The activities of three enzymes of haem synthesis during hepatic erythropoiesis in the mouse embryo

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SUMMARY

Aminolaevulinate synthetase, aminolaevulinate dehydratase, and haem synthetase, three enzymes which may have a regulatory role in haem synthesis, have been determined in liver extracts from different foetal stages of the mouse.

Haemoglobin synthesis increases rapidly from early on the 14th day, after fertilization, to reach a maximum late on the 15th day. Aminolaevulinate synthetase reaches a maximum on the 14th day, 24–36 h before the peak of haemoglobin synthesis, aminolaevulinate dehydratase on the 15th day, about 12 h before the peak of haemoglobin synthesis, and haem synthetase on the 17th day. Maximal activity of aminolaevulinate synthetase and aminolaevulinate dehydratase is of only a few hours' duration. Throughout embryonic development the activities of all three enzymes are higher than in the adult liver.

The absence of a correlation of enzyme activity with foetal liver cell population changes implies that fluctuations in enzyme activity cannot be explained solely by changes in the proportions of different cell types. The high levels of activity relative to those of adult liver may be related to the high proportion of erythroid cells in the foetal liver.

It is concluded that these enzymes are unlikely to form rate-limiting steps during the increase in haemoglobin synthesis between 14 and 15 days.

INTRODUCTION

Erythropoiesis in the embryonic mouse occurs first in the yolk sac. From the 12th day onwards it becomes established in the liver and this is the major site of erythropoiesis until a few days before birth. Thereafter erythropoiesis is located principally in the spleen and bone marrow (Rugh, 1968). During the hepatic phase of embryonic erythropoiesis the most rapid increase in the rate of haemoglobin synthesis occurs between the 14th and 15th days of gestation (Cole & Paul, 1966). This is evident both as an increase in the rate of synthesis of protein-bound haem and in the synthesis of the adult type of haemoglobin (Cole, Hunter & Paul, 1968).

The activity of haem-synthesizing enzymes, in particular aminolaevulinate synthetase (ALAS), may be rate-limiting in the synthesis of haemoglobin (Levere, Kappas & Granick, 1967). It has been shown that avian yolk sac can
synthesize haemoglobin precociously if supplied with aminolaevulinate, implying that ALAS is the rate-limiting step (Levere & Granick, 1965; Wainwright & Wainwright, 1970; Wilt, 1965).

As the haem-synthesizing enzymes may be involved in the control of the initiation of hepatic erythropoiesis, the activities of these enzymes have been measured at different stages of gestation and correlated with the rate of haemoglobin synthesis.

MATERIALS AND METHODS

Source of erythroid cells

Embryos from Porton White Swiss mice were used. The stage of gestation was determined by counting the detection of a vaginal plug as day zero. Pregnant mice were killed by cervical fracture and the embryonic livers dissected into Hanks' balanced salt solution. Livers were used fresh for ALAS determinations; storage at -70 °C did not reduce either aminolaevulinate dehydratase (ALAD) or haem synthetase activity.

Enzyme assays

The activity of ALAS was determined by incubating a fresh enzyme extract for 30 min with 8 μCi/ml [2-14C]glycine (Radiochemical Centre, Amersham; 21.8 mCi/mM) in 0.04 M-phosphate buffer at pH 7.0 in the presence of mM-glycine, 0.27 mM-pyridoxal phosphate, 3.3 mM-sodium malate and 1.3 mM-MgCl2. The [5-14C]aminolaevulinate so formed was separated from [14C]-glycine precursor by thin-layer electrophoresis, eluted, and counted on a scintillation counter (Freshney & Paul, 1970). Since rapid homogenization of the small volumes involved caused frothing, ALAS extracts were prepared by lysis in an equal amount of a hypotonic buffer containing 20 mM-MgCl2, 10 mM EDTA, 1 mM-glycine, 4 mM-mercaptoethanol and 0.01 M-phosphate, pH 7.0. Though this preparation gave variable results with adult liver (Freshney & Paul, 1970) the yields of activity from embryonic liver were reproducible and were greater than with homogenization in buffered sucrose.

The rate of aminolaevulinate synthesis in an adult liver homogenate is linear up to at least 4 h (Freshney & Paul, 1970); however, in a 15-day embryonic liver lysate the rate decreased after 30 min (Fig. 1). Consequently in the present studies the time of incubation was restricted to 30 min.

ALAD activity was measured in 0.15 M-KCl homogenates by colorimetric estimation of porphobilinogen (PBG) produced from aminolaevulinate (Gibson, Neuberger & Scott, 1955; Shemin, 1962) using Ehrlich's reagent as modified by Mauzerall & Granick (1956).

Haem synthetase was measured as follows. Enzyme extract was prepared by homogenization at 50 mg wet weight/ml in 0.15 M-KCl containing 0.4% (v/v) Tween 20; 0.1 ml of this extract was incubated for 20 min at 37 °C under N2 with 0.4 ml 0.1 M-glutathione in 0.23 M-Tris-HCl, pH 7.4; 0.4 ml of a solution containing 0.25 μCi/ml 59FeCl3 (0.25–1.00 Ci/mM) 0.05 mM-FeCl3, and 0.5 mM-
protoporphyrin IX (substrate mixture) were then added to each tube. After a further hour at 37 °C under N₂ the reaction was terminated with 0·1 ml 1 N-HCl and the haem extracted once with 2·0 ml and once with 1·5 ml butanone at 0 °C. In blanks the substrate mixture was added after acidification; 0·5 ml of the pooled butanone extracts was dried on glass-fibre discs and the radioactivity determined in toluene-based scintillator in a Nuclear Chicago mark I scintillation counter.

It was found that high counts sometimes detected in blanks were due to inorganic ⁵⁹Fe and could be greatly reduced by further extraction of the pooled butanone phases with 3·0 ml of 0·1 N-HCl.

Protoporphyrin IX was prepared from the dimethyl ester by overnight hydrolysis, at 20 °C in 25% HCl. The free protoporphyrin was dried on a
rotary evaporator and redissolved in 1.2% sodium bicarbonate. Though some precipitation occurred during storage at $-20\, ^\circ C$, this redissolved completely in the substrate mixture.

When incubations were performed in the absence of exogenous protoporphyrin the activity was reduced by 99%.

As the reaction rate increased linearly with the concentration of homogenate from 10 to 200 mg/ml it was assumed that cellular iron does not contribute significantly to the reaction rate.

**Measurement of haemoglobin synthesis**

Single cell suspensions were prepared by overnight trypsinization at 4 °C as described by Cole & Paul (1966). In some cases the livers were suspended directly without trypsinization. Aliquots were incubated at $10^6$ total cells per ml in Waymouth's medium MB 752/1 supplemented with 10% (v/v) foetal bovine serum (Flow Laboratories, Irvine). $^{59}$FeCl$_3$ (0.25–1.00 Ci/mm) was incubated overnight at 37 °C with mouse serum to equilibrate with transferrin; 0.15 ml was then added to 1 ml test-tube cultures to give 1 μCi/ml and 6.5% (v/v) of mouse serum.

After 30–60 min incubation at 37 °C unincorporated isotope was removed by centrifugation in two separate washes of 10 ml ice cold Hanks' balanced salt solution. One ml Drabkins solution was added to the cell pellet to produce stable cyanmethaemoglobin which could then be stored at $-20\, ^\circ C$. The suspension of cells in Drabkins solution was frozen and thawed three times to complete lysis and the haem extracted from the lysate by acidifying with 0.1 ml N-HCl and mixing with 1.2 ml butanone (ice-cold) (Teale, 1959); 0.3 ml of the butanone layer, which contained the haem, was dried on to glass-fibre discs and counted in toluene-based scintillator (Cole & Paul, 1966).

**RESULTS**

**Development of haem-synthesizing enzymes**

ALAS, ALAD and haem synthetase activities were determined separately in different samples of embryonic liver, collected in the mornings of the 12th or 13th day of gestation till birth. ALAS activity (Fig. 2) was five- to sixfold higher than that of adult liver, expressed per mg of liver protein, for most of the gestational period. A peak of activity occurred at 14 days, when the maximum was about four times the level at other times, and about 20 times the adult level. In subsequent experiments, where samples were collected in the afternoon, 13-day activities ranged from 2.77 to 5.25 and 14-day from 3.70 to 6.50 p-moles ALA/min/mg protein. It is possible that minimal 13-day activities and maximal 14-day activities are only detectable early on the appropriate day. ALAD activity (Fig. 3) declined between 12 and 13 days, increased about threefold from 13 to 15 days, and declined again after 15 days, to reach the adult level at
Embryonic haem synthesis

birth. This general pattern was confirmed in two separate series of assays and confirmed from 13 to 16 days in later experiments (see below). ALAD activity increased and decreased less rapidly than ALAS activity.

Fig. 2. ALAS activity at different developmental stages. Foetal and newborn livers were collected at the times indicated and homogenized slowly for 15–20 sec in hypotonic buffer (see Materials and Methods). Adult liver was homogenized for 2 min in 0.05 M-phosphate buffer, pH 7.0, containing 0.02 M-MgCl₂, 0.01 M-EDTA, 4 mM-mercaptoethanol and 1 mM-glycine. ALAS assays were performed as described in the Materials and Methods section. Each horizontal bar represents one observation and each circle is the mean of assays performed on livers from one litter. NB = New Born; Ad = Adult. The adult value is taken from Freshney & Paul (1970).

Fig. 3. ALAD activity at different developmental stages. Livers were collected at the times indicated and ALAD activity estimated (Gibson et al. 1955). Data points and means as in Fig. 2. The adult point is the average of the means of two livers each assayed in triplicate. Other data points and means as in Fig. 2.

Fig. 4. Haem synthetase activity at different developmental stages. Livers were collected at the times indicated and haem synthetase activity estimated as described in the Materials and Methods section. Data points and means as in Fig. 2.
Haem synthetase activity (Fig. 4) declined between 13 and 14 days, increased between 14 and 15 days, and continued to rise to reach a maximum at 17 days, which was about double the activity at 14 days. It then declined to the adult level at birth.

Correlation of enzyme activity with haemoglobin synthesis

The rate of haemoglobin synthesis per cell in the foetal mouse liver reaches a maximum at 15 days (Cole & Paul, 1966), which corresponds approximately to the maxima observed in ALAS and ALAD activities. In order to correlate precisely the activity of these two haem-synthesizing enzymes with the rate of haemoglobin synthesis, cell suspensions were prepared from the livers of 13 to

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Fig. 5. Relative activities of ALAS and haemoglobin synthesis. Litters were collected between 13 and 16 days, and the age was determined from the date of plugging and by morphological characteristics. ALAS activity was determined directly on lysates prepared from the livers of some of the embryos. The remaining livers from each litter were placed in 0.25 % trypsin, 0.3 % carboxymethylcellulose, in isotonic sodium citrate (Cole & Paul, 1966). These were kept at 4 °C overnight, raised to 37 °C for 5 min the following day and disaggregated. Haemoglobin synthesis was then measured over 1 h in each cell suspension as described in Materials and Methods. Each open or closed circle is the mean of five replicate observations on one litter, with standard deviations represented as vertical bars. The solid line and open circles represent ALAS activity; closed circles and the broken line represent the rate of haemoglobin synthesis.
16-day embryos. The rate of haemoglobin synthesis was measured as described above, and either ALAS or ALAD activities determined in the livers of littermates. The stages of development were determined by morphological criteria.

ALAS activity reached a maximum 24–36 h before haemoglobin synthesis (Fig. 5), while ALAD activity reached a maximum about 12 h before haemoglobin synthesis (Fig. 6). Both enzymes returned to a low level by 16 days. When enzyme activities are expressed as units per $10^6$ cells the sequence of maximum enzyme activities and maximum rate of haemoglobin synthesis is not altered. The level of ALAS activity at 13 days in Fig. 5 is higher than that observed in Fig. 2; this may be due to time of sampling (see above). The 13-day sample in

![Graph](image)

Fig. 6. Relative activity of ALAD and haemoglobin synthesis from 14–16 days. Litters were collected as for Fig. 5, and ALAD activity determined directly (Shemin, 1962) in homogenates of some of the livers from each litter. The remaining livers were disaggregated directly, by pipetting with Waymouth’s medium without previous trypsinization. Haemoglobin synthesis was then measured over 30 min as described in the Materials and Methods section. The solid line and open circles represent ALAD activity; the broken line and closed circles represent the rate of haemoglobin synthesis. All the haemoglobin synthesis values are means of ten replicates with standard deviations represented as a solid vertical bar. The first ALAD point is a mean of five replicates with standard deviation (solid vertical bar). The remaining ALAD points are means of duplicate or triplicate observations with each individual observation represented as a short horizontal bar connected to the mean by a dotted vertical line.
Fig. 2 was collected at about 10.00 a.m., while that in Fig. 5 was collected at about 4.00 p.m.

The maximum ALAS activity was determined precisely only after assaying many samples at 14 days and is probably of only a few hours' duration. The beginning of the rise in the rate of haemoglobin synthesis did not occur until ALAS had already started to decline; no difference in haemoglobin synthesis was recorded between the 13th day and early on the 14th day.

**DISCUSSION**

The incorporation of $^{59}$Fe$^{3+}$ into haemoglobin in the present series of experiments confirms the observation of Cole & Paul (1966) that the rate of haemoglobin synthesis per cell increases rapidly between 14 and 15 days in Porton White Swiss mice and not before this. Paul, Conkie & Freshney (1969) showed an increase in both hepatogenic haemoglobinized cells and total embryonic haemoglobin between the 13th and 14th day. These data were concerned with average differences in day-to-day values and not with rate changes within a 24 h period. The present data, though showing no increase in haemoglobin synthesis between the 13th and early on the 14th day, do show an increase during the 14th day and are therefore compatible with the results of Paul et al. (1969).

The appearance of the complete range of erythroid precursors in the foetal liver before the rise in haemoglobin synthesis suggests that erythropoiesis may occur in two phases: (1) initiation of differentiation of erythroid precursors, which is already detectable by 12 days; (2) acceleration of maturation and haemoglobin synthesis at 14 days, possibly by erythropoietin.

When the changes in enzyme activity are compared with changes in the cell population of the liver during development (Paul et al. 1969; Djaldetti, Chui, Marks & Rifkind, 1970; Tarbutt & Cole, 1970) few increases in the proportions of specific cell types are seen which can be correlated with the changes in enzyme activity observed. Such correlations as can be made suggest that an increase in total erythroblasts may precede the increases of enzyme activity and haemoglobin synthesis. Enzyme fluctuations may therefore result from changes of enzyme activity within all or some of the cell compartments, rather than changes in the proportions of cells with characteristic enzyme activities. The high activity of these enzymes, relative to the adult, may be correlated with high proportions of erythroid elements in the foetal liver. Preliminary observations on suspensions of foetal liver cells separated by velocity sedimentation at unit gravity indicate that aminolaevulinate dehydratase activity is almost exclusively located in erythroblasts (unpublished results).

From the data presented it would appear possible that the synthesis of ALAS, ALAD and globin might be sequentially induced, and that haem synthetase might be co-induced with globin synthesis.
Embryonic haem synthesis

The separation in time between the maximum ALAS activity and the maximum rate of haemoglobin synthesis makes it unlikely that ALAS is a controlling step in haemoglobin synthesis, but cannot exclude this possibility entirely. In the case of ALAS the temporal separation from the maximum rate of haemoglobin synthesis is much less and it is more difficult to exclude ALAD as a controlling step.

According to Fantoni, de la Chapelle, Rifkind & Marks (1968), Djaldetti et al. (1970) the haemoglobin synthesizing system becomes stable between the 12th and 13th day of gestation when $^{55}$Fe and $[^{3}H]$leucine incorporation become resistant to the action of actinomycin D. These findings suggest that unstable RNA’s or proteins no longer form rate-limiting steps. If this is so the transient increases of ALAS and ALAD activity, which implies an unstable mechanism, is unlikely to be rate-limiting at this stage of the development of haemoglobin biosynthesis.

The authors wish to express their gratitude to Miss Sheila Brown for skilled technical assistance, and to Mrs M. G. Freshney for assistance in drawing diagrams. The work was supported by grants from the Medical Research Council and from the Cancer Research Campaign.

REFERENCES


(Manuscript received 1 April 1971)