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OXYGENATIONAL PROPERTIES AND PHOSPHORYLATED METABOLIC INTERMEDIATES IN BLOOD AND ERYTHROCYTES OF THE DOGFISH, *SQUALUS ACANTHIAS*

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SUMMARY

A typical whole blood O_2-equilibrium curve from *Squalus acanthias* had a P_{50} of 13·2 mmHg and was slightly sigmoidal, having an n value of 1·6 at 15 °C, $P_{CO_2} = 2{\cdot}2$ mmHg (pH = 7·85). A small Bohr effect was present ($\phi = -0{\cdot}28$) together with a weak Haldane effect and no Root shift.

The predominant trinucleotide, determined by thin layer chromatography, was ATP (0·44 ± 0·13 S.D. mmol l^{-1} blood) with smaller amounts of GTP present (0·07 ± 0·02 S.D. mmol l^{-1}). Total nucleotide concentrations, determined enzymatically, were low by comparison with teleosts. Incubation of erythrocytes with or without oxygen, or in the presence of a metabolite-enriched 'cocktail' showed limited potential for phosphate cofactor regulation of blood oxygen affinity.

INTRODUCTION

Regulation of blood oxygen affinity of lower vertebrates in response to changes in the partial pressure of inspired oxygen is primarily attributable to changes in the levels of erythrocytic trinucleotides – adenosine triphosphate (ATP) and particularly guanosine triphosphate (GTP) when present. ATP and GTP depress the oxygen affinity of haemoglobin directly by allosteric interaction and indirectly by decreasing intraerythrocytic pH through modification of the Donnan distribution of protons across the red cell membranes (Wood & Johansen, 1972; Weber, Lykkeboe & Johansen, 1976; Johansen, Lykkeboe, Weber & Maloiy, 1976; Greaney & Powers, 1977; Qvist, Weber, DeVries & Zapol, 1977; Weber, Johansen, Lykkeboe & Maloiy, 1977; Weber & Lykkeboe, 1978).

The allosteric phosphate cofactors occurring in the erythrocytes of the various classes of vertebrates, and their modulatory influences on the oxygen affinity of the isolated haemoglobins have been well documented in recent years; few studies have, however, been directed towards evaluating to what extent the *in vivo* response in blood oxygen affinity to environmental hypoxia is located in the erythrocytes themselves (see reviews by Johansen & Weber, 1976; Bartlett, 1980; Isaacks & Harkness, 1980; Powers, 1980; Weber, 1982). Unlike mammalian erythrocytes where ATP

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concentrations are low, the nucleated erythrocytes of fish derive energy mainly fro oxidative phosphorylation and have high nucleotide concentrations. This suggests a regulatory feedback, with low oxygen tensions increasing blood oxygen affinity via decreased concentration of erythrocytic trinucleotides, resulting in compensatory increases in oxygen loading in the gills under hypoxic conditions.

No comparable studies appear to have been carried out for any of the elasmobranchs. Whereas the erythrocytes of most teleosts contain relatively high concentrations of ATP, GTP is the major erythrocytic trinucleotide in a Japanese shark and the smooth dogfish, *Mustelus canis* (Kono & Hashimoto, 1977; Borgese *et al.* 1978). Moreover, the erythrocytes of two sharks and a ray from Australia contain unusually high concentrations of inositol monophosphate (Coates, Paton & Thompson, 1978).

We have investigated the efficiency of the internal oxygen transporting system of an elasmobranch, the dogfish *Squalus acanthias*. A primary objective was to investigate the influence of perturbations in oxidative phosphorylation (induced by decreased oxygen tension) on the steady state level of erythrocytic trinucleotides with the view of evaluating the allosteric control of blood oxygen affinity. A parallel study (Weber, Wells & Rossetti, 1983) focuses on the oxygen and carbon dioxide equilibria of the isolated haemoglobin.

MATERIALS AND METHODS

Maintenance of animals and blood collection

Spiny dogfish, *Squalus acanthias* L., weighing 2–7 kg, were obtained from North Sea fishermen through the Esbjerg Fisheries Aquarium in Denmark. (*S. acanthias* is now considered to be the same as *S. suckleyi*, a name used in some earlier literature for the Pacific specimens.) Fish were kept at the aquarium or in large tanks containing aerated sea water at 15 °C, after transferring them to the laboratory in Odense.

Some fish were cannulated with Intramedic (Clay Adams) PE-50 tubing to avoid the traumatic effects of acute venepuncture which might elevate haematocrit and decrease blood pH (cf. Soivio & Oikari, 1976). Consistent results were, however, subsequently obtained by acute venepuncture provided that sampling was completed within 15–30 s.

Between 1 and 5 ml blood was taken from an animal, usually only once.

Haemoglobin and haematocrit

The cyanmethaemoglobin method for estimating haemoglobin concentration (see Dacie & Lewis, 1975) is recommended for use with fish blood (Blaxhall, 1972). Thus, 20 μl of fresh whole blood was added to 4·0 ml Drabkin's reagent, shaken, and left to stand for 20 min to ensure complete conversion of the haemoglobin to cyanmethaemoglobin.

The packed cell volume of erythrocytes in 75-μl samples of fresh, anticoagulated blood was determined by centrifugation at 8000 ***g*** for 5 min in microhaematocrit tubes.

Acid-soluble phosphates

Freshly drawn blood was diluted 1 : 1 v/v with 120 g l^{-1} trichloroacetic acid, shaken vigorously, and placed on ice for 5 min. The protein precipitate was removed by centrifugation and the clear extract was tested using three techniques.

(1) Total nucleoside triphosphate (NTP) was measured spectrophotometrically using an enzymatic test-combination kit for ATP (Boehringer, Mannheim). The technique cannot discriminate between ATP and other trinucleotides.

(2) ATP and guanosine triphosphate (GTP) were distinguished enzymatically using a two-step reaction with guanosine monophosphokinase and nucleoside diphosphokinase using u.v.-spectroscopy (J. B. Jørgensen, T. Mustafa & R. E. Weber, unpublished observations).

(3) Nucleotide mixtures were resolved by thin layer chromatography (TLC) on cellulose plates impregnated with polyethyleneimine (Cashel, Lazzarini & Kalbacher, 1969) with modifications by Johansen *et al.* (1976). Following one-dimensional development in phosphate buffer, pH 3·5, 'spots' were identified and circumsected under u.v. light, and eluted with Tris-HCl-Mg^{2+} buffer, pH 7·5. ATP and GTP concentrations were ascertained from absorption maxima using the appropriate extinction coefficients. Relative mobilities were compared using standard nucleotide compounds obtained from Boehringer.

Oxygen equilibrium

Continuous, whole blood oxygen equilibrium curves were registered from thin blood films using a Hemoscan instrument (Aminco, U.S.A.). The instrument was modified by reducing the rate of air leakage into the sample compartment. Equilibration gases were forwarded from two Wösthoff mixing pumps (Bochum, F.R.G.) arranged in series. The validity of the technique was tested by comparing the data with discontinuous curves obtained by thoroughly purging the films with pre-set oxygen mixtures, thus ensuring complete equilibration of the blood film and at the same time confirming P_{O_2} – electrode linearity. Oxygen affinity, evaluated by half-saturation tension, P_{50}, and cooperativity, evaluated by Hill's (1910) sigmoid coefficient, n, showed no significant differences using the two techniques. The carbon dioxide Bohr effect was obtained by measuring P_{50} at different P_{CO_2} values embracing the normoxic pH range of arterial and mixed venous blood (Butler & Taylor, 1975; Short, Taylor & Butler, 1979; Truchot, Toulmond & Dejours, 1980).

At the conclusion of a run, blood films were scanned in a Unicam SP-1800 recording spectrophotometer to discern oxidation to methaemoglobin. Levels of methaemoglobin were always less than 5 % of total haemoglobin when the equilibria were obtained below 20 °C.

pH

Blood samples of 75 µl volume were equilibrated with the Hb-O_2 equilibrium gas mixes for 12–15 min in a Radiometer BMS-2 for pH measurement. pH was also measured in aliquots equilibrated to various P_{CO_2} values in oxy and deoxy states. All gas mixtures were forwarded from Wösthoff pumps.

Intra-erythrocytic pH was obtained after whole blood tonometry with air/CO_2 mixtures. Red cells packed under paraffin were freeze-thawed three times with liquid N_2 and brought to 15 °C. The slurry was then sucked into the pH capillary electrode of the BMS2 and pH recorded in the usual manner.

In vitro *incubation of erythrocytes*

Approximately 3 ml blood pooled from three fish was placed in each of two tonometers (Eschweiler, Kiel) and equilibrated at 10 °C under a continuous stream of humidified gas supplied from Wösthoff mixing pumps. One stream contained $P_{CO_2} = 2{\cdot}2$ mmHg, balance N_2. The other stream contained $P_{CO_2} = 2{\cdot}2$ mmHg, $P_{O_2} = 97$ mmHg, balance N_2. Aliquots were taken anaerobically at hourly intervals for 5 h, then at 18 h for measurements of haematocrit, pH, ATP, GTP, methaemoglobin and P_{50}. Deproteinized NTP extracts were prepared for thin layer chromatography as before, and P_{50} and pH determined at $P_{CO_2} = 2{\cdot}2$ mmHg and 10 °C. Preliminary experiments conducted at 15 °C were adequate for up to 2 h incubation but by 18 h resulted in the formation of > 20 % methaemoglobin and appreciable haemolysis. At 10 °C methaemoglobin amounted to approx. 7 % with negligible haemolysis at 18 h and this was considered acceptable. Measurements of fresh blood at 0 h were taken as controls and values following 18 h on ice in stoppered tubes were used for comparison. Each measurement was performed in duplicate and the average taken.

A second equilibration was carried out on another pool from two animals. To this pool was added a 'cocktail' containing the following concentration of metabolites in distilled water: 100 mM-pyruvate; 100 mM-inosine; 10 mM-adenine; 100 mM-dextrose; 200 mM-$Na_2HPO_4.2H_2O$ and 170 mM-NaCl. A similar brew was effective in raising ATP and 2,3-diphosphoglycerate in incubated mammalian erythrocytes (Wells, Hill & Woodfield, 1981). The solution had a pH of 7·90 and an osmolarity of 1120 mosM, close to values obtained from dogfish plasma. One volume of the 'cocktail' was added to 10 volumes of whole blood. The osmolarity of test solutions and separated plasma was measured using a Knauer osmometer.

Pooled blood was used, eliminating individual variation and thus analysis of covariance could not be undertaken.

RESULTS

Intra-erythrocytic trinucleotide concentrations and haematological data are summarized in Table 1. The three methods used for measuring NTP are in close agreement. The predominant trinucleotide is ATP, which is about six times as abundant as GTP. Animals in captivity which were not feeding regularly may have had lower haematocrits, but insufficient numbers were available to test the hypothesis.

Oxygen equilibrium

A typical O_2-equilibrium curve obtained at 15 °C and $P_{CO_2} = 2{\cdot}2$ mmHg (pH = 7·85) is shown in Fig. 1. The curve has a P_{50} of 13·2 mmHg and is slightly sigmoidal having an n value of 1·65. All equilibrium curves were digitized with up to 30 data pairs and analysed for n_{50} and P_{50} according to Hill's (1910) equation using an Apple II computer.

Table 1. *Nucleoside triphosphate and haemoglobin concentrations in* Squalus acanthias

Mean values ± S.D., N = number of measurements

	Method*	N	Concentration (mmol l^{-1}) Blood	Red cells**
ATP	TLC	5	0·44 ± 0·13	3·70 ± 1·14
	E, s	1	0·39	3·27
GTP	TLC	5	0·07 ± 0·02	0·60 ± 0·18
	E, s	1	0·08	0·68
ATP + GTP	TLC	5	0·51 ± 0·15	4·31 ± 1·26
	E, s	1	0·47	3·96
NTP	E, ns	5	0·48 ± 0·16	4·04 ± 1·35
Hb		5	0·42 ± 0·14	

* TLC = thin layer chromatography; E, s = specific enzymic method; E, ns = non-specific enzymic method (see Materials & Methods).

** Cellular concentrations were calculated from blood values and haematocrits, assuming all measured nucleotides and Hb are intracellular. Mean haematocrit (N = 8) was 11·55 ± 3·65 %.

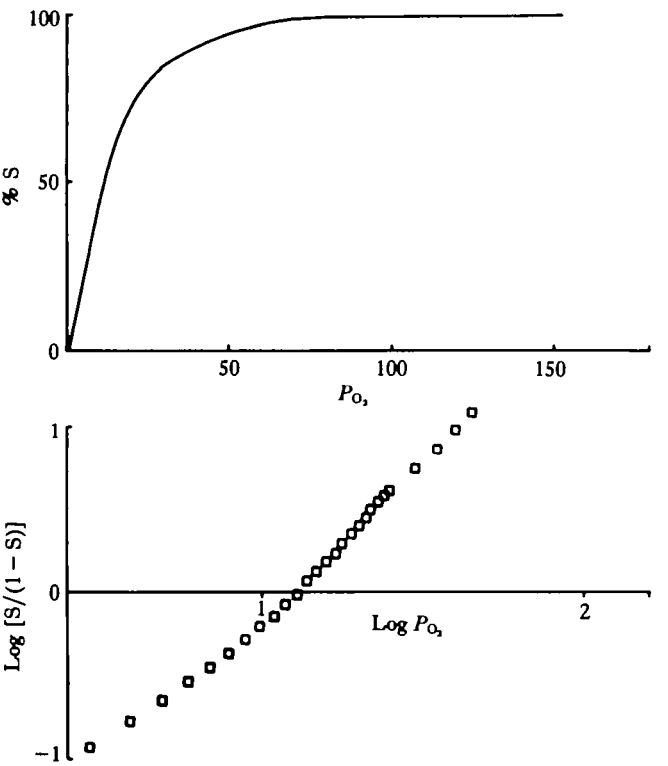

Fig. 1. Representative O_2-equilibrium curve and double log Hill transformation of whole blood from *Squalus acanthias*. Equilibrium conditions established at 15 °C, P_{CO_2} = 2·2 mmHg, pH = 7·85. The Hill plot indicates that O_2 binding is weakly cooperative (slope, n = 1·6) and O_2 affinity is moderately high (P_{50} = 12·6 mmHg).

The carbon dioxide Bohr effect is shown in Fig. 2. Assuming a linear relationshi between pH and P_{50} for the pH range tested, the Bohr coefficient was estimated from the slope of the regression equation, $\log P_{50} = -0{\cdot}28\,\mathrm{pH} + 3{\cdot}45$ ($r = 0{\cdot}92$) as $-0{\cdot}28$. Hill's coefficient n, did not show significant pH variation in the range tested (Fig. 2). Oxygenated and deoxygenated blood equilibrated with CO_2 showed a small pH difference, indicating a Haldane effect of minor magnitude (Fig. 3). The relatively weak influence of pH on oxygen affinity of the blood (the Bohr effect) may thus be viewed as a reciprocal effect of the poor ability of *Squalus* haemoglobin to remove hydrated carbon dioxide. The accompanying paper (Weber *et al.* 1983) considers the implication of the Haldane effect in blood CO_2 transport and buffering capacity.

During the course of our study some dogfish were born in captivity. In two, approximately 2-week-old specimens we found no detectable GTP, variable amounts of ATP (1·8 and 2·6 mmol l^{-1} blood cells) and haematocrits of 10%. Whole blood P_{50} was 11·5 mmHg (cf. 13·2 mmHg in maternal) and $n \approx 1{\cdot}1$ at 15 °C.

Intracellular pH

Intraerythrocytic pH was maintained acidic relative to plasma pH following equilibration to different P_{CO_2}, but the relationship between pH_i and pH_e was not linear (Fig. 4). As might be expected from the charge carried by red cell membranes, the cell-free plasma pH values were slightly higher than those from whole blood (cf. Fig. 3).

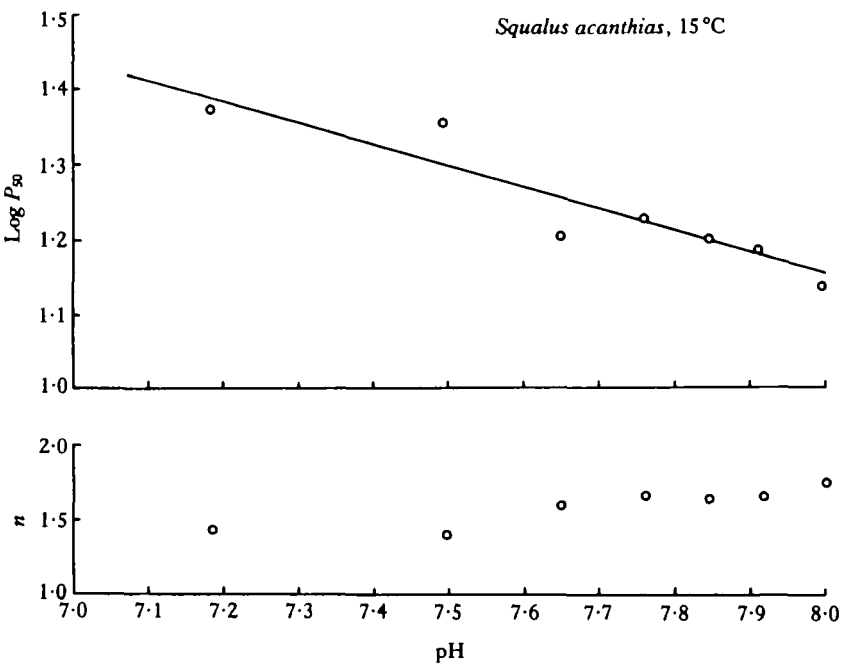

Fig. 2. Dependence of oxygen affinity (P_{50}) and Hill's cooperativity coefficient, n, on pH for *S. acanthias* whole blood at 15 °C. An average Bohr coefficient was calculated from the slope of the regression line assuming linearity of the data points. Each point represents duplicate determinations.

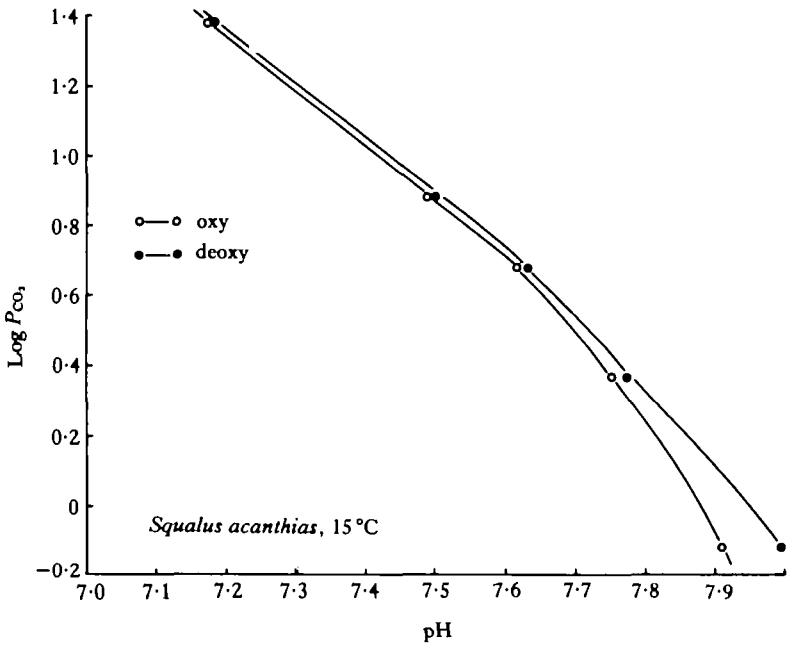

Fig. 3. pH values of blood from *S. acanthias*, 15 °C equilibrated at various CO_2 tensions. Data from a single animal with each point averaged from quadruple measurements.

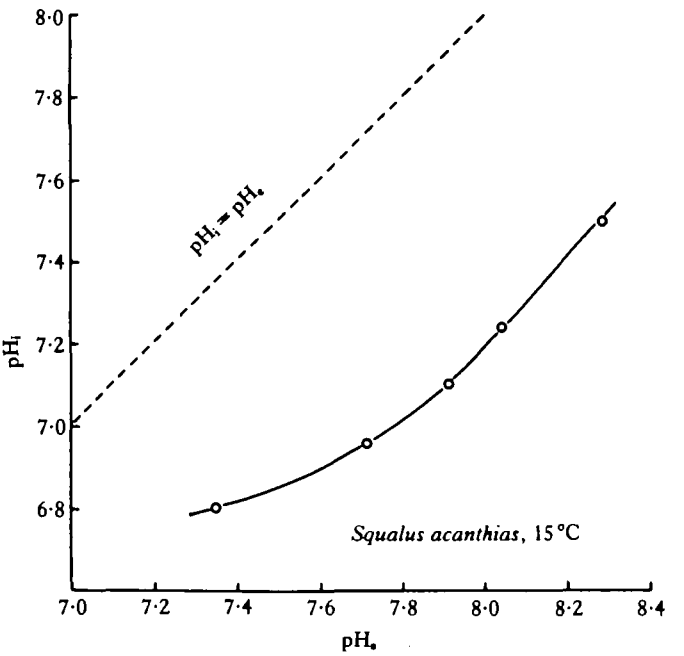

Fig. 4. Relationship between intraerythrocytic pH (pH_i) and cell-free plasma pH (pH_e) for *S. acanthias* blood equilibrated at 15 °C to different CO_2 tensions. An iso-pH line (dashed) is shown for reference. All points represent the average from quadruple measurements from a single animal.

In vitro *studies on erythrocytes*

Tonometry of whole blood resulted in a slight (approx. 2 mmHg) increase in oxygen affinity during the first hour, and thereafter no changes were discerned during 5 h continuous tonometry at 10 °C (see Fig. 5). A slight increase in affinity was seen at 18 h in the deoxygenated sample. Storage of blood for 18 h at 0 °C in the oxy state did not increase the oxygen affinity of the blood (see C in Fig. 5). ATP levels remained constant throughout the period of tonometry when in the oxy state, but fell slightly in the absence of oxygen. This would be anticipated from the major pathway to ATP

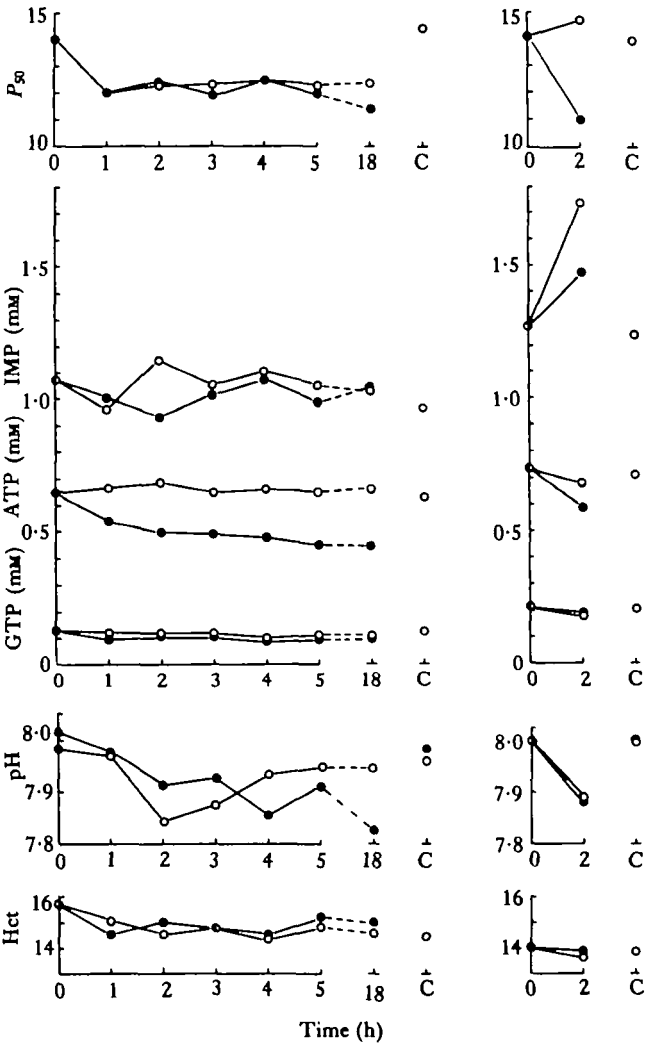

Fig. 5. Time course of parameters during 18 h (with no added metabolites) and 2 h (added metabolites) erythrocyte incubation in oxygenated (○——○) and deoxygenated (●——●) *S. acanthias* blood at 10 °C. Comparisons (C) for blood stored on ice for the duration of incubation are also given. Parameters are: O_2 affinity (P_{50}, mmHg), nucleotide concentrations (IMP, ATP, GTP), pH (CO_2-equilibrated), and haematocrit (Hct). For details and further explanation, see text.

synthesis through oxidative phosphorylation. There were no changes in GTP concentration. The fall in ATP in the deoxy state did not appear to be accompanied by a comparable increase in oxygen affinity or any alteration to the Bohr effect. This suggests that either the major fraction of ATP is not cytoplasmic in origin, or there is compensation by other cofactors which we have not identified. Longer incubation times could not be maintained without high (> 10 %) methaemoglobin formation.

When blood was tonometered with the metabolic 'cocktail' for 2 h, (see Methods) both ATP and pH decreased. The haematocrit values remained close to control levels throughout the 18 h and during incubation with metabolites. The influence of metabolites on intracellular pH was not ascertained.

A forward running spot of inositol monophosphate (IMP) in TLC was identified and distinguished from other mono- and dinucleotides by comparison of its relative mobility with standards, and its u.v.-maximum of 220 nm. The IMP pool showed no consistent change during 18 h tonometry (Fig. 5). During incubation with metabolites, IMP increased. Conceivably, ATP in the presence of inosine is released as ADP + IMP. Monophosphate nucleotides, however, do not appear to have an allosteric role in haemoglobin function (Chanutin & Curnish, 1967); nor does IMP seem to be a precursor for trinucleotide formation.

DISCUSSION

Increased ventilation volume is a primary response to environmental hypoxia in both elasmobranchs and teleosts (Satchell, 1961; Hughes, 1973). The failure of the dogfish to survive prolonged exposure to severe hypoxia ($P_{I,O_2} < 50$ mmHg) is probably related to their inability to increase oxygen transport by substantially increasing ventilation volume or blood O_2 affinity. Recent studies with elasmobranchs have shown some degree of respiratory control in response to moderate hypoxia (Butler, Taylor & Davison, 1979; Short *et al.* 1979) and hyperoxia (Truchot *et al.* 1980). The possible regulation of oxygen release by haemoglobin has not been previously studied.

In normoxic conditions, *Squalus acanthias* has a haematocrit of one half to two-thirds the value of most temperate marine teleosts. The haematocrit value (11·55 ± 3·65 %) is typical of elasmobranchs in being substantially lower than values from most teleosts (cf. Larsson, Johansson-Sjöbeck & Fänge, 1976). The haemoglobin concentration is correspondingly low, reflecting a relatively low oxygen carrying capacity. Mean cell haemoglobin concentration (3·64 ± 1·2 mmol l^{-1} blood) falls within the range of values from cold temperate teleosts (see Wells, Ashby, Duncan & Macdonald, 1980). A low O_2 carrying capacity and relatively high O_2 affinity were not found in fishes with high resting and routine metabolic rates. Thus it is particularly noteworthy that *S. acanthias* has a resting metabolic rate among the lowest of all fishes (Brett & Blackburn, 1978).

The P_{50} and n value of *S. acanthias* in Fig. 1 accord with a P_{50} value of 17 mmHg and low cooperativity reported by Lenfant & Johansen (1966) for '*S. suckleyi*' (see Methods) blood at 11 °C and $P_{CO_2} = 0{\cdot}5$ mmHg. Moreover, *S. acanthias* shares with other elasmobranchs blood-oxygenation properties characterized by small Bohr and Haldane effects, the absence of a Root effect, and weakly cooperative oxygen binding (Bill, Edwards & Florkin, 1932; McCutcheon, 1947; Manwell, 1958*a*,*b*; Lenfant &

Johansen, 1966; Pennelly, Noble & Riggs, 1975). The small Bohr effect accords with weak oxygenation-linked proton binding (Fig. 3 and Albers & Pleschka, 1967). It is also associated with a small arterial-mixed venous pH differences noted in dogfish circulation (0·07 units by Baumgarten-Schumann & Piiper, 1968; 0·05 units by Short *et al.* 1979).

Whereas synthesis of ATP in fish red blood cells is oxygen dependent (Tetens & Lykkeboe, 1981), GTP is synthesized in the Krebs cycle and its metabolic control may be independent of ATP production. In fish (including *S. acanthias*) GTP is more potent than ATP in regulating haemoglobin-oxygen affinity (Weber *et al.* 1976, 1983). At high plasma pH (within the physiological range) the indirect effect of organic phosphate is more important than its allosteric effect (Wood & Johansen, 1972; 1973*a*,*b*) since the binding constants of the phosphate-haemoglobin reaction are pH dependent (Fyhn & Sullivan, 1975).

We have shown that the concentration of trinucleotides in *S. acanthias* is low by comparison with teleosts and similar to concentrations in other elasmobranchs, even though the molar ratio of ATP to haemoglobin is around unity (cf. Bricker *et al.* 1968; Coates, 1975; Kono & Hashimoto, 1977; Borgese *et al.* 1978; Bartlett, 1980). These studies are not in agreement with results from Leray (1982) who finds *S. acanthias* to have higher NTP levels than many teleosts. This is possibly explained by the use of stressed fish as evidenced by very high haematocrit values. The agreement between the sum of ATP and GTP concentrations and the enzymatically determined NTP values excludes the presence of significant levels of other trinucleotides. *Squalus acanthias* is exceptional among the elasmobranchs in having ATP as the dominant phosphate with smaller amounts of GTP present (Table 1; confirming Bartlett, 1980, his Table 1; Leray, 1982). In most elasmobranchs GTP predominates (Kono & Hashimoto, 1977; Borgese *et al.* 1978; Coates *et al.* 1978; Bartlett, 1980). Remarkably Borgese & Nagel (1978) observed that erythrocytes of *S. acanthias* contain inositol pentaphosphate (IPP, the cofactor characteristic of bird erythrocytes) at concentrations of about half of that of ATP, as well as small concentrations of uridine triphosphate (UTP) and IMP.

Purified shark haemoglobins are sensitive to ATP, DPG and inositol hexaphosphate (IHP) though the sensitivity to phosphates is less than for teleosts (Andersen, Olsen, Gibson & Carey, 1973). Urea, which occurs in high concentration in the erythrocytes of elasmobranchs (Smith, 1936; Browning, 1978) has a slight, depressant effect on the oxygen affinity of purified skate haemoglobin (Bonaventura, Bonaventura & Sullivan, 1974). Coates (1975) reports that it inhibits phosphate-binding to haemoglobin, suggesting that erythrocytic phosphates have little importance as regulators of O_2 affinity in elasmobranchs. The following study (Weber *et al.* 1983) shows that urea increases the O_2 affinity of *S. acanthias* haemoglobin and reduces its ATP sensitivity.

An important question concerns the ability of elasmobranchs to regulate oxygen affinity in response to declining oxygen tension by parallel reductions in phosphate cofactors. The fall in P_{50} observed in deoxy tonometered blood (cf. Fig. 5) can be attributed to decreased ATP because the observed pH decrease should raise P_{50}. By contrast human blood incubated with the 'cocktail' at physiological temperature has been found to result in a dramatic rise in both organic phosphates and P_{50} (Wells *et al.* 1981)

Since ATP is present in low concentration in *S. acanthias* erythrocytes, and since the O_2 affinity of the haemoglobin shows modest NTP sensitivity (Weber *et al.* 1983), we surmise the *in vivo* response to decreased oxygen availability to be very limited. The data do not suggest a role for other agents (such as IPP and mononucleotides) in controlling oxygen affinity. In fact, the whole oxygen transport system, with its essential lack of phosphate regulation, Bohr, and Haldane effects, constitutes an inflexible haemoglobin-oxygen delivery mechanism which may, nevertheless, adequately meet the metabolic demands of the animal in the absence of substantial perturbations in environmental oxygen tension. This is in sharp contrast to the data from teleosts. The control of oxygen affinity by GTP in response to hypoxia is known for eels (Wood & Johansen, 1972, 1973*a*,*b*; Weber *et al.* 1975, 1976) and other fresh water fishes (Lykkeboe & Weber, 1978; Weber, Wood & Davis, 1979), and by ATP in trout (Soivio, Nikinmaa & Westman, 1980; Tetens & Lykkeboe, 1981). There are few data from salt water teleosts but the indications are that a similar control system operates (Wood, Johansen & Weber, 1975; Greaney & Powers, 1978; Greaney *et al.* 1980). The reviews of Johansen & Weber (1976), Powers (1980), Wood (1980) and Weber (1982) clearly indicate the need for further work in marine species, especially the elasmobranchs, in order to discern the generality of the findings in our study.

The higher blood oxygen affinity of neonatal dogfish is also evident in the maternal-foetal shift in purified haemoglobin solutions from '*S. suckleyi*' (Manwell, 1958*b*) and two other species of sharks (Pennelly *et al.* 1975), although in the latter case the shift was not evident in the presence of saturating quantities of ATP.

Several problems need to be highlighted in the control of haemoglobin oxygen affinity in fishes. Firstly, there is a conflict in roles for the high energy phosphate, ATP. If all the erythrocytic ATP in *S. acanthias* is membrane bound, and is deployed as an energy supply for maintaining the integrity of erythrocytic structure, then it may be unavailable for an allosteric role, despite the demonstration of its effect in purified solution (cf. Andersen *et al.* 1973; Weber *et al.* 1983). Indirect evidence for this is provided by our unsuccessful attempts to deplete cells of all ATP. The role of ATP in maintaining erythrocyte structure has also been considered for enucleated mammalian cells (Feo & Mohandas, 1977). ATP would have to be mainly cytoplasmic in order to effect either an allosteric role, or to influence intracellular pH.

Secondly, of the phosphate compounds identified from *Squalus* erythrocytes – ATP, GTP, UTP, IMP, ADP, IPP – we are ignorant of their distribution within the cell. Significant quantities of these would be expected to occur in the nucleus of the erythrocyte resulting from the biosynthesis and degradation of nucleic acids. Communication of these substances from the nucleus to the cytoplasm has not been investigated. This problem was compounded by our unpublished observation that haemoglobin from *S. acanthias* is distributed in the nucleus as well as in the cytoplasm. In other poikilotherms, up to 40% of the haemoglobin may be locked in the nucleus (Davies, 1962; Tooze & Davies, 1963). The physiological significance of these findings is entirely a matter for conjecture.

Finally, in order to assess accurately the magnitude of the Bohr effect in fishes, it is necessary to make assumptions about intraerythrocytic pH. Unfortunately, the existing methods for measuring intracellular pH only provide information on the average intraerythrocytic pH, and will include a high activity of hydrogen ions from

the nucleic acids. In view of the distribution of some portion of haemoglobin within the nuclear envelope, the physiological significance of the measured intraerythrocytic pH remains clouded.

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