J. exp. Biol. (1980), 86, 19–37
With 10 figures
Printed in Great Britain

HAEMOGLOBIN-OXYGEN BINDING PROPERTIES IN THE BLOOD OF *XENOPUS LAEVIS*, WITH SPECIAL REFERENCE TO THE INFLUENCES OF AESTIVATION AND OF TEMPERATURE AND SALINITY ACCLIMATION

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(*Received* 11 *August* 1979)

SUMMARY

The oxygen equilibrium properties of blood and of solutions of haemoglobin from *Xenopus laevis* are reported. At pH 7·6 the oxygen affinity of the blood, expressed as half saturation oxygen tension (P_{50}) amounts to 27·0 mm and 13·7 mmHg (3·60 and 1·83 kPa) when measured at 25 and 10 °C, while the Bohr factor ($\Delta\log P_{50}/\Delta pH$) was $-0{\cdot}40$, and the Hill's cooperativity coefficient, n, averaged 2·1. These data reflect an overall heat of oxygenation, ΔH, of $-7{\cdot}9$ kcal.mol^{-1}, which decreased to $-6{\cdot}3$ kcal.mol^{-1} when the live animals were acclimated to each measuring temperature. *Xenopus* blood showed a high O_2 capacity (15 vol.%) compared to that of other amphibians.

Acclimation to water of increased salinity (12‰), and aestivation, raised blood O_2 affinity; at 25 °C and pH 7·6, P_{50} decreased to 21·1 and 25·2 mmHg (2·81 and 3·36 kPa), respectively. These changes were concomitant with increases in the blood levels of urea. In contrast to NaCl and ATP, urea increased O_2 affinity of the purified haemoglobin, suggesting that oxygenation-linked binding to haemoglobin is involved in the modulations of the blood O_2 affinity during aestivation and acclimation to salt water.

Xenopus haemoglobin consists of two components. The major component is electrophoretically anodal, and has O_2 binding properties similar to those of the haemolysate; the minor component is cathodal, and shows extremely low P_{50}, pH sensitivity and cooperativity.

INTRODUCTION

Xenopus laevis is neither a frog nor a toad, but is one of the ancient Aglossans (tongueless anurans). *Xenopus* is completely aquatic and lives in ponds and small lakes at physical conditions (i.e. temperature, CO_2 and O_2 tensions and dissolved solutes) that vary widely both diurnally and seasonally. During droughts *Xenopus* may escape dehydration and death by burrowing into the substrate and entering aestivation until the rainy season returns (Deuchar, 1975).

Xenopus, unlike other amphibians, does not change from ammonotelism to ureotelism during metamorphosis, which accords with its purely aquatic habitat (Cragg,

Balinsky & Baldwin, 1961). However, adults become increasingly ureotelic under arid conditions or on exposure to saline water (Balinsky, Cragg & Baldwin, 1961; Ireland, 1973; Funkhouser & Goldstein, 1973; Romspert, 1976; Hillman, 1978; McBean & Goldstein, 1970). During aestivation high concentrations of urea accumulate in the blood of *Xenopus* (Balinsky *et al.* 1967) as is the case with dormant spadefoot toads, *Scaphiopus* (Shoemaker, McClannahan & Ruibal, 1969) and with aestivating lungfish (Jansens & Cohen, 1968).

Xenopus is exposed to wide seasonal temperature variations. The effects of temperature acclimation on aerobic metabolism in poikilothermic vertebrates are well known, but only a few investigations on the effect of temperature acclimation on O_2-binding properties of the blood have been carried out on amphibians. Oxygen equilibrium curves of blood from cold-acclimated (15 °C) *Rana esculenta* are shifted slightly to the right of those of warm-acclimated (25 °C) specimens, when measured at either temperature (Gahlenbeck & Bartels, 1968). In a fish and a reptile, a similar change in O_2 affinity following temperature acclimation has been ascribed to changes in the concentrations of intra-erythrocytic cofactors (Grigg, 1969, 1972; Wood *et al.* 1978).

This paper reports on O_2 affinity of blood from *Xenopus laevis*, and concerns the possible adaptive changes in the blood respiratory properties during aestivation and acclimation to altered salinity and temperature. Mechanisms responsible for the changes in the O_2 binding properties of the blood were also studied in experiments on the influences of temperature, pH, urea, ATP, CO_2 and inorganic ions on the oxygenation properties of the stripped (cofactor-free) haemoglobin in solution.

MATERIALS AND METHODS

Animals

Specimens of *Xenopus laevis*, of both sexes and of various size (most weighing 50–100 g), were obtained from a South African animal supplier (Thomas Cook, Johannesburg). The animals were kept at 25 °C, in groups of ten, in 60 l plastic containers with circulating, aerated fresh water for at least one month before experiments. They were fed chopped liver weekly.

Acclimation

Acclimation to water of increased salinity. Animals were first transferred to 2‰ salt water (Tropic Marine Export, Fulda) at 25 °C. Changing the water weekly, the salinity was then increased in 0·5–1 ‰ increments to 12 ‰ over a 3-month period. Animals were acclimated to this salinity for 2 months before measurements were performed.

Cold acclimation. Animals that were pre-conditioned to 25 °C were used. Their water was changed weekly while the temperature was lowered 2 °C each time until 10 °C was attained. At 10 °C cold acclimation was continued for an additional two month period.

Aestivation. Aestivation was induced by placing animals in glass cylinders (60 cm high, 20 cm diameter) half filled with mud and containing about 10 cm supernatant water. An infra-red lamp placed about 50 cm above the cylinders caused gradual

increase in dehydration. The animals burrowed until they were completely covered with mud. In preliminary experiments the animals died when the mud became dry and hard. The mud was therefore kept moist during aestivation. The data reported here are based on results from two specimens that survived for two months in the aestivating condition.

Blood sampling

Blood was sampled into heparinized syringes from the ventricle after exposing the heart. Sampling was carried out as rapidly as possible (within 5 min) to minimize acidification due to handling stress. With rapid sampling the lactate concentration was less than 5 mM.

Analysis of blood samples

Immediately following sampling the blood samples were subdivided and subjected to the following procedures. *Blood pH* was measured in duplicate by means of Radiometer equipment BMS-2 MK-2 connected to a PHM 64 using precision buffers for calibration. *Haematocrit* values were determined in duplicate by centrifugation in glass capillaries (3 min at 12000 rev/min). Plasma and blood samples were frozen at −18 °C for *urea* assay. Proteins were precipitated (as described below) in preparation for determination of *blood lactate* and *red cell phosphates* and the protein-free preparations were frozen at −18 °C.

Haemoglobin contents in whole blood (quoted in Table 1) were calculated from O_2-capacity measurements, obtained using a Tucker chamber (below). In experiments on haemolysates, the concentrations were determined spectrophotometrically with a Unicam SP 1800 spectrophotometer after diluting 10 μl samples in 1 ml water. Pigment concentrations were calculated from the millimolar extinction coefficients for oxygenated Hb of 14·6 and 13·8 at 577 and 541 nm, respectively (Antonini & Brunori, 1971).

Nucleoside triphosphate (NTP) concentrations were assayed by thin layer chromatography (Johansen *et al.* 1976). Prior to measurement 50 μl blood samples were deproteinized by addition of an equal volume of 12% trichloracetic acid (TCA). Concentrations inside the erythrocytes were calculated from the haematocrit values assuming that all NTP is erythrocytic.

Lactate was measured in 100 μl blood samples deproteinized with 200 μl 0·6 N perchloric acid (PCA) according to the lactate dehydrogenase enzymic method (Boehringer–Mannheim).

Urea concentrations in plasma and blood were determined by two methods: (*a*) the Barthelot's urease method (Boehringer), reading optical densities at 546 nm (this results in interference from haemoglobin and the method was only used in initial determinations on the separated plasma), and (*b*) the urease GIDH method (Boehringer). This method rests on O.D. measurements at 340 nm and was used with plasma and whole blood. Erythrocytic concentrations (C_e) of urea were calculated from blood and plasma levels (C_b and C_p, respectively) and haematocrit (Hct) using the formula (Weber & Lykkeboe, 1978):

$$C_e = \left[C_b - \left(C_p \times \frac{100\text{-Hct}}{100}\right) \times \frac{100}{\text{Hct}}\right].$$

Chloride concentrations in whole blood and solutions were measured by titration with a Radiometer CMT 10 chloride titrator.

The *osmotic concentrations* in blood, plasma and acclimation water were determined with a Knauer semi-micro osmometer, type M.

Intra-erythrocytic pH was measured in 70 μl blood samples which had been equilibrated for 20 min in a Radiometer BMS-2 MK-2 with mixtures of CO_2 (1–5%) and atmospheric air delivered by Wösthoff gas mixing pumps. After blood pH measurement, the samples were returned from the pH electrode into the tonometers. The blood was then layered below paraffin oil contained in 1·5 ml Eppendorf tubes using a haematocrit suction adapter and capillary tubes that had been rinsed in the equilibration gas mixture. The plasma and red cells were subsequently separated in an Eppendorf 3200 microcentrifuge, and pH values in the plasma (drawn from under the paraffin oil into the BMS pH electrode) were measured. The pH values of blood and of plasma were not significantly different. After removal of the plasma the red blood cells were lysed by three times freezing (immersing the tubes in alcohol at −18 °C) and thawing, and the cellular pH was then measured.

Whole blood oxygen equilibria were measured by equilibrating 50 μl samples for 20 min periods to different gas tensions and measuring O_2 content using a Tucker chamber (Tucker, 1967) modified after Bridges *et al.* (1979). O_2 capacity (i.e. 100% saturation) was measured in samples equilibrated with a gas mixture having a p_{O_2} of 650 mmHg. Haemoglobin-bound oxygen was obtained by subtraction of dissolved oxygen calculated from the Bunsen O_2 solubility coefficients (Christoforides & Hedley-Whyte, 1969).

Preparation of haemoglobin solutions. Red cells were washed three times in 0·9% NaCl and lysed in thrice their volume of 0·01 M Tris HCl buffer at pH 8. The haemoglobin was stripped of cofactors and other ions, firstly by passage through mixed ion exchange resin (Amberlite MB-3) and subsequently by chromatography on a 30 cm column of Sephadex G-25 superfine gel eluting with 0·05 M Tris buffer pH 7·5 containing 0·1 M-NaCl (Berman, Benesch & Benesch, 1971). Curiously, the haemoglobin denatured when Sephadex stripping preceded the MB-3 treatment, and the absorption peak near 577 nm decreased strongly with CO, so CO treatment was avoided. The haemolysate was dialysed against four changes of 0·01 M Tris buffer pH 7·5, containing 5×10^{-4} M EDTA for 48 h. The haemoglobin solutions were concentrated on Amicon B15 concentrators (Oosterhout, Holland) to a tetrameric concentration of about 0·4 mM. (Control experiments showed that O_2 affinity of the stripped haemoglobin was virtually independent of concentration in the range of 0·15–0·7 mM tetramer.)

To determine heterogeneity of the haemoglobin and to isolate the component haemoglobins, preparative isoelectric focussing was carried out at +4 °C in 110 ml columns using ampholytes of pH ranges 5–8 (0·6%) and 3–10 (0·2%) (LKB, Sweden).

All preparative procedures were carried out at 0–5 °C.

Oxygen equilibria of haemoglobin solutions were measured at 436 nm using a modified gas diffusion chamber technique (Sick & Gersonde, 1969; Weber, Lykkeboe & Johansen, 1976). When measuring the effect of CO_2 on O_2 affinity, separate samples of the haemoglobin solutions were equilibrated to the same gas mixtures in a BMS-2 for pH measurement. The effects of ATP and urea were investigated by addition of

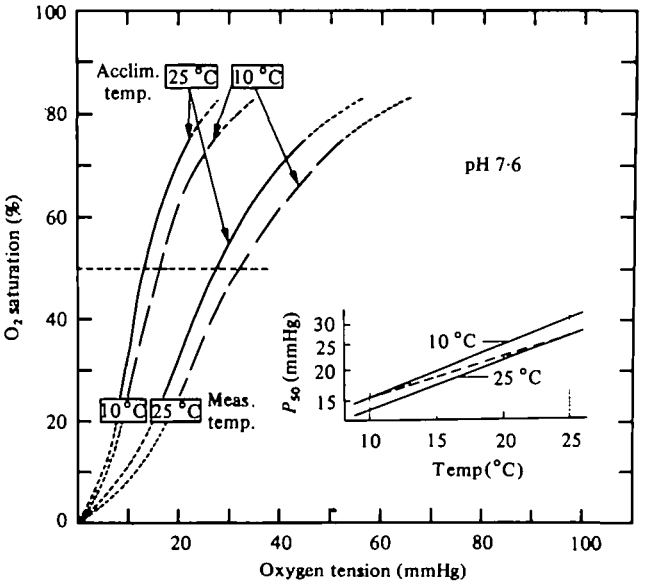

Fig. 1. Oxygen equilibrium curves of *Xenopus laevis* acclimated to 10 (dashed curve) and 25 °C (continuous curve). Inset: the influence of temperature on P_{50} of *Xenopus* blood, interpolated from Fig. 2. Dotted line indicates the temperature sensitivity in blood of acclimated animals.

stock solutions assayed with Boehringer test chemicals. The urea solutions used were first deionized with mixed ion-exchange resin. In view of the specific effect of chloride on oxygen affinity and its interactions with organic phosphates, care was taken to keep the chloride concentration constant when studying effects of other parameters. The effect of the anion (Cl^-) and/or cations (Na^+, Mg^{2+}) were investigated by addition of Cl^- assayed stock solutions.

Values of P_{50} and n were obtained from calculated regressions of double logarithmic plots of (Oxy-Hb]/[Total Hb] versus p_{O_2} (Hill plots). Due to heterophasic cooperativity in O_2 binding by *Xenopus laevis* Hb the regressions were calculated using only values between 25–75 % saturation, where no change in Hill coefficient, n, was evident. Equilibrium curves were constructed from the Hill equation:

$$\log (y/(1-y)) = n \log p_{O_2} + a,$$

where $a = -n \log P_{50}$ if the cooperativity coefficient is constant (above).

RESULTS

Oxygen equilibrium curves of blood of *Xenopus* acclimated to 10 °C and 25 °C and measured at both temperatures are given in Fig. 1. For 25 °C acclimated animals the P_{50} values at pH 7·6 are 27·0 and 13·7 mmHg (3·60 and 1·83 kPa) at 25 and 10 °C, respectively. Oxygen capacity averaged about 15 vol%, which is unusually high compared to other amphibians (Wood *et al.* 1975).

It is seen that acclimation to lower temperature (10 °C) decreases O_2 affinity; P_{50} values increased to 31·3 and 15·6 mm (4·18 and 2·08 kPa) at 25 and 10 °C. It follows

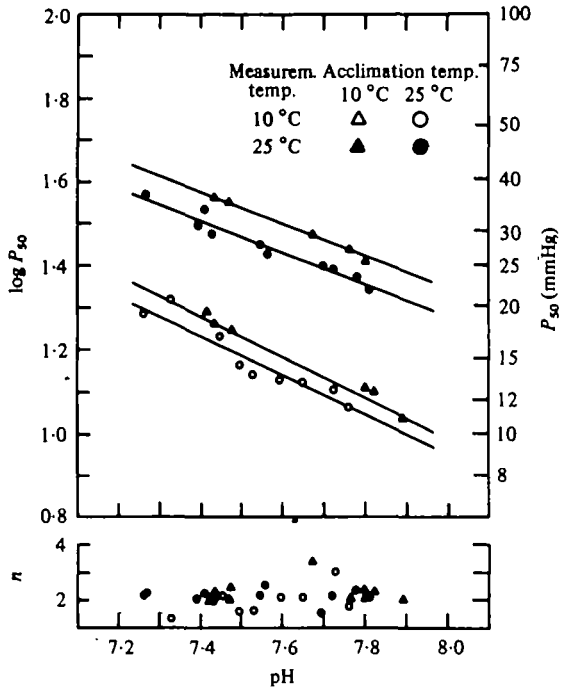

Fig. 2. Values of P_{50} and Hill's coefficient, n, and their pH dependence measured at 10 (open symbols) and 25 °C (solid symbols) of blood of *Xenopus* acclimated to 10 (triangles) and 25 °C (circles). Regression equations (calculated by the method of least squares) are: *10 °C acclimation:* $\log P_{50} = -0{\cdot}47\ \mathrm{pH} + 4{\cdot}77$ ($r = -0{\cdot}98$) (△); $\log P_{50} = -0{\cdot}39\ \mathrm{pH} + 4{\cdot}46$ ($r = -0{\cdot}99$) (▲); *25 °C acclimation:* $\log P_{50} = -0{\cdot}38\ \mathrm{pH} + 4{\cdot}32$ ($r = -0{\cdot}96$) (●); $\log P_{50} = -0{\cdot}47\ \mathrm{pH} + 4{\cdot}71$ ($r = -0{\cdot}92$) (○).

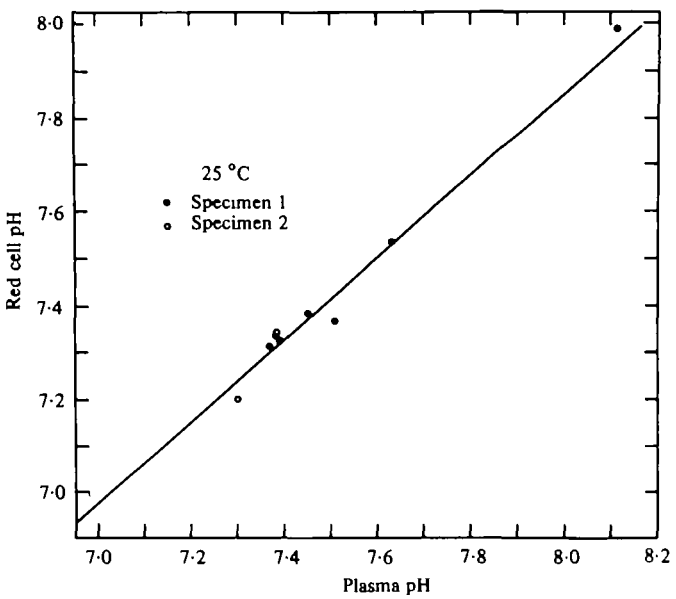

Fig. 3. Relationship between pH in plasma and red cells of *Xenopus* measured in two experiments (●, ○). Regression equation: $\mathrm{pH}_{\text{red cells}} = 0{\cdot}88\mathrm{pH}_{\text{plasma}} + 0{\cdot}86$ ($r = +0{\cdot}98$).

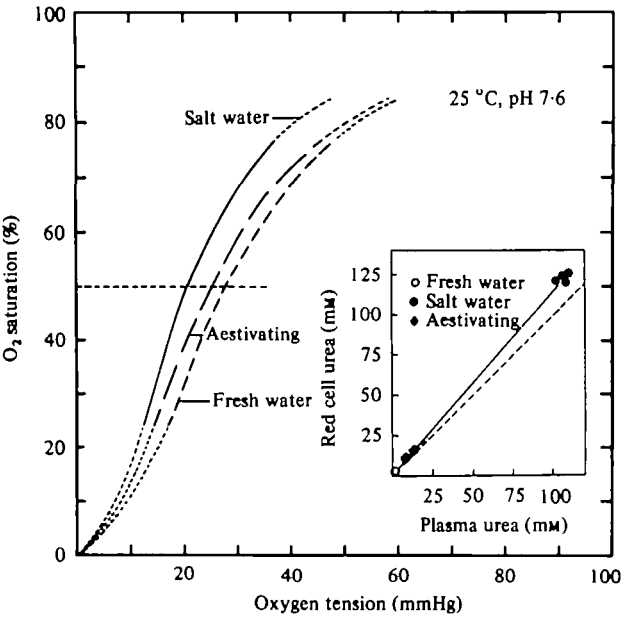

Fig. 4. O_2 equilibrium curves at 25 °C of blood from aestivating *Xenopus* (dashed curve) and from specimens acclimated to 12°/oo salt water (continuous curve) compared to that in fresh water specimens (dotted curve). (*Inset*) Relationship between urea concentration in plasma and red cells of active *Xenopus* (○), aestivating (◆) and salt-water-acclimated specimens (●). Dotted line indicates isocline. Regression equation: $[\text{Urea}]_{\text{red cells}} = 1{\cdot}14\ [\text{Urea}]_{\text{plasma}} - 0{\cdot}002$ ($r = +0{\cdot}999$).

that the effect of temperature is greater in animals subjected to acute temperature changes than in those acclimated to different environmental temperatures. The Bohr factors were −0·39 and −0·47 when measured at 25 and 10 °C, respectively (Fig. 2).

Interpolation from Fig. 2 at pH 7·6 shows that O_2 affinity of *Xenopus* blood has low temperature sensitivity; the ΔH values, calculated from the Van't Hoff equation as $-2{\cdot}303 . R . \Delta\log P_{50}/\Delta T^{-1}$, where T is the absolute temperature and R is the gas constant (Wyman, 1964), were −7·8 and −8·0 kcal.mol^{-1} for warm and cold-acclimated animals, respectively. In 10 °C animals, blood pH and red cell urea concentrations were significantly higher than those at 25 °C (Table 1). Intraerythrocytic pH values were less than 0·2 units lower than those in plasma (Fig. 3).

Salt water acclimation as well as aestivation resulted in higher O_2 affinities than in active, freshwater specimens (Fig. 4, 5). At pH 7·6 P_{50} values were 21·1 and 25·2 mm-Hg, (2·81 and 3·36 kPa) respectively. Also, salt water acclimation and aestivation significantly raised the levels of urea in red cells, blood and plasma (Table 1 and Fig. 4)

Higher salinity and aestivation lowered the haemotocrit values. In salt water, blood chloride increased about 20% (Table 1), while body weights decreased by 20–25% during aestivation and salinity acclimation. As expected, the blood and plasma osmotic concentrations were essentially identical (equivalent to 12·6 and 12·4 g/l NaCl, respectively), whereas the Cl^- levels were about 16% higher in the plasma than in whole blood (equivalent to 6·6 and 5·7 g/l NaCl, respectively) Non-chloride compounds were therefore correspondingly higher in blood and in the cells than in plasma (equivalent to 6·9 and 5·8 g/l NaCl, respectively).

Table 1. *Hematological parameters*

(Means ± S.D. measured in the blood (B), plasma (P) and red blood cells (RBC) of *Xenopus laevis* acclimated to fresh water at 25 and 10 °C and to saline water, and in aestivation. Numbers in parentheses = number of measurements when not performed on all specimens.)

	Fresh water, 25 °C (*A*)	Saline water 25 °C (*B*)	Fresh water 10 °C (*C*)	Aestivating (*D*)	Sign* of difference between			
					A−*B*	*A*−*C*	*A*−*D*	*B*−*D*
N	35	4	3	2	—	—	—	—
Hct (%)	38.7 ± 6.4	31.8 ± 3.5	37.7 ± 3.2	32.0 ± 7.1	0.005	N.S.	N.S.	N.S.
Hb (mM.l^{-1} (B))	1.76 ± 0.42 (14)	1.57 ± 0.18	1.89 ± 0.2	1.53 ± 0.2	N.S.	N.S.	N.S.	N.S.
ATP (mM.l^{-1} (B))	0.62 ± 0.27 (21)	0.59 ± 0.11	0.53 ± 0.09	0.55 ± 0.20	N.S.	N.S.	N.S.	N.S.
ATP (mM.l^{-1} (RBC))	1.59 ± 0.69 (21)	1.84 ± 0.14	1.43 ± 0.33	1.69 ± 0.52	N.S.	N.S.	N.S.	N.S.
ATP:Hb	0.35 ± 0.12 (14)	0.38 ± 0.07	0.29 ± 0.07	0.36 ± 0.08	N.S.	N.S.	N.S.	N.S.
Cl^- (m-equiv.l^{-1} (B))	74.1 ± 9.1 (12)	88.5 ± 5.45	—	—	< 0.005	—	—	—
Urea (mM.l^{-1} (P))	1.88 ± 0.88 (18)†	109.14 ± 3.13	1.39 ± 1.43	10.69 ± 2.87	< 0.0005	N.S.	< 0.0005	< 0.0005
Urea (mM.l^{-1} (B))	1.84 ± 1.39 (8)	113.42 ± 2.43	2.11 ± 1.26	11.76 ± 2.69	< 0.0005	N.S.	< 0.0005	< 0.0005
Urea (mM.l^{-1}) (RBC))	1.09 ± 0.75 (6)	124.02 ± 1.94	3.28 ± 1.65	14.23 ± 1.50	< 0.005	< 0.05	< 0.0005	< 0.0005
O_2-cap (vol %)	15.80 ± 3.78 (14)	14.09 ± 1.61	16.94 ± 2.38	13.75 ± 1.87	N.S.	N.S.	N.S.	N.S.
pH (B)‡	7.516 ± 0.134 (21)	7.533 ± 0.015 (3)	7.875 ± 0.08	7.667 ± 0.235	N.S.	< 0.001	N.S.	N.S.

* *P* values for paired tests (N.S. = not significant).

† Eleven determinations were done according to Barthelot's urease method and 7 samples were assayed using GIDH method (see Methods). As values were within the same range, they are pooled in the table.

‡ pH values were measured at 25 °C, except those of cold acclimated specimens, which were measured at 10 °C.

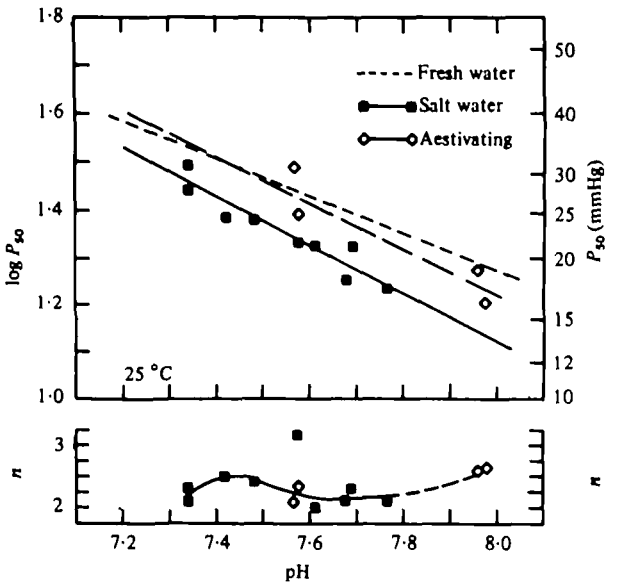

Fig. 5. Variation in P_{50} and n in blood from aestivating (◇), and 12 °/oo salt-water acclimated specimens (■) with pH, measured at 25 °C. Regression equations: $\log P_{50} = -0{\cdot}51\ \mathrm{pH} + 5{\cdot}20$ ($r = -0{\cdot}90$) (■); and $\log P_{50} = -0{\cdot}48\ \mathrm{pH} + 5{\cdot}05$ ($r = -0{\cdot}91$) (◇) for salt water and aestivating specimens, respectively. Dotted line, fresh water specimens (from Fig. 2).

There is no evidence for a significant effect of aestivation and salinity acclimation on blood haemoglobin concentrations (range, 1·5–1·9 mM tetramer.l^{-1} (B)) nor on the ATP levels in blood and red cells. The relationship between O_2 affinity and pH in stripped haemoglobin in the absence and presence of urea and NaCl, is shown in Fig. 6. At 10 and 25 °C the Bohr factor, $\phi = \Delta \log P_{50}/\Delta\mathrm{pH}$, was $-0{\cdot}37$ to $-0{\cdot}40$. The inverse relation between measurement temperature and O_2 affinity (Fig. 6) reflects a ΔH value of $-5{\cdot}6$ kcal.mol^{-1} for the stripped pigment. Urea has a strong positive effect on O_2 affinity at all pH values studied, while NaCl decreases O_2 affinity. Urea and NaCl, however, exert no significant effect on the cooperativity coefficient, n (Fig. 6).

With the view of investigating their possible roles in the acclimation, the effects of increasing concentrations of urea and chloride were studied (Fig. 7). Raising the urea concentration (Fig. 7*A*) from 0 to 500 mM decreases P_{50} from about 10 to 4·5 mm Hg (1·33–6·00 kPa) at pH 7·65. Cl^- decreases the O_2 affinity of *in vitro* haemoglobin preparations most strongly at low concentrations (Fig. 7*B*) as found in the red cells (cf. Table 1); at pH 7·6 an increase from 0·05 to 0·5 M raises P_{50} from about 10–21 mm (1·33–2·80 kPa). Equimolar concentrations of Cl^- added as NaCl and $MgCl_2$ respectively, resulted in similar P_{50} values, suggesting that the valency of the cation has no distinctive effect on O_2 affinity.

The organic phosphate, ATP, has a comparatively small effect on O_2 affinity; at pH 7·55, P_{50} is raised from 10·7 to only 13·4 mmHg (1·43–1·79 kPa) as the molar ratio of ATP to haemoglobin is increased from 0–3, while n increases slightly from 2·4–2·6 (Fig. 7*C*). Comparative data are available for few other amphibians. The fossorial apoda, *Boulengerula taitanus*, showed a similar low ATP sensitivity; at

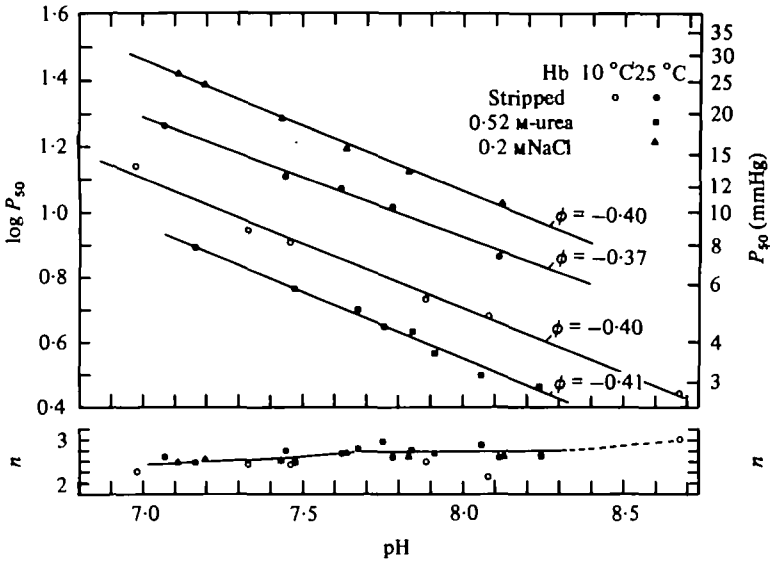

Fig. 6. P_{50} and n values and their pH dependence in stripped *Xenopus* haemoglobin in the absence (○, ●) and presence of 0·2 M-NaCl (▲) and 0·52 M urea (■), measured at 10 °C (○) and 25 °C (●, ▲, ■). Regression equations: *10 °C:* log P_{50} = −0·40 pH + 3·90 (r = −0·996): *25 °C, stripped:* log P_{50} = −0·37 pH + 3·88 (r = −0·99); *25 °C, 0·52 M urea:* log P_{50} = −0·41 pH + 3·83 (r = −0·99); *25 °C, 0·2 M-NaCl:* log P_{50} = −0·40 pH + 4·26 (r = −0·997). Tetrameric Hb concentration, 0·4 mM; Tris buffer I, 0·05.

pH 7·1, 50 times excess ATP to Hb increased P_{50} from 5·0–7·6 mmHg (0·67–1·01 kPa) (Wood *et al.* 1975). Hb of the anuran, *Rana esculenta*, lacks significant ATP sensitivity (R. E. Weber & A. Jokumsen, unpublished data). In the viviparous caecilian *Typhlonectes compressicauda* there is, however, a large ATP effect. At pH 7·1 a 1 mM concentration changed P_{50} from 1·9–5·6 mmHg (0·25–0·75 kPa) (Garlick *et al.* 1979).

The influence of CO_2 and ATP, singly and in combination, on O_2 affinity and cooperativity in *Xenopus* haemoglobin is shown in Fig. 8. A specific, pH-independent effect of CO_2 is demonstrated, decreasing O_2 affinity of the stripped haemoglobin. At p_{CO_2} levels near 7 and 30 mm (0·93 and 4·00 kPa), these effects were manifest only above pH 7·4 and 7·26 respectively. The specific CO_2 effect is markedly decreased in the presence of ATP. This indicates competition between ATP and CO_2 for common amino groups as in mammals and shows that ATP is bound by the haemoglobin despite its low oxygenation effects. It also suggests that the specific effect of CO_2 will have limited influence on the *in vivo* P_{50}. A CO_2 effect, however, persists in the presence of ATP when pH is high (above about 7·4) reflecting increased carbamate formation as protonation of the amino groups and competition from the anionic ATP decrease.

Isoelectric focusing resolves *Xenopus* haemoglobin in one minor and one major component (I and II in Fig. 9), which are isoelectric near pH 9·3 and 7·0, respectively, and account for about 3·9 and 96·1 % of total haemoglobin. The major component has approximately similar oxygen affinities and n values as the haemolysate (Fig. 10). ATP has the strongest effect on O_2 affinity at lower pH, as expected from a greater protonation of the binding groups on the molecule under these conditions. Remarkably, Hb I has a significantly higher oxygen affinity, lower cooperativity and a

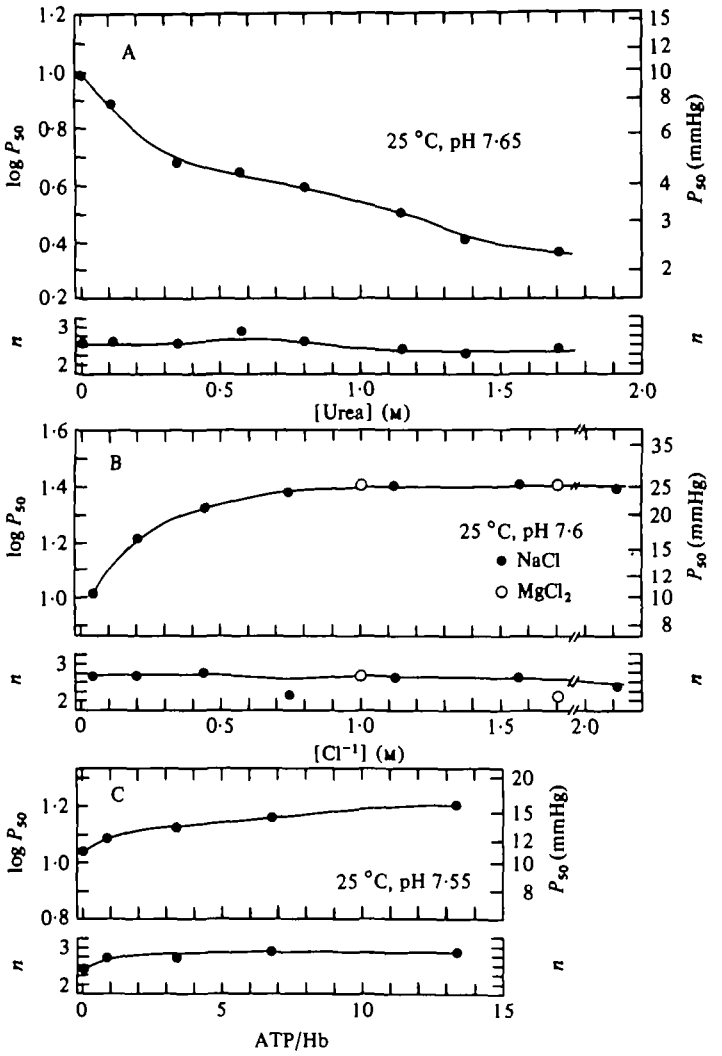

Fig. 7. (A) Effect of increasing urea concentrations on P_{50} and n values of *Xenopus* haemoglobin at 25 °C. pH, 7·65; Hb, 0·43 mM; Tris buffer I, 0·05. (B) Effects of NaCl and $MgCl_2$ on P_{50} and n values of *Xenopus* haemoglobin at 25 °C. pH, 7·6; Hb, 0·7 mM; Tris buffer I, 0·05. (C) Effects of ATP on P_{50} and n values of *Xenopus* haemoglobin at 25 °C, pH 7·55. Small corrections of P_{50} values (to pH 7·55) were done using Bohr factors estimated for the given ATP:Hb ratios from Figs. 6 and 8. Hb, 0·2 mM; I, 0·05.

smaller Bohr factor than Hb II; at pH 7·6, P_{50} and n are 4·3 mmHg (0·57 kPa) and 1·0, respectively, and $\phi = -0{\cdot}11$. Only a small part of this difference may be attributable to the lower concentration in Hb I (see p. 22).

DISCUSSION

The O_2 affinity (P_{50} = 27 mmHg or 3.60 kPa at 25 °C and pH 7·6) and the Bohr factor (ϕ, −0·39 to −0·47) of *Xenopus* blood are higher than those found in most

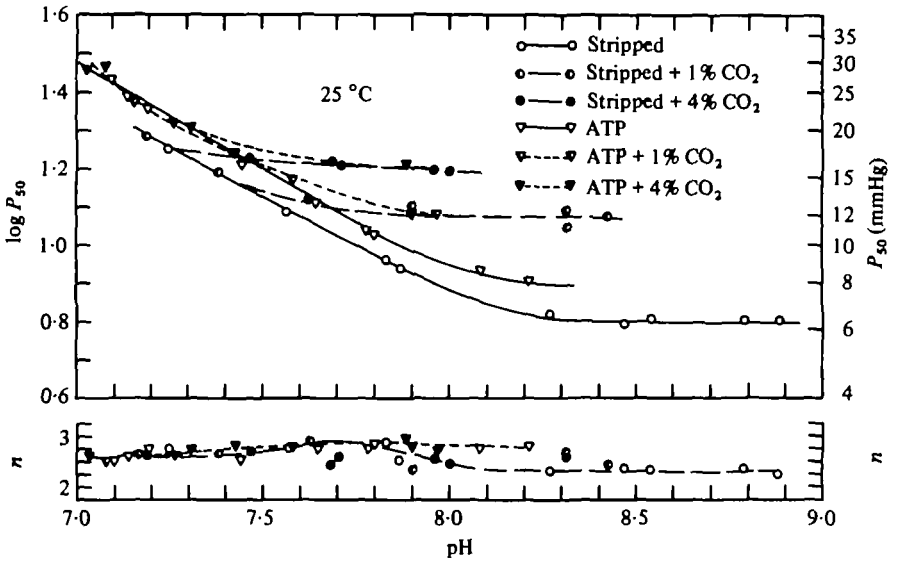

Fig. 8. Effects of ATP (triangles) on P_{50} and n values of stripped *Xenopus* haemoglobin (circles) in the absence of CO_2 (open symbols) and the presence of 1 % (~ 7·4 mm Hg) CO_2 (half-filled symbols) and 4 % (~ 30 mm Hg) CO_2 (solid symbols). Temperature, 25 °C; Hb, 0·37 mM; I, 0·05; ATP:Hb, 1·53.

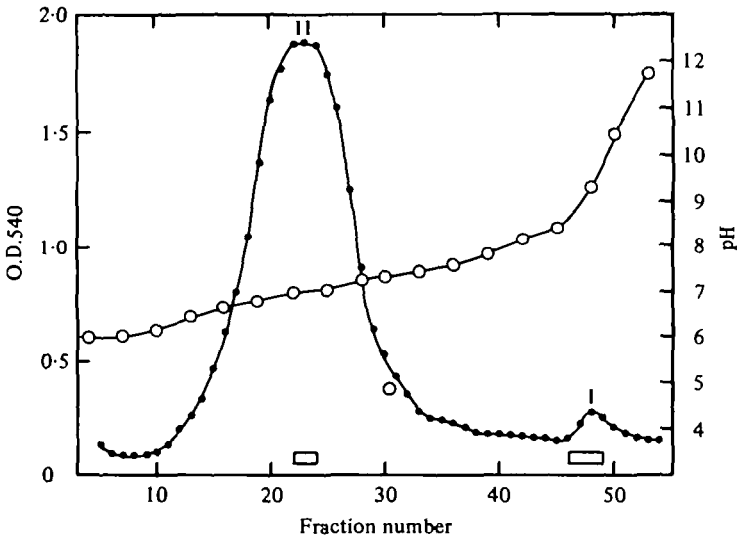

Fig. 9. Isolation of haemoglobin components (I and II) from *Xenopus* haemolysate using isoelectric focusing. ●, optical density at 540 nm; ○, pH at 10 °C; horizontal bars, fractions pooled for measurements of oxygen equilibria (cf. Fig. 10).

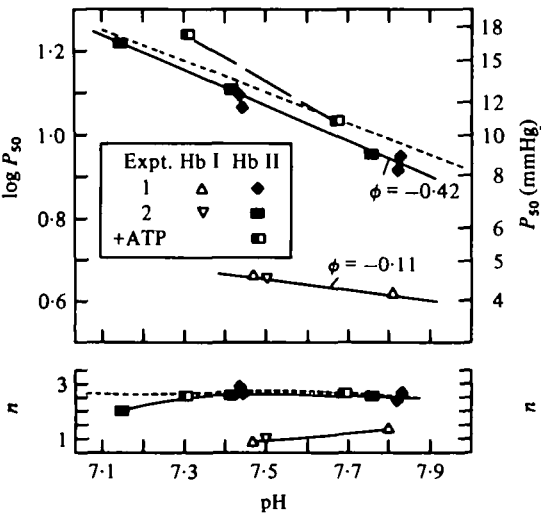

Fig. 10. Effects of pH on P_{50} and n of two haemoglobin components isolated separately from haemolysates of two *Xenopus* specimens measured at 25 °C; △, ▽, Hb I; ◆, ■, Hb II; ◧ Hb II (expt. 2) in presence of excess 13 times molar excess of ATP/Hb. Dotted line, haemolysate (from Fig. 6). Hb I, 0·05 mM; Hb II, 0·2 mM.

other amphibians, where P_{50} values range from 18–50 mm (2.4–6·67 kPa) and ϕ from −0·13 to −0·29; the O_2 affinity is particularly high compared to values for other anurans (cf. Wood *et al.* 1975). These properties may be adaptive to the aquatic existence. The high affinity will favour cutaneous O_2 uptake, whereas the high pH sensitivity and the specific CO_2 effect will facilitate O_2 unloading in the tissues as P_{CO_2} rises during diving (cf. Emilio & Shelton, 1974). A comparison of the Australian and South American lungfish shows a decrease in Bohr factor with increasing dependence on air as a respiratory medium (Lenfant, Johansen & Grigg, 1966/7; Johansen & Lenfant, 1967), whereas a similar relation between O_2 affinity and air dependence was found in a series of amphibians studied by Lenfant & Johansen (1967).

Our data show that the O_2 affinity of blood of *Xenopus* is subject to environmental modulation; O_2 affinity is increased following acclimation to high temperature and saline water, and during aestivation. ATP, the main allosteric modulator of blood O_2 affinity in lower vertebrates, however, does not seem to play a major role, in view of its small direct effect on the O_2 affinity of the haemoglobin and the small changes in its concentration. The latter, moreover, excludes a significant indirect effect of ATP via changes in intracellular pH coupled with its influence on the Donnan equilibrium of protons across the red cell membranes (Wood & Johansen, 1973).

The low values for the overall heat of oxygenation of the whole blood and the cofactor-free haemoglobin of *Xenopus* (ΔH, −7·9 and −5·6 kcal.mol^{-1}, respectively, compared to −10 to −14 in most other vertebrates) suggest relatively constant O_2 affinities in the face of varying external temperatures. In acclimated animals the temperature sensitivity is even lower than the acute *in vivo* effect; measured at the acclimation temperature, ΔH approximates −6·3 kcal.mol^{-1} (Fig. 1 inset; dashed

line). These results agree qualitatively with those found for *Rana esculenta* by Kirberger (1952), Straub (1957) and Gahlenbeck & Bartels (1968).

In air-breathing vertebrates with an efficient pulmonary gas exchange, the arterial blood will be essentially saturated with O_2, so a decrease in O_2 affinity is the only way in which tissue O_2 delivery can be enhanced through affinity modulation. A high temperature sensitivity will normally thus be favourable, enhancing O_2 unloading as O_2 demand increases at high temperature. Although *Xenopus* primarily relies on lung breathing, its skin plays an important role in gas exchange. Emilio & Shelton (1974) showed that it may account for 30% of O_2 uptake and 80% of CO_2 elimination, and Jones (1967) found that during diving O_2 uptake is 43 $ml.kg^{-1}.h^{-1}$, compared to pre- and post-dive rates of 63 and 91 $ml.kg^{-1}.h^{-1}$, respectively. The low temperature sensitivity of *Xenopus* haemoglobin may thus be adaptive in the sense that it safeguards O_2 loading in ponds where O_2 tension may be low and temperature high. This property may also favour O_2 uptake when similar conditions prevail in the microenvironment of the aestivating animals.

During air breathing, when pulmocutaneous vasodilation accompanies increased pulmonary blood flow (Shelton, 1970) the O_2 tensions approximate 80 mm in the systematic and 60 mmHg in the pulmocutaneous blood of *Xenopus* (Emilio & Shelton, 1974) providing evidence for the functional separation between blood flows through the single heart ventricle (De Graaf, 1957). However, pulmocutaneous blood is mixed due to a shunt of blood from the left to the right ventricle cavity (De Graff, 1957). During diving, however, when O_2 tensions fall in all parts of the circulation to about 45 mmHg (Emilio & Shelton, 1974), the above cardiovascular adjustments during lung ventilation are reversed (Shelton, 1970). The functional significance of this regulation of blood flow in conjunction with conveying mixed blood in the pulmocutaneous circulation may be that it allows for a gradual depletion of the lung O_2 stores during submersion or whenever *Xenopus* experiences hypoxic habitats.

Our data do not permit inferences on the mechanisms underlying temperature-induced changes in O_2 affinity. It is, however, likely that changes in the erythrocytic concentrations of inorganic ions are implicated. Straub (1957) and Gahlenbeck & Bartels (1968) report that whereas the erythrocytic concentration of K^+ is higher in warm- than in cold-adapted *Rana esculenta*, the opposite applies to the concentrations of Na^+, K^+ and Cl^- in the plasma. Thermoacclimatory responses in inorganic erythrocyte ions are also known in fish. Recently (1979) Houston & Smeda showed that with increasing acclimation temperature, the K^+: haemoglobin ratio in rainbow trout and the Na^+: haemoglobin and Cl^-: haemoglobin ratios in carp increased whereas the Mg^{2+}: haemoglobin in carp decreased. The inverse effect of NaCl on O_2 affinity of *Xenopus* haemoglobin corresponds to that generally encountered in vertebrates (Antonini, Amiconi & Brunori, 1972). For intact human erythrocytes Sommerkamp *et al.* (1961) have shown an inverse relation between cation content and O_2 affinity.

The increases in O_2 affinity induced by osmotic stress and aestivation were accompanied by significant increases in the urea levels in blood, plasma and red cells. In conjunction with the specific effect of urea on the affinity of *Xenopus* haemoglobin (Fig. 7A), this suggests that the changes in blood O_2 affinity result from the increased urea levels. The fact that salts and ATP decrease O_2 affinity (Fig. 7B, C) shows that

the higher affinities cannot be attributed to increases in the concentrations of these factors. On the contrary, changes in their concentrations may have reduced the urea effect. The concentrations of urea measured in the aestivating specimens ($\sim$ 11 mM (P) – Table 1) were, moreover, much lower than those ($\sim$ 30 m-mol.kg^{-1}(P)) Balinsky *et al.* (1967) found in naturally aestivating specimens. It follows that in nature the possible effects of urea on the functional properties of the blood will be greater than those we could document here.

Seymour (1973) found a similar increase in the O_2 affinity of the blood of the spadefoot toad during dormancy, and related the change (measured under unspecified pH conditions) to an O_2 storage function of the haemoglobin. As with *Xenopus*, the affinity increase may have been caused by raised blood urea levels.

What may be the biological significance of an increased O_2 affinity during aestivation? A high O_2 affinity implies that the haemoglobin will only unload when O_2 tension is low. In the absence of compensatory increases in the circulation rate (cf. Coulson, Hernandez & Herbert, 1977), a high affinity could result in a decrease in the rate of O_2 delivery and thus lower O_2 tensions at the mitochondria, which will favour the lower metabolic rates that characterize dormancy. In lungfish, a significant increase in blood O_2 affinity during aestivation is ascribable to a marked fall in the erythrocytic concentration of the cofactor guanosine triphosphate (Johansen *et al.* 1976). Studies on squirrels and hamsters have correspondingly showed 29–48% decreases in erythrocytic diphosphoglycerates during hibernation (Burlington & Whitten, 1971; Tempel & Musacchia, 1975). The present contention that the increased O_2 affinity is adaptive to lower tissue O_2 tensions rather than to high O_2 saturation of the pigment at the respiratory surfaces is analogous to the suggestion of Manwell (1959) that the high affinities of some invertebrate haemoglobins may protect their tissues from injurious effects of high O_2 tension (O_2 poisoning). A similar mechanism may be basic to the findings (Wood *et al.* 1975) that blood P_{50} and ATP values increase when plaice from hypoxic water is subjected to atmospheric O_2 tensions, but then decrease again as P_{O_2} increases to hyperoxic levels.

The high sensitivity of O_2 affinity of *Xenopus* haemoglobin to urea is similar to that of Human Hb (Rossi-Fanelli, Antonini & Caputo, 1961, 1964), but contrasts with the low sensitivity seen in Hb from aestivating lungfish (Weber *et al.* 1977) and the skate (Bonaventura, Bonaventura & Sullivan, 1974), which in life similarly experience high concentrations of urea. Urea causes dimerization of human haemoglobin (Bonaventura *et al.* 1974) whereas the quaternary structure of the elasmobranch Hb appears only slightly affected by this compound. This correlates with the different effects of Cl^- on O_2 affinity which indicates that the urea insensitivity in the skate results from stronger electrostatic interactions between the Hb subunits. However, *Xenopus* Hb shows Cl^- sensitivity similar to that of human haemoglobin indicating that different molecular adaptations are basic to the tolerance of *Xenopus* and skate haemoglobins to urea.

The unequal distribution of urea between erythrocytes and the plasma (Fig. 4, inset) in *Xenopus* is in agreement with that found in human and skate blood (Ralls, 1943; Browning, 1978). The higher red cell urea concentration may be due to binding of urea to the haemoglobin molecule. The cationic properties and the amide structure of urea particularly favour hydrogen binding of urea to the normally

dissociated and hence negative terminal carboxyl groups of the subunits of haemoglobin (Krichevskaya, Lukash & Kartasheva, 1973). This suggests that urea may act as a positively charged cofactor undergoing O_2-linked binding of the *Xenopus* haemoglobin molecule, in analogous fashion to anionic phosphate cofactors from most vertebrate erythrocytes.

We demonstrate a direct pH independence effect of CO_2 on O_2 binding of *Xenopus* Hb. As HCO_3^- is always present in experiments with CO_2, this anion could be contributing to the effect attributed to CO_2. However, the absence of CO_2 and substitution of HCO_3^- by acetate, an ion of similar size and charge distribution, resulted in unchanged O_2 affinity in lungfish haemoglobin (Farmer, 1979) indicating that the specific CO_2 effect is due to carbamino CO_2. In human haemoglobin, CO_2 binds at the α and β chain amino termini competing with DGP for the latter sites. Competition between CO_2 and the organic phosphate in *Xenopus* haemoglobin suggests qualitatively similar interaction to that which occurs in human haemoglobin A, and that carbamino formation at the free α amino terminal groups mediates the CO_2 effect in *Xenopus*, In conjunction with the low levels of ATP in *Xenopus* blood, and its small influence on oxygen affinity, this competition also indicates that oxylabile carbamates may contribute significantly to CO_2 transport in *Xenopus* blood. Farmer (1979) suggested that a CO_2-induced decrease in O_2 affinity in three fishes and one amphibian of only half of that found in human Hb A may be due to blocked α chain amino termini. Acetylation of the α chain amino terminal groups has in fact been reported in some fish and amphibians (Riggs, 1970; De Witt & Ingram, 1967; Sullivan, 1974).

Though most CO_2 in *Xenopus* is eliminated through the skin, p_{CO_2} of blood fluctuates (7–13 mmHg) in relation to the breathing rhythm, with lower values during breathing periods and higher ones during immersion (Emilio & Shelton, 1974). During diving CO_2 may thus increase the O_2 unloading pressure, resulting in larger oxygen gradients from the blood capillaries to the cellular metabolic sites.

The higher O_2 affinity found in the haemolysate compared to that in the whole blood may be attributed to various interacting factors, including lower intracellular pH, different concentrations of interacting ions, a specific effect of CO_2 and the lower haemoglobin concentration. Although the haemoglobin concentration effect was insignificant in the stripped haemolysate (p. 22) it may increase drastically at higher pigment concentrations in the presence of cofactor as was recently demonstrated for carp haemoglobin (Lykkeboe & Weber, 1978).

The presence of one minor component, Hb I, and one major component, Hb II, (3·9 and 96·1 %, respectively) agrees with what has been found by Maclean & Jurd (1971). Gel-electrophoretic studies of Just, Schwager & Weber (1977) revealed two Hb components of different molecular weights, evidencing dimerization of the minor Hb component. The distinctly different O_2 binding properties of the two fractions is reminiscent of many fish where an electrophoretically cathodal Hb (cf. Hb I) has high O_2 affinity and lacks cooperativity and pH sensitivity, while anodal Hbs (cf. Hb II) have low O_2 affinity and distinct cooperativity and pH sensitivity. In fish (Hashimoto, Yamaguchi & Matsuura, 1960; Powers, 1972; Weber & De Wilde, 1976), these differences may represent a division of labour, the cathodal component ensuring O_2 uptake and transport when O_2 tensions or pH is low. Despite its low relative concentration the properties of component I of *Xenopus* foreshadows a similar role.

Advice criticism and help from Dr Helmut Schröck, Karlsruhe, West Germany and Professor Kjell Johansen, Gunnar Lykkeboe and Winnie Heidemann, Aarhus, Denmark, are gratefully acknowledged. The work was supported by the Danish Natural Science Council.

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