Iron incorporation and haemoglobin synthesis in erythropoietic cells during the ontogenesis of the mouse

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SUMMARY

Iron incorporation (59Fe) into erythropoietic cells from adult and foetal (11- to 15-day) peripheral blood and from foetal (12- to 15-day) livers has been investigated. Ion-exchange chromatography of haemolysates from such cells revealed two groups of 59Fe-containing proteins. The first group (X-fraction) was eluted from CMC-columns in the void volume and was highest in lysates of immature erythropoietic cells. This fraction contained a radiolabelled haemprotein of high molecular weight as well as other 59Fe-containing proteins. The haemprotein does not appear to be related to haemoglobin.

The second group consisted of haemoglobins. One major (A1) and two minor (A2 and A3) haemoglobins were found in adult peripheral blood. In foetal liver lysates two major (F1 and A1) and two minor (F2 and A3) haemoglobins were present. The relative proportion of the major haemoglobins changed during development. Haemoglobin F1 was highest in the more mature livers. F1 proved to be different from A1 by chromatographic behaviour, in polypeptide chain composition and in fingerprints. A unique foetal polypeptide chain, intermediate in electrophoretic behaviour between the adult α- and β-chain, was identified. In young foetal peripheral blood (11-day), in which 95 % of the cells are of yolk-sac origin, one major (E1), two intermediate (E2 and E3) and one minor (E4) haemoglobin were demonstrable. Haemolysates of the peripheral blood of older embryos contain haemoglobins from erythroid cells of both yolk-sac and foetal liver origin. The haemoglobin pattern of such lysates is explicable in terms of the decreasing amount of embryonic haemoglobins (E1, E2 and E3) and the increasing amount of foetal haemoglobins (F1 and A1). Since A1 and E1 are the most prominent haemoglobins of livers from young embryos and yolk-sac erythrocytes respectively, and since they are very similar in chromatographic behaviour, foetal peripheral blood at all stages contains one dominant haemoglobin peak in the A1-E1 region. Most authors have neglected the relatively slight elevation of the foetal haemoglobin peak (F1) in front of A1-E1, more because the F1-A1 region has been suspected sometimes to contain artificial haemoglobin components (Riggs, 1965). This probably explains why no foetal haemoglobin (F1) has been reported previously in the peripheral blood of foetal mice.

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INTRODUCTION

Erythroid cell differentiation represents the process whereby a multipotential precursor cell becomes committed to devoting most of its synthetic machinery to the production of a specific protein, haemoglobin. This process involves the co-ordinated control of many biochemical and morphological events, some of which have been elucidated using in vitro cultures of erythropoietic cells stimulated to differentiate by the hormone erythropoietin (for review, see Harrison, Conkie & Paul, 1973). A relatively early event in such cultures is the stimulation of iron incorporation and the synthesis of haem (Krantz & Fried, 1968; Hrinda & Goldwasser, 1969; Gross & Goldwasser, 1970; Shepp, Toff, Yamada & Gabuzda, 1972). This is then followed at a later stage by the synthesis of haemoglobin. However, the mechanism whereby iron is transported from the cell surface, where it is delivered by serum transferrin (Katz & Jandl, 1964), to the mitochondrion, where it is utilized for haem synthesis, has not yet been elucidated. Furthermore, very little is known about the factors involved in transport of haem to the globin chains. Thus more information would be valuable concerning the role of iron and haem-iron-containing proteins, including the haemoglobins, during erythroid cell development. This paper describes experiments concerning the relative proportions and characteristics of iron- and haemproteins in erythroid cells of yolk-sac and foetal liver origin in the mouse. It is likely that the relative proportions of the iron- and haemproteins differ greatly in mature and immature cells. Information about this may be useful, not only as information about embryonic development, but also to explain discrepancies which may arise when calculations of radioactive iron or haem-iron incorporation into haemopoietic cells are used to quantitate haemoglobin synthesis (Harrison et al. 1973).

MATERIALS AND METHODS

Collection and lysis of cells. Adult Porton white Swiss mice were bled by decapitation. Foetuses of the same strain aged 11–15 days (the day on which vaginal plugs were observed being taken as day zero) were washed thoroughly and bled from the umbilical vessels for peripheral blood cells. The livers were excised, washed thoroughly and disaggregated by pipetting. Isotonic ice-cold saline was used for washings and cell collection. The blood cell suspensions were washed three times in at least a tenfold volume of saline. Following the final washing the cells were resuspended in a buffer containing 0·81 % NaCl, 0·12 % Tris and 0·03 % Mg acetate, pH 7·0. When all clumps were dissociated, NP<sub>40</sub> (Nonidet P<sub>40</sub>, Shell Chemicals) was added to 0·5 %. Lysis was allowed for about 30 min. In some experiments the cells were lysed by adding glass-distilled water, but, especially with foetal liver cells, the NP<sub>40</sub> method gave better results. The haemoglobins were converted to the CO-form, centrifuged and dialysed overnight against the starting buffer. All manipulations were done at 4 °C. No
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Freezing-and-thawing, ammonium sulphate precipitation, conversion to cyanmethaemoglobin or ageing of the haemolysate (more than 24 h) was allowed, as all this is known to promote extra haemoglobin peaks (Riggs, 1965; Manwell, Baker & Betz, 1966).

An occasional experiment was done with Friend cells (clone M2) grown in bulk cultures as described by Conkie, Affara, Harrison & Paul (1974). Erythroid differentiation in Friend cells was induced by culturing for 5 days in the presence of 1.5% dimethyl sulphoxide.

Preparation and estimation of radiolabelled proteins. Ten μCi [59Fe]chloride or citrate (spec. act. 2-10 μCi/μg Fe) was injected intraperitoneally into adult and pregnant animals, 24 h before death. In one experiment [3H]leucine (250 μCi) was added to a cell culture of mouse erythropoietic cells, 5 days before cell harvest. The radioactivity of the 59Fe-labelled fractions was measured by adding aliquots of the separated fractions to Triton X-100/toluene-based scintillator (1:2, v/v) and counting them in a liquid scintillation counter (Beckman LS-100). [3H]leucine-labelled fractions were acid-precipitated on to glass-fibre discs and counted in a toluene-based scintillator.

Chromatography of proteins. Anion-exchange chromatography was performed over carboxymethyl cellulose (Whatman CM 52) columns using 0.01 M sodium phosphate buffer in a linear gradient from pH 6.7 to 8.2 for elution. Cation-exchange chromatography was performed over diethylaminoethyl-Sephadex (A 50, Pharmacia) columns, using 0.05 M TrisHCl buffer in a linear gradient from pH 8.2 to 7.2. Gel-filtration columns were made of Sephadex G-50, G-100 and G-200 (Pharmacia) and developed with 0.01 M sodium phosphate buffer pH 6.7.

Chromatography of polypeptide chains. Acid-acetone precipitated globins of isolated lyophilized haemoglobin fractions were analysed on CM 52 columns made up in 8 M urea and 50 mM mercaptoethanol. The columns were developed with sodium phosphate buffer pH 6.9 in a linear gradient from 0.01 to 0.1 M, these buffers also containing the same amounts of urea and mercaptoethanol (Lingrel, 1972).

Starch-gel electrophoresis of polypeptide chains. Isolated haemoglobins A1, A2, A3 and F1 were submitted to vertical starch-gel electrophoresis according to Gilman & Smithies (1968), using 8 M urea in barium-lactate buffer (pH 3.2) containing mercaptoethanol.

Digestion of globins. Isolated haemoglobins A1 (from adult animals) and F1 (from foetal livers) were denatured in 8 M urea at 65 °C for 45 min, dialysed against water and treated at pH 8.5 with 0.5% trypsin (recrystallized three times, Worthington), the trypsin/protein ratio being 1:50. After 30 min the same amount of trypsin was added. The digestion was terminated after 2 h, by adjusting the pH to 6.0 with HCl and lyophilization.

Peptide mapping. About 300 μg lyophilized trypsinized A1 or F1 was applied together with 5 μg leucine and 5 μg argHCl to activated 200×200×0.25 mm
Fig. 1. Haemolsates prepared from mouse adult peripheral blood cells. (A) CM-cellulose chromatogram. (B) DEAE-Sephadex chromatogram. The elution pattern of optical density at 280 nm (---) for protein and at 419 nm (----) for haemprotein are shown. The pH-gradient is indicated (—).

RESULTS

Adult haemolsates (Fig. 1A, B). Three haemoglobins, one major (A1) and two minor (A2 and A3), were found by chromatography on both DEAE-Sephadex precoated TLC plates (Silicagel 60, without fluorescent indicator, Merck). Ascending chromatography in phenol–H2O (3 + 1 (w/w) containing 20 mg NaCN per 100 ml solution) for 16 h at room temperature, was followed by horizontal electrophoresis in pyridine–glacial acetic acid–water (10 + 5 + 485 (v/v), pH 5.2) at 500 V for 1.5 h at 0 °C. Spots were visualized by spraying with ninhydrin (pH 5.0).

Spectrophotometry. The fractions of each column were measured in a Sp 400 spectrophotometer (Unicam) at 280 nm for protein and at 419 and/or 540 nm for haemprotein content. Approximate relative concentrations of (haem) protein were calculated from these readings. Absorption spectra of some of the fractions were recorded in a Sp 800 spectrophotometer (Unicam).

Haem extraction. 59Fe-labelled haem was extracted in butanone from an acidified aliquot of the fractions in Drabkins solution (Cole & Paul, 1966) and counted on glass-fibre discs in a toluene-based scintillator.

Catalase activity was determined by the rate of breakdown of hydrogen peroxide (Chance & Herbert, 1950) as recorded by the decrease in optical density at 240 nm in an automatically-recording Gilford spectrophotometer. A catalase standard was used to calculate the absolute amounts of catalase.

Ammonium sulphate precipitation was performed according to the method of Scher, Holland & Friend (1971), in which haemoglobin precipitates between 60 % and 85 % saturated ammonium sulphate.

Treatment with iodoacetamide was performed as described by Riggs (1965).
Fig. 2. Polypeptide chain composition, as revealed by urea starch-gel electrophoresis, of adult and foetal haemoglobins isolated by CM-cellulose chromatography. A_1, A_2 and A_3: haemoglobin peaks derived from adult peripheral blood. A_1 and F_1 (foetal): haemoglobin peaks derived from 14-day foetal livers.

and CM-cellulose. Apart from their chromatographic behaviour these haemoglobins were different in polypeptide chain composition as judged by urea starch-gel electrophoresis (Fig. 2). All adult haemoglobins seemed to contain the adult α-chain; however, a normal adult β-chain seemed not to be present in haemoglobins A_2 and A_3. Whether or not the other zones visible represent a different chain, or are due to degradation products, is not clear, owing to the atypical aspect of these zones. A small proportion of the total protein was eluted from CM-cellulose in the void volume (X-fraction). The relative amounts
Table 1. Percentages of the proteins found in haemolysates of adult peripheral blood cells, foetal liver cells and foetal peripheral blood cells

<table>
<thead>
<tr>
<th>Origin of haemolysate</th>
<th>No. of anal.</th>
<th>Protein fractions</th>
<th></th>
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<tr>
<td></td>
<td>X</td>
<td>Y</td>
<td>F₂</td>
<td>A₂/F₁</td>
<td>A₁</td>
<td>E₁</td>
<td>A₃/E₂</td>
<td>E₃</td>
<td></td>
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<tr>
<td>Ad. periph. bl. CM-cell.</td>
<td>5</td>
<td>5 &lt; 1</td>
<td>—</td>
<td>—</td>
<td>5</td>
<td>7</td>
<td>—</td>
<td>2*</td>
<td>4*</td>
<td>—</td>
<td>—</td>
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<tr>
<td>DEAE-Seph.</td>
<td>5</td>
<td>6 &lt; 1</td>
<td>—</td>
<td>—</td>
<td>4</td>
<td>6</td>
<td>—</td>
<td>7</td>
<td>8</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Foetal liver CM-cell.</td>
<td>13 days</td>
<td>1 88</td>
<td>30</td>
<td>58</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>6</td>
<td>35</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>4</td>
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<tr>
<td></td>
<td>14 days</td>
<td>3 87</td>
<td>38</td>
<td>62</td>
<td>1</td>
<td>7</td>
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<td></td>
<td>15 days</td>
<td>3 87</td>
<td>30</td>
<td>66</td>
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<td>4</td>
<td>6</td>
<td>39</td>
<td>4</td>
<td>19</td>
<td>10</td>
<td>2</td>
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<tr>
<td>Foetal periph. blood CM-cell.</td>
<td>11 days</td>
<td>1 —</td>
<td>50</td>
<td>—</td>
<td>22</td>
<td>—</td>
<td>—</td>
<td>1</td>
<td>—</td>
<td>12</td>
<td>7</td>
<td>8</td>
<td>—</td>
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<tr>
<td></td>
<td>12 days</td>
<td>1 78</td>
<td>26</td>
<td>39</td>
<td>2</td>
<td>16</td>
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<td>1</td>
<td>6</td>
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<td></td>
<td>13 days</td>
<td>2 58</td>
<td>5</td>
<td>28</td>
<td>6</td>
<td>13</td>
<td>11</td>
<td>—</td>
<td>3</td>
<td>8</td>
<td>6</td>
<td>21</td>
<td>51</td>
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<tr>
<td></td>
<td>14 days</td>
<td>2 56</td>
<td>7</td>
<td>30</td>
<td>7</td>
<td>10</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>8</td>
<td>17</td>
<td>15</td>
<td>40</td>
</tr>
<tr>
<td>Components as % of the total amount of protein (280), haemprotein (419) and ⁵⁹Fe incorporation (cpm).</td>
<td></td>
<td>A₁ + E₁</td>
<td></td>
<td>280</td>
<td>419</td>
<td>cpm</td>
<td></td>
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<td>33</td>
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<td>6</td>
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<td>7</td>
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</table>

* Resolution of A₃ on CM-cellulose not as good as on DEAE-Sephadex.
Fig. 3. CM-cellulose chromatography of foetal livers. The patterns of (A) lysate of 13-day radiolabelled livers and (B) lysate of 14-day radiolabelled livers with added unlabelled haemolysate prepared from adult peripheral blood cells. The elution patterns of optical density at 280 nm (—) for protein, at 419 nm (—) for haemprotein and of radioactivity (-----) are shown. In (B) only the pattern of radioactivity is representative for the foetal material, the optical density being recorded also from the added adult lysate. The discrepancy in the curves for optical density and radioactivity indicates the position of the unlabelled major adult haemoglobin (A1).

of these fractions are summarized in Table 1. The three isolated haemoglobin fractions showed a typical CO-haemoglobin absorption spectrum and an absorbance ratio (o.D. 419/280 nm) of approximately three, which is well in accordance with their haemoglobin nature. The first eluted fraction (X) showed a somewhat different absorbance spectrum in that the 260–280 nm region was elevated, which accounted for the inverse absorbance ratio of less than one. However, the peaks in the visible region and the Sorret peak were also present, which means that some haem-containing substance must be present in this fraction.

Foetal livers (Fig. 3 A, B). Livers from embryos aged 12–15 days were analysed by CM-cellulose chromatography. The presence of two major and two minor haemoglobins was noted. In order to determine which of the haemoglobins was chromatographically comparable to the major adult haemoglobin, CMC-columns were loaded with a radiolabelled foetal liver lysate plus a non-labelled lysate from adult peripheral blood cells. The position of the major adult haemoglobin can therefore be deduced from the ratio of the radioactivity and the o.D. tracings. In this way the later-eluted major foetal peak was found to co-chromatograph with the major adult haemoglobin and was therefore assumed to be A1 (Fig. 3B). The proportion of the first major foetal haemoglobin eluted from CMC-columns was found to increase during foetal development (Table 1). As this peak was high in mature foetal livers it was called F1, although it eluted in the same chromatographic region as adult A2.

Urea starch gels (Fig. 2) revealed that the polypeptide chain pattern of the
haemoglobin $F_1$ was different from the adult haemoglobins $A_1$, $A_2$ or $A_3$. Apart from the adult $\beta$-chain, a non-adult polypeptide chain intermediate in electrophoretic behaviour between the adult $\alpha$- and $\beta$-chain was present in foetal haemoglobin $F_1$. Preliminary peptide maps of haemoglobins $A_1$ (adult) and $F_1$, although not ideal because whole haem-containing haemoglobins were mapped, also revealed differences between these haemoglobins. Surprisingly the $A_1$ fraction from foetal livers was not identical to that from the adult $A_1$ haemoglobin, in that it also contained the extra non-adult polypeptide chain. This could be due to contamination of this fraction with $F_1$; however, if this were the case, the intensity of the $\beta$-chain (which is common to both $A_1$ and $F_1$) should be relatively higher. An alternative explanation is that the non-adult chain in haemoglobin $A_1$ from foetal livers originates from traces of the embryonic haemoglobin $E_1$ (see below) from small numbers of yolk-sac cells in young foetal livers.

The minor foetal haemoglobins which were recognized as small shoulders on $F_1$ and $A_1$ are designated $F_2$ and $A_3$ respectively. All the haemoglobins described showed normal CO-haemoglobin absorption spectra. The absorption spectrum of the $X$-fraction from foetal livers was the same as described for the $X$-fraction of adult haemolysates.

A very high proportion of the total iron- and haem-containing proteins eluted in the $X$-fraction, which was sometimes subdivided into more peaks (Fig. 3; cf. the situation in adult blood, Fig. 1, Table 1). The proportion eluting in the $X$-fraction was greater the younger the embryos from which the livers were derived.

**Foetal peripheral blood** (Fig. 4A, B). Samples from 11- to 15-day-old embryos were analysed using CMC-chromatography. During this period a change takes place in the erythroid cells of which the blood is composed (Russell & Bernstein,
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Fig. 5. Sephadex G-200 chromatogram of an X-fraction prepared from radio-labelled foetal livers. The elution patterns of the optical density of 280 nm (——) for protein and 419 nm (— —) for haemprotein; the radioactivity (……) and the catalase activity (— — — — —) are shown.

In 11-day-old foetuses the peripheral blood cells originate entirely (over 95% in our samples) from the yolk-sac, whereas in later stages an increasing number of erythropoietic cells from the foetal liver is present. The chromatographic pattern of the haemolysates of 11-day peripheral blood is complicated (Fig. 4). Six peaks with a haemoglobin-like absorption spectrum were found. The chromatographic behaviour of the major embryonic peak was very much like that of A1; the peaks of the A1 and of the major embryonic peak (Ex) are separated by only one fraction. However, as the A1/Ex peak is high in embryonic peripheral blood, lower in foetal livers and higher again in adult blood cells it is likely that E1 is in fact different from the major adult haemoglobin A1. A minor haemoglobin fraction eluted immediately prior to E1 and may be the major foetal haemoglobin Fv. Two further substantial haemoglobin peaks (E2 and E3) elute after E1; E2 elutes between A1 and A3; E3 eluted after A3. Between the X-fraction and the region where the haemoglobins usually elute, two more haemoglobin fractions (Y1 and Y2) were consistently found. They were visible in all embryonic peripheral blood samples, the amount decreasing as the age increased.

The peripheral blood of the older embryos (14- and 15-day-old) investigated showed a major peak in the A1/Ex haemoglobin region. This peak is thought to be composed of a continuously decreasing amount of Ex, which is gradually replaced by an increasing amount of A1. The haemoglobin eluting in front of this
peak is relatively greater in amount than in 11-day haemolysates and is thought to contain mainly F₁ derived from foetal liver cells. The peaks eluting after A₁/E₁ are most probably E₂ and E₃ derived from the embryonic yolk-sac cells. The X-fraction in haemolysates of older embryos is considerably smaller than at the younger stage. Absorption spectra of the peaks were similar to the corresponding foetal ones. Quantitative data of the fractions in these haemolysates are shown in Table 1.

The X-fraction. In an attempt to assess the proportion of iron and haem-containing proteins, some X-fractions from immature liver erythropoietic cells were taken up in acidified Drabkins solution and extracted with butanone. It was found that 30–50% of the ⁵⁹Fe counts were haem-bound. Since all haemolysates were dialysed, the non-haem ⁵⁹Fe counts were not due to the presence of free iron. The haem component was not free haem since no ⁵⁹Fe counts were extracted by butanone from a non-acidified Drabkins solution of the X-fraction. The molecular weight of the protein in the X-fraction was estimated by chromatography on Sephadex G-200 columns, using myoglobin as a molecular weight marker (Fig. 5). In only one of the analyses – a 12-day liver lysate – were ⁵⁹Fe counts found in the myoglobin region. The bulk of the ⁵⁹Fe-labelled proteins are of high molecular weight. The possibility that haemoglobin aggregates might be formed was eliminated since treatment of haemolysates with iodoacetamide (Riggs, 1965) did not affect the chromatographic behaviour of the
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$^{59}\text{Fe}$ proteins in the X-fraction on G-200. Single chains thus may represent a small amount of the iron counts in the X-fraction but the majority of the iron counts in this peak must consist of another haemprotein. To exclude the possibility that the haemprotein under investigation was simply haemoglobin absorbed to a very large non-haemprotein, the X-fraction was subjected to ammonium sulphate precipitation. Less than 0.5% of the $^{59}\text{Fe}$ counts in this fraction precipitated with 60–85% ammonium sulphate, i.e. the conditions under which haemoglobin is precipitated. Finally, to determine whether any globin chains could be prepared from this peak, the X-fraction was isolated from erythroid cells (Friend cells) cultivated in the presence of $[^3\text{H}]\text{leucine}$. This labelled X-fraction was mixed with non-radiolabelled $\alpha_1$ haemoglobin, precipitated with acid-acetone and the polypeptide chains were separated as described (Fig. 6). The radioactivity pattern was clearly different from the O.D. 280 nm tracing of globin chains from isolated adult haemoglobin $\alpha_v$. Calculations based on the total radioactivity in the regions where $\alpha$- and $\beta$-globins elute showed that less than 5% of the X-fraction represented such chains. Since radioactivity eluting in the region of $\alpha$- and $\beta$-globins represents shoulders on the major, non-globin peak, this value for the proportion of globin chains in the X-fraction is clearly over-estimated.

A further possibility is that the haemprotein in the X-fraction is catalase, which is a large protein (M.W. 230000) and contains four haem groups. Moreover, catalase is known to be present in larger amounts in immature than in mature cells. We therefore determined the amount of catalase in the X-fraction after chromatography on Sephadex G-200 (Fig. 5). Assuming that the haem in catalase has the same specific activity as the haem in the haemoglobin from the same haemolysate, we calculated that all of the $^{59}\text{Fe}$ radioactivity in the high M.W. component of the X-fraction eluted from G-200 could be due to catalase. However, the amount of catalase may represent some but not all the $^{59}\text{Fe}$ radioactivity in the lower M.W. components of the X-fraction eluted from G-200. Since catalase activity is not restricted to the catalase protein, but is a property of all iron porphyrin complexes (Jones, Robson & Brown, 1973), the amount of catalase will be over-estimated in these calculations.

DISCUSSION

Chromatographic analysis of the $^{59}\text{Fe}$-containing proteins in adult and embryonic mouse red blood cells revealed the existence of two separate groups of proteins. The first group eluting in the initial phase of CMC-chromatography apparently consists of a variety of proteins, gathered in this study under the name X-fraction. The second group eluting later consists of haemoglobins. Both groups of proteins show changes during foetal development.

Adult haemoglobins. The haemoglobins from various strains of mice have been termed either 'single' or 'diffuse' according to their electrophoretic pattern in
starch gels. However, using other techniques such as polyacrylamide gels, the so-called ‘single’ strain haemoglobin sometimes splits into two components (Barker, 1968). The Porton white Swiss mice have the ‘diffuse’ haemoglobin type, with three haemoglobins. The difference between the haemoglobins may be due to the presence of more α-chain types (Popp & Cosgrove, 1959; Popp, 1965, 1967; Hilse & Popp, 1968) or more β-chain types (Hutton, Bishop, Schweet & Russell, 1962a, b). In our experiments the three haemoglobins display a common α-chain.

Embryonic haemoglobins in mice have been described by several authors (Table 2). Although the nomenclature is very confusing it may be concluded that specific embryonic haemoglobins are certainly present in erythroid cells from the yolk sac and peripheral blood of 11- to 14-day-old embryos.

Foetal haemoglobins in the peripheral blood of mice have not been reported previously (Table 2), although fraction 6 reported by Barker (1968) may correspond to our haemoglobin F₁. It is not surprising that the existence of a foetal haemoglobin was never noticed in haemolysates from peripheral blood, since here the haemoglobins are derived from both yolk sac and foetal liver erythroid cell types. This confuses any investigation on foetal haemoglobins, as haemoglobins F₁ and A₂ are very similar in chromatographic and electrophoretic behaviour. Moreover the elevation of the F₁/A₂ peak is relatively low, because the major haemoglobin peaks E₁ and A₁ are superimposed with most of the techniques applied. An extra difficulty is that the F₁/A₂ region is notorious in being the region where artificial components, due to degradation of haemoglobins (Riggs, 1965; Bonaventura & Riggs, 1967), are known to chromatograph. Relatively small alterations in the concentrations of this peak in haemolysates of peripheral blood are usually neglected for these reasons.

More reliable information concerning foetal haemoglobins may be obtained from analyses of haemolysates from erythroid cells derived from foetal livers or other erythropoietic organs. In this study a foetal haemoglobin was resolved in haemolysates of foetal-liver origin using chromatography. With this technique it has proved possible to isolate the haemoglobin and to establish that one of its polypeptides is not of adult type, both by urea starch-gel electrophoresis and preliminary fingerprinting of tryptic digests. It has not yet been possible to determine whether the foetal haemoglobin F₁ consists of the adult β-chain together with one of the embryonic chains common to yolk-sac erythropoiesis. The presence of a foetal haemoglobin in foetal livers has not been reported previously either (Table 2), with the possible exception again of fraction 6 reported by Barker (1968) in liver cell haemolysates. In a study of the haemoglobin pattern of erythroid cells from adult spleen, bone marrow and peripheral blood, Kraus (1970) noticed that the relative amount of the haemoglobins was not the same. In particular, the most anodic haemoglobin component was most pronounced in haemolysates originating from spleen and bone marrow. Significantly, phenylhydrazine stimulation of erythropoiesis changed the haemo-
Table 2. Haemproteins found in erythroid cells from mouse embryos

<table>
<thead>
<tr>
<th>Relative mobilities or elution order of haemproteins</th>
<th>Origin (organ)</th>
<th>Age (days)</th>
<th>Analytical method</th>
<th>Authors</th>
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<tbody>
<tr>
<td>( E_1 ) — — ( E_{II} ) ( E_{II} ) —</td>
<td>Yolk-sac</td>
<td>8-12</td>
<td>Chromatography of Hb's and chains</td>
<td>Fantoni, Bank &amp; Marks, 1967</td>
</tr>
<tr>
<td>6 4 2 1</td>
<td>Yolk-sac</td>
<td>9-10</td>
<td>Polyacrylamide gel</td>
<td>Barker, 1968</td>
</tr>
<tr>
<td>( X ) ( F_2 ) ( F_1 ) ( A_1 ) ( A_3 ) —</td>
<td>Foetal liver</td>
<td>13-15</td>
<td>Chromatography of Hb's, urea starch-gel of chains</td>
<td>This paper</td>
</tr>
<tr>
<td>— ( A ) — —</td>
<td>Foetal liver</td>
<td>?</td>
<td>Chromatography of Hb's and chains</td>
<td>Fantoni et al., 1967</td>
</tr>
<tr>
<td>— ( A(B) ) ( A(C) ) —</td>
<td>Foetal liver</td>
<td>15-16</td>
<td>Polyacrylamide gel</td>
<td>Kovach, Marks, Russell &amp; Epler, 1967</td>
</tr>
<tr>
<td>6 5 3 —</td>
<td>Foetal liver</td>
<td>12</td>
<td>Polyacrylamide gel</td>
<td>Barker, 1968</td>
</tr>
<tr>
<td>( X ) ( F_2 ) ( F_1 ) ( A_1/E_1 ) ( E_2 ) ( E_3 )</td>
<td>Periph. blood</td>
<td>11-14</td>
<td>Chromatography of Hb's Starch gel</td>
<td>This paper</td>
</tr>
<tr>
<td>— 1 2 3 4</td>
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<td>11-15</td>
<td>Starch gel</td>
<td>Craig &amp; Russell, 1963, 1964</td>
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<tr>
<td>— 2 3 4</td>
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<td>13</td>
<td>Starch gel</td>
<td>Morton, 1966</td>
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<tr>
<td>— ( A ) ( B ) ( C ) —</td>
<td>Periph. blood</td>
<td>12-16</td>
<td>Polyacrylamide gel</td>
<td>Kovach et al. 1967</td>
</tr>
<tr>
<td>— ( A ) ( E_1 ) ( E_2 ) ( E_3 ) —</td>
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<td>12-14</td>
<td>Starch gel of Hb's and chains</td>
<td>Gilman &amp; Smithies, 1968</td>
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<tr>
<td>6 5 4 3 2 1</td>
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<td>10-17</td>
<td>Polyacrylamide gel</td>
<td>Barker, 1968</td>
</tr>
<tr>
<td>— ( A ) ( E_1 ) ( E_{II} ) ( E_{III} ) —</td>
<td>Periph. blood</td>
<td>10-14</td>
<td>Polyacrylamide gel, chromatography of chains</td>
<td>Fantoni, De La Chapelle &amp; Marks, 1969</td>
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<tr>
<td>( E_1 ) — — ( E_{II} ) Minor ( E_{III} ) —</td>
<td>Periph. blood</td>
<td>12-13</td>
<td>Chromatography of Hb's and chains</td>
<td>Steinheider, Melderis &amp; Ostertag, 1972</td>
</tr>
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</table>

Positions correlated mainly to major adult haemoglobin peak. ( ) means not present in all samples. ? = age not given in paper.
globin pattern in that the same anodic component in haemolysates from peripheral blood became more pronounced. Since the most anodic component would be expected to elute early from CMC-columns, this component might well correspond to our Fv. It is quite likely that this change in the type of haemoglobin synthesized during anaemia is analogous to the situation in sheep (Blunt & Evans, 1963; Van Vliet & Huisman, 1964; Garrick, Reichlin, Mattioli & Manning, 1973) and in man (Huisman, 1969), in which the foetal haemoglobin is shown to reappear under such circumstances.

The X-fraction. The X-fraction contains several proteins, some of them carrying iron in haem-form or otherwise. In a study on chicken haemoglobins (Schalekamp, Schalekamp, Van Goor & Slingerland, 1972) we found a similar peak and showed it to contain eight proteins. In the same study immunological evidence was produced that the proteins originated from cell stroma and not from contaminating serum. Extracellular proteins such as serum transferrin therefore do not seem to contribute to the peak. The iron counts are bound to proteins of high molecular weight, 30–50% being in the form of haem. One of the non-haem iron carriers may be ferritin (m.w. 450000). Although ferritin is mainly thought to participate at a later stage of erythropoiesis (as a latent store for iron from degraded haemoglobin), it is known (Shepp et al. 1972; Theil, 1973) that, especially in erythroid precursor cells, an isotype of ferritin is present which is clearly involved in the early iron uptake. This anabolic ferritin fraction increased when haemoglobin synthesis was stimulated. Hrinda & Goldwasser (1969) found, in addition to ferritin, another not yet identified non-haem iron protein in bone-marrow cell lysates. The production of both iron-containing proteins was enhanced when erythropoiesis was induced by erythropoietin, this process being dependent on continued RNA and protein synthesis.

In addition to non-haem iron-proteins we found 59Fe to be present as haemprotein in the X-fraction. A possible haemprotein which might be expected in lysates is catalase. Considerable catalase activity was shown to be present in the X-fraction. Nevertheless, even if all the activity was due to catalase, this would not account for all the haemprotein counts in this peak.

Our finding of a haemprotein other than haemoglobin in cell lysates differs from that of Gallien-Lartigue & Goldwasser (1964), who claimed that essentially all the haem in lysates of nucleated bone-marrow cells was derived from haemoglobin. This paper is often used to state that the determination of haem in lysates would represent the amount of haemoglobin present. Although Gallien-Lartigue and Goldwasser worked with a system in which cultivated cells were labelled in vitro, allowing differential selection of cell types, and our finding was established on in vivo labelled whole-liver lysates, we feel that the authors may have over-estimated the fraction of total haem synthesis represented by haemoglobin for various other reasons (Harrison et al. 1973). Even in more pure erythroblast preparations derived from peripheral blood of mouse foetuses, we
found a relatively high quantity of non-haemoglobin haemprotein in the X-fraction.

Our non-haemoglobin haemprotein could be somewhat similar to serum haemopexin, which may transport haem from the mitochondrion (where it is synthesized) to the nascent globin chains on the polysomes. Alternatively it may represent the translational repressor substance which prevents the initiation of globin chain production unless haem is present (Balkow, Mizuno, Fisher & Rabinovitz, 1973; Gross, 1974). This haem-controlled repressor and its precursor are proteins with an approximate m.w. of $4 \times 10^5$ (Gross & Rabinovitz, 1973) and thus may well be present in our X-fraction. Our finding of a high X-fraction in immature foetal livers, before haemoglobin synthesis is fully established, is suggestive for the presence of haem synthesizing and regulating proteins in this peak.

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Fe incorporation in erythropoietic cells


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