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ALLOSTERIC CONTROL OF OXYGEN BINDING BY HAEMOGLOBIN DURING EMBRYONIC DEVELOPMENT IN THE CROCODILE *CROCODYLUS POROSUS*: THE ROLE OF RED CELL ORGANIC PHOSPHATES AND CARBON DIOXIDE

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Summary

The P_{50} of whole blood [30°C, P_{CO_2}=2.08kPa (15.6mmHg)] decreases during embryonic development from approximately 6.7kPa (50mmHg) at 15 days to about half this value at hatching (86 days), paralleling a decrease in ATP from 100 to 5–10 μmol g^{-1} Hb. There is also a progressive changeover from embryonic to adult haemoglobin (HbA). A pulse of 2,3-diphosphoglycerate (2,3-DPG) (18 μmol g^{-1} Hb) occurs late in embryonic life. It has no effect on whole-blood oxygen-affinity and falls rapidly at hatching to values typical of post-hatchling crocodilians in general (<1.0 μmol g^{-1} Hb). ATP has a marked effect on the oxygen affinity of embryonic haemoglobin (HbE) but not on HbA. 2,3-DPG has only very small effects on the oxygen affinities of HbE and HbA. CO_2 has a small effect on the oxygen affinity of HbE but a marked effect on that of HbA. Values of P_{O_2} measured in the chorio-allantoic artery [2.9kPa (22mmHg)] and vein [5.9kPa (52mmHg)] imply an increase in saturation from approximately 30% to more than 80%. Neither whole-blood oxygen-affinity nor ATP level was altered in response to an experimental 7-day exposure to low ambient oxygen levels [10.7kPa (80mmHg)]. The results do not lend themselves easily to the pan-selectionist paradigm in which all physiological traits are viewed as being adaptive.

Introduction

Crocodiles and alligators form an ancient group of vertebrates surviving relatively unchanged from the Mesozoic age and, from evidence based on haemoglobin sequencing and on immuno-electrophoresis, show little variation between species (Schneck *et al.* 1984; Densmore and Owen, 1989). The functional properties of their haemoglobin and mechanisms of allosteric control have therefore attracted interest. Juvenile and adult crocodilians have very low levels of red-cell organic phosphates (Bauer and Jelkmann, 1977; Grigg and Gruca, 1979) and appear to be unique because bicarbonate undertakes the allosteric role that organic phosphate compounds play in regulating haemoglobin oxygen-affinity in most vertebrates (Bauer *et al.* 1978; Brittain and Wells, 1991). This

Key words: crocodile, haemoglobin, red cell organic phosphates, embryo, *Crocodylus porosus.*

unusual mechanism for regulating haemoglobin function has been interpreted in terms of adaptations to diving (Grigg and Gruca, 1979; Grigg and Cairncross, 1980; Weber and White, 1986) and the unique cardiovascular responses associated with this behaviour (Grigg and Johansen, 1987; Grigg, 1991). Crocodiles, like a small number of mammalian species (ungulates and felids), have low or zero red-cell ATP and 2,3-DPG concentrations, a contrast with most other vertebrates, including fish, amphibians, other reptiles and birds, which employ red-cell organic phosphates (RCOP) as modulators of oxygen affinity.

Grigg and Gruca (1979) have pointed out that, in general, low RCOP concentrations are associated with a reduction of the fixed-acid Bohr effect and an enhanced CO_2 Bohr effect. Accordingly, they have interpreted the low levels of RCOP in crocodilians and other low-RCOP vertebrates in functional terms, as conferring an advantage which offsets the effects of low pH experienced at the end of a prolonged dive or active period.

Regardless of what the functional significance may be, if any, the occurrence of only low levels of RCOP in crocodilians poses the question of whether the bicarbonate-mediated control of oxygen delivery offsets the loss of organic phosphate sensitivity. Alternatively, the phosphate cofactors may also be absent from the embryo. A second question is whether the crocodilian embryo regulates oxygen transport during the course of development and has the potential to improve oxygen supply to tissues in the face of altered environmental circumstances.

We report here the patterns of changes in blood oxygen-affinity and concentrations of red cell organic phosphates, ATP and 2,3-DPG in the estuarine crocodile *Crocodylus porosus* (Schneider) from early in embryonic development through to hatching and juvenility. We report also the effects of ATP, 2,3-DPG and CO_2 on the oxygen affinity of haemolysates and the apparent unresponsiveness of embryonic whole-blood oxygen affinity to low ambient oxygen levels. Further, in order to understand better the evolutionary origins of allosteric regulation, we examine whether the oxygen-binding traits of the red cells in early embryonic development appear to have been subject to selection pressures.

Materials and methods

We studied blood from embryonic (15–86 days), hatchling and juvenile crocodiles in a makeshift laboratory at the Edward River Crocodile Farm, Pormpuraaw, Queensland, Australia (14˚54′S, 141˚36′E) in March 1990 and April 1991, late in the nesting season. Additional eggs were transported from Pormpuraaw to Brisbane by light aircraft, the eggs travelling for the day-long journey in moist vermiculite in a polystyrene box while being handled with great care in order to avoid bumps which may have ruptured fragile attachment membranes. Some of these were sampled as embryos and some were allowed to hatch to provide known-age hatchlings. The work was conducted under a permit from the Queensland National Parks and Wildlife Service and followed guidelines approved by The University of Queensland's Animal Ethics Committee.

To sample embryonic blood, an emery cutting wheel (Dremel Tool Co.) was used to remove the upper third of the egg shell, exposing the embryo. Blood was then drawn into

a heparinized syringe from either a chorio-allantoic vein or the heart, using a 30-gauge needle. Blood from the smallest embryos was collected from the heart into a heparinized glass tube drawn in a match flame to a fine point. In this way, we were able to obtain samples from embryos as early as 15 days through to hatching (approximately 86 days). Samples from hatchlings were drawn by cardiac puncture using a 26-gauge needle. Blood from juveniles was collected at the farm from individuals shot in the medulla at close range (0–1cm) with a .22Z-calibre bullet, the standard method of farm slaughter. A deep incision was made immediately, behind the skull platform, severing the cervical artery. A large sample of clean blood could easily be taken into a heparinized syringe from the profuse flow welling from the cut.

In a number of full-term embryos we were able to draw samples anaerobically from the chorio-allantoic artery and vein, for measurement of 'arterial' and 'venous' O_2 and CO_2 partial pressures using Radiometer BMS3 and PHM71 or PHM73 gas analysis equipment stabilized at 30°C. In these cases, removal of the scribed shell section was accomplished rapidly, and sampling was completed with the assumption that blood gases were not significantly contaminated.

Red cell analysis

Blood was subdivided immediately after sampling for determinations of the oxygen equilibrium curve (2 μl), haematocrit (10 μl) and haemoglobin content (5 μl) by the method of Dacie and Lewis (1984). Haematocrit measurements using microcap capillaries were validated against conventional 75mm tubes. 2,3-DPG (50 μl) and ATP (100 μl) were assayed using Sigma enzymatic test chemicals and an LKB Ultraspec spectrophotometer. It must be noted that the enzymatic method cannot distinguish ATP from other nucleotide triphosphates. In some of the youngest embryos, not all determinations could be made because there was insufficient blood.

Nucleotide phosphate fractions were characterized further by high-performance liquid chromatography (HPLC) (Ryder, 1985). Samples of whole blood from 40- and 70-day embryos and from juvenile animals were diluted sixfold with 0.6mol l^{-1} perchloric acid and the extracts neutralized with 5mol l^{-1} K_2CO_3. Filtered extracts were chromatographed on a Waters Associates reverse-phase 250mm 5μ C-18 Lichrosorb column (Merck) eluted with 0.1mol l^{-1} phosphate buffer at pH7.2. Peaks were identified from the column retention times of a series of standard nucleotide solutions and their areas were integrated.

Oxygen equilibrium curves

Oxygen equilibrium curves were determined at 30°C, the temperature of incubation at the farm, using an Aminco Hem-O-Scan (American Instrument Co.) taking particular care to ensure the chamber was kept fully moist and using other precautions described by Wells and Weber (1989). Gases for equilibration were pre-mixed and pre-analysed; 2% CO_2 in air, 2% CO_2 in N_2, 6% CO_2 in air and 6% CO_2 in N_2 (Commonwealth Industrial Gases, Australia). This afforded determination of whole blood or haemolysate oxygen equilibria at 2.08kPa (15.6mmHg) and 5.91kPa (44.3mmHg) CO_2, respectively. pH was

calculated from pH–P_{CO_2} diagrams for *C. porosus* (Seymour *et al.* 1985). This has not been confirmed for early embryos, because insufficient volumes of blood were collected. Full saturation was confirmed with brief exposure to 100% oxygen and the absence of haem oxidation was checked spectrophotometrically (see Dacie and Lewis, 1984).

Molecular studies

Haemoglobin componentry

Lysates were prepared by freeze-thawing erythrocytes in 5mmol l^{-1} Tris–HCl (pH8.2) buffer containing 100mmol l^{-1} NaCl and 10mmol l^{-1} dithiothreitol, protected from oxidation by carbon monoxide. Polyacrylamide gel isoelectric focusing (IEF) was performed according to the basic procedures described by Riggs (1981) in order to establish the pattern of isohaemoglobins during development. Tube gels containing 5% ampholines mixed in the ranges pH5–8 and pH6–9 (1:1) were prefocused for 30min prior to sample application. Anodal and cathodal electrolyte solutions were 0.01mol l^{-1} phosphoric acid and 0.01mol l^{-1} NaOH respectively. Ten 1 μl samples of 60 μmol l^{-1} Hb-lysate were applied and IEF was initiated at 3mA per tube, maximum power 0.25W per tube, for approximately 5h, until the current dropped to 0.6mA per tube. After focusing was complete the gels were scanned in a densitometer, then removed from the rods. Bands were circumsected and the haemoglobins eluted overnight in cold 10mmol l^{-1} KCl. Concentrations were estimated spectrophotometrically and the proportions calculated.

Oxygen-binding reactions

A lysate of embryonic Hb was prepared from pooled blood taken from embryos less than 40 days old and the absence of HbA was confirmed by IEF. Red cells were washed in 1mmol l^{-1} Tris–HCl buffer (pH8.2) and brought to 100mmol l^{-1} with NaCl. Stroma were removed by centrifugation and the clear lysate was 'stripped' of organic phosphates by Sephadex gel-filtration as described by Jelkmann and Bauer (1976). Lysates were adjusted to 0.6mmol l^{-1} (Hb-tetramer) with distilled water and combined with equal volumes of 100mmol l^{-1} Tris–HCl or Bis–Tris–HCl with a final concentration of 0.3mmol l^{-1} Hb and 100mmol l^{-1} chloride. Buffers were also prepared containing ATP (disodium salt) or 2,3,-DPG (pentacyclohexammonium salt) in 10-fold molar excess to compensate for dissociation of the Hb–phosphate complex in dilute solution. Experiments were carried out in the presence and absence of CO_2.

Experimental exposure of embryos to low ambient oxygen levels

Five control and five experimental eggs, 40 days old, were exposed at 30°C in separate containers to humidified gas mixtures for 7 days. The flow rate through each chamber was held at 400ml min^{-1}. The controls received humidified air while the experimental eggs received air supplemented with nitrogen to reduce P_{O_2} to approximately 10.7kPa (80mmHg). This was achieved using precision flowmeters (GAP and Meter Rule, UK) and the mix was confirmed by periodic measurements of oxygen partial pressure in the excurrent gas.

Results

Haematology

Haematocrits were higher during embryonic life, with a conspicuous fall immediately after hatching (Fig. 1). Haemoglobin concentration, in contrast, rose during development, followed by a similarly conspicuous drop at hatching (Fig. 2). These results imply a steady increase in mean corpuscular haemoglobin concentration during embryonic life to

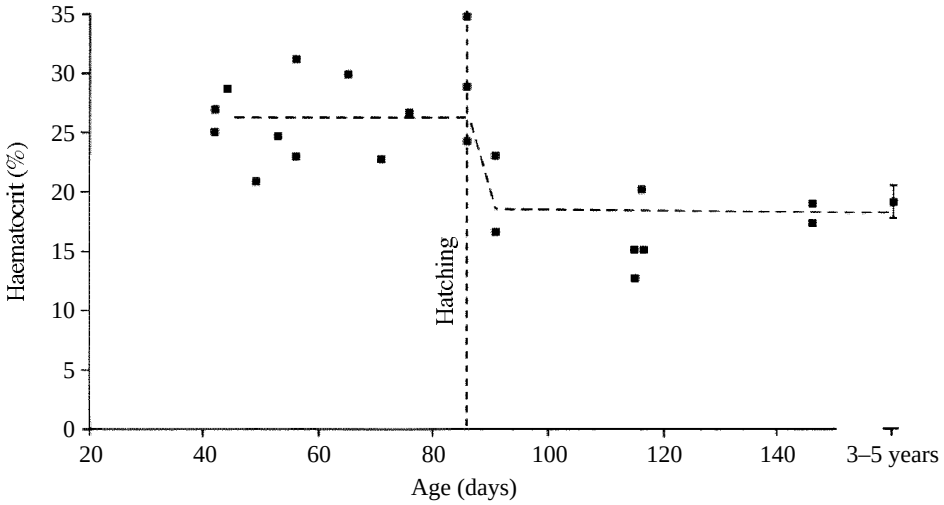

Fig. 1. Changes in the haematocrit of whole blood of *Crocodylus porosus* during embryonic life and into juvenility. Values are individual samples except for that at 3–5 years, which is mean ± S.D. (*N*=10). In this and other figures the lines were fitted by eye.

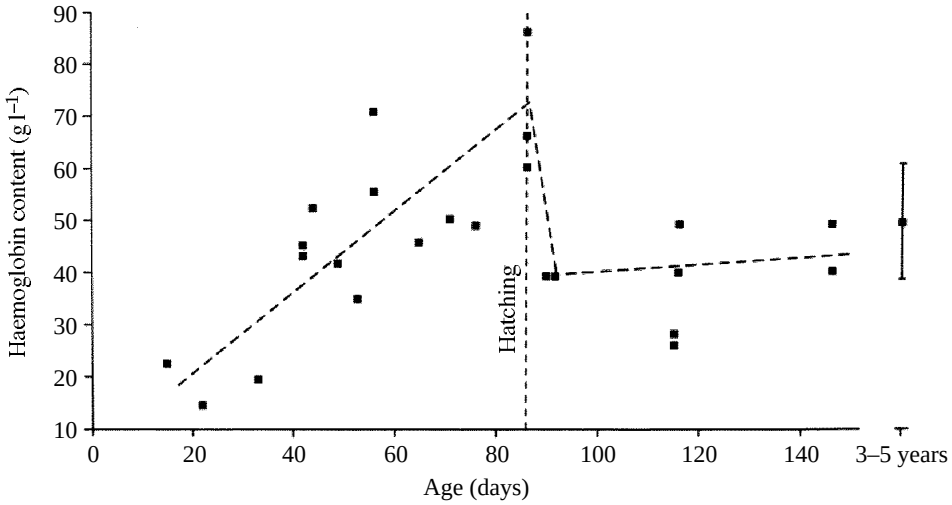

Fig. 2. Changes in the haemoglobin content of whole blood of *Crocodylus porosus* throughout embryonic life and into juvenility. Values are individual samples except for that at 3–5 years, which is mean ± S.D. (*N*=10).

the values seen in juveniles (262±72 g l^{-1}, S.D., *N*=10) and a decrease in blood viscosity without compromising oxygen transport capacity (Wells *et al.* 1991).

Red cell organic phosphates

The pattern of changing levels of organic phosphates during development is shown in Fig. 3. For the first half of development, ATP (Fig. 3A) is the dominant RCOP, with levels near 100 μmol g^{-1} Hb at 15 days (as early as measurement was practical). There is an exponential fall in ATP concentration to a plateau of 5–10 μmol g^{-1} Hb at hatching, a

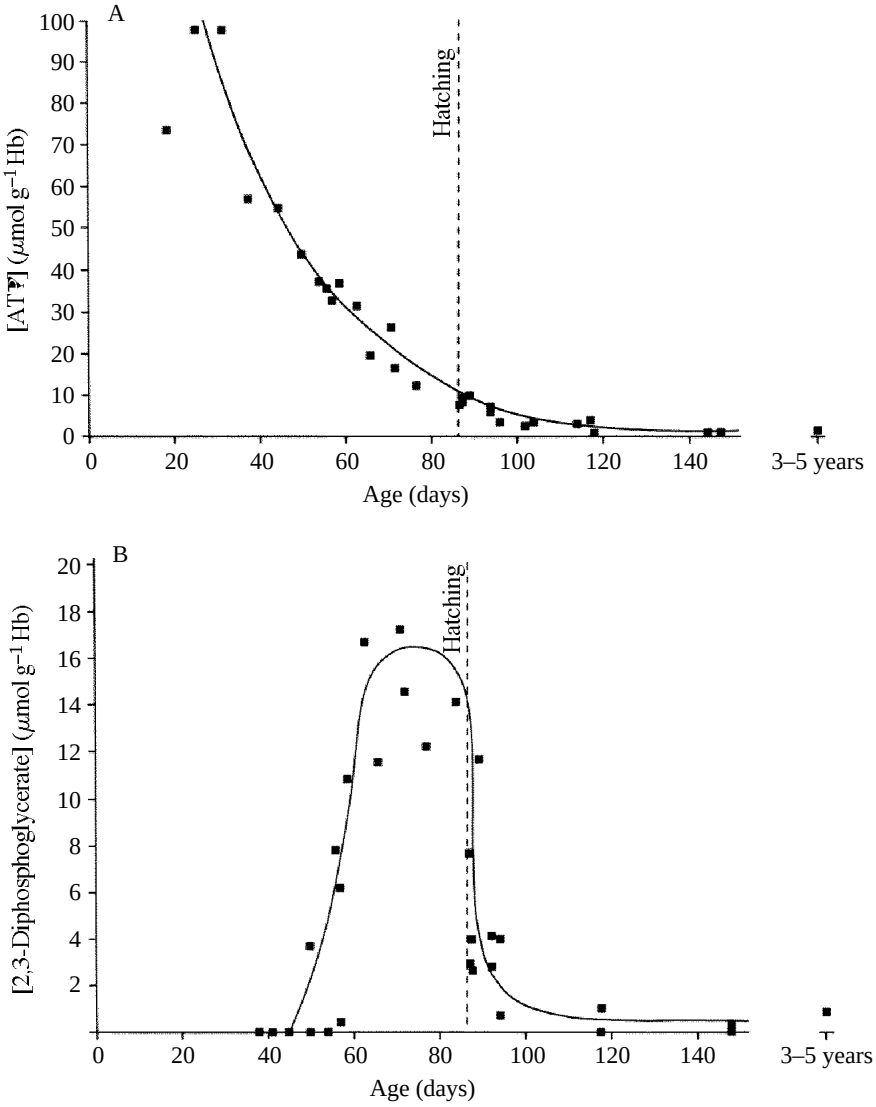

Fig. 3. Changes in red cell organic phosphate concentrations of *Crocodylus porosus* during development. (A) ATP concentration; (B) 2,3-DPG concentration. Values are individual samples except for that at 3–5 years, which is mean ± S.D. (*N*=10).

level which persists past hatching into juvenile life. Assuming a relative molecular mass of 65000 for both embryonic and adult tetrameric Hb, these values correspond to a fall from 6.7 to 0.3molATPmol^{-1}Hb. Levels of 2,3-DPG (Fig. 3B) are initially undetectable but rise dramatically after day 40, so that late in embryonic life there is a marked pulse rising to about 18 μmol g^{-1}Hb (≈1.2molmol^{-1}Hb), a result which confirms the high levels of 2,3-DPG found in embryos of *C. porosus* close to hatching (Hinchliffe, 1980). This pulse decays within 10 days after hatching to the low levels (<1 μmol g^{-1}Hb) typical of posthatching crocodilians in general.

Identification of the components in the nucleotide phosphate pools in erythrocytes from early and late embryos and from juveniles was made using HPLC chromatograms. The percentage contribution of each is shown in Table 1. ATP is the principal erythrocyte

Table 1. *Nucleotide phosphate components in 40-day and 70-day embryo and juvenile* Crocodylus porosus

	40-day embryo	70-day embryo	Juvenile
GTP	12.3	8.0	23.7
IMP	10.4	38.7	12.6
ATP	25.2	14.7	63.7
ADP	16.3	13.2	–
AMP	14.2	8.4	–
Hypoxanthine	21.5	17.4	–

Components were separated by HPLC and are shown as a percentage of the total pool.

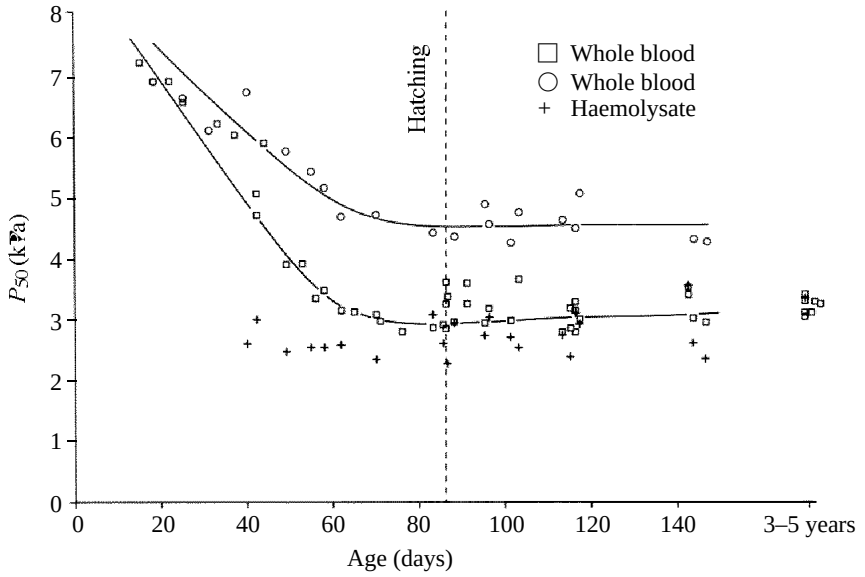

Fig. 4. Changes in oxygen affinity of whole blood during the development of *Crocodylus porosus*, measured at 30°C and CO_2 partial pressures of 2.08 and 5.91kPa (16 and 44mmHg), and of crude haemolysates at 2.08kPa (16mmHg).

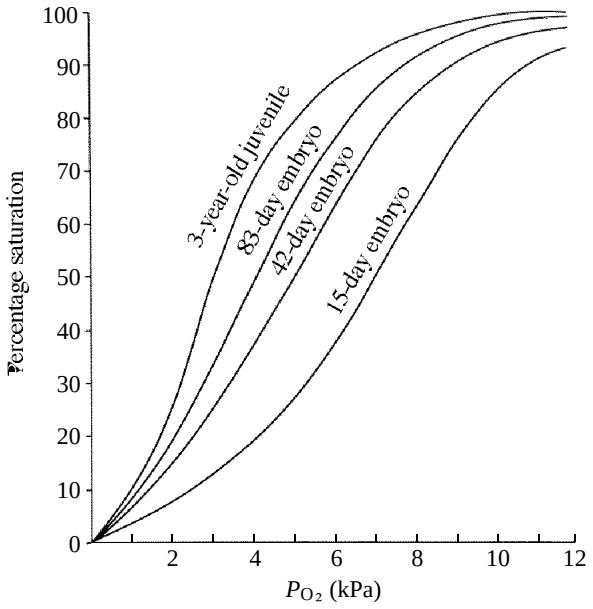

Fig. 5. Progressive changes in the oxygen equilibrium curve of the whole blood of *Crocodylus porosus*, measured at 30˚C and 2.08kPa (16mmHg) CO_2. Data are representative and are continuous curves.

nucleotide throughout development, but GTP makes up a significant fraction of the nucleotide triphosphate pool; approximately 27% in the juvenile and 33% in the early embryo, with the balance ATP. Since the enzymatic method for detecting ATP also detects GTP, the pattern in Fig. 3 must be interpreted accordingly. Small fractions of inosine monophosphate (IMP) occur in the erythrocytes from embryos and juveniles. Appreciable fractions of adenine nucleotides in the embryonic samples are consistent with metabolically active erythrocytes in the embryos.

Oxygen equilibrium curves of whole and haemolysed blood

Oxygen affinity

Early in embryonic life the blood has a strikingly low affinity for oxygen [P_{50} at 30˚C and P_{CO_2} of 20.08kPa (15.6mmHg) is approximately 6.7kPa (50mmHg) at 15 days]. P_{50} decreases as development proceeds (Fig. 4) to about half this value by about day 80, an affinity typical of hatchlings and juveniles. Fig. 5 shows the progressive change in the position of the oxygen equilibrium curve from early embryonic life through to that seen in juveniles. The increase in oxygen affinity parallels the fall in ATP levels (Fig. 3). P_{50} tends to be slightly lower before hatching than after hatching, as in birds (Lomholt, 1975), but there is no suggestion of any effect from the pulse of 2,3-DPG at 60–80 days.

The CO_2 Bohr effect increases gradually throughout development (Fig. 4). Fig. 4 also shows that the oxygen affinity of crude haemolysates of whole blood is high and remains

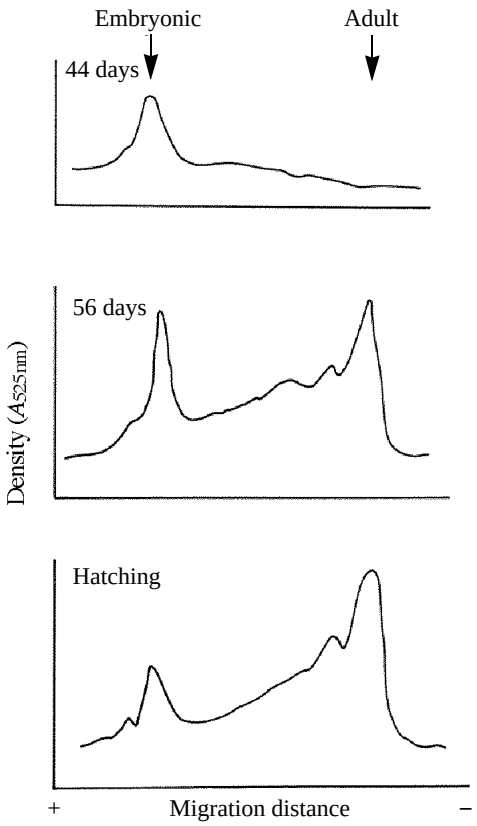

Fig. 6. Densitometric scans of IEF tube gels showing embryonic and adult haemoglobin components at 44 and 56 days of embryonic incubation and at the time of hatching.

essentially unchanged during development, implying that the observed changes in whole-blood oxygen-affinity are a consequence of the allosteric effects of the changing levels of red-cell organic phosphates, rather than a consequence of a changing population of haemoglobin components.

Haemoglobin complement

The developmental profile of the Hb components was resolved by isoelectric focusing of lysates protected from autoxidation with carbon monoxide, in which a well-defined embryonic band (HbE), a poorly focused adult component (HbA) and several trace bands were revealed (Fig. 6). In the early developmental stages, HbE predominates and it is gradually replaced by HbA so that by hatching only traces of HbE persist (Fig. 7).

The molecular integrity of HbE and HbA was confirmed at 500–700nm and the pH of the eluted fractions indicated isoelectric points of 7.2 and 7.8 for HbE and HbA, respectively. The trace components were assumed to be derived artefacts as they were absent in fresh samples of oxyHb taken from eggs and from hatchlings returned to the University for analysis. Further, the fuzzy component observed previously in crocodiles

(Bauer and Jelkmann, 1977) appeared to increase in stored lysates, but resolved into a sharp band when treated with 10mmol l^{-1} dithiothreitol. This result suggested that polymerization by disulphide linkage of native tetramers occurs very readily in HbA.

Oxygen-binding properties of HbE and HbA and the influence of ATP and 2,3-DPG

Pooled erythrocytes harvested from embryos younger than 40 days were shown by isoelectric focusing to be free of HbA and HbE was absent from erythrocytes of juvenile crocodiles. Preparative procedures did not promote significant formation of oxidized Hb derivatives as judged from spectral ratios (Benesch *et al.* 1973).

The oxygen-binding properties of HbE and HbA are compared in Fig. 8A,B. The intrinsic affinity of HbE for oxygen is higher than that of HbA throughout the pH range tested, and it is slightly less sensitive to pH as quantified by the fixed-acid Bohr factor, $\Phi=\Delta\log P_{50}/\Delta\mathrm{pH}$, of -0.21 *vs* -0.25 calculated linearly between pH7.1 and 7.7. Furthermore, HbE shows an almost hyperbolic equilibrium curve (Hill coefficient, n_{50}=1.1–1.3) compared with the significantly sigmoidal curve (n_{50}=2.2–2.8) seen in HbA.

The most striking difference between HbE and HbA lies in their relative responsiveness to organic phosphates and CO_2 modulators. Whereas HbA shows a small, insignificant decrease in oxygen affinity in the presence of ATP or 2,3-DPG, the affinity is decreased dramatically by molecular CO_2 (Fig. 8). Conversely, HbE is most responsive to ATP and much less so to 2,3-DPG. The CO_2 effect is significant in HbE but decreases oxygen affinity less than in HbA.

Assuming a linear Bohr relationship between pH7.1 and 7.7, the fixed-acid value of

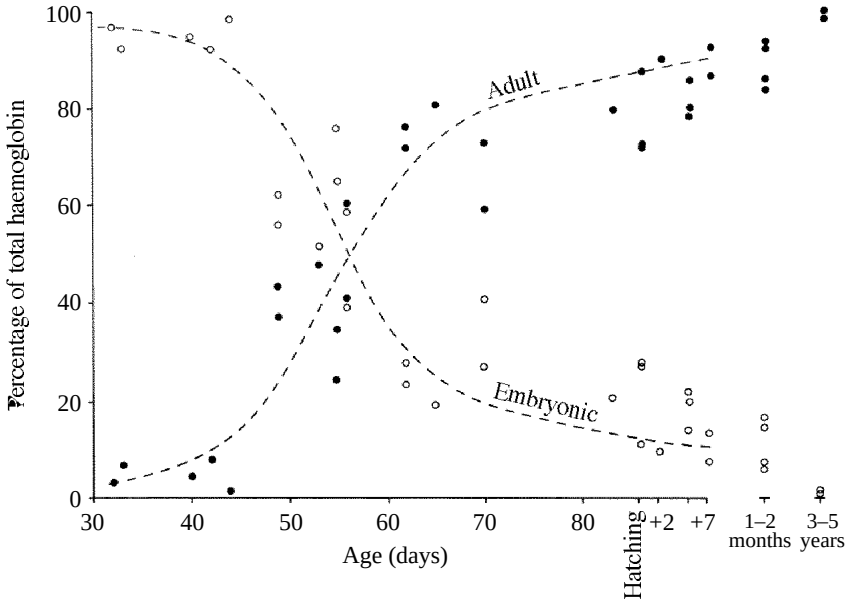

Fig. 7. Embryonic and adult haemoglobin components as a percentage of total major components from age 30 days of incubation to hatching and beyond, to juveniles aged 3–5 years.

Φ=−0.21 for HbE was essentially similar with either added 2,3-DPG (−0.20) or ATP (−0.14) in the absence of CO_2. Both phosphates increased the cooperativity of HbE and the effect was more marked at alkaline pH. Molecular CO_2 superimposed on varying

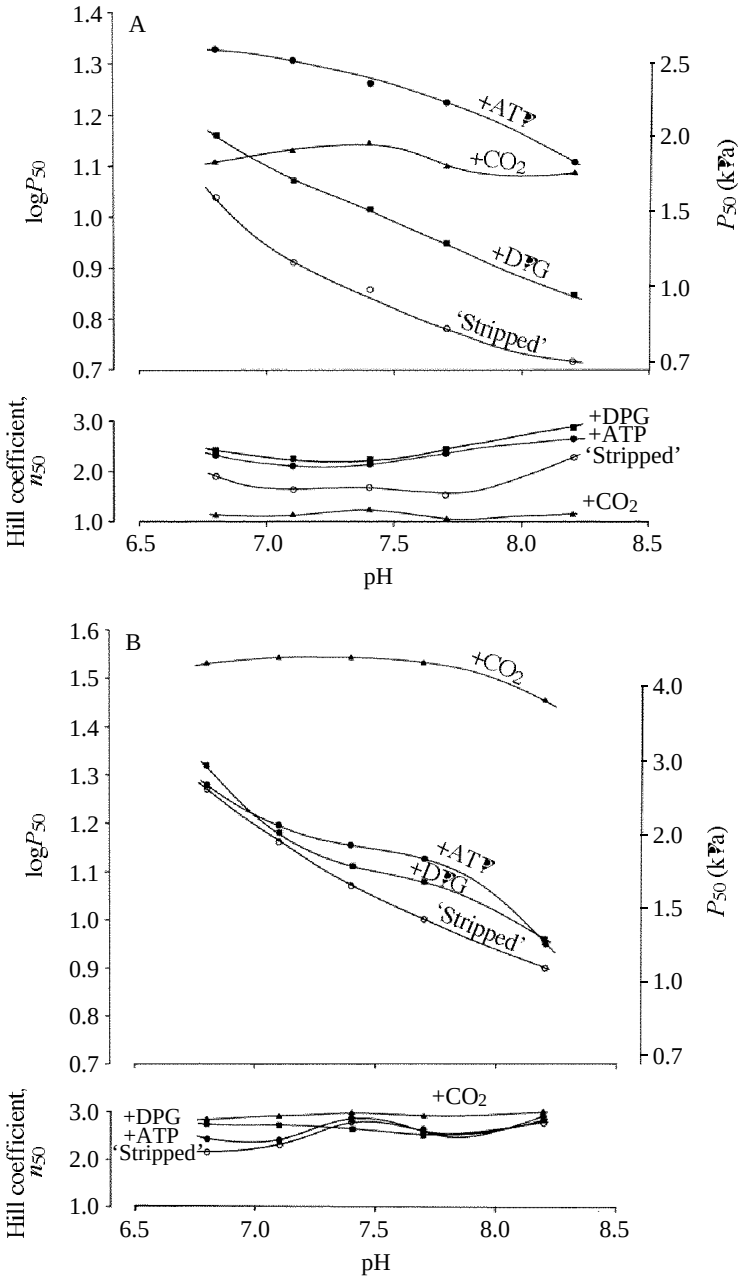

Fig. 8. Comparative effects of ATP, 2,3-DPG (CO_2-free) and CO_2 on the oxygen affinity and the Hill coefficient of 'stripped' Hb over a range of pH values, in pooled embryonic (A) and adult (B) *Crocodylus porosus* haemoglobin 0.3 mmol l^{-1} tetramer and 100 mmol l^{-1} Cl^- at 31 °C.

fixed acid loads (pH) appeared to reduce considerably the Bohr effect of both HbE and HbA but, whereas cooperativity was decreased in the former, it was enhanced in the latter.

The essential regulating effects of ATP and CO_2 on the shape and position of the oxygen equilibrium curve at pH7.7 are illustrated in Fig. 9.

Oxygen affinity of embryos exposed to low ambient oxygen

Exposure of 40-day embryos to a low-oxygen environment [10.7kPa (80mmHg)] for 7 days did not result in either an increased oxygen-affinity of embryonic whole blood or

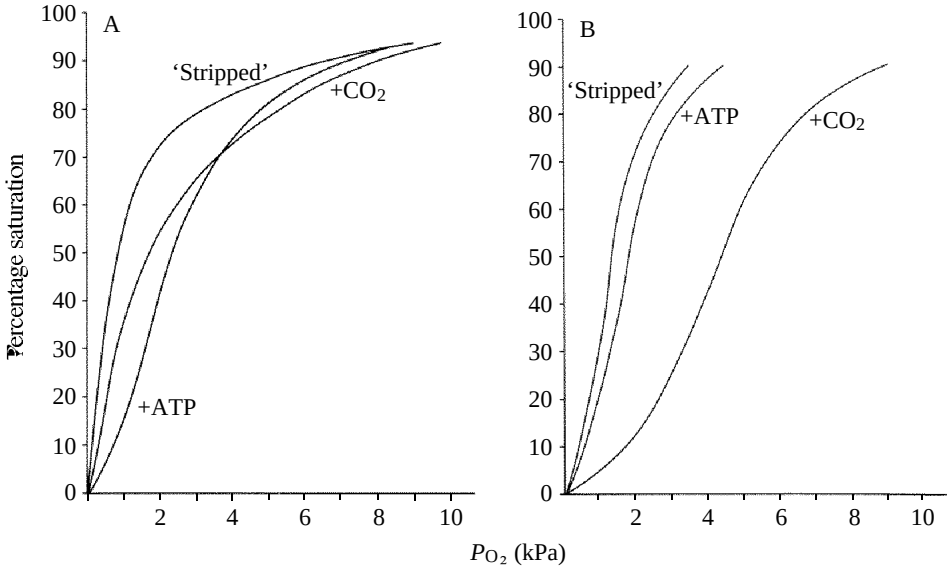

Fig. 9. Comparative effects of ATP and CO_2 on the oxygen equilibrium curves of purified embryonic (A) and adult (B) haemoglobin from *Crocodylus porosus*, measured at 31˚C and pH7.7.

Table 2. *Oxygen and carbon dioxide levels in chorio-allantoic artery ('venous' blood) and vein ('arterial' blood) in full-term embryos of* Crocodylus porosus

	'Venous' blood		'Arterial' blood	
	P_{O_2} (kPa)	P_{CO_2} (kPa)	P_{O_2} (kPa)	P_{CO_2} (kPa)
	3.1	4.3	6.7	1.6
	2.8	3.7	7.6	1.1
	3.1	4.7	7.5	1.9
	2.9	–	6.4	1.3
	–	–	7.5	1.9
	–	–	6.4	1.9
	–	–	6.3	1.5
Mean	3.0	4.2	6.9	1.6
Range	2.8–3.1	3.7–4.7	6.3–7.6	1.1–1.9

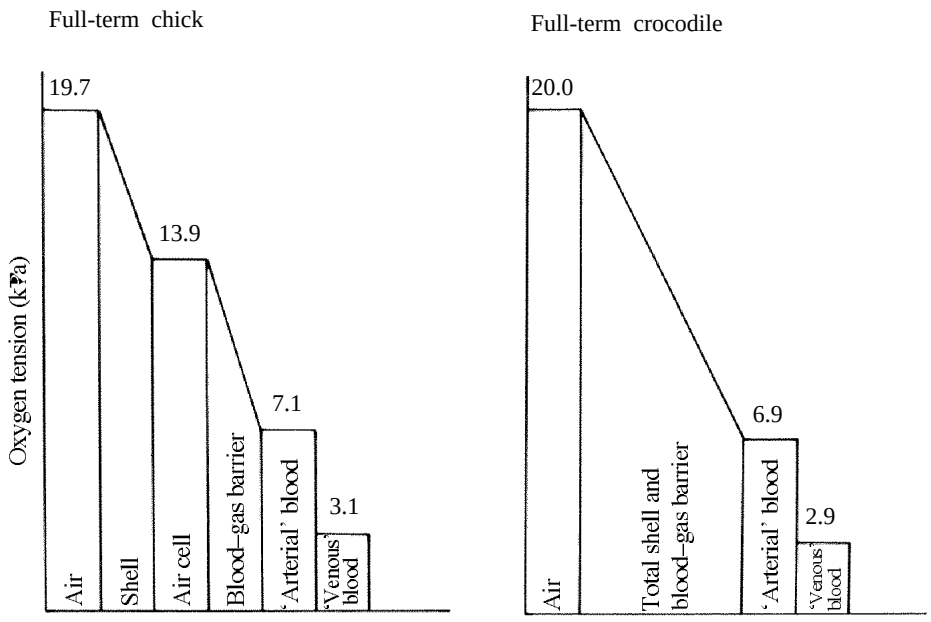

Fig. 10. Comparison of oxygen 'cascades' between crocodilian and chicken embryos, just prior to hatching (chicken data from Wangensteen, 1972).

any change in red-cell ATP levels, the dominant organic phosphate at 40 days. Mean oxygen affinities (± S.D.) of the whole blood in the hypoxic and control groups were 4.49kPa (33.7mmHg) (±0.69kPa, *N*=5) and 4.78kPa (35.9mmHg) (±0.75kPa, *N*=5) respectively. Mean ATP levels in the hypoxic and control groups were, respectively, 53.4±12.8 μmol g^{-1} Hb, *N*=4, and 54.6±8.2, μmol g^{-1} Hb, *N*=5.

Oxygen and carbon dioxide partial pressures in 'arterial' and 'venous' blood in full-term embryos

Obtaining adequate blood volumes to make these measurements was technically difficult. The results are shown in Table 2 and, with reference to Fig. 5, imply an increase from approximately 30% to above 80% in the saturation of the blood as it traverses the chorio-allantois. A comparison of the 'crocodile egg oxygen cascade' with that of chickens is shown in Fig. 10.

Discussion

Changes in blood oxygen-affinity during development

The affinity of *C. porosus* blood for oxygen increased throughout the embryonic phase (Fig. 4). It was correlated with both the progressive replacement of embryonic haemoglobin by adult haemoglobin (HbA) (Fig. 6), which was essentially complete soon after hatching (Fig. 7), and with the progressive fall in red-cell ATP concentration

(Fig. 3). Of particular note is the apparent lack of response of the whole-blood oxygen-affinity to the peak in 2,3-DPG prior to hatching.

The replacement of one Hb type by another during development parallels that in several species of precocial birds (Borgese and Nagel, 1977; Baumann *et al.* 1982) and in turtles (Isaacks *et al.* 1978). These events have been interpreted in terms of falling P_{O_2} at the chorio-allantoic surface immediately prior to hatching (Lomholt, 1975; Snyder *et al.* 1982; Lapennas and Reeves, 1983) and on the basis of measurements of metabolic rates in embryos (Whitehead and Seymour 1990). A similar argument may be advanced for *C. porosus.* Reptiles belonging to the Squamata, in contrast, do not appear to have similar ontogenetic changes in haemoglobin components, and oxygen affinity appears to be regulated allosterically (Korsgaard and Weber, 1989). The general pattern of change in blood oxygen-affinity during embryonic development is geared to the regulation of oxygen supply in response to metabolism. There is no evidence that oxygen becomes limiting in the nest environment of *C. porosus* (G. C. Grigg and L. A. Beard, unpublished observations).

Allosteric regulation of oxygen affinity during development

The higher oxygen affinity of haemolysates compared to whole blood in embryos of *C. porosus* supports an allosteric role for ATP in regulating embryonic oxygen transport, but not apparently for 2,3-DPG (Figs 8, 9). This poses a riddle in seeking an adaptive explanation for the function of 2,3-DPG in early development. 2,3-DPG is an allosteric modulator that generally *decreases* blood oxygen-affinity, and yet we found no change in whole-blood oxygen-affinity during development when the 2,3-DPG level peaks and when embryonic Hb is present in significant proportion. Similar results have been obtained in other species: a flush of 2,3-DPG appears late in the embryonic development of several birds and turtles (Isaacks and Harkness, 1975; Bartlett, 1976, 1978, 1982; Snyder *et al.* 1982) and, prior to hatching, the 2,3-DPG peak in embryonic red cells of the green sea turtle has little effect on the oxygen-transporting properties of the embryonic haemoglobin (Isaacks *et al.* 1978).

Of particular interest in relation to any adaptive interpretation of the role of organic phosphates is the lack of an increase in whole-blood oxygen-affinity in response to an exposure to low ambient oxygen levels for 7 days. This contrasts with results in many fish such as *Pagothenia borchgrevinki* and in other vertebrates (Wells *et al.* 1989*a*). Ingermann *et al.* (1983) observed that hypoxia in the chick embryo led to an increase in 2,3-DPG and a fall in ATP levels, suggesting a regulatory role for 2,3-DPG near the end of the incubation period when oxygen is limited. 2,3-DPG is, however, a feeble effector in the allosteric regulation of oxygen affinity in chick and goose embryos (Baumann *et al.* 1982; Snyder *et al.* 1982), despite the presence of the requisite erythrocyte metabolites and phosphoglyceromutase enzymes providing for a potential regulatory role (Harkness *et al.* 1980).

The observation of fluxes in 2,3-DPG levels in the early development of birds and reptiles may be extended to mammals: 2,3-DPG appears in high concentrations in the early development of the marsupial wallaby (Baudinette *et al.* 1988), in rabbits (Jelkmann and Bauer, 1977) and in mice (Wells, 1979). In mammals, however, a correlation of

falling 2,3-DPG levels with increased oxygen affinity during intrauterine development has been established (Brittain and Wells, 1983). More puzzling is the peak of 2,3-DPG appearing during sheep development (Aufderheide *et al.* 1980) because, as in crocodiles, adult sheep lack 2,3-DPG and there is no direct allosteric effect of 2,3-DPG on either foetal or adult sheep haemoglobin (Baumann *et al.* 1972). Similar patterns of development occur in goats (Blunt, 1972) and in cattle, although the latter retain traces of 2,3-DPG in the adult erythrocytes (Zinkl and Kaneko, 1973).

The embryonic erythrocytes of *C. porosus* contained equivalent amounts of ATP and DPG at 70 days. In late alligator and turtle embryos, ATP is about 5–7 times as abundant as 2,3-DPG, and in crocodiles inositol polyphosphate (IPP) is absent (Bartlett, 1978). An allosteric role for ATP in *C. porosus* during development therefore seems likely. Nucleotide triphosphates also regulate the foetal–maternal shift in an ovoviviparous snake (Birchard *et al.* 1984) and are implicated in the development of viviparous lizards (Grigg and Harlow, 1981).

The progressive shift from organic phosphate to CO_2 modulation of oxygen affinity parallels the replacement of the embryonic component by adult haemoglobin, and the process is fully completed soon after hatching.

Structuralism and the adaptationist argument

Physiologists often view traits in haemoglobin function as the optimized result of natural selection, when a different kind of explanation may be warranted (Wells, 1990). Both reptiles and birds have developed separate and distinct strategies in allosteric regulation of haemoglobin function in adults which may reasonably be interpreted in adaptationist terms (see Weber and Wells, 1989, for a review). Embryonic development, in contrast, shows remarkable uniformity in that blood oxygen affinity is increased near to the time of hatching, and appreciable quantities of 2,3-DPG are accumulated. This occurs despite the fact that 2,3-DPG has less effect on chicken HbA than does either ATP or IPP, little effect on turtle HbA (Lutz and Lapennas, 1982) and no effect on HbA from *C. porosus* (Bauer *et al.* 1978). Moreover, the embryonic haemoglobins of green sea turtles and loggerhead turtles appear to be functionally indistinguishable, despite marked differences in the oxygen affinity of the adult blood (Isaacks *et al.* 1978).

These observations have alerted some authors to the dangers of pan-selectionism. Bartlett (1982, p. 134) stated that 2,3-DPG in bird embryos '... has not been shown to have any clear functional purpose' and, of turtles, Bartlett (1978, p. 197) wrote '... the pool of DPG in the late embryos is a non-functional vestige...'. In a similar vein, Isaacks *et al.* (1978), writing about marine turtles, stated, '... phosphate modulators have little effect upon the mechanisms regulating haemoglobin function...'. These comments invite us to consider the possibility that Hb function in embryonic development has not been scrutinized by natural selection and support the contention of Von Baer (1828) that early development is a highly conserved process.

2,3-DPG accumulates when glycolysis predominates and ATP accumulates with oxidative phosphorylation. Viewed in this way, the period of ATP depletion and 2,3-DPG accumulation in the erythrocytes of the crocodile egg just prior to hatching represents a fully discharged energy unit and need not be seen in adaptationist terms. We may

therefore envisage embryonic development as an orderly metabolic integration in which ATP is the 'currency' of metabolic economy, its accumulation in early embryos being an index of the extent of phosphorylation, or adenylate energy charge (cf. Atkinson, 1968; Wells *et al.* 1989*b*). Although few people would seriously wish to resurrect Haekel's Biogenetic Law ('ontogeny recapitulates phylogeny'), this structuralist approach to embryonic development may be preferable to a functionalist one (Wells, 1990).

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