# PHYSIOLOGICAL PROPERTIES OF
# EEL HAEMOGLOBIN: HYPOXIC ACCLIMATION,
# PHOSPHATE EFFECTS AND MULTIPLICITY

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## SUMMARY

Unlike the whole blood oxygen affinity, which adapts readily to environmental oxygen tensions, haemoglobins prepared from normoxic- and hypoxic-acclimated eels (*Anguilla anguilla*) show no adaptive changes in oxygenation properties or in multiplicity. Hypoxic acclimation is, however, accompanied by a strong decrease in red cell nucleoside triphosphates, particularly guanosine triphosphate (GTP), which depresses oxygen affinity of the composite and component haemoglobins more strongly than does the concurring ATP. The effects of pH, temperature and salts on the oxygenation properties of the (isolated) haemoglobins are reported, discussed in relation to the varying environmental conditions encountered by eels, and compared with data on American and Japanese eels (*A. rostrata* and *A. japonica*, respectively).

## INTRODUCTION

The functional properties of fish haemoglobins are strongly adapted to environmental conditions (Willmer, 1934; Grigg, 1974; Johansen & Weber, 1975). Eels migrate between fresh and sea waters that vary greatly in temperature, salinity, pH and content of dissolved gases – factors that generally influence the oxygen-binding properties of haemoglobin. Eel haemoglobin is thus of particular interest in the study of environmental adaptation of haemoglobins in fishes. This paper deals with a number of specific questions provoked by previous investigations on oxygen-binding of eel haemoglobin and its significance to oxygen transport in the natural environment.

Wood & Johansen (1972) demonstrated that subjection of the eel *Anguilla anguilla* to hypoxic conditions results in increased blood oxygen affinity. This is associated with a decreased concentration of organic triphosphates which, as with DPG in mammals (Benesch & Benesch, 1967), are potent regulators of blood oxygen affinity in fish (see also Gillen & Riggs (1971), Wood, Johansen & Weber (1975)). Co-factor interaction is, however, not the sole mechanism by which oxygen affinity could adapt to environmental conditions, for changes in the pigment itself could also occur. In goldfish and rainbow trout, for example, the thermo-acclimatory processes

are accompanied by changes in the patterns of heterogeneity of the haemoglobins (Houston & Cyr, 1974; Weber, Wood & Lomholt, 1976). The fact that the individual haemoglobin components in the Japanese eel, *Anguilla japonica* (Yamaguchi *et al.* 1962; Yoshioka *et al.* 1968; Okazaki, Misawa & Shukuya, 1974), and the American eel, *A. rostrata* (Gillen & Riggs, 1973), have distinctly different oxygenation properties, suggests that haemoglobin multiplicity could be involved in the adaptation of *A. anguilla* haemoglobin to environmental conditions. We thus investigated the structural and functional heterogeneity of *A. anguilla* haemoglobin following acclimation to hypoxia. Data on heterogeneity in the three eel species provides information on the differentiation of haemoglobin systems in closely related fish. In addition comparison of *A. rostrata* and *A. anguilla* is of interest in view of the controversy as to whether they are distinct species, or merely morphological variants of the same species resulting from different environmental interaction during their migrations from the breeding grounds to Europe and North America, respectively (Poluhowich, 1970).

Geoghegan & Poluhowich (1974) report that in the American eel, *A. rostrata*, the major erythrocytic phosphate is guanosine triphosphate (GTP) while ATP is the second most abundant. The GTP and ATP concentrations were thus measured in the blood of eels acclimated to hypoxic and normoxic conditions. The relative influences of both co-factors on oxygen affinity of the composite and component haemoglobins were also determined. Finally the temperature and salt sensitivities of the oxygen affinities of *A. anguilla* haemoglobins were investigated, since the low sensitivities to temperature and salinity reported for the haemoglobins of Japanese and european eels, respectively, have been interpreted as compensatory mechanisms that favour a constancy in blood-oxygen affinity (Kawamoto, 1929; Poluhowich, 1972).

### MATERIALS AND METHODS

#### Animals and acclimation

Specimens of *Anguilla anguilla* (Linnaeus), from 46 to 52 cm in length (240–260 grams, wet weight), were obtained from Dejret near Aarhus, Denmark. The eels were maintained in sea water at 15 °C, and were acclimated to oxygen tensions of 30 and 150 mmHg (i.e. hypoxic and normoxic conditions, respectively) for three weeks as described by Wood, Johansen & Weber (1975).

#### Blood collection and phosphate assay

Blood was collected from the caudal vein into a 10 ml syringe of which the dead space was filled with heparin (5000 IE units). The concentration of total nucleoside triphosphate (NTP) in whole blood, and of ATP (disodium salt, Sigma, St Louis) and GTP (trisodium salt, Boehringer, Mannheim) and diphosphoglycerate, DPG, in stock solutions, were determined by Sigma enzymic procedures. The concentrations of ATP and GTP in eel blood (deproteinized with equal volumes of 10 % trichloracetic acid) were also determined by thin-layer chromatography, as described by Cashel, Lazzarini & Kalbachu (1968) but with the following modifications. Samples of 0·1 ml were applied along a 2 cm line on the cellulose carrier plates (Cel 300 PEI – Macherey, Nagel & Co., Düren, West Germany) and the phosphates were developed

In 1·5 M potassium dihydrogen phosphate, pH 3·5 (ascending chromatography) in a closed chamber at room temperature. GTP and ATP were identified under ultraviolet light on the basis of their positions and those of the pure phosphates that were run in parallel. Areas containing the separated phosphates were cut out and extracted in 4 ml aliquots of 2 N-NaOH, and the nucleoside triphosphates were determined spectrophotometrically (230–280 nm), taking as zero the values obtained from similarly treated NTP-free carrier of the same area and at the same distance from the starting line as the phosphate spots.

*Haemoglobin preparation*

The red cells were washed twice in 2·0% NaCl and lysed in five times their volume of 0·1 N-Tris buffer, pH 8·0. The cell debris was separated by centrifugation and the red supernatant dialysed for 20 h against 3 changes of 0·01 N-Tris-HCl buffer, pH 7·8, containing $5 \times 10^{-4}$ M-EDTA, which stabilizes the reduced form of haemoglobin (Giovenco *et al.* 1970). All preparative steps were carried out at 0–5 °C.

Haemoglobin solutions were 'stripped' of organic and inorganic ions using Amberlite MB-3 mixed ion exchanger.

All spectroscopic observations were carried out using a Unicam SP 1800 spectrophotometer. Where partial oxidation of the haemoglobin was seen, the haemoglobin was reduced by addition of a pinch of solid dithionite and dialysis against three changes of CO-saturated Tris-EDTA for at least two days during rapid stirring. Haemoglobin concentrations were estimated after reduction with solid dithionite and saturation with carbon monoxide, taking a millimolar extinction coefficient of 13·4 at 540 and 569 nm. Chloride concentrations were measured using a Radiometer CMT 10 chloride titrator.

*Isoelectric focusing*

The component composition of the carboxy-haemoglobins was studied by isoelectric focussing in 110 ml LKB isoelectric focussing columns (LKB Produkter, Bromma, Sweden) containing 1% Ampholine solutions, pH 3–10, at 5 °C, essentially as described by the LKB manual. At the end of focussing, the column contents were collected in 0·8 ml fractions, and optical density and pH values of the fractions were read using respectively a Zeiss PMQ II spectrophotometer and a Radiometer model 26 pH meter with microelectrode unit (type E5021).

Haemoglobin fractions from isoelectric focussing columns were pooled and dialysed for 72 h against four changes of 0·01 N-Tris HCl buffer, pH 7·8, after which the pH of the haemoglobin solution had assumed that of the buffer. It is unlikely that residual ampholines could influence oxygen-binding properties of the haemoglobins; in control experiments (unpublished) the presence of 1 per cent added ampholines only slightly changed the oxygen affinity of a stripped eel haemolysate at 20 °C and pH 7·43 (P50 decreased from 2·2 to 2·1 torr).

Table 1. *Concentrations of the total nucleoside triphosphates (NTP) and of ATP and guanosine triphosphate (GTP), in mM.l⁻¹ red cells, in blood of eels acclimated to hypoxic and normoxic conditions*

| | | Phosphate concentration ($\pm$ S.D.) | | Significance of difference* | |
| | Method of | | | | |
| Phosphate | determination | Normoxic ($N = 6$) | Hypoxic ($N = 7$) | $t$ | $P$ |
|---|---|---|---|---|---|
| NTP | Enzymic | 10·43 ± 2·62 | 8·39 ± 1·74 | 1·67 | N.S. |
| ATP + GTP | Thin layer | 11·06 ± 0·71 | 7·32 ± 1·01 | 7·54 | < 0·001 |
| ATP | Thin layer | 2·31 ± 0·38 | 1·84 ± 0·31 | 2·48 | N.S. |
| GTP | Thin layer | 8·75 ± 0·85 | 5·84 ± 1·10 | 5·88 | < 0·001 |
| GTP/ATP | Thin layer | 3·78 | 2·98 | | |

* Using Student's $t$ test; N.S. = not significant.

## Oxygen equilibrium determination

Haemoglobin-oxygen equilibria were determined using an oxygen diffusion chamber modified after Sick & Gersonde (1969). In the procedure the oxygen tension of gas mixtures, used to equilibrate a thin smear of deoxygenated haemoglobin solution, was increased in stepwise fashion using a Wösthoff model 201/a-F gas mixing pump. The changes in light absorption at 436 nm were recorded with a photomultiplier tube (model RCA 931-A) and an Eppendorf model 1100M photometer coupled to a potentiometric linear recorder.

Values of the haem-haem cooperativity coefficient $n$, which reflects the sigmoid character of the haemoglobin-oxygen equilibrium curve, were determined graphically at the $P_{50}$ value (the oxygen tension which half-saturates the haemoglobin), using the Hill plot: $\log[\text{Oxy Hb}]/[\text{Hb}]$ vs $\log p_{O_2}$.

### RESULTS AND COMPARATIVE INTERPRETATION

#### (a) Organic phosphate concentrations

The concentrations of total nucleoside triphosphates (NTP) and of ATP and GTP were lower in eels with hypoxic history than in normoxic ones (Table 1), in agreement with previous measurements of the organic triphosphates (Wood & Johansen, 1972). Significantly, the GTP concentrations were about 3–4 times higher than the ATP concentrations (Weber, Lykkeboe & Johansen, 1975). This accords with the high GTP/ATP ratios (4·4 in seawater eels, and 2·7 in freshwater eels) found by Geoghegan & Poluhowich (1974) for *A. rostrata*. In hypoxic eels, the GTP:ATP ratio was lower than in normoxic animals (Table 1), indicating that GTP plays a major role in adapting oxygen affinity of the blood to ambient oxygen tensions.

#### (b) Oxygen equilibria of haemoglobin in solution

In freshly prepared haemoglobin solutions, at pH 7·4 and 15 °C, $P_{50}$ was around 1·7 torr and the Bohr effect ($\phi = \Delta \log P_{50}/\Delta \text{pH}$), was −0·46 (Fig. 1). Remarkably the Bohr effect was reversed at high pH, assuming a value of +0·07 between pH 8·0 and 8·2. A reversed Bohr effect at high pH has been reported in carp haemoglobin, but not in that of *A. rostrata* (Gillen & Riggs, 1972, 1973).
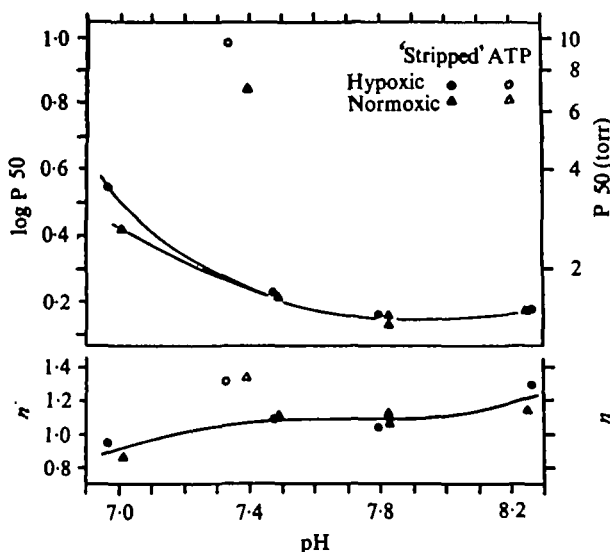
Fig. 1. pH dependence of the half-saturation oxygen tensions (P50) and Hill's sigmoid coefficients, $n$, of 'stripped' haemoglobin from hypoxic- and normoxic-acclimated eels, *A. anguilla*, in 0·1 M Tris and bis Tris buffers, and in the absence and presence (23-fold excess over haemoglobin tetramers) of ATP. Temperature, 15 °C; Hb conc., 0·21 mM.

The P50 and $n$ values of stripped haemoglobins from hypoxic and normoxic eels were virtually identical at pH values above 7·2 (Fig. 1). Near neutrality, however, slightly higher P50 and Bohr effect values were obtained in the haemoglobin of hypoxic eels. In repeat measurements on the haemoglobins that had been kept in deep-frozen storage for 2 weeks, the same differences between hypoxic and normoxic eels were found, although all P50 values were lower after freezing. (Similar effects of freezing have been observed in turtle haemoglobins – Sullivan & Riggs, 1967.) The difference in P50 between hypoxic and normoxic eels was, however, small, and opposite to that observed in whole blood (Wood & Johansen, 1972, 1973).

The $n$ values of the stripped haemoglobins were low (0·9–1·3), and similar in haemoglobins from hypoxic and normoxic animals. They showed a positive correlation with pH between 7·0 and 8·2. Saturation with ATP drastically reduced oxygen affinity and increased the $n$ value in haemoglobins from both groups of eels (Fig. 1).

We investigated the responses of eel haemoglobin to the major organic phosphates in the red cells (GTP and ATP) and other organic and inorganic phosphates. Significantly, GTP was found to decrease oxygen affinity much more strongly than ATP at the same phosphate:haemoglobin concentration ratios (Fig. 2A). The affinity of eel haemoglobin was also strongly reduced by IHP, whereas the DPG effect was of the same order of magnitude as the ATP effect (Fig. 2B). The organic phosphates which had the greatest effect on P50, also exerted the greatest influence on cooperativity (Fig. 2A, B). Inorganic phosphates elicited a large P50 increase (comparable to that of GTP and IHP). A diphosphate (sodium pyrophosphate) was, however, more effective in reducing P50 at low concentration than the monophosphate buffer salts (Fig. 2C). Particularly noteworthy was the finding that in the presence of inorganic phosphates saturating concentrations of GTP caused a
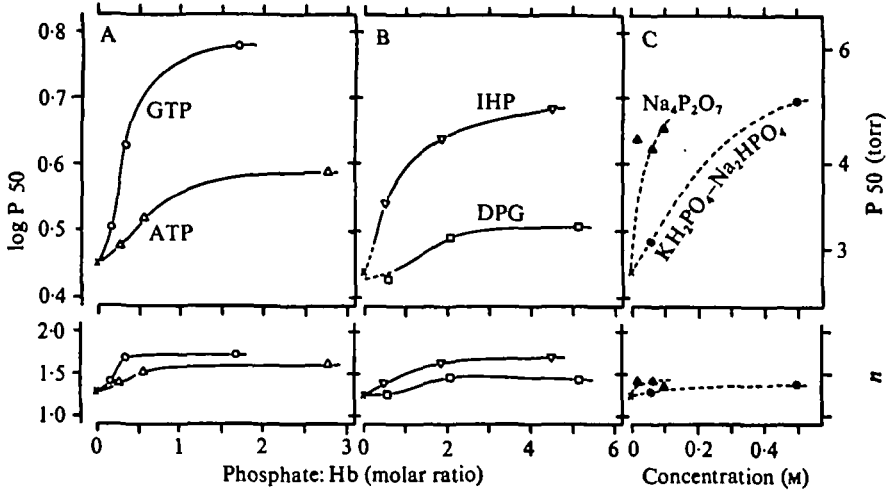
Fig. 2. Effects of the organic phosphates ATP, GTP (guanosine triphosphate), DPG (2,3-diphosphoglycerate) and of the inorganic phosphates $Na_4P_2O_7$ (sodium pyrophosphate) and $KH_2PO_4$-$Na_2HPO_4$ (phosphate buffer salts) on P 50 and $n$ values of stripped eel haemoglobin in 0·1 M Tris buffer. pH 7·55; temperature, 20·0 °C, Hb conc., 0·22 mM.

Table 2. *Effect of various combinations of phosphate buffer salts and GTP and ATP on P 50 and n of stripped eel haemoglobin in* 0·1 M *Tris buffer; pH* 7·47; *temperature,* 20 °C; *haemoglobin concentration, approximately* 0·22 mM (*tetramer*)

| Phosphate buffer concentration (M) | Organic phosphate to haemoglobin ratio | | log P 50 | $n$ |
|---|---|---|---|---|
| | ATP | GTP | | |
| 0·0 | 0 | 0 | 0·47 | 1·14 |
| 0·5 | 0 | 0 | 0·69 | 1·38 |
| 0·5 | 26 | 0 | 0·65 | 1·55 |
| 0·5 | 0 | 16 | 0·82 | 1·41 |
| 0·5 | 13 | 8 | 0·80 | 1·81 |

further increase in P 50 (Table 2), whereas this was not found with ATP. All curves yielded monophasic Hill plots, except when both GTP and inorganic phosphates occurred at high concentration, in which case $n$ was higher at high than at low oxygen saturation.

The effect of temperature on haemoglobin-oxygen affinity was measured at high pH (8·63 at 20 °C) to exclude contributions from the Bohr effect. Between 5 and 25 °C, P 50 was related to absolute temperature ($T$) according to the linear regression:

$$\log P\,50 = 3198(1/T) + 11·40 \quad (N=9; \text{ correlation coefficient, } -0·994).$$

The apparent heat of oxygenation ($\Delta H$), calculated from the modified Van 't Hoff equation: $\Delta H = 2·303R.\Delta \log P\,50/\Delta(1/T)$ (Wyman, 1964), was $-14·6$ kcal.mole$^{-1}$, which falls within the range generally found for vertebrate haemoglobins.

### (c) Haemoglobin heterogeneity

#### (i) Component haemoglobins

In isoelectric focussing eel carboxy-haemoglobins resolved into six components, with isoelectric points at 5 °C near 9·4, 7·7, 6·7, 6·1, 5·5 and 5·4. No significant
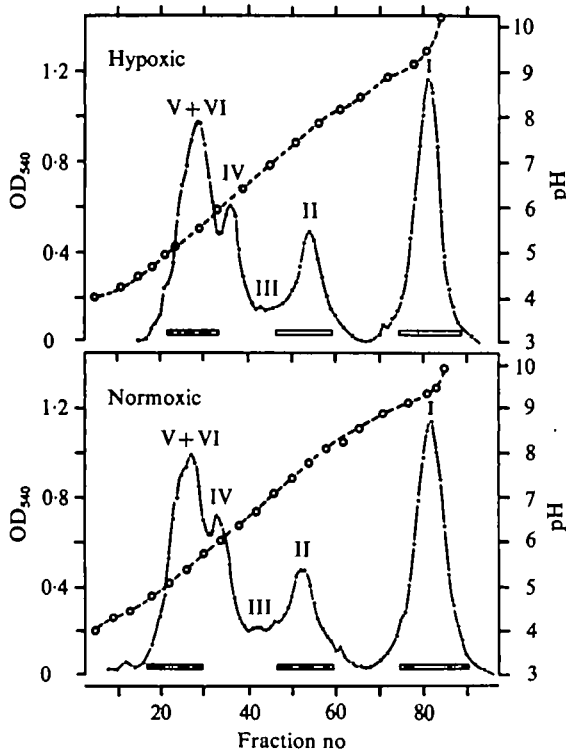
Fig. 3. Separation of multiple haemoglobins of eels, *A. anguilla*, acclimated to hypoxic (upper panel) and normoxic conditions (lower panel), by preparative isoelectric focussing. ○, pH values at 5 °C; ●, optical densities at 540 nm of fractions collected from the isoelectric focussing column; horizontal bars, fractions pooled for oxygen equilibrium determination.

difference was found in the number and relative concentrations of components in haemoglobins originating from hypoxic and normoxic eels (Fig. 3). Planimetric analysis of the optical density diagrams shows that components I, II and IV + V + VI comprised about 33, 15 and 48% respectively of the total haemoglobin, both in eels acclimated to air saturated water and in hypoxic eels.

### (ii) *Functional characterization of haemoglobin components*

*pH effects.* The P50 and *n* values of the major haemoglobin components and their pH dependencies are shown in Fig. 4. Component I, which had the highest iso-electric point (cathodic), differed significantly in its oxygen-binding properties from the other major (anodic) components. As in *A. rostrata* (Gillen & Riggs, 1973) and *A. japonica* (Yamaguchi *et al.* 1962; Yoshioka *et al.* 1968) it showed higher oxygen affinity and cooperativity than the anodic components. Remarkably Hb I showed a 'reverse' Bohr effect with a Bohr factor of about +0·34 at pH 7·4. In Hbs II and IV + V the Bohr effects were 'normal' with $\phi$ values of −0·40 and −0·51, respectively. In stripped solutions of the cathodic component of *A. rostrata*, Gillen & Riggs (1973) similarly found a reverse Bohr effect. This, however, disappeared in the presence of ATP, suggesting that the absence of a Bohr effect previously reported in Hb I of
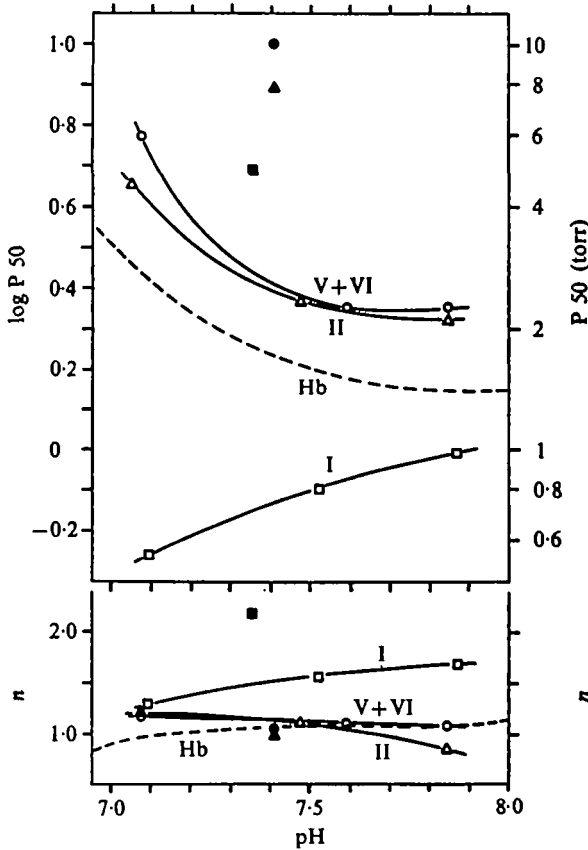
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Fig. 4. P50 and *n* values in 0·1 M Tris and bis Tris buffer of the main haemoglobin components (isolated from hypoxia-acclimated eels, *A. anguilla*) and their pH and ATP sensitivities. Roman figures, haemoglobin components in order of decreasing isoelectric point (cf. Fig. 3). Dashed curves, data for stripped whole haemolysate (cf. Fig. 1); open symbols, absence of added phosphate; closed symbols, presence of saturating concentrations of added ATP (ATP/haemoglobin ratio, approximately 50); temperature, 15 °C; Hb conc., approximately 0·1 mM.

*A. japonica* by Yamaguchi *et al.* (1962) and Yoshioka (1968) is attributable to their use of phosphate buffers as solvents.

*Organic phosphates.* ATP significantly depressed the oxygen affinities of the cathodic and the main anodic components, but the decrease was greater in Hb I (Figs. 4, 5).

The relative sensitivities of Hb I and Hb V+VI to ATP and GTP are shown in Fig. 5. As for the haemolysate, GTP depressed oxygen affinity more strongly than ATP in both the haemoglobin fractions studied.

*Inorganic salts.* Increasing sodium chloride concentrations increased both the P50 and *n* values of stripped solutions of Hb I and of Hb V+VI of *A. anguilla*, but as with the organic anions the effects were more pronounced in the cathodic than in the anodic fractions (Fig. 6). Between NaCl concentrations of 0·13 and 0·17 M (corresponding to the milliosmol values of 250 and 315 which characterize plasma concentrations of *A. rostrata* in fresh and sea water, respectively – Poluhowich &
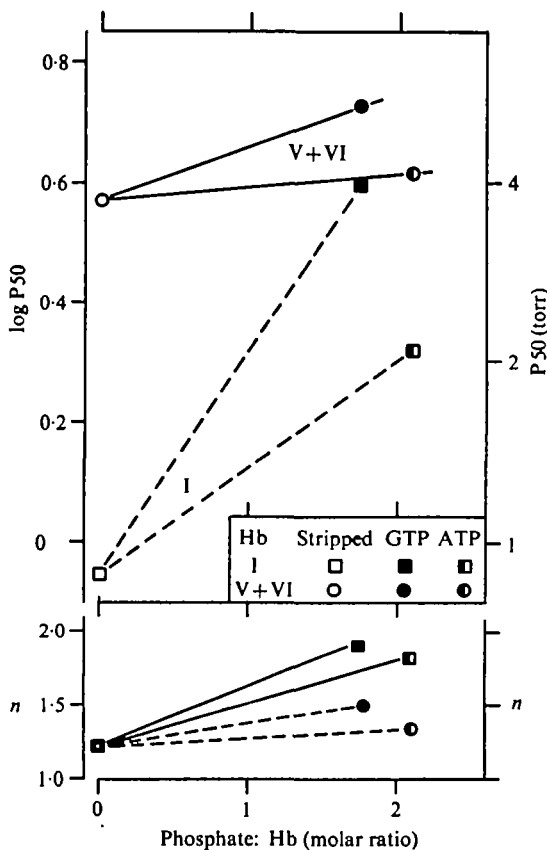
Fig. 5. Effects of GTP (guanosine triphosphate) and ATP on $P_{50}$ and $n$ values of the main cathodic (Hb I) and anodic (Hb V+VI) eel haemoglobin components (isolated from normoxia-acclimated eels – Fig. 3) in Tris buffer containing 50 mM NaCl. pH, 7·56; temperature, 20 °C.

Parks, 1971) the salt effect values ($\Delta \log P_{50}/\Delta \log [\mathrm{NaCl}]$) amounted to 0·19 and 0·12 for Hb I and Hb V+VI, respectively. The higher NaCl effect in the cathodic than in the anodic Hb is directly opposite to the data reported for *A. japonica* (Yamaguchi *et al.* 1962) and salmon (Hashimoto, Yamaguchi & Matsuura, 1960) haemoglobins. The difference may be related to the presence in the earlier studies of phosphates, which mask the intrinsic salt effects, particularly that of the cathodic component which shows greater phosphate sensitivity.

*Temperature effects.* The effects of temperature on the oxygen equilibria of Hb I and Hb V+VI of *A. anguilla* (Fig. 7) are consistent with apparent $\Delta H$ values of approximately $-13\cdot3$ kcal.mole$^{-1}$ and $-16\cdot0$ kcal.mole$^{-1}$, respectively, which are in good agreement with that of the haemolysate. They are, however, higher than those (approximately $-7$ and $-13$ kcal.mole$^{-1}$) found by Yamaguchi *et al.* (1962) for *A. japonica* cathodic and anodic haemoglobins in phosphate buffers. These data suggest that the low temperature sensitivity of whole blood (Kawamoto, 1929) is attributable to phosphate interaction in accordance with the exothermic nature of organic phosphate binding to haemoglobin (Benesch & Benesch, 1968).
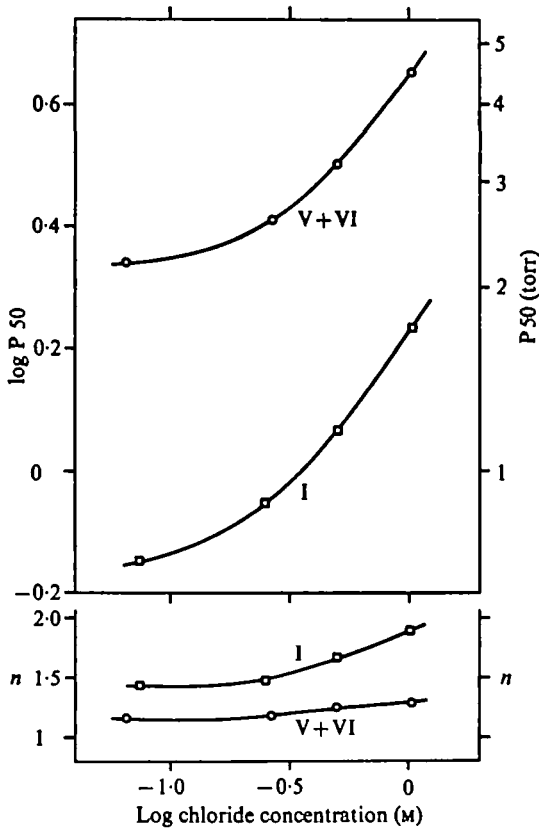
Fig. 6. Effects of NaCl on P 50 and $n$ values of the main cathodic (Hb I) and anodic (Hb V+VI) eel (*A. anguilla*) haemoglobin components, in 0·025 M Tris buffer. pH, 7·60; temperature, 15 °C; Hb conc., approximately 0·1 mM.

## DISCUSSION

It is well established that haemoglobin-oxygen equilibria in fish whole blood adapt to environmental oxygen tension by changed co-factor interaction. However, it is not known whether the functional properties of the haemoglobin adjust to environmental conditions through synthesis of new haemoglobins, or through changes in relative concentrations of component haemoglobins, either in the differentiating or in the circulating red cells. Unlike mammals, fish red cells possess nuclei, a necessary prerequisite for protein synthesis. The identical multiplicity patterns of haemoglobins from hypoxic and normoxic eels (separated by isoelectric focussing) plus the similarity in P50 and $n$ values of 'stripped' haemoglobins from each group shows, however, that the haemoglobin itself does not contribute to the adaptation of blood oxygen affinity to ambient oxygen tensions, demonstrated previously by Wood & Johansen (1972).

The findings that GTP is the most abundant red cell phosphate in *A. anguilla* (as in *A. rostrata*; Geoghegan & Poluhowich, 1974), that the GTP levels decrease more significantly than ATP levels during subjection to hypoxia and that GTP is the more potent regulator of oxygen affinity, demonstrate that this triphosphate
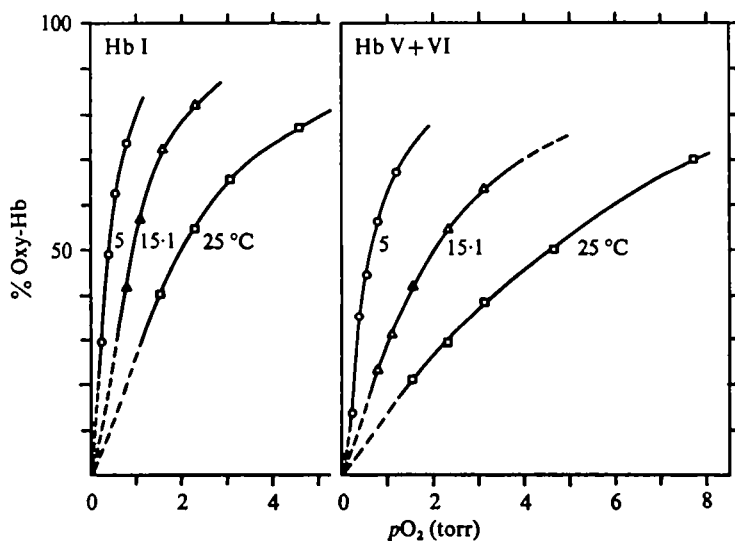
Fig. 7. Effect of temperature on oxygen equilibrium curves of eel (*A. anguilla*) haemoglobin components I and V+VI (cf. Fig. 3), in o·1 M Tris buffer. pH, 8·00 to 8·5 (decreasing with increasing temperature); Hb conc., approximately o·1 mM.

must play the major role in the adaptation of eel blood to environmental oxygen levels. An important unanswered question is whether, apart from variation in the total NTP, transfer of phosphate between the two nucleosides could form the basis for the rapid and exact attunement of blood P 50 in eels to the varying oxygen tensions in their habitats.

It has been shown (Benesch & Benesch, 1968; Arnone, 1972) that the organic phosphates' polyanions bind at a well-defined protein site of the tetrameric vertebrate haemoglobin molecule (i.e. at the entrance to the central cavity on the diad axis of the molecule, where they react with the basic $N$-terminal residues of the $\beta$ chains). The greater effect of IHP than of ATP and DPG on eel haemoglobin tallies with the fact that IHP has eight negative charges at neutral pH (Benesch & Benesch, 1974) compared with three to four in ATP and DPG. It is, therefore, able to neutralize the positive charges at the binding site more fully, thus leading to tighter salt-bridge complex formation and more effective oxygenation linkage. The greater effect of GTP compared to ATP can, however, not be explained in this way since both co-factors have the same negativity at neutral pH. Evidently the eel haemoglobin molecule is sensitive to the small stereochemical differences which occur between ATP and GTP and is highly selective to GTP, or GTP binds at an additional or different site than ATP, which is more strongly oxygenation linked. The latter possibility is indicated by the finding that in the presence of inorganic phosphate, saturating concentrations of GTP decrease the oxygen affinity of stripped haemoglobin more strongly than the inorganic phosphate alone, whereas this is not the case for ATP. This is reminiscent of the observations (reviewed by Benesch & Benesch, 1974) that pyridoxal phosphate (PLP) like DPG, reacts electrostatically with the $N$ terminal residues of the $\beta$ chains and also binds covalently to the $N$ terminal residue of the $\alpha$ chains, which results in a permanent lowering of oxygen

affinity. GTP differs from ATP in its increased ability to form hydrogen-bonding with protein, which may form the basis for the stronger linkage. The mechanism of the phosphate effects in eel haemoglobin is being further investigated.

The fact that the oxygen-binding properties of the whole lysate approximate the mean of the components suggests that the individual haemoglobins function independently *in vivo*. The higher sensitivity of Hb I to ATP and GTP, compared to the anodic components, shows that this haemoglobin is primarily implicated in the adaptation of the whole blood to ambient oxygen levels. It is significant that in the presence of triphosphate:haemoglobin ratios exceeding 1·5 (which occur in eels acclimated to well-aerated water – Wood & Johansen, 1972), the oxygen affinities of the cathodic and anodic components became similar (cf. Fig. 5). The present data thus support the hypothesis that both the anodic and the cathodic haemoglobins serve in oxygen transport in well-aerated water. During hypoxia the decreased NTP levels significantly increase the oxygen affinity of the cathodic haemoglobin, and cause a shift in the equilibrium curve of whole blood into range with the lower arterial and venous oxygen tensions found under these conditions (Wood & Johansen, 1973). In the presence of low NTP levels, as in hypoxia, the functional heterogeneity of eel haemoglobin is thus increased, enabling the pigment to function under a wider range of oxygen tensions.

The oxygenation properties of the isolated component haemoglobins of *A. anguilla* show general agreement with those of corresponding components in *A. rostrata* (Gillen & Riggs, 1973) and *A. japonica* (Yamaguchi *et al.* 1962; Yoshioka *et al.* 1968; Okazaki, Misawa & Shukuya, 1974), when account is taken of the specific effects of phosphates in the earlier studies on *A. japonica*. The functional heterogeneity in eels contrasts sharply with the situation in flatfish (*Pleuronectes platessa* and *Platichthys flesus*) where all components have similar oxygenation properties (Weber & de Wilde, 1975). Interspecific differences which may bear on the questioned validity of according separate species status to european and american eels are slight and include the occurrence of reversed Bohr effect at high pH (absent in *rostrata*, present in *anguilla*) and the salt effect. In *A. rostrata*, Poluhowich (1972) found that whereas the P50 of the anodic haemoglobin increases when the concentration of phosphate buffer salts is increased from 250 to 315 m-osmoles (i.e. the osmotic concentrations of eel plasma in fresh water and sea water, respectively), a 'reverse' salt effect was present in the cathodic haemoglobin, and he suggests that these effects favour a relatively constant oxygen affinity in the blood of eels during migration between fresh and sea water. A 'reverse' effect of neutral salts has similarly been found in the cathodic haemoglobin of salmon, in the presence of phosphate buffer (Hashimoto, Yamaguchi & Matsuura, 1960). The present results suggest that this could result from a competition for the anion-binding sites, between the phosphates and the neutral salts, and that at the concentrations used the latter are less effective inhibitors of oxygenation. The greater effect of neutral salts on the cathodic than on the anodic P50 values of stripped haemoglobin found for *A. anguilla* in this study (Fig. 6) accords with the relative effects of organic phosphates on these haemoglobins, and thus with the qualitative similarity of the effects of organic phosphates and inorganic salts (Benesch & Benesch, 1974).

At physiological osmolalities, the salt sensitivity indices of the cathodic and

anodic haemoglobin of *A. anguilla* ($\Delta \log P_{50}/\Delta \log [\mathrm{NaCl}]$ = 0·19 and 0·12, respectively) are appreciably lower than the corresponding value of about 0·5 for human haemoglobin (Antonini, Amiconi & Brunori, 1972). Since plasma osmotic concentrations in eels vary significantly during migration between fresh and sea water (Poluhowich & Parks, 1971), the low salt sensitivity of eel haemoglobins will serve to reduce the dependence of blood oxygen affinity on environmental salinity.

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