlled matings and obtain pedigrees of eels. The technique applied, however, will serve the purpose of obtaining data on the frequencies of the various phenotypes. These data may provide evidence of the genetic mechanisms accounting for them. At the same time, the data should, as in the case of haemoglobins, demonstrate similarities or differences in phenotypic frequencies in samples from the two sides of the Atlantic, and thus contribute to the elucidation of the 'Atlantic eel problem'.

SUMMARY

Electrophoretic studies on starch gel demonstrated the existence in the European eel of four haemoglobins, not two as believed from agar gel studies. The two minor fractions have been shown to be neither polymers nor methaemoglobin derivatives of the two principal fractions.

The esterase zymograms of eel sera exhibit up to eight fractions. The individual differences observed suggest genetic polymorphism and the same applies to the esterase pattern of various tissues.

This approach is of value for the collection of phenotypic frequency data with a view to solving the Atlantic eel problem.

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