

# HEMOGLOBIN SWITCHING IN SHEEP AND GOATS

## V. Effect of Erythropoietin Concentration on In Vitro Erythroid Colony Growth and Globin Synthesis

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### ABSTRACT

Erythroid colonies were generated in response to erythropoietin in plasma clot cultures of sheep and goat bone marrow cells. At low concentration of erythropoietin only hemoglobin A ( $\beta^A$  globin) was synthesized in goat cultures, but at high concentrations 50% of the hemoglobin synthesized was hemoglobin C ( $\beta^C$  globin). This effect of erythropoietin on the expression of a specific beta globin gene was manifested only after 72 h in vitro and followed the development of erythroid colonies. Sheep colonies behaved differently from those of goat in that little or no  $\beta^C$  globin synthesis occurred even at high erythropoietin concentration. To investigate this difference, sheep marrow cells were fractionated by unit gravity sedimentation. The erythroid colony-forming cells sedimented more rapidly (3.5–6 mm/h) than the hemoglobinized erythroid precursors (1–3.5 mm/h), suggesting that the colonies were formed from an early erythroid precursor. However, the colonies formed from the sheep marrow fractions synthesized only  $\beta^A$  globin even at concentrations of erythropoietin sufficient to stimulate  $\beta^C$  globin synthesis in goat colonies. Morphologically, the goat colonies were larger and more mature than those of the sheep. By 96 h in vitro three-fourths of the goat colonies contained enucleated red cells compared to only 3% of the sheep colonies. Thus, erythropoietin had an equivalent effect in stimulating erythroid colony growth from the marrow of both species although there were both biochemical and morphological differences between the colonies. Hemoglobin switching appeared to require exposure of an early precursor to high erythropoietin concentration, but the results with sheep marrow suggested that the rate of colony growth and cellular maturation might also be important.

A unique opportunity to analyze the regulation of globin gene expression is provided by studying erythropoiesis in goats and certain sheep since these animals respond to an anemic stress or to injection of erythropoietin (ESF), a glycoprotein hormone which stimulates hemopoiesis, by synthesizing a new hemoglobin (4, 15–17, 29). Hemoglo-

bin C ( $\alpha_2\beta_2^C$ ) differs from the usual adult hemoglobins only in its nonalpha globin, and hence the switch represents activation of  $\beta^C$  globin synthesis with concomitant suppression of the synthesis of the usual adult beta chains. During the first few months of life, newborn animals synthesize  $\beta^C$  globin. Hemoglobin C may reach 20–30% of the

total circulating hemoglobin in lambs and up to 100% in young goats, but by three months of age it normally falls to very low concentrations and is replaced by the adult hemoglobin.

The ability to stimulate hemoglobin C synthesis *in vivo* (9, 17, 28), and more recently *in vitro* (1, 3), has permitted analysis of several aspects of this switch. Gabuzda et al. (9) have found that hemoglobin C is synthesized in sheep marrow *in vivo* 3–5 days after injection of erythropoietin or plasma from anemic animals. This result has been interpreted to indicate that the switch is controlled in an early erythroid precursor cell. A similar conclusion has been suggested by our finding that erythropoietin-dependent hemoglobin C synthesis is detectable in goat marrow suspension cultures only after 72 h *in vitro* (3).

Erythropoietin appears to stimulate erythroid cell proliferation both *in vivo* (14) and *in vitro* (5, 19, 25), and may have specific effects on RNA synthesis (11, 13) and mRNA production (12). The effects of erythropoietin on the molecular and cellular control of hemoglobin C synthesis are unknown. The switch appears to be regulated by an intracellular mechanism since both hemoglobins A and C are found in the same red cell (10, 24). Control of specific beta globin synthesis does not appear to be at the translational level (cytoplasmic protein synthesis) since no evidence for the selective translation of mRNA by unique initiation mechanisms or by limiting amounts of tRNA species has been found (2, 8, 18, 23).

To quantitate the effect of erythropoietin in stimulating proliferation of an early, but presumably committed, erythroid precursor, we have grown erythroid colonies from goat and sheep marrow in plasma clot cultures (19, 26). With this technique we have been able to compare the concentration of erythropoietin required to stimulate proliferation and maturation of goat erythroid colonies *in vitro* with that which stimulates synthesis of beta<sup>c</sup> globin. Beta<sup>c</sup> globin synthesis appears in goat cultures only after colony growth has occurred. Sheep colonies differ from those of the goat in that little or no beta<sup>c</sup> globin synthesis occurs even at high erythropoietin concentration. The relative size and degree of maturation of sheep and goat colonies have been examined to provide further insight into the cellular aspects of hemoglobin switching in these animals. Furthermore, sheep marrow has been fractionated by unit gravity sedimentation in an attempt to characterize the

cells capable of generating erythroid colonies.

## MATERIALS AND METHODS

### *Cell Culture*

Sheep and goats were selected for homozygosity of hemoglobin A by preliminary screening of peripheral blood hemolysates either by electrophoresis on cellulose acetate (sheep)-or by column chromatography of globin chains on carboxymethylcellulose (CM-cellulose, goats) (23). The animals were anesthetized by intravenous injection of sodium pentobarbital (30 mg/kg) and exsanguinated via a catheter placed in the femoral artery. The femurs and humeri were immediately dissected out, the ends of the bones were cut off, and the marrow was gently removed with a sterile spatula and placed in Eagle's minimal essential media (MEM). The marrow was disaggregated by flushing it through a catheter-tipped 50-ml syringe. The cells were washed once, resuspended in MEM, and dispersed into a suspension of single cells by expulsion through a 20-gauge syringe needle.

The technique utilized for generation of the clot culture was similar to that described by MacLeod et al. (19). The concentrated cell suspension was diluted to a final concentration of  $0.5-1 \times 10^7$  cells/ml using a solution prepared as follows: 10 ml of  $\times 10$  Eagle's basal medium with Hanks' balanced salts, 1 ml of  $\times 100$  Eagle's basal medium amino acid solution, 1 ml of 100 mM sodium pyruvate, 1 ml of 200 mM L-glutamine, 1 ml of 7.5% NaHCO<sub>3</sub>, and 2 ml of penicillin-streptomycin (5,000 U of each/ml) were mixed with sufficient sterile distilled water to give a final volume of 100 ml. After dilution, 1 vol of the cell suspension was mixed with 1 vol each of 0.8% beef embryo extract, 35% bovine serum albumin (BSA), and 0.2 mg/ml L-asparagine, 0.5 vol of either isologous lamb serum (sheep cultures) or heat inactivated fetal calf serum (goat cultures), and 4.5 vol of NCTC-109 (Hyland Laboratories, Los Angeles, Calif., containing penicillin and streptomycin—100 U of each/ml). Erythropoietin dissolved in 0.5 vol NCTC-109 was added to the medium to achieve the desired final erythropoietin concentration. The final proportions of the various components of the incubation medium are given in Table 1.

Plasma clots were generated by adding 0.05 vol of citrated bovine plasma to 0.95 vol of the final cell suspension in culture medium and pipetting 0.1 ml into individual 6-mm diam plastic microwells. Three plastic sections, each containing six wells, were placed in a 20  $\times$  100-mm petri dish along with a 10  $\times$  35-mm petri dish containing 1 ml of sterile distilled water. The clot cultures were incubated in 5% CO<sub>2</sub>-95% air with 95% relative humidity at 37°C.

Erythropoietin utilized in this study was derived from anemic sheep plasma (Step III—4.7 U/mg supplied by Connaught Medical Laboratories, Toronto, Canada) or human urine (Lot no. H-6-TaLSL—36.3 U/mg supplied

TABLE I  
Requirements for Erythroid Colony Growth

Components	ml of component per ml of medium
NCTC-109	0.45
Bovine serum albumin (35%)	0.10
Beef embryo extract (0.8%)	0.10
L-Asparagine (0.2 mg/ml)	0.10
Citrated bovine plasma	0.05
Serum (isologous or fetal calf)*	0.05
Erythropoietin (10 U/ml)†	0.05
Diluted cell suspension	0.10

\* Isologous serum was used for sheep cultures and fetal calf serum for goat cultures except as otherwise indicated.

† The amount of erythropoietin added was varied for each individual experiment.

by Dr. Peter Dukes through the Erythropoietin Committee, Division of Blood Diseases and Resources, National Heart and Lung Institute). Highly purified human urinary erythropoietin (H4H-36, 12,000 U/mg<sup>1</sup>) was also made available through the courtesy of the Erythropoietin Committee and was used in certain of our experiments. The disposable microwells were obtained from Cooke Engineering Co., Alexandria, Va. and were sterilized by exposure to UV light. Eagle's basal medium amino acid solution, sodium pyruvate, beef embryo extract, and citrated bovine plasma were purchased from Grand Island Biological Co., Grand Island, N. Y.; while L-glutamine, sodium bicarbonate, penicillin-streptomycin, NCTC-109, and Eagle's minimal essential media were obtained from Microbiological Associates of Bethesda, Md. Fisher Chemical Co. supplied the L-asparagine, and Sigma Chemical Co., St. Louis, Mo., supplied the bovine serum albumin solution.

### Histological Characterization

The clot cultures were examined for colony growth at the desired time intervals by study of six microwells. The wells were rimmed with a spatula and three clots were placed on each of two glass slides. The clots were then fixed by covering each slide with a strip of no. 1 Whatman filter paper to which was applied 6 drops of 5% glutaraldehyde in 0.01 M sodium phosphate buffer, pH

<sup>1</sup> The activity of this preparation was 12,000 U/mg as assayed by stimulation of <sup>59</sup>Fe incorporation into red cells of the polycythemic mouse. Two in vitro assays, one measuring incorporation of [<sup>14</sup>C]glucosamine and the other <sup>59</sup>Fe incorporation by rat marrow cells, gave values of 237 and 2512 U/mg respectively (data supplied by the Erythropoietin Committee, National Heart and Lung Institute).

7.2. Excess glutaraldehyde was removed after 5-min fixation by blotting with an additional filter paper strip. The filter papers were removed and the slides washed in distilled water for 10 min. After drying, the slides were stained in 1% 3,3'-dimethoxybenzidine in methanol and counterstained with Harris-Lily hematoxylin (Fisher Scientific Co., Pittsburgh, Pa.). Each clot was examined and the number of benzidine-positive colonies quantitated by counting those which contained eight or more cells.

### Fractionation of Bone Marrow Cells

Sheep marrow cells were fractionated by unit gravity sedimentation on a shallow 1 to 2% bovine serum albumin gradient at 4°C (7). The "Staput" apparatus (Johns Corp., Toronto, Canada), consisting of a sequence of two reservoirs flowing into a mixing chamber with an outlet to a separatory chamber, was employed in a manner similar to the apparatus described by Miller and Phillips (21). 20 ml of 0.5% bovine serum albumin in phosphate-buffered saline (PBS, Grand Island Biological Co.) was allowed to flow from the mixing chamber into the separatory chamber followed by  $1 \times 10^6$  total cells (nonnucleated/nucleated = 5/1) suspended in 100 ml of PBS in 5% serum. The mixing chamber was rinsed and filled with 0.5% BSA in PBS. The gradient was generated by placing 800 ml of 1% BSA in PBS in the reservoir adjacent to the mixing cylinder and 800 ml of 2% BSA in PBS in the second reservoir, and allowing the gradient to flow through the mixing chamber and upward into the separatory chamber over a 1-h period. After the cells were allowed to sediment for 6 h, 95-ml fractions were collected by drawing off fluid from the base of the separatory chamber. The cells were recovered by centrifugation and suspended in MEM containing 5% fetal calf serum. The cell number was ascertained by counting a diluted fraction in a hemocytometer. A small aliquot of each fraction was also layered onto a slide with the Shandon cytocentrifuge, stained with benzidine-hematoxylin as described above, and the percentage of hemoglobinized cells determined. Plasma clot cultures (containing  $10^6$  nucleated cells/ml) of each fraction were incubated for 96 h in the presence of varying quantities of erythropoietin. The number of benzidine-positive colonies were determined, and the synthesis of the individual globin chains was quantitated by CM-cellulose chromatography.

### Radioactive Labeling of Globin

To label the cultures, 10  $\mu$ Ci of [<sup>3</sup>H]leucine (sp act 30-50 Ci/mmol, New England Nuclear, Boston, Mass.) in 10  $\mu$ l of NCTC-109 was added to each microwell and incubation continued for 24 h. At the end of the labeling period the individual clots were removed from the microwells using a sterile spatula. The clots were digested in 0.05% trypsin (Worthington Biochemical Corp., Free-

hold, N. J.) in Ca-Mg-free PBS (Grand Island Biological Co.). 12–14 clots were added to 5 ml of the trypsin solution in a 15 × 60-mm petri dish. The clots were reduced in size mechanically by triturating with a Pasteur pipette. After 5-min incubation at room temperature, the suspension was transferred to a centrifuge tube and the petri dish washed with 1 ml of serum. The serum was added to the suspension (providing a substrate for further trypsin action), and the cells were collected by centrifugation, washed once in 0.9% saline, and lysed with 1 ml of distilled water. The lysate was centrifuged and the resulting stroma-free solution was stored at -20°C. 40–60 individual 0.1-ml clots were used for analysis of the incorporation of radioactivity into globin chains.

#### *Analysis of Radioactive Globin by Polyacrylamide Gel Electrophoresis*

The method described by Moss and Ingram (22) was used for the rapid analysis of sheep globin chains. A stock urea solution was prepared by dissolving 240 g urea (Schwarz/Mann Div. Becton, Dickinson and Co., Orangeburg, N. Y., ultrapure) and 77 mg dithiothreitol (Calbiochem, San Diego, Calif.) in sufficient water to give a final volume of 450 ml. Each of the following solutions was prepared: (a) acrylamide: 4.24 g acrylamide and 112 mg *N,N'*-methylene-bis-acrylamide (each supplied by Bio-Rad Laboratories, Richmond, Calif.) in 20 ml of stock urea; (b) gel buffer: 1.74 g Tris base (Schwarz/Mann, ultrapure), 130 mg glycine (J. T. Baker Chemical Co., Phillipsburg, N. J.), and 0.012 ml TEMED (Bio-Rad) in 20 ml of stock urea; and (c) polymerizing agent: 56 mg ammonium persulfate in 20 ml stock urea. Equal volumes of acrylamide solution, gel buffer, and the polymerizing agent were mixed, deaerated, and poured into gel tubes 5 mm wide by 10.5 cm long (12 gels could be conveniently made if 9 ml of each of the solutions were used). The gels were allowed to polymerize at room temperature for 30 min and were then preelectrophoresed at 1 mA (constant current)/gel for 0.5 h. Both the anode and the cathode chambers contained buffer prepared by dissolving 15.2 g Tris base and 1.12 g glycine in sufficient stock urea to give a final volume of 350 ml.

The stroma-free lysates to be analyzed were lyophilized and dissolved in 0.3 ml of reservoir buffer containing 5% sucrose and carrier sheep globin (containing unlabeled beta<sup>c</sup> and beta<sup>a</sup> globins prepared from a red cell lysate by acid-acetone extraction) at a concentration of 5 mg/ml. 0.05 ml was applied to each gel and electrophoresis was performed for 4–5 h at 2 mA/gel. Samples of the unlabeled globin alone were electrophoresed in each set of gels to serve as a marker for the position of the globin chains. These gels were fixed and stained in 7% acetic acid containing amido black (0.5 g/ml). After 6–8 h the gels were electrically destained. Gels containing radioactive plus nonradioactive carrier globin were frozen and then sliced in sections 2 mm thick. Each section was placed in 1 ml of a mix of 9 parts NCS

(New England Nuclear) and 1 part water and heated for 2 h at 60°C to swell the gel and elute the labeled globin. 10 ml of a mix of 9 parts LSC complete and 1 part Hydromix (both from Yorktown Research) were then added, and radioactivity in the samples was measured at an efficiency for <sup>3</sup>H of approximately 20% in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.)

#### *Analysis of Radioactive Globin by CM-Cellulose Chromatography*

CM-cellulose chromatography was used for analysis of goat globin since the beta<sup>a</sup> and beta<sup>c</sup> globin of this species could not be resolved by the acrylamide gel procedure. It was also used for the sheep globin chain when precise quantitation of incorporation of [<sup>3</sup>H]leucine into the individual globins was desired. Chromatography was performed in 8 M urea phosphate buffer as previously described (6, 23). Sheep or goat hemoglobin to be used as markers was labeled by incubation of sheep or goat reticulocytes with [<sup>14</sup>C]leucine. 50 mg (as the stroma-free lysate) was added to the [<sup>3</sup>H]leucine-labeled globin chains to be analyzed. Heme was then extracted by treatment with acid-acetone, and the globin obtained was dried under a stream of N<sub>2</sub>. It was dissolved in 0.01 M NaPO<sub>4</sub>, pH 6.8, in 8 M urea and applied to the CM-cellulose column. The column was eluted with a nonlinear gradient of increasing sodium concentration (6, 23), and 1.5 ml fractions were collected directly into scintillation vials. 10 ml of Riafluor (New England Nuclear) were added to the vials, and double label liquid scintillation counting was performed with an efficiency of 56% for <sup>14</sup>C and 19% for <sup>3</sup>H.

## RESULTS

### *Erythroid Colony Growth*

Erythroid colonies were grown from both sheep and goat bone marrow cells in response to erythropoietin. The number of erythroid colonies was quantitated by counting those which contained eight or more benzidine-positive cells. At 48 h the erythroid cells were immature in appearance with vesicular nuclei and cytoplasm which was only weakly stained with benzidine (Fig. 1 a). At 72 h the colonies were larger and more deeply stained with benzidine (Fig. 1 b). After 96 h in vitro many of the goat colonies contained fully mature red cells from which the nuclei had been extruded, although there were still large colonies containing quite immature-appearing erythroid cells (Fig. 1 c). In general the sheep colonies contained fewer cells, and these cells were more immature in appearance than goat colonies at equivalent times in vitro (Fig. 1 d).

The number of colonies formed was propor-

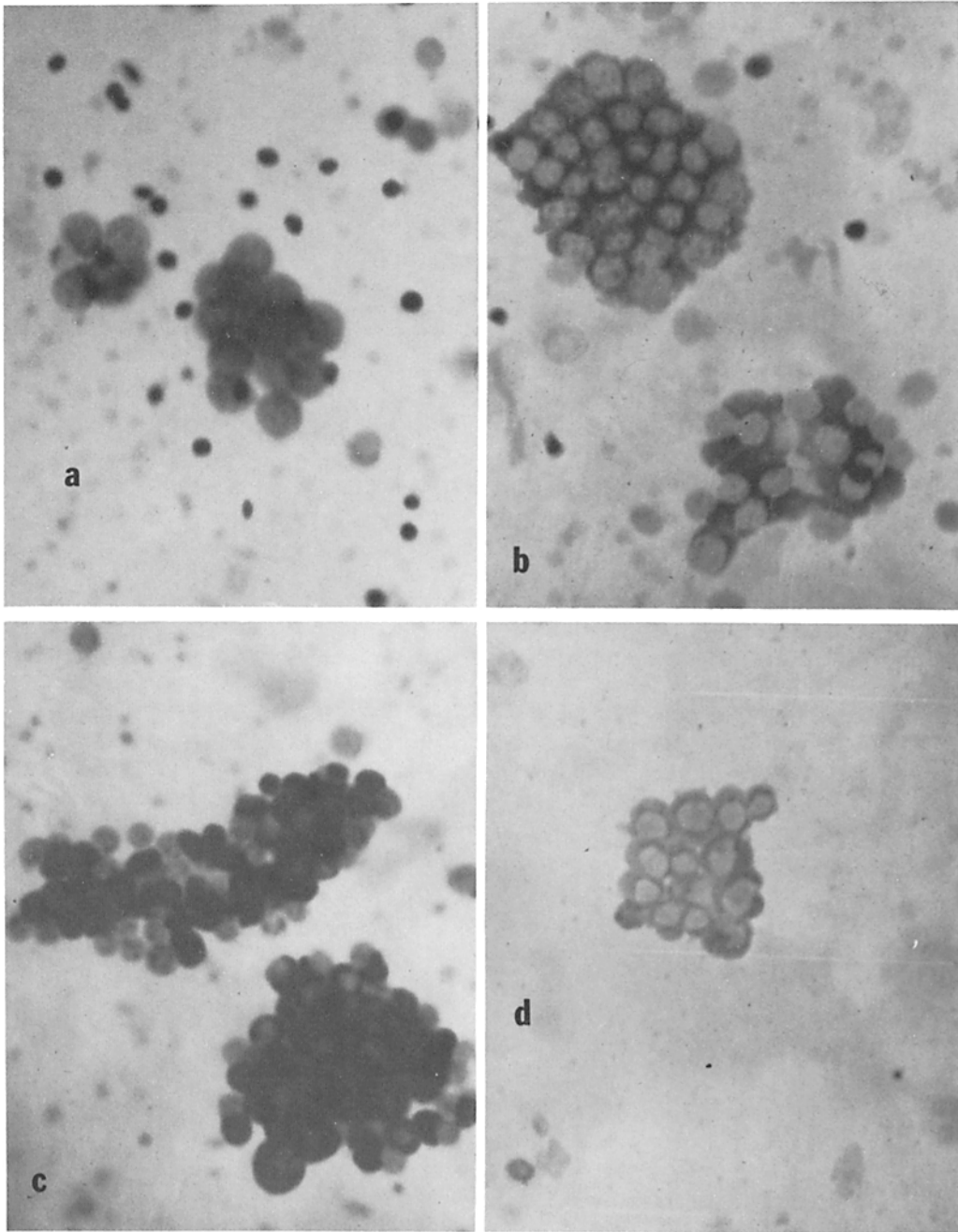


FIGURE 1 Morphology of erythropoietin-dependent sheep and goat erythroid colonies. 1 *a*. Goat colonies after 48 h in vitro. Illustrated are one 8-cell colony and one 4-cell group which would not yet be considered a colony. 1 *b*. Goat colonies after 72 h in vitro. The colonies are larger and some of the red cells have begun to undergo enucleation. 1 *c*. Goat colonies after 96 h in vitro. Further maturation has occurred. 1 *d*. A sheep colony at 96 h. The colony is smaller and the cells are more immature. All cultures contain 0.5 U/ml (sheep plasma, Step III) erythropoietin.  $\times 630$ .

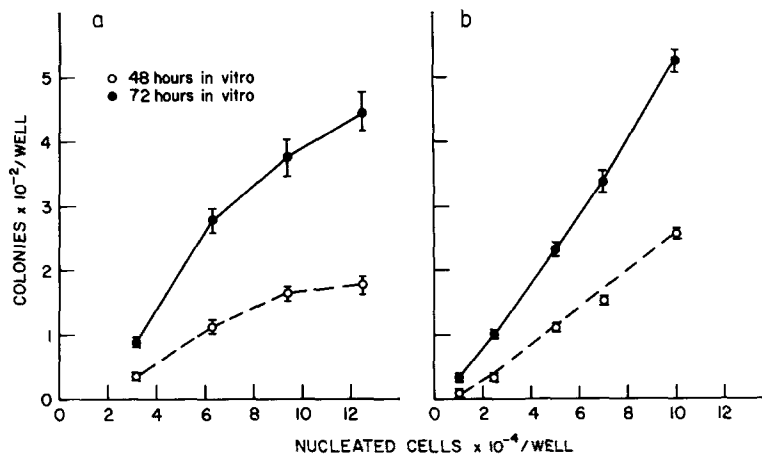


FIGURE 2 The relationship between the number of nucleated cells and the number of colonies in each microwell. Both goat and sheep marrow cells were cultured in the presence of 0.50 U/ml (sheep plasma, Step III) ESF. (a) Goat; (b) sheep. Bars indicate  $\pm$  standard error of the mean.

tional to the number of nucleated marrow cells incubated in the individual 0.1 ml plasma clots. This relationship was found for both goats (Fig. 2 a) and sheep (Fig. 2 b) and permitted the calculation that 0.4–0.6% of the total nucleated cells of adult goats and young sheep (2–4 mo of age) were capable of developing into erythroid colonies in vitro. Several adult sheep were also studied in the course of these experiments. The percentage of cells forming erythroid colonies in their marrow was lower, averaging 0.1% in six animals. There was an increase in the number of colonies between 48 and 72 h (Fig. 2 a and b) which appeared to reflect an increase in the number having eight or more cells rather than initiation of new colonies. There was no further increase in the number of colonies between 72 and 96 h (data not shown).

#### Globin Synthesis in Goat Marrow Cultures

The effect of increasing the concentration of erythropoietin on the synthesis of the individual globin chains in goat marrow culture was analyzed as illustrated in Fig. 3. After 96 h in vitro at an erythropoietin concentration of 0.025 units/ml, [<sup>3</sup>H]leucine was incorporated into the beta<sup>A</sup> and alpha chain, but only a minimal amount of radioactivity was eluted in the position of the beta<sup>C</sup> chain (Fig. 3 a). Increasing the erythropoietin concentration to 0.125 U/ml resulted in a twofold increase in incorporation of radioactivity into total globin (Fig. 4 c) and substantial synthesis of beta<sup>C</sup> globin (Fig. 3 b). At a concentration of 2.0 U/ml there was a further increase in total globin synthe-

sis and 54% of the [<sup>3</sup>H]leucine incorporated into beta globins was now in the beta<sup>C</sup> peak (Fig. 3 c). From the data in Fig. 3 we determined that, as the percent [ $\beta^C/\beta^A + \beta^C \times 100$ ] of beta<sup>C</sup> globin synthesis increased with increasing erythropoietin concentration from approximately 10% at low ESF concentrations to 50% at 2 U/ml, there was a proportionate decrease in the percentage of beta<sup>A</sup> globin synthesized.

The effect of erythropoietin concentration on goat colony growth, total globin synthesis, and specifically beta<sup>C</sup> globin synthesis was determined by the experiment illustrated in Fig. 4 a, c, and e. Even at the lowest concentration tested there were nearly maximal numbers of colonies generated (Fig. 4 a). In the absence of the hormone, generally there were fewer than 40 colonies per well, although in this experiment 70 colonies were found. Total hemoglobin synthesis increased almost three times over the 2-log range of ESF concentration (Fig. 4 c). Very little beta<sup>C</sup> synthesis occurred at the lowest concentration of ESF (Fig. 4 e). Between 0.125 and 2.0 U ESF/ml there was a dramatic increase in the amount of beta<sup>C</sup> formed. It reached 54% of the total beta globin synthesized at the highest ESF concentration. The ESF used in these experiments was Step III (4.7 U/mg) derived from anemic sheep plasma.

#### Globin Synthesis in Sheep Marrow Cultures

The response of sheep marrow cultures differed in several respects from that of the goat. Colony growth was dependent on the presence of erythro-

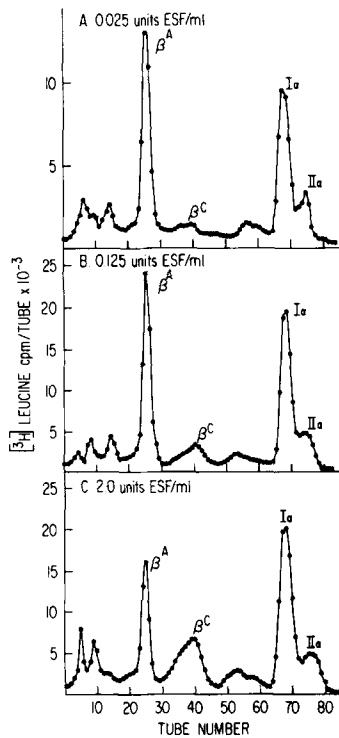


FIGURE 3 CM-cellulose column chromatography of radioactive globin chains synthesized by goat erythroid colonies between 72 and 96 h *in vitro*. At the end of the culture period the cells were recovered by trypsinization of the clots and prepared for chromatography as indicated in Materials and Methods. The position of the individual globin peaks was ascertained by the absorbance profile of the globin and by including  $^{14}\text{C}$ -labeled carrier globin prepared by incubation of reticulocytes (6). (A) Globin from cultures containing 0.025 U/ml of (sheep plasma, Step III) erythropoietin; (B) globin from cultures containing 0.125 U/ml of erythropoietin; (C) globin from cultures containing 2.0 U/ml of erythropoietin.

poietin with less than 20 colonies present in the absence of ESF, but there was no apparent increase in the number of colonies at the higher erythropoietin concentrations tested (Fig. 4 *b*). Over the range of erythropoietin concentration (from 0.025 to 2.0 U/ml), the total amount of globin synthesized was practically unchanged (Fig. 4 *d*). Beta<sup>C</sup> globin synthesis was negligible at all concentrations of erythropoietin (Fig. 4 *f*), never constituting more than 6% of the total beta globin. In attempts to induce an *in vitro* switch in sheep marrow cultures, cells were incubated in various types of serum including heat inactivated, dialysed, or untreated fetal calf; isologous or nonisologous

sheep; and fetal bovine serum. Human urinary ESF (H-6-TalSL) was tested at several concentrations up to 10 U/ml. Colony growth and globin synthesis were substantial, but beta<sup>C</sup> synthesis did not occur. A representative gel separation of the globins synthesized under these conditions is indicated in Fig. 5 *a*.

Bone marrow was studied from a sheep already synthesizing hemoglobin C. This animal had been phlebotomized for 3 wk during which time its hematocrit was maintained at 12–14%. At the end of this period the bone marrow was cultured with increasing concentrations of erythropoietin. The relative proportions of beta<sup>A</sup> and beta<sup>C</sup> globin from clot cultures labeled for 24 h were analyzed by polyacrylamide electrophoresis. There was no difference between four successive 24-h labeling periods at the various ESF concentrations. The result for 0.5 U ESF/ml at 96 h is illustrated in Fig. 5 *b*.

#### Globin Synthesis in Cultures of Fractionated Sheep Marrow

In sheep marrow separated in an albumin gradient, the erythroid colony-forming cells sedimented more rapidly than most of the hemoglobinized erythroid precursors (Fig. 6). Two pools of cells were obtained, the first sedimenting between 1 and 3 mm/h which contained 95% of the hemoglobinized cells, and the second sedimenting between 3.5 and 6 mm/h which contained 98% of the erythroid colony-forming cells. Clot cultures of each pool (54 microwells) were incubated *in vitro* for 96 h and labeled for the last 24 h with [ $^3\text{H}$ ]leucine. Globin synthesis in the cultures containing colonies was 100 times that found in the cultures which included previously differentiated hemoglobinized cells but lacked colonies (data not shown). Three different concentrations of ESF were tested (0.025, 0.125, and 0.5 U/ml), and beta<sup>C</sup> globin synthesis was less than 5% at each concentration both in those cultures from the pool containing the colony-forming cells and in those from the pool of hemoglobinized cells.

#### Comparison of Sheep and Goat Cultures

Colony number, globin synthesis, and beta<sup>C</sup> synthesis in the sheep cultures at 96 h were compared to those in goat cultures as illustrated in Fig. 4. At 96 h, sheep colonies were found to be generally smaller than goat colonies, two-thirds being in the range of 8–12 cells compared to one-third for the goat (Fig. 7). One-third of the

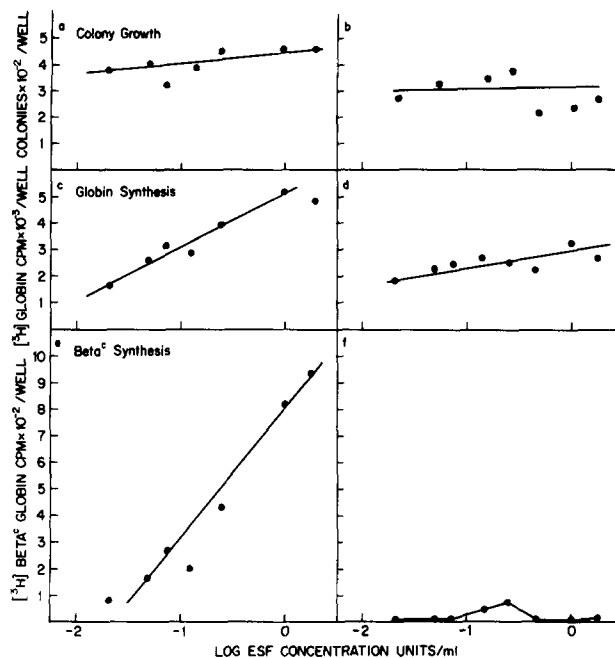


FIGURE 4 Relationship of colony growth, total globin synthesis, and beta<sup>C</sup> globin synthesis to erythropoietin concentration. The radioactivity in globin was calculated by summing the counts per minute which eluted in the individual peaks. Inclusion of the same amount of [<sup>14</sup>C]globin carrier in each chromatographic separation permitted correction for losses during both preparation and chromatography of the globin; these amounted to 10-30%. *a*, *c*, and *e*: Goat cultures; *b*, *d*, and *f*: sheep cultures.

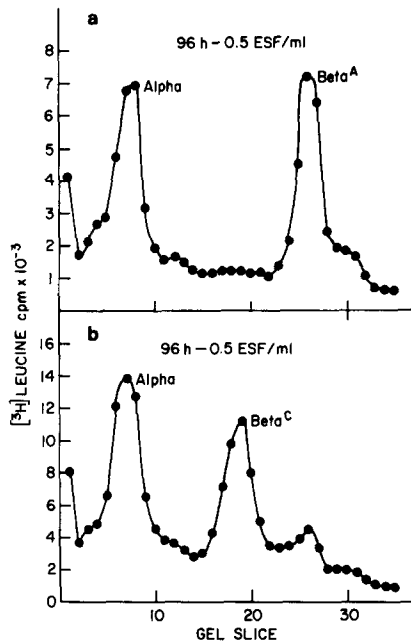


FIGURE 5 Polyacrylamide gel electrophoresis of sheep globin labeled during the period of 72-96 h *in vitro*. Details of the technique are given in Materials and Methods. The position of the individual chains was

goat colonies were larger than 28 cells, but only 15% of the sheep colonies attained this size. By 96 h nearly all of the goat colonies contained fully mature enucleated red cells, but only 3% of the sheep colonies had reached this level of maturity.

The time course of colony growth and globin synthesis was compared in cultures of goat and sheep marrow (Fig. 8 *a* and *b*). Colonies appeared in each at 48 h and increased approximately twofold by 72 h with essentially no further change at 96 h. The behavior of the two species with respect to total globin synthesis was quite different, however. Maximal globin synthesis in the goat cultures coincided with the maximal colony growth, whereas globin synthesis in sheep cultures declined after 48 h. Beta<sup>C</sup> globin was synthesized in the goat colonies grown in the presence of higher

determined by electrophoresis of labeled standards. (*a*) Globin synthesized in the cultures grown from the marrow of a normal sheep; (*b*) globin synthesized in the cultures of a sheep which had been made anemic by phlebotomy in order to stimulate synthesis of hemoglobin C.



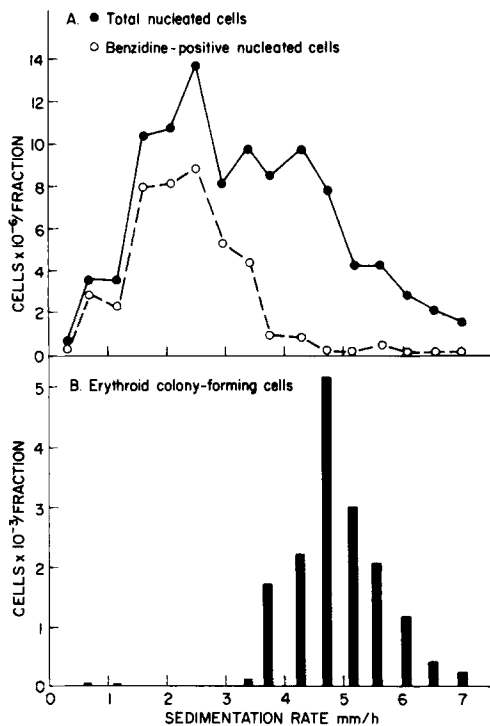


FIGURE 6 Fractionation of sheep bone marrow by unit gravity sedimentation. Sixteen 95-ml fractions were collected. The data for each fraction are plotted at the midpoint of its sedimentation rate. (A) Distribution of total nucleated and benzidine-positive nucleated cells. (B) Distribution of erythroid colony-forming cells.

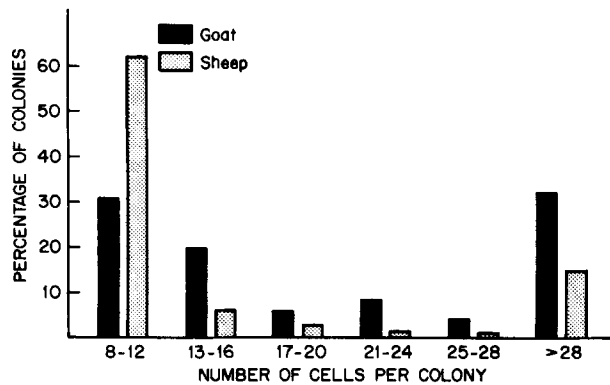
concentrations of ESF, whereas little or no beta<sup>c</sup> globin was synthesized in sheep cultures.

### Globin Synthesis in Colonies Generated by Human Erythropoietin of High Specific Activity

Both sheep and goat marrow were cultured in the presence of the highly purified human urinary erythropoietin. Because of the limited amount available, only one concentration was tested. At 2.0 U/ml the number of colonies and the synthesis of beta<sup>c</sup> did not differ significantly from the results obtained with the crude hormone obtained from sheep plasma, but total globin synthesis was slightly less (data not shown). Thus, 50% of the total beta globin synthesized in goat colonies was beta<sup>c</sup>, but no incorporation of radioactivity into beta<sup>c</sup> globin was detected in the sheep cultures.

### DISCUSSION

Erythropoietin seems to have several effects on erythropoiesis. One of the earliest responses is stimulation of RNA synthesis (11, 13), perhaps specifically mRNA (12), but the hormone also stimulates proliferation of committed erythroid precursors in vivo (14) and in vitro (15, 19, 26) and may enhance hemoglobin synthesis in cells already making hemoglobin (14). Our studies have shown two well-defined effects of erythropoietin in vitro



#### PERCENTAGE OF COLONIES WITH ENUCLEATED RED CELLS

Goat	78.0%
Sheep	2.7%

0.5 units ESF/ml  
 96 hours in vitro

FIGURE 7 Comparison of the size and maturity of sheep and goat colonies. The number of cells in 150 individual colonies grown in vitro for 96 h at 0.50 U/ml (sheep plasma, Step III) ESF were enumerated and a similar number were examined for evidence of enucleation.

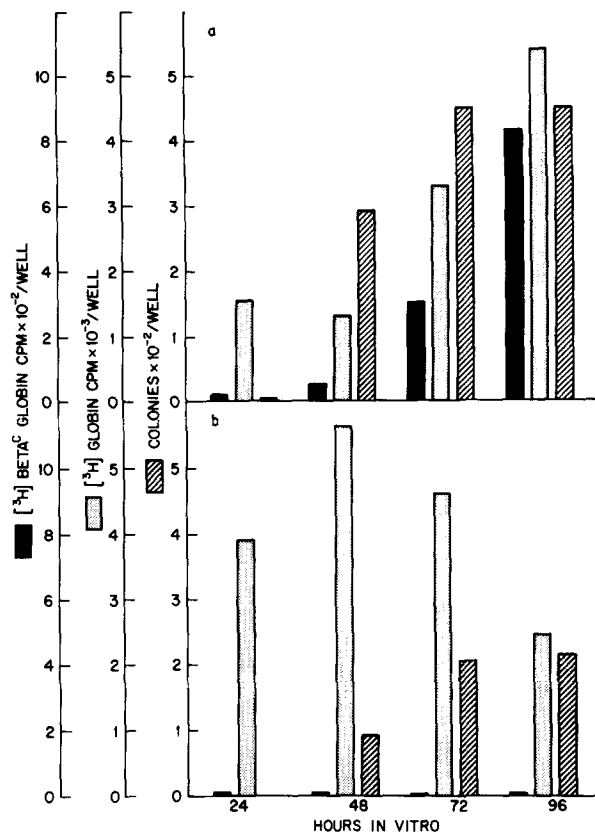


FIGURE 8 Comparison of goat and sheep colonies as to the time course of colony development, globin synthesis, and beta<sup>c</sup> globin synthesis. Labeling of the cultures was for 24-h intervals. The erythropoietin concentration was 0.5 U/ml (sheep plasma, Step III) and the nucleated cell concentration 10<sup>6</sup>/ml. (a) Goat; (b) sheep.

on goat erythroid cells. These are (a) proliferation to form colonies of hemoglobinized cells, an effect which occurs at low (and high) concentrations (Fig. 4 a), and (b) stimulation of beta<sup>c</sup> globin synthesis, an effect which occurs to a significant extent only at high concentrations of ESF (Fig. 4 e). That the same substance exerts both effects is suggested by the similar result obtained with crude (4.7 U/mg) ESF prepared from sheep plasma and highly purified (12,000 U/mg) material from human urine. A similar conclusion has been suggested by studies in which ESF of various degrees of purity has been injected into intact sheep (28).

The action of erythropoietin on cell proliferation and on specific intracellular biochemical events is partially clarified by our studies. Both effects appear to be exerted primarily on an early precursor cell. Cell proliferation in response to ESF to form multiple hemoglobinized progeny has been shown *in vivo* to be a property of an undifferenti-

ated responsive cell in spleens of plethoric mice (14) and also *in vitro* as shown with purified fractions of undifferentiated mouse fetal liver cells (5). Sedimentation velocity analysis of rat marrow has led to the identification of a large, rapidly sedimenting cell which responds to ESF by cell division (20). We also found that the colony-forming cell from sheep marrow is rapidly sedimenting, indicating that it is a large and immature cell (Fig. 6). The sequential development of goat colonies from small clusters of weakly benzidine-positive cells with large vesicular nuclei (Fig. 1 a) to completely hemoglobinized colonies (Fig. 1 c) has further substantiated the concept that we have stimulated proliferation of an early precursor cell to give erythroid colonies.

That the action of ESF on specific biochemical events occurs on an early precursor cell has been suggested by our finding that synthesis of beta<sup>c</sup> globin in goat cultures occurs only after the

appearance of differentiated colonies (Fig. 8 *a*). Exposure of differentiated cells already active in hemoglobin synthesis to high concentrations of the hormone for periods of up to 48 h does not result in beta<sup>c</sup> globin synthesis either in the plasma clot (Fig. 8 *a*) or in suspension cultures (3). Previous studies in these and other laboratories have shown that both hemoglobins A and C are found in the same red cell (10, 23), indicating that the switch must be mediated by an intracellular mechanism. Regulation at the level of mRNA translation into specific globin chains does not appear to occur (2, 8, 18, 23). Thus ESF at high concentrations seems to act on an early erythroid precursor in goat marrow determining which of the specific beta globin genes is transcribed. Alternatively, there might be selective processing of the immediate gene transcription product to produce beta<sup>A</sup> and/or beta<sup>c</sup> mRNA depending on the concentration of erythropoietin to which the cell is exposed. That this action of ESF becomes apparent only after proliferation of the cell to form differentiated progeny is consistent with the requirement for DNA synthesis and mitosis for hormone-dependent differentiation in other systems (27). Whether there is early accumulation of mRNA for the specific beta globins which, later in differentiation, is translated into globin or whether production of the specific beta globin mRNA continues during differentiation can only be a matter of speculation at this time.

Sheep erythroid colonies have been grown under conditions very similar to those employed for the goat marrow, but we have detected little or no beta<sup>c</sup> synthesis. Several explanations might account for this finding. Perhaps expression of the individual beta globin genes is determined earlier in erythropoiesis in the sheep than in the goat so that the erythroid colony-forming cell is already committed to synthesis of particular globin chains. The result obtained with the phlebotomized sheep (Fig. 5 *b*) appears to support this notion, although the similar time course of development of beta<sup>c</sup> synthesis in intact sheep and goats is against it (9, 15). In contrast to our results, Adamson and Stamatoyannopoulos (1) have induced the synthesis of substantial amounts of hemoglobin C in short-term suspension cultures of sheep marrow. Thus, they have demonstrated the potential for hemoglobin C activation. The reason for the difference between their results and ours is not immediately apparent.

The differing appearance of sheep and goat

colonies (Fig. 1 *c* and *d*) has led us to compare more precisely several aspects of their growth. Total globin synthesis increases dramatically in goat cultures as the colonies mature (Fig. 8 *a*) but actually declines in sheep cultures after 48 h (Fig. 8 *b*). Since 100 times more globin is synthesized between 72 and 96 h in gradient separated sheep marrow fractions which have colonies, we conclude that most of the globin produced is derived from these colonies. Thus, exposure of the immature sheep erythroid precursor cell, capable of colony growth, to high concentrations of ESF is insufficient to activate the synthesis of beta<sup>c</sup> globin. The lack of beta<sup>c</sup> globin synthesis in sheep colonies can be correlated morphologically with their relatively small size and their failure to mature completely in 96 h to enucleated red cells (Fig. 7). Thus, although the amount of ESF added to the sheep and goat cultures is equivalent, the differing morphological and biochemical behavior of the two types of colonies suggests that the effect of the hormone on intracellular phenomenon in the two species might be different. Further studies will be needed to clarify whether this is a property of the erythropoietin preparation or intrinsic to the erythroid cells of the two species.

Expression of specific globin genes cannot be studied profitably without consideration of the processes of cellular proliferation and maturation which occur during erythroid differentiation. The biochemical action of ESF in inducing expression of specific globin genes appears to be exerted early in erythropoiesis, indicating the need to use purified populations of early precursor cells to study the molecular mechanism of this action of the hormone. It is hoped that definition of the growth behavior of the erythroid cells necessary for expression of specific globin genes will permit a search for factors responsible for the switch from fetal to adult hemoglobin in man and other species.

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