*Aust. J. Biol. Sci.*, 1978, **31**, 337–44

# Oxygen Binding of Monotrene Haemoglobins II.* Fixation of Functionally Non-equivalent Haemoglobins in the Echidna Population

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*Abstract*

The echidna, *Tachyglossus aculeatus*, has two haemoglobins and polymorphic variants of both have been described. Haemoglobins Hb-IA, Hb-IB and Hb-IIA have been separated and their oxygen binding parameters studied. Hb-IIA has a markedly higher oxygen affinity and lower cooperativity than the polymorphs of haemoglbin I. In phosphate buffer, pH 7·1 and 25°C, Hb-IIA has an oxygen half-saturation pressure ($P_{50}$) of 1·3 kPa and a Hill coefficient of 2·4, whereas the polymorphic forms of Hb-I have $P_{50}$ values of about 1·6 kPa and Hill coefficients of about 2·9. Differences in diphosphoglyceric acid interactions account for some of these differences. When the polymorphic forms of haemoglobin I were compared at pH 7·1, Hb-IA had a $\Delta H^0$ value of −27·9 kJ/mol and a Hill coefficient of 2·9, whereas Hb-IB had a $\Delta H^0$ value of −31·7 kJ/mol and a Hill coefficient of 3·0.

These results are discussed in terms of selection value to the animal and in relation to the molecular structure of haemoglobins.

## Introduction

The echidna, *Tachyglossus aculeatus*, has two haemoglobins and polymorphic variants of both have been described (Cooper *et al.* 1973). Polymorphic variants of the minor component Hb-II are found in Tasmania while polymorphic forms of the major component Hb-I are found on the Australian mainland. Hb-IB is found mainly in eastern Australia, while Hb-IA is found in central and western Australia. In central coastal New South Wales echidnas are found which have Hb-IIA and are heterozygous for Hb-IA and Hb-IB. In a previous study oxygen binding properties of platypus and echidna haemolysates (Hb-IB, Hb-IIA type) were described (Hosken 1978). It is of interest to determine whether the distribution of echidna haemoglobins is the result of natural selection, in which case differences in oxygen binding properties might be found, or due to random genetic drift, in which case the haemoglobins would be selectively neutral (Kimura 1968; King and Jukes 1969; Ohta 1974).

The amino acid sequences have been determined for the α- and β-chains of echidna Hb-IB (Whittaker *et al.* 1972, 1973), Hb-IA (Dodgson *et al.* 1974) and for the minor component Hb-IIA (Thompson *et al.* 1973). In the present study Hb-IA, Hb-IB and Hb-IIA were isolated and the oxygen binding parameters determined. These results are interpreted in relation to the selection value to the animal and in relation to the stereochemistry of the haemoglobin molecule.

* Part I, *Aust. J. Biol. Sci.*, 1978, **31**, 327–36.

## Materials and Methods

Blood samples of about 5 ml each were taken from three echidnas found in the Hunter Valley region of New South Wales. Starch–gel electrophoresis of the echidna haemolysates was carried out at pH 8·6 as described by Hosken (1978). When the resulting bands were compared with those described by Cooper *et al.* (1973), the blood of one animal contained Hb-IA and Hb-IIA, the second animal had Hb-IB and Hb-IIA, and the third had Hb-IA, Hb-IB and Hb-IIA. Cells were collected from the blood by centrifugation, washed in 0·9% (w/v) saline and the haemolysate prepared as described by Hosken (1978).

Haemoglobins Hb-IB, Hb-IA and Hb-IIA were separated on a DEAE–Sephadex column (2·2 by 15 cm) using a gradient buffer of 0·05 M tris–HCl, pH 8·2 gradually decreasing to a limit of pH 7·5 (Whittaker *et al.* 1972).

Oxygen dissociation curves of the dialysed haemoglobin fractions were determined at various temperatures and pH values using the spectrophotometric method described previously (Hosken 1978).

2,3-Diphosphoglycerate (Sigma) was converted to the free acid (DPG) by passing it through a 3 by 12 mm column of Dowex 50W X8 in the $H^+$ form. A 5 mM solution of DPG in 0·05 M bis–tris was used for the addition of DPG to haemoglobin 'stripped' of DPG (Benesch *et al.* 1969).

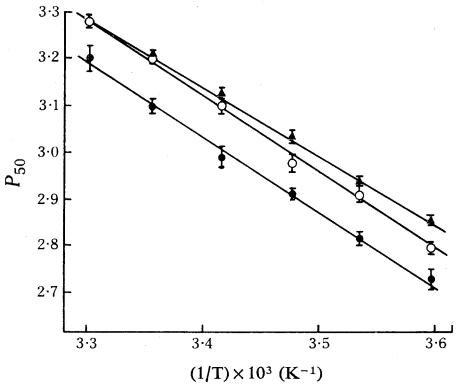


**Fig. 1.** Effect of temperature on oxygen affinity of echidna haemoglobins measured as $\log P_{50}$ (Pa). Oxygen equilibria were determined in phosphate buffer, ionic strength 0·2 and pH 7·1.
▲ Echidna Hb-IA.
○ Echidna Hb-IB.
● Echidna Hb-IIA.

## Results

The isolated echidna haemoglobins, Hb-IA, Hb-IB and Hb-IIA, were dialysed against phosphate buffer, ionic strength 0·2 and pH 7·1, and oxygen dissociation curves were determined at temperatures between 5 and 30°C. For each temperature the fractional saturation ($Y$) for each increment in partial pressure of oxygen was plotted as $\log Y/(1-Y)$ versus $\log p_{O_2}$. A series of straight lines was obtained and from the line of best fit at each temperature the pressure at which the haemoglobin is 50% saturated with oxygen ($\log P_{50}$) and Hill coefficient ($n$) were determined. Hb-IIA with a Hill coefficient of $2{\cdot}44\pm0{\cdot}24$ ($\pm$s.d.) had a significantly lower level of cooperativity than Hb-IA ($n = 2{\cdot}94\pm0{\cdot}13$) and Hb-IB ($n = 3{\cdot}03\pm0{\cdot}16$). The Van't Hoff plot of $(1/T)\times 10^3$ versus $\log P_{50}$ at each temperature yielded the three straight lines shown in Fig. 1. The results in Fig. 1 show that at all temperatures Hb-IIA has a higher oxygen affinity than either Hb-IA or Hb-IB. While there is some overlap of points in Hb-IA and Hb-IB, Hb-IA generally has a slightly lower oxygen affinity than Hb-IB at all temperatures examined.

Applying the equations $\Delta G^0 = \Delta H^0 - T\Delta S^0$ and $\Delta G^0 = -RT\ln K$ to the lines of best fit, values for enthalpy $\Delta H^0$, entropy $\Delta S^0$ and free energy $\Delta G^0$ were obtained and are shown in Table 1.

The results in Table 1 show that Hb-IIA with a $P_{50}$ value at 25°C of 1·3 kPa* and a $\Delta G^0$ value of 5·6 kJ/mol has a higher oxygen affinity than either of the polymorphs of Hb-I which have a $P_{50}$ value at 25°C of about 1·6 kPa and a $\Delta G^0$ value of 6·2 kJ/mol. Oxygen binds to Hb-IA with a $\Delta H^0$ value of −27·9 kJ/mol, which is significantly less exothermic than the reactions with Hb-IB and Hb-IIA. As Hb-IA and Hb-IB were isolated from different animals, the possibility must be considered that slight differences in preparative techniques may contribute to these differences.

**Table 1. Oxygen binding parameters for echidna haemoglobins**

Oxygen equilibria were determined in phosphate buffer, ionic strength 0·2 and pH 7·1

| | Hb-IA | Hb-IB | Hb-IIA |
|---|---|---|---|
| $P_{50}$ (kPa at 25°C) | 1·64 | 1·60 | 1·29 |
| $\Delta H^0$ (kJ/mol) | −27·95±0·46 | −31·71±0·92 | −31·46±2·05 |
| $\Delta G^0$ (kJ/mol) | 6·23±0·04 | 6·15±0·08 | 5·65±0·17 |
| $\Delta S^0$ (J/deg) | 114·68±1·59 | 127·11±3·05 | 124·56±6·99 |

In order to examine the effects of heterotropic interaction on the thermodynamics of oxygen binding, oxygen dissociation curves at different temperatures were determined for haemoglobins 'stripped' of organic phosphates. They were also determined at pH 9·2 which is above the p$K$ values of ionisable groups involved in heterotropic interactions and so the heat contribution of these interactions need not be considered.

**Table 2. Effect of absence of heterotropic interactions on the oxygen affinity of echidna haemoglobins**

For the normal haemoglobins, oxygen equilibria were determined in 0·1 M tris, pH 9·2. For the 'stripped' haemoglobins, oxygen equilibria were determined in 0·05 M bis-tris, pH 7·1

| Haemoglobin | $P_{50}$ (kPa at 25°C) | $\Delta H^0$ (kJ/mol) | Haemoglobin | $P_{50}$ (kPa at 25°C) | $\Delta H^0$ (kJ/mol) |
|---|---|---|---|---|---|
| (*a*) Normal haemoglobins | | | (*b*) Stripped haemoglobins | | |
| Hb-IB | 0·31 | −41·00±2·93 | Hb-IB | 0·41 | −42·26±2·93 |
| Hb-IIA | 0·27 | −42·68±4·60 | Hb-IA | 0·60 | −41·00±2·93 |
| | | | Hb-IIA | 0·57 | −41·00±2·51 |

The results of these experiments are shown in Table 2. The results in Table 2 also show that when the haemoglobins are stripped of organic phosphate the enthalpy is reduced from 8·4 to 12·6 kJ/mol as a result of reduced heterotropic interactions, and the oxygen affinity increases markedly. It is uncertain whether the slight differences between the oxygen affinities of the stripped preparations are of functional significance or whether they are due to differences in experimental technique. At pH 9·2 the affinity of the haemoglobins is increased slightly compared with the values obtained when stripped of organic phosphates, while $\Delta H^0$ is the same as that obtained for the stripped haemoglobins. In the presence of organic phosphate Hb-IA and Hb-IB have comparable oxygen affinities ($P_{50}$ at 20°C = 1·3 kPa). To examine the effect of DPG on modulating oxygen affinity of echidna haemoglobins, increasing

* 1 kPa = 7·5 mm Hg.

amounts of DPG were added to preparations of Hb-IA and Hb-IIA stripped of organic phosphate and the $P_{50}$ value was estimated for each increase in the number of moles of DPG per mole of haemoglobin. These results are shown in Fig. 2. The results suggest that DPG decreased the oxygen affinity of Hb-IA and Hb-IIA to much the same extent until they have about 0·75 mol of DPG per mole of haemoglobin. At this point DPG has little further effect on Hb-IIA but with increasing amounts of DPG the affinity of Hb-IA is decreased further.

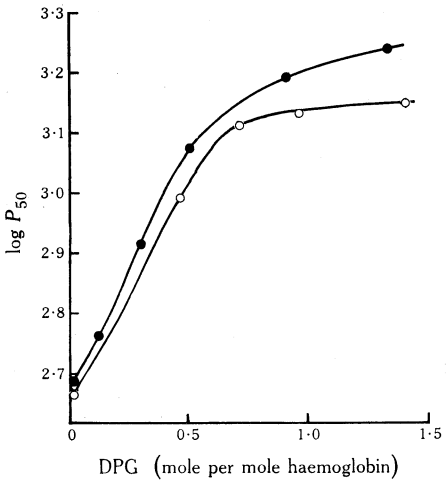


**Fig. 2.** Effect of addition of DPG on oxygen affinity of echidna haemoglobin expressed as log $P_{50}$ (Pa). Oxygen equilibria were determined in 0·05 M bis–tris buffer, pH 7·1, 20°C.
● Hb-IA.
○ Hb-IIA.

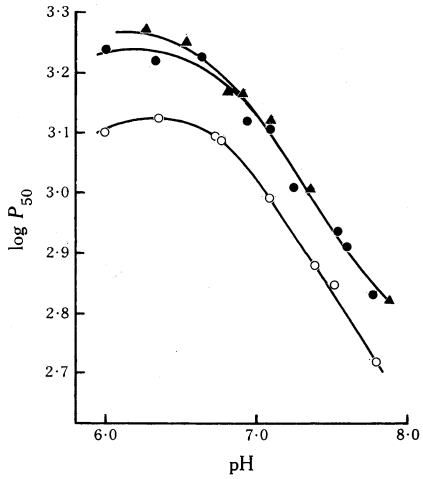


**Fig. 3.** Bohr curve of echidna haemoglobins. Oxygen affinity is expressed as log $P_{50}$ (Pa), the pressure at which the haemoglobins are 50% saturated with oxygen at measured pH values. Oxygen equilibria were determined in phosphate buffer, ionic strength 0·2, at 20°C.
▲ Hb-IA.
● Hb-IB.
○ Hb-IIA.

The effect of hydrogen ions on the oxygen affinity of echidna haemoglobins Hb-IA, Hb-IB and Hb-IIA was examined by adjusting haemoglobin solutions dialysed against phosphate buffer, ionic strength 0·2 and pH 7·1, to various pH values between 6·0 and 8·0, using 0·05 M HCl or 0·5 M NaOH. Oxygen dissociation curves were determined and $P_{50}$ values were estimated at each pH from the Hill plot of log $Y/(1-Y)$ versus log $p_{O_2}$. The Bohr curve for the echidna haemoglobins is shown in Fig. 3. Hb-IIA shows a higher oxygen affinity at all pH values between 6·0 and 8·0. The slope of the alkaline Bohr curve, $\Delta \log P_{50}/\Delta \mathrm{pH}$, is shown in Table 3 together with average values for the Hill coefficient ($n$).

Table 3 shows that the slope of the alkaline Bohr curve from pH 6·8 to pH 8·0 is similar in Hb-IA, Hb-1B and Hb-IIA. At pH values of about 6·5, Hb-IB appeared to have a slightly higher oxygen affinity than Hb-IA (Fig. 3), whilst the affinity of Hb-IIA was considerably higher. Hb-IIA again had a low level of cooperativity as indicated by a Hill coefficient of 2·3. In both the thermodynamic experiments and the Bohr effect experiments the Hill coefficient of Hb-IA ($n = 2·8$) was slightly less than that of Hb-IB ($n = 3·0$).

**Table 3. Bohr effect of echidna haemoglobins measured as $\Delta\log P_{50}/\Delta pH$**

Pressure was measured in pascals. Cooperativity is expressed as the Hill coefficient ($n$)

| | $n$ | $\Delta\log P_{50}/\Delta pH$ |
|---|---|---|
| Hb-IA | 2·8±0·3 | 0·34±0·03 |
| Hb-IB | 3·0±0·3 | 0·38±0·03 |
| Hb-IIA | 2·3±0·1 | 0·32±0·03 |

## Discussion

Echidnas have two haemoglobins, Hb-I and Hb-II, both of which exist in polymorphic forms. Haemoglobin Hb-IIA comprises 20% of the total haemoglobin and Hb-I the remaining 80%. Echidnas from central and western areas of Australia have Hb-IA, whilst those in south-eastern Australia have Hb-IB. A hybrid zone exists, extending outwards from central coastal New South Wales, in which echidnas have either Hb-IA or Hb-IB or are heterozygous for Hb-IA and Hb-IB. Echidnas on mainland Australia have Hb-IIA, but polymorphism of this haemoglobin occurs in Tasmania (Cooper *et al.* 1973). Haemoglobin Hb-IIA has a markedly higher oxygen affinity than Hb-IA and Hb-IB; this higher affinity is related to a difference in the DPG interaction. It seems that Hb-IIA has arisen by an $\alpha$ gene duplication and in the course of time mutations have occurred leading to functional divergence. The presence of 20% Hb-IIA in echidna blood suggests that either there has not been sufficient time for the effect of genetic load to select either for or against Hb-IIA, or alternatively Hb-IIA has some specialized function essential to echidna respiratory physiology. The observed distribution of the polymorphic forms of echidna Hb-I on the Australian mainland may have occurred due to the influence of natural selection or it may have occurred by fixation of mutants resulting from random genetic drift (Kimura 1968). If differences in functional properties of the polymorphic form of echidna Hb-I can be shown, strong support will be given to the concept that the observed geographical distribution is the result of natural selection.

In the present study slight differences in the properties of Hb-IA and Hb-IB were detected. These differences border on the limits of experimental accuracy used in their determination. The enthalpy for Hb-IA was slightly higher than that for Hb-IB, the level of cooperativity of Hb-IA was slightly lower in the range of temperature and pH examined, and the oxygen affinity of Hb-IA was slightly lower than that of Hb-IB at pH 6·5. It seems reasonable that these differences would be selected for and could account for the observed geographical distribution of Hb-I. South-eastern Australia in general has a cooler climate than the dry warmer central and western parts of the continent. Temperature regulation of echidnas deteriorates at

ambient temperatures of 35°C and above (Griffiths 1968) and the higher enthalpy of Hb-IA compared to Hb-IB infers that variation in temperature would not affect the oxygen affinity of Hb-IA as much as that of Hb-IB. This may have some selective advantage.

The amino acid sequence has been determined for echidna Hb-IB $\beta$-chain (Whittaker *et al.* 1972) and $\alpha$-chain (Whittaker *et al.* 1973), as well as for the minor component Hb-IIA $\alpha$- and $\beta$-chains (Thompson *et al.* 1973). The $\beta$-chain is common to both Hb-I and Hb-II, whereas the $\alpha$-chain of echidna Hb-IIA differs at nine positions from that of Hb-IB. The amino acid sequence has also been determined for Hb-IA from a Western Australian echidna (Dodgson *et al.* 1974) and while the $\beta$-chain was again identical to that of Hb-IB and Hb-IIA, the $\alpha$-chain had four residues different from that of Hb-IB and 10 residues different from that of Hb-IIA. These differences are shown in Tables 4 and 5.

**Table 4. Amino acid residue differences between Hb-IA and Hb-IIA $\alpha$-chains**

| Position | | Hb-IA | Hb-IIA | Significance |
|---|---|---|---|---|
| $\alpha$ 7 | A5 | Lys | Arg | Usually Lys |
| $\alpha$ 12 | A10 | Gly | Ser | |
| $\alpha$ 23 | B4 | Glu | Asp | |
| $\alpha$ 54 | E3 | Gln | His | Usually Gln or Asp |
| $\alpha$ 56 | E5 | Lys | Arg | Usually Lys<br>Chicken A1 Arg |
| $\alpha$ 61 | E10 | Arg | Lys | Haem contact—invariant Lys |
| $\alpha$ 70 | E19 | Ala | Val | Internal position |
| $\alpha$ 78 | EF7 | Asn | Gly | |
| $\alpha$ 82 | F3 | Ala | Asp | |
| $\alpha$ 102 | G9 | Ser | Ala | |

**Table 5. Amino acid residues different between Hb-IA and Hb-IB $\alpha$-chains**

| Position | | Hb-IA | Hb-IB | Hb-IIA |
|---|---|---|---|---|
| 12 | A10 | Gly | Ser | Ser |
| 78 | EF7 | Asn | Ser | Gly |
| 102 | G9 | Ser | Ala | Ala |
| 115 | GH3 | Glu | Ala | Glu |

The echidna haemoglobins are unusual in that all haemoglobins have the same $\beta$-chain but have considerable variation in amino acid sequence in the $\alpha$-chain. Eutherian animals usually show a greater variability of the $\beta$-chain.

Studies on human haemoglobin mutants have shown that mutants with increased oxygen affinity and lowered cooperativity usually have substitutions at either the $\alpha_1\beta_2$ contact, the *C*-terminal end of the $\beta$-chain, or the DPG binding site between the $\beta$-chains (Bellingham 1976). When the differences in Hb-IA and Hb-IIA are examined (Table 4), the only substitution involving residues with defined functional roles (Goodman *et al.* 1975) is $\alpha$70 E19. Man and most species have Val at this position. It is doubtful that this Ala substitution in an internal position, although having different stereochemical properties, would account for all the differences in functional properties between Hb-IA and Hb-IIA.

Echidna Hb-IA and Hb-IIA show an unusually high degree of substitution in the vicinity of the E helix of the α-chain, suggesting rapid evolution of this part of the molecule. The propionate side chain of the haem makes important contacts at CD3 His and E10 Lys and there are further haem contacts at E7 His and E11 Val (Perutz 1976). It is probable that the α-chain amino acid substitutions at A5, E3, E5, E10, and E19 are collectively related to changes in the stereochemical relation between the haem and the E helix. The most significant substitution is E3 Gln→His which involves the replacement of a neutral residue with a more basic residue. These substitutions could well lead to a reduction of the displacement of the iron from the haem plane so biasing the T→R equilibrium more towards the high affinity R state in Hb-IIA than in Hb-IA.

The substitution F3 Ala→Asp would account for the different electrophoretic mobility of Hb-IA and Hb-IIA, but it is unlikely to have any functional significance.

Table 5 shows only four amino acid substitutions which could possibly account for the marginal differences in oxygen binding functions between Hb-IA and Hb-IB. None of these substitutions are at known functional sites. The substitutions GH3 and A10, although involving residues with different stereochemical properties, show significant species variation (Dayhoff 1972), and are therefore unlikely to have functional significance. The substitutions at EF7 Asn→Ser and G9 Ser→Ala usually show structural constraints and are therefore more likely to account for the physiological and selective differences between Hb-IA and Hb-IB.

It therefore appears that amino acid substitutions that have occurred in echidna Hb-IIA since gene duplication have led to structural and functional divergence of the molecule from both polymorphic forms of Hb-I. The geographical distribution, apparent functional differences, and structural constraints on amino acid substitution suggest that the distribution of polymorphic forms of Hb-I are maintained by natural selection.

## Acknowledgments

This work was supported in part by the Australian Research Grants Committee. The permission of the National Parks and Wildlife to take blood samples from echidnas is gratefully acknowledged.

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Manuscript received 14 November 1977