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MOLECULAR BASIS OF THE DIFFERENCE IN OXYGEN AFFINITY BETWEEN MATERNAL AND FOETAL RED BLOOD CELLS IN THE VIVIPAROUS GARTER SNAKE *THAMNOPHIS ELEGANS*

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Summary

Molecular mechanisms that may explain why oxygen affinity is higher in foetal than in maternal red blood cells were studied in the viviparous garter snake, *Thamnophis elegans* (Baird and Girard). Foetal and adult haemoglobins were structurally indistinguishable, as demonstrated by native polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulphate PAGE, low pH/urea PAGE, and gel filtration column chromatography. Oxygen-binding studies of haemoglobin in the absence of organic phosphates showed that adult and foetal haemoglobins had relatively high affinities for oxygen, low Bohr coefficients, and Hill coefficients of about 4·0 at pH 7·0. Adenosine triphosphate (ATP) lowered the oxygen affinity of the haemoglobins from about 3·6 to 9·6 mmHg (1 mmHg = 133·3 Pa) at pH 6·8. Maternal red cells contained more nucleoside triphosphate (NTP) (primarily ATP) than did foetal cells by about 0·9 mol NTP mol^{-1} haemoglobin tetramer. No 2,3-diphosphoglycerate was detected in the cells. Combined levels of magnesium and calcium were comparable in maternal and foetal red cells. Mean corpuscular haemoglobin concentrations (MCHC) in foetal red cells were about 79 % of maternal values. There were no significant differences in maternal and foetal methaemoglobin levels. It appears that a difference in maternal and foetal red cell organic phosphate concentrations, and possibly MCHC values, rather than a difference in haemoglobin structures, explain why oxygen affinity is higher in foetal than in maternal red blood cells in *T. elegans*.

Introduction

Viviparity has apparently evolved independently in many lineages of the squamate reptiles (Shine, 1984, 1985). Therefore, a comparative examination of the physiological strategies used by viviparous reptiles should indicate the range of physiological mechanisms which successfully support foetal development in viviparous vertebrates. One important aspect of reproductive physiology involves the transfer of oxygen from mother to foetus and the molecular mechanisms which

Key words: Reptilia, *Thamnophis*, ATP, oxygen affinity, haemoglobin, foetus.

facilitate that transfer. Although such facilitation has been studied extensively in mammals, relatively little work has focused on the lower vertebrates, especially reptiles.

Manwell (1960) reported that suspensions of foetal red cells from the viviparous garter snake *Thamnophis elegans* had higher oxygen affinities than did suspensions of maternal red cells. The basis for this difference has not been elucidated. However, almost all viviparous vertebrates show a higher blood oxygen affinity in foetal than in maternal blood. In mammals, this difference appears to facilitate the transfer of oxygen from mother to foetus and promote normal foetal growth and development (Battaglia *et al.* 1969; Hebbel *et al.* 1980; Bauer *et al.* 1981). Molecular mechanisms responsible for this higher foetal blood oxygen affinity vary among vertebrate species, and include the production of high oxygen affinity foetal haemoglobins and/or differing maternal and foetal concentrations of red cell organic phosphates (e.g. Manwell, 1963; Tyuma & Shimizu, 1969; Blunt *et al.* 1971; Novy *et al.* 1973; Garlick *et al.* 1979; Weber & Hartvig, 1984).

Among the viviparous reptiles, the oxygen affinity of maternal blood is lower than that of foetal blood in the lizard, *Sphenomorphus quoyii*, and the cottonmouth snake, *Agkistrodon piscivorus*, although the maternal and foetal haemoglobins are electrophoretically indistinguishable (Grigg & Harlow, 1981; Birchard *et al.* 1984). The difference in *A. piscivorus* appears to be mediated by organic phosphate (nucleoside triphosphate) concentrations, which are lower in foetal than in maternal red cells.

A different situation may exist in the garter snake. Pough (1977) found that blood from the neonates of *T. sirtalis* had a higher oxygen affinity than that of adults. He also found an ontogenetic change in the electrophoretic pattern of the haemoglobin and a decrease in the organic phosphate to haemoglobin ratio with development. (Foetuses were not examined in his study.) These results suggest that the foetal–maternal difference in oxygen affinity noted by Manwell (1960) in *T. elegans* may be associated with differences in haemoglobin structure rather than differences in organic phosphate concentrations. Consequently, we studied *T. elegans* with respect to structure and function of haemoglobins from the adult and from foetuses at several developmental stages. We also examined the organic phosphates of maternal and foetal *T. elegans* red cells, and the influence of adenosine triphosphate (ATP) on haemoglobin function. The concentrations of various divalent cations, mean corpuscular haemoglobin concentration (MCHC), and the level of nonfunctional haemoglobin (methaemoglobin) can influence oxygen-binding properties of haemoglobins. Therefore, we examined these factors in maternal and foetal bloods. The results of our studies are reported in this paper, and a molecular mechanism is proposed to explain why the oxygen affinity is higher in foetal than in maternal red blood cells of *Thamnophis elegans*.

Materials and methods

Adult garter snakes were collected in early summer in Latah Co., Idaho, and

identified as *Thamnophis elegans* according to Nussbaum *et al.* (1983). Pregnancy was determined by abdominal palpation. The developmental stage of the foetuses was determined according to Zehr (1962). Foetuses of developmental stages 30–36 were examined in this study.

Snakes were bled into ice-cold heparinized 150 mmol l^{-1} NaCl. All following preparative procedures were performed at 4 °C. Red cells were washed three times with 150 mmol l^{-1} NaCl by centrifugation at 1000 *g* for 5 min. Cells were lysed with distilled water and a single freeze–thaw cycle. The haemolysate was centrifuged at 5000 *g* for 10 min. Subsequently, the supernatant was passed through an 85 cm × 1·6 cm column of Sephadex G 100–120 equilibrated with 100 mmol l^{-1} NaCl, 10 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} Tris and titrated to pH 8·0 with HCl.

Relative molecular masses of the native haemoglobins were determined on the Sephadex G 100–120 column. Elution was monitored spectrophotometrically at 280 nm. Relative molecular mass standards used were bovine serum albumin ($M_r = 68\,000$), human haemoglobin ($M_r = 64\,500$), ovalbumin ($M_r = 43\,000$) and sperm whale myoglobin ($M_r = 17\,800$). Blue dextran and tryptophan were used to indicate the column void volume and salt peak, respectively.

Native polyacrylamide gel electrophoresis (PAGE) was carried out in tubes using the method of Davis (1964). Resolving gels were 7·5 % (w/v) acrylamide, pH 8·9 and stacking gels were 3·0 % acrylamide, pH 7·2. The acrylamide to bisacrylamide mass ratio was 30:0·8. Separate electrophoretic experiments were carried out on carboxy- and cyanmethaemoglobin. Haemoglobin samples were converted to carboxyhaemoglobin by addition of sodium dithionite and bubbling with carbon monoxide, and to cyanmethaemoglobin by the addition of potassium cyanide and potassium ferricyanide.

Sodium dodecyl sulphate (SDS)–PAGE of the globin chains was carried out by the method of Laemmli (1970) on 1·5 mm slab gels. Resolving gels were 15 % acrylamide, 1·0 mmol l^{-1} dithiothreitol, 0·1 % SDS (w/v), pH 8·8 with an acrylamide to bisacrylamide ratio of 30:0·8. Globin chains were denatured by incubation at 40 °C for 1·5 h in a solution of 62·5 mmol l^{-1} Tris, 2·0 % SDS, 5 % 2-mercaptoethanol (v/v), 1·4 mmol l^{-1} phenylmethylsulphonyl fluoride and titrated to pH 6·8 with HCl. Samples were stored frozen at −20 °C for up to 1 month. Relative molecular mass standards used were bovine serum albumin, human haemoglobin, ovalbumin, sperm whale myoglobin and lysozyme ($M_r = 14\,300$).

Gel electrophoresis of the globin chains was carried out in the presence of 6·25 mol l^{-1} urea (mixed fresh to minimize cyanate formation), 1·0 mmol l^{-1} dithiothreitol, pH 2·2 (established with glacial acetic acid) on 6·0 % acrylamide gels having an acrylamide to bisacrylamide ratio of 20:1·2 (Panyim & Chalkley, 1969; Poole *et al.* 1974). The samples to be electrophoresed were first carboxymethylated using iodoacetamide as described by Poole *et al.* (1974) to block sulphhydryl bridge formation.

For oxygen-binding studies, haemoglobins were first purified on the Sephadex G 100–120 column as described above. The purified haemoglobin solution was

concentrated over sucrose before being further stripped of organic phosphates by passage through a 35 cm × 1·6 cm column of Sephadex G 25–50 equilibrated with 100 mmol l^{-1} NaCl, 50 mmol l^{-1} HCl, titrated to pH 8·5 with Tris (Garlick *et al.* 1979). Haemoglobins were dialysed to the desired pH for 2 h immediately before binding studies. Oxygen-binding buffers were 100 mmol l^{-1} NaCl, 50 mmol l^{-1} HCl, titrated to pH 7·0 and below with Bistris and above pH 7·0 with Tris. Oxygen equilibrium measurements were made at 20°C in glass tonometers (from Ryan Scientific Glass, Pullman, WA) by monitoring the change in absorbance at 540, 560, 575 and 580 nm as deoxygenated haemoglobin was reoxygenated (Benesch *et al.* 1965). Four tonometers were run simultaneously. For these functional studies, the haemoglobin tetramer concentration was 12–18 μmol l^{-1} determined spectrophotometrically as the cyanmet-derivative using a millimolar haem extinction coefficient of 11·0. The values of P_{50} (the oxygen tension for half saturation of the haemoglobin) and the Hill coefficient (used as an indicator of haem–haem interaction) were determined from a Hill plot using 3–6 data points corresponding to haemoglobin which was 25–75 % saturated with oxygen. The P_{50} value calculated for each tonometer represents $n = 1$ in the calculations of Bohr coefficients and in Fig. 6. Oxygen-binding studies were performed at various pH values in the absence and presence of ATP. ATP was added to a final concentration of 2 mmol l^{-1} from a concentrated stock solution prepared immediately before use.

For determination of red cell organic phosphate concentrations, snakes were bled into ice-cold heparinized Ringer's saline (110 mmol l^{-1} NaCl, 1·9 mmol l^{-1} KCl, 1·1 mmol l^{-1} $CaCl_2$, 2·4 mmol l^{-1} $NaHCO_3$, pH 7·6). Red cells were washed three times in this saline by centrifugation at 1000 *g* for 5 min at 4°C. Washed and resuspended cells were extracted with an equal volume of ice-cold 12 % trichloroacetic acid (TCA), within 30 min of blood collection, and centrifuged for 10 min at 5000 *g*. Total red cell nucleoside triphosphate (NTP) concentrations were determined in the supernatant using an enzymatic assay kit from Sigma Chemical Co. (no. 366-UV). Red cell concentrations of 2,3-diphosphoglycerate (2,3-DPG) were also determined using an enzymatic assay kit from Sigma (no. 35-UV) on TCA extracts of red cell suspensions. NTP concentrations are expressed as mole NTP per mole haemoglobin tetramer.

For analysis of ATP to guanosine triphosphate (GTP) ratios, rinsed, packed red cells were extracted with perchloric acid and supernatants were neutralized with concentrated KOH using the method of Bartlett (1978). Extracts were then analysed by HPLC with a Varian AX-10 column using a gradient of 0·01 mol l^{-1} KH_2PO_4, pH 3·0 to 0·75 mol l^{-1} KH_2PO_4, pH 5·0.

For analysis of red cell magnesium and calcium concentrations, maternal and foetal cells were collected in ice-cold, heparinized 150 mmol l^{-1} NaCl and washed as described above. Washed cell pellets were lysed with distilled water, centrifuged at 20 000 *g* for 30 min and the supernatants were used for analysis. Magnesium and calcium levels were determined using inductively coupled plasma atomic emission spectrometry by monitoring atomic emission with an Applied Research Labora-

tories 35000 C ICP. Results are expressed as mole ion per mole haemoglobin tetramer.

Haematocrit values of red cell suspensions were obtained using 10 μl micropipette capillary tubes; these tubes were centrifuged in an IEC MB haematocrit centrifuge for 4 min. MCHC values were calculated from red cell suspension haemoglobin concentrations and haematocrit values.

Methaemoglobin concentrations were determined as described by Dubowski (1960), with the modifications suggested by Gruca & Grigg (1980). Red cell suspensions were lysed using approximately one drop of Triton X-100 per 10 ml of suspension. As snake red cells are nucleated, it was necessary first to remove cell debris from the haemolysates to obtain consistent results; this was accomplished by centrifugation at 1000 *g* for 5 min. Methaemoglobin concentrations were then determined spectrophotometrically on the supernatant fractions.

All chemicals and relative molecular mass standards were from Sigma Chemical Co. (St Louis, MO) with the exception of SDS which was from Bio-Rad (Richmond, CA).

Results

The apparent relative molecular mass of haemoglobins from adult and foetal *T. elegans* was approximately 54000, on the basis of the elution of protein standards from the Sephadex G 100–120 column. However, when run together, adult garter snake haemoglobin co-eluted with adult human haemoglobin (Fig. 1),

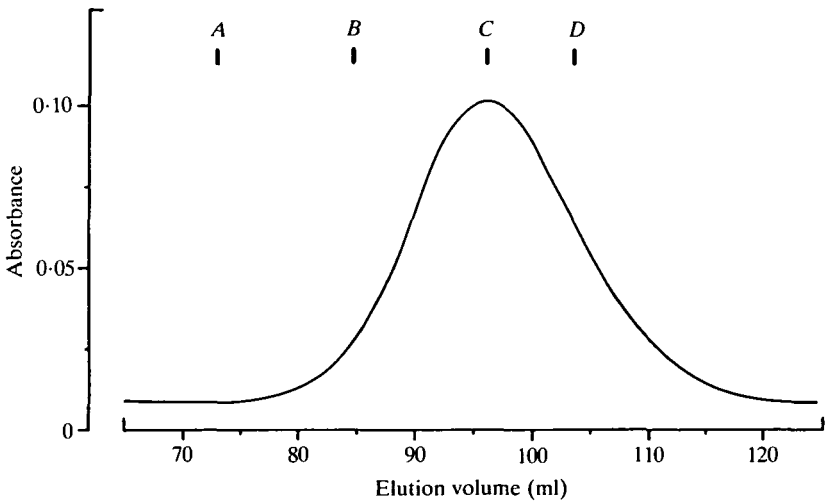

Fig. 1. Sephadex G 100–120 column chromatography of an equimolar mixture of adult *Thamnophis elegans* and human haemoglobins monitored for absorbance at 280 nm. Calibrants: (*A*) blue dextran; (*B*) bovine serum albumin; (*C*) adult human haemoglobin; (*D*) ovalbumin. Not shown are the elution volumes of myoglobin (135 ml) and tryptophan (160 ml).

and the haemoglobin from stage 30 and 36 foetuses co-eluted with adult snake haemoglobin.

Electrophoresis of the native cyanmethaemoglobins of adult and stage 30 and 36 foetuses resulted in one relatively broad electrophoretic band for each (Fig. 2). When mixed and run together, these haemoglobins co-electrophoresed. Comparable results (not shown) were obtained with carboxyhaemoglobin. SDS slab gel electrophoresis of globin chains from adult and stage 30 and 36 foetuses showed a single band for each (Fig. 3) with a relative molecular mass of 15 400 based on the standards. However, when snake and human globins were electrophoresed in the same lane of the gel, the snake globin co-electrophoresed with the human beta-globin chain. Electrophoresis of carboxymethylated globin chains from adults and stage 30 foetuses in the presence of urea at low pH resulted in three electrophoretically separable bands for each (Fig. 4). Globins from stage 30 foetuses co-electrophoresed with those of the adult. Comparable results (not shown) were obtained with stage 36 foetal globins. Human haemoglobin, run as a control, showed two bands.

Oxygen-binding studies yielded very similar functional characteristics for stripped adult and foetal haemoglobins in the absence and presence of 2 mmol l^{-1} ATP. In the absence of ATP, the Hill coefficient for each haemoglobin averaged about 3·0 over most of the pH range examined but increased to about 4·0 at pH values between 6·8 and 7·2 (Figs 5 and 6). P_{50} values ranged from 2·0 to 5·0 mmHg between pH 6·5 and 8·5. Adult and foetal haemoglobins showed little Bohr effect with Bohr coefficients, $\Delta \log P_{50}/\Delta pH$, of −0·06 ($N = 24$) and −0·01 ($N = 12$), respectively (with 95 % confidence intervals of −0·10 to −0·02 and −0·12 to 0·09, respectively), over all pH values tested (Fig. 6). These Bohr coefficients were significantly different ($P < 0·05$, F-test, Snedecor & Cochran, 1967).

In the presence of 2 mmol l^{-1} ATP, adult and foetal stripped haemoglobins had a lower oxygen affinity at pH values below 8·0 (Fig. 6). Adult and foetal haemoglobins showed significant Bohr effects with Bohr coefficients of −0·37 ($N = 24$) and −0·39 ($N = 12$), respectively (with 95 % confidence intervals of −0·44 to −0·29 and −0·60 to −0·18, respectively), over pH values of 7·0–8·0. These Bohr coefficients were not significantly different. Over this range of pH, Hill coefficients did not exceed 3·4 (Figs 5 and 6).

Maternal red cells had higher NTP levels than the red cells of their foetuses in all cases, with a maternal–foetal difference of 0·93 ± 0·68 mol NTP mol^{-1} haemoglobin tetramer (Table 1). As determined by HPLC, the primary NTP in the red cells of both maternal and foetal bloods was ATP (Table 2). No 2,3-DPG was detected in either adult or foetal red cells.

Whereas maternal and foetal haemolysates had similar molar magnesium to haemoglobin ratios, foetal haemolysates had significantly higher calcium to haemoglobin ratios (Table 2). Maternal snakes had significantly higher MCHC values than foetal snakes, with a maternal–foetal difference of 0·76 ± 0·62 mmol l^{-1} (Table 2). The methaemoglobin levels in adult and foetal cells were low and not significantly different for centrifuged lysates (Table 2).

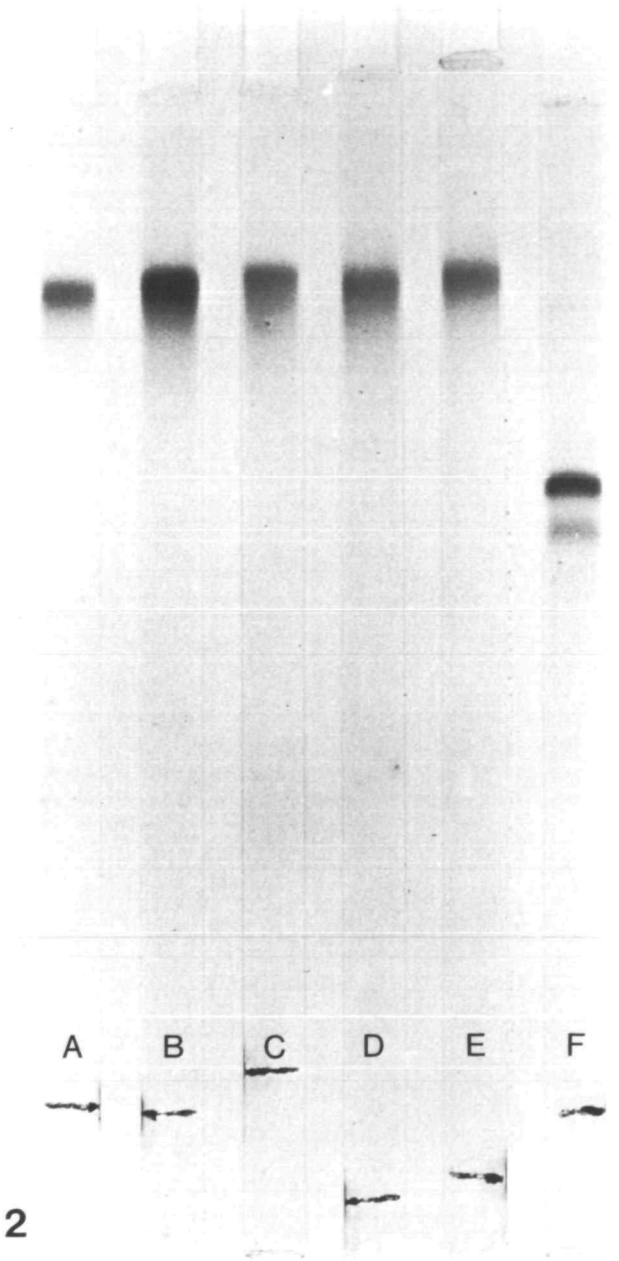

Fig. 2. Native PAGE of cyanmethaemoglobins of *Thamnophis elegans* stage 30 foetus (A); stage 30 foetus + adult (B); adult (C); stage 36 foetus + adult (D); stage 36 foetus (E); and adult human haemoglobin (F). Gels were 7·5% acrylamide, pH 8·9.

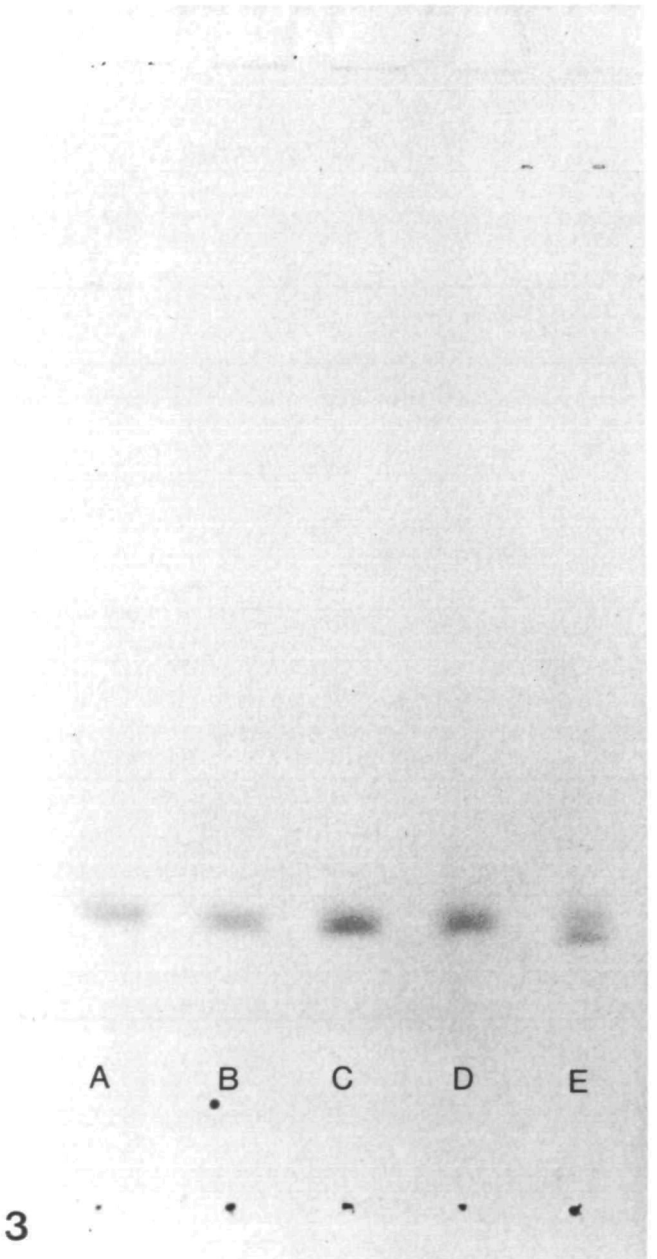

Fig. 3. SDS–PAGE of the globin chains of *Thamnophis elegans* stage 30 foetus + adult (A); stage 30 foetus (B); stage 36 foetus (C); adult (D); and adult human haemoglobin (E). Gels were 15 % acrylamide, pH 8·8.

Discussion

In some viviparous vertebrates, maternal–foetal oxygen transfer may be facilitated by the presence of a unique foetal haemoglobin which has an

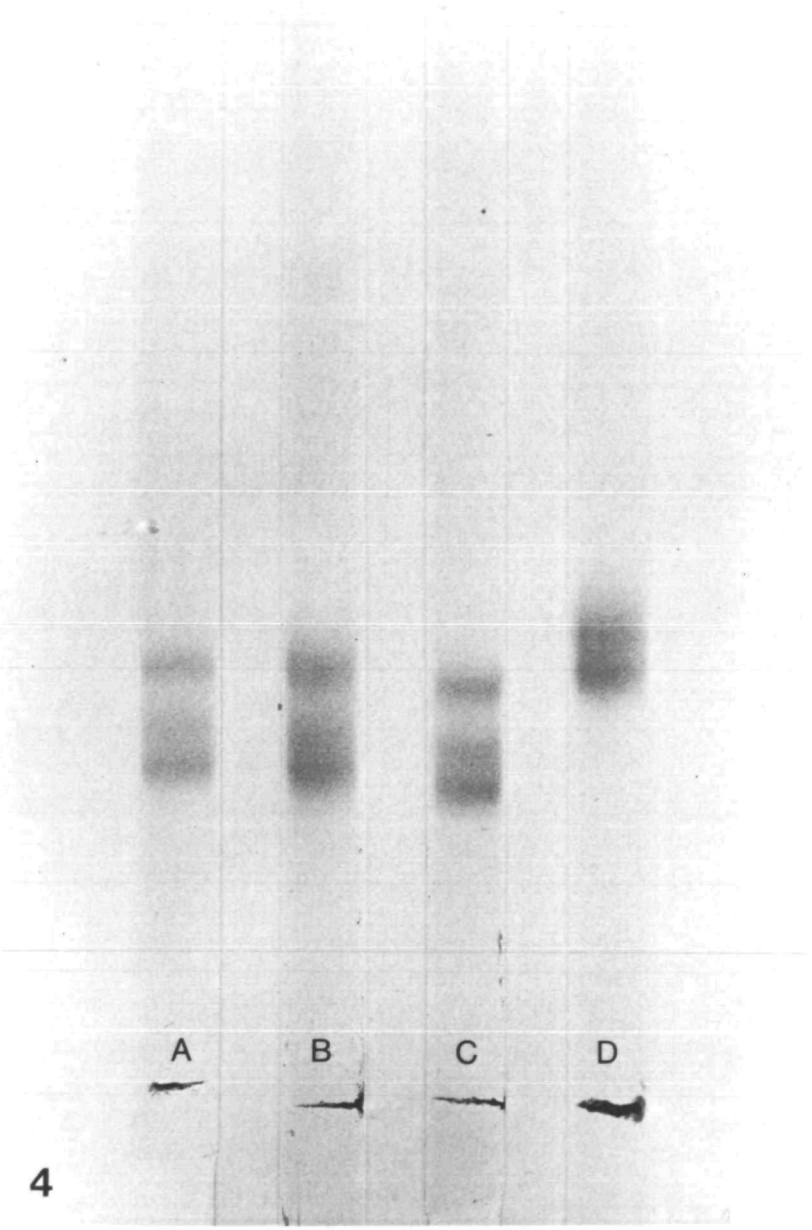

Fig. 4. Low pH/urea PAGE of the globin chains of *Thamnophis elegans* stage 30 foetus (A); stage 30 foetus + adult (B); adult (C); and adult human haemoglobin (D). Gels were 6·25 mol l^{-1} urea, 6·0 % acrylamide, pH 2·2.

intrinsically higher affinity for oxygen than does the adult haemoglobin. Since Pough (1977) reported ontogenetic changes in the haemoglobins of *T. sirtalis*, it is possible that different foetal and maternal haemoglobins underlie the difference in

red cell oxygen affinities of foetal and maternal *T. elegans* found by Manwell (1960). The data from the present study, however, do not support this hypothesis.

Adult and foetal haemoglobins were structurally indistinguishable by several forms of electrophoresis and by gel filtration chromatography. Furthermore, based on SDS–PAGE and chromatography, these native molecules appear to be tetrameric with a subunit relative molecular mass of about 16000. [Based on the elution of protein standards from the gel filtration column, the apparent relative molecular mass of these molecules was 54000. This value appears low for a vertebrate haemoglobin, and may be due to the tendency of some haemoglobins to adsorb to Sephadex gel thereby yielding lower values than expected (Wilkins, 1970; Iuchi, 1973). However, *T. elegans* haemoglobin co-eluted with adult human haemoglobin when chromatographed together, suggesting a native relative molecular mass of about 64000.]

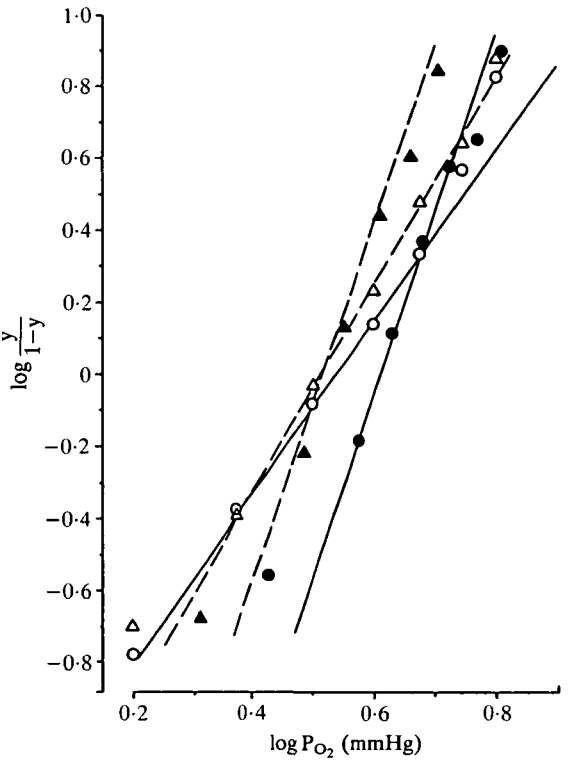

Fig. 5. Typical Hill plots for *Thamnophis elegans* maternal (circles) and foetal (triangles) haemoglobins at pH 6·8 (filled symbols) and pH 8·5 (open symbols), where y is the fraction of total haemoglobin that is oxygenated. Lines were drawn by linear regression of data for haemoglobin which was 25–75 % saturated with oxygen. Slopes of the lines for adult and foetal haemoglobin, representing the Hill coefficient, at pH 6·8 were 5·1 and 5·3, respectively, and at pH 8·5 were 2·4 and 2·8, respectively. These data were also used to determine haemoglobin P_{50} values (i.e.) P_{O_2} value where $y = 0{\cdot}5$ and $\log[y/(1-y)] = 0$ in Fig. 6.

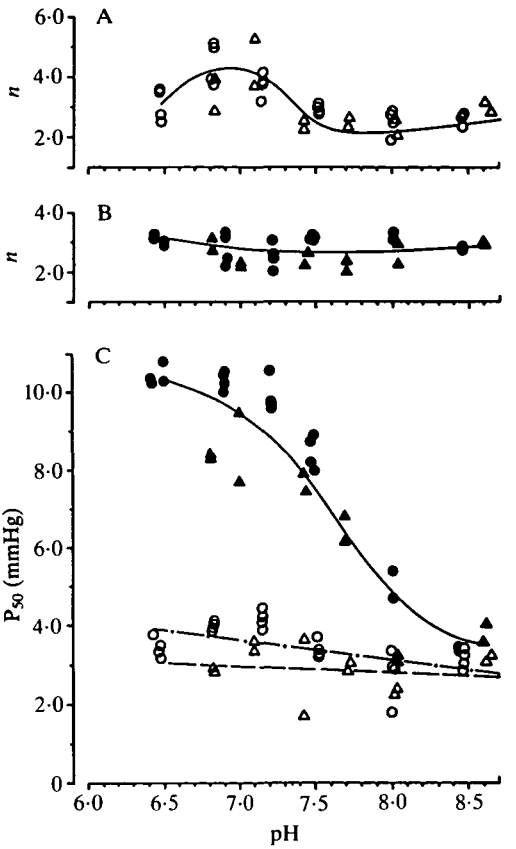

Fig. 6. Hill coefficient (n) (A,B) and P_{50} (C) plotted *vs* pH for maternal and foetal haemoglobins where circles are maternal values and triangles are foetal values in the absence (open symbols) and presence (closed symbols) of 2 mmol l^{-1} ATP. Curves were fitted to data by eye. Straight lines were drawn by linear regression of data generated for adult (—·—) and for foetal (— —) haemoglobins in the absence of ATP. Slopes of these lines were −0·44 and −0·10, respectively, with 95% confidence intervals of −0·74 to −0·14 and −0·74 to 0·54, respectively.

The results of the native PAGE and low pH/urea PAGE together suggested that the snake haemoglobin may exist as an asymmetrical tetramer, as has been suggested for the haemoglobins of some salmon and sharks (Tsuyuki & Ronald, 1971; Andersen *et al.* 1973). However, as vertebrate haemoglobins are generally symmetrical tetramers (Coates, 1975), it is more likely that the snake haemoglobin actually exists as two symmetrical tetramers. This could explain the relatively broad band seen by native PAGE (Fig. 2).

Adult and foetal haemoglobins had relatively high oxygen affinities and low sensitivities to pH in the absence of ATP (Fig. 6). Furthermore, both haemoglobins showed strikingly high Hill coefficients near pH 7·0 in the absence of ATP (Fig. 6). Although similar values have been reported for whole blood from two

Table 1. *Moles of NTP per mole of haemoglobin tetramer (Hb) of maternal and foetal red cells*

	Foetal NTP/Hb	Maternal NTP/Hb	Maternal–foetal difference
	2·45*	2·78	0·33
	2·24*	3·15	0·91
	2·34*	3·45	1·11
	3·21	3·27	0·06
	1·96	2·49	0·53
	1·94	2·87	0·93
	2·22	3·54	1·32
	1·83	4·09	2·26
Mean ± S.D.	2·27 ± 0·43	3·21 ± 0·50	0·93 ± 0·68†

* Values for foetuses at stage 30–32 of development. All other values are for foetuses at stage 35–36.

† Significant at $P < 0{\cdot}005$ by paired difference test.

Data are paired for mothers and blood pooled from their own foetuses.

No differences were noted between stage 30–32 and 35–36 foetuses.

Table 2. *Maternal and foetal erythrocytic mole ATP per mole GTP, mole Mg^{2+} per mole haemoglobin (Hb) tetramer, mole Ca^{2+} per mole Hb tetramer, mean corpuscular haemoglobin concentrations (MCHC), and percentage of total haemoglobin that is methaemoglobin (MetHb)*

	ATP/GTP‡	Mg^{2+}/Hb	Ca^{2+}/Hb*	MCHC†·‡ ($mmol\,l^{-1}$)	MetHb (%)
Maternal	6·8, 6·9	1·73 ± 0·32 ($N = 6$)	0·02 ± 0·01 ($N = 6$)	3·62 ± 0·25 ($N = 6$)	4·31 ± 2·55 ($N = 6$)
Foetal	7·1, 7·6	1·34 ± 0·51 ($N = 4$)	0·24 ± 0·24 ($N = 4$)	2·86 ± 0·51 ($N = 6$)	2·40 ± 1·52 ($N = 3$)

* Significant at $P < 0{\cdot}005$ by Student's *t*-test.

† Significant at $P < 0{\cdot}025$ by paired difference test.

‡ Data represent determinations for mothers and *their* foetuses.

lizards (Pough, 1969) and a rattlesnake (MacMahon & Hamer, 1975), this is a very unusual finding. Since human sickle cell haemoglobin shows a Hill coefficient of 5–6 owing to haemoglobin polymerization (Gill *et al.* 1978), our data suggest that *T. elegans* haemoglobins may also show some tetramer–tetramer interaction at pH 7·0. In the presence of ATP, the haemoglobins from both sources showed an appreciably reduced oxygen affinity, a marked pH sensitivity and a decreased Hill coefficient near pH 7·0 (Fig. 6). Thus, adult and foetal haemoglobins had similar functional characteristics and both were similarly responsive to ATP.

That maternal red cell levels of ATP were appreciably higher than foetal levels

(Table 1) suggests that a difference in organic phosphate concentrations is an important molecular strategy underlying the difference in oxygen affinities of maternal and foetal red cells. A doubt about this interpretation arises from a consideration of the stoichiometry of the organic phosphate–haemoglobin interaction: one polyanion interacts with one tetrameric haemoglobin. In both foetal and maternal *T. elegans* red cells, the ATP to haemoglobin ratio was significantly greater than one, suggesting that the haemoglobin in both cell types may be saturated with ATP. Therefore, it becomes necessary to consider intracellular levels of divalent cations.

Magnesium and calcium ions bind to ATP in a 1:1 molar ratio forming a fairly stable complex which binds to haemoglobin more weakly than does ATP alone. Thus, these cations increase the oxygen affinity of haemoglobin by decreasing the ATP–haemoglobin interaction (White *et al.* 1964; Lehninger, 1975; Weber & Lykkeboe, 1978; Burton, 1980; Ingermann & Terwilliger, 1981). *T. elegans* foetal and maternal NTP/haemoglobin ratios were about 2·3 and 3·2, respectively, and total divalent cation/haemoglobin ratios were about 1·6 and 1·8, respectively (Table 2). If complete complexing were to occur between the divalent cations and NTP, foetal but not maternal red cells would have less than saturating levels of free NTP available to interact with haemoglobin (0·7 and 1·4 NTP haemoglobin^{-1}, respectively). This should promote a higher oxygen affinity in foetal red cells. Magnesium and calcium ions interact with membrane phospholipids and sedimentable material (Rose, 1968; Sanui, 1970; Rubin, 1975; Sanui & Rubin, 1979). Since the divalent cation concentrations were determined on haemolysates in this study, the values did not include divalent cations bound to the intra- and extracellular faces of the red cell membranes. Therefore, the divalent cation levels may be underestimates; those cations bound to the extracellular face would probably contribute to the nonmembrane-bound intracellular concentrations *in situ*. If so, the amount of free NTP should be lowered. This should enhance the functional effect of the maternal–foetal difference in red cell organic phosphate concentrations.

High concentrations of organic phosphates are associated with high concentrations of protons in the cell; the relatively low intracellular pH exists because of the Gibbs–Donnan equilibrium (Duhm, 1971, 1976). Since there were appreciably higher organic phosphate concentrations in maternal than in foetal red cells, this should result in lower intracellular pH values in maternal than in foetal red cells. In the absence of ATP, *T. elegans* haemoglobin was relatively insensitive to changes in pH. However, in the presence of ATP, the haemoglobin was much more sensitive to pH (Fig. 6). Therefore, the higher maternal than foetal red cell organic phosphate levels may promote the lower oxygen affinity of maternal red cells *via* the Bohr effect in the presence of organic phosphate.

MCHC values of maternal and foetal *T. elegans* red cells were significantly different (Table 2). Such a maternal–foetal difference has also been reported for the teleost, *Embiotoca lateralis* (Ingermann & Terwilliger, 1982) and the cottonmouth snake, *Agkistrodon piscivorus* (Birchard *et al.* 1984). A decrease in MCHC

has been correlated with an increase in red cell oxygen affinity apparently due to a lessened haemoglobin–organic phosphate interaction (Forster, 1972; Sinet *et al.* 1976; Lykkeboe & Weber, 1978). Since the foetal *T. elegans* red cell had a lower MCHC than did the maternal cell, the foetal red cell should have a higher affinity because of this difference. This should enhance the difference in oxygen affinity between maternal and foetal bloods.

Although methaemoglobin does not reversibly bind oxygen, it does tend to increase the oxygen affinity of the other, nonmethaemoglobin subunits (Darling & Roughton, 1942; Enoki *et al.* 1969). Therefore, methaemoglobin tends to increase the overall oxygen affinity but it decreases the oxygen-carrying capacity of the blood. Several reports have suggested that reptilian red cells possess exceptionally high proportions of methaemoglobin (Dessauer, 1970; Wood & Lenfant, 1976; Pough, 1977). This interpretation has been challenged by Gruca & Grigg (1980). As the amount of methaemoglobin in maternal and foetal *T. elegans* bloods could affect maternal–foetal oxygen transfer, methaemoglobin levels were quantified. Levels of methaemoglobin were relatively low and similar in foetal and adult blood samples (Table 2).

The oxygen affinity difference noted by Manwell (1960) between maternal and foetal *T. elegans* red cell suspensions does not appear to be due to different maternal and foetal haemoglobins. Rather, it is likely to be due primarily to different red cell concentrations of ATP, and possibly also to different MCHC values. In this respect, the molecular strategies underlying maternal–foetal oxygen transfer in *T. elegans* are similar to those of the viviparous snake *A. piscivorus* (Birchard *et al.* 1984). As ATP levels in nucleated red cells respond quickly to oxygen availability (Greaney & Powers, 1977; Ingermann *et al.* 1983), this set of molecular strategies may be particularly well-suited to allow the blood of the *T. elegans* neonate rapidly to assume functional characteristics of adult blood and thus facilitate the onset of independent life.

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