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# Disturbed haem and globin synthesis in reticulocytes of prenatal flexed-tailed (*f*/*f*) anaemic mice

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SUMMARY

Haem synthetase activity and co-ordination of $\alpha$- and $\beta$-globin chain synthesis have been investigated in prenatal reticulocytes of congenic FL/4 Re +/+ $Lv^b/Lv^b$ and FL/1 Re*f*/*f* $Lv^b/Lv^b$, mice, which have a marked hypochromic, microcytic, siderocytic anaemia, with reduced erythrocyte numbers at birth, and also in other stocks bearing the *f* lesion. Haem synthetase activity in *f*/*f* reticulocyte homogenates was similar to that in normal cells but was markedly dependent on protoporphyrin added to the homogenate, while activity in normal cell homogenates was relatively independent of added precursor. In cultured normal prenatal reticulocytes $\alpha$- and $\beta$-globin was synthesized in approximately equal amounts during a 4 h labelling period, but in *f*/*f* reticulocytes there was an approximate 50% deficiency in $\beta$-globin chain synthesis. This deficiency could be repaired by added haem but not by protoporphyrin. Such a lesion is quantitatively consistent with the observed hypochromia of neonatal *f*/*f* erythrocytes. The relationship of this abnormality to effects of the *f* locus on early erythropoietic precursor cells is discussed.

## 1. INTRODUCTION

In both pre- and postnatal mammals the rate of production of erythrocytes is homeostatically regulated to maintain an optimum supply of oxygen to the tissues. The glycoprotein hormone, erythropoietin, has a central role in this regulation and *in vivo* exerts its primary effect by increasing recruitment of differentiating erythroblasts from a class of cells intermediate, on kinetic evidence, between the pluripotent stem cell, and the earliest recognizable erythroid precursor, the pro-erythroblast. *In vitro* experiments show that erythropoietin increases DNA, RNA, haem and protein synthesis in sensitive cells from both foetal and adult erythroid organs and also maintains the presence of early erythroblasts which disappear without continued exposure to hormone. Erythropoietin has no direct effect on haemoglobin synthesis in late erythroblasts or reticulocytes.

A number of inherited anaemias, each causing a discrete disruption of erythroid regulation, are available as experimental tools to aid analysis of developmental and physiological control of erythropoiesis.

Prenatal and neonatal flexed-tailed (*f*/*f*) mice are severely anaemic. The number of red cells/ml in *f*/*f* blood is approximately 80% that of normals and individual



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red cells contain only 60–70% of the normal complement of haemoglobin, so that newborn *f/f* mice contain about half the normal amount of haemoglobin. Most of the erythrocytes of newborn *f/f* mice are siderocytes, containing non-haem iron granules. These symptoms disappear progressively after birth and *f/f* adults have normal blood values, but are slow to respond to anaemia. Several studies (Coleman, Russel & Levin, 1969; Thompson *et al.* 1966, Fowler *et al.* 1967) have indicated that the primary effect of the *f/f* lesion is on the rates of proliferation or differentiation of early erythroid precursor cells, restricting the flow of cells through the 'erythropoietin responsive cell' compartment. However, it is now clear that the haemoglobin deficiency of pre- and neonatal *f/f* mice arises from complex interactions between disturbed proliferation of precursor cells, and disturbed haemoglobin synthesis in terminally differentiated cells.

Up to day 16 of gestation fewer colony forming cells are found in the livers of *f/f* foetuses than in normal foetuses (Bateman & Cole, 1972), and early *f/f* foetal livers contain fewer erythroblasts. Maximum numbers of erythroid cells are reached later, and characteristic changes in proportions of early and late erythroblasts are retarded. However, the relative rate of increase of erythroblasts in the early *f/f* foetal liver is higher than that in normal livers (Bateman *et al.* 1972), and *in vivo* analysis of the kinetics of erythroblasts of 14-day livers shows that the cell cycle of *f/f* late erythroblasts is shorter than normal (Tarbutt & Cole, 1972). These findings suggest that enhanced proliferation occurs in *f/f* foetal liver erythroblasts in compensation for the anaemia.

Erythroblasts from early *f/f* foetal livers show greater enhancement of DNA and RNA synthesis *in vitro* in response to erythropoietin than wild type cells, and similar increments in haem synthesis (Bateman *et al.* 1972). In contrast, haem synthesis in *f/f* prenatal reticulocytes is severely reduced, probably causing the appearance of siderotic granules. While iron uptake into *f/f* prenatal reticulocytes is normal, utilization of absorbed iron for haem synthesis is reduced to less than half normal level, and, in addition, the pool of iron available for haem synthesis in *f/f* late prenatal reticulocytes is less than half that in normal reticulocytes, suggesting a possible lesion in the mobilization of intracellular iron stores.

Glycine is a precursor of both haem and globin. Changes in the co-ordinated synthesis of these molecules can therefore be detected by changes in relative incorporation. In *f/f* prenatal reticulocytes incorporation of glycine into haem is reduced, relative to that incorporated into globin, indicating derangement of haem synthesis, but the total level of protein synthesis is similar in both mutant and wild-type reticulocytes (Cole, Regan & Tarbutt, 1972).

The present report presents further data on the abnormality of *f/f* prenatal reticulocytes.

## 2. MATERIALS AND METHODS

### (i) *Animals*

Two strains of mice were used in this study, the 'Edinburgh' *f/f* and +/+ stocks previously described, and the highly congenic FL/1 Re *f/f* $Lv^b/Lv^b$ and FL/4 Re +/+ $Lv^b/Lv^b$ obtained from the Jackson Laboratory, Bar Harbor, U.S.A. The

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*Lv* locus determines the level of the haem synthetic enzyme δ-amino-laevulinate-dehydratase in a variety of tissues, $Lv^a/Lv^a$ having high tissue levels and $Lv^b/Lv^b$ having low levels. The expression of the *f* locus is basically similar in the Edinburgh and FL/Re strains. Foetuses were obtained from overnight natural matings; day 0 is the morning on which mating plugs were present.

### (ii) *Haem synthetase assay*

Circulating foetal blood cells were harvested in heparinized normal saline, washed by centrifugation and stored at −40 °C. Reticulocytosis was measured after staining with brilliant cresyl blue, and RNA content determined by a modified Schmidt–Tannhauser procedure. The enzyme assay procedure was slightly modified from that of Freshney & Paul (1971). Aliquots of $1 \times 10^8$ to $6 \times 10^8$ cells were homogenized in 1 ml. 0·15 M-KCl containing 0·4% (v/v) Tween 20, or disrupted by freezing and thawing without detergent; 0·1 ml of extract was incubated under nitrogen at 37 °C with 0·2 ml 0·1 M glutathione in 0·23 M Tris HCl, pH 7·4, for 20 min. 0·05 μCi of $^{59}FeCl_3$ (1 μCi/0·2 μg to 1 μCi/0·75 μg) were then added in 0·2 ml reaction mixture containing 0·5 mM protoporphyrin as appropriate. After 1 h incubation the reaction was terminated with 0·05 ml normal HCl, and haem extracted with butanone for counting on a gas flow counter. Blanks were prepared by addition of substrate after acidification.

### (iii) *Preparation of labelled globins*

Circulating foetal blood cells were harvested in heparinized culture medium (Waymouths MB752/1 plus 10% calf serum containing 0·5 μg Fe/ml). Five ml cultures were set up with 100 μCi/ml tritiated leucine (DL-leucine-4,5$^3$H, 26·8 Ci/mmol) for 4 h at 37·5 °C. Labelled cells were washed with Hanks balanced salt solution, lysed, and globin precipitated from the total haemolysate in 15 vols. 2% acid acetone at −20 °C. Acetone/ether washed, dialysed globins were then separated on CM-cellulose columns using 8 M urea-mercaptoethanol buffers, according to the method of Clegg, Naughton & Weatherall (1966). Eluates of each chain were pooled, dialysed, their protein content determined, and 2 ml aliquots precipitated with 6 ml 2 N perchloric acid, and collected on glass-fibre filters for scintillation counting. Four to six samples were counted for each chain.

Protoporphyrin was prepared from the dimethyl ester by treatment with 2% HCl for 24 h at room temperature, and checked for aggregation before use (Adamson, Herbert & Kemp, 1969).

## 3. RESULTS

### (i) *Haem synthetase activity in reticulocyte homogenates*

Haem synthetase, usually bound in mitochondria, inserts iron into protoporphyrin to form haem. Its activity in erythroid tissue is therefore dependent on the activity of δ-ALA-synthetase, δ-ALA-dehydratase and other steps in the haem synthetic pathway, and on the availability of iron. Previous studies (Fowler &

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Table 1. *Haem synthetase activity in homogenates of circulating blood cells of prenatal mice*

| Strain | Day | Reticulocytes (%) | cpm/$10^8$ reticulocytes | | cpm protoporphyrin / cpm + protoporphyrin | cpm/ODU RNA | | ODU RNA/$10^7$ reticulocytes | Specific activity of non-reticulocyte Fe |
|---|---|---|---|---|---|---|---|---|---|
| | | | + protoporphyrin | − protoporphyrin | | + protoporphyrin | − protoporphyrin | | |
| FL/4Re +/+ | 17 | 85 | 3138 | 2588 | 0·82 | 980 | 809 | 0·32 | 1 μCi/0·5 μg |
| FL/1Re *f*/*f* | 17 | 100 | 2119 | 1329 | 0·62 | 573 | 359 | 0·37 | 1 μCi/0·5 μg |
| FL/4Re +/+ | 18 | 75 | 1073 | 520 | 0·48 | 715 | 346 | 0·15 | 1 μCi/0·75 μg |
| FL/1Re *f*/*f* | 18 | 100 | 1492 | 0 | 0 | 678 | 0 | 0·22 | |
| Edin. *f* + | 17 | 85 | 1128 | 1094 | 0·96 | 342 | 331 | 0·33 } | 1 μCi/0·2 μg |
| Edin. *f f* | 17 | 100 | 167 | 13 | 0·08 | 48 | 4 | 0·35 } | |
| Edin. *f* + | 18 | 75 | 168 | 129 | 0·77 | 99 | 76 | 0·17 } | 1 μCi/0·6 μg |
| Edin. *f f* | 18 | 100 | 0 | 0 | 0 | 0 | 0 | 0·27 } | |

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Russel, 1968; Cole *et al.* 1972) have shown reduced utilization of intracellular iron by intact *f/f* prenatal reticulocytes.

Haem synthesis in homogenates of 17-day wild-type FL/Re reticulocytes appears to be only slightly dependent on added protoporphyrin (Table 1) suggesting that an adequate pre-formed pool exists, or that sufficient protoporphyrin synthesis can occur in homogenates. However, in 17-day FL/Re *f/f* reticulocyte homogenates haem synthesis is considerably restricted in the absence of added protoporphyrin. On day 18, when the foetal circulation contains a higher proportion of more mature reticulocytes, the wild-type cells without added protoporphyrin are still able to synthesize about half the amount of haem found in the presence of added precursor, while no haem synthesis occurs in *f/f* reticulocyte homogenates without additional protoporphyrin. Since, in the presence of additional protoporphyrin, on days 17 and 18 wild-type and mutant reticulocyte homogenates incorporate similar amounts of labelled iron into haem, it appears that haem synthetase activity in *f/f* reticulocytes is unimpaired.

Similar dependence on added protoporphyrin is seen in 17-day reticulocyte homogenates from the 'Edinburgh' stock. However, in both 17- and 18-day *f/f* reticulocytes of this stock haem synthetase activity is reduced, relative to the normals, even when protoporphyrin is added. Although the RNA content/$10^7$ reticulocytes is similar between the different strains on each day, the reticulocytes of the Edinburgh stock may be relatively more mature, since the gestation period of the FL/Re stocks is 1 day longer in each case, and the corpuscular haemoglobin content on day 18 is about 30% higher in the 'Edinburgh' stock. Intact early 18-day reticulocytes of 'Edinburgh' *f/f* mice actively incorporate iron into haem *in vitro* (Cole *et al.* 1972).

Table 2. *Haem synthetase activity in* f/f *and normal foetal livers*

| Genotype | Age (days) | mg/liver wet weight | cpm $^{59}$Fe/liver in haem: + protoporphyrin | cpm $^{59}$Fe/liver in haem: − protoporphyrin | cpm/g wet weight/liver: + protoporphyrin | cpm/g wet weight/liver: − protoporphyrin |
|---|---|---|---|---|---|---|
| *f/f* | 16 | 40 | 3937 | 5270 | 98000 | 131000 |
| *f/+* | 15 | 30 | 1920 | 2795 | 59000 | 86000 |

Although the endogenous pool of iron available for haem synthesis in intact prenatal reticulocytes has been determined (18-day *f/f* 0·2 $\mu$g/$10^7$ cells, 18-day wild-type 0·5 $\mu$g/$10^7$ cells), the amount released on homogenization is not known, so that absolute amounts of haem synthesized cannot be determined by this method.

Previous studies of haem synthesis by *f/f* and normal foetal liver erythroblasts *in vitro* indicate little or no difference in the haem synthetic ability of individual *f/f* erythroblasts (Bateman *et al.* 1972). Haem synthetase assays were therefore performed on foetal liver homogenates comparing 16-day *f/f* livers with 15-day normal livers. Livers of both genotypes then contained similar proportions of erythroid and non-erythroid cells, and similar distributions of erythroblast types.

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These results (Table 2) show that both on a per liver and on a wet-weight basis haem synthetase activity in *f/f* liver homogenates exceeds that in normal homogenates. Addition of protoporphyrin caused a slight reduction in iron incorporation into haem in foetal liver homogenates from both genotypes, indicating that it is unlikely that haem synthetase activity in *f/f* foetal livers is restricted by shortage of precursor.

(ii) *Synthesis of globin chains in* f/f *and normal prenatal reticulocytes*

Erythroid cells derived from the mouse foetal liver synthesize adult haemoglobin, $\alpha_2\beta_2$ (Fig. 1), so that normally, prenatal reticulocytes should show net synthesis of approximately equal quantities of $\alpha$- and $\beta$-globin chains (Fantoni, Bank & Marks, 1967).

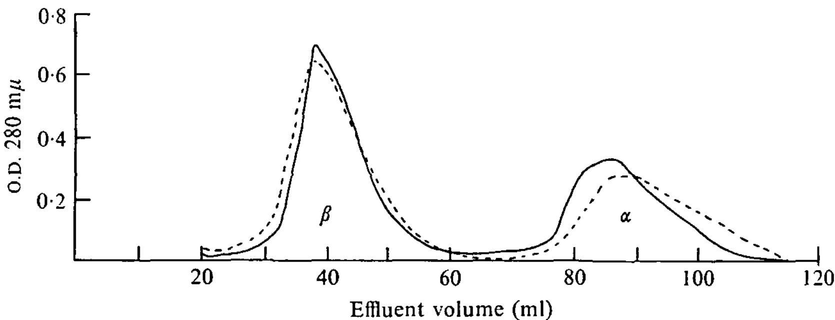


Fig. 1. CM cellulose chromatography of globins from total haemolystates of 18-day FL/4 Re +/+ (------) and FL/1 Re *f/f* (——) prenatal peripheral blood cells. Globins prepared by acid–acetone treatment of whole haemolysates were loaded onto CM cellulose columns in 0·005 M-$Na_2HPO_4$, containing 8 M urea and 0·05 M 2-mercaptoethanol, pH 6·7. Elution was by means of a linear $Na^+$-ion gradient formed by mixing starting buffer with 0·03 M-$Na_2HPO_4$ containing 8 M urea and 0·05 M 2-mercaptoethanol, pH 6·7.

This was found in cultures of 17- and 18-day FL/Re4 +/+ reticulocytes, where the ratios of total radioactivity incorporated, and specific activities of $\alpha$- and $\beta$-chains are near unity. However, in 17- and 18-day FL/Re1 *f/f* reticulocytes there is a marked deficiency in $\beta$-chain synthesis. This imbalance is not corrected by protoporphyrin added to the culture medium but is repaired by the addition of haem. A similar deficiency in $\beta$-globin synthesis is seen in the 'Edinburgh' strain *f/f* reticulocytes.

On day 18 *f/f* blood cells contain only about two-thirds of the haemoglobin found in wild-type cells (Edinburgh stock *f/f* 2·4 mg/$10^8$, wild-type 3·8 mg/$10^8$, FL/Re 1·7 and 2·7 mg/$10^8$ respectively) and a similar ratio is found in mature erythrocytes. About half (Fantoni *et al.* 1968) to two-thirds (Paul, Conkey & Freshney, 1969) of the total haemoglobin found in mature erythrocytes derived from the foetal liver is synthesized in the reticulocyte stage. Previous studies (Bateman *et al.* 1972) have indicated that haem and probably haemoglobin synthesis in *f/f* nucleated erythroid cells is almost normal, and that total protein synthesis in *f/f* and wild-type prenatal reticulocytes is similar (Cole *et al.* 1972). The observed

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Table 3. *Synthesis of α- and β-globin chains in* f/f *and normal prenatal reticulocytes*

| Strain | Day | cpm/total chain | | m.g. total globin/column | α:β | Specific activity cpm/mg | | |
|---|---|---|---|---|---|---|---|---|
| | | α | β | | | α | β | α:β |
| FL/4 Re +/+ | 17 | 665 183 | 650 232 | 8·8 | 1·02 | 154 494 | 144 955 | 1·07 |
| FL/1 Re *f/f* + protoporphyrin | 17 | 2 232 860 | 1 452 041 | 13·5 | 1·60 | 352 106 | 210 434 | 1·67 |
| FL/1 Re *f/f* + haem | 17 | 1 045 759 | 1 003 838 | 12·8 | 1·05 | 155 118 | 164 573 | 0·94 |
| FL/4 Re +/+ | 18 | 34 856 | 40 022 | 13·6 | 0·87 | 5 714 | 5 336 | 1·07 |
| FL/1 Re *f/f* | 18 | 156 020 | 102 060 | 13·2 | 1·53 | 26 234 | 14 019 | 1.87 |
| FL/1 Re *f/f* + haem | 18 | 57 627 | 72 768 | 10·1 | 0·79 | 11 299 | 14 554 | 0·78 |
| Edinburgh *f*/+ | 18 | 107 838 | 86 200 | 7·4 | 1·25 | 27 650 | 24 629 | 1·12 |
| Edinburgh *f/f* | 18 | 1 394 524 | 659 321 | 9·0 | 2·12 | 296 702 | 153 324 | 1.94 |

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hypochromia of *f/f* neonatal erythrocytes therefore leads to a prediction that haemoglobin formation in *f/f* reticulocytes will be reduced by about 50%, and this is consistent with the present observation that only about 50% of $\alpha$-chains will be able to combine with $\beta$-chains to form haemoglobin.

## 4. DISCUSSION

The deficiency in globin chain synthesis observed in prenatal *f/f* reticulocytes is superficially similar to that in human $\beta$-thalassaemia, which is also characterized by disturbances in proliferation of erythroblasts, and abnormalities of iron utilization and haem synthesis. However, $\beta$-thalassaemia appears to result from decreased availability of $\beta$-globin mRNA (Nathan *et al.* 1971), whereas the synthetic lesion of the prenatal *f/f* reticulocytes appears to be due to failure of haem synthesis. Current studies of thalassaemic $\beta$-globin mRNA in reticulocytes do not exclude the possibility that it is damaged during maturation of erythroid precursors, since normal globin ratios have been observed in narrow cultures of thalassaemic patients whose reticulocytes showed marked imbalance of $\alpha$- and $\beta$-globin synthesis (Schwarz, 1970). Abnormal patterns of globin synthesis have also been observed in reticulocytes of human sideroblastic anaemia, but in this case there is a deficiency of $\alpha$-chain formation (White, Brain & Ali, 1971).

In normal rabbit reticulocytes $\alpha$- and $\beta$-globin chains appear to be translated and terminated at the same rate, although initiation on $\beta$-chain mRNA is 65% faster than that on $\alpha$-chain mRNA (Lodish & Jacobsen, 1972). While almost all $\beta$-chain mRNA is found associated with polysomes there is an excess of $\alpha$-chain mRNA, not associated with ribosomes, suggesting that there is over-production of $\alpha$-chain mRNA early in erythroid differentiation (Jacobs-Lorena & Baglioni, 1972).

The presence of an $\alpha$-globin chain pool in reticulocytes suggests a continuously inbalanced globin synthesis, but since the pool does not continue to increase in size there must be continuous destruction of excess $\alpha$-chains, resulting in net production of nearly equal quantities of $\alpha$- and $\beta$-globin. Recent studies with intact rabbit reticulocytes (Rabinowitz *et al.* 1969) show that each haemoglobin subunit can be synthesized independently of the other, so that neither subunit can regulate its own synthesis by feedback inhibition, nor is one subunit required for the synthesis of the other. Therefore it appears that regulation of the numbers of globin subunits to be synthesized is determined during transcription in erythroid precursor cells. In both $\alpha$- and $\beta$-thalassaemia in humans, haemoglobin subunits are also synthesized independently (Clegg & Weatherall, 1967; Bank, Braverman & O'Donnell, 1968).

In normal reticulocytes the level of globin synthesis is co-ordinated with the level of haem present. In rabbit reticulocyte lysates haemin promotes globin synthesis by maintaining polysome integrity (Zucker & Schulman, 1968), its main effect being to promote peptide chain initiation (Waxman, Freedman & Rabinovitz, 1967; Hunt, Vanderhoff & London, 1972). Globin chains are unstable in the absence of haemin and aberrant $\alpha$-chains are formed in haemin-deficient rabbit

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reticulocytes (Tavill *et al.* 1968). The level of haemin may also regulate the rate at which an inhibitor of chain initiation is formed in reticulocytes and therefore determine the overall rate of haemoglobin synthesis (Rabinovitz *et al.* 1969). Haem appears to limit its synthesis by end-product inhibition or repression of $\delta$-amino-laevulinic acid synthetase (Kappas & Granick, 1965) so that normally haem and globin synthesis remain co-ordinated.

In human $\beta$-thalassaemia excess $\alpha$-globin chains precipitate and may be demonstrated as inclusion bodies by light and electron microscopy as well as chemical methods. These inclusion bodies are subject to proteolytic degradation in intact reticulocytes (Marks & Bank, 1971). Similar intracellular inclusions are present in erythrocytes of neonatal *f/f* mice stained with brilliant cresyl blue, suggesting that the fate of the excess $\alpha$-globin chains is similar in both *f/f* mouse cells and $\beta$-thalassaemic human cells.

There appear to be two possible explanations for the observed effects of the *f* gene in causing a reduction in the number of colony forming cells in the early liver, together with aberrant haem and globin synthesis in reticulocytes. Both abnormalities may result from effects of the *f* gene on an enzyme associated with haem synthesis, in which case it is necessary to suppose that both differentiation of colony forming cells and synthesis in reticulocytes are critically dependent on the products of this enzyme, while adequate levels exist to permit differentiation of erythroblasts even in *f/f* foetuses. Alternatively, the defective haem synthesis observed in *f/f* prenatal reticulocytes may be a consequence of abnormal precursor cell proliferation, although the causal link between the two phenotypic expressions of the *f* gene is unclear. Earlier results which suggested deficiencies in $\delta$-amino-laevulinate dehydratase in nucleated erythroid cells of *f/f* mice (Margolis & Russell, 1965) were almost certainly due to differences at the *Lv* locus (Coleman *et al.* 1969) and the enzymic lesion caused by the *f* gene is predominately effective only after the loss of a functional erythroblast nucleus.

Haemoglobin synthesis in cells derived from early foetal livers depends on unstable mRNA species, which later become stabilized (Fantoni *et al.* 1968), and early foetal liver erythroblasts show characteristic patterns of cell cycle parameters which change with development (Tarbutt & Cole, 1970). Stabilization of a critical mRNA species may therefore depend on certain cell cycle parameters being achieved within developing erythroblasts which are prevented by the *f/f* lesion. Haem and globin synthesis appear to be normal in reticulocytes of even severely anaemic adult *f/f* mice (Cole *et al.* 1972), and cannot be disturbed by a combination of $\gamma$-irradiation preceding phenylhydrazine-induced anaemia, designed to mimic precursor cell deficiency (Cole & Tarbutt, unpublished). The normality of adult *f/f* reticulocytes may result from the fact that a considerably smaller proportion of the final erythrocyte complement of haemoglobin is synthesized after the cessation of transcription than is the case in erythrocytes derived from the foetal liver, i.e. in adult reticulocytes the bulk of haemoglobin synthesis may depend on 'younger' mRNA molecules. Alternatively the micro-environment in which pre- and post-natal erythroid cells develop may exert an effect or the pattern of synthesis in

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terminally differentiated cells be influenced by the different age distribution of 'erythropoietin-responsive cells' in pre- and post-natal erythropoietic organs. Decreased haem synthesis, resulting from reduced formation of protoporphyrin or its precursors and aberrant regulation of globin chain synthesis at the translational level are therefore major causes of the anaemia of neonatal *flexed* mice.

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