

TEMPERATURE ACCLIMATION AND OXYGEN-BINDING PROPERTIES OF BLOOD AND MULTIPLE HAEMOGLOBINS OF RAINBOW TROUT

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SUMMARY

Acclimation of rainbow trout to 5, 15 and 22 °C for periods exceeding 4 months had no significant effect on the oxygen affinity of whole blood or on the concentration of ATP, which is the main organic phosphate in red cells.

Slight differences were, however, found in the oxygenation properties of the haemolysates, which correlate with changes in the relative concentration of the multiple haemoglobins. The oxygen-binding properties of the main haemoglobin components account for the observed differences in the haemolysates. The possible thermoacclimatory significance of changes in haemoglobin multiplicity and co-factor concentrations is discussed.

INTRODUCTION

The effects of temperature on the oxidative metabolism of poikilothermic vertebrates are well known. However, the mechanism by which fish cope with the temperature-induced variations in oxygen demand are imperfectly understood (cf. Houston & De Wilde, 1968; Johansen & Weber, 1976).

Rainbow trout frequently surface-breathe at the beginning of summer, suggesting that they have thermo-acclimatory respiratory responses. Heath & Hughes (1973) found that both ventilation and heart rates of rainbow trout increase with rising temperature (by about 34% between 15 and 24 °C) but that this is insufficient to compensate for a nearly threefold increase in O₂ consumption observed. The occurrence of significant compensatory responses in heart stroke volume is unlikely (stroke volume is temperature-independent in the lingcod *Ophiodon elongatus* - Randall, 1968). Heath & Hughes (1973), however, report that with increasing temperature there is a decrease in venous O₂ saturation (which approaches zero near 24 °C), reflecting an increased oxygen delivery to the tissues.

Several studies (de Wilde & Houston, 1967; Houston & de Wilde, 1968, 1969; Cameron, 1970; Houston & Cyr, 1974) report increases in blood haemoglobin content in trout and other fish during adaptation to increased ambient temperatures. Although the associated augmentation in blood oxygen capacity may contribute to a

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seasonal adjustment in oxygen transport, this is far less than the amount of change in respiratory demand (Cameron, 1970). In the trout, moreover, seasonal changes in haemoglobin content have also been observed at constant temperature (Denton & Yousef, 1975), indicating that this response may not be temperature-induced.

The published data suggest that changes in oxygen affinity of fish blood may also be implicated in temperature adaptation. In the brown bullhead *Ictalurus nebulosus*, the oxygen affinity of the blood of warm-acclimated fish exceeds that in cold-acclimated fish, a difference ascribed to a change in the concentration of intracellular factors (Grigg, 1969, 1972). In the bowfin *Amia calva*, changes in the cellular organic phosphate levels, however, fail to explain a similar reduction in the temperature-sensitivity of blood of temperature-acclimated fish (Johansen & Lenfant, 1972). Recent investigations (Wood & Johansen, 1972, 1973; Wood, Johansen & Weber, 1975; Weber, Lykkeboe & Johansen, 1976) have demonstrated the important role of phosphorylated glycolytic intermediates in adjusting red-cell oxygen affinities to another environmental factor, oxygen tension.

That the haemoglobin itself (as opposed to its co-factor interaction) may change during thermoacclimation follows from the observations that trout haemoglobin is structurally and functionally heterogeneous (Binotti *et al.* 1971) and that environmental temperatures can induce variation in the relative concentrations of the individual components (Houston & Cyr, 1974).

This present paper traces the possible adaptations of blood oxygen affinity of rainbow trout to environmental temperature, following prolonged temperature acclimations, and investigates the possible thermoacclimatory roles of glycolytic phosphorylated intermediates and of changes in the relative concentrations of the individual haemoglobin components.

MATERIALS AND METHODS

Specimens of *Salmo gairdneri*, about 30–35 cm in length, were obtained from the experimental trout farm, Forsøgsdambruget, at Brøns, Denmark. All fish came from the same stock, which had been bred for more than 20 years at the farm.

In a first experiment the fish were divided into two groups which were acclimated to 5 °C and 15 °C for 4–6 weeks. In a second experiment the 5 °C-fish from the first experiment were maintained at 5 °C, and the 15 °C-fish were acclimated to 22 °C (i.e. close to the upper incipient lethal temperature) for a further 12–30 weeks before blood sampling. A slow influx of fresh water provided gradual turnover of the water in each tank. The fish were fed daily *ad libitum* on a commercial pellet feed (Trouvit, Produits Trouw, Vervins, France) and remained active and healthy throughout the investigation.

Each individual fish was bled only once. Blood samples were drawn into a heparinized syringe, from the caudal vessels ventral to the vertebral column.

The half-saturation oxygen tension (P_{50}) of the whole blood was determined by a method of mixing known proportions of deoxygenated and fully oxygenated blood in a 125 μ l capillary (Edwards & Martin, 1967), and measuring the P_{O_2} and pH with Radiometer electrodes. A Radiometer BMS-2 was used for tonometry of the blood.

The concentrations of ATP and other nucleoside triphosphates (NTP) in the blood and in stock phosphate solutions were assayed enzymically using Sigma ATP test chemicals (St Louis, U.S.A.). The contribution of different nucleoside triphosphates

Table 1. Haematocrit (Hct) and the concentrations of haemoglobin and nucleoside triphosphates (NTP) in the blood of trout acclimated to 5, 15 and 22 °C

Expt.	Minimum acclimation	Acclimation temp.	N	Hct	NTP	Hb	NTP/Hb	Significance of difference†			
								Hct	NTP	Hb	NTP/Hb
1	4 weeks	5 °C	10	23* (±5)	1·37 (±0·40)	0·97 (±0·21)	1·41 (±0·32)	N.S.	N.S.	N.S.	N.S.
1	4 weeks	15 °C	8	23* (±6)	1·39 (±0·17)	0·91 (±0·16)	1·57 (±0·30)				
2	12 weeks	5 °C	9	24* (±3)	1·36 (±0·39)	1·04 (±0·15)	1·31 (±0·42)	< 0·001	N.S.	< 0·05	N.S.
2	12 weeks	22 °C	7	34* (±3)	1·43 (±0·22)	1·19 (±0·12)	1·21 (±0·21)				

* $N = 5$. † = Student's t -test. N.S. = not significant.

to the measured values was estimated by thinlayer chromatography as previously described (Weber, Lykkeboe & Johansen, 1976).

Haemoglobin in solution was prepared by washing the cells twice in 1% NaCl and lysis, under rapid stirring, with three times their volume of 0·1 N bis-Tris buffer, pH 7·5. Where methaemoglobin could be detected spectrophotometrically, the samples were reduced by the addition of a trace of solid sodium dithionite, saturated with carbon monoxide (CO) and dialysed for at least 36 h against two to three changes of CO-saturated 0·01-N Tris buffer pH 7·5, containing 5×10^{-4} M-EDTA (which stabilizes the reduced form - Giovenco *et al.* 1970). All preparative steps were carried out at 0-4 °C.

Haemoglobin concentrations were estimated spectrophotometrically with a Unicam SP 1800 recording spectrophotometer using the extinction coefficients of human haemoglobin (Antonini & Brunori, 1971). Haemoglobin solutions were stripped of organic and inorganic ions using Amberlite MB3 mixed-bed ion exchange resin.

The composite haemoglobins of the haemolysates were separated by isoelectric focusing in 110 ml LKB columns containing 1% solutions of CO-saturated LKB ampholines in the pH range 3·5-10. pH values of the fractionated column contents were measured at 5 °C (Radiometer microelectrode type E 5021a). The isolated haemoglobin components were pooled and dialysed for a further 36 hours against several changes of the Tris EDTA buffer.

Oxygen equilibria of haemoglobin solutions were measured by an oxygen diffusion chamber method (Sick & Gersonde, 1969) modified as described (Weber, Lykkeboe & Johansen, 1976).

RESULTS

(a) Haematocrit, haemoglobin and ATP concentrations

Table 1 shows that the blood of trout acclimated to 5, 15 and 22 °C respectively, showed no significant differences in NTP concentrations or in the molar NTP: haemoglobin ratios. While the haemoglobin concentrations were not significantly different in 5 °C- and 15 °C-acclimated fish, a difference ($P < 0·05$) was found between 5 °C and 22 °C fish. The haematocrit values of 22 °C-acclimated fish were, however,

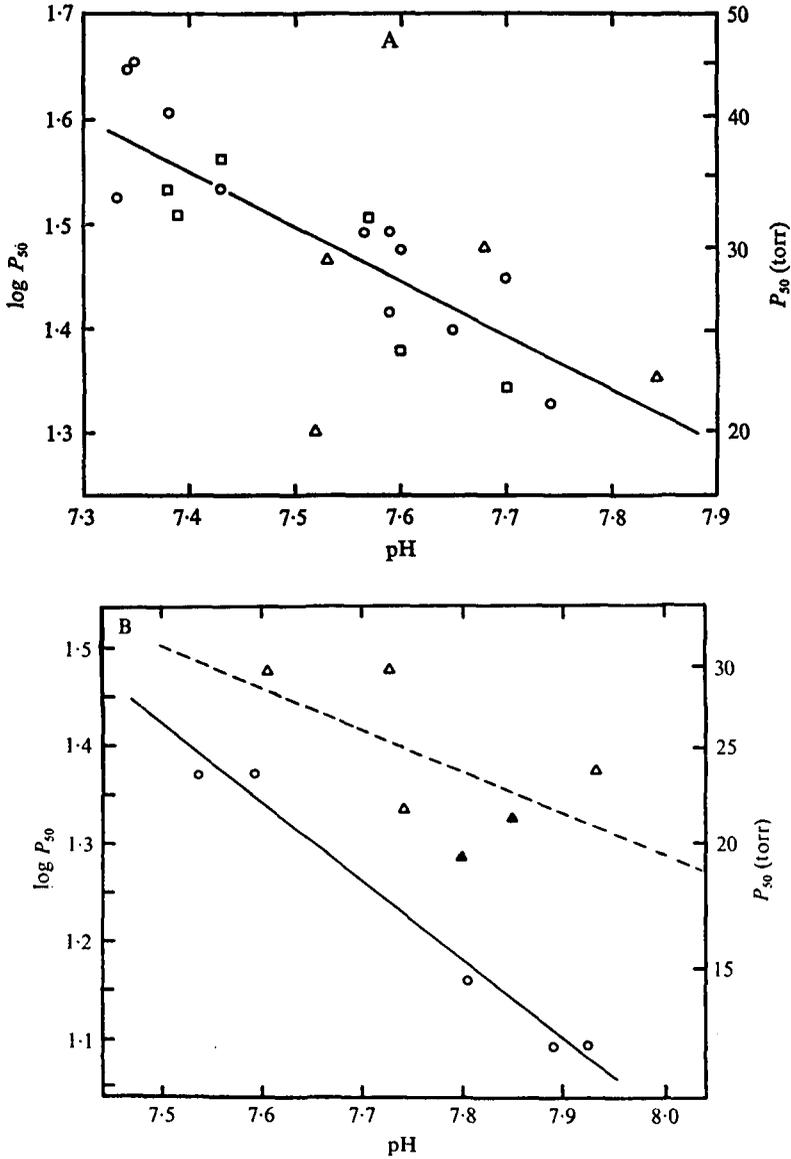


Fig. 1. *Salmo gairdneri* (A). Half-saturation oxygen tensions, P_{50} , at 15 °C and at different pH values, of whole blood from trout acclimated to 5 °C (O), 15 °C (□) and 22 °C (Δ). (B) P_{50} values of blood from trout acclimated to 5 °C (O, Δ) and 22 °C (▲) measured at 5 °C (circles) and 22 °C (triangles).

significantly higher ($P < 0.001$) than those in 5 °C-acclimated fish, reflecting significant decreases in the cellular concentrations of haemoglobin (from 4.3 to 3.5 mM) and NTP (from 5.7 to 4.2 mM).

Thin layer chromatography experiments revealed that the NTP in trout blood consists mainly of ATP, and that less than 10 % is present as guanosine triphosphate (GTP). Thus in five specimens acclimated to 5 °C, the ATP and GTP concentrations (mean \pm S.D.) were 1.29 ± 0.24 and 0.13 ± 0.06 . In five 22 °C-acclimated trout the

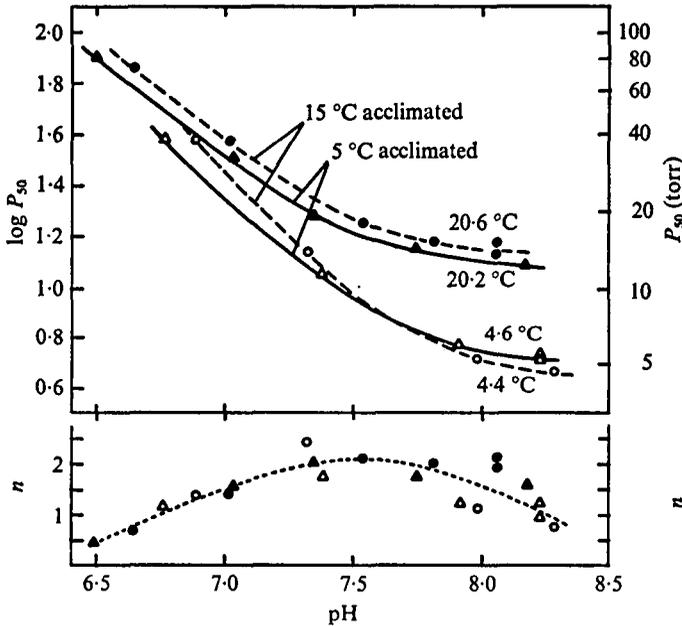


Fig. 2. *Salmo gairdneri*. P_{50} and Hill's coefficients (n) of stripped haemolysates of trout acclimated to 5 °C (Δ , \blacktriangle) and to 15 °C (\circ , \bullet) measured near 5 °C (open symbols) and 20 °C (closed symbols), in 0.1 M Tris and bis Tris buffers. Hb concentration approximately 0.16 mM.

corresponding values were 1.36 ± 0.09 and 0.13 ± 0.06 . In this respect trout differs from the American and European eel (Geoghegan & Poluhowich, 1974; Weber, Lykkeboe & Johansen, 1976) where GTP is the major red-cell organic phosphate.

(b) Oxygen-equilibria

1. Whole blood

Measured at 15 °C, the blood of 5 °-, 15 °- and 22 °C-acclimated trout showed no significant mutual differences in oxygen affinity (Fig. 1A). A large individual variation in half-saturation oxygen tension (P_{50}) was seen within each acclimation group. The data show a P_{50} of 22 torr at pH 7.8 and a Bohr factor ($\Delta \log P_{50} / \Delta \text{pH}$) of -0.52 in the pH range measured. The oxygen affinities are in good agreement with data of Eddy (1971) who, however, found a slightly larger Bohr factor (-0.57 at 15 °C). Fig. 1B shows the P_{50} values at both acclimation temperatures, of 5 °C- and 22 °C-acclimated fish. The 5 and 15 °C data (Fig. 1A, B) indicate values for the apparent heat of oxygenation, ΔH (calculated as $2.303 R \Delta \log P_{50} / \Delta (1/T)$ - Wyman, 1964) of $-8.4 \text{ kcal mole}^{-1}$ at pH 7.8 and $-2.7 \text{ kcal mole}^{-1}$ at pH 7.5. All these ΔH values include the heat of solution of oxygen ($-3.1 \text{ kcal mole}^{-1}$ at 25 °C).

2. Stripped haemolysates

Very similar oxygen affinities and Bohr effects were obtained for the stripped haemolysates of trout acclimated to 5 and 15 °C, respectively (Fig. 2). At the lower pH values, however, the P_{50} values and Bohr factors were higher in the warm than in the cold-adapted fish. In the whole blood, these differences were evidently masked

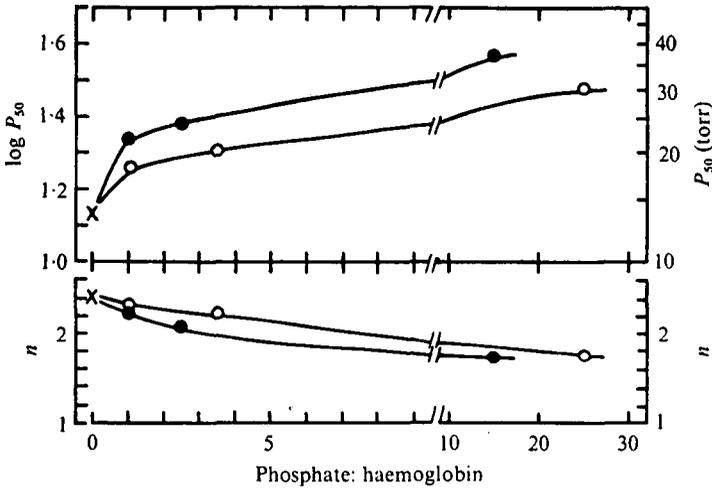


Fig. 3. *Salmo gairdneri*. Effects of ATP (○) and GTP (●) on P_{50} and n values of stripped haemolysate (from 15 °C-acclimated trout), measured in 0.05 M Tris buffer; Hb concentration 0.18 mM, pH 7.48. Temperature, 15 °C.

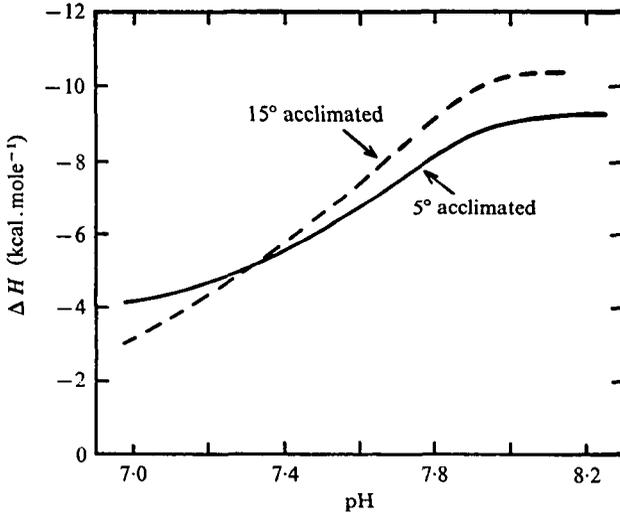


Fig. 4. *Salmo gairdneri*. Apparent heat of oxygenation, ΔH , of stripped haemolysates of 5- and 15 °C-acclimated trout (calculated from data in Fig. 3).

by the large variation in P_{50} values (Fig. 1A), which in turn correlates with the variation in red cell nucleoside triphosphate levels (cf. Table 1). In haemoglobins from both acclimation groups, the Hill coefficient, n , characterizing the sigmoidicity of the oxygen equilibrium curve was maximal near pH 7.5, decreasing at higher and lower pH values.

The haemolysates from warm- and cold-acclimated fish showed similar ATP sensitivity; at pH 7.3, saturating concentrations of ATP (about a 30-fold molar excess over haemoglobin) raised the $\log P_{50}$ values of both haemolysates by about 0.48 units. We also compared the sensitivity of haemoglobin obtained from 15 °C-acclimated fish to ATP and GTP. Interestingly, GTP depresses oxygen affinity more potently than does ATP at the same molar concentration (Fig. 3).

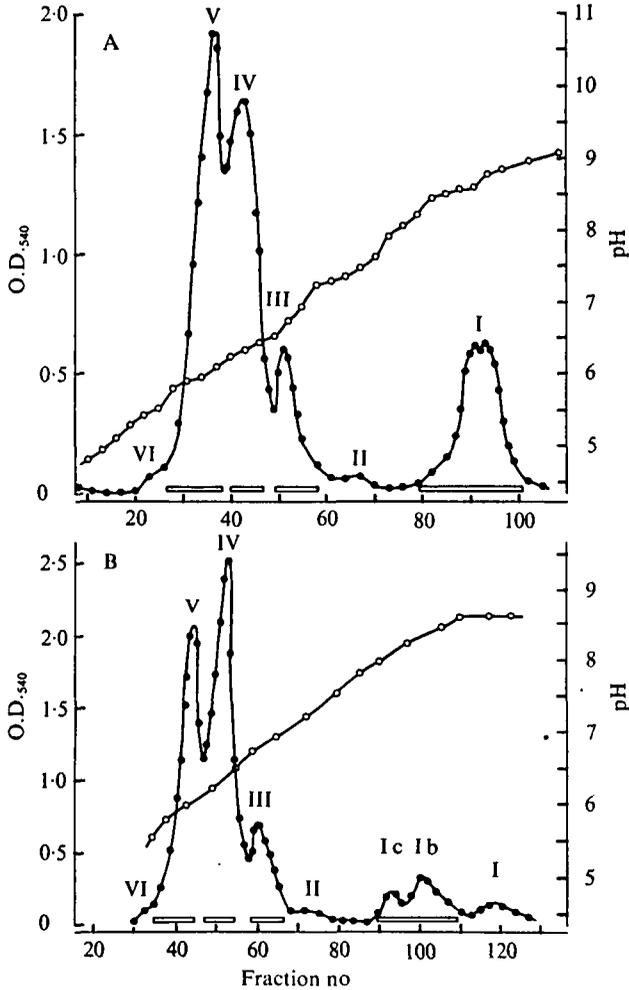


Fig. 5. Isoelectric focusing of haemoglobins from (A) 5 °C-acclimated, and (B) 15 °C-acclimated trout. ●, optical density at 540 nm; ○, pH at 5 °C. About 80 and 200 mg haemoglobin were applied in A and B, respectively. Horizontal bars, fractions pooled for functional studies.

The ΔH values and their pH dependence of warm- and cold-adapted trout (interpolated from the curves in Fig. 2) are given in Fig. 4. The decrease in ΔH with decreasing pH reflects the contribution of the heat of ionization of the oxygenation-linked groups, which are responsible for the Bohr effect (Wyman, 1964). The lower ΔH values in whole blood, compared to that of the haemoglobin in solution, can be ascribed partly to an increased binding of organic phosphates, which lowers the apparent temperature sensitivity of haemoglobin-oxygen affinity (Benesch, Benesch & Yu 1969), and partly to the fact that the intracellular pH values will be significantly lower than those measured for whole blood (cf. Wood & Johansen, 1973).

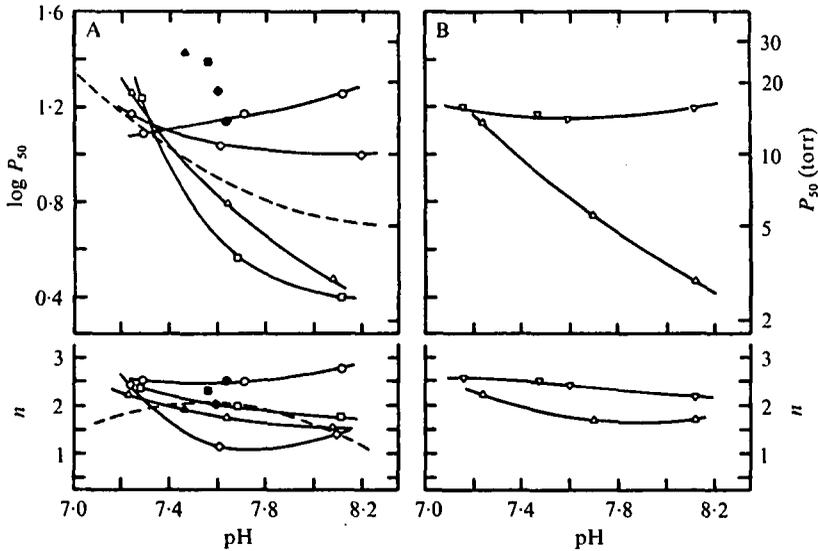


Fig. 6. P_{50} and n values of haemoglobin components from trout acclimated to 5 °C (Fig. 6A) and 15 °C (Fig. 6B) isolated by isoelectric focusing (Figs 5A and 5B, respectively) measured in 0.1 M Tris buffer. \circ \bullet , Hb I; \diamond \blacklozenge , Hb III; \triangle \blacktriangle , Hb IV; \square \blacksquare , Hb V; ∇ , Hb Ia+Ib (cf. Fig. 5B). Open symbols, absence of organic phosphates. Closed symbols, ATP present in approximately 40-fold molar excess over haemoglobin. Temperature, 4.2 °C; Hb concentration, 0.07–0.12 mM.

3. Multiple haemoglobins

The haemoglobins of cold- and warm-acclimated trout resolved into several components during isoelectric focusing (Fig. 5). There was no evidence that hybrid oxidation contributed to the multiplicity observed. In 5 °C-acclimated specimens, the carboxy forms of the main components were isoelectric near pH values of 8.6, 7.4, 6.7, 6.3, 6.1 and 5.4 at 5 °C (Hbs I to VI, respectively, Fig. 5A). In 15 °C-acclimated fish (Fig. 5B) components II–VI could readily be aligned with corresponding haemoglobins in the 5 °C-animals, on the basis of isoelectric points (pI), but slight changes in the relative concentrations were seen. In the warm-adapted fish, HbI was markedly reduced compared to the cold-adapted fish, and two other components, Ib and Ic, became more abundant. It is unlikely that the differences represent genetic polymorphism in view of the genetic homogeneity of the fish used and the fact that both patterns were duplicated in repeat experiments.

The oxygen equilibrium properties of the isolated haemoglobins from warm- and cold-acclimated trout are given in Fig. 6. The different components isolated from 5 °C-acclimated trout show a large variation on oxygen affinity and its pH sensitivity, both parameters tending to increase as the pI of the components decreases (Fig. 6A). At pH 7.8 the Bohr factors of haemoglobins I, III, IV and V were +0.19, 0.10, -0.73 and -0.48, respectively. It is seen that the oxygen affinity and cooperativity of the composite haemolysate appears to be a good average of those of the isolated components, indicating the absence of interaction between concurring components. The oxygen affinity of all components except Hb I was sensitive to ATP (Fig. 6A).

The 'reverse' Bohr effect (increased oxygen affinity with decreasing pH) found for HbI contrasts with Binotti *et al.*'s (1971) data, which were obtained in the presence of

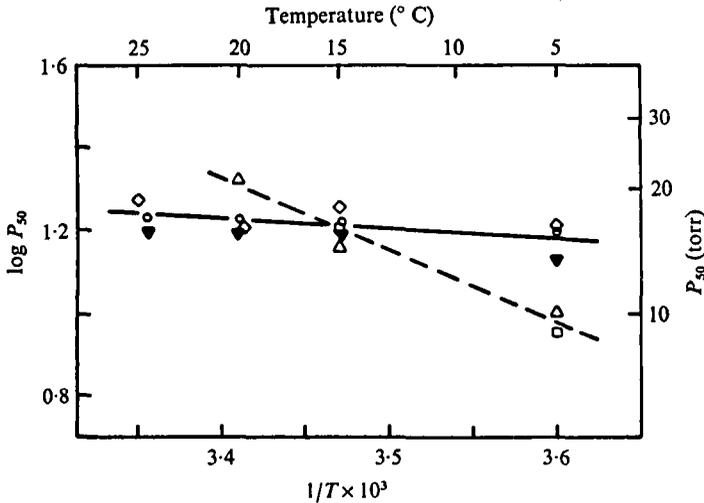


Fig. 7. *Salmo gairdneri*. Effect of temperature on P_{50} values of haemoglobin components isolated by isoelectric focusing (cf. Figs. 5 A, B). Hb I (○), Hb III (◇), Hb IV (△) and Hb V (□) from 5 °C-acclimated trout, and of Hb Ib + Ic (▲) from 15 °C-acclimated trout measured in 0.1 M Tris buffer. The pH of the components haemoglobin solutions was measured at each experimental temperature, and the P_{50} values were corrected to pH 7.4 using the Bohr factors of the respective components at pH 4.2 °C (cf. Fig. 6). Hb concentration, 0.04–0.06 mM.

phosphate buffer and show the absence of a Bohr effect. A 'reverse' Bohr effect is, however, also found in the phosphate-free, but not in the crude, haemolysates, from the eels *Anguilla rostrata* and *A. anguilla* (Gillen & Riggs, 1973; Weber, Lykkeboe & Johansen, 1976), suggesting that anions in solution depressed this pH sensitivity in the study of Binotti and coworkers.

Fig. 6B depicts data for two haemoglobin fractions isolated from haemolysates of 15 °C-acclimated fish. It is seen that Hb IV has identical properties to that of the 5 °C animals, indicating the functional independency of the individual components and justifying the interpolation of the functional properties of the composite haemolysate from those of the individual components. Significantly, Hb Ib + Ic (from warm-acclimated fish) show similar P_{50} values as Hb I (from warm-acclimated fish) but lack a Bohr effect. This difference is in qualitative agreement with that between the haemolysates of cold-compared to warm-acclimated fish (Fig. 3).

Both in Hb I and Hb Ib + Ic the oxygen affinities are virtually temperature insensitive (ΔH approximating -1.0 and -1.5 kcal mole $^{-1}$, respectively – Fig. 7). Hb III exhibits similar temperature independence, whereas the more abundant anodal components have values near -8.5 kcal mole $^{-1}$ at pH 7.4. (These ΔH values include the heat of solution of oxygen.)

DISCUSSION

The data show that alterations in the respiratory properties of the blood of rainbow trout are not implicated in the acclimatory responses to environmental temperature even after acclimation periods of up to six months. This accords with the absence of temperature-induced changes in NTP or in NTP/haemoglobin ratio. Our recent findings (unpublished) show that dilution of haemoglobin of the carp *Cyprinus carpio* increases its oxygen affinity, even when the phosphate/haemoglobin ratio is kept

constant. The increase in haematocrit but not in NTP/haemoglobin of the 22 °C-acclimated trout (Table 1) thus predicts a higher oxygen affinity in this fish than in cold-acclimated ones, i.e. a response similar to those seen in the brown bullhead (Grigg, 1969) and the bowfin (Johansen & Lenfant, 1972). The present investigation agrees with an earlier observation (Cameron, 1971) indicating the absence of a thermoacclimatory response in oxygen affinity of rainbow trout blood after a shorter acclimation period (three weeks). In conjunction with Black, Kirkpatrick & Tucker's (1966) finding that the P_{50} and Bohr effect are the same in winter- and summer-acclimated brook-trout *Salvelinus fontinalis*, these data suggest that as far as the respiratory function of blood is concerned, salmonids must rely almost completely on alternative mechanisms for seasonal temperature adaptations, such as changes in the degree of utilization of the haemoglobin-bound oxygen, and its delivery rate to the tissues (circulatory variations).

The possible adaptive significance of the temperature-induced changes in the heterogeneity of trout haemoglobin is not clear. It is, however, suggestive that the heterogeneity pattern of the haemoglobin of a 'pure genetic line' of *Salmo irideus* – which is now considered to be the same species as *S. gairdneri* – from a hatchery in Macerata, Italy (Binotti *et al.* 1971; Giovenco *et al.* 1970; Brunori *et al.* 1973) resembles that which we obtained for warm-acclimated fish (the cathodal haemoglobin resembling Hb *Ib* + *Ic* with regard to isoelectric point). That the relative amount of the cathodal haemoglobin could form a basis for adaptation moreover follows from the observations that *S. gairdneri* from Buffalo, New York, have a much higher concentration of cathodal haemoglobin (exceeding the combined concentration of the other components – Lau *et al.* 1975), than the Danish and Italian material, and that the oxygen-binding properties of this component differ significantly from the other ones (being less sensitive to pH, temperature and organic phosphates). At the comparative level these data suggest that intraspecific adaptation to environmental temperature might be more readily discernible at the whole blood level in genetically isolated stocks that had become acclimated to different climatic conditions for many years.

Where the acclimatory response of the fish to ambient temperature involves the synthesis of new haemoglobins, the adaptation will depend on the duration of temperature acclimation period and the rate of haemoglobin synthesis, which will in turn be dependent on whether new haemoglobins are formed within circulating erythrocytes or only during erythropoiesis. In the latter case the adaptation rate will be slow since fish red cells have a long life span; in the tench, *Tinca vulgaris*, Hevesy *et al.* (1964) found a life span of about 150 days. Data from these authors, however, indicate that at 18 °C the kidneys (the main erythropoietic organ in fish) take up only about 6% of injected iron, of which only about 1% is incorporated in haem, whereas more than 70% of injected iron is taken up by the erythrocytes, which can synthesize about 0.05% of the haemoglobin per day. It is, however, also possible that acclimatory changes in haemoglobin components may result from alterations in aggregations of pre-existing subunits of the molecules.

The possibility cannot be excluded that the temperature-induced changes in haemoglobin heterogeneity may be related to functions other than oxygen transport, since adaptive variation in oxygenation properties of the blood could, it would appear, be

affected more readily by varying the red cell levels of organic phosphates. It is also remarkable that ATP is the main co-factor in the cells, although GTP is a more potent modulator of oxygen affinity.

While the oxygenation properties of trout haemoglobin components I, II and IV have been extensively studied (cf. Binotti *et al.* 1971; Brunori *et al.* 1973, 1975) we are not aware of previous data on Hb III and V. In its functional properties, Hb V is very similar to Hb IV. Hb III on the other hand appears to be intermediate between the cathodal and anodal components. Thus, whereas its low-temperature sensitivity resembles HbI its distinct Bohr and ATP effects resemble Hb IV. These sensitivities suggest that in Hb III the histidine residues at positions 143 and 146 of the β chains (which are present in most haemoglobins, and are involved in the binding of phosphates and protons, respectively – Arnone, 1974) are unsubstituted. This contrasts with trout Hb I, where the replacement of the histidine residues by serine (at 143) and phenyl alanine (at 146) correlates with the loss of phosphate and pH sensitivities (Barra, Bossa & Bonaventura, 1973; Brunori *et al.* 1975).

The cathodal haemoglobins of fish that possess structurally and functionally different components generally have a lower temperature sensitivity of P_{50} than the anodal components. In the presence of phosphate buffers the cathodal and anodal haemoglobins of salmon have ΔH values of about -2.4 and -7.2 kcal mole⁻¹, respectively (Hashimoto, Yamaguchi & Matsuura, 1960), whereas the corresponding haemoglobins in *Anguilla japonica* have values of -7 and -13 kcal mole⁻¹ (Yamaguchi *et al.* 1962). In the absence of phosphates, a higher temperature dependence can be expected (Benesch, Benesch & Yu, 1969) and accordingly values of -13.3 and -16.0 kcal mole⁻¹, respectively, were found in the stripped cathodal and anodal haemoglobins of *A. anguilla* (Weber, Lykkeboe & Johansen, 1976). The cathodal haemoglobin of trout (ΔH , approximately -1 kcal mole⁻¹ for stripped haemoglobin) exemplifies extreme reduction in temperature sensitivity. This temperature independence is similar to that in tuna haemolysate (ΔH , -1.8 kcal mole⁻¹ – Rossi-Fanelli & Antonini, 1960) where it appears to be adaptive to the heterothermic condition of tuna tissues (Carey & Teal, 1966), serving to avoid gas bubble formation when cool blood from the gills enters the warm swimming musculature (Hochachka & Somero, 1973). Curiously, the cathodal haemoglobin of the primitive holostean bowfin *Amia calva* demonstrates the other extreme, in having a higher temperature sensitivity than the whole haemolysate (-12.6 and -11.4 kcal mole⁻¹, respectively – Weber *et al.* 1976). It is evident that the cathodal haemoglobins in trout will tend to stabilize the temperature-induced variations in oxygen affinity of whole blood. This is illustrated by the lower oxygen affinity between pH 7.4 and 7.8 of the cathodal than of the anodal haemoglobins at 5 °C (Fig. 6), whereas the reverse is seen at 20 °C (Binotti *et al.* 1971).

In further research, the effect of the rightward shift of the oxygen equilibrium curve at higher temperatures on the oxygen unloading by blood in the tissues will be investigated.

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