The effects of erythropoietin on haem synthesis in mouse yolk sac and cultured foetal liver cells

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Erythropoiesis in the foetal mouse is reported to occur initially in the yolk sac, followed by the liver, the spleen and finally the bone marrow (Snell, 1956; Borghese, 1959). At 12 days of gestation 97% of the circulating erythrocytes are nucleated (Craig & Russell, 1964) and presumably arise from the yolk sac blood islands. The cells of this generation persist in decreasing numbers until the 16th day of gestation. Haemoglobin can first be recognized by histochemical methods (O'Brien, 1961) in the yolk sac before somite formation at the early head fold stage and a network of blood islands, almost covering the embryo, rapidly develops (Plate 1, figs. A–F).

The rate of erythropoiesis in the adult mammal is controlled by the hormone erythropoietin, which is released from the kidney as a response to lowered oxygen tension in the arterial blood supply (Fisher, Schofield & Porteus, 1965). Erythropoietin, which is glycoprotein, and not species specific, appears to exert its effect at several levels in the erythropoietic system (Lajtha, 1964). It affects the erythropoietic stem cell population directly, causing an increased number of cells to enter the erythrocytic pathway of differentiation (Filaminovicz & Gurney, 1961; Krantz, Gallien-Lartigue & Goldwasser, 1963), and also increases haemoglobin synthesis in existing normoblasts (Stahlman, Brecher & Moores, 1962). Further, increases in the absolute number of normoblasts present (Fisher, Roh, Couch & Nightingale, 1964) and increased release of reticulocytes from the marrow pool (Fisher, Lajtha, Buttoo & Porteus, 1965) have been observed.

It has recently been found (Gallien-Lartigue, personal communication) that cells of the erythropoietic series can be maintained in organ cultures of mouse foetal liver in the presence of anaemic serum but disappear if cultures are grown with normal serum.

The present communication describes the effects of purified sheep erythropoietin on intact mouse embryos at the yolk sac stages and on cells from foetal livers disaggregated at various stages of development, both in vitro.

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MATERIALS AND METHODS

All embryos were obtained from random-bred 10- to 12-week-old Swiss albino mice (Porton strain) after induction of ovulation and mating with 'Gestyl' and 'Pregnyl' (Organon, Ltd.). This permits accurate timing of developmental ages and provides large numbers of embryos of the required stage. All embryos are timed from ovulation (midnight following the Pregnyl injection) and the morning on which mating plugs are present is day 0.

Embryos to be cultured intact were isolated on the 8th and 9th days of gestation. The uteri were excised aseptically, the decidual masses removed from the uterine musculature in culture medium based on tris/citrate buffered balanced salt solution (Paul, 1965) and the embryo, surrounded by trophoblastic giant cells and Reichert’s membrane, isolated (New & Stein, 1964). The embryos were prepared for culture by opening Reichert’s membrane but leaving all the adhering trophoblast attached to the ectoplacental cone. The embryos were cultured in 3.5 cm diam. Falcon polystyrene dishes in 5% CO2 in air at 36.5°C. Each dish contained 1 ml of medium, i.e. just enough to cover the larger embryos. The developmental stage of each embryo was noted and the main axes were measured, at the beginning and end of the culture period, which was 16–21 h. Any markedly abnormal or retarded embryos were rejected from the final analysis of the data.

Livers from more advanced embryos were removed aseptically in Hanks’s balanced salt solution (Paul, 1965) after checking the developmental stage of the embryo by its external appearance (Grüneberg, 1943). The livers were disaggregated by exposing them to Difco trypsin 1/250 in isotonic sodium chloride/sodium citrate, pH 7.8 (Paul, 1965), containing 0.3% sodium carboxymethyl cellulose (to protect the cell membranes), at 4°C overnight. The supernatant trypsin was then removed and the tubes warmed to 36.5°C for 5 min. Culture medium containing 5% calf serum was added and disaggregation completed by pipetting. The cells were washed once in medium, checked for clumping and for viability (by dye exclusion with 0.1% naphthalene black) and counted with a Coulter electronic cell counter, Model D (Threshold 50, Aper-
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ture current 2). This technique regularly produces single cell suspensions of 80–90% viability at all stages of liver development studied.

The medium used for culturing both the intact embryos and liver cells was based on Waymouth's MB752/1 (Waymouth, 1959), which has been found especially satisfactory for early mammalian embryonic material (Cole & Paul, 1966). This was modified by the addition of 5% neonatal calf serum, 5% female mouse serum, and 0.01 mM-FeCl₃, so that the final iron concentration of the medium was between 1 and 2 µg/ml. Iron determinations on sera and complete media, were carried out by the dipyridyl method of Ramsay (1951).

Foetal liver cells were cultured in suspensions of 0.5–1 × 10⁶ cells/ml in 1 ml aliquots in 10 ml Excello test tubes covered with an Oxoid cap, in 5% CO₂ in air at 36-5°C.

Haem synthesized in vitro was labelled by the addition of ⁵⁹FeCl₃ previously equilibrated with 50% mouse serum in Hanks's BSS, for at least 12 h at 36-5°C at a concentration of 10 µc/ml. The specific activity of the ⁵⁹Fe used in these experiments varied from 3.0 to 12.5 µc/µg Fe and was added to the cultures at the level of 1–2 µc/ml. At the end of the incubation time the embryos or cells were washed with Hanks's BSS, and 2 parts H₂O:1 part Drabkin's solution (Wintrobe, 1961) was added (1 ml final volume per embryo or per cell aliquot). The samples were lysed by freezing and thawing three times, the debris sedimented by centrifugation and the supernatant decanted and acidified with 0.1 ml of n-HCl. Total haem was extracted from the supernatant with 1.2 ml of ethyl methyl ketone (Teale, 1959; Krantz et al. 1963). Aliquots of 0.5 ml were dried on stainless steel planchettes and counted in a Nuclear Chicago gas-flow counter.

The lysed debris, and the Drabkin's solution supernatant and remaining ethyl methyl ketone from each intact embryo were recombined, hydrolysed with perchloric acid and the deoxyribose content of the hydrolysate measured by the diphenylamine method of Burton (1956). (The presence of ethyl methyl ketone interferes with the estimation if trichloroacetic acid is used.) This enabled haem synthesis to be expressed against deoxyribose content per embryo, which provided a reliable basis for quantitative analysis and overcame the problem of the range of developmental stages found at the same time of gestation.

The erythropoietin used in these experiments (N.I.H. Haematology Study Section, step 4, Lot K 103, 217A) was prepared from plasma of phenylhydrazine-treated sheep (White, Gurney, Goldwasser & Jacobson, 1960) and had a specific activity of 22 units/mg protein. It was used at concentrations between 0.15 and 1 unit/ml.

RESULTS

Yolk-sac erythropoiesis

During this early period of development, when morphological changes are especially rapid, considerable variations in the stages of development reached occur both within litters and between litters at the same times after ovulation.
Each experiment therefore contained embryos of various stages, and the haem synthesized was labelled throughout the culture period. The yolk sac contains undifferentiated, or differentiating erythroid precursor cells before the first

**Text-fig. 1.** Haem synthesis during yolk-sac erythropoiesis, labelled with $^{59}$Fe for 21 h in vitro showing relationship between deoxyribose content and haem synthesized in: (1) control embryos, and (2) embryos exposed to 0·4 unit/ml erythropoietin. (3A) and (3B) are the 95% confidence limits of the pooled data. O, Control embryo; x, embryo exposed to erythropoietin. (A–E) Developmental stages of embryos when explanted, relative to deoxyribose content after 21 h development in vitro. A, Primitive streak; B, 4-somite; C, 8-somite; D, 14-somite; E, 20-somite.
appearance of haemoglobin in the embryo; hence factors which stimulate erythropoiesis might bring about haemoglobin synthesis earlier than normal, or increase the rate of synthesis, or both.

The relationship between the amount of haem synthesized and stage of development, estimated by deoxyribose content/embryo, is shown in Text-fig. 1. The thirty embryos used in this experiment were derived from seven litters, isolated on the late 8th or early 9th day after ovulation, and ranged from presomite to early heart-beat stages. The presence of erythropoietin at 0.2 unit/ml did not affect either the total amount of haem synthesized, or cause it to appear at an earlier stage of development. The stage at which $^{57}$Fe incorporation into haem first occurred, in embryos containing 4–5 $\mu$g deoxyribose at the end of the culture period, is in agreement with the stage at which haemoglobin can first be detected by histochemical methods. Increasing the concentration of erythropoietin to 1 unit/ml is similarly ineffective (Text-fig. 2). This experiment utilized twenty embryos from three litters, ranging from pre-somite to 6–8 somite stages at the time of explantation. The difference in the regression coefficients of the treated and untreated embryos is not significant and haem synthesis was again
initiated at similar embryonic stages. The background values in these experiments were equivalent to less than 1 μm haem formed/embryo.

**Foetal liver erythropoiesis**

Haem synthesis in duplicate aliquots of the same cell suspension was measured by determining 59Fe incorporation over periods of 2–4 h and calculated as μm haem formed per hour per million cells present at time 0, and plotted at the mid point of the exposure to 59Fe. As far as possible embryos from single litters

Table 1. *The numbers of cells per liver obtained after disaggregation with trypsin, at different developmental stages*

<table>
<thead>
<tr>
<th>Days of gestation</th>
<th>Yield of cells/liver × 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>10½</td>
<td>0-4</td>
</tr>
<tr>
<td>12½</td>
<td>0-75</td>
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<tr>
<td>14½</td>
<td>5</td>
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<tr>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>Neonatal</td>
<td>10</td>
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</tbody>
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Text-fig. 3. Haem synthesis in 10½-day foetal mouse liver cultures: × —— ×, with 0-15 unit/ml erythropoietin; ○—— ○, control. Solid bars on the time ordinate indicate period of exposure to 59Fe.
were used for each experiment, although this was not possible with the earlier developmental stages (Table 1). Any obviously retarded or abnormal embryos were rejected.

The earliest livers studied were from 10½-day-old embryos. Text-fig. 3 shows the results obtained from pooled livers from thirty-one embryos from three litters, all at comparable stages of development, exposed to 0·15 unit erythropoietin/ml. At this stage the livers contain a few faintly pink foci and obviously contain little haemoglobin. In the first 8 h of culture both control and erythropoietin-treated cells show a rising rate of haem synthesis. This rise in rate is, however, only maintained in the presence of erythropoietin, when it reaches a maximum after approximately 28 h and then declines. The rate of synthesis in control cultures declines continuously after 8 h. At its maximum, the rate of haem synthesis in the erythropoietin-treated cells is 19 times that in the control.
cells at the same time, and more than 6 times the maximum reached by the control. The failure of late yolk-sac stage embryos to respond to erythropoietin indicates that the response detected in these liver cultures is by cells which were an inherent part of the liver structure at the time of explantation and not maturing cells of the yolk-sac generation of erythropoiesis, reaching it via the circulation.

Text-fig. 5. Haem synthesis in 14½-day foetal liver cultures. Legend as Text-fig. 2.

A similar response was seen with liver cells from 12½-day embryos exposed to 0·4 unit of erythropoietin/ml. Text-fig. 4 shows the results obtained from fifty-six embryos from four litters. A maximum rate of incorporation is again reached after approximately 28 h; this maximum rate is nearly 16 times the control value at equivalent time, but only 3 times the initial rate.

The rate of haem synthesis in cells from 14½-day embryos (Text-fig. 5) declines during the first 12 h in culture in the presence of erythropoietin as well as in control cultures. In the presence of erythropoietin this decline is subsequently reversed, and a maximum rate of synthesis occurs after 24–28 h; in contrast, it remains very low in the absence of erythropoietin. In these cultures, stimulated with 0·4 unit of erythropoietin/ml, the maximum rate of synthesis
observed is 45 times that of controls at equivalent times, and approximately 9 times the initial rate.

A marked change in this pattern of response occurs in cultures derived from 15½-day embryonic livers (Text-fig. 6). The mean rate of haem synthesis in the first 4 h is slightly higher than the maximum erythropoietin-stimulated rate observed in 14½-day embryonic livers. However, the rate of haem synthesis in both erythropoietin-treated and control cultures falls from the time of explantation and erythropoietin has no significant effect.

A similar decline in the rate of haem synthesis and lack of responsiveness to erythropoietin has been observed in 16½-day, 18½-day, immediately prenatal and 1st day post-natal embryos (Text-figs. 7–10). Cells of the 16-day liver

Text-fig. 6. Haem synthesis in 15½-day foetal liver cultures. Legend as Text-fig. 2.
showed only very slow rates of haem synthesis and this declined in culture even in the presence of erythropoietin. The initial rate of synthesis increased with stage of development until in 19-day embryos, immediately before birth, it was approximately \( \frac{3}{8} \) that of the highest rate observed, i.e. in cultures from 15\( \frac{1}{2} \)-day livers.

Text-fig. 7. Haem synthesis in 16\( \frac{1}{2} \)-day foetal liver cultures. Legend as Text-fig. 2.
Text-fig. 8. Haem synthesis in 18-day foetal liver cultures. Legend as Text-fig. 2.
Text-fig. 9. Haem synthesis in immediately prenatal liver cultures. Legend as Text-fig. 2.
Text-fig. 10. Haem synthesis in 1st day postnatal liver cultures. Legend as Text-fig. 2.
Erythropoietin and foetal erythropoiesis

It has been reported (Borghese, 1959) that in the foetal mouse the spleen becomes an erythropoietic organ at 16 days of gestation. However, no significant incorporation of $^{59}$Fe into haem could be detected in cultures derived from 16½- or 18-day foetal spleen, or neonatal spleen, either initially or after exposure to erythropoietin. The background values in this series of experiments were equivalent to 1 $\mu$g haem/h/10$^6$ cells.

DISCUSSION

Several recent studies have suggested that globin synthesis, and hence haemoglobin formation, is controlled at the polysome level by the availability of haem. The addition of hemin and other tetrapyrrolys to isolated avian erythrocyte nuclei increases the rate of globin synthesis and reverses the inhibition caused by high $O_2$ tensions (Hammel & Bessman, 1964, 1965). An increased incorporation of $^{14}$C valine into haemoglobin in the presence of hemin in rabbit reticulocytes has also been observed (Bruns & London, 1965). The addition of the haem precursor $\delta$-aminolaevulinic acid to de-embryonated chick blastoderms causes increased haemoglobin synthesis and this effect is actinomycin D-resistant but puromycin-sensitive (Levere & Granick, 1965). Erythropoietin, acting on potential haemoglobin synthesizing cells in vitro, causes an increase in the rate of synthesis of RNA (which is probably messenger RNA) within 15 min (Krantz & Goldwasser, 1965). The primary effect of erythropoietin action may therefore be to induce synthesis of messenger RNA for an enzyme or enzymes responsible for haem synthesis.

In the chick blastoderm, synthesis of messenger RNAs essential for haemoglobin synthesis has occurred before the formation of the head fold stage, several hours before haemoglobin first becomes detectable, at the 7–8-somite stage (Wilt, 1965). In even the earliest mouse embryos used in the present study, transcription of messenger RNA necessary for the initiation of haemoglobin synthesis may therefore have occurred before the time of explantation and exposure to erythropoietin in vitro. Since no increase in haem synthesis occurs in the presence of erythropoietin during the yolk-sac stage, the majority, if not all, of the potentially erythropoietic cells available at this stage must previously have been stimulated to differentiate. Whether this initial phase of haemoglobin synthesis is independent of an erythropoietin-like stimulus, or depends on an endogenous or exogenous, i.e. maternal, hormone supply cannot yet be ascertained.

The results of the experiments with foetal liver cells are summarized in Text-fig. 11. These show that as early as 10½ days of gestation, soon after the liver can first be recognized as a discrete organ, at least on gross examination, it contains cells capable of responding to erythropoietin by synthesizing haem. The maximum rates of synthesis observed, following erythropoietin stimulation in 12½- and 14½-day embryos and the initial spontaneous rate in 15-day embryos, are all similar. This suggests that, with the doses of erythropoietin used,
the sensitive cell population is responding to a similar extent as *in vivo*, and that this represents the maximum obtainable rate of synthesis in these conditions. The temporal pattern of response to erythropoietin seen in the 10–14-day embryonic livers is very similar to that observed in adult rat bone marrow cells cultured under similar conditions (Krantz *et al.* 1963), with a maximum rate of haem synthesis occurring 28–30 h after the initial stimulation by erythropoietin. However, the maximum rate of haem synthesis in the liver cells is 3–4 times higher than that in rat marrow cultures on a per cell basis, with equal doses of erythropoietin. The lag period in the response to erythropoietin of approximately 10 h, observed in the cultures of 14½-day liver, was also consistently found in the rat marrow cultures. Induction of erythropoiesis *in vivo* in the polycythemic mouse takes from 12–18 h and suggests that an initial period of synthesis of precursors occurs, before haem appears. In this case failure to demonstrate a similar lag in cultures from 10½-day livers might indicate that these precursors were present at this stage of embryonic development.

These results suggest that erythropoietin production may be a prerequisite for hepatic erythropoiesis in the mouse foetus. From the kinetics of the response to erythropoietin it is likely that erythropoietin production rapidly reaches a maximum or is actually initiated during the 13th to 14th days of development.
Examination of livers from foetuses between 16 days and birth, and from neonatal animals shows a second phase of haem synthesis with a low rate on the 16th day but increasing until birth. This may indicate cytochrome synthesis in the liver parenchyma, or as it is generally accepted that bone marrow erythropoiesis begins in the mouse foetus at 16 days it is possible that this secondary haem synthesizing cell population is derived from circulating maturing cells produced by the bone marrow.

The stem cell population in a haemopoietic organ may be endogenous in origin ('extra vascular'), or, alternatively have originated in another site, and migrated via the circulation to the site of differentiation. Examination of spatial relationships in fixed material cannot decide between these alternatives, because the stem cells may possess invasive properties assisting their migration.

Taylor (1965) has shown the presence of pluripotential haemopoietic cells in 12- and 15-day foetal mouse liver, with lymphopoietic capacity in the thymus of irradiated recipients. Since pluripotential cells cannot be recovered from the thymus, it is considered that the cells are irreversibly stimulated to differentiate just before or just after entry. Cells from immediately neonatal mouse liver can form spleen colonies in irradiated recipients but with only one-third of the efficiency found with adult marrow (McCulloch & Till, 1963). However, the proportion of colonies derived from this material representing the different pathways of haemopoietic differentiation is not stated and the present study indicates that only few erythropoietin-sensitive stem cells could remain in the liver at this stage. Therefore, two alternative explanations for the change in the pattern of haem synthesis, between 14½- and 15½-day foetal mouse livers, observed in the present study, are possible. The erythropoietin-sensitive stem-cell population remaining in the liver may migrate during the 14th day of development, leaving behind only those cells which have received the stimulus to differentiate. Alternatively, the stem-cell population originally present, and its descendants, are entirely switched into the erythropoietic pathway at this stage of development and the stem cells of the subsequent haemopoietic sites have a separate origin.

**SUMMARY**

1. The effects of erythropoietin on haem synthesis during yolk sac and hepatic erythropoiesis in the foetal mouse have been studied *in vitro*.

2. Cultures were maintained in Waymouth's medium MB725/1 with 5 % calf serum, 5 % mouse serum and 0.01 mM-FeCl₃. Yolk-sac erythropoiesis was studied in embryos explanted at stages between primitive streak and heart beat and cultured intact, and hepatic erythropoiesis in cell suspensions obtained by trypsinizing foetal livers.

3. Haem synthesized *in vitro* was labelled by exposing cultures to ⁵⁹Fe equilibrated with mouse transferin, and was separated chemically with ethyl methyl ketone.
4. Haem synthesis does not occur in embryos containing less than 4–5 μg deoxyribose. Neither the stage at which synthesis is initiated, nor the rate of synthesis in the yolk sac is affected by erythropoietin.

5. Foetal livers from 10½-, 12½- and 14½-day foetuses contain cells responsive to erythropoietin by increased rates of haem synthesis. Synthesis is only maintained in vitro in the presence of erythropoietin and maximum rates occur approximately 28 h after initial exposure.

6. The rate of haem synthesis in 15½-day liver cells is not responsive to erythropoietin and declines in culture, but the initial rate is similar to the maximum rate obtained from earlier liver cells stimulated by erythropoietin in vitro.

7. The rate of haem synthesis in 16-day liver cells is very low, but rises in later foetuses until birth, although no response to erythropoietin occurs.

8. The kinetics of response to erythropoietin in 12–14-day mouse liver cells is similar to that of adult rat bone marrow in vitro.

9. No haem synthesis could be detected in cultures from spleens from 16½- or 18-day foetuses, or from neonatal animals, with or without erythropoietin.

RÉSUMÉ

Les effets de l'érythropoïétine sur l'hémosynthèse dans la vésicule ombilicale des souris et dans des cultures de cellules hépatiques foetales

1. On a étudié in vitro les effets de l'érythropoïétine sur l'hémosynthèse pendant l'érythropoïèse dans la vésicule ombilicale et dans le foie des souris foetales.

2. Les cultures étaient maintenues dans le milieu de Waymouth, MB725/1 avec 5% de sérum de veau, 5% de sérum de souris et 0·01 mM FeCl₃. On a étudié l'érythropoïèse dans la vésicule ombilicale d'embryons prélevés à diverses étapes entre la ligne primitive et le battement du cœur et maintenus intacts en la culture. On a étudié l'érythropoïèse hépatique au moyen de la suspension de cellules, obtenue par l'action de la trypsine sur le foie foetal.

3. On a suivi l'hémosynthèse in vitro en exposant les cultures au ⁵⁹Fe, équilibré par de la transférine de souris, et ensuite on sépara le hémé chimiquement au moyen d'éthyle méthylique cétoné.

4. L'hémosynthèse n'a pas lieu dans les embryons qui contiennent moins de 4–5 μg de désoxyribose. L'érythropoïétine n'a aucun effet ni sur l'étape à laquelle commence la synthèse ni sur la vitesse de synthèse dans la vésicule ombilicale.

5. Les foies foetaux de 10½ jours, de 12½ jours et de 14½ jours, contiennent des cellules, qui réagissent l'érythropoïétine par l'accélération de leur vitesse d'hémosynthèse. La synthèse ne se maintient in vitro qu'en la présence de l'érythropoïétine et les vitesses maximum sont atteintes 28 heures, à peu près, après l'exposition originelle.

6. La vitesse de l'hémosynthèse dans les cellules de foie de 15½ jours ne montre pas de réaction à l'érythropoïétine, et cette vitesse diminue dans les
cultures. Pourtant, la vitesse primaire est semblable à la vitesse maximum obtenue dans des cellules hépatiques moins développées, stimulées par l’érithropoïétine in vitro.

7. La vitesse de l’hémosynthèse dans les cellules de foie de 16 jours reste très basse, mais elle s’accélère dans des foetus plus avancés et persiste jusqu’à la naissance bien qu’aucune réaction à l’érithropoïétine n’ait lieu.

8. La cinétique de réponse à l’érithropoïétine dans des cellules de foie de souris de 12–14 jours est semblable à celle qui a lieu dans la moelle des rats adultes in vitro.

9. Il est impossible de constater l’hémosynthèse ni dans les cultures de rate des foetus de 16\(\frac{1}{2}\) ou de 18 jours, ni dans la rate des animaux nouveau-nés, que l’érithropoïétine soit présente ou non.

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