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Molecular aspects of embryonic mouse haemoglobin ontogeny

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Embryos from C57BL/6J mice between the gestational ages of 9 and 16 days possess three embryonic haemoglobins EI, EII and EIII, the proportions of which change as a function of gestational age. Component EI, originally present at approx. 65% at day 9, decreases to approx. 20% by day 16, while component EII increases in an inverse manner to that of component EI. During this period component EIII remains essentially constant at approx. 25%. Separation of these species by ion-exchange chromatography has allowed the characterization of the Hill coefficient, Bohr effect, heat of oxygenation and binding of allosterically active organic phosphates for each component. The three components show marked functional heterogeneity and also differ from maternal haemoglobin. Oxygenation curves for whole embryonic blood show distinct deviations from simple binding behaviour. The presence of a high-affinity component within the blood samples may be accounted for by the presence of haemoglobin EI. By using parameters obtained from the study of the isolated components it has been possible to synthesize mathematically the O_2-binding curves, obtained experimentally, throughout the gestational period. The characteristics of the isolated haemoglobin components of embryonic mouse blood are discussed in terms of the changing demands for O_2 likely to be encountered by the developing embryo.

During the early stages of development in mice three types of embryonic haemoglobins (EI, EII and EIII) have been identified as being present in the nucleated erythrocytes from a number of strains of mice (Craig & Russell, 1964; Rifkind *et al.*, 1974; Popp *et al.*, 1981). The pattern of development of the three components has been determined in the F_1 hybrid of the B6TSJ strain by Brotherton *et al.* (1979*a*).

Some of the O_2-binding properties of early embryonic blood from mice have been studied by Bauer *et al.* (1975) at 12.5 days' gestation by the use of p_{50} measurements over a limited range of conditions on the unfractionated haemoglobin system. It is noteworthy that in none of these cases was a deviation from a simple O_2-binding pattern noted. In a subsequent report by Wells & Brittain (1981), non-standard equilibrium curves were noted in whole embryonic mouse blood, and attention was focused on the inadequacy of conventional O_2-binding parameters in describing functionally heterogeneous systems. A method for the chromatographic separation of the embryonic components from mice has appeared (Fantoni *et al.*, 1967), but no subsequent functional studies on the individual components were reported.

We now report on studies of the developmental pattern of embryonic haemoglobin production in a pure strain of mice (C57BL/6J). The component haemoglobins have been isolated and quantified during early development. An analysis of the O_2-binding parameters for each of the isolated components has allowed us to simulate mathematically the anomalous O_2-binding curves of the whole-blood system in terms of the contributions of each of the embryonic components. The properties of the individual components and their relative contributions to the whole embryonic erythrocyte system have been rationalized on the basis of the changing physiological demands for O_2 encountered by the embryo in its changing intra-uterine environment.

Materials and methods

Mice of the strain C57BL/6J were obtained from the Animal Laboratory of the Cancer Society of New Zealand and housed under normal conditions. Timed matings were secured by the usual methods (Whitten, 1958). Pregnant female mice were killed and embryos dissected in cold saline solution (0.9% NaCl) as quickly as possible. For embryos of gestational age greater than 11 days it was possible to separate the umbilical blood vessels from the placenta and obtain blood by capillary sampling. Where appropriate, the erythrocytes were separated

by centrifugation and lysed by freeze–thawing after three washings in cold saline solution. Haemoglobin solutions were used immediately or stored, until required, at −80°C as the carbonmonoxy derivative, in which case, before use, the CO was removed by repeated evacuation and oxygenation in the presence of strong light. In these cases complete removal of the ligand was monitored by measuring the Soret-band/α-band absorption ratios (Antonini & Brunori, 1971; Kilmartin & Rossi-Bernardi, 1971).

Electrophoresis in polyacrylamide gels was performed by the procedure of Barker (1968), and isoelectric focusing was performed as described by Riggs (1981). Quantification of each haemoglobin component in gels was achieved by using a Helina Quickscan scanning densitometer. Analysis of the densitometer scans by a Gaussian-fit non-linear least-squares procedure showed the bands to be symmetric and Gaussian in form. The presence of maternal haemoglobin in whole blood samples was identified by comparison with parallel-run gels containing authentic maternal haemoglobin as well as by adding authentic sample to the experimental sample.

Embryonic haemoglobins were isolated by ion-exchange chromatography on CM-cellulose with gradient elution as outlined by Fantoni *et al.* (1967).

O_2-binding curves were obtained by using an Aminco Hemoscan oxygen-dissociation analyser (Aminco, Silver Spring, MD, U.S.A.) fitted with Wösthoff gas-mixing pumps (Bochum, W. Germany) to allow accurate control of gas mixtures. The apparatus was modified to decrease gas leakage and flow rate (Lapennas *et al.*, 1981). Temperature control was provided with a Haake F3-C (Berlin, W. Germany) circulating-water bath.

Mathematical simulations were made by using non-linear least-squares fitting routines based on those published by Wilkins *et al.* (1974). All calculations were performed on a Compucorp 625 (Los Angeles, CA, U.S.A.) mini-computer.

In order to allow comparison with previous, less-detailed, studies, the data reported here are presented in terms of the empirical parameter p_{50} for material haemoglobin and the embryonic whole-blood haemolysate. In these cases p_{50} was determined experimentally as that O_2 concentration which leads to half-saturation. Full-saturation values were obtained in the presence of pure O_2.

Endogenous 2,3-bisphosphoglycerate was removed from haemoglobin samples by using the method of Berman *et al.* (1971) with the modifications suggested by Jelkman & Baur (1976).

Results

Whole-blood studies show that maternal blood has a lower affinity for O_2 than does whole embryonic blood, under all conditions studied. Furthermore, both types of blood show a marked increase in O_2 affinity when stripped of their endogenous organic phosphates by means of gel-filtration chromatography.

Whole-haemolysate studies

The maternal haemoglobin shows a minimum O_2 affinity at approx. pH 6.2 and presents a value of the Bohr coefficient $[\Delta(\log p_{50})/\Delta pH]$ of −0.65 between pH 7 and pH 8. Under the same conditions the sample of mixed embryonic haemoglobins obtained at 13 days' gestation shows an affinity minimum at approx. pH 6.2 and a Bohr coefficient of approx. −0.4 (Fig. 1).

The effect of temperature on the two systems is quite different. The maternal haemoglobin shows a heat of oxygenation, uncorrected for the heat of solution of O_2, of $-53\,kJ \cdot K^{-1} \cdot mol^{-1}$ (Fig. 2). The addition of organic phosphates, in the form of 2,3-bisphosphoglycerate, in each case produces a lowering of O_2 affinity, which becomes saturating at

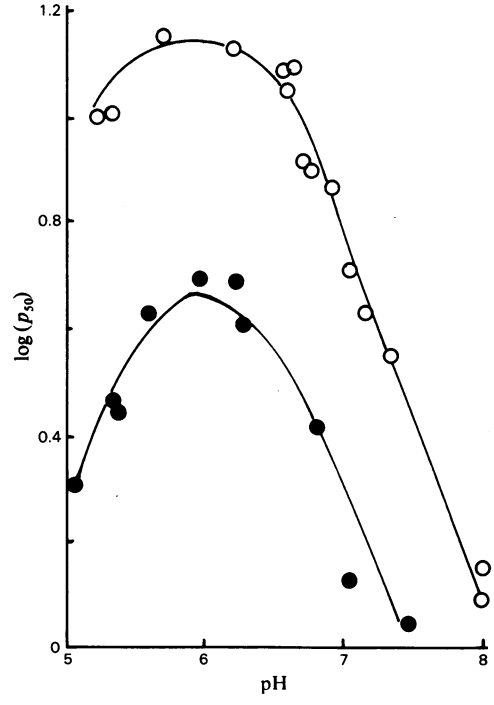

Fig. 1. *Comparison of the Bohr effect for maternal and embryonic haemolysates*

O_2-binding curves for maternal (○) and unfractionated 13-day-embryonic haemolysates (●) were obtained at 20°C in 0.1 M-Tris/HCl buffer, over the pH range shown, after removal of endogenous 2,3-bisphosphoglycerate. For full details see the text.

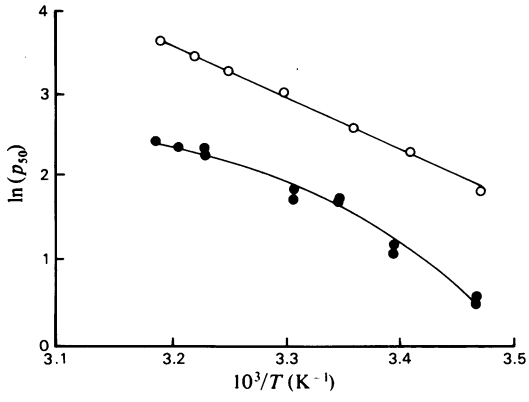

Fig. 2. *Effect of temperature on the oxygen affinity of maternal and embryonic haemolysates*

O_2 affinity was measured for both maternal (○) and unfractionated 13-day-embryonic haemolysates (●) in 0.1 M-Tris/HCl buffer, pH 6.7, after removal of endogenous 2,3-bisphosphoglycerate. For full details see the text.

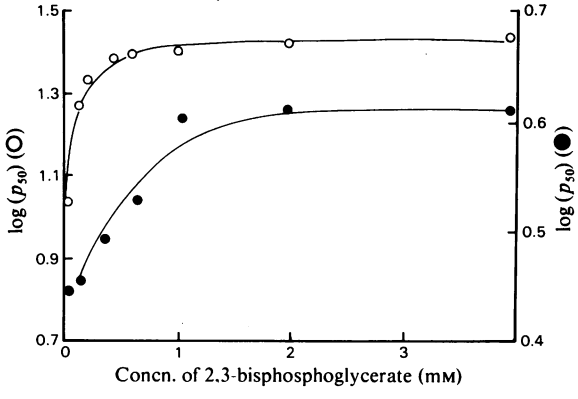

Fig. 3. *Effect of addition of 2,3-bisphosphoglycerate on the oxygen affinity of maternal and whole embryonic haemolysates*

2,3-Bisphosphoglycerate was added as a neutralized solution to samples of maternal (○) and 13-day-embryonic haemolysates (●) in 0.1 M-Tris/HCl buffer, pH 6.5, at 20°C. For full details see the text.

approximately stoichiometric concentrations (Fig. 3).

Developmental studies

When subjected to electrophoresis on polyacrylamide gel the blood of mouse embryos at 10–16 days' gestation shows the presence of three embryonic species, which on isoelectric focusing have

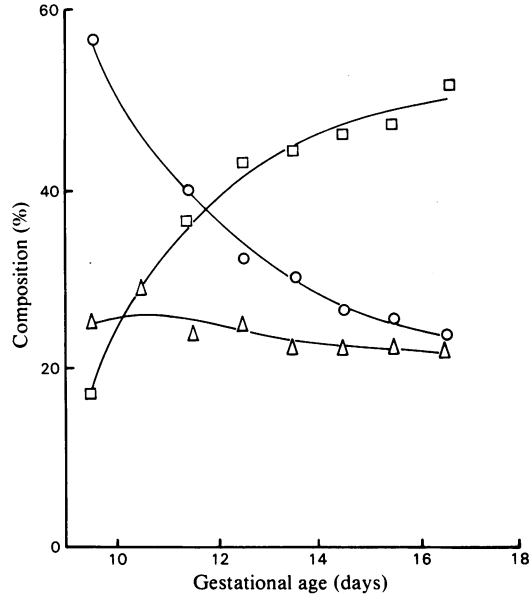

Fig. 4. *Developmental changes in the relative composition of embryonic blood in terms of its haemoglobin components*

The percentage composition of embryonic blood in terms of components EI (○), EII (□) and EIII (△) was determined by scanning densitometry of gel electrophoretograms as a function of gestational age. The lines represent visual smoothing of the data, added only for clarity. For full details see the text.

isoelectric points of 6.6, 7.1 and 7.3 for components EI, EII and EIII respectively.

Quantification of the embryonic species as a function of age by means of scanning densitometry of polyacrylamide gels shows a marked alteration of the relative composition of embryonic blood with age. At the earliest stages component EI predominates, only to be replaced by component EII, while component EIII remains essentially constant at approx. 25% (Fig. 4).

Studies on isolated embryonic haemoglobins

Preparative isolation of each of the embryonic components by pH-gradient elution from CM-cellulose gave samples suitable for subsequent O_2-binding studies (Fig. 5).

O_2-binding curves obtained for components EII and EIII showed co-operative binding with Hill coefficients of 2.3 and 2.6 respectively. Component EI showed extremely high-affinity binding, with an associated Hill coefficient of approx. 1. As the p_{50} for component EI was typically of the order of 1 mmHg of O_2 and non-dissociating conditions of

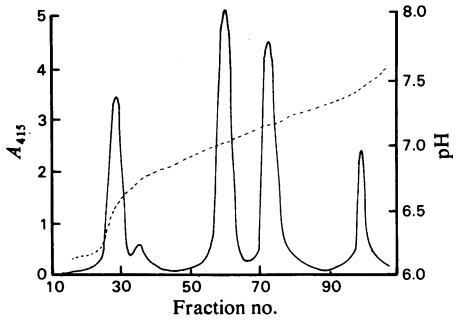

Fig. 5. *Typical ion-exchange elution profile of the embryonic-haemoglobin components*
The haemolysate from 13-day embryos was eluted from a column of CM-cellulose by using gradient elution from pH6 to pH7.5. ——, A_{415}; ------, pH. The major peaks represent, from left to right, haemoglobins EI, A, EII and EIII. For full details see the text.

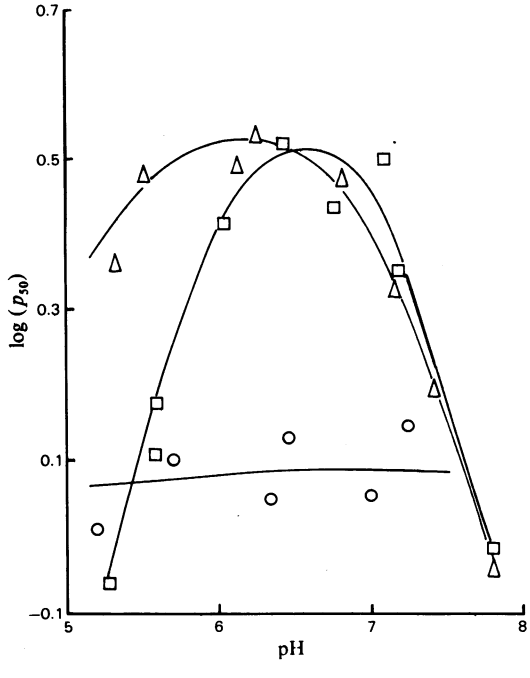

Fig. 6. *Bohr effect for separated embryonic haemoglobins*
O_2-binding curves were obtained for components EI (○), EII (□) and EIII (△) at 20°C in 0.1 M-Tris/HCl buffer, over the pH range shown. For full details see the text.

high protein concentration were employed, values of the parameters associated with this component are less accurate than those associated with the other components.

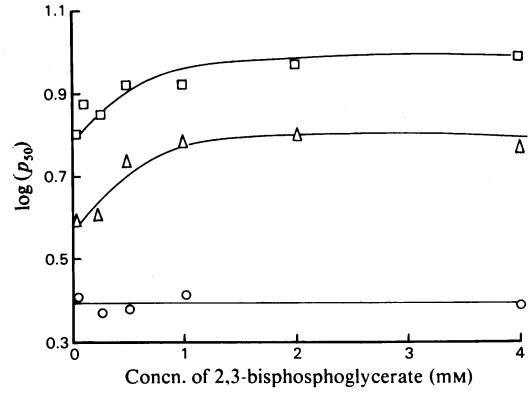

Fig. 7. *Effect of added 2,3-bisphosphoglycerate on the oxygen affinity of purified embryonic haemoglobins*
2,3-Bisphosphoglycerate was added at pH5.7 at 20°C to components EI (○), EII (□) and EIII (△) under the same conditions. For full details see the text.

The Bohr effects of components EII and EIII are qualitatively similar, particularly in the alkaline region. In contrast with these findings, component EI was found to show, within experimental error, pH-independent O_2 binding in the range pH5–8 (Fig. 6).

In terms of their response to changes in temperature, each embryonic component exhibits essentially simple Van't Hoff character with associated heats of oxygenation of −60, −34 and $-36\,kJ\cdot K^{-1}\cdot mol^{-1}$ for components EI, EII and EIII respectively.

Components EII and EIII show saturating 2,3-bisphosphoglycerate binding at approximately stoichiometric concentration, whereas component EI is insensitive to the presence of this organic phosphate (Fig. 7).

Simulation of whole-embryonic-blood binding curves

As no reliable data are available concerning the presence and concentrations of possible allosteric effectors in the early mouse erythrocyte, it is not possible to define precisely the physiological intra-erythrocyte environment at each stage of development. It is thus inappropriate to take data directly from measurements on isolated components, under various conditions, and simply sum their O_2-binding curves in the appropriate proportions under some arbitrary conditions. In order to overcome these problems we have employed a modified and extended approach of the form reported previously by Wells & Brittain (1981). In this method an iterative least-squares procedure has been used to obtain a best fit between variable parameters and the curves obtained experimentally over the range of gestation explored. Fitting was initiated by using proportions

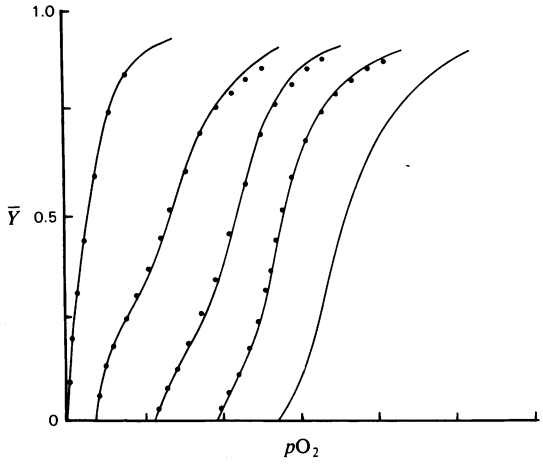

Fig. 8. *Oxygen-binding curves for whole embryonic blood as a function of gestational age*
O_2-binding curves for whole embryonic blood at gestational ages of 10, 12, 14 and 16 days and maternal blood (left to right) are shown together with best-fit curves (●) synthesized mathematically by using eqn. (1). $\bar{Y}$ represents fractional saturation. Each unit on the abscissa represents 20 mmHg pO_2. All curves are displaced for clarity.

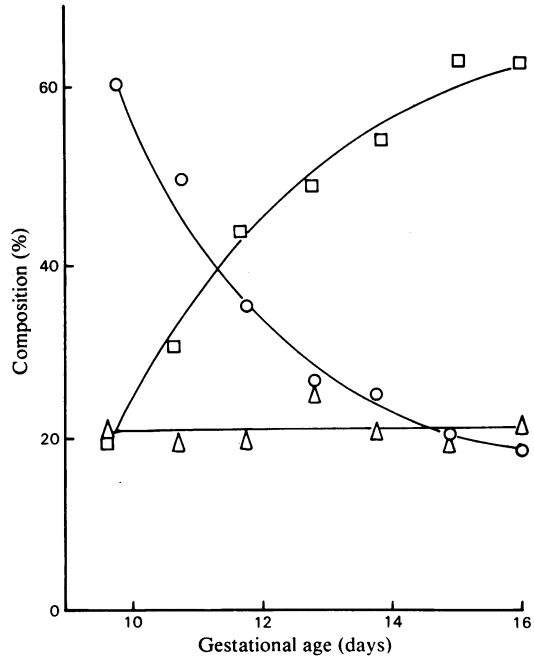

Fig. 9. *Computer-generated developmental changes in the composition of embryonic blood*
The percentage composition of embryonic blood in terms of components EI (○), EII (□) and EIII (△) predicted by best-fit computer-fitting of eqn. (1) to experimentally measured O_2-binding curves is shown as a function of gestational age. The lines represent a visual smoothing of the data, added only for clarity.

of each component obtained from Fig. 4 and Hill coefficients and affinity constants obtained at 20°C and pH 7.2 (the conditions under which the whole-blood curves were obtained). Data on each individual curve were fitted up to 0.95 saturation, assigning equal weighting to each region of the curve. By using computer programs adapted from those reported by Wilkins *et al.* (1974) the best-fit parameters for the anomalous embryonic whole-blood O_2-binding curves were obtained from the equation:

$$\bar{Y} = EI \cdot \frac{K_1 pO_2}{1 + K_1 pO_2} + EII \cdot \frac{K_2 pO_2^{N_1}}{1 + K_2 pO_2^{N_1}} + EIII \cdot \frac{K_3 pO_2^{N_2}}{1 + K_3 pO_2^{N_2}} \quad (1)$$

where $\bar{Y}$ is the fractional saturation, *EI*, *EII* and *EIII* give the percentage composition in terms of components EI, EII and EIII, K_1, K_2 and K_3 are the oxygen affinity constants associated with components EI, EII and EIII, N_1 and N_2 are the Hill coefficients and pO_2 is the partial pressure of O_2 (Fig. 8). By using this procedure it has been possible to obtain mathematically the change in relative composition as a function of age that best fits the O_2-binding curves (Fig. 9). The set of curves obtained at daily intervals between days 9 and 16 were best fitted by values of $K_1 = 0.11$ (± 0.02), $K_2 = 2 \times 10^{-4}$ ($\pm 0.2 \times 10^{-4}$), $K_3 = 7 \times 10^{-4}$ ($\pm 0.1 \times 10^{-4}$), $N_1 = 2.85$ (± 0.1) and $N_2 = 2.5$ (± 0.1).

Discussion

A comparison of the O_2-binding characteristics of maternal and embryonic whole blood shows that, although the embryonic system exhibits qualitatively similar responses to solution conditions, the quantitative response is such that the embryonic system maintains a higher affinity under all the conditions studied. Moreover, the embryonic system shows a much smaller response to solution conditions and would therefore be expected to decrease the effects of any alterations in the maternal environment. The whole-blood studies reported in the present paper represent the response of an embryonic system, at day 13 of gestation, but a clear insight into the functional requirements of the embryo *in utero* can only be obtained by the study of its individual component haemoglobins and their individual responses to solution conditions. The

switching of production from embryonic to adult haemoglobin and the explanation of the changing proportions of the embryonic haemoglobin during gestation are still open to considerable speculation. From days 8 to 16 of gestation embryonic haemoglobin EI decreases in proportion relative to component EII, which increases over the same period, while the proportion of component EIII remains relatively constant. These changes in the relative proportions of the three components could be due to different rates of synthesis for each component or to variation in rates of degradation of the embryonic haemoglobins. The synthesis of each of the haemoglobin peptide chains is in the first instance under separate genetic control, and the factors promoting the onset of synthesis of embryonic haemoglobin and later the synthesis of adult haemoglobin have not yet been determined. There are indications that the factor(s) involved is/are relatively independent of other developmental processes, as the appropriate haemoglobins appear at the expected stages of gestation even in developmentally retarded mice (Brotherton *et al.*, 1979*b*). To date, no measurements have been made of the survival times of the three embryonic haemoglobins. There are indications that the relative rates of synthesis of embryonic haemoglobins, in cells incubated *in vitro*, are consistent with the relative changes in content of the haemoglobins occurring *in vivo* (Fantoni *et al.*, 1969). It seems probable that variation in rates of synthesis of the haemoglobins is the major factor in determining their changes in relative proportions during gestation. Haemoglobin synthesis in the embryonic haemopoietic cells has been shown to be resistant to actinomycin D (Fantoni *et al.*, 1968), suggesting that the mechanisms regulating the rates of formation of haemoglobins EI, EII and EIII do not depend on immediate or continued RNA synthesis. A mechanism by which the mRNA was selectively degraded could, however, explain the decrease in production of one haemoglobin relative to the others. Farkas & Marks (1968) have provided evidence that RNA degradation may be the controlling factor in rabbits.

Haemoglobin EI presents a hyperbolic O_2-binding curve with a Hill coefficient of about 1. This haemoglobin is the major component present during the earliest stages of O_2 transport within the developing embryo, and as such its major role could be the direct transfer of O_2 from the maternal interstitial fluid to the embryo. Changes in pH and the concentration of the allosteric effector 2,3-bisphosphoglycerate do not appear to have a significant effect on the p_{50} value of component EI. Petschow *et al.* (1978) report very low concentrations of 2,3-bisphosphoglycerate in mouse embryonic erythrocytes, and it is likely that at the earliest stages of gestation sensitivity of a haemoglobin to 2,3-bisphosphoglycerate would not be of any advantage, as the system controlling the production of this organic phosphate might not yet be fully operational and the quantities of 2,3-bisphosphoglycerate present would be negligible. The otherwise low release of O_2 to the tissue at the earliest stages of development may be to some extent alleviated by the unusually high haemoglobin concentration within the developing embryo. The haemoglobin content of adult mice and humans is approx. 0.1% of the body weight. In mouse embryos at day 12 of gestation the total haemoglobin content is about 0.3% of the body volume (Fantoni *et al.*, 1981).

Functional analysis of the components EII and EIII reveals that they both present sigmoidal O_2-binding curves with Hill coefficients >2. The affinity of component EII for O_2 is higher than that of maternal haemoglobin, presumably because of the requirement of the transfer of O_2 across the placenta. It displays co-operativity and thus sensitivity to changes in pH and 2,3-bisphosphoglycerate concentration. It seems plausible that, as the circulatory system of the foetus develops, the limited functional capabilities of component EI should be replaced by a functionally competent component amenable to control by other molecules within the erythrocyte. Component EII fulfils this role, but continues to shield the foetus from any drastic changes in environmental conditions, as its response to changes in concentration of allosteric effectors is smaller than that of maternal haemoglobin.

The functional properties of component EIII are broadly similar to those of component EII in that it is a co-operative system and is sensitive to changes in the quantity of H^+ ions and 2,3-bisphosphoglycerate in its environment. The Bohr effect of component EIII does not appear to have the same degree of reverse Bohr shift as does component EII and it may protect the embryo during brief episodes of acidosis. The developmental role of component EIII within the developing foetus is not so apparent. This component is present in approximately constant proportion between days 8 and 16 of gestation. Whether component EIII is a product of gene duplication of component EII is debatable, but it is unlikely that it would be retained unless it possessed some functional advantage. It is possible that the significance of component EIII is not demonstrated in the series of experiments reported in the present paper, and it may prove to be sensitive to ATP or some other allosteric effector. Alternatively component EIII may simply act as a 'back-up system' for component EII and has sufficiently different properties to compensate in any situations in which the efficiency of the functioning of component EII was impaired. Hill plots produced from the O_2-binding curves of haemoglobins EII and EIII suggest that the two components have slightly different N

values, which might imply that they have slightly different O_2-transporting capacities (Neville, 1977).

The fitting of the O_2-binding curves for embryonic mouse blood to eqn. (1) implies a number of important points. Discounting the systematic relatively poor fit to data at $\bar{Y} > 0.8$, which may well represent the operation of positive asymmetry in the system, as alluded to by Weber (1982) and Peller (1982), the fact that the curves may be defined by the simple sum of the contributions of the three components suggests that no appreciable hybridization occurs in the system. Furthermore, the constant nature of the best-fitting binding parameters again suggests that the only major change in the embryonic system that occurs on gestation is a change in the relative proportions of the three embryonic haemoglobins. The predicted change in composition closely follows that obtained analytically, and moreover provides strong evidence that the hyperbolic parts of the oxygenation curves, apparent particularly in the earliest stages of development, arises from the presence of component EI and not from other possible causes, such as the presence of less-than-saturating amounts of allosteric effectors such as 2,3-bisphosphoglycerate (Stanley *et al.*, 1978).

In summary, although the existence of the three embryonic mouse haemoglobins in significant quantities in the embryonic erythrocyte has been known for some time, it is only recently that their effect on the O_2-binding curve of whole embryonic erythrocytes has been noted (Wells & Brittain, 1981).

The lack of information in the literature on the anomalous O_2-binding characteristics of whole embryonic blood may arise from the dearth of investigations that include studies at the earliest stages of development or from analysis of equilibrium data over a restricted range of saturation. From this study and that previously reported by Wells & Brittain (1981) it now appears that the hyperbolic character of the earliest oxygenation curves may well prove to be a general characteristic, at least for mice. We await further studies to ascertain if this phenomenon is general to all mammalian species.

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