ERYTHROPOIETIN EFFECTS ON
FETAL MOUSE ERYTHROID CELLS

I. Cell Population and Hemoglobin Synthesis

DAVID H. K. CHUI, MEIR DJALDETTI, PAUL A. MARKS,
and RICHARD A. RIFKIND

From the Department of Human Genetics and Development and the Department of Medicine, Columbia University, College of Physicians and Surgeons, New York 10032. Dr. Chui's present address is the Department of Pathology, McMaster University Faculty of Medicine, Hamilton, Ontario, Canada.

ABSTRACT

The effect of the hormone, erythropoietin, on cultures of erythroblasts derived from the livers of fetal C57BL/6J mice was examined. An increase both in the content and in the rate of synthesis of normal adult mouse globin chains was detected in hormone-treated cultures. The rate of protein synthesis by individual erythroblasts does not increase in response to the hormone, whereas the absolute number of hemoglobin-synthesizing cells does increase and accounts for the observed stimulation of hemoglobin synthesis. The principal effect of erythropoietin appears to be upon the population of immature erythroid precursor cells which persists in the presence of the hormone, the cells maintaining their ability to replicate, and their capacity to differentiate into hemoglobinizing erythroblasts. In the absence of hormone, already committed erythroblasts continue their development, but erythropoiesis is not sustained.

INTRODUCTION

Erythropoiesis in the mammalian fetus has provided a useful model for the study of the regulation of normal mammalian cell differentiation (Marks and Kovach, 1966). In the C57BL/6J mouse, with a gestational period of 20-21 days, the first population of erythroblasts, synthesizing embryonic hemoglobin, appears in the yolk sac blood islands at about the 8th day of gestation. These cells continue to divide, and mature into nucleated erythrocytes in the circulation where they persist until about day 17 (Craig and Russell, 1964; Fantoni et al., 1967). By the latter part of day 10 of gestation, hepatic erythropoiesis begins (Rifkind et al., 1969 a), and the sequential differentiation and development of proerythroblasts, basophilic, polychromatophilic, and orthochromatoid erythroblasts, and reticulocytes can be observed in this fetal organ at least until birth, at which time the major site of erythropoiesis is transferred to spleen and marrow. The hemoglobin formed by fetal hepatic erythroid cells is the same as that of adult red blood cells (Fantoni et al., 1967).

In the mammalian adult, erythropoietin is a major factor regulating the rate of erythropoiesis in response to physiological demand (Gurney, 1968). The role of this hormone in the regulation of neonatal and fetal erythropoiesis, however,
embryos were subsequently dissected out, the livers

Biological Co. [Gibco], Grand Island, N.Y.). The

globulin-free newborn calf serum (Grand Island

mixture of 50% Tyrode's solution and 50% gamma

releasing the pear-shaped decidual swellings into a

to a sterile Petri dish, and the uterine wall was opened,

day zero of the gestation.

et al., 1965). The morning after mating is designated

to hormonally primed immature females (Southard

strain. Fetuses were obtained by the timed mating of

were mated with males (3-5 months old) of the same

Animals

MATERIALS AND METHODS

Animals

Three- to four-week-old female C57BL/6J inbred mice (The Jackson Laboratory, Bar Harbor, Maine) were mated with males (3-5 months old) of the same strain. Fetuses were obtained by the timed mating of hormonally primed immature females (Southard et al., 1965). The morning after mating is designated day zero of the gestation.

Cells

On the morning of the 13th day of gestation, pregnant mice were sacrificed by cervical dislocation. The rest of the operative procedures were performed aseptically. Each uterine horn was transferred to a sterile Petri dish, and the uterine wall was opened, releasing the pear-shaped decidual swellings into a mixture of 50% Tyrode's solution and 50% gamma globulin-free newborn calf serum (Grand Island Biological Co. [Gibco], Grand Island, N.Y.). The embryos were subsequently dissected out, the livers

were removed, placed in complete culture medium (see below), and kept in an atmosphere of 5% CO₂ in air at 4°C. Pooled livers were cut into small pieces, and after 3 min exposure to 0.25% trypsin in Gibco Solution A, a cell suspension was obtained by repeated aspiration through a siliconized, fine-tipped Pasteur pipette, as previously described (Djaldetti et al., 1970). Cell suspensions were washed twice with Waymouth medium, and resuspended in complete culture medium. Between 7 and 12 X 10⁶ cells were obtained from each batch of approximately 30 fetal livers prepared for culture. Approximately 4 hr, from the time of sacrifice of the pregnant mice, were required to prepare cell suspensions for culture.

Culture Conditions

Freshly prepared complete sterile culture medium consisted of 87% Waymouth medium (MB752/1; Microbiological Associates, Inc., Bethesda, Md.), 9% fetal bovine serum (Microbiological Associates, Inc.), 3% chick embryo extract (Gibco) and penicillin and streptomycin (1%). 3 µg of FeCl₃ were incubated with 10 ml of adult mouse serum at room temperature for 2 hr, sterilized by Millipore filtration (Millipore Corp., Bedford, Mass.), and stored frozen until use. Enough serum-FeCl₃ mixture was added to the culture medium so that the final concentration was 3 µg of FeCl₃/ml. Cell suspensions from disaggregated fetal livers were suspended in this medium at a concentration of 2.0-4.0 X 10⁶ cells/ml; 3.0 ml of cell suspension were put in each sterile cellulose nitrate tube (size 1 X 3½ inch) and incubated with and without erythropoietin at 37°C, without agitation, in a humidified atmosphere of 5% CO₂ in air. The pH of the medium was between 7.2 and 7.4 during culture. Human urinary erythropoietin, obtained from the Committee on Erythropoietin of the National Heart Institute (pool A-1-TaLSL, 26.4 units/mg of protein), was dissolved in sterile 0.85% saline, stored frozen in small portions until use, and added to cell suspensions at onset of each culture, at a final concentration of 0.5 unit/ml.

Hemoglobin Synthesis and Content

For determination of the rate of synthesis of hemoglobin, cells were collected and resuspended in 1 ml of complete culture medium or 1 ml of 90% leucine-free modified Krebs-Ringer bicarbonate medium (Kovach et al., 1967) and 10% fetal bovine

1 Erythropoietin procured by the Department of Physiology, University of the Northeast, Corrientes, Argentina, processed by the Hematology Research Laboratories, Children's Hospital of Los Angeles for distribution by the National Heart Institute under Research Grant HE-10880.
serum. After 5 min preincubation at 37°C, 50 μCi of L-leucine-4,5-3H (SA 55.5 Ci/m mole) was added, and incubation continued for 10 min. The cells were washed twice with cold Waymouth medium, saturated with carbon monoxide, and lysed by three cycles of freeze-thaw in 0.5–1.0 ml of 10–3 M Na2HPO4. Stroma was removed by centrifugation at 27,000 g for 15 min, and 1.0–2.0 mg of freshly prepared adult hemoglobin was added to the lysate. Hemoglobin was purified from the hemolysate by carboxymethyl cellulose (CMC) column chromatography as previously described (Fantoni et al., 1968). Hemoglobin recovery, by this method, was approximately 50%. For determination of radioactivity in the hemoglobin fraction, the eluate was precipitated with cold acid acetone, and was heated at 90°C for 30 min. Precipitates were collected on Millipore filters, and radioactivity was determined in a liquid scintillation counter (Bank, 1968). In preliminary experiments, it was established that the rate of incorporation of leucine-3H into purified hemoglobin was linear for at least 30 min and over the range of concentration of cells employed in these studies. Hemoglobin concentration and content was determined by the method of Drabkin (1932).

**Globin Synthesis**

After culture, cells were pooled, pelleted, and resuspended in 100 μl of freshly prepared culture medium, to which 50 μCi of L-leucine-4,5-3H (SA 55.5 Ci/m mole) were added, and incubated at 37°C for 2 hr. Subsequently, the cells were washed, 20 μg of adult mouse hemoglobin was added, and globin was prepared by dripping the mixture into 40 ml of cold acid acetone as previously described (Braverman and Bank, 1969). Globin chains were separated by column chromatography on carboxymethyl cellulose in 8 M urea as described by Fantoni et al. (1967). Radioactivity was determined by liquid scintillation counting.

**Cytology**

After culture, cells were pooled and cell counts were obtained in duplicate with a Levy hemocytometer (Max Levy & Company, Inc., Philadelphia, Pa.). Unfixed cells were deposited on slides by centrifugation (cytocentrifuge; Shandon Scientific Co., London, England), stained with benzidine for hemoglobin (Pearse, 1960), and counterstained with Wright-Giemsa. Portions of cells were also fixed for 2 hr in cold 1% phosphate-buffered, redistilled glutaraldehyde, postfixed with 1% osmium tetroxide, dehydrated in graded alcohol, and embedded in Epon 812. Thick sections (0.5 μ) were prepared for light microscopy or radioautography. For electron microscopy, thin sections (500–800 Å) were stained with uranyl acetate and lead citrate, and examined in either a Hitachi HS-7S or HU-11-C electron microscope.

**DNA Synthesis**

Triplicate 1 ml cultures of 3 × 106 cells were incubated for 20 min in Waymouth medium with 2 μCi of thymidine-2-14C (SA 59.1 mCi/m mole). The cells were then washed twice with cold Tyrode’s solution, were precipitated with cold perchloric acid, and DNA was extracted from the precipitate by two cycles of acid hydrolysis with 2 N perchloric acid at 70°C (Hutchison et al., 1962). After neutralization with KOH, portions of the deoxyribonucleotide extract were used for the determination of radioactivity (Bray, 1960). In preliminary studies, it was established that further acid hydrolysis did not yield significant additional radioactivity. In addition, incorporation of thymidine-14C was found to be linear for at least the period of incubation.

**Radioautography**

Cells incubated with leucine-3H or with 50 μCi (methyl-3H)-thymidine (SA 6.7 Ci/m mole) for 20 min, under the same conditions as indicated in section immediately above (DNA synthesis), were prepared for quantitative radioautography as previously described (Djaldetti et al., 1970). 0.5 μ sections of Epon-embedded cells mounted on glass slides were coated by dipping in a 1:1 dilution of melted Ilford K-5 photographic emulsion (Ilford Ltd., Ilford, England), dried, and stored at 4°C in a light tight box for the appropriate time. Slides were developed for 3 min in Kodak D-19 at 17°C, fixed, and stained with 1% methylene blue. All experimental points compared by quantitative radioautography were processed together to insure uniformity of conditions. As prepared in this manner, background grain counts were virtually zero. At least 100 cells of each cytologic type and maturational stage were counted, and the results are expressed as mean grains per cell. For determination of the thymidine labeling index, cells with three or more grains were scored as labeled.

**RESULTS**

**Hemoglobin Synthesis**

The first series of experiments were designed to determine the effect of erythropoietin on hemoglobin synthesis by fetal hepatic erythroblasts in culture. The rate of hemoglobin synthesis by
Effect of Erythropoietin on Rate of Hemoglobin Synthesis by 13-Day Fetal Erythroid Cells in Culture

| Conditions of incubation with | Zero time | 24 hr culture with erythropoietin | 24 hr culture without erythropoietin |
|------------------------------|-----------|----------------------------------|-------------------------------------|
| culture medium               |           |                                  |                                     |
| KRB§                         | 273       | 720                              | 264                                 |
| KRB§                         | 3980      | 7780                             | 195                                 |
| KRB¶                         | 5000      | 7000                             | 140                                 |
| KRB¶                         | 3000      | 5080                             | 169                                 |

* Hemoglobin synthetic rate per 10⁶ cells present at onset of culture, expressed as cpm of leucine-³H incorporated into purified hemoglobin per 10 min period of incubation.
† Calculated as (cpm after 24 hr culture per cpm at onset of culture) × 100.
§ Complete culture medium (see Materials and Methods).
¶ Modified, leucine-free Krebs-Ringer bicarbonate (KRB) (see Materials and Methods).
¶ Cell suspension obtained solely by mechanical disaggregation without aid of brief period of trypsinization (see text).

13-day fetal hepatic erythroblasts, before and after 24 hr of culture with and without erythropoietin, was determined by measuring the incorporation of radioactive leucine into hemoglobin purified from lysates of erythroid cells (Table I). In the absence of added hormone, the rate of synthesis of hemoglobin per culture increased by 1.4- to 2.6-fold within a 24 hr period, as compared to the zero time rates (a statistically significant difference by the two tailed t test; $P < 0.05$). This accelerated rate of hemoglobin synthesis could be maintained, occasionally, for as long as 48 hr but deteriorated thereafter, despite addition of fresh erythropoietin. In the absence of added hormone, the rate of synthesis of hemoglobin per culture decreased markedly. The observed variability in the response to erythropoietin suggests a variable degree of cell injury during preparative procedures. The contribution of one potentially injurious agent, trypsin, to this variability, was explored. Omission of trypsin from the cell disaggregation step, thereby subjecting the fetal livers solely to mechanical disruption, had no significant effect on the response of cultured erythroblasts to erythropoietin.

In order to ascertain whether the observed acceleration of the rate of hemoglobin synthesis was reflected in a net increase in the hemoglobin content of the developing erythroid cells, the hemoglobin content of two 24 hr cultures with and without erythropoietin was determined. The hemoglobin content of cultures with and without the hormone was increased when compared to the level at onset of culture. Although statistical treatment is not possible, the increment in the presence of hormone (119 and 55%) was in both instances greater than in its absence (66 and 32%, respectively). Taken together, the observed increase in the rate of synthesis of hemoglobin and the net increment in hemoglobin content indicate that the effect of erythropoietin on cultured fetal liver erythroblasts is comparable to the effect of the hormone on hemoglobin production by adult bone marrow erythroid cells in vitro (Goldwasser, 1966).

The globin composition of the hemoglobin synthesized under conditions of in vitro culture was analyzed by CMC-urea column chromatography (Fig. 1). After 24 hr in culture with or without erythropoietin, fetal hepatic erythroblasts continued to synthesize alpha and beta globin chains indistinguishable, chromatographically, from the alpha and beta globin chains of adult mouse hemoglobin. Radioactivity incorporated into isolated globin was inhibited by over 98% if puromycin ($2 \times 10^{-8}$ M) was added to the cell culture for a 30 min preincubation period. This suggests that leucine-³H incorporation into globin represents new protein synthesis.

Protein Synthesis by Individual Polychromatophilic and Orthochromatic Erythroblasts

The rate of protein synthesis by individual polychromatophilic and orthochromatic erythroblasts, both before culture and after culture with and without erythropoietin, was determined by
radioautography (Table II). The uptake of leucine-3H by fetal hepatic erythroblasts is linear for at least 2 hr, as determined by quantitative radioautography under the conditions employed in these experiments (Djaldetti et al., 1970). As previously reported (Grasso et al., 1963; Djaldetti et al., 1970), polychromatophilic erythroblasts are more active in protein synthesis than orthochromatic erythroblasts and this relationship is not altered after 24 hr culture either with or without the hormone. Moreover, the moderate fall in isotope incorporation which occurs in cells of both developmental stages after culture is independent of the presence or absence of the hormone. The data suggest that erythropoietin does not exert its effect on hemoglobin synthesis by means of an effect on the rate of protein synthesis by individual polychromatophilic and orthochromatic erythroblasts.

**Changes in the Erythroblast Population**

During 24 hr of culture there is an increase in the total size of the erythroblast population, and the increment in cell number is greater in cultures with erythropoietin. In 15 experiments, the mean cell count after 24 hr of culture with hormone was 161 ± 44% of the total cell number at onset of culture, while in 10 cultures without the hormone the mean cell count was 122 ± 20%. The difference between these mean cell counts is highly significant (P < 0.01).

In addition to its effect on the total erythroblast cell population, erythropoietin also exerts a profound influence on the distribution of cells among the several stages of development. Erythroblasts

![Figure 1](image-url)

**Figure 1** Chromatography of the globins synthesized in 24 hr culture of 13-day fetal hepatic erythroblasts, with erythropoietin. The cells were labeled with leucine-3H for 2 hr after culture, as described in text. Adult mouse hemoglobin was added as carrier and marker. Dashed line, radioactivity; solid line, optical density at 280 mµ of the marker adult hemoglobin.

**Table II**

| Experiment number | Zero time  | 24 hr culture with erythropoietin | 24 hr culture without erythropoietin |
|-------------------|------------|----------------------------------|--------------------------------------|
|                   | avg grains/cell | avg grains/cell | % | avg grains/cell | % |
| Polychromatophilic erythroblast | | | | | |
| 1 ‡ | 20.8 | 19.2 | 92 | 16.8 | 81 |
| 2 § | 14.6 | 12.2 | 84 | 12.9 | 88 |
| 3 § | 12.7 | 11.1 | 87 | 10.1 | 80 |
| Orthochromatic erythroblast | | | | | |
| 1 ‡ | 13.3 | 13.4 | 101 | 13.0 | 98 |
| 2 § | 10.2 | 6.9 | 68 | 8.8 | 86 |
| 3 § | 9.2 | 6.5 | 71 | 6.1 | 66 |

* Calculated as (average grains per cell after 24 hr culture per average grains per cell at onset of culture) × 100. Radioautographic technique as described in Materials and Methods.
‡ Cells incubated with leucine-3H for 2 hr in complete culture medium (see Materials and Methods). The low rate of isotope uptake by these cells was compensated by a proportionate prolongation of radioautographic exposure time.
§ Cells incubated with leucine-3H for 10 min in modified, leucine-free Krebs-Ringer bicarbonate (see Materials and Methods).
FIGURE 2 Erythroblasts from the 13 day fetal liver at the onset of culture. Identified in this field are: a proerythroblast (Pr) displaying a prominent nucleolus, fine chromatin and moderately basophilic, benzidine-negative cytoplasm; a basophilic erythroblast (B) with a more condensed nucleolus and chromatin and intensely basophilic cytoplasm; several polychromatophilic erythroblasts (Po) at progressive stages of maturation evidenced by increasing benzidine reactivity (yellow stain); an orthochromatic erythroblast (O). Wright-Giemsa–benzidine stain. X 1200.

FIGURE 3 Erythroid cells after 24 hr culture with erythropoietin and prepared as in Fig. 2. A mitotic pro- or possibly basophilic erythroblast, several basophilic erythroblasts in interphase, polychromatophilic erythroblasts, and an orthochromatic erythroblast are seen. Nonnucleated erythrocytes observed include several with residual basophilia (probably reticulocytes) and a mature, intensely benzidine-reactive erythrocyte. X 1200.

FIGURE 4 Erythroid cells after 24 hr culture in the absence of erythropoietin. The majority of cells are orthochromatic erythroblasts displaying intensely benzidine-reactive cytoplasm and the characteristic eccentric pycnotic nucleus. A single late stage polychromatophil (Po) is seen, as well as several non-nucleated erythrocytes. X 1200.

comprise at least 95% of the cells present in the disaggregated 13 day fetal liver (Djaldetti et al., 1970), and these erythroblasts are distributed among the four developmental stages of erythropoiesis, i.e., proerythroblasts, basophilic, polychromatophilic, and orthochromatic erythroblasts (Fig. 2). The latter, by extrusion of their nuclei, become reticulocytes which rapidly enter the peripheral circulation. The cytological and ultrastructural characteristics of each maturational stage have been described previously (Fantoni et al., 1968; Rifkind et al., 1969 b) and differed in no way in the present studies. The proportion of cells at each stage depends upon the fetal age at time of sacrifice (Silini et al., 1967; Fantoni et al., 1968; Paul et al., 1969), and upon the presence or absence of the hormone, erythropoietin, during the 24 hr of culture in vitro (Table III). Cells of the most immature stages, proerythroblasts and basophilic erythroblasts, constitute almost 40% of the 13 day fetal hepatic erythroid cell population at the onset of the culture (Fig. 2). After 24 hr in culture in the presence of erythropoietin, these still constitute over 20% of the total (Fig. 3), whereas in the absence of the hormone, immature erythroblasts virtually disappear (Fig. 4). Well-differentiated, hemoglobinized erythroid cells, the polychromatophilic and orthochromatic...
TABLE III

| Experiment                        | Proerythroblast | Basophilic erythroblast | Polychromatophilic erythroblast | Orthochromatic erythroblast |
|-----------------------------------|-----------------|-------------------------|---------------------------------|-----------------------------|
| Zero time                         | %               | %                       | %                               | %                           |
| 24 hr culture with erythropoietin | 13 ± 5          | 26 ± 6                  | 46 ± 8                          | 15 ± 6                      |
| 24 hr culture without erythropoietin | <1             | 3 ± 1                   | 46 ± 20                         | 50 ± 20                     |

Values are expressed as mean ±1 sd of five independent sets of experiments.

TABLE IV

| Experiment                        | Number of erythroblasts* |
|-----------------------------------|--------------------------|
|                                  | Total | Proerythroblasts | Basophilic erythroblast | Polychromatophilic erythroblast | Orthochromatic erythroblast |
| No. 1                             |      |                 |                       |                                |                             |
| Zero time                         | 180   | 32              | 54                    | 62                             | 30                          |
| 24 hr culture with erythropoietin | 329   | 13              | 40                    | 190                            | 87                          |
| 24 hr culture without erythropoietin | 197   | 3               | 5                     | 118                            | 71                          |
| No. 2                             |      |                 |                       |                                |                             |
| Zero time                         | 174   | 27              | 44                    | 85                             | 18                          |
| 24 hr culture with erythropoietin | 220   | 12              | 39                    | 139                            | 30                          |
| 24 hr culture without erythropoietin | 118   | 1               | 5                     | 85                             | 27                          |

* Expressed as 10^5 cells.

erythroblasts, concomitantly increase from 62% at zero time of culture to 79% after 24 hr of culture with the hormone (Fig. 3), and to over 96% after 24 hr of culture without the hormone (Fig. 4).

The absolute numbers of erythroblasts of each developmental stage were determined (Table IV). Erythropoietin in the culture results in two major changes in the composition of the erythroid cell population. These are: (a) preservation of a large, although reduced, population of immature erythroblasts; and (b) an increase in the number of polychromatophilic erythroblasts, accounting for the bulk of the erythropoietin-induced increase in total cell number. Morphologically, all these cells closely resemble cells of analogous stages of maturation at the onset of culture, as determined both by light microscopy (Figs. 2 and 3) and by electron microscopy. Mitotic figures may be found among both the immature and the hemoglobinized erythroblasts, in zero-time cultures and after 24 hr with erythropoietin. In the absence of the hormone, immature precursors not only decrease strikingly in number, but the remaining few appear morphologically deteriorated, displaying excessively clumped nuclear chromatin and a vacuolated cytoplasm. The erythroblast population consists principally of a small number of polychromatophilic erythroblasts and a preponderance of orthochromatic erythroblasts (Fig. 4). These cells display a uniformly advanced degree of nuclear heteropycnosis and cytoplasmic hemoglobinization (intensity of benzidine reactivity), while stages of nuclear extrusion (reticulocyti...
cyte formation) are commonly observed. These observations suggest that they represent a distinctly more mature population of erythroblasts than that present at the onset of culture or after incubation with erythropoietin.

**DNA Synthesis**

**BIOCHEMICAL STUDIES:** DNA synthesis per culture, measured by the incorporation of thymidine-14C, was determined at the onset of culture and after 10 and 24 hr in culture with and without erythropoietin (Fig. 5). In the presence of the hormone, thymidine incorporation per culture remained either unchanged, or was decreased slightly at 10 or 24 hr compared to zero time of culture. In the absence of the hormone, the rate of synthesis of DNA decreased gradually during the first 10 hr and much more rapidly during the subsequent 14 hr, such that by 24 hr the hormone-treated culture incorporated radioactive thymidine at a rate almost six times that of the hormone-free culture.

**THYMIDINE-3H LABELING INDEX:** Radioautographic studies of the thymidine-3H labeling index were performed in order to determine whether the erythropoietin-induced maintenance of the capacity to synthesize DNA by cultured erythroblasts could be attributed to an increase in the proportion of DNA-synthesizing cells confined to one or another of the developmental stages of erythropoiesis (Table V). Consistent with previous report from this laboratory (Djaldetti et al., 1970), immature erythroblasts from 13-day fetal livers display a high labeling index, while a much smaller proportion of polychromatophilic erythroblasts incorporated radioactive thymidine during the 20 min labeling period. Orthochromatotic erythroblasts demonstrate no significant thymidine-3H uptake and are mitotically inactive. In erythropoietin-treated cultures, despite accumulation of large numbers of polychromatophilic erythroblasts, the labeling index of these cells and of their more immature precursors, the proerythroblasts and basophilic erythroblasts, does not increase, as compared with the labeling indices of cells of analogous stages of maturation at the zero time of the culture.

In the absence of erythropoietin, proerythroblasts virtually disappear, while the labeling indices of basophilic and polychromatophilic erythroblasts decrease, consistent with their more mature cytological features, already described. Taken together, these observations on total DNA synthesis and the labeling indices of individual classes of erythroblasts suggest that erythropoietin induces an increase in the rate of hemoglobin synthesis and an increase in the size of the erythroid cell population in culture by maintaining the capacity of proerythroblasts to synthesize DNA, to divide and to yield differentiated erythroid cells for a more prolonged period of time than occurs in cultures without added hormone.

**Figure 5** The rate of DNA synthesis of each culture, determined in triplicate, at the onset and after 10 and 24 hr of culture, with and without erythropoietin. The results are expressed as counts per minute of thymidine-14C incorporated into DNA during a 20 min pulse/10⁶ cells introduced into the culture at zero time.

**Table V**

| Labeled Cells* | Pro-erythroblasts | Basophilic Erythroblasts | Polychromatophilic Erythroblasts | Orthochromatotic Erythroblasts |
|----------------|-------------------|-------------------------|---------------------------------|-------------------------------|
| Zero time      | 84                | 65                      | 22                              | 4                             |
| 24 hr with erythropoietin | 85 | 55                      | 13                              | < 1                           |
| 24 hr without erythropoietin | -- | 56                      | < 1                             | < 1                           |

* Incubations and radioautography were performed as described in the text (see Materials and Methods).
DISCUSSION

These studies were designed to determine the effect of erythropoietin on hemoglobin synthesis by cultured fetal hepatic erythroblasts, and to examine, at the cellular and biochemical level, the basis for this response. The present observations establish that the hormone causes cultures of erythroid cells to form increased amounts of globin chains which are indistinguishable, chromatographically, from the alpha and beta globin chains which are synthesized by these erythroblasts in vivo. Measurement of the rate of synthesis of globin, rather than the heme moiety of hemoglobin, obviates the considerable difficulties associated with the use of iron uptake as an index of hemoglobin synthesis (Cole and Paul, 1966; Felicetti et al., 1966; Bunn and Jandl, 1968; Djaldetti et al., 1970). Although it has been suggested that erythropoietin is involved in producing alterations in the types of hemoglobins synthesized in a variety of species (Moore et al., 1966; Bertles and Borgese, 1968; Gabuzda et al., 1968; Kraus et al., 1968; Lewis et al., 1968), there has been no direct demonstration of this effect. Direct observations in the rat (Hunter and Paul, 1969) of the effect of erythropoietin on the types of hemoglobin formed showed, as in the present case, no alteration in the type of hemoglobin synthesized. It is possible that apparent species differences in this regard depend, in part, upon the presence of minor hemoglobins in the red cell population whose synthesis is regulated by the intensity of erythropoietic stress (Marks et al., 1964).

The erythropoietin-mediated stimulation of the rate of hemoglobin synthesis per culture of fetal hepatic erythroblasts may reflect an increased rate of hemoglobin synthesis by individual erythroblasts, an increase in the number of hemoglobin-producing cells, or both of these mechanisms. The present observations demonstrate that the hormone does not increase the total protein-synthetic capacity of individual polychromatophilic and orthochromatic erythroblasts. Since hemoglobin is the major protein synthesized by these cells, these data suggest that the hormone-induced increase in hemoglobin synthesis per culture does not reflect an increase in the rate of synthesis of hemoglobin per polychromatophilic or orthochromatic erythroblasts. Most of the hemoglobin synthesis which occurs in the erythroid cell population in culture is accomplished by cells of these stages whereas protein synthesis by proerythroblasts and basophilic erythroblasts consists principally of nonhemoglobin proteins (Thorell, 1947; Grasso et al., 1963; Djaldetti et al., 1970). Taken together, these data indicate that the roughly twofold increase in rate of synthesis of hemoglobin per culture induced by erythropoietin is the result of the increased number of polychromatophilic erythroblasts and, to a lesser degree, orthochromatic erythroblasts, in the erythropoietin-treated cultures.

The observed accumulation of large numbers of polychromatophilic erythroblasts in cultures incubated with erythropoietin may reflect either an accelerated rate of cell division among these cells, an increased rate of differentiation from early precursors, or prolongation of the period during which immature precursors are maintained in culture and are contributing to the yield of polychromatophilic and orthochromatic erythroblasts. An increase in the number of immature precursors maintained in a population of erythroid cells is one major effect of the hormone, as documented by the response of splenic colonies in the irradiated, plethoric animal (O'Grady et al., 1968), and of the spleen in polycythemic mice both in vivo (Orlic et al., 1965), and in vitro (Nakao et al., 1966), as well as by direct cytologic demonstration of a stimulation of RNA synthesis which is confined to the most immature erythroid cell precursors in mouse fetal livers (Rifkind et al., 1969 a). The present findings support the hypothesis that a principal effect of the hormone is exerted upon a population of very immature precursor cells. These cells are morphologically indistinguishable from and are included within the class of proerythroblasts, according to criteria previously described (Djaldetti et al., 1970). Nevertheless, present methods do not exclude the possibility of functional subpopulations, within the class of proerythroblasts, which differ in their responsiveness to erythropoietin and may have a precursor-progeny relationship. Differentiation among these precursor cells could account for the observed increase in total number of hemoglobin producing erythroblasts, and, in turn, for the increased rate of hemoglobin synthesis per culture. Failure to sustain the rate of hemoglobin synthesis beyond 24–48 hr of culture, and a moderate reduction in the number of immature precursors, may indicate that the present culture system, despite addition of erythropoietin, fails to provide all those condi-
tions or factors required for normally maintained erythropoiesis. Nevertheless, changes in the proportion of immature erythroid precursors and hemoglobinized erythroblasts similar to those observed in these cultures have been reported in fetal mouse livers developing in vivo (Fantoni et al., 1968; Djaldetti et al., 1970). Loss of precursors for the erythropoietin sensitive cell itself may be entertained as one possible explanation for this deterioration in vitro. A comparable situation, equally unexplained, is provided by the physiological disappearance of erythropoiesis from the liver which takes place at the time of birth, despite the likelihood of continued or even elevated levels of endogenous erythropoietin (Zanjani et al., 1969).

In the absence of erythropoietin relatively mature hemoglobinized erythroblasts accumulate and reticulocyte formation continues, with a modest increase in hemoglobin content. Nevertheless, immature precursors and young polychromatophilic erythroblasts almost disappear from the culture, accounting for a marked decrease in the over-all rate of DNA synthesis in hormone-free cultures. Taken together, these observations suggest that the maturation of erythroid cells already committed, by exposure in vivo to a factor yet undefined but possibly erythropoietin, proceeds even in the absence of additional erythropoietin in vitro. Nevertheless, the renewal of immature erythroid precursors and sustained erythropoiesis during the period of these cultures requires the presence of added erythropoietin. This interpretation is consistent with previous studies which have demonstrated that the continuous presence of erythropoietin is not necessary for the temporary maintenance of erythroblast maturation, although it does stimulate erythropoiesis (Alpen and Cranmore, 1969; Bruce and McCulloch, 1964; Bleiberg et al., 1967; Erslev, 1959; Filmanowicz and Gurney, 1961; Lajtha and Oliver, 1960; Stohlman, 1961).

It has also been proposed that a major effect of erythropoietin is to accelerate the rate of division and to shorten the development time of the maturing population of hemoglobinizing erythroid cells (Borsok et al., 1968; Blackett, 1968; Fischer, 1962; Fisher et al., 1965; Gallagher et al., 1963; Gordon et al., 1962). The present data do not exclude such an effect, but an accelerated rate of maturation does not, alone, adequately explain the observed preservation of replicating precursor cells in the hormone-treated cultures. Recent studies (McCool et al., 1970) employing fractionated cell populations likewise suggested that stimulation of a hemoglobinizing population of maturing erythroid cells constitutes at best a minor portion of the erythropoietic response to erythropoietin.

The authors gratefully acknowledge the technical assistance of Mrs. Robin George and Miss Beth Craig.

Portions of this work were supported by grants from the National Science Foundation (GB-4631), the United States Public Health Service (National Institutes of Health) (GM-14552), the American Cancer Society (Institutional Grant 315-3070-2675), and the RGK Foundation. Dr. Djaldetti was an International Fellow (1-F03-TW-1223) of the United States Public Health Service (National Institutes of Health); Dr. Rifkind is a Career Investigator of the Health Research Council of New York under contract 1-336.

Received for publication 7 January 1971, and in revised form 23 April 1971.

BIBLIOGRAPHY

Alpen, E. L., and D. Cranmore. 1959. In The Kinetics of Cellular Proliferation. F. Stohlman, Jr., editor. Grune & Stratton, Inc. New York. 290.

Bank, A. 1968. J. Clin. Invest. 47:860.

Bertles, J. F., and T. A. Borgese. 1968. J. Clin. Invest. 47:2679.

Blackett, N. M. 1968. Changes in proliferation rate and maturation time of erythroid precursors in response to anemia and ionizing radiation. Effects of Radiation on Cellular Proliferation and Differentiation. International Atomic Energy Agency, Vienna. 235.

Bleiberg, I., M. Liron, and M. Feldman. 1967. Blood. 29:469.

Borsok, H., K. Ratner, B. Tattrie, D. Teigler, and L. G. Lajtha. 1968. Nature (London). 217:1024.

Braverman, A. S., and A. Bank. 1969. J. Mol. Biol. 42:57.

Bray, G. A. 1960. Anal. Biochem. 1:279.

Bruce, W. R., and E. A. McCulloch. 1964. Blood. 23:216.

Bunn, H. F., and J. Jandl. 1968. J. Biol. Chem. 243:465.

Carmena, A. O., D. Howard, and F. Stohlman, Jr. 1968. Blood. 32:376.

Cole, R. J., and J. Paul. 1966. J. Embryol. Exp. Morphol. 13:245.

Craig, M., and E. S. Russell. 1964. Develop. Biol. 10:191.
Djalдети, М., А. Банк, и Р. А. Рифкинд. 1967. J. Mol. Biol. 50:345.

Драбкин, Д. Л., и Дж. Шум. 1932. J. Biol. Chem. 98:312.

Эрслев, А. И. 1959. In The Kinetics of Cellular Proliferation. F. Stohlman, Jr., editor. Grune & Stratton, Inc., New York. 313.

Фанони, А., А. де ла Чапель, Р. А. Рифкинд, и П. А. Маркс. 1968. J. Mol. Biol. 33:79.

Фельдкин, Л., Б. Коломбо, и С. Баглиони. 1966. Biochim. Biophys. Acta. 129:380.

Филмановиц, С., и С. В. Герн. 1961. J. Lab. Clin. Med. 57:65.

Фишер, С. И. 1962. In Erythropoiesis. L. О. Якобсон и М. Дойл, editors. Grune & Stratton, Inc., New York. 204.

Фишер, Дж., Л. Г. Лайтха, А. С. Буттоо, и Д. Д. Портеос. 1965. Brit. J. Haematol. 11:242.

Габуэда, Т. Г., М. А. Шумман, Р. К. Сайлер, и Х. Б. Люис. 1968. J. Clin. Invest. 47:1895.

Галлазер, Н. И., Г. Л. Сейферт, Ж. Л. Кальина, А. А. Маркс, и Р. Д. Ланге. 1963. J. Lab. Clin. Med. 61:258.

Галлени-Лартигур, О. 1967. C. R. H. Acad. Sci. Ser. D. 264:1066.

Голдуэй, В. 1966. In Current Topics in Developmental Biology. A. Монро и А. А. Масона, editors. Academic Press Inc., New York. 1173.

Гордон, А. С., Я. Лобус, Б. С. Дорнфест, и Г. У. Коoper. 1962. In Erythropoiesis. Л. О. Якобсон и М. Дойл, editors. Grune & Stratton, Inc., New York. 321.

Грансо, А. Дж., Я. В. Уоудворд, и Х. Спитф. 1963. Proc. Nat. Acad. Sci. U.S.A. 50:134.

Герн, С. 1968. Vitamins Hormones. 26:619.

Галворсен, С. 1963. Acta Paediat. Scand. 52:425.

Хантер, Дж. А., и Ж. Пол. 1969. J. Embryol. Exp. Morphol. 21:361.

Хатчинсон, В. С., Е. Д. Довирай, и И. Н. Мунро. 1962. Biochem. Biophys. Acta. 55:561.

Кованж, Ж. С., П. А. Маркс, Е. С. Ремсдал, и И. Феллер. 1967. J. Mol. Biol. 25:131.

Крауз, Л. М., И. Ичич, и Т. Крауз. 1968. Ann. N.Y. Acad. Sci. 149:423.

Лайтха, Л. Г., и Р. Оливер. 1960. Haemopoiesis: Cell Prod. Its Regul. Ciba Found. Symp. 289.

Леви, Я., Д. А. Альфред, И. Е. Смит, Б. Ф. Хортон, и Л. Смит. 1968. Brit. J. Haematol. 14:457.

Люкарелли, Г., А. Порцилла, С. Карневали, А. Кармена, и Ф. Стохлман. 1968. Ann. N.Y. Acad. Sci. 149:544.

Маркс, П. А., Е. Р. Брика, и Р. А. Рифкинд. 1964. Medicine (Baltimore). 43:769.

Маркс, П. А., и Й. о. Ковач. 1966. In Current Topics in Developmental Biology. А. Монро и А. А. Масона, editors. Academic Press Inc., New York. 1213.

Маккал, Д., Р. Й. Миллер, Р. Х. Перри, и В. Р. Брук. 1970. Cell Tissue Kinet. 3:55.

Муро, Й. Л., В. С. Годдил, Г. Ван Вийт, Й. Л. Льюис, Е. Бойд, и Т. Х. І. Гарсман. 1966. Blood. 28:314.

Накао, Н., Й. Мира, и Ф. Такаку. 1966. Blood. 27:646.

О’Гради, Л. Ф., Я. П. Льюис, Р. Д. Ланге, и Ф. Е. Тробау. 1968. Amer. J. Physiol. 215:176.

Орл, Д., А. С. Гордин, и Й. А. Брод. 1965. J. Ultrastruct. Res. 15:516.

Пол, Н., Р. К. Конни, и Р. И. Фрешней. 1969. Cell Tissue Kinet. 2:283.

Пол, Й., и Й. А. Гаттер. 1969. J. Mol. Biol. 42:31.

Парсет, А. Е. 1960. In Histochemistry. Литтл, Браун, и Компани, Бостон. 903.

Рифкинд, Р. А., Д. Чу, М. Джалдетти, и П. А. Маркс. 1969 а. Trans. Ass. Amer. Physicians Philadelphia. 82:380.

Рифкинд, Р. А., Д. Чу, и Х. Еплер. 1969 б. J. Cell Biol. 40:343.

Салваторелли, Г., А. М. Гулинати, и П. Д. Драндже. 1969. Blood. 34:472.

Силни, Г., Л. В. Поззи, и С. Пон. 1967. J. Embryol. Exp. Morphol. 17:303.

Саутхард, Й. Л., Й. Г. Вольф, и Е. С. Рассел. 1965. Nature (London). 208:1126.

Стохлман, Ф. 1961. Proc. Soc. Exp. Biol. Med. 107:751.

Тхорел, Б. 1947. Studies on the Formation of Cellular Substances during Blood Cell Production. Henry Kimpton, London.

Танжан, Е. Д., Е. О. Хоргер III, А. С. Гордин, Л. Н. Кантор, и Д. Хатчинсон. 1969. J. Lab. Clin. Med. 74:782.