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TRANSITION TO COOPERATIVE OXYGEN-BINDING BY EMBRYONIC HAEMOGLOBIN IN MICE

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During the course of mammalian development access and the demand for oxygen in the uterus are likely to change. Accordingly, the type of haemoglobin changes with the shift of erythropoietic site from the yolk sac and its nucleated red cells, to those from the liver, spleen, and ultimately, the bone marrow (see Kitchen & Brett, 1974). Embryonic haemoglobin (Hb-Eb) originating from the yolk sac is superseded by foetal haemoglobin (Hb-F), or in the mouse directly by adult haemoglobin (Hb-A) arising in hepatic tissues. Nucleated erythrocytes from the yolk sac are the only source of haemoglobin in the mouse from the 7th day to the 12th day of gestation. By this time the embryo has implanted and three embryonic haemoglobins are evident (Melderis, Steinheider & Ostertag, 1974; Shimizu & Watanabe, 1978; Brotherton *et al.* 1979). The proportions of these components vary slightly according to the strain of mice, but the beginning of the foetal phase is marked by the onset of Hb-A synthesis in the liver (Shimizu & Watanabe, 1978; Brotherton *et al.* 1979).

It might be predicted that in the earliest stages of development, the embryonic erythrocytes will have a high affinity for oxygen to take up oxygen which diffuses into the maternal interstitial fluid. A diffusion-dependent supply of oxygen might not be adequate when the oxygen uptake rises during development and the embryonic tissues must be adapted to function at lower tensions. This situation changes when perfusion of oxygen develops across the placenta later on.

Only two studies have focused on the mammalian embryo at a time when post-embryonic Hb comprized an insignificant proportion of the total (Bauer *et al.* 1975; Wells, 1979). Purified haemolysates from 12·5 day old mouse embryos had intrinsically higher oxygen affinity than in later development (Bauer *et al.* 1975). Moreover, this property was also manifested in intact erythrocytes at 11·5 days in the presence of 2,3-DPG which acts *in vivo* as an allosteric modulator of oxygen affinity (Wells, 1979). In 10- and 11-day old mouse embryos there is no Hb-A but by 12 days Hb-A comprises 5·4% of the total haemoglobin (Brotherton *et al.* 1979). By day 16, Hb-A dominates and its oxygen affinity is regulated by 2,3-DPG (Petschow *et al.* 1978).

Haemoglobin-oxygen equilibrium curves have not yet been reported for any mammal prior to implantation. The earliest curves obtained by Wells (1979) showed an interesting departure from the classical sigmoid curve. 11·5 day old mouse embryos had a high oxygen affinity at low saturations, suggesting the presence of an early embryonic component with very high oxygen affinity and lacking co-operativity. We now record oxygen equilibrium curves for whole blood from 7 day

old mouse embryos, (a stage when erythrocytes first appear in the blood islands) and follow the equilibrium to day 13 (when Hb-Eb becomes rapidly diluted by Hb-A).

Pregnant mice of the CD-1 strain were sacrificed from day 7 to day 13 of gestation. Uteri were quickly excised and the embryos released into 4 °C isotonic saline. After blotting, blood was sampled from the embryos with a finely drawn pipette. Approximately 10 nl of blood was obtained from the earliest embryo.

Ten nl of blood was spread in a thin film on the centre of an 18 mm diameter glass coverslip. The film was covered with a transparent silicone copolymer membrane and placed in the humidified test chamber of a Hemoscan Oxygen Dissociation Analyser (Aminco, U.S.A.). A progressively oxygenating gas mixture containing 5·6% CO_2 was passed over the blood film and monitored with an oxygen electrode on the X-channel of a Philips PM 8041 X–Y recorder. The saturation of the haemoglobin was monitored using dual wavelength spectroscopy and expansion of the optical signal of the Y recorder. In this way within about 10 min a continuous equilibrium curve was generated. Spectrophotometric scans of the blood films showed that < 1% of the total Hb became oxidized during oxygenation.

The curves were digitized into twenty data pairs, with points taken every 2 mm Hg P_{O_2}, at low saturations, and every 5 or 10 mm Hg in the latter stages of oxygenation. By this means, conventional Hill plots were made from

$$\log (Y/1-Y) = n \log (P_{O_2}) - \log K,$$

where Y is the fractional saturation of the haemoglobin; n, or Hill's coefficient, is a measure of the sigmoidal shape of the equilibrium, and K the oxygen affinity constant.

From the Hill plots, two n values were obtained for the slope of each phase, and an upper limit of these by the O_2 saturations C_1' and C_2' respectively from linear least squares analysis of the different slopes. 'C' is a concentration term for each of the two graphical components expressed as a percentage of the total haemoglobin. All lines had correlation coefficients of 0·99 or better.

Having established these parameters, the initial phase of each of the equilibrium curves was fitted to the hyperbolic function:

$$Y_1 = (K_1 P_{O_2}/1 + K_1 P_{O_2})\, C_1''$$

The hyperbolic phase of the curve was next 'stripped' from the total curve to yield a sigmoidal curve which was fitted to:

$$Y_2 = (K_2 (P_{O_2})^{n2}/1 + K_2 (P_{O_2})^{n2})\, C_2''$$

yielding values for P_{50}'', n_2 and C_2''. Finally, to discount accumulated errors, the parameters derived from the non-linear least squares procedures were used to synthesize oxygen equilibrium curves as the sum of the two phases:

$$Y = C_1'' (K_1 P_{O_2}/1 + K_1 P_{O_2}) + C_2'' (K_2 (P_{O_2})^{n2}/1 + K_2 (P_{O_2})^{n2}).$$

The programmes used for curve fitting were iterative non-linear least squares routines employing matrix inversion by Gauss–Jordan elimination.

Whole blood oxygen P_{50} values showed a decrease in oxygen affinity between days 7 and 13 of gestation (Fig. 1). However, P_{50} is not a reliable index of t

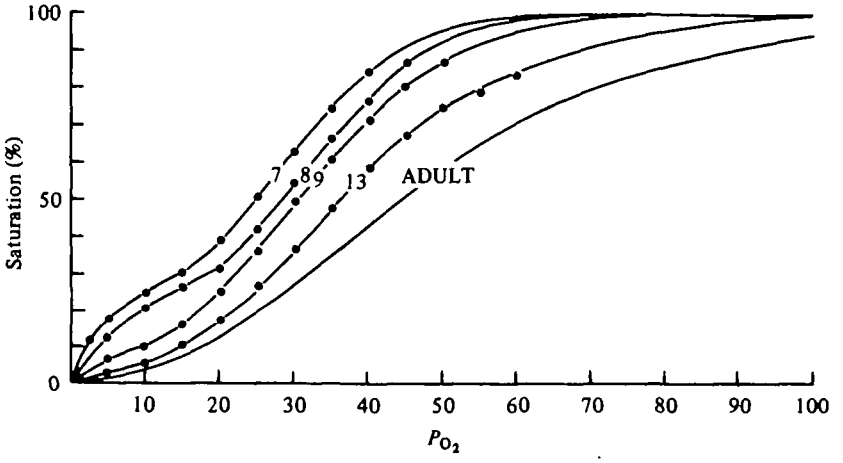

Fig. 1. Oxygenation curves of embryonic erythrocytes from 7 to 13 days gestation at 37 °C and P_{CO_2} = 41 mm Hg. The solid lines are the actual curves obtained as a continuous X–Y plot and the points from synthesized data in Table 1.

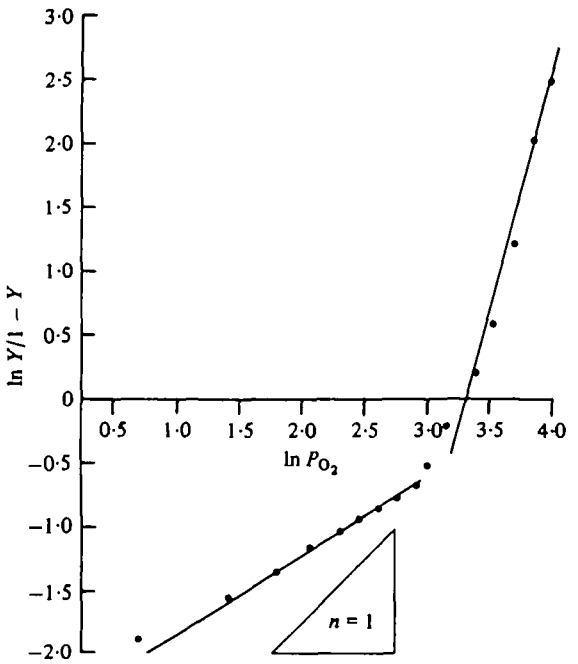

Fig. 2. Hill plot of 8-day embryonic blood illustrating the presence of two oxygenational phases.

oxygen-binding potential of embryonic blood because the curves are distorted by a high affinity component at low oxygen saturations. The Hill plots resolved into two distinct phases, as illustrated by the day 8 transformation in Fig. 2. Human haemoglobins containing normal Hb and high O_2 affinity may also show biphasic Hill plots (see Antonini & Brunori, 1971).

When the two embryonic components were examined separately as a function gestational age, it was found that over the range 7–13 days the character of each

Table 1. *Parameters derived from analysis of oxygenation curves. P_{50} is the apparent affinity for a whole curve. Other terms are defined in the text. Where * indicated, minor component represents < 5% total*

Age	P_{50}	n_1	C_1'	n_2	C_2'	P_{50}'	C_1''	C_2''	P_{50}''
Maternal	45·0	—	—	3·0	100	—	—	—	44·0
7 days	23·0	1·3	38	3·2	62	*	44	56	24·5
8	27·5	0·7	32	3·4	68	4·0	36	64	29·9
9	28·0	0·8	15	3·3	85	6·6	16	84	27·1
10	33·6	1·0	12	3·5	88	10·0	11	89	33·1
11	36·6	0·7	11	3·2	89	6·6	13	87	24·5
13	37·0	1·2	7	3·0	93	*	10	93	33·1
Mean	—	0·96	—	3·3	—	6·8	—	—	28·6

did not alter: their Hill numbers and P_{50}'s were independent of age (Table 1). However, their proportion changed markedly, the high O_2 affinity component declining from ~ 45 to ~ 10%. The observed increase of P_{50} with time for the complete curves is thus merely a reflexion of their composition. Our analytical approach is apparently validated by the close agreement obtained between synthesized and experimental data (Fig. 1).

The functional significance of these findings must be speculative in the absence of information about the blood chemistry and metabolic demands of the embryo *in vivo*. However, a high affinity haemoglobin component with hyperbolic binding characteristics might act as a store and facilitate diffusion within the cell, and, in addition, buffer the embryo against unfavourable fluctuations in oxygen supply as does myoglobin in diving mammals. This component may turn out to be Hb-Eb1 which has an X_2Y_2 structure and thus differs from II–III which have α-chains (Fantoni, Bank & Marks, 1967; Melderis, Steinheider & Ostertag, 1974; Huehns & Farooqui, 1975; Kamuzora & Lehmann, 1975; Tuchinda, Nagai & Lehmann, 1975; Brotherton *et al.* 1979). While it is true that a degree of cooperativity in haemoglobin requires two different pairs of chains in the tetramer, our results do not necessarily imply a tetramer of like chains.

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REFERENCES

ANTONINI, E. & BRUNORI, M. (1971). Hemoglobin and myoglobin in their reaction with ligands. *Frontiers of Biology* 21 (ed. A. Neuberger and E. L. Tatum), Amsterdam, North-Holland.

BAUER, C., TAMM, R., PETSCHOW, D., BARTELS, R. & BARTELS, H. (1975). Oxygen affinity and allosteric effects of embryonic mouse haemoglobin. *Nature, Lond.* **257**, 333–334.

BROTHERTON, T. W., CHUI, D. H. K., GAULDIE, J. & PATTERSON, M. (1979). Hemoglobin ontogeny during normal mouse fetal development. *Proc. Natl. Acad. Sci.* **76**, 2853–2857.

FANTONI, A., BANK, A. & MARKS, P. A. (1967). Globin composition and synthesis of hemoglobins in developing fetal mice erythroid cells. *Science* **157**, 1327–1329.

HILL, A. V. (1910). The possible effects of aggregation of molecules of hemoglobin on its dissociation curve. *J. Physiol., Lond.* **40**, iv–vii.

HUEHNS, E. R. & FAROOQUI, A. M. (1975). Oxygen dissociated properties of human embryonic r[illegible] cells. *Nature, Lond.* **254**, 335–337.

KAMUZORA, H. & LEHMANN, H. (1975). Human embryonic haemoglobins including a comparison by homology of the human ζ and α chains. *Nature, Lond.* **256**, 511–513.

KITCHEN, H. & BRETT, I. (1974). Embryonic and fetal haemoglobin in animals. *Ann. N.Y. Acad. Sci.* **241**, 653–671.

MELDERIS, H., STEINHEIDER, G. & OSTERTAG, W. (1974). Evidence for a unique kind of α-type globin chain in early mammalian embryos. *Nature, Lond.* **250**, 774–776.

PETSCHOW, R., PETSCHOW, D., BARTELS, R., BAUMANN, R. & BARTELS, H. (1978). Regulation of oxygen affinity in blood of fetal new born and adult mouse. *Respir. Physiol.* **35**, 271–282.

SHIMIZU, K. & WATANABE, T. (1978). Re-evaluation of the presence of multiple haemoglobins during the ontogeny of the mouse. *J. Embryol. exp. Morph.* **43**, 17–28.

TUCHINDA, S., NAGAI, K. & LEHMANN, H. (1975). Oxygen dissociation curve of Haemoglobin Portland. *FEBS Letts.* **49**, 390–391.

WELLS, R. M. G. (1979). Haemoglobin-oxygen affinity in developing embryonic erythroid cells of the mouse. *J. comp. Physiol.* **129**, 333–338.