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ALLOSTERIC INTERACTIONS GOVERNING OXYGEN EQUILIBRIA IN THE HAEMOGLOBIN SYSTEM OF THE SPINY DOGFISH, *SQUALUS ACANTHIAS*

BY ROY E. WEBER, RUFUS M. G. WELLS* AND JOHN E. ROSSETTI

Institute of Biology, Odense University, Campusvej 55, DK-5230 Odense M, Denmark

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SUMMARY

The oxygenation-linked, allosteric interactions of erythrocytic organic phosphates and urea with the haemoglobin (Hb), and the functional significance of the Hb multiplicity, were studied in an elasmobranch, *Squalus acanthias*.

The autochthonous red cell nucleoside triphosphates (NTP) ATP and GTP (guanosine triphosphate) strongly depress O_2 affinity of the stripped (cofactor-free) Hb and increase cooperativity in O_2 binding. As previously found in teleost Hbs, GTP exerts a greater effect than ATP at the same concentration. Urea, in contrast, increases O_2 affinity and depresses cooperativity. It also antagonizes the modulator effectivity of NTP at physiological NTP/Hb concentration ratios.

Deoxygenation of the Hb raises blood pH. This Haldane effect contrasts with earlier findings for Pacific specimens, but accords with the presence of a Bohr effect ($\phi = \Delta \log P_{50}/\Delta pH$).

S. acanthias Hb resolves into six main components (three pairs) on the basis of isoelectric point. There is no evidence for radical functional differentiation as found in teleosts with electrophoretically anodal and cathodal Hb components.

The physiological implications of the findings and the possible molecular mechanisms basic to the NTP and urea effects are discussed.

INTRODUCTION

The red cells of teleost fish contain high concentrations of ATP, often in concurrence with guanosine triphosphate (GTP) (Parks *et al.* 1973; Geoghegan & Poluhowich, 1974; Weber & Lykkeboe, 1978; Bartlett, 1980). These nucleoside triphosphates (NTP) depress the O_2 affinity of the haemoglobin (Hb) by allosteric interaction as does 2,3-diphosphoglycerate (DPG) in human red blood cells (Benesch & Benesch, 1967; Chanutin & Curnish, 1967). Changes in their concentrations correlate neatly with adaptive modulation of blood O_2 affinity in teleosts in response to changed environmental or metabolic stimuli (reviewed by Weber, 1982). Where

* Present address: Department of Zoology, University of Auckland, Auckland, New Zealand.
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present, GTP appears to play the greater role, as evident from its greater depressant effect on O_2 affinity and its lesser inhibition by divalent cations compared to ATP under identical *in vitro* conditions (Weber, Lykkeboe & Johansen, 1975; Weber & Lykkeboe, 1978).

Very little is known about the effects of erythrocytic cofactors on Hb function in the elasmobranchs, despite a tantalizing variation in type and concentration of potential ligands encountered. The erythrocytic ATP/GTP ratios vary tremendously – from less than unity in the school shark, *Galeorhinus australis*, to about 6 and 12 in the dogfishes *Squalus acanthias* and *Scyliorhinus canicula*, respectively (Coates, Paton & Thompson, 1978; Leray, 1979; Wells & Weber, 1983). Some species, e.g. the ray *Narcacion nobiliana* and *Squalus acanthias*, additionally contain small concentrations of inositol pentaphosphate (IPP) (Borgese & Nagel, 1978), the allosteric cofactor common in birds. Importantly, the erythrocytes of marine elasmobranchs also contain high concentrations of urea (near 0·5 M, Browning, 1978) where it serves an osmotic function. Urea, however, also binds to carboxyl groups of proteins like Hb (Krichevskaya, Lukash & Kartasheva, 1973) whereby it may influence its oxygenation properties.

Fish and other ectothermic vertebrates frequently possess multiple Hb systems. Many teleost fish possess anodal Hb components, whose O_2 affinities generally are highly sensitive to organic phosphates, pH and temperature, and cathodal ones with higher O_2 affinities and lower sensitivities to these factors (Binotti *et al.* 1971; Brunori, 1975; Weber, Wood & Lomholt, 1976; Weber, 1982). No information on the functional consequences of Hb multiplicity seems to be available for elasmobranchs, although they have highly variable patterns of Hb heterogeneity (Fyhn & Sullivan, 1975) which frequently also are polymorphic.

We studied the influences of potential erythrocytic modifiers of O_2 affinity (NTP and urea) on the composite and isolated Hbs, and the functional significance of Hb heterogeneity in the dogfish, *Squalus acanthias*, where the allosteric interactions are of additional interest in view of the almost complete dissociation of the tetrameric deoxygenated Hb molecules to dimeric halves upon oxygenation *in vitro* (Fyhn & Sullivan, 1975). We also report the dependence of blood pH on oxygenation at constant CO_2 tension, and the O_2 affinity in the Hb from juvenile specimens. The preceding paper (Wells & Weber, 1983) deals with the oxygenational properties of the whole blood and the regulation of O_2 affinity during *in vitro* incubation of the red cells.

MATERIALS AND METHODS

The study is based on seven specimens of the spiny dogfish, *Squalus acanthias* L., about 70–90 cm in length and 4–7 kg in weight. The sharks were caught by Esbjerg fishermen and kept at Esbjerg Marine Aquarium ('Fiskerimuseet') in sea water of about 35‰ salinity and at 10–15 °C until use. We also used blood from a batch of 2-week-old, 30–40 g juveniles that were born in the aquarium.

Whole blood samples were obtained by acute venepuncture. This technique, together with those used for estimating haematocrit and for enzymic and chromatographic assay of the concentrations of ATP and guanosine triphosphate (GTP), are detailed elsewhere (Wells & Weber, 1983).

The relationship between CO_2 tension and pH in oxy- and deoxygenated blood was investigated by equilibrating 75 µl aliquots of blood in Radiometer BMS-II tonometers with gas mixtures containing varying CO_2 tensions, and O_2 tensions of either zero or 148–155 mm, balance N_2, for 20 min at 20 °C. These gas mixtures were prepared with Wösthoff gas mixing pumps using 99·995 % pure N_2. Millimolar bicarbonate concentrations were calculated from the Henderson-Hasselbalch equation as $10^{(pH-pK')} \alpha_{CO2} P_{CO2}$ using a value for the CO_2 solubility coefficient (α_{CO2}) of 0·04400 mmol l^{-1} $mmHg^{-1}$, calculated from Pleschka & Wittenbrock's (1971) data for dogfish, and pK'_1 values interpolated from those given by Albers & Pleschka (1967) for elasmobranchs where pK'_1 at 20 °C and pH 7·4 (corresponding to 77 % CO_2 solubility in pure water) equals 6·042, and where $pK'_1/°C = -0·008$ and $pK'_1/pH = -0·097$.

Hb solutions were prepared by osmotic-shock and ultrasonification of red cells that had been washed 2–3 times in filtered sea water or 2 % saline. The Hb was stripped of ionic cofactors by chromatography on Sephadex G25 Superfine, as preliminary attempts to strip samples with Amberlite MB 3 mixed ion exchange resin resulted in methaemoglobin formation. Other procedures for preparing haemolysates, administering cofactors and separating multiple Hbs were carried out as described earlier (Weber, Lykkeboe & Johansen, 1976; Weber & Lykkeboe, 1978) except that some O_2 equilibria were measured in Na-Hepes instead of Tris-Cl buffer (specified in legends).

A modified diffusion chamber technique (Weber, 1981) was used for measuring O_2 equilibria, evaluating cooperativity coefficients at half saturation (n) from the slopes of Hill plots (Hill, 1910). The effects of urea at varying ATP concentrations were investigated by preparing stock solutions of stripped Hb, ATP, urea, and Na-Hepes buffer, each containing 0·09 M of the buffer and 0·09 M-NaCl, then mixing these in the desired proportions. The apparent heat of oxygenation (ΔH_{app}) was calculated from the derived van't Hoff equation (Wyman, 1948) as $R\Delta ln P_{50}/\Delta(1/T)$ where R is the gas constant (8·3143 J K^{-1} mol^{-1}) and T the absolute temperature.

RESULTS

O_2 affinity and its dependence on protons, organic phosphates, urea and temperature

The stripped Hb showed a high O_2 affinity, a weak Bohr effect (at 10 °C and pH 7·85, $P_{50} = 2·3$ and $\phi = -0·21$) and very slight cooperativity ($n = 1·05$) which increased slightly with falling pH (Fig. 1). The O_2 affinity was much higher than that found in the whole blood ($P_{50} = 13$ mmHg – Wells & Weber, 1983).

The Bohr effect of the stripped Hb was slightly greater at 2 °C than at 15 °C ($\phi =$ 0·26 and −0·21 respectively – Fig. 2). The overall heat of oxygenation (ΔH_{app}, which includes the heat of solution of oxygen) was −35 and −44 KJ $(mol\ O_2)^{-1}$ at pH 7·0 and 7·9. This pH dependence of ΔH is compatible with greater proton binding at low pH. The haemolysate from juvenile specimens had a higher intrinsic O_2 affinity than the cofactor-free maternal Hb (Fig. 2), indicating that this difference contributes to the maternal-foetal O_2 transfer in *S. acanthias*.

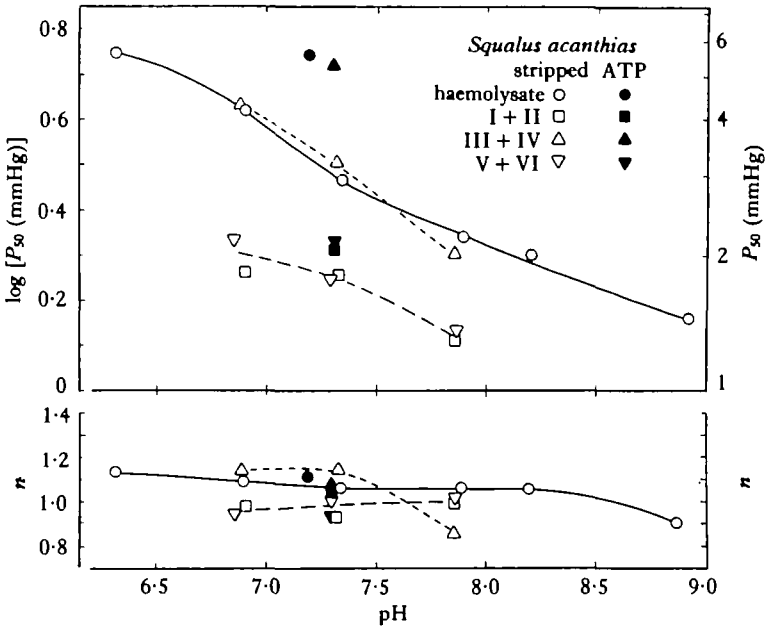

Fig. 1. O_2 tensions and cooperativity coefficients at half-saturation (P_{50} and n_{50}, respectively) and their pH dependence, in the stripped, composite haemolysate (○, ●) and in Hb fractions I + II (□, ■), III + IV (△, ▲) and V + VI (▽, ▼) measured in Tris/bis Tris buffer, ionic strength 0·05. Haem concentration, 0·3–0·4 mM (haemolysate and Hbs III + IV) and 0·1 mM (Hbs I + II and V + VI). Open and closed symbols, Hbs in the absence and presence of ATP, respectively. ATP: Hb ratios (mol/mol), 1·0.

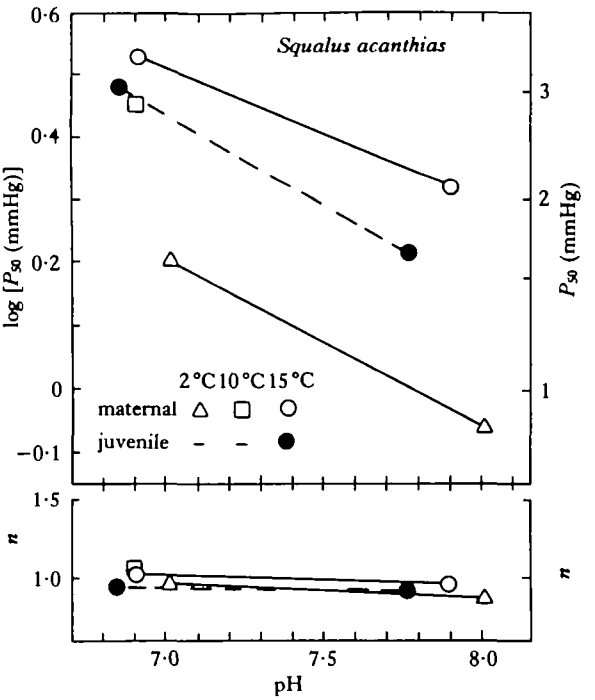

Fig. 2. P_{50} and n_{50} values of the stripped haemolysate of adult specimens at 2, 10 and 15 °C (△, □ and ○) compared with that of juvenile specimens at 15 °C (●), measured in 0·1 M-Na-Hepes buffer. Haem concentrations, 0·12 mM.

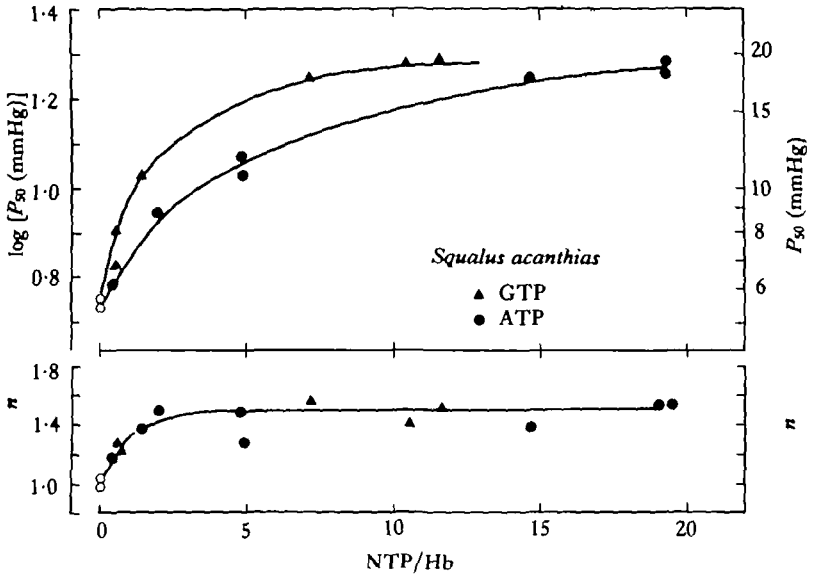

Fig. 3. Effects of ATP (●) and GTP (▲) on P_{50} and n_{50} of the stripped haemolysate measured at 15 °C, pH 7·28 in 0·10 M-Na-Hepes buffer. Haem concentration, 0·4 mM.

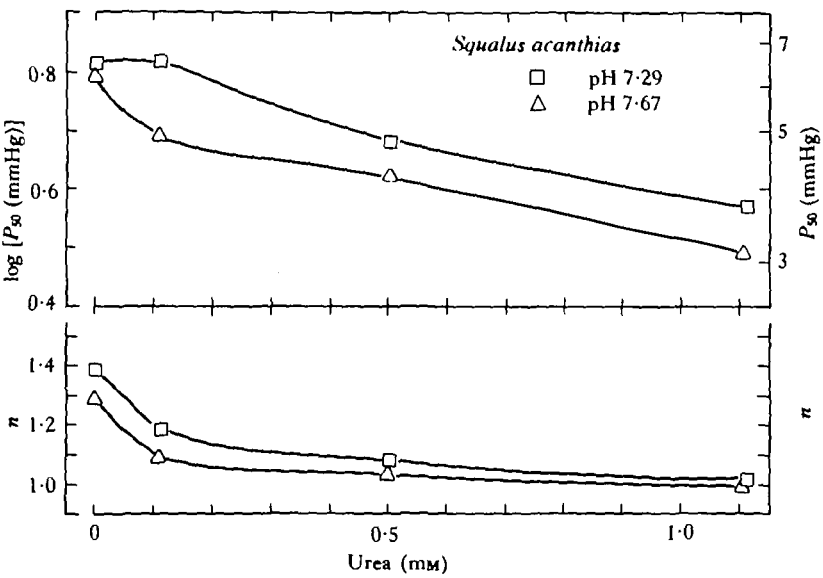

Fig. 4. Effects of urea on P_{50} and n_{50} of the stripped haemolysate at pH 7·29 (□) and 7·67 (△) measured at 15 °C in 0·10 M-Na-Hepes buffer. Haem concentration, 0·31 mM.

GTP decreased O_2 affinity more than ATP at the same phosphate: Hb ratio, as previously established in teleost fish, where both these factors are found (cf. Weber *et al.* 1975). The phosphates exerted greater effect on P_{50} at lower concentration ratios and raised n from about 1 to 1·5 (Fig. 3).

The influence of urea on Hb oxygenation and the implications of its concurrence with ATP are illustrated in Figs 4, 5 and 6. Urea increased the O_2 affinity of *Squalus*

Hb and decreased its cooperativity (Fig. 4); the affinity effect was greater at pH 7·1 than pH 7·3. Urea also reduced the sensitivity of O_2 affinity to ATP (Fig. 5). Thus, in the absence of urea, ATP (at 1·5 molar excess over Hb tetramers) decreased O_2 affinity below pH 7·5; in the presence of 0·4 M-urea this effect was evident only at pH below ~7·25 (Fig. 6). These observations suggest antagonism between oxygenation-linked binding of the two metabolites. Curiously, ATP increased O_2-affinity of the Hb at high pH (in the absence or presence of urea – Fig. 6).

Influence of Hb oxygenation on blood bicarbonate and buffer capacity

Fig. 7A, B shows the variations of CO_2 tension and bicarbonate concentration with pH for oxygenated and deoxygenated *S. acanthias* blood. The higher pH in the deoxygenated Hb at the same CO_2 tension (the Haldane effect) reflects binding of the Bohr protons to the Hb upon liberation of O_2 and a resultant increase in blood bicarbonate concentration ($\Delta HCO_3^- = 1{\cdot}4$ mmol l^{-1} at pH 7·9). The buffer capacity ($\Delta HCO_3^-/\Delta pH$) decreased from about 10 and 11·8 mol (pH unit)$^{-1}$ for deoxy- and oxygenated blood at pH 7·85, to about 2·4 and 4·0 respectively, at pH 7·0.

Hb multiplicity

Electrophoresis revealed the presence of three main anodal Hb bands in the adult as well as juvenile stages and showed no evidence for polymorphic variation in the material used. In preparative iso-electric focusing each of these bands resolved into two components; these six components accounted for at least 95 % of total haem (Fig. 8). At 10 °C the carboxy derivatives of the three pairs of components (I + II, III + IV and V + VI in Fig. 8) were isoelectric near pH values of 7·7, 7·4 and 6·9, respectively. O_2 equilibrium properties (incorporated into Fig. 1) showed that in the pH range of 7·0 to 7·9, which embraces physiological conditions, the most abundant fraction

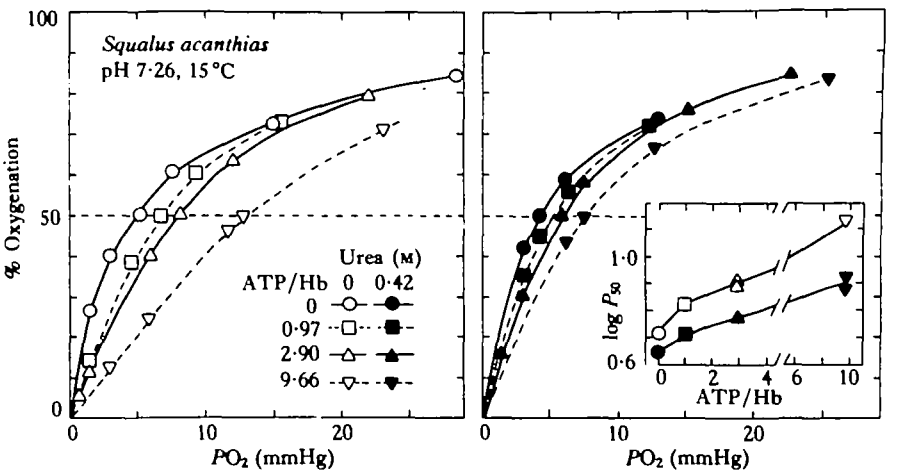

Fig. 5. Effects of urea on O_2 equilibria of the stripped haemolysate measured at 15 °C and pH 7·26 in the absence (open symbols) and presence (closed symbols) of 0·42 M-urea, in 0·1 M-Na-Hepes buffer. Haem concentration, 0·41 mM; ATP/Hb (mol/mol) ratios, 0 (○, ●), 0·97 (□, ■), 2·90 (△, ▲) and 9·66 (▽, ▼).

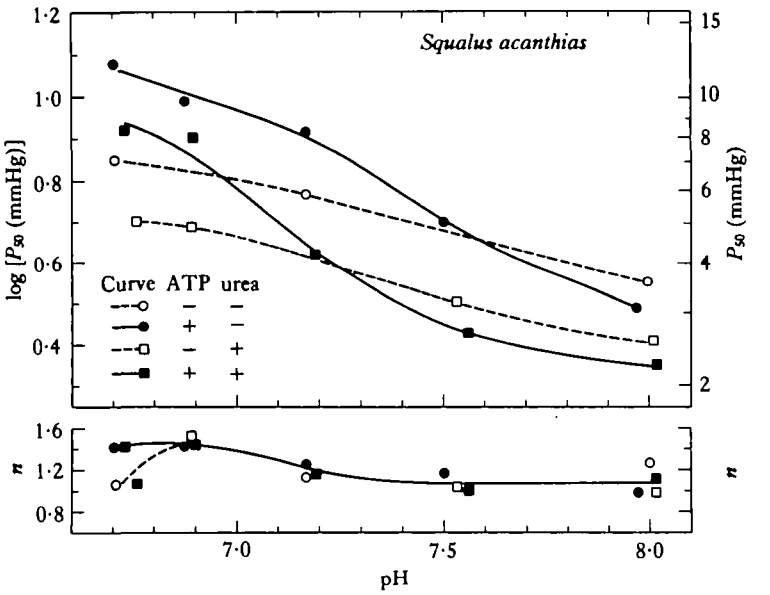

Fig. 6. Dependence of P_{50} and n_{50} of the stripped haemolysate on pH in the absence of added ligands (○) and in the presence of ATP (●), urea (□) and ATP + urea (■) measured at 15 °C in 0·1 M-Na-Hepes buffer. Haem concentration, 0·30 mM; urea (where present), 0·46 M; ATP/Hb (mol/mol) ratio, 1·51.

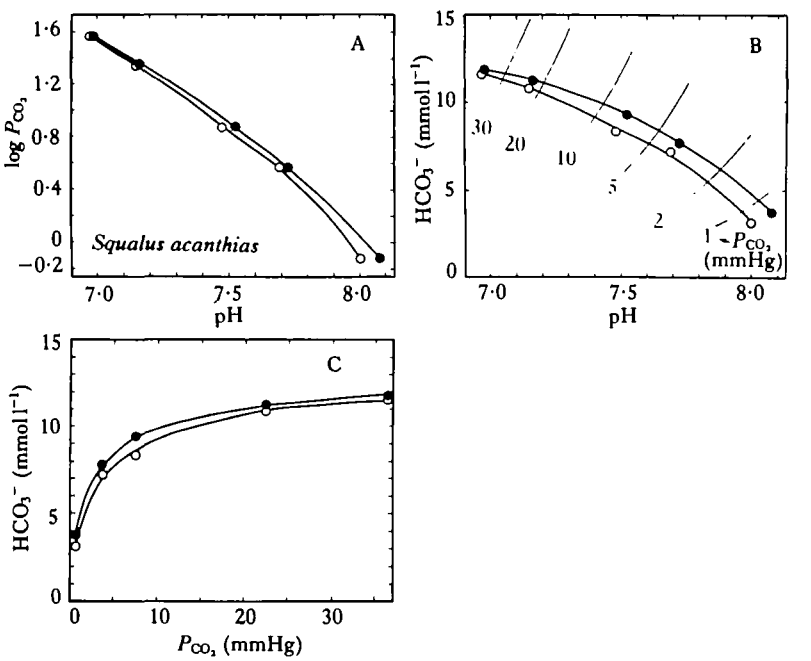

Fig. 7. Influence of haemoglobin oxygenation on (A) the P_{CO_2}-pH relationship, (B) the bicarbonate–pH relationship and (C) the bicarbonate–P_{CO_2} relationship, measured in a blood sample containing 0·93 mM-haem at 20 °C. ○, oxygenated Hb; ●, deoxygenated Hb.

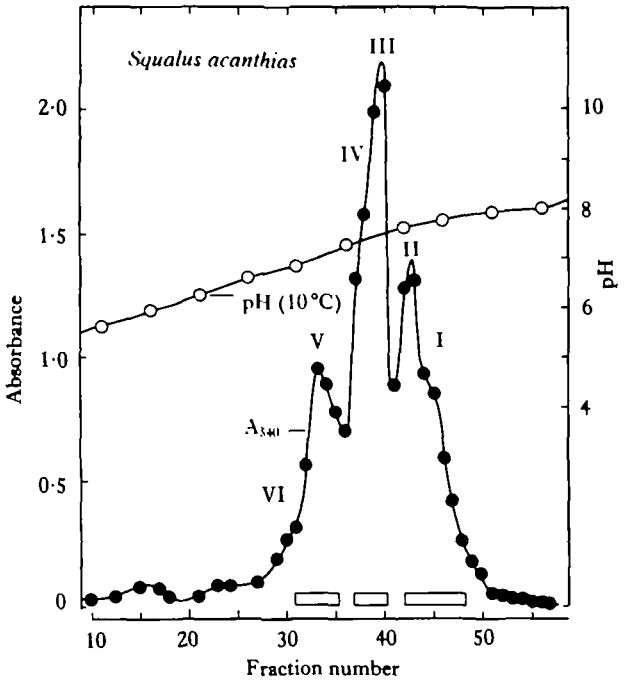

Fig. 8. Isolation of component Hbs by isoelectric focusing in solutions of ampholines (LKB, Sweden) with pH ranges of 5 to 8 (0·42 %) and 3·5 to 10 (0·28 %). Open horizontal bars, fractions pooled for O_2 equilibrium determinations (given in Fig. 1).

(III + IV) had a similar O_2 affinity to the haemolysate but a slightly larger Bohr effect ($\phi = -0{\cdot}35$), whereas fractions I + II and V + VI had smaller pH effects (ϕ near $-0{\cdot}18$) and higher O_2 affinities. Again, ATP had a lesser effect on O_2 affinity of fractions I + II and V + VI than on those of the main fraction (III + IV) or the whole haemolysate. The closeness of the isoelectric points and the resultant difficulty in completely separating the main fraction (III + IV) from the smaller adjacent ones (cf. Fig. 8) suggest that the above differences will be more pronounced in pure, isolated components.

DISCUSSION

The high O_2 affinity ($P_{50} = 2{\cdot}3$ at pH 7·85 and 10 to 15 °C), low cooperativity ($n = 1{\cdot}05$) and small Bohr factor ($\phi = -0{\cdot}20$) of stripped *Squalus acanthias* Hb conform broadly with corresponding earlier findings for other elasmobranchs (Manwell, 1963; Pennelly, Noble & Riggs, 1975; Mumm, Atha & Riggs, 1978; Martin *et al.* 1979). The available information thus suggests that homotropic and heterotropic intramolecular interactions will only play a modest role in adapting O_2 transport by haemoglobin in sharks, skates and rays to environmental and metabolic changes.

Unlike in teleost fish, where the alkaline Bohr effect disappears at high and low pH, that of *Squalus acanthias* is manifest over an extremely wide pH range (6·3 to 8·9), reflecting the implication of several acid groups with widely different pK values. Th

was also evident in stingray, *Dasyatis*, Hb (Mumm *et al.* 1978). The higher Bohr factor seen in the whole blood ($\phi = -0{\cdot}28$, Wells & Weber, 1983) is consistent with the allosteric interaction of anionic erythrocytic phosphates, which raise ϕ by increased binding to Hb at low pH.

The rise in the Bohr effect as pH decreases from 8 to 7 (Fig. 1) correlates with the concomitant decrease in the temperature effect ($\Delta H = -44$ and $-36\,KJ\,mol^{-1}$ at pH 8 and 7 – cf. Fig. 2), suggesting that the latter phenomenon results from endothermic protonation reactions that subtract from the exothermic oxygenation reaction proper. The overall heats of oxygenation fall within the range for Hbs from other vertebrate classes (Rossi-Fanelli, Antonini & Caputo, 1964) but not for the Hb from the porbeagle shark, *Lamna nasus*, where a ΔH value near zero reflects the occurrence of two types of functional subunits with different sensitivities within the same tetramers (Andersen, Olson & Gibson, 1973).

The pronounced sensitivity of the O_2 affinity of *S. acanthias* Hb to ATP and GTP contrasts sharply with ATP insensitivities observed in the stingrays, *Dasyatis* and *Potamogon*, and the torpedo ray, *Torpedo nobiliana*, (Mumm *et al.* 1978; Martin *et al.* 1979; Bonaventura, Bonaventura & Sullivan, 1974*a*,*b*), but accords with findings for the Japanese shark *Triakis scyllia* (Kono & Hashimoto, 1977). The available information thus suggests that ATP sensitivity may differ between the selachian (sharks) and batoidean (skates and rays) elasmobranchs.

The greater sensitivity of O_2 affinity of *Squalus* Hb to GTP than to ATP aligns the elasmobranchs with the teleost fish (cf. Weber *et al.* 1975; Petersen & Poluhowich, 1976). GTP may be formed by successive phosphate transfers from ATP to GMP, catalysed by guanosine monophosphokinase (GMPK) and nucleodiphosphokinase (NDPK) (cf. Weber, 1982). Both these enzymes occur in *Squalus acanthias* erythrocytes (0·05 and 25 units/ml cells, respectively, Parks *et al.* 1973). Such ATP to GTP conversion would increase the modulator effectivity of the erythrocytic NTP, and may moreover account for the remarkable intraspecific variability in ATP/GTP ratio encountered in some species (e.g. the smooth dogfish, *Mustelus canis*, Borgese *et al.* 1978). Studies on the kinetic rates of ligand reactions in the Hb of the stingray *Potamotrygon* (Martin *et al.* 1979) show that ATP decreases O_2 affinity by raising the rate of O_2 dissociation without affecting that of ligand association, while proton binding (the Bohr effect) affects both rates. This picture is complicated by an observation of kinetic heterogeneity in Hb from the carpet shark, *Cephaloscyllium*, in the absence of phosphate cofactors. This appears to be a point of difference between elasmobranch and teleost haemoglobins (Brittain, Barber, Greenwood & Wells, 1982).

Urea markedly increases the O_2 affinity of *Squalus acanthias* Hb, as observed in Hbs of man (Rossi-Fanelli *et al.* 1964) and the toad, *Xenopus laevis*, where urea levels rise in response to salt-water acclimation and aestivation (Jokumsen & Weber, 1980). In contrast, Hb-O_2 affinity is virtually independent of urea concentration in the clearnose skate, *Raja*, the Amazonian stingray, *Potamotrygon*, (Bonaventura *et al.* 1974*a*,*b*; Martin *et al.* 1979) and in the African lungfish, *Protopterus*, which experiences high urea concentrations during aestivation (Weber, Johansen, Lykkeboe & Maloiy, 1977). Within elasmobranchs these data thus show a dichotomy which coincides with the selachian-batoidean differentiation.

Kinetic carbon monoxide binding studies with *Raja* Hb indicate that urea insensitivity is dependent on the integrity of the tetrameric molecular structure of the Hb, whereas the urea-sensitive, human Hb dimerizes extensively in the presence of urea (Bonaventura *et al.* 1974*a*,*b*). In this light the large urea sensitivity of *Squalus* Hb correlates with evidence that 94% of non-oxidized pigment in solution consists of dimers, which aggregate to tetramers upon deoxygenation (Fyhn & Sullivan, 1975). That molecular dissociation alone will not account for the urea-induced increase in O_2-affinity, however, follows from the persistence of the ATP effect in the presence of urea, since ATP modulation, in analogy with mammalian Hb and DPG, is dependent upon the integrity of the tetrameric structure.

Urea decreases the effect of ATP on O_2 affinity (Figs 5, 6). This suggests that *in vitro* oxygenation studies conducted in the absence of urea overestimate the modulator role of the phosphate in elasmobranchs. Urea, however, does not obliterate NTP sensitivity completely (see Fig. 5). Hypoxic incubation of intact *S. acanthias* erythrocytes decreases cellular NTP and increases O_2-affinity (Wells & Weber, 1983).

The effect of urea on the oxygen equilibria may be due to oxygenation linked binding of this cationic compound to the negatively charged carboxyl termini of the polypeptide chains of Hb. Binding to Hb and other intracellular proteins at these sites is consistent with the greater effect of urea on O_2 affinity at high pH (Fig. 4), where the carboxyl groups will show greater ionization. Such binding may explain the higher urea concentrations in erythrocytes than in plasma (Krichevskaya *et al.* 1973; Browning, 1978) since urea diffuses freely through the erythrocyte membrane (Hunter, 1976). Urea binding at the carboxyl groups might thus increase affinity by hindering the oxygenation-linked binding at these sites of the Bohr protons which have a negative effect on O_2-affinity (removal of the C-terminal histidines of the β chains of human Hb decreases its Bohr effect by half – Kilmartin & Wootton, 1970). Urea may also affect O_2 affinity of Hbs due to its spontaneous conversion to cyanate, resulting in the slow, irreversible carbamylation of the NH_2-terminal valine residues of the Hb protein chains (Cerami *et al.* 1973; Bonaventura *et al.* 1974*a*,*b*). Carbamylation of these α-amino N-termini would explain the urea induced reduction of the ATP-effect (since those of the β-chains contribute two of the seven positively charged sites where DPG interacts in human Hb – cf. Arnone, 1972). The irreversibility of the carbamylation reaction and the fact that the amino terminal residue of stingray Hb is 'uncarbamylated' valine (Mumm *et al.* 1978), however, appear to be evidence against the implication of this reaction in the urea effect.

In whole blood, the Hb of *Squalus acanthias* shows a distinct Haldane effect. Curiously, Lenfant & Johansen (1966) found no such effect in the Pacific *S. suckleyi* (which now is considered to be *S. acanthias*). A Haldane effect is also lacking in the dogfish, *Scyliorhinus stellaris* and *Mustelus mustelus*, and in the electric ray, *Torpedo ocellata* (Albers & Pleschka, 1967). These data predict the absence of a Bohr effect in the latter three species (as recorded for *S. suckleyi*, Lenfant & Johansen, 1966). Since the Haldane effect is a thermodynamic consequence of the Bohr effect the presence of both effects in the Hb of *S. acanthias* appears to be internally consistent. The steepness of the $(HCO_3^-)/P_{CO_2}$ curve at low, physiological CO_2 tensions suggests transport of significant blood CO_2 as bicarbonate. Our values for the log P_{CO_2}/pH relationsh

■−1·9 to −2·1) and buffering capacity (10–12 mM-HCO_3^-/pH unit at pH 7·85 – Fig. 6A, B) agree well with those in other elasmobranchs (Albers & Pleschka, 1967).

We find at least six Hb components in *Squalus acanthias* compared to previous electrophoretic studies showing one and two components (Buhler & Shanks, 1959; Manwell, 1963). Although the shark haemolysate does not display such striking functional heterogeneity as teleosts with cathodal Hbs, there is evidence for some degree of molecular 'division of labour' – the main components showing higher heterotropic interactions (associated with proton and ATP binding) than the other components, with the composite haemolysate having intermediate properties (reflecting the absence of hybridization). The absence of radical functional heterogeneity and of large heterotropic interactions (see Results) in elasmobranch Hb may contribute to the low tolerance of elasmobranchs to variation in water O_2 tension, and to their low capacity for maintaining constant O_2 uptake rates as O_2 tension falls (cf. Piiper, Baumgarten & Meyer, 1970).

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