Rat embryonic and foetal erythrocytes

High 2,3-bisphosphoglycerate and ATP and low oxygen affinity *in vitro* for nucleated embryonic cells

John G. GILMAN*

Naval Medical Research Institute, Bethesda, MD 20014, U.S.A.

(Received 30 June 1980/Accepted 8 July 1980)

Embryonic nucleated red cells of the rat have high ATP and 2,3-bisphosphoglycerate and relatively low oxygen affinity. During foetal life they are replaced by large non-nucleated red cells with high ATP, low bisphosphoglycerate and high oxygen affinity. After birth, small non-nucleated red cells with high bisphosphoglycerate and low oxygen affinity rapidly predominate.

Early embryos of all mammals studied to date have nucleated red cells and embryonic haemoglobins (Kitchen & Brett, 1974). The late foetus has non-nucleated red cells with higher oxygen affinity than the non-nucleated red cells of the mother. The oxygen-affinity differential between maternal and foetal blood facilitates oxygen transfer to foetal blood under the conditions of low oxygen tension in the placenta (Jelkmann & Bauer, 1977).

By analogy with foetal red cells, embryonic nucleated red cells were expected to have higher oxygen affinity than adult cells. Indeed, mouse and rabbit embryonic haemoglobins have higher oxygen affinity than the respective adult haemoglobins (Bauer *et al.*, 1975; Jelkmann & Bauer, 1977). However, studies on intact nucleated embryonic red cells have given differing results. Human embryonic red cells have the same oxygen affinity as foetal red cells (Huehns & Farooqui, 1975). Mouse embryonic red cells have higher oxygen affinity than maternal red cells (Wells, 1979). Rabbit embryonic red cells have relatively low oxygen affinity, almost as low as for maternal red cells (Jelkmann & Bauer, 1977).

The present paper reports studies on red-cell oxygen affinity, red-cell ATP and bisphosphoglycerate content, and proportions of different red-cell types, during rat development from early embryo to several weeks after birth. The data show that embryonic nucleated red cells of the rat have affinity for oxygen only slightly higher than that of maternal red cells under the experimental conditions employed.

Abbreviations used: *p*50, partial pressure of oxygen to attain half-saturation of haemoglobin with oxygen.

* Present address: Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, GA 30912, U.S.A.

Materials and methods

Animals

Rats (*Rattus norvegicus*) were of the Long-Evans strain (Charles River Breeding Laboratories, Wilmington, MA, U.S.A.). They were chosen to have haemoglobin type B and the high-bisphosphoglycerate genotypes *DD* or *Dd* (Brewer *et al.*, 1978). Animals were mated naturally, and day 0 of gestation was counted as the day of discovery of the vaginal plug. Both males and females were used for experiments, except in the case of adult rats, when only females between 4 and 8 months of age were used.

Oxygen-dissociation-curve determinations

From day 18 of gestation to 2 weeks of age, individual foetuses or young were killed by decapitation to obtain blood. Older animals were bled from the tail. Blood was washed once and resuspended in cold bicarbonate buffer [0.25 M-NaHCO₃/0.122 M-NaCl/glucose (2 g/litre)/bovine serum albumin (30 mg/ml)/0.1 mM-EDTA]. At 37°C, in air/CO₂ (19:1), the pH of the cell suspension was 7.45. The haemoglobin concentration of the suspension was 1–2 mM in tetramers, as determined spectrophotometrically on cyanmethaemoglobin (van Kampen & Zijlstra, 1965). Oxygen-dissociation-curve determinations were begun as soon as the cell suspension was prepared, and were completed 50 min later. For embryos younger than day 18 of gestation the procedure was similar, except that red cells from a single litter were pooled; experiments were begun within 1 h of killing the mother.

Oxygen-dissociation-curve measurements were performed at 37°C by using a Hemo-Scan

instrument (American Instrument Co., Silver Spring, MD, U.S.A.). The two equilibration gases had the following compositions: N_2/CO_2 (19:1) and $N_2/O_2/CO_2$ (14:5:1). Values of the Hill coefficient (h) were calculated (Kilmartin & Rossi-Bernardi, 1971) by using points between 30 and 70% saturation with oxygen.

ATP, bisphosphoglycerate, and red-cell-morphology determinations

For ATP analyses, a neutralized $HClO_4$ extract (Beutler, 1975) was prepared as soon as the blood or red-cell suspension had been obtained. The extract was frozen, and ATP was determined within 24 h by an end-point type of enzymic assay (Lamprecht & Trautschold, 1958). For bisphosphoglycerate determinations on samples with less than 2.5 mM-bisphosphoglycerate, an automated procedure involving a kinetic-rate enzymic assay was used (Hickey *et al.*, 1979). For samples with higher bisphosphoglycerate concentrations, the end-point enzymic assay of Keitt (1971) was used.

To determine the percentage of nucleated red cells, a count of about 300 cells was made by using Wright-stained red-cell smears. If less than 10% of the red cells were nucleated, about 1000 cells were counted. Size distributions of non-nucleated red cells were found by measuring to the nearest $0.5 \mu m$ the diameters of about 100 cells of a Wright-stained smear, by using a calibrated ocular micrometer.

Results and discussion

Oxygen-dissociation curves and $p50$

Fig. 1 shows representative oxygen-dissociation curves for red-cell suspensions from a pregnant rat (embryos at 13 days of gestation), a newborn rat, and a pooled litter of rat embryos at 14 days of gestation. All determinations are for pH 7.45 at $37^\circ C$, with CO_2 at a partial pressure of about 4.8 kPa (36 torr).

For the maternal sample, $p50$ was 4.45 kPa (33.4 torr). $p50$ values are a measure of the oxygen affinity of the red cells; high $p50$ values indicate low oxygen affinities. For this sample, the Hill coefficient (a measure of haemoglobin co-operativity) was 2.54.

Red cells of the newborn rat gave a $p50$ of 3.43 kPa (25.7 torr; $h = 2.52$). This is much lower than the $p50$ of the maternal red cells. Therefore the red cells of the newborn rat have a much higher affinity for oxygen than have maternal red cells.

The curve for the 14-day embryonic red cells shown in Fig. 1 gave a $p50$ value of 4.21 kPa (31.6 torr; $h = 3.11$). Red cells of 13-day embryos, from the mother whose oxygen-dissociation curve is shown in Fig. 1, gave a $p50$ of 4.17 kPa (31.3 torr; $h = 3.40$). Both of the embryonic samples had about 5% contamination with maternal red cells; the former had 2% foetal non-nucleated red cells, whereas the latter had none. One should note that the oxygen-dissociation curve shown in Fig. 1 for the embryonic red cells has an atypical shape, which

Fig. 1. Oxygen-dissociation curves and $p50$ for red cells of embryonic, newborn, and adult rats

Complete oxygen-dissociation curves were obtained for red-cell suspensions at $37^\circ C$, pH 7.45, in the presence of CO_2 , as described in the text. —, Embryonic-rat red cells at 14 days of gestation; ···, newborn-rat red cells; ----, red cells of a pregnant adult rat carrying 13-day embryos. The insert to the Figure summarizes oxygen-dissociation-curve data for the developing rat. Values of $p50$ are plotted against the rat's prenatal or postnatal age. Means $\pm$ s.d. are shown for more than two experiments at a given age. Data are for experiments on 27 red-cell samples for developing rats of 15 different ages, plus eight adult samples.

leads to a high calculated value for h . The reason for this atypical shape is unknown. However, it was reproducible with fresh embryonic samples; the average value of h for five independent 13- or 14-day embryonic samples was 3.20.

The inset to Fig. 1 summarizes all of the $p50$ data obtained from embryonic, foetal, and young-rat red cells. For the early embryos, $p50$ is between 3.9 and 4.3 kPa (29 and 32 torr), and it falls to 3.5 kPa (26 torr) by the time of birth. $p50$ rises after birth to about 4.8 kPa (36 torr), and then gradually falls off to the adult value of 4.3–4.5 kPa (32–34 torr).

These data show that rat embryonic nucleated red cells have $p50$ values almost as high as the maternal values, at 37°C and pH 7.45, in the presence of CO_2 ($p\text{CO}_2 = 4.8$ kPa). Jelkmann & Bauer (1977) found a similar situation in the rabbit, and they suggested that the low oxygen affinity of early embryonic red cells aids in oxygen delivery to the rapidly metabolizing embryonic tissues. High oxygen affinity of late-foetal and newborn-animal red cells is generally seen in mammalian development. In the late-foetal stage, presumably, the limiting factor in oxygen delivery to foetal tissues is the ability of foetal blood to achieve sufficient degree of oxygen saturation in the placenta.

ATP and bisphosphoglycerate concentrations and red-cell types

ATP, bisphosphoglycerate and other organic phosphates interact with haemoglobin and reduce its affinity for oxygen. The presence of large amounts of red-cell bisphosphoglycerate causes the low red-cell oxygen affinity of many mammals (Bunn, 1971). Conversely, in mammals that lack distinctly foetal

hemoglobins, such as the pig, rabbit and mouse, the high foetal and newborn red-cell oxygen affinity is due to low amounts of red-cell bisphosphoglycerate (Kim & Duhm, 1974; Jelkmann & Bauer, 1977; Wells, 1979).

Fig. 2 shows the changing pattern of red-cell ATP and bisphosphoglycerate concentrations during rat development. At 13 and 14 days of gestation, bisphosphoglycerate concentrations are very high relative to haemoglobin concentration. At these ages, red-cell smears showed 100 and 98% nucleated red cells respectively.

The early rat embryo has embryonic haemoglobins (Stein *et al.*, 1971), which in the mouse and rabbit are known to have very high affinities for oxygen (Bauer *et al.*, 1975; Jelkmann & Bauer, 1977). The high ATP and bisphosphoglycerate concentrations in the nucleated embryonic red cells are therefore the likely reason for their low oxygen affinity. Low intracellular pH has also been hypothesized to be a contributing factor to low oxygen affinity in rabbit embryonic red cells (Jelkmann & Bauer, 1977).

At day 15, non-nucleated red cells enter the circulation in force, and account for 35% of total circulating red cells. At this time, ATP, bisphosphoglycerate and $p50$ have begun to fall, as shown by Figs 1 and 2. From day 15 on, the decline of nucleated red cells is exponential, with a half life of 0.7 day. ATP, bisphosphoglycerate, and $p50$ continue to fall until stable values are reached at about 19 days of gestation. At this time, more than 98% of the red cells in the foetal circulation are non-nucleated.

Examination of Fig. 2 shows that the foetal

Fig. 2. Red-cell ATP and bisphosphoglycerate concentrations during rat development

ATP, bisphosphoglycerate and haemoglobin concentrations of blood or red-cell suspensions (for prenatal ages up to day 18 of gestation) were determined as described in the text. O, ATP; ●, bisphosphoglycerate. Means $\pm$ S.D. are shown for more than two experiments at a given age. Solid and dotted lines connect the mean values at each age for bisphosphoglycerate and ATP respectively. Data are for experiments on 70 samples at 21 ages for bisphosphoglycerate and 83 samples at 21 ages for ATP, plus nine adult samples for each.

non-nucleated red cells have 0.8 mol of ATP/mol of haemoglobin tetramer, but very little bisphosphoglycerate. They are large cells, with 85% of them between 8 and 10 μm in diameter (the rest are slightly larger or slightly smaller). This compares with 6.5 μm in diameter and smaller for the adult-red cell. As shown by Trávníčková & Šulc (1970) and Stein *et al.* (1971), the foetal- and newborn-rat red cell has adult haemoglobins rather than distinctly foetal haemoglobins.

After birth, red-cell ATP and bisphosphoglycerate concentrations increase relative to haemoglobin. The ATP concentration reaches a peak at 3–6 days after birth. Thereafter it declines gradually to the adult value of about 0.25 mol/mol of haemoglobin. Bisphosphoglycerate increases to a maximum of over 2 mol/mol of haemoglobin at 4 weeks of age. A gradual decline then occurs to the adult value of about 1.7/mol of haemoglobin.

A postnatal decrease in red-cell diameter accompanies the increase in bisphosphoglycerate concentration; by 3 weeks of age, over 80% of the red cells in circulation are smaller than 8 μm in diameter. These data on red-cell diameter agree with observations of Lucarelli *et al.* (1968), who reported that the mean cell volume of rat red cells decreases from 136 μm^3 at birth to 67 μm^3 by 20 days of age. A more detailed analysis of red-cell-volume changes (Valet *et al.*, 1972) showed that the red-cell populations initially present at a rat's birth are replaced by populations of smaller red cells by 27 days of age.

Concluding comments

The present results show that bisphosphoglycerate and ATP concentrations are very high in early embryonic nucleated red cells of the rat. Both organic phosphates may therefore play a role in lowering the oxygen affinity of rat embryonic red cells to close to the maternal value at 37°C, pH 7.45, in the presence of CO_2 . The late-foetal non-nucleated red cell has very little bisphosphoglycerate, so that at this stage, ATP is probably the organic phosphate primarily responsible for modulating haemoglobin function. Oswald & Dassler (1967) showed that UTP is the only other organic phosphate present in significant amounts in the late-foetal red cell, and there are three times as much ATP. After birth, bisphosphoglycerate rapidly becomes the predominant organic phosphate in the rat red cell. A similar sort of programming of organic phosphates has been shown for the developing chicken; bisphosphoglycerate is the major red-cell organic phosphate several days before hatching; ATP predominates shortly after hatching, followed by inositol pentaphosphate beyond 1 week of age (Isaacks *et al.*, 1976).

The programming of organic-phosphate content of the rat red cell is related to the organ site of erythropoiesis. The early embryonic nucleated red cells of mammals are produced in the yolk sac (Metcalfe & Moore, 1971). Lucarelli *et al.* (1968) showed that the liver is the primary haematopoietic organ in the foetal rat, although the spleen is active at birth and possibly before; the bone marrow becomes haematopoietic soon after birth, when it shows an 'explosive increase' in erythroid activity. The liver ceases its haematopoietic function between 2 and 10 days after birth (Greengard *et al.*, 1972). Thus the foetal liver is primarily responsible for producing the large non-nucleated red cells with high ATP and low bisphosphoglycerate reported in the present paper. The bone marrow is primarily responsible for production of the smaller non-nucleated red cells with high bisphosphoglycerate and low ATP, which predominate by 3–4 weeks of age.

Foetal red cells of humans are biochemically different from adult red cells with regard to metabolism and membrane properties (Osaki & Schwartz, 1972), and in possessing a specifically foetal haemoglobin. Although foetal rats do not have distinctly foetal haemoglobins, the present results suggest that phosphate metabolism of rat foetal red cells differs from that of adult-rat red cells. Red-cell-membrane properties also appear to differ between newborn and adult rats (Seaman *et al.*, 1980). One may therefore hypothesize a clonally and biochemically distinct non-nucleated red cell for the foetal rat, compared with the non-nucleated red cell of the rat after birth.

I thank Dr. L. A. Kiesow for valuable discussions and technical assistance. This work was supported by the Naval Medical Research and Development Command, Research Work Unit no. MR 000101.1228, and by U.S. National Institutes of Health (grant no. HL 25803).

References

- Bauer, C., Tamm, R., Petschow, D., Bartels, R. & Bartels, H. (1975) *Nature (London)* **257**, 333–334
- Beutler, E. (1975) *Red Cell Metabolism*, 2nd edn., pp. 16–17, Grune and Stratton, New York
- Brewer, G., Gilman, J., Noble, N. & Crews, V. (1978) *Biochem. Genet.* **16**, 697–707
- Bunn, H. F. (1971) *Science* **172**, 1049–1050
- Greengard, O., Federman, M. & Knox, W. E. (1972) *J. Cell Biol.* **52**, 262–272
- Hickey, T. M., Uddin, D. E. & Kiesow, L. A. (1979) *Clin. Chem.* **25**, 1314–1317
- Huehns, E. R. & Farooqui, A. M. (1975) *Nature (London)* **254**, 335–337
- Isaacks, R. E., Harkness, D. R., Froeman, G. A., Goldman, P. H., Adler, J. L., Sussman, S. A. & Roth, S. (1976) *Comp. Biochem. Physiol.* **53A**, 151–156

- Jelkmann, W. & Bauer, C. (1977) *Pflügers Arch.* **372**, 149–156
- Keitt, A. S. (1971) *J. Lab. Clin. Med.* **77**, 470–475
- Kilmartin, J. V. & Rossi-Bernardi, L. (1971) *Biochem. J.* **124**, 31–45
- Kim, H. D. & Duhm, J. (1974) *Am. J. Physiol.* **226**, 1001–1006
- Kitchen, H. & Brett, I. (1974) *Ann. N.Y. Acad. Sci.* **241**, 653–671
- Lamprecht, W. & Trautschold, I. (1958) *Hoppe-Seyler's Z. Physiol. Chem.* **311**, 245–255
- Lucarelli, G., Porcellini, A., Carnevali, C., Carmena, A. & Stohlman, F., Jr. (1968) *Ann. N.Y. Acad. Sci.* **149**, 544–559
- Metcalf, D. & Moore, M. A. S. (1971) *Haematopoietic Cells*, pp. 172–195, North-Holland Publishing Co., Amsterdam and London
- Oski, F. A. & Schwartz, E. (1972) in *Hematology* (Williams, W. J., Beutler, E., Erslev, A. J. & Rundles, R. W., eds.), pp. 52–59, McGraw-Hill, New York
- Oswald, B. & Dassler, G. (1967) *Acta Biol. Med. Germ.* **18**, 163–175
- Seaman, G. V. F., Tamblyn, C. H., Colvin, N. & Walter, H. (1980) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39**, 1917
- Stein, S., Chernian, M. G. & Mazur, A. (1971) *J. Biol. Chem.* **246**, 5287–5293
- Trávníčková, E. & Šulc, K. (1970) *Physiol. Bohemoslov.* **19**, 243–250
- Valet, G., Metzger, H., Kachel, V. & Ruhlenstroth-Bauer, G. (1972) *Blut* **24**, 42–53
- van Kampen, E. J. & Zijlstra, W. G. (1965) *Adv. Clin. Chem.* **8**, 141–186
- Wells, R. M. G. (1979) *J. Comp. Physiol.* **129**, 333–338