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BLOOD OXYGEN TRANSPORT IN THE FREE-SWIMMING HAGFISH, *EPTATRETUS CIRRHATUS*

BY R. M. G. WELLS

Department of Zoology, University of Auckland, Auckland

M. E. FORSTER, W. DAVISON, H. H. TAYLOR

Department of Zoology, University of Canterbury, Christchurch

P. S. DAVIE

Department of Physiology and Anatomy, Massey University, Palmerston North

AND G. H. SATCHELL

Department of Physiology, University of Otago, Dunedin, New Zealand

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SUMMARY

1. Arterial and mixed venous blood were sampled through chronically implanted cannulae from rested and swimming hagfish. Pa_{O_2} remained high when hagfish were swum for 15 min at a velocity of 20 cm s^{-1}. $P\bar{v}_{O_2}$ fell from 17·2 mmHg at rest to 3·5 mmHg after swimming, and the arteriovenous pH difference increased from 0·15 to 0·25 pH units.

2. Whole blood oxygen equilibrium curves were essentially hyperbolic (Hill's n value = 1·38) and gave a half-saturation P_{O_2} (P_{50}) value of 12·3 mmHg at pH 7·8 and 16°C. A CO_2-Bohr factor ($\phi = \Delta \log P_{50}/\Delta pH$) of −0·43 and a limited buffering capacity of the blood, amounting to approx. 4 slykes, were observed.

3. The role of the blood in transporting oxygen and carbon dioxide both at rest and after swimming is established by *in vivo* blood gas measurements and *in vitro* oxygen-binding data. The low internal $P\bar{v}_{O_2}$ at rest is close to the P_{50} measured under similar conditions and the hyperbolic equilibrium curve permits further oxygen unloading when $P\bar{v}_{O_2}$ falls during swimming.

INTRODUCTION

Hagfish are primitive jawless vertebrates whose antecedents, first appearing more than 550 million years ago, are now preserved in Upper Cambrian deposits. The composition of myxiniform plasma, quite unlike that of any other vertebrates, scarcely differs from that of sea water (Robertson, 1976). In a low pressure circulation, a series of pumps propels the blood through a specialized blood system containing large venous sinuses (Johansen, 1963; Satchell, 1971, 1984). The erythrocytes contain haemoglobin, which when isolated shows an absence of cooperative oxygen binding, lack of appreciable Bohr and allosteric effects, high affinity for

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oxygen, and no evidence for the tetrameric association of subunits to be found in higher vertebrates (Manwell, 1963; Bannai, Sugita & Yoneyama, 1972; Li, Tomita & Riggs, 1972; Bauer, Engels & Paleus, 1975).

Manwell (1963) speculated that the blood might function as a storage system during hypoxic episodes, and it was suggested that if the blood had a transport function, then oxygen unloading must occur at remarkably low internal tensions (Johansen & Strahan, 1963). In relation to speculations on hypoxic tolerance in hagfish, it is worth noting that agnathans evolved at a time when the earth's atmosphere contained less than one-twentieth of the present oxygen concentration (Rutten, 1970).

This paper describes, for the first time, the respiratory properties of whole hagfish blood and relates these data to *in vivo* blood gas tensions. Here we have recorded oxygen equilibrium curves from the blood of the polybranchiate species, *Eptatretus cirrhatus*, and measured arterial and venous blood gas tensions in both resting and swimming hagfish.

MATERIALS AND METHODS

Capture and containment

Hagfish were collected in baited crayfish pots off Motunau Beach, North Canterbury (43° 05′ S, 173° 06′ E) and were held in a mesh trap prior to transfer to Christchurch. Here they were housed in aquaria containing running sea water at 16 °C, the temperature at which all experiments were performed. Animals were held for at least 1 week before use and were not fed during this period. The hagfish ranged in weight from 628 to 914 g.

Blood sampling

Eight hagfish were anaesthetized in a solution of 0·04 % Benzocaine in sea water. Under anaesthesia, a cannula was placed into the 6th or 7th afferent branchial artery on one side for the collection of venous blood. Arterial blood was collected through a second cannula inserted into the dorsal aorta *via* a segmental artery close to the vent. Polyethylene cannulae (Portex) were used, of internal diameter greater than 1 mm, and they were filled with a heparinized saline based on the analyses of *Myxine* serum (Morris, 1965). Unless otherwise stated, hagfish were allowed to recover for at least 16 h before experimental manipulations and blood sampling.

Haematology

Approximately 0·5 ml of blood was collected into a heparinized tube and placed on ice. Haematocrit (Hct) was estimated by centrifugation at 3250 ***g*** for 6 min and haemoglobin (Hb) concentration determined spectrophotometrically from Drabkin's cyanmetHb derivative (ICSH, 1978) with the added precaution of centrifuging the extract to remove particulate matter. A relative molecular mass of 16 000/monomer was assumed. Erythrocytes were counted in a Neubauer chamber filled with a

suspension of cells at a 1:50 dilution. Mean corpuscular haemoglobin concentration (MCHC) was calculated as Hb concentration×100/Hct.

Plasma lactate, erythrocyte ATP (non-specific nucleoside triphosphate), and 2,3-diphosphoglycerate concentrations were determined spectrophotometrically using the enzymatic test procedures of the Sigma Chemical Co.

Swimming

Hagfish were forced to swim in a flume at 20 cm s^{-1} (approx. 0·4 body lengths s^{-1}). The P_{O_2} in inspired water did not fall below 137 mmHg. Blood samples were withdrawn after 10–15 min swimming.

Blood gases and acid–base balance

Blood gas tensions and pH were measured in heparinized blood using Instrumentation Laboratory and Radiometer electrodes thermostatted at 16 °C and connected to IL and Radiometer gas monitors. The pH electrode was calibrated using precision buffer solutions and the gas electrodes with certified mixtures. Stable readings were obtained after 4 min. Blood oxygen contents were measured using Tucker's (1967) method.

Blood buffering capacity was estimated by tonometering 3 ml of blood at 16 °C at known P_{CO_2}, with $P_{O_2} > 140$ mmHg or $P_{O_2} = 0$ mmHg in an IL 237 tonometer. Samples of 50 μl were taken for measurement of pH and total CO_2 using the method of Cameron (1971). The solubility of CO_2 in hagfish blood was taken to be the same as that in sea water at the same temperature, and dissolved CO_2 was subtracted from the measured total CO_2 to give the non-bicarbonate buffering capacity of whole blood. Regression lines were fitted to the data by the method of least squares.

Oxygen equilibrium

Whole blood oxygen equilibrium curves (OEC) were generated from thin blood films using the dual wavelength optical system of an Aminco Hemoscan Analyzer and modified for gas supply with Wösthoff gas mixing pumps (Wells & Weber, 1985). Dynamic error was estimated from static conditions whereby gases were admitted to the apparatus in a stepwise manner enabling the blood film to come into equilibrium with the gas. Dynamic error was found to be negligible at the slow rate and extreme pH ranges at which the OEC were registered. The CO_2-Bohr effect ($\phi = \Delta \log P_{50}/\Delta pH$) was determined by varying the P_{CO_2} in the equilibrium gases. The equilibrium pH was measured following tonometry for 10–12 min in a Radiometer BMS2 system with a gas blend closely matching the P_{50} value determined for each oxygen-binding curve. The possibility of a Root effect (reduction in O_2-binding capacity at low pH) was investigated by monitoring, in the Hemoscan, the saturation of a blood film equilibrated consecutively with pure O_2, 5 % CO_2 in air, and finally O_2. A haemolysate was prepared by osmotic shock, stripped of organic phosphates, and the OEC measured as described earlier (Wells, 1982). OEC data were analysed according to Hill's (1910) equation. Detailed Hill plots were constructed by computer using digitized data pairs and analysing slopes at 20, 50 and 80 % saturation.

Table 1. *Blood gas tensions in swimming hagfish*

	Pre-swim	Swimming	Recovery
Pa_{O_2} (mmHg)	94·0 ± 9·6	91·6 ± 6·9	111·3 ± 3·5*
Pa_{CO_2} (mmHg)	0·7 ± 0·3	1·2 ± 0·5	0·9 ± 0·3
pHa	7·92 ± 0·02	7·95 ± 0·02	8·03 ± 0·04
$P\bar{v}_{O_2}$ (mmHg)	17·2 ± 3·0	3·5 ± 1·6*	4·2 ± 1·5
$P\bar{v}_{CO_2}$ (mmHg)	1·2 ± 0·4	1·5 ± 0·8	1·2 ± 0·4
pHv̄	7·77 ± 0·14	7·71 ± 0·09	7·72 ± 0·06

* Significantly different from pre-swimming values ($P < 0·05$).
Hagfish were swum for 10–15 min before blood samples were drawn. Recovery values were taken approx. 20 min after swimming.
Data expressed as mean ± S.E.M., $N = 3$.

All blood films were analysed after equilibrium runs to check for oxidation to methaemoglobin using a Pye Unicam SP 1750 recording spectrophotometer and the extinction coefficients given by Benesch, Benesch & Yung (1973). All data are expressed as mean ± S.E.M. and significances were calculated from Student's *t* statistic, where appropriate.

RESULTS

In vivo *observations*

At rest, the P_{O_2} of dorsal aortic (DA) blood was 90·5 ± 6·5 mmHg (mean ± S.E.M., $N = 8$) and ventral aortic (VA) P_{O_2} was 16·8 ± 3·1 mmHg ($N = 7$). For dorsal and ventral aortic blood samples taken within a few minutes of each other, the arterio-venous difference averaged 0·25 pH units (DA 7·92 ± 0·01 mmHg, VA 7·67 ± 0·07) and P_{CO_2} was 0·7 mmHg lower in arterial blood ($P < 0·05$, $N = 6$). In three cases where it was possible to sample VA and DA blood successively from resting, swimming and recovered hagfish, it was found that Pa_{O_2} was maintained during swimming and even rose during recovery (Table 1). The P_{O_2} of venous blood ($P\bar{v}_{O_2}$) returning to the gills fell dramatically (Table 1) and was not restored to pre-swimming levels even after 20 min recovery.

The hagfish swam well at the low velocities tested and were not exhausted by 15 min of exercise. Measured lactate concentrations in VA and DA blood taken after 20 min recovery averaged only 0·43 ± 0·11 mmol l^{-1}, compared with the pre-swimming value of 0·35 ± 0·06 mmol l^{-1} ($N = 6$). By contrast, considerably higher blood lactate concentrations of 2·7 and 7·1 mmol l^{-1} were measured in two other specimens 3 h after anaesthesia, where ventilation had been suppressed. These two high blood lactate concentrations were associated with a marked acidaemia with pHv̄ at 7·46 and 7·49, respectively.

Oxygen contents were lower in ventral aortic than in dorsal aortic blood (1·09 ± 0·08 ml dl^{-1} at $P\bar{v}_{O_2}$ of 18·9 ± 3·2 mmHg and 2·22 ± 0·15 ml dl^{-1} at Pa_{O_2} of 82·3 ± 1·46 mmHg, $N = 5$). The arterial blood was almost fully saturated with

oxygen, being close to the measured blood oxygen capacity of 2·29 ml dl^{-1} at a mean haematocrit of 8·5 %. If we assume that plasma has an oxygen capacitance identical to that of sea water, it can be calculated that 15·3 % of the total oxygen is carried in solution at the high P_{O_2} found in arterial blood.

In vitro *observations*

Haematological measurements for dorsal aortic blood are given in Table 2. Erythrocyte ATP concentrations varied from 4·53 to 7·59 μmol g^{-1} haemoglobin and no 2,3-diphosphoglycerate was detected. Erythrocytes were few in number ($210–280 \times 10^3$ μl^{-1}) and large in size (446–555 fl). It was not possible to measure all variables in all fish at the same time.

Table 2. *Haematological parameters of dorsal aortic blood from* Eptatretus cirrhatus

Haematocrit (%)	8·5 ± 1·0
[Haemoglobin] (g dl^{-1})	1·61 ± 0·15
Red blood cells ($\times 10^6$ μl^{-1})	0·26 ± 0·04
Mean corpuscular haemoglobin concentration (g dl^{-1})	18·95 ± 1·11
Mean cell volume (fl)	507 ± 43
[ATP] (μmol g^{-1}Hb)	6·1 ± 1·3

Data are mean values ± S.E.M., $N = 6$.

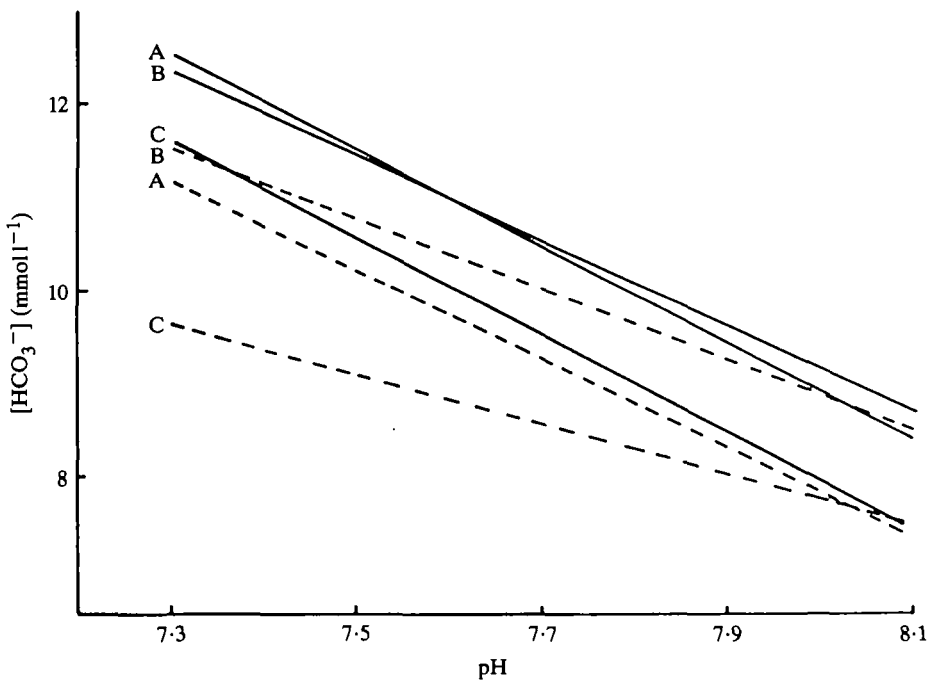

Fig. 1. *In vitro* buffer lines for deoxygenated (——) and oxygenated (– – –) blood at 16 °C from three hagfish with haematocrits of (A) 10·8 %, (B) 11·9 % and (C) 5·1 %.

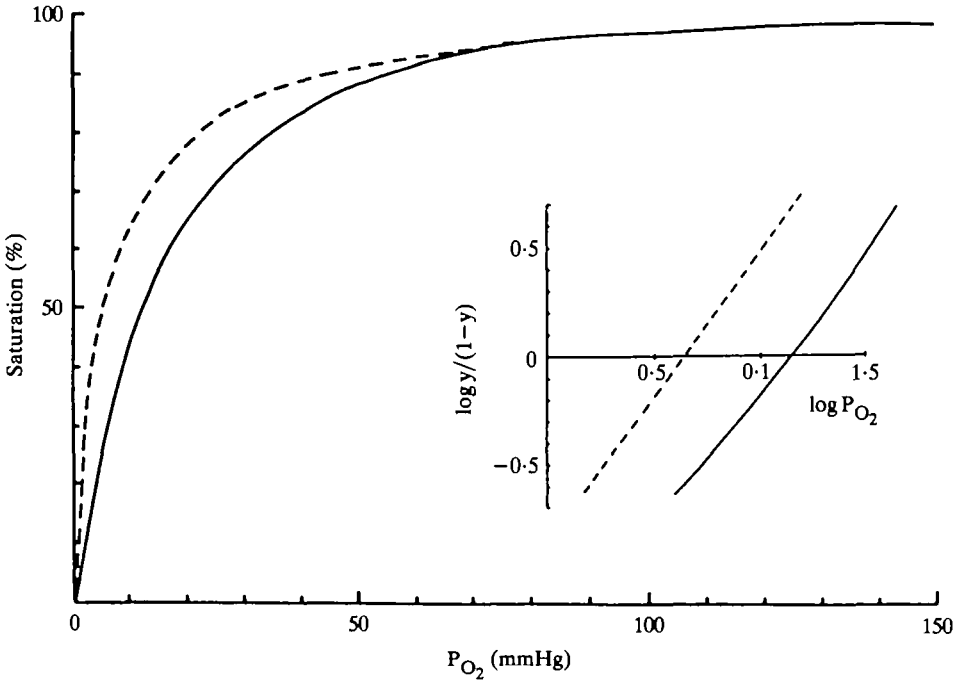

Fig. 2. Oxygen equilibrium curves for whole blood (——) at 16°C and pH 7·8 with equilibrium $P_{CO_2} = 2{\cdot}3$ mmHg, and for Tris-buffered 2 mmol l^{-1} haemoglobin solution (– – –) at 16°C, pH 7·8. Hill plots of the data are shown in the inset to indicate the low degree of cooperativity.

The *in vitro* buffer lines showed a low buffering capacity and a significant Haldane effect (Fig. 1), with values for deoxygenated and oxygenated bloods averaging 4·6 and 3·5 slykes, respectively.

Oxygen-combining properties of hagfish blood

The OEC of hagfish blood under standard conditions at 16°C and pH = 7·80 showed a moderately high oxygen affinity, $P_{50} = 12{\cdot}3 \pm 0{\cdot}55$ mmHg, with mild cooperativity, Hill's $n = 1{\cdot}38 \pm 0{\cdot}08$ ($N = 5$). The oxygen affinity was increased in the purified haemoglobin (Fig. 2). The pH sensitivity of the equilibrium is shown in Fig. 3, where the Bohr factor, ϕ, $= \Delta \log P_{50}/\Delta pH = -0{\cdot}43$ and the pH sensitivity is greater at P_{50} than at P_{20} or P_{80}. Hill's cooperativity coefficient, n, was independent of saturation and thus the average n values were recorded as a function of pH (Fig. 3). There was no evidence for a Root effect.

DISCUSSION

Blood gases

Arterial P_{O_2} in both resting and swimming hagfish was maintained sufficiently high to ensure virtually full saturation, with oxygen tensions close to 100 mmHg and a relatively high blood oxygen affinity. This finding contrasts with the earlier estimate

of less than 50% arterial saturation at $Pa_{O_2} = 5$ mmHg in an eptatrid hagfish (Manwell, 1963) but corresponds with high arterial saturations in lamprey cyclostomes (Johansen, Lenfant & Hansen, 1973). Similarly high arterial P_{O_2} was observed in the swimming shark *Negaprion* (Bushnell *et al.* 1982) which has blood O_2-binding properties comparable with those of *E. cirrhatus*. Oxygen tensions in mixed venous blood from the ventral aorta was, by contrast, low in resting fish ($P\bar{v}_{O_2} = 17{\cdot}2 \pm 3{\cdot}0$ mmHg) and even lower in swimming fish ($3{\cdot}5 \pm 1{\cdot}6$ mmHg). These measurements, taken together with the low estimates of plasma lactate,

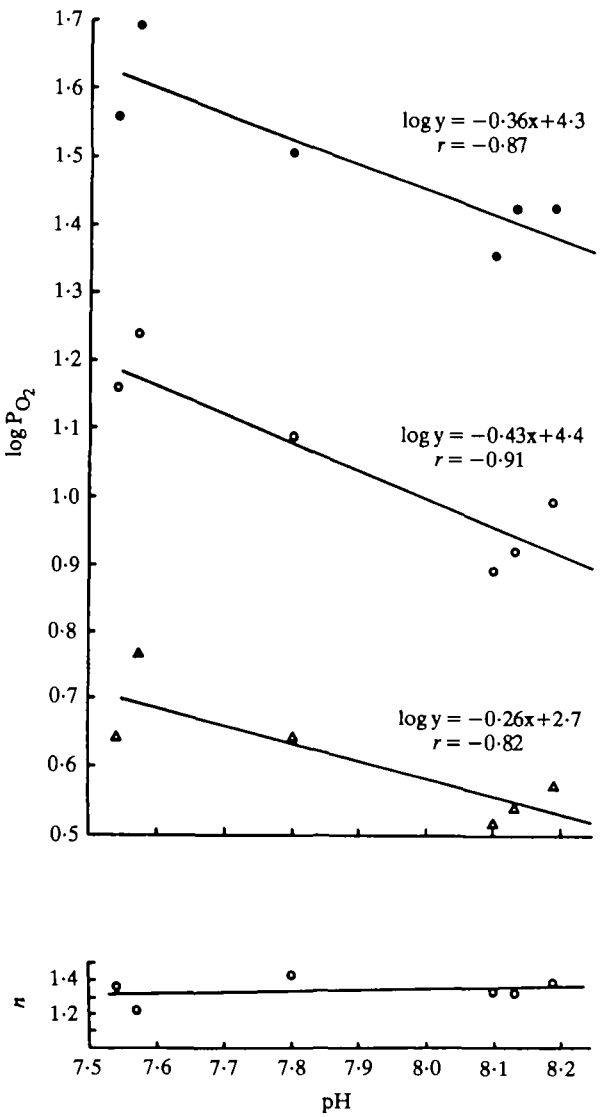

Fig. 3. Oxygen equilibrium data for whole blood presented as CO_2-Bohr plots at three saturations: P_{80} (●), P_{50} (○) and P_{20} (△) at 16°C together with their regression equations. Average values of Hill's coefficient, n, as a function of pH are also shown.

confirm that the blood has an important role in transporting oxygen to the tissues both at rest and during swimming. At rest, hagfish have very low metabolic rates (Smith & Hessler, 1974), though the limits to aerobic scope for activity have not been determined.

The haematocrit, haemoglobin concentration and oxygen-carrying capacity of the blood was low compared with measurements from lampreys (Beamish & Potter, 1972) and other fish species when expressed on a unit volume basis. Dissolved oxygen is a significant component of the total blood oxygen content and the high resting arterial P_{O_2} of *Eptatretus* blood will increase this fraction.

Oxygen equilibrium

The blood of *E. cirrhatus* has a relatively high affinity for oxygen ($P_{50} = 12{\cdot}3 \pm 0{\cdot}6$ mmHg at pH 7·8 and 16°C), exhibits weakly cooperative oxygen binding (Hill's $n = 1{\cdot}38 \pm 0{\cdot}08$ at pH 7·8 and 16°C) and has a modest CO_2-Bohr effect ($\phi = -0{\cdot}43$). At P_{O_2} approaching 100 mmHg, i.e. that obtained in the dorsal aorta, the haemoglobin will obviously be saturated with oxygen. This conclusion differs from that of Manwell (1958) who found low saturation and P_{O_2} in the dorsal aorta of *E. stouti*. It should be pointed out, however, that he collected blood from a 'sliming' fish nailed to a board, and this cannot be related to any natural physiological situation.

In our study, the resting $P\bar{v}_{O_2}$ of $17{\cdot}2 \pm 7{\cdot}0$ mmHg at pH 7·77 corresponds to a saturation of approximately 58 % from the appropriate oxygen equilibrium curve. The unloading tension in the resting hagfish is therefore close to the P_{50}. Venous P_{O_2} falls further during swimming ($P\bar{v}_{O_2} = 3{\cdot}5 \pm 1{\cdot}6$ mmHg) and as a consequence of the modest degree of cooperative oxygen binding, a further 40 % bound oxygen may be released at the prevailing pH of 7·7.

The oxygen affinity, cooperativity and Bohr effect in whole blood from *E. cirrhatus* are strikingly similar to equilibrium data from elasmobranchs such asthe dogfish, *Squalus acanthias* (Wells & Weber, 1983) and the carpet shark, *Cephaloscyllium isabella* (Tetens & Wells, 1984). The P_{50} and Bohr factors were also similar to values obtained for whole blood in the lamprey, *Lampetra fluviatilis* (Nikinmaa & Weber, 1984). There are, surprisingly, no published data of cooperativity coefficients from whole blood in lampreys or hagfish, although buffered erythrocyte suspensions from the lampreys, *Geotria australis* and *L. fluviatilis* yielded n values which rose from close to 1·0 at low saturation to 2·0 and 1·5 at 80 % saturation (Macey & Potter, 1982; Bird, Lutz & Potter, 1976). In this respect, hagfish blood differs because cooperativity is less marked and the linear Hill plots indicated that n values were independent of saturation. As in the lamprey erythrocytes (Bird *et al.* 1976), cooperativity in hagfish blood was independent of pH over the range tested.

Hyperbolic oxygen-binding curves of relatively high affinity are an expected feature of fish with either a low demand for oxygen or with poor access to oxygen in the environment, and thus oxygen transfer may be effective over a relatively wide range of P_{O_2} values. Highly active fish with good access to oxygen tend to have sigmoidal oxygen-binding curves and lower affinities (Riggs, 1970; Powers, 1980).

A particularly high affinity for oxygen and n value of 1·1 in the tuna (Cech, Laurs & Graham, 1984) appears to be an extraordinary exception.

The role of phosphorylated metabolic intermediates in the erythrocytes of hagfish is not known. The principal phosphate isolated from *Eptatretus stouti* was ATP (Bartlett, 1982), as we have found for *E. cirrhatus*. A clue to the possible significance of erythrocyte organic phosphates in modulating haemoglobin function in the Cyclostomata is known only from the study of *Lampetra*, in which an indirect role of ATP in intra-erythrocytic pH was demonstrated, rather than an allosteric role (Nikinmaa & Weber, 1984). In our hagfish, the stripped haemolysate had a markedly higher affinity than that of whole blood at the same pH (Fig. 2), which might also reflect a relatively lower intra-erythrocytic pH. Hagfish haemoglobins appear to be monomeric (Li *et al.* 1972; Paleus & Liljeqvist, 1972) and thus the presence of a Bohr effect and weak cooperative oxygen binding is unusual. Our demonstration of a Bohr effect and weak cooperativity in whole hagfish blood is corroborated by the haemolysate studies of Bauer *et al.* (1975) who further found that CO_2 appeared to increase the cooperativity of stripped lysate from *Myxine*.

Carbon dioxide transport

The blood of *Eptatretus* is poorly buffered in comparison with elasmobranch and teleost blood (Albers, 1970; Eddy, 1976; Heisler, 1980; Albers, Gotz & Welbers, 1981), which presumably reflects primarily the low concentration of haemoglobin. However, at rest DA blood is relatively alkaline when compared to elasmobranch and teleost blood at the same temperature, although lying within the range of values compiled by Heisler (1984). Using the mean pH values of VA and DA blood given above, from the fitted regression lines for oxygenated and deoxygenated blood (Fig. 1), it can be calculated that the Haldane effect is responsible for the loss of from 1·5 to 2·4 mmol l^{-1} of bicarbonate as CO_2 at the gills, or from 14 to 22% of total bicarbonate. This represents a substantial Haldane effect when the low haemoglobin concentration is considered.

The hagfish *Eptatretus cirrhatus* occurs in southern cooler waters to depths of over 1000 m (Ayling & Cox, 1982) but may also occur in shallow water. The habits of hagfish are poorly known, although it is claimed that myxinoids inhabit muddy burrows, whereas eptatrids are frequently found on rocky substrata (Hardisty, 1979). Our underwater field observations indicate that the animals prefer to inhabit rocky crevices and that they are not seen in sandy or muddy sites where other burrowing forms are observed. *Myxine* survives well with the nostril occluded (Johansen & Strahan, 1963) and uses the skin as the major respiratory exchange surface (Steffensen *et al.* 1984). Being a considerably larger animal, the lower surface area to volume ratio of *E. cirrhatus* will not favour transcutaneous oxygen uptake. When feeding within the body of dead or dying fish (Hardisty, 1979), gill oxygen uptake might be impaired and at such times the blood might function as an oxygen store. The comparatively high affinity of its blood for oxygen and weakly cooperative oxygen binding are features which may facilitate oxygen transfer in an animal with a very low metabolic rate and low internal oxygen pressures. These properties,

together with a weak Bohr effect, apparently enable the blood to transport oxygen and CO_2 both at rest and when swimming. Our demonstration of appreciable venous desaturation during aerobic activity shows that *E. cirrhatus* exploits the full range of its equilibrium curve in a blood circulation with large venous sinuses and allows for an efficient uptake of oxygen at the gill surfaces.

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