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pH AND HAEMOGLOBIN OXYGEN AFFINITY IN BLOOD FROM THE ANTARCTIC COD *DISSOSTICHUS MAWSONI*

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SUMMARY

Blood pH in the antarctic cod (*Dissostichus mawsoni*) and in two *Trematomus* species, occurring at −1·9 °C, is extremely high (~ 8·2 to 8·3). This supports and extends Rahn's (1966) model for the temperature–pH relationship in cold-blooded vertebrates.

The blood of *D. mawsoni* shows a low oxygen affinity ($P_{50} \simeq 14{\cdot}5$ mmHg at pH 8·16 and −1·9 °C). Despite normal *in vitro* temperature and pH sensitivities, blood P_{50} increases only slightly when live fish are temperature-stressed (+4·0 °C), or become acidotic as a result of agitational stress (blood pH 7·71), primarily as a result of compensatory decreases in blood ATP levels.

Oxygen-binding properties of 'stripped' (cofactor-free) solutions of *D. mawsoni* haemoglobin were measured in attempts to elucidate the molecular mechanisms involved in the function of the pigment.

INTRODUCTION

Fishes live in environments that vary greatly in physical and chemical properties. Their blood thus transports oxygen under very different conditions. Accordingly, the respiratory properties of the blood in fishes vary widely, both in intrinsic oxygen-binding characteristics of the haemoglobin and in the intracellular factors which influence its oxygenation properties.

Oxygen transport in the blood of antarctic fishes is of considerable physiological interest. The physico-chemical conditions in antarctic waters are characterized by greater stability than the temperate regions. In McMurdo Sound, Antarctica, where the fishes used in this study originate, the water temperature is low (mean value, −1·9 °C) and shows only slight variation (−1·4 to −2·0 °C) and the water remains virtually fully saturated with oxygen (Littlepage, 1965).

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Few published data deal with the blood respiratory properties of fish from the arctic and antarctic regions. Grigg (1967) reported oxygen affinities in blood from four species of the antarctic genus *Trematomus*, quoting half-saturation oxygen tensions (P_{50}) from about 8 to 21 mmHg at $-1{\cdot}5$ °C. Assuming a normal temperature sensitivity, these findings suggest that, at equivalent temperatures, the oxygen affinities are markedly lower than those of fishes from temperate regions. Grigg (1967), however, did not study the *in vivo* blood pH values in the fish, the temperature-induced variation in blood pH, or the role of intracellular organic phosphates such as ATP, which have more recently been shown to be involved in modulating haemoglobin–oxygen affinity in fish blood (Wood & Johansen, 1972, 1973; Wood, Johansen & Weber, 1975; Weber, Lykkeboe & Johansen, 1975, 1976).

This paper focuses on blood and haemoglobin of the stenothermal and benthic antarctic fish, *Dissostichus mawsoni*. Special attention is paid to blood pH at the low body temperature, to ascertain how far this conforms to the model of relative alkalinity (Rahn, 1966), which predicts that *in vivo* pH varies in parallel with the thermal change in pN (the neutral point of water.) This paper also reports blood oxygen affinity and how it is influenced by changes in pH and temperature, both when these changes are induced *in vivo* (live fish) and *in vitro* (drawn blood samples) and the accompanying changes in ATP concentrations are recorded. Lastly, an attempt is made to understand some of the molecular mechanisms involved in the function of the haemoglobin in the blood, by studying the oxygen-binding properties of the isolated haemoglobin in solution and its sensitivites to pH, ATP and temperature.

MATERIAL AND METHODS

(*a*) *Materials*

Specimens of the antarctic cod (*Dissostichus mawsoni* Norman, 1937) weighing 15 to 70 kg were caught in McMurdo Sound, Antarctica (77° 30′ S, 165° E), during an expedition in October 1974. The fish were caught by hook, bait and winched set-line from the bottom of the Sound (about 500 m deep) after a hole 85 cm in diameter was drilled through the 2 m thick sea-ice layer.

Antarctic cods were percutaneously cannulated without anaesthesia (using 18-gauge spinal needles and PE 50 polyethylene tubing) immediately after landing and were then placed for a few hours in well-aerated sea water containers in which they were transported to McMurdo Base and transferred to circular tanks (2·5 m in diameter). For comparative measurements of blood pH, 100–200 g specimens of the smaller antarctic fish belonging to the genus *Trematomus* (*T. borchgrevinki* and *T. bernacchii* Boulenger, 1902), were catheterized in the dorsal aorta (using PE 10 polyethylene tubing) after anaesthesia with Sandoz MS 222. The fish were kept in running sea water at $-1{\cdot}9$ °C for 2 to 21 days before measurements were made.

The effects of sustained 'agitational stress' on the blood respiratory properties of antarctic cod were also investigated. To this effect freshly caught specimens were re-attached to the set-line by means of a steel wire loop passed through a cauterized hole in the lower jaw. The fish were then lowered into the sea for a 24 h period before blood sampling.

(*b*) *Methods*

(1) *Whole blood studies*

Blood pH and PO_2 measurements were performed with Radiometer microelectrodes (types G 297 and E 5046, respectively) which were jacketed at $-1{\cdot}9$ and $+4{\cdot}0$ °C and coupled to a Radiometer millivolt meter (PHM 72) and recorder (REC 51). At $-1{\cdot}9$ °C the average response times to determine pH and PO_2 were 6 and 20 min, respectively. The oxygen electrode was frequently zeroed with oxygen-free solutions. Before introduction of the blood samples, the oxygen electrode was flushed with nitrogen to bring the PO_2 of the electrode close to the PO_2 of the sample to be measured. To ensure complete temperature equilibration, blood samples were passed through thin polyethylene tubing coiled inside a thermostatted glass jacket before entering the oxygen electrode.

Oxygen equilibrium curves of whole blood were determined by the 'mixing' method whereby PO_2 and pH are measured (as above) after mixing set volumes of fully deoxygenated and oxygenated blood (Nørgaard-Pedersen, Siggaard-Andersen & Rem, 1972). The gases used to oxygenate and deoxygenate the blood samples were compressed air and nitrogen, respectively, each containing 0·25 % carbon dioxide. These gases were also used to calibrate the oxygen electrodes. The time lapse between blood sampling from the tonometers and injection into the electrodes was always less than 90 s.

The concentrations of ATP and lactic acid in whole blood, and of ATP in stock solutions, were measured using Sigma enzymatic test chemicals (Sigma Technical Bulletin no. 366 (1974) and 826 (1968) (Missouri)).

(2) *Haemoglobin solution studies*

Studies on the oxygenation properties of the haemoglobin in solution were conducted using compacted red cell samples that had been deep frozen.

Haemoglobin was extracted by mixing three volumes of 0·1 M-Tris buffer (pH 7·5) with one of thawed red cells. The mixture was thoroughly stirred and the cell debris was precipitated by centrifugation. The haemoglobin in the red supernatant was reduced with sodium dithionite as described by Sullivan & Riggs (1967). The solutions were then dialysed in partially filled Erlenmeyer flasks with carbon monoxide (CO) as the gas phase for at least 24 h against three changes of CO-saturated 0·1M-Tris buffer (pH 7·5) containing 5×10^{-4} M-EDTA at $+5{\cdot}0$ °C. Haemoglobin concentrations were estimated spectrophotometrically using extinction coefficients for human haemoglobin (Antonini & Brunori, 1971) which agree well with the corresponding values in eels (Yamaguchi *et al.* 1962). Haemoglobins were freed from organic and inorganic ions ('stripped') by passage through Amberlite MB-3 mixed ion exchanger. Where needed the haemoglobin solutions were concentrated using Minicon B 15 concentrators (Amicon, Oosterhout, Holland).

Oxygen equilibrium curves of the haemoglobin were measured using a diffusion chamber technique modified after Sick & Gersonde (1969) (Weber, Lykkeboe & Johansen, 1976). To trace the influence of ATP on oxygen binding, appropriate amounts of stock solutions of the disodium salts of this cofactor were added.

Isoelectric focusing of the haemoglobin was carried out at $+5{\cdot}0$ °C, using LKB

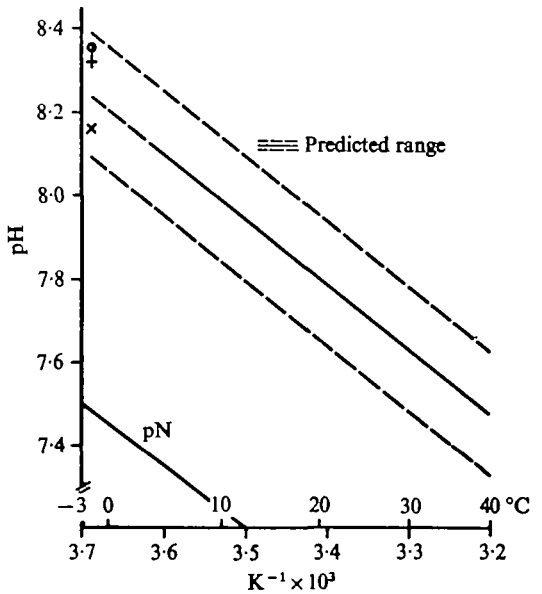

Fig. 1. Mean blood pH values of three antarctic fishes. The dashed lines indicate limits that have been established by Rahn (1966) and by Garey (1972) for temperatures down to +3·0 °C. ●, *T. borchgrevinki*; +, *T. bernacchii*; ×, *D. mawsoni*.

ampholytes giving a pH gradient of 3 to 10, in 110 ml preparative LKB columns (Bromma, Sweden), after saturation of the ampholyte and haemoglobin solutions with CO.

RESULTS AND INTERPRETATION

(*a*) *Whole blood*

(1) *pH data*

Nine measurements on three specimens of *D. mawsoni* that were allowed to recover in the sea water tanks for 3 days after catheterization, showed an average blood pH of 8·16 (S.D. ± 0·04). The measured blood pH values were about 0·1 pH unit more alkaline than the environmental water. The fish were calm and apparently undisturbed when the blood was sampled through the catheters.

Measurements were also made of blood pH in freshly surfaced fish, which had not been more than 1 h on the set-line. Six measurements on separate fish showed an average value of 7·71 (S.D. ± 0·09). This pH was unchanged (7·71 (S.D. ± 0·07)) after the same specimens had been 'agitationally stressed' (relowered on the set-line – see page 79) for a further 24 h.

The data obtained indicate that *D. mawsoni* is remarkably tolerant to shifts in blood pH. In one specimen, which recovered completely, the pH decreased to 7·33 during anaesthesia despite irrigation of the gills with a sea water hose. On the other hand, values of up to 8·34 were observed when fish acclimated for 4 days to +4·0 °C were suddenly cooled to −1·9 °C.

Blood pH values of undisturbed catheterized specimens of *Trematomus borchgrevinki* and *T. bernacchii* at −1·9 °C were 8·35 (± 0·04, $N = 4$) and 8·32 (± 0·04, $N = 2$), respectively. When the blood was sampled by heart puncture within 60 s after hooking,

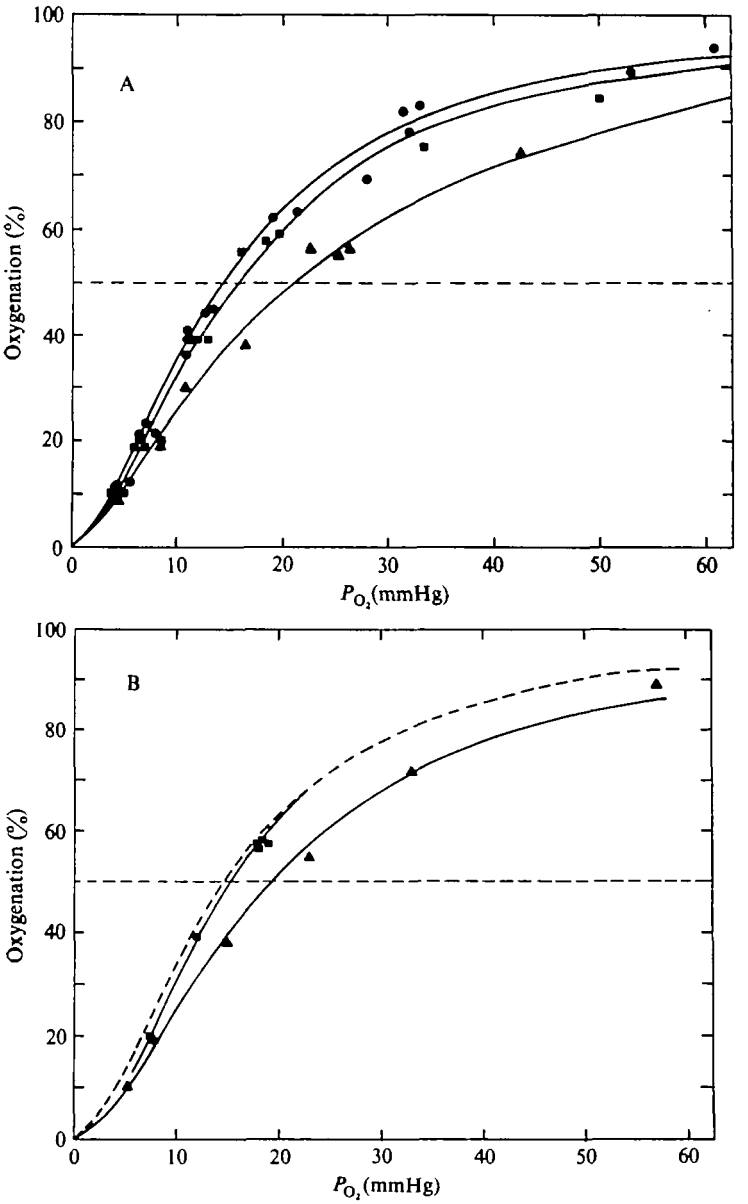

Fig. 2. (A), Influence of pH on blood–oxygen equilibrium curves of *D. mawsoni* at −1·9 °C. The drawn curves were interpolated from linear Hill plots (log oxyHb/Hb *vs.* log PO_2). ●——●, undisturbed fish acclimated to −1·9 °C, blood pH = 8·16, P_{50} = 14·5 mmHg, n = 1·75; ■——■, fish subjected to agitational stress (left on the set-line for 24 h), blood pH = 7·71, P_{50} = 15·5 mmHg, n = 1·70; ▲——▲, blood from undisturbed fish titrated to pH 7·75 with lactic acid, P_{50} = 20·9 mmHg, n = 1·38. (*B*), Influence of temperature on blood–oxygen equilibrium curve of *D. mawsoni*. – – –, undisturbed fish acclimated to −1·9 °C (the data points and oxygenation characteristics are the same as the corresponding curve in Fig. 2A); ■——■, fish 'warm'-acclimated for 4 days to +4 °C, P_{50} = 15·4 mmHg, n = 1·94, measured at +4 °C; ▲——▲, blood of fish acclimated to −1·9 °C measured at 4·0 °C, P_{50} = 19·1 mmHg, n = 1·68.

Table 1. *Blood respiratory properties in* Dissostichus mawsoni *during steady state condition (−1·9 °C, at rest), metabolic acidosis and after acclimation to +4·0 °C*

Sample	Temp. (°C)	pH	ATP/Hb (mole/mole)	P_{50} (mm Hg)	Lactic acid (mmol/l)	Hct (%)
(1) Confined, dorsal artery catheter	−1·9	8·16	1·82	14·5	0·2	23·0
(2) At sea, dorsal artery puncture:						
1 h on set-line	−1·9	7·71	1·32	15·8	2·0	32·8
24 h on set-line	−1·9	7·71	1·25	15·3	2·2	20·8
(3) As (1) + lactic acid addition	−1·9	7·75	1·78	20·9	2·0	23·0
(4) As (1) but fish adapted 4 days to +4·0 °C	+4·0	8·06	1·60	19·1	—	23·0
(5) As (1) but measured at +4·0 °C	+4·0	8·05	1·09	15·4	0·3	18·0

the pH values in these fish were 7·9 to 8·1. Fig. 1 shows that the pH values of bloods of *D. mawsoni* and *Trematomus* at −1·9 °C fall well within the range predicted for this temperature by data of Rahn & Baumgardner (1972) and Garey (1972) which were obtained at higher temperatures.

(2) *Oxygen equilibria*

Oxygen equilibrium data determined on blood collected via the catheters of undisturbed *D. mawsoni* are given in Fig. 2A and 2B and Table 1.

1. *Influence of pH.* The effect of pH on oxygen binding of the blood was studied both by provoking lactic acidosis in live fish (agitational stress) and by addition of lactic acid to the blood *in vitro*.

At −1·9 °C and pH 8·16 the blood oxygen affinity is relatively low (P_{50} = 14·5 mmHg) despite the low temperature and high pH to which it is exposed (Fig. 2A). *In vitro* addition of lactic acid, to decrease the pH to 7·75 (near the blood pH induced by agitational stress), resulted in an increase of P_{50} to 20·9 mmHg. During this *in vitro* treatment the ATP/Hb ratio stayed almost unchanged near 1·8 (Table 1). This pH-induced shift in P_{50} is compatible with a Bohr factor ($\Delta \log P_{50}/\Delta pH$) of −0·39.

Surprisingly, the blood oxygen affinity of *D. mawsoni* subjected to the agitational stress associated with hooking and reattachment to the set-line decreased only slightly (P_{50} from 14·5 to 15·5 mmHg; Fig. 2A) although the blood pH was significantly lowered (8·16 to 7·71). The maintainance of this affinity for oxygen was, however, correlated with a marked reduction in ATP/Hb ratio (from about 1·8 to about 1·3; Table 1). This P_{50} shift corresponds to an apparent '*in vivo* Bohr shift' of only −0·06.

2. *Influence of temperature.* The effects of temperature on P_{50} of the blood were determined both when the temperature of the intact animal and of *in vitro* blood samples were raised from −1·9 to +4·0 °C (see Fig. 2B and Table 1).

A marked increase in the P_{50} is seen when blood from a fish at −1·9 °C is measured at +4·0 °C. The P_{50} changed from 14·5 to 19·1 mmHg, indicating an apparent heat of

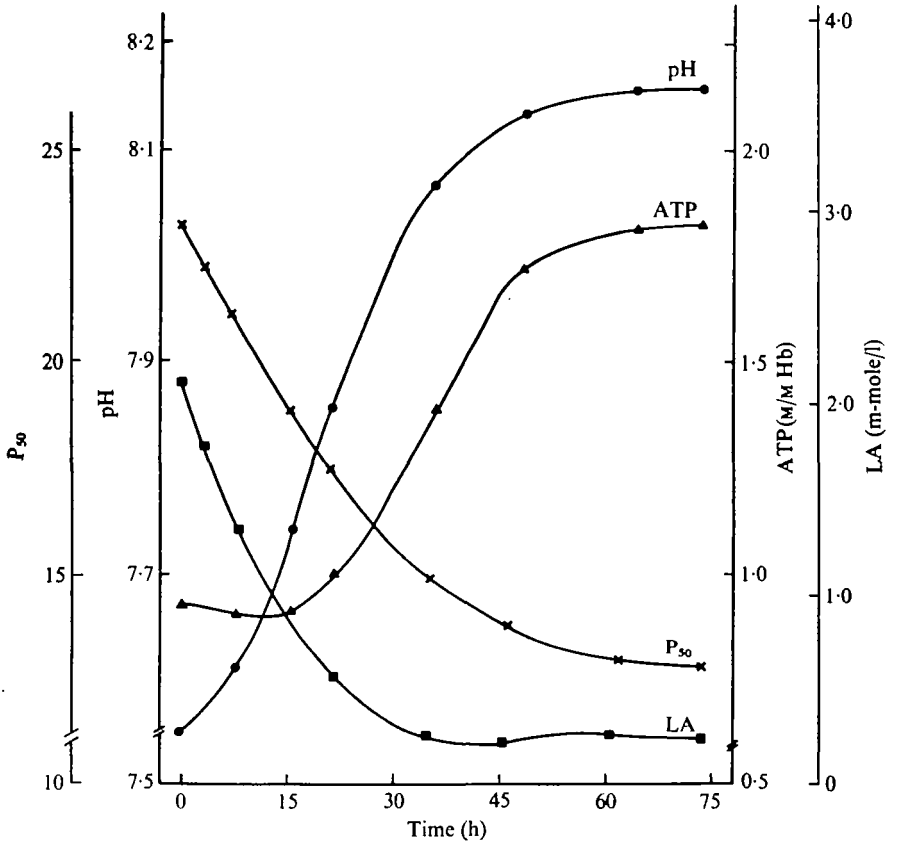

Fig. 3. Blood pH, P_{50}, ATP and lactic acid (LA) concentrations of *D. mawsoni* during recovery from agitational stress in two fish at −1·9 °C (see text for details).

oxygenation, ΔH of −7·24 kcal mole^{-1} (calculated as 2·303 $R.\Delta \log P_{50}/\Delta$ (K^{-1}), where R is the gas constant, K is degrees Kelvin: Wyman, 1964). In contrast, when the live fish were acclimated to +4·0 °C, the P_{50} rose only slightly (from 14·5 to 15·4 mmHg) reflecting an apparent ΔH of −1·52 kcal mole^{-1}. The pH was the same (8·05 to 8·06; Table 1) for blood equilibrated to +4·0 °C *in vitro* and *in vivo* indicating that another factor is responsible for limiting the P_{50} change in the intact fish. Again, it is noteworthy that the ATP/Hb ratio in fish acclimated at +4·0 °C for 4 days is markedly lower than in fish acclimated to −1·9 °C. Separate serial measurements indicate that the change in blood P_{50} values occur rapidly (within 8 h of the temperature change).

ATP appears to be the major organic phosphate in *D. mawsoni* red cells. There was no evidence for the presence of either 2·3 diphosphoglycerate (2·3 DPG) (tested by Sigma's enzymatic method) or guanosine triphosphate (GTP) which is the major cofactor in some other fish species (thin layer chromatographical determination – Geoghegan & Poluhowich, 1974; Lykkeboe, Johansen & Maloiy, 1975; Weber, Lykkeboe & Johansen 1975; Lykkeboe, unpublished).

Figure 3 depicts some changes in blood variables following recovery of fish after catching and cannulation. It is seen that agitational stress is associated with high lactic acid concentrations in the blood which presumably cause the low pH values and the

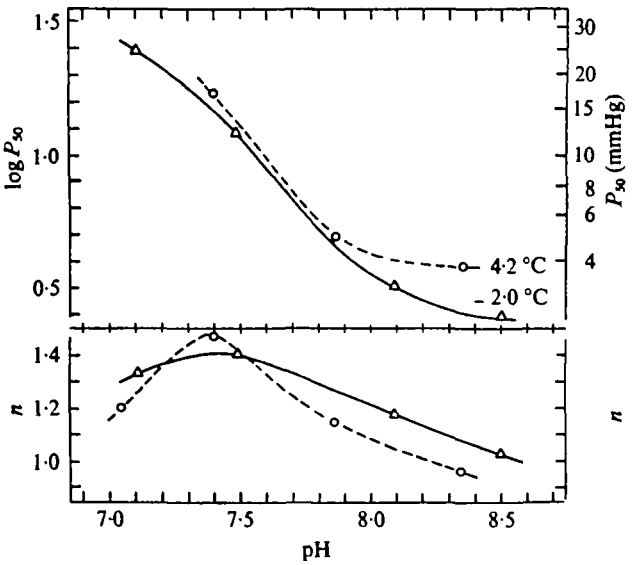

Fig. 4. Values of half-saturation oxygen tensions (P_{50}) and Hill's cooperativity coefficients (n) of stripped *D. mawsoni* haemoglobin at −2·0 °C (△) and at +4·2 °C (○). Solvent, 0·05 M-Tris buffer; Hb concentration, 0·05 mM.

low blood–oxygen affinity (initial P_{50} = 23 mmHg). During the recovery period, ATP initially shows no response but increases after about 15 h. Between 0 and 15 h (where ATP concentration is constant) P_{50} decreases from 23 to ± 18 as pH increases from 7·6 to 7·8. This represents a Bohr shift of −0·48, which shows general agreement with the value of −0·39 for *in vitro* blood samples and thus indicates that the change in P_{50} is largely attributable to pH change. In the subsequent period (after 15 h), P_{50} decreases as pH increases despite a rise in ATP, showing that the increase in the concentration of this cofactor does not have a tangible influence on P_{50}.

(*b*) *Haemoglobin solutions*

The oxygen-binding properties of stripped *D. mawsoni* haemoglobin (Fig. 4) were measured at −2·0 and +4·2 °C (i.e. temperatures close to that of the natural habitat, and the upper lethal temperature, respectively). At −2·0 °C and pH 8·1, the haemoglobin shows a high oxygen affinity (P_{50} is about 3 mmHg; Fig. 4). The Bohr factor is pronounced and pH dependent; at −2·0 °C it increases from about −0·5 at pH 8·1 to about −1·1 at pH 7·7. The difference between the P_{50} values at −2·0 °C and +4·2 °C (indicative of the heat of oxygenation), is reduced as the Bohr effect increases at decreasing pH, reflecting contributions of opposite sign from the heats of ionization of oxygen-linked groups responsible for the Bohr effect.

The Hill's haem–haem cooperativity constant, n, increases as pH decreases in the physiological range, signifying a more sigmoid shape of the haemoglobin–oxygen equilibrium curve and thus more efficient oxygen unloading in the tissues. The effects of ATP on P_{50} and n values of *D. mawsoni* haemoglobin at two pH values is given in Fig. 5. It is evident that the observed *in vivo* variations in the ATP/Hb ratio (from about 1 to 2; Table 1) will markedly influence haemoglobin–oxygen affinity. The

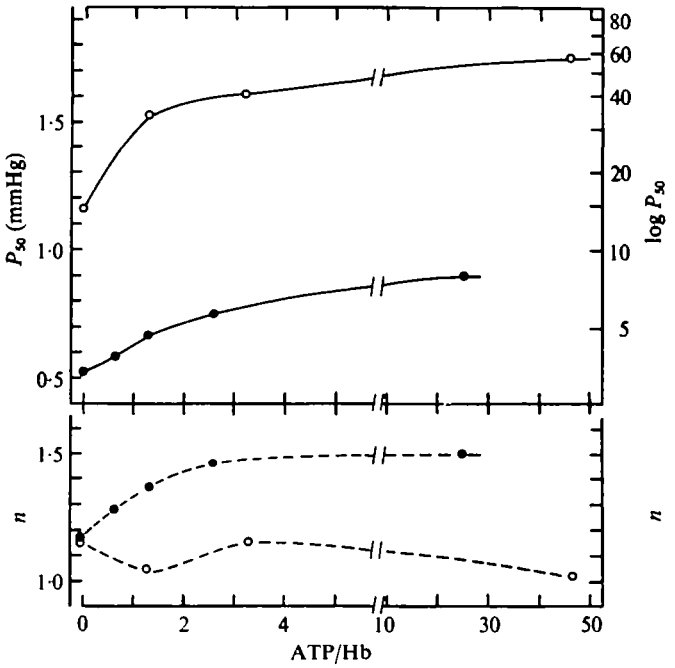

Fig. 5. Effect of ATP on P_{50} and n values of *D. mawsoni* haemoglobin at pH 7·5 (○) and 8·1 (●), measured in 0·05 M-Tris buffer. $t = -2{\cdot}0$ °C. Hb concentration, 0·06 mM.

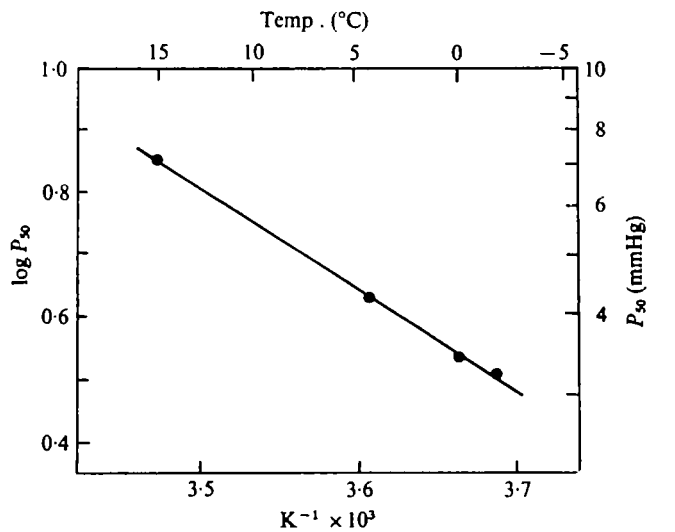

Fig. 6. Effect of temperature on P_{50} value of *D. mawsoni* haemoglobin. Hb concentration, 0·05 mM; solvent, 0·05 M-Tris buffer, pH 8·1.

effect of the phosphate is greater at the lower pH, in accordance with binding of phosphate polyanion at the positively charged binding site of vertebrate haemoglobin (Perutz, 1970). Thus under the same conditions (Fig. 5) saturating concentrations of ATP increase log P_{50} of stripped haemoglobin by 0·36 at pH 8·1, compared to 0·58 at pH 7·5.

ATP increases n at pH 8·1, but exerts no significant effect on n at pH 7·5 (Fig. 5). demonstrating a pH dependency of the effect of phosphate on n as previously noted with cichlid and carp haemoglobins–Gillen & Riggs (1971) and Tan & Noble (1973).

The temperature dependence of P_{50} was measured by suspending haemoglobin from a stock solution in different buffers so that the pH was constant at 8·1 at the different measurement temperatures (Fig. 6). The influence of temperature exhibited by *D. mawsoni* haemoglobin under these conditions is compatible with ΔH value of $-7{\cdot}4$ kcal mole^{-1}.

Isoelectric focusing experiments showed that the haemoglobin of *D. mawsoni* is virtually homogeneous, consisting of a single major component which (on the basis of optical densities at 540 mm) comprises more than 95 % of the total haem, and two very small components. At +5·0 °C the major component was isoelectric at near pH 6·9, and the minor components near pH 8·2 and 5·3.

DISCUSSION

This study demonstrates the occurrence of extremely high blood pH values, ~8·2 – 8·3, in *Dissostichus mawsoni* and in two other fish (*Trematomus borchgrevinki* and *T. bernacchii*) at low ambient temperature (−1·9 °C). The present data confirm and extend the relation between blood pH and environmental temperature as predicted by previous data obtained at higher temperatures (Fig. 1). Interestingly, the comparative measurement (Qvist *et al.*, unpublished) of haemolymph pH in the antarctic isopod, *Glyptonotus antarcticus*, showed similarly high pH values (~8·1 to 8·2) at −1·9 °C, conforming to the relative alkalinity model previously established for lower vertebrates (Howell *et al.* 1970; Rahn & Baumgardner, 1972). The *in vivo* pH values reported in the present investigation suggest that oxygen affinities earlier reported for extracted *Trematomus* blood at pH 7·4 to 7·5 (Grigg, 1967) are too low.

The oxygen affinity of *D. mawsoni* blood (P_{50} = 14·5 mmHg at pH 8·16 and −1·9 °C) is extremely low compared to that in temperate fish under similar conditions, and presumably relates to the high oxygen tension in the antarctic environment (Littlepage, 1965).

The oxygen affinity of the stripped haemoglobin solutions is much higher (P_{50} ~ 3 mmHg at pH 8·1 and −2·0 °C; see Fig. 4). This is attributable to the absence of ATP (and other intraerythrocytic factors that depress oxygen affinity), to the lower haemoglobin concentrations and the fact that the intraerythrocytic pH in fish may be considerably lower than those in the plasma and whole blood (Wood & Johansen, 1973).

The lower Bohr effect of the whole blood *in vitro* (ϕ = −0·39 at −1·9 °C; cf. Fig. 4) than in stripped haemoglobin solutions is at variance with the finding that phosphate binding, and hence the apparent Bohr effect, increases with a pH decrease in the physiological range (Fig. 5). The temperature sensitivity of oxygen binding of cofactor-free haemoglobin solutions, however, accords with that found in the whole blood (ΔH, about −7·4 and 7·2 kcal mole^{-1}, respectively). These values include the heat of solution of oxygen, and are low compared to values usually obtained in vertebrate haemoglobins (−9 to −14 kcal mole^{-1}; Rossi-Fanelli, Antonini & Caputo, 1964), reflecting a relative independency of the P_{50} of *Dissostichus* haemoglobin of environmental temperature. They are, however, higher than that (−1·8 kcal mole^{-1})

found for the virtually temperature-insensitive haemoglobin from tunny (Rossi-Fanelli & Antonini, 1960).

The haemoglobin of *D. mawsoni* is virtually electrophoretically homogeneous. In this respect it differs from those of some active fish from temperate regions, such as salmon, trout, eel, catfish and the bowfin (Hashimoto, Yamaguchi & Matsuura, 1960; Binotti *et al.* 1971; Weber, Wood, & Lomholt, 1976; Gillen & Riggs, 1973; Weber, Lykkeboe & Johansen, 1976; Powers, 1972; Weber *et al.* 1976). In these latter species functional differentiation among haemoglobin components appears to provide a basis for functioning of the composite pigment under diverse physico-chemical conditions. The present data thus indicate that for its adaptation to changes in operating conditions, *D. mawsoni* haemoglobin is solely dependent on changes in its cellular environment in the live fish.

It is evident that both during agitational stress (associated with marked decrease in blood pH) and during heat stress (+4·0 °C), the oxygen affinity of the blood in the fish remains at virtually the same level as in undisturbed fish at −1·9 °C. Both the lactic acidosis and the rise in temperature decrease oxygen affinity of the haemoglobin, and the presence of the compensatory reactions in the blood may thus serve to prevent decreases in the oxygen affinity which could adversely affect oxygen loading in the gills.

Our data show that ATP is the major factor implicated in both compensatory responses occurring in the live animal (cf. Table 1). Similar decreases in temperature sensitivity of haemoglobin–oxygen affinity following temperature acclimation have been observed by Grigg (1969) in brown bullhead fishes, and by Johansen & Lenfant (1972) in the bowfin. However, whereas the organic phosphates were not measured in the former study, the latter one shows lower ATP and DPG concentrations in the cold-adapted as compared to warm-adapted fish. These data thus fail to explain the changed temperature sensitivity.

While previous data on fish have by now amply illustrated the role of the nucleotide triphosphates ATP and GTP in increasing oxygen affinity in response to environmental hypoxia (Wood & Johansen, 1972, 1973; Wood, Johansen & Weber, 1975; Weber, Lykkeboe & Johansen, 1975) the present data demonstrate that in antarctic cod ATP is also implicated in modifying oxygen affinity during lactic acidosis and thermal stress.

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