

# STUDIES OF MURINE ERYTHROID CELL DEVELOPMENT

## Synthesis of Heme and Hemoglobin

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### ABSTRACT

Techniques of cell separation were used to isolate murine erythroid precursors at different stages of maturation. Cells were studied before and after short-term incubation in the presence or absence of erythropoietin. Complementary results were obtained by direct examination of the cell fractions and by the short-term culture experiments. Indices of heme synthesis, including incorporation of  $^{59}\text{Fe}$  or  $[2\text{-}^{14}\text{C}]\text{glycine}$  into heme and activity of  $\delta$ -aminolevulinic acid synthetase, were already well developed in the least mature cells, chiefly pronormoblasts. Activity then rose moderately in the cell fractions consisting primarily of basophilic and polychromatophilic normoblasts, and fell off with further increases in cell maturity. On short-term culture in the presence of erythropoietin, activity declined with increasing cell maturation except in the least mature fraction where the original level of activity was maintained. By contrast, synthesis of labeled hemoglobin ( $^3\text{H}$ )leucine) was very low in the least mature cell fractions and rose progressively with increasing cell maturity. The rate of hemoglobin synthesis increased in cells at all stages of maturation when cultured in the presence of erythropoietin. Despite the different patterns observed for heme synthesis and hemoglobin synthesis, both synthetic activities were consistently higher in cells cultured with erythropoietin as compared to controls.

These findings suggest that erythropoietin stimulates biochemical differentiation of erythroid precursors at various stages of maturation. They also demonstrate an asynchronism between heme synthesis and hemoglobin synthesis; heme synthesis is already well developed in the least mature erythroid cells and begins to diminish as the capacity for hemoglobin synthesis continues to rise.

Gene expression in mammalian erythropoietic cells is influenced by the glycoprotein hormone, erythropoietin. Studies *in vivo* have suggested that erythropoietin stimulates the proliferation of erythropoietin-responsive cells and accelerates erythroid cell maturation (1-3). Culture of erythropoietic cells has been used to demonstrate erythro-

poietin stimulation of structural RNA synthesis, (4, 5), globin mRNA development (6),<sup>1</sup> iron-uptake (7), heme synthesis (8-10),  $\delta$ -aminolevulinic acid (ALA) synthesis activity (11),

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hemoglobin synthesis (10, 12), and synthesis of erythrocyte stroma (13). There appear to be temporal relationships among these effects (7, 14) but a more precise description of these relationships should be possible through analysis of the various parameters of heme and hemoglobin syntheses in erythroid cells at different stages of maturation. The present study utilizes techniques of cell separation to obtain murine erythroid cells at different stages of development which undergo morphological maturation in short-term culture. This system is used to study the pattern of development and response to erythropoietin of several aspects of hemoglobin synthesis.

## MATERIALS AND METHODS

### *Techniques of Cell Separation*

Cell populations highly enriched for the different stages of erythroid cell maturation were prepared by three sequential operations: harvesting of erythroid cells after induction of erythroid hyperplasia in the spleens of mice; elimination of the more mature erythrocytes by immunological techniques; and separation of the residual nucleated erythroid cells as a function of size by the velocity sedimentation technique.

Hemolytic anemia was induced in CD-1 virgin female mice (Charles River Breeding Laboratories, Wilmington, Mass.) with intraperitoneal injections of phenylhydrazine, 30 mg/kg, on days 0, 1, and 3. Spleens were removed on day 4, minced with fine scissors, and forced through stainless steel mesh. Single cell suspensions were prepared by passage through 35- $\mu$ m mesh Nitex cloth (Tobler, Ernst, & Traber, Inc., New York). Cells were routinely prepared in phosphate-buffered saline (PBS) containing 15% fetal calf serum (FCS).

Immunological hemolysis was accomplished according to the method of Cantor et al. (15). The titers of rabbit antimouse erythrocytes and complement were selected so as to hemolyze virtually all enucleated red cells, many orthochromatophilic normoblasts, and some polychromatophilic normoblasts. The resulting cell suspension was further separated by the velocity sedimentation technique described by Miller and Phillips (16), using a Staput cell separator (O. H. Johns Scientific Company, Toronto, Ont.) with a bowl diameter of 18 cm. The cone volume (250 ml) was discarded and 60-ml samples were collected sequentially. The first three samples were pooled to form fraction I because of the limited number of cells.

### *Techniques of Cell Culture*

The pelleted velocity sedimentation fractions were resuspended in modified McCoy's 5A medium containing 15% FCS, 100 U/ml penicillin, and 100  $\mu$ g/ml

streptomycin. Replicate samples were plated either as 0.2-ml aliquots in microtiter plates or as 2-ml aliquots in 30  $\times$  15-mm tissue culture dishes (Falcon Plastics, Oxnard, Calif.) at concentrations of 2–6  $\times$  10<sup>6</sup> cells/ml. Human urinary erythropoietin<sup>2</sup> was added to cultures at a concentration of 0.2 U/ml. Trial experiments indicated that this yielded near optimal stimulation of cell proliferation and hemoglobin synthesis, similar to the changes observed with concentrations of 0.5 U/ml. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were examined before and after culture for 18 h.

### *Cell Number and Differential Counts*

Nucleated cell counts were performed in hemacytometers with 3% acetic acid-1% gentian violet as diluent. Slides were prepared with a cytocentrifuge (Shandon Scientific Co., London, England) and stained with benzidine and Wright-Giemsa stain (17). Erythroid cells were classified morphologically as follows: pronormoblasts—large cells with densely blue cytoplasm, a finely reticulated nucleus, and nucleoli; basophilic normoblasts—smaller cells with a more compact chromatin pattern and without nucleoli; polychromatophilic normoblasts—cells with cytoplasm becoming positive to benzidine and a further contracted nucleus; orthochromatophilic normoblasts—cells with densely benzidine-positive cytoplasm and fully contracted nuclear chromatin. 400 cells were counted per point. Enucleated erythrocytes were enumerated per 100 nucleated cells during differential counts.

### *Heme Synthesis*

Heme synthesis was measured by <sup>59</sup>Fe incorporation into heme (18). <sup>59</sup>Ferrous citrate (New England Nuclear, Boston, Mass., 14–30 mCi/mg) was added to FCS in quantities that would just saturate the previously measured unsaturated iron-binding capacity. The solution was incubated at 37°C for 1 h to insure binding of the iron to transferrin. A volume of this solution containing 1  $\mu$ Ci <sup>59</sup>Fe, usually 0.1–0.2 ml, was added to 1 ml of the erythroid cell suspension in tissue culture dishes. Duplicate samples were incubated for 4 h. Cells were then harvested and washed three times in PBS, the heme was extracted into cyclohexanone (18), and an aliquot was counted in a Nuclear Chicago well-type gamma scintillation counter (Nuclear Chicago Corp., Des Plaines, Ill.). Results were corrected for variations in specific activity

<sup>2</sup> Human urinary erythropoietin (H-4-S4-6SL, 153.0 U/mg) was supplied by the National Institutes of Health. The erythropoietin was collected and concentrated by the Department of Physiology, University of the Northwest, Corrientes, Argentina, and further processed and assayed by the Hematology Research Laboratories, Children's Hospital of Los Angeles, under research grant HE-10880.

and for radioactive decay of  $^{59}\text{Fe}$  and were expressed as counts per minute of  $^{59}\text{Fe}$  incorporated into heme per  $10^6$  erythroid cells per hour.

To confirm the findings with  $^{59}\text{Fe}$  and to insure that they were not influenced by changes in cellular iron pools as a function of cell maturation or time in culture, incorporation of  $[2\text{-}^{14}\text{C}]\text{glycine}$  into heme was measured in two experiments. 5–10  $\mu\text{Ci}$   $[^{14}\text{C}]\text{glycine}$  (New England Nuclear, 92 mCi/mmol) were incubated for 2 h with 2 ml of the cell suspension as described above for  $^{59}\text{Fe}$ . After the cells were washed with PBS, an excess of nonradioactive carrier mouse red cells containing a measured quantity of hemoglobin was added and hemoglobin hemin was crystallized (19), recrystallized (20), and its specific activity measured after combustion to  $^{14}\text{CO}_2$  (21). Glycine incorporation into heme was calculated from the specific activity of the  $[^{14}\text{C}]\text{hemin}$  and the total hemin present in the added carrier red cells, since there was a negligible quantity of hemin present in the original cell suspension.

### *ALA Synthetase Activity*

Duplicate samples of cells were washed in PBS, resuspended in 1 ml of 10 mM Tris-0.9% NaCl, pH 7.4, and lysed by freeze-thawing twice. The lysate was assayed immediately for ALA synthetase (ALA-S) activity as described by Ebert et al., measuring the conversion of  $[\alpha\text{-}^{14}\text{C}]\text{ketoglutarate}$  ( $[\alpha\text{-}^{14}\text{C}]\text{KGA}$ ) to ALA (23).  $[\alpha\text{-}^{14}\text{C}]\text{KGA}$ , sp act about 13 mCi/mmol, was obtained from Amersham/Searle Corp., Arlington Heights, Ill. The described procedure was modified slightly by eluting the ALA in 4 ml 1 M  $\text{NH}_4\text{OH}$  and counting in Bray's solution (23) in a liquid scintillation counter.

### *Labeled Hemoglobin Synthesis*

Replicate samples of cells were washed in PBS-10% dialyzed FCS and resuspended in MEM minus leucine (Grand Island Biological Co., Grand Island, N. Y.) containing 10% dialyzed FCS, 2 mM L-glutamine, 2  $\mu\text{g}/\text{ml}$  iron citrate, and 50  $\mu\text{Ci}$   $[^3\text{H}]\text{leucine}$  (New England Nuclear, sp act 30–82 Ci/mmol). Cells were incubated at 37°C in an atmosphere containing 5%  $\text{CO}_2$  for 1–2 h; labeled protein synthesis increased linearly during this time. The cells were washed, resuspended in 2 ml 0.01 M KCN, and lysed by freeze-thawing. Cellular debris was removed by centrifugation at 15,000 rpm for 10 min in a Sorvall model R2-B centrifuge (DuPont Instruments, Sorvall Operations, Newtown, Conn.), and the supernate was dialyzed overnight against 0.01 M KCN. An aliquot of the lysate was mixed with about 100  $\mu\text{g}$  of unlabeled mouse hemoglobin and the hemoglobin was then separated by isoelectric focusing in polyacrylamide gels (24). The samples were dispersed in  $0.5 \times 10$  cm gels composed of 4.8% acrylamide, 0.2% bisacrylamide, 2.9% ampholines of pH 6–8 (LKB Instruments, Rockville,

Md.), 4.8% glycerol, and 0.08%  $N,N,N',N'$ -tetramethylethylenediamine (TEMED). The mixture was gelled with ammonium persulfate. Gels were focused in a Metaloglass isofocusing apparatus (Metaloglass, Inc., Boston), with circulating ice water as a coolant, at 1 mA/gel until a voltage of 400 was reached; the focusing was then continued at this voltage for 5 h. 1-mm gel slices were counted in 3% Protosol in Liquiflor, both obtained from New England Nuclear. The radioactivity coelectrophoresing with the cold carrier hemoglobin was used to calculate the rate of hemoglobin synthesis, expressed as cpm/hour/ $10^6$  erythroid cells.

## RESULTS

### *Cell Separation*

The erythroid populations resolved by the velocity sedimentation technique are shown in Fig. 1. Since the rate of sedimentation is primarily a function of cell volume (25), the most rapidly sedimenting fraction, fraction I, contains the most immature cells, mainly pronormoblasts (67.5%) and basophilic normoblasts (18%), with only about 14% benzidine-positive cells. In contrast, fraction III contains primarily basophilic and polychromatophilic normoblasts, while fractions V and VI are composed largely of benzidine-positive cells.

Proliferation of erythroid cells was observed in all of the less mature velocity sedimentation fractions cultured for 18 h in the presence of erythropoietin (Fig. 1). The total number of erythroid cells increased by 31–70% in fractions I–IV. In contrast, with cultures incubated in the absence of erythropoietin, the number of erythroid cells fell off moderately in fraction I and remained roughly constant or perhaps rose slightly in the higher fractions. Fig. 1 also illustrates that erythroid cell maturation occurred on overnight culture of all of the velocity sedimentation fractions. The extent of differentiation in the presence of erythropoietin was such that the cells in fraction I matured to about the level observed in fraction III before culture, and corresponding changes were seen in the other fractions. Replicate samples cultured in the absence of erythropoietin also showed a shift to greater maturity and the absolute numbers of more mature cells were similar to those in the cultures containing erythropoietin. Thus, the increase in cell number in the erythropoietin-containing cultures was due to greater numbers of early precursors (pronormoblasts and basophilic and polychromatophilic normoblasts) after 18 h.

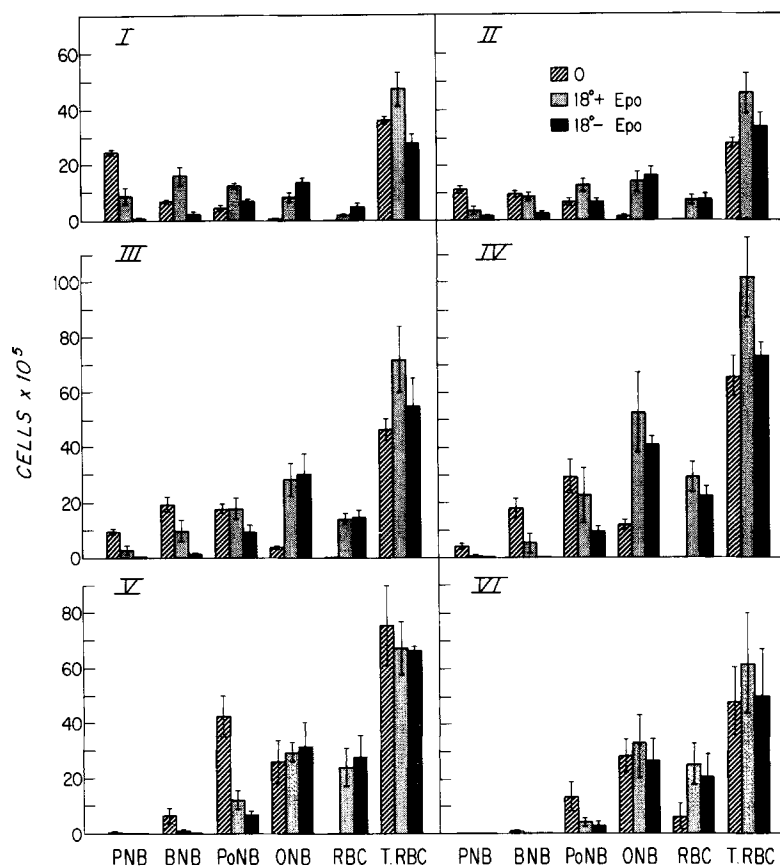


FIGURE 1 Erythroid cell populations resolved by velocity sedimentation and the effects of short-term culture. Cell counts and differentials were performed on replicate aliquots of the velocity sedimentation fractions before culture (0 h) and after 18 h of culture in the presence (18 h + Epo) or absence (18 h - Epo) of erythropoietin. Results are expressed as mean cell number  $\pm$  SE for six experiments. *PNB*, pronormoblasts; *BNB*, basophilic normoblasts; *PoNB*, polychromatophilic normoblasts; *ONB*, orthochromatophilic normoblasts; *RBC*, enucleated erythroblasts; *tRBC*, total erythroid cells.

### Heme Synthesis

Iron incorporation into heme was proportionate both to cell concentration, over the range of concentrations studied, and to the length of incubation with <sup>59</sup>Fe. The findings for the six velocity sedimentation fractions are shown in Fig. 2. The earliest cells were capable of active heme synthesis, at a rate of about 120 cpm/h/10<sup>6</sup> erythroid cells. The heme synthetic capacity was more than two-fold higher in fractions III and IV, which were enriched for basophilic and polychromatophilic normoblasts, and then decreased with further cell maturation.

The changes in <sup>59</sup>Fe incorporation into heme after overnight culture of the same velocity sedimentation fractions are illustrated in Fig. 3. When cultured in the presence of erythropoietin, the cells

in fraction I retained approximately the same heme synthetic rate as found on initial isolation, but the rate fell in the absence of erythropoietin. In all the more mature fractions, the rate of <sup>59</sup>Fe incorporation decreased with culture, although the decrease in fractions II, III, and IV was consistently smaller if the cells were cultured in the presence of erythropoietin.

When incorporation of [2-<sup>14</sup>C]glycine rather than of <sup>59</sup>Fe into heme was measured, roughly similar patterns of heme synthesis were observed both in the original isolates and after overnight culture (Fig. 4).

### ALA-S Activity

Conversion of [ $\alpha$ -<sup>14</sup>C]KGA to ALA increased linearly over a wide range of cell numbers from

$2 \times 10^6$ , the lower limit of the assay's sensitivity, to at least  $1 \times 10^8$  erythroid cells. ALA production was also proportionate to the length of incubation and the substrate concentration. The pattern of ALA-S activity in the velocity sedimentation fractions, shown in Fig. 5, resembled that observed for labeled heme synthesis. Enzyme activity varied among experiments, accounting for the large standard error; however, changes in enzyme activity were consistent within each experiment. Enzyme activity was already well developed in the earliest precursor cells, rose by a factor of about two with increasing cell maturity, and then fell slightly in the most mature cell fractions. The changes observed

after culture also corresponded to those found for heme synthesis. ALA-S activity was maintained at its original level in the cells of fraction I cultured in the presence of erythropoietin, and decreased to 36% of the original in the absence of erythropoietin. In fractions III and V, activity was 37% and 6% of the original, respectively, in the presence of erythropoietin, and 21% and 6% in its absence.

#### Labeled Hemoglobin Synthesis

The validity of isoelectric focusing as a means of separating mouse hemoglobin was documented as follows: a single hemoglobin band was observed that coincided with a single peak of absorbancy at

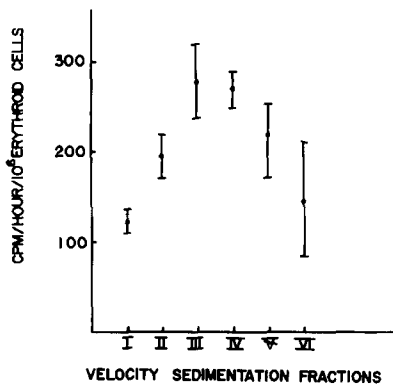


FIGURE 2  $^{59}\text{Fe}$  incorporation into heme in the velocity sedimentation fractions. Results are means  $\pm$  SE for six experiments. Incorporation of  $^{59}\text{Fe}$  into heme by peripheral red blood cells obtained from the donor mice after the same course of phenylhydrazine was 24 cpm/h/ $10^6$  erythroid cells.

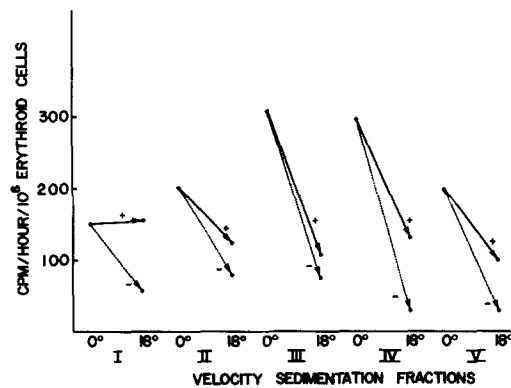


FIGURE 4  $^{14}\text{C}$ glycine incorporation into heme by velocity sedimentation fractions before and after culture in the presence or absence of erythropoietin. Data shown are for one experiment; similar results were obtained in two additional studies.

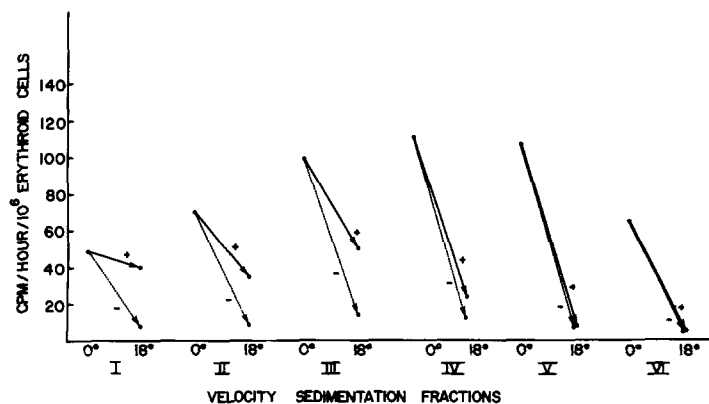


FIGURE 3  $^{59}\text{Fe}$  incorporation into heme in the velocity sedimentation fractions before and after 18 h of culture in the presence or absence of erythropoietin. Data are from one experiment. One additional experiment gave similar results.

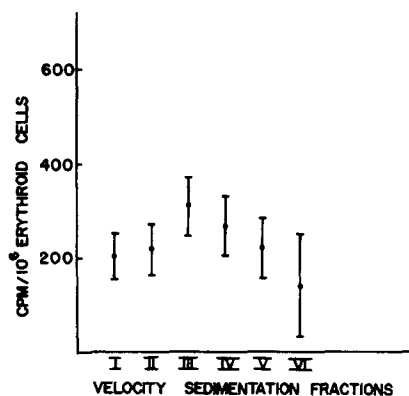


FIGURE 5 ALA synthetase activity measured by incorporation of  $[\alpha\text{-}^{14}\text{C}]\text{KGA}$  into ALA by whole cell lysates of the velocity sedimentation fractions (see Materials and Methods). Results are means  $\pm$  SE over 40 min of incubation for six experiments. Activity for peripheral blood erythrocytes after the standard course of phenylhydrazine was 25 cpm/10<sup>6</sup> erythroid cells.

540 nm, a discrete band staining with benzidine (26), and a distinct peak of radioactivity from hemoglobin from the peripheral blood of mice given <sup>59</sup>Fe 3 days earlier. The amount of <sup>59</sup>Fe in this band was equal to the amount of <sup>59</sup>Fe extractable as heme from an equivalent aliquot of hemoglobin.

Representative electropherograms are shown in Fig. 6. The rates of labeled hemoglobin synthesis from [<sup>3</sup>H]leucine in the velocity sedimentation fractions were calculated from the radioactivity migrating with hemoglobin and are illustrated in Fig. 7. The developmental pattern for labeled hemoglobin synthesis was different from that observed for <sup>59</sup>Fe incorporation into heme and ALA-S activity. In all experiments the rate of [<sup>3</sup>H]hemoglobin production was somewhat lower in fraction II than in fraction I, and then increased progressively in all experiments, so that the maximum value was observed in fraction VI. The values for fraction VI exceeded the minimal value in fraction II by a factor of 4–5.

The changes in labeled hemoglobin synthesis after short-term culture (Fig. 8) also differed from those found for labeled heme synthesis and ALA-S activity. The rate of [<sup>3</sup>H]hemoglobin production increased in all cell fractions cultured with erythropoietin. An increase was also observed in fractions I–III in the absence of erythropoietin, but to a much smaller extent than in the presence of the hormone. Activity fell in the more mature cell fractions when cultured without erythropoietin.

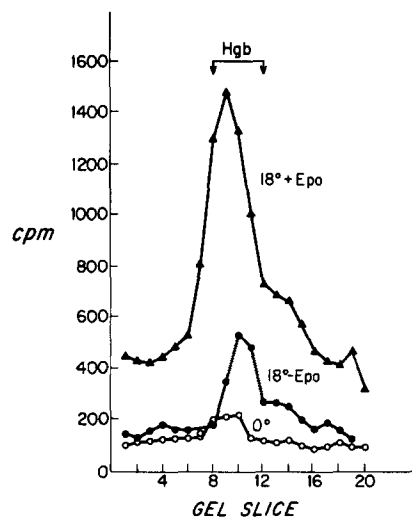


FIGURE 6 Representative electropherograms from replicate samples of cells from fraction I incubated with [<sup>3</sup>H]leucine before culture (0 h) and after culture in the presence (18 h + Epo) or absence (18 h - Epo) of erythropoietin. Hemolysates from equivalent numbers of erythroid cells were focused with cold carrier hemoglobin; the region of the hemoglobin band (*Hgb*) is shown for each gel.

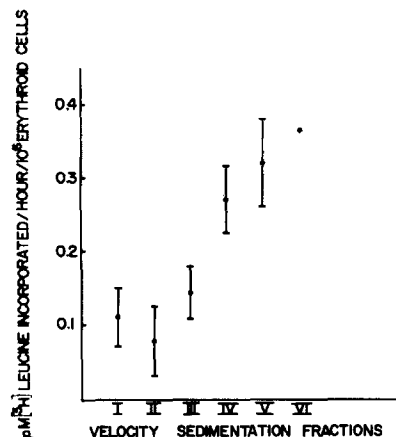


FIGURE 7 [<sup>3</sup>H]leucine incorporation into hemoglobin for the velocity sedimentation fractions. Results are means  $\pm$  SE for four experiments; those for fraction VI are from one experiment. Erythrocytes from peripheral blood obtained after the standard course of phenylhydrazine incorporated 0.15 pM [<sup>3</sup>H]leucine/10<sup>6</sup> erythroid cells/hour. Qualitatively similar patterns were obtained in two additional experiments, but the magnitude of isotope incorporation was larger, presumably because a different lot of FCS was used.

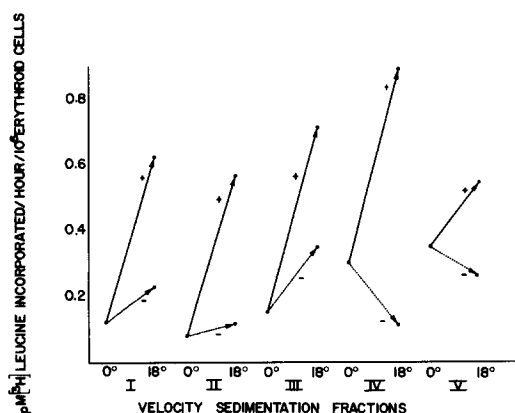


FIGURE 8 Hemoglobin synthesis by velocity sedimentation fractions before and after culture in the presence or absence of erythropoietin. Results are the means of three experiments.

## DISCUSSION

The techniques described provide a means of studying erythroid cells at different stages of maturation, both by direct examination of cell fractions separated according to size by velocity sedimentation and by study of these same cell fractions as they differentiate in short-term culture. The ability to study erythroid precursors at different stages of maturation allows measurement of biochemical aspects of erythroid cell differentiation. The present study demonstrates that heme and hemoglobin synthesis develop asynchronously and have different responses to erythropoietin. In addition, erythropoietin was found to stimulate hemoglobin synthesis in late as well as early erythroid precursors.

A variety of techniques have been utilized previously to study the different phases of erythroid cell development. Generally, these methods have been directed toward isolating the earliest precursor cells and observing their subsequent maturation *in vivo* or *in vitro*. The present experimental system in part employs a similar approach but has the additional advantage that complementary studies can be performed on cell fractions representing virtually all phases of the maturation process, isolated from a uniform source at one time. Fetal mouse yolk sac erythroid cells mature as a cohort and have provided a system for study of the synthesis of macromolecules as a function of differentiation *in vivo* (28). The liver in the fetal mouse is a site of erythropoiesis from day 11 of gestation; while not proceeding as a cohort, the

erythroid cell population does undergo progressive maturation, and provides another system for examining erythroid differentiation *in vitro* (28) or *in vivo* (29). In adult rabbits a cohort of early erythroid cells may be obtained as the bone marrow recovers from damage by actinomycin (30). These cells pass sequentially through the stages of erythroid differentiation, but differentiation proceeds with only minimal proliferation (30). Borsook and co-workers (17) have used density gradient centrifugation and immune hemolysis to separate erythroid cells at different stages of maturation from rabbit bone marrow. In contrast to the present findings, rabbit erythroid cells generated in response to phenylhydrazine appear resistant to immune lysis and separate poorly on density gradients (17). Finally, velocity sedimentation at unit gravity has been employed to obtain separations of erythroid cells (31-33). By this technique it has been possible to separate both spleen colony-forming cells (31) and mature cells capable of heme synthesis (32) from immature erythropoietin-responsive cells, and to study RNA and protein synthesis as a function of erythroid cell maturation (33). However, this method has not previously been exploited for examination of the developmental aspects of erythropoiesis and hemoglobin synthesis, as in the present experiments.

Mention should be given to potential liabilities in the techniques used in this study. Phenylhydrazine-induced hemolysis proved to be the most consistent and reliable means of achieving a luxuriant population of erythroid cells. However, the potency of this stimulus meant that the cells were bathed in high levels of erythropoietin before isolation and conceivably could have been subjected to some membrane oxidation by the drug; the time intervals involved and the fact that newly developed erythroid cells were studied make the latter possibility highly unlikely. In addition, the immune lysis technique used to eliminate the more mature erythroid cells possibly may have affected the remaining red cell precursors which apparently share similar antigens in lesser density (34). Nevertheless, the cells isolated by these techniques carried out a variety of functions related to hemoglobin synthesis and responded to erythropoietin *in vitro*.

Cell maturation and hemoglobin synthesis, as judged by morphological criteria, took place in all cell fractions whether or not they were cultured with erythropoietin (Fig. 1); the absolute numbers of orthochromatophilic normoblasts and non-

nucleated erythrocytes were similar in cultures incubated with or without this hormone. Thus, the increase in cell number found in the cultures of the early fractions containing erythropoietin was due to the larger number of more immature precursors.

The studies of  $^{59}\text{Fe}$  incorporation into heme (Fig. 2) indicated that the earliest red cell precursors in fraction I synthesized heme at about one-half of the maximum rate observed, the latter occurring in the fraction enriched primarily for basophilic and polychromatophilic normoblasts. Activity then fell off moderately with increasing cell maturity. The substantial capacity for heme synthesis found in the youngest cell fractions could be only partly explained by contamination with more mature cells since vigorous immunological lysis to eliminate all but the most immature cells failed to suppress heme synthesis or ALA-S activity to a significant extent. That a similar developmental pattern of heme synthesis was observed with  $[2\text{-}^{14}\text{C}]\text{glycine}$  as precursor indicates that the changes in  $^{59}\text{Fe}$  incorporation were not the result of alterations in metabolic iron pools as a function of cell differentiation. Izak and Kaisai (10) have made similar observations with cultures of adult rabbit marrow cells; they found a threefold increase in heme synthesis as a cohort of pronormoblasts differentiated to the level of basophilic and polychromatophilic normoblasts, and a subsequent decrease in still more mature cells. The pattern of ALA-S activity in the velocity sedimentation fractions (Fig. 5) reinforces the findings for labeled heme synthesis. ALA-S is the rate-limiting enzyme in heme biosynthesis (35) and, not surprisingly, enzyme activity and the rate of  $^{59}\text{Fe}$  incorporation change more or less in parallel with increasing cell maturity.

The results of the culture experiments are also consistent with the pattern of change of heme synthesis and ALA-S activity in the velocity sedimentation fractions. As the cells in fraction I matured in the presence of erythropoietin, the capacity for heme synthesis in different experiments either decreased slightly or remained the same (Figs. 3, 4). The level of cell differentiation was now similar to that of the cells initially present in fraction III, and a higher rate of heme synthesis might have been anticipated (Fig. 2). However, comparison of the morphology of cells isolated by velocity sedimentation with that of cells grown *in vitro* may be misleading; it is also possible that some loss of synthetic function occurs under the

conditions of cell culture. With all of the mature velocity sedimentation fractions, cellular heme synthesis decreased with culture as increasing cell maturation took place. Activity fell more rapidly in the absence of erythropoietin, when the differential cell counts were shifted to an even greater degree of cell maturity, emphasizing that the heme synthesis rate is greater in the more immature cells.

The pattern of labeled hemoglobin synthesis from  $[^3\text{H}]\text{leucine}$  in the velocity sedimentation fractions markedly contrasted with those observed for heme synthesis and ALA-S activity. Small amounts of  $[^3\text{H}]\text{leucine}$  incorporation into hemoglobin were observed with the immature cells in fractions I and II; the rate of hemoglobin synthesis then increased progressively in the more mature fractions, with highest activity in the population containing primarily poly- and orthochromatophilic normoblasts. Activity would then doubtless have fallen had still more mature cell fractions been studied; indeed, lower levels of leucine incorporation were observed in similar experiments with reticulocytes from the peripheral blood of phenylhydrazine-treated mice. Rough calculations indicate that the few benzidine-positive cells contaminating fraction I could have accounted for about one-half of the labeled hemoglobin synthesis detected in this cell population. In experiments in which smaller fractions of the sedimentation gradient were sampled, populations consisting almost entirely of pronormoblasts were obtained and demonstrated only negligible hemoglobin synthesis. The consistently lower synthesis rates observed with fraction II are difficult to explain, especially since the percentage of benzidine-positive cells was twice that in fraction I. Since cell size is determined in part by the phase of the cell cycle (36), it is possible that the cells in fraction II were isolated at a position of the cell cycle in which hemoglobin synthesis is reduced.

As in the studies of labeled heme synthesis and ALA-S activity, the culture experiments (Fig. 8) confirmed the pattern of development of hemoglobin synthesis observed on direct assay of the velocity sedimentation fractions. With increasing maturation in culture, the rate of labeled hemoglobin synthesis measured after 18 h of culture rose for all fractions cultured with erythropoietin. In the absence of erythropoietin, synthesis rose only in the earliest fractions and then to a much smaller extent than in the presence of erythropoietin. The definite effect of erythropoietin on the higher cell



fractions provides evidence that this material stimulates biochemical differentiation in more mature as well as primitive erythroid precursors. The stimulation of hemoglobin synthesis per cell in fraction V suggests that this effect of erythropoietin is not necessarily associated with enhancement of cell proliferation, since no increase in cell number was observed in cultures of this fraction (Fig. 1). Moreover, the extent of erythroid differentiation was similar in fraction V cultured with or without erythropoietin. Hence the changes noted with erythropoietin treatment of fraction V appear to reflect stimulation of hemoglobin synthesis in individual erythroid cells at this phase in the maturation sequence. Recently, Priesler and Giladi (37) have demonstrated a similar effect in mature, benzidine-positive erythroleukemic cells. If the cellular hemoglobin content of the mature erythrocyte is to be normal, then these findings suggest that there is a decrease in net hemoglobin synthesis at still later stages of maturation. Alternatively, these changes might account for the production of the large cells with increased hemoglobin observed in response to erythropoietin (39, 40).

In separate experiments the evolution of globin mRNA was found in most respects to parallel that of hemoglobin synthesis.<sup>1</sup> Globin mRNA levels rose progressively with increasing cell maturity in the sedimentation fractions, and increased in fraction I when cultured with erythropoietin. However, globin mRNA content remained stable in erythropoietin-containing cultures of the more mature fractions, as might be anticipated in view of the long half-life of this material in mammalian erythroid cells (38). Hence the increased hemoglobin synthesis in the later fractions appears to reflect post-transcriptional control of globin synthesis. The nature of such control is a matter of speculation and might possibly reflect a nonspecific nutritive effect of erythropoietin on cells in culture. Alternatively, it is intriguing to speculate that heme synthesis stimulated by erythropoietin in turn increases globin synthesis by the known ability of heme to stimulate the initiation of translation of globin mRNA (41, 42).

The different patterns observed for heme synthesis and hemoglobin synthesis in these experiments imply an asynchrony between the development of heme and globin synthesis during erythroid cell differentiation. Such a disparity may also be inferred from the observations of Izak (10). In addition, Goldwasser (7) noted that erythropoietin

stimulates iron transport before affecting hemoglobin synthesis in cultures of rat marrow cells. In the present experiments, heme synthesis was already well developed in early erythroid cells and began to diminish as the capacity for hemoglobin synthesis continued to rise. It is thus possible that heme synthesized by early erythroid cells is, at least initially, unrelated to hemoglobin synthesis and may serve to regulate the development of hemoglobin synthesis. This sequence of development is compatible with recent information indicating that heme may act as a derepressor of the synthesis of globin as well as numerous other proteins (41). It is possible, then, that there is a pool of unassigned heme in early erythroid cells similar to that proposed for hepatic heme synthesis (43).

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