HEMOGLOBIN SWITCHING IN SHEEP AND GOATS

VI. Commitment of Erythroid Colony-Forming Cells to the Synthesis of Beta C Globin

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ABSTRACT

Bone marrow from mature goats and sheep was cultured in plasma clots, and three erythropoietin (ESF)-dependent responses—growth (colony formation), differentiation (globin production), and initiation of hemoglobin C (αβ C) synthesis—were quantitated. ESF concentrations below 0.01 U/ml supported colony growth and adult hemoglobin production in cultures of goat marrow, while maximal hemoglobin C synthesis (70%), as measured between 72 and 96 h in culture, required a 100-fold higher ESF concentration. Sheep marrow was cultured in a medium enriched to enhance growth and to permit complete maturation of colonies. These colonies active in hemoglobin synthesis between 24 and 96 h produced mainly adult hemoglobin, and only between 96 and 120 h did sheep colonies develop which produced mainly hemoglobin C (up to 70%). A similar heterogeneity may exist among goat colonies. Thus, when goat bone marrow was fractionated by unit gravity sedimentation, more hemoglobin C synthesis was observed in colonies derived from cells of intermediate sedimentation velocity than in colonies derived from the most rapidly sedimenting cells. Brief exposure of sheep (in vivo) and goat (in vitro) bone marrow to a high ESF concentration committed precursor cells to the generation of colonies which, even at low ESF concentration, produced hemoglobin C. Commitment to hemoglobin phenotype appears to be an early and probably irreversible event in the development of an erythroid cell.

The hormone erythropoietin supports red cell production by accelerating growth, differentiation, and maturation of erythroid cells. In addition to its function as an erythropoietic stimulating factor (ESF), it also induces synthesis of hemoglobin C (αβ C) in sheep (23) and goats (10). Hemoglobin C is normally generated during the later stages of development, accounting for 20-30% of the hemoglobin in certain newborn sheep (those homozygous for hemoglobin A) and 95% in goats. As the animal matures, hemoglobin C synthesis ceases and is replaced by the adult hemoglobin; hemoglobin A (αβ A) in sheep and hemoglobin A, D, or E (containing β A, β B, and β E, respectively) in goats. In bone marrow cells of adult animals, hemoglobin C production can be reinitiated in vivo by anemic stress or injection of ESF (7, 10, 23) and in vitro by exposure to ESF (1, 3, 4).

We have previously analyzed hemoglobin C
production during erythroid cell development, employing the plasma clot culture system (14, 21) for generating erythroid colonies. The goat and sheep erythroid colonies were derived from a population of nonhemoglobinized precursors (3). The number of colonies generated was shown to be directly proportional to cell input and to be related to ESF concentration. While maximal colony number was reached by 72 h in vitro, globin synthesis continued thereafter. In cultures of goat bone marrow, hemoglobin C (\(\beta^c\) globin) synthesis occurred only after colonies developed and increased directly with ESF concentration over a range from 0.01 to 2.0 U ESF/ml. Erythroid colonies also developed in cultures of sheep bone marrow, but they were smaller and less differentiated (fewer cells exhibited nuclear extrusion) than the goat colonies and no significant \(\beta^c\) synthesis was obtained.

In further studies described herein, using an improved culture medium, we have observed fully developed sheep erythroid colonies. Nonetheless, synthesis of significant amounts of \(\beta^c\) globin was delayed until after 4 days in vitro, implying that the colonies actively synthesizing globin earlier during the culture were derived from precursors already committed with respect to hemoglobin phenotype. Additional evidence implying heterogeneity of the colony-forming population was obtained by culturing fractions of goat bone marrow separated by unit gravity sedimentation. We have also obtained evidence that commitment of colony-forming cells to \(\beta^c\) synthesis may be obtained in sheep (in vivo) and goats (in vitro) by brief exposure to high ESF concentration.

### MATERIALS AND METHODS

#### Cell Culture Methods

Sheep, homozygous for hemoglobin A, and goats, lacking hemoglobin E, were selected for these experiments by prescreening of blood hemolysates by cellulose acetate electrophoresis of hemoglobin (sheep) or carboxymethyl cellulose chromatography (goats) (3, 16). The animals were anesthetized and exsanguinated, and single cell suspensions of marrow were prepared for culture as previously described (3). The medium for culture of goat marrow was unchanged from that employed before (3), while the medium found optimal for growth of sheep erythroid colonies differed in several respects (see Table I). Bovine serum albumin was routinely deionized by stirring with a mixed bed resin, AG 501-X8(D) (BioRad Laboratories, Richmond, Calif.) (5). In certain experiments, beta-mercaptoethanol

#### Table I

| Component: medium (ml/ml) | Goat | Sheep |
|--------------------------|------|-------|
| NCTC-109                 | 0.45 | 0.1   |
| F-12M                    |      | 0.35  |
| Bovine serum albumin (10%) |    | 0.1   |
| Bovine serum albumin (35%) | 0.1 |      |
| Beef embryo extract (0.8%) | 0.1 | 0.1   |
| L-Asparagine             | 0.1  |      |
| Citrated bovine plasma   | 0.05 | 0.1   |
| Serum (fetal calf)       | 0.05 | 0.05  |
| Serum (isologous)        |      | 0.05  |
| Erythropoietin (10 U/ml)* | 0.05| 0.05  |
| Diluted cell suspension† | 0.1  | 0.1   |

* ESF was dissolved in NCTC-109 at a concentration appropriate for each individual experiment.
† Sheep cells and goat cells were suspended in a medium previously described at 10 times the concentration desired in the final clot culture (3).

(Sigma Chemical Co., St. Louis, Mo.) was added to a concentration of \(1.4 \times 10^{-4}\) M. The source of culture materials was as previously indicated (3). F12 M medium was obtained from Grand Island Biological Co., Grand Island, N. Y.

For certain experiments, goat bone marrow cells were preincubated in suspension culture (4) at a cell concentration of \(1.8 \times 10^7\) cells/ml. After incubation for varying time intervals (0-18 h), the cells were washed twice with minimal essential Eagle's medium (MEM), resuspended in MEM at a cell concentration of \(10^7\) cells/ml, and set up in clot culture as described previously (3). Zero-time cells were put in the medium at the indicated ESF concentration and then immediately spun down and washed. The total time of the cells in vitro was 96 h including both that spent in suspension and that in clot culture.

#### Analysis of Colony Number and Globin Synthesis

0.1-ml clot cultures were fixed in 5% glutaraldehyde on glass slides, stained with benzidine, and counterstained with Harris-Lilly hematoxylin, and the number of colonies containing eight or more cells were counted microscopically (3). To quantitate the amount and type of globin synthesized in colonies, 10 \(\mu\)l of MEM containing 10 \(\mu\)Ci of \([\text{H}]\)leucine (sp act 30-50 Ci/mmol) were added to each individual 0.1-ml clot. After an additional 24 h of incubation, the colonies were retrieved by trypsinization of the clots from 48 to 60 wells, washed, and lysed, and their products were analyzed by carboxymeth-
y cellulose chromatography in 8 M urea phosphate buffer (3, 16). To determine the pattern of globin synthesis in differentiating erythroid cells, bone marrow was placed directly in suspension culture and labeled between 0 and 11 h in vitro by addition of 300 μCi of [3H]leucine (4). The cells were recovered and prepared for chromatography as previously described (16).

**Sephadex G-100 Filtration of Erythropoietin**

Erythropoietin (Connaught Medical Research Laboratories, Willowdale, Ontario, lot no. 3004-3) was made up to a final concentration of 250 U/ml in Dulbecco’s phosphate-buffered saline, pH 7.4, lacking calcium and magnesium. 500 U were applied to a Sephadex G-100 (superfine) column (1.5 × 70 cm) previously equilibrated at 4°C with the same buffer. Two protein peaks were eluted: one in the void volume and a second included in the column. The included peak was collected, concentrated by ultrafiltration through a UM 10 filter (Amicon Corp., Lexington, Mass.) and lyophilized. The powder, solubilized in NCTC-109 medium at an estimated concentration of 100 U/ml, was titrated against an erythropoietin of known activity by using mouse marrow in the erythroid colony-forming assay (14).

**Fractionation of Bone Marrow Cells**

Bone marrow was fractionated on the basis of cell size by unit gravity sedimentation on a 1-2% bovine serum albumin gradient (6, 13, 15) as indicated previously (3). The number of cells in each fraction was determined, and adjacent fractions were pooled to provide a sufficient number of cells so that both colony number and globin synthesis could be analyzed at a single ESF concentration in clot culture.

**Bone Marrow Aspiration and ESF Injection in an Anesthetized Sheep**

A 60-day old lamb was anesthetized with 2-5% Halothane, intubated, and maintained at a surgical plane of anesthesia by a closed system with 1.5-2% Halothane. The animal was positioned in lateral recumbency and the hind or the front leg prepared for surgery. A 4-6-cm incision was made longitudinally over the proximal long bone, and the muscles were retracted to expose the femoral or humeral shaft. A 4-mm hole was drilled into the marrow cavity with a 5/32 inch bit attached to a Collison orthopedic hand drill held at a 60° angle to the bone and directed towards its proximal end. No more than two holes were drilled in each bone to avoid the possibility of later fracture. A Coke pressure injection monitoring tube with a female tip (no. 40-101, Coke Labs, Inc., Lakewood, Colo.), cut to give a tip with a bevel of 45°, was attached to a 10-ml heparinized syringe and inserted into the hole. A marrow sample was removed by advancing the tubing and gently retracting the syringe plunger. The number of nucleated and total cells (including enucleated red cells) was immediately determined. If the nucleated cells were less than 10% of the total, the sample was discarded and another obtained. The holes were sealed with bone wax (Ethicon Inc., Somerville, N. J., no. W38G), the parted muscles were released and returned to normal apposition, and the incision was sutured.

After the initial bone marrow sample was obtained, the animal was injected with ESF. 200 ml of blood were removed via a jugular venous catheter, and 150 ml of plasma from an anemic sheep (maintained at a hematocrit of 10% for 10 days by phenylhydrazine injection [16]) were infused. Then 1,500 U ESF (Connaught, lot no. 3003-11) in 50 ml of phosphate-buffered saline were immediately injected. The animal was allowed to recover and was reanesthetized each time a marrow sample was required. At the end of the experiment, the animal was sacrificed and the long bones were removed in the usual manner.

**RESULTS**

**Effect of ESF Concentration on Colony Growth and Globin Synthesis**

**GOAT:** The effect of increasing ESF concentration on three parameters, colony number, total globin synthesis, and βc globin synthesis, was measured 96 h after initiation of clot cultures of goat bone marrow (Fig. 1). The number of erythroid colonies increased from 25 to 380 colonies per clot over the range 0.00025-5 U ESF/ml, with a significant (P > 0.03) decrease at 10 U ESF/ml. Total globin synthesis (labeling period 72-96 h) was augmented 30-fold over the same ESF concentration range. Adult β-globins, βa and βb, were the only globins produced at ESF concentrations below 0.01 U/ml and increased to a maximum at an ESF concentration of 0.05 U/ml, with no significant change at higher ESF concentrations. βc globin synthesis was first discernible at an ESF concentration of 0.005 U/ml and increased to a maximum at 5 U/ml.

**SHEEP:** Sheep marrow cells, cultivated in an improved medium, generated large colonies with well-differentiated erythroid cells (Fig. 2). Concentrations of ESF greater than 0.5 U/ml markedly reduced the number of colonies formed from sheep marrow (Fig. 3). ESF was also inhibitory to mouse erythroid colony development but only at concentrations above 5 U/ml. Chromatography on G-100 Sephadex abolished the inhibitory effect of ESF at high concentration on mouse colony formation as previously reported by Iscove et al.
Colony Growth and Globin Synthesis in Fractions of Goat Bone Marrow

Marrow fractionation by unit gravity sedimentation provided separation of the smaller benzidine-positive nucleated cells in the top 800 ml of the gradient from the larger predominantly nonhemoglobinized cells in the bottom 800-1,600 ml of gradient (Fig. 6). The cells from each of eight fractions were cultured in plasma clots with 1 U ESF/ml. Colonies (dark bars) were derived only from those cells in the lower portion of the gradient. The colony-forming efficiency of the individual fractions ranged between one colony per 200 cells (I) and one colony per 300 cells (V), while that for unfractionated marrow was one colony per 700 cells. Globin was synthesized between 72 and 96 h only in cultures derived from fractions containing colony-forming cells. In fractions I-III, 35% of the total β globin synthesized was βc, while in fractions IV and V, 50% of the total was βc.

Commitment of Goat Colony-Forming Cells to βc Globin Synthesis In Vitro

In an effort to commit colony-forming cells to βc globin synthesis, goat marrow was established in suspension culture with 0.5 U ESF/ml, incubated for various periods, washed, and then cultured in plasma clots at low ESF concentration. [3H]Leucine was present during the final 24 h in culture. Brief exposure of the marrow to medium containing 0.5 U ESF/ml (0 time) was insufficient to initiate βc globin synthesis since subsequent transfer of these cells to 0 or 0.005 U ESF/ml resulted in synthesis of only 2.0 and 3.8%, respectively, of βc globin (Table II). Increasing the duration of exposure of the marrow cells to 0.5 U ESF/ml resulted in synthesis of more βc, up to 19.1% at 18 h. In these particular experiments, marrow grown continuously in plasma clot culture at 0.5 U ESF/ml synthesized 19.8% βc globin. If the cells were incubated in suspension culture at 0.5 U/ml of ESF for 18 h and then cultured in plasma clots at this concentration of ESF, there was a twofold increase in βc synthesis (35% of the total β-globin).

Commitment of Sheep Colony-Forming Cells to βc Globin Synthesis In Vivo

To determine the effects of transient exposure of sheep marrow to high ESF concentration, the
FIGURE 2  Morphology of colonies from sheep marrow after 96 h in vitro at 1.0 U ESF/ml. × 200.

FIGURE 3  Titration of ESF. Comparison of mouse and sheep erythroid colony growth at various concentrations of ESF before (●) and after (○) filtration of the ESF on a G-100 column. Enumeration of colonies was done after 96 h in culture for sheep and after 48 h for mouse.
procedure outlined in Fig. 7 was followed. Bone marrow aspirates were removed from an anesthetized animal 0.5 h before and 9, 18, 24, and 139 h after intravenous injection of ESF (see Materials and Methods). Globin synthesis was monitored both in the cells already actively synthesizing globin (differentiating cells) at the time of explantation and also in cells which formed erythroid colonies (CFU-E) in vitro. Radiochromatograms of
globin synthesized by these two cell populations are depicted in Fig. 8. Before ESF injection, both the differentiating cells labeled from 0 to 11 h in vitro and the colonies generated by exposure to 1 U ESF/ml and labeled from 72 to 96 h in vitro, were synthesizing predominantly $\beta^A$. 9 h after the ESF injection, $\beta^C$ globin synthesis was negligible in the differentiating cells but accounted for 30% of the total $\beta$ globin in the erythroid colonies. By 139 h after injection, the differentiating cells as well as the colonies were synthesizing 50% $\beta^C$ globin.

The results of the entire experiment are summarized in Fig. 9. Differentiating cells synthesized 10% or less $\beta^C$ globin (of the total $\beta$) before and for 24 h after the in vivo ESF injection, but by 139 h had switched toward $\beta^C$ production. Colonies derived from bone marrow obtained before ESF injection produced 11% $\beta^C$ globin at an ESF concentration of 0.1 U/ml and 20% at an ESF concentration of 10.0 U/ml between 72 and 96 h in vitro. 300 colonies developed per $10^5$ bone marrow cells before in vivo ESF injection. Both parameters increased linearly over the next 24 h
after ESF injection, colony number exhibiting a threefold augmentation while the percent of \( \beta^c \) synthesis increased sixfold. Bone marrow cultured at a high dose of ESF (10 U/ml) produced 1.1-1.8 times more \( \beta^c \) globin but fewer colonies than marrow cultured at 0.1 U ESF/ml.

**DISCUSSION**

The differential expression of the individual \( \beta \)-globins responsible for hemoglobin switching in sheep and goats provides an opportunity to study the interrelationship between erythropoietin action, erythroid differentiation, and gene regulation. Our experience with sheep marrow illustrates the critical importance of providing conditions optimal for erythroid development in order to obtain expression of the genes for \( \beta^c \) globin. Previous studies had shown that, under conditions satisfactory for goat colony formation, sheep marrow produced poorly developed colonies of which 60% had only 8-12 cells even at a high ESF concentration (3). No \( \beta^c \) synthesis was observed. In improved medium (Table I), sheep marrow was found to generate large, well-differentiated colonies (Fig. 2) which synthesized up to 60% \( \beta^c \) globin between 96 and 120 h in vitro. At this time, it is not possible rationally to predict which components will optimally support growth of erythroid colonies from marrow of different species. Several investigators (14, 22) as well as ourselves (5) have found that albumin, although important for colony formation, must be carefully delonized to avoid a toxic effect on colony formation. But, in addition, a considerable effort must be invested in a process of trial and error in order to find components which optimize colony formation from marrow of each species. Obtaining satisfactory cell growth in vitro in order to study differential expression of individual globin genes, as illustrated by our experience with sheep marrow, is likely to be of importance for the in vitro analysis of fetal to adult switching in human marrow as well.

Several aspects of our data imply heterogeneity with respect to the potential for \( \beta^c \) synthesis within the population of cells capable of giving rise to erythroid colonies in vitro. Colonies whose cells contain abundant hemoglobin were present in cultures of sheep marrow 48-72 h after explantation.

| Time as suspension (h) | Erythropoietin conc. (U/ml) | \( \beta^c \) globin (%) |
|-----------------------|-----------------------------|------------------------|
| 0                     | 0.50                        | 2.0                    |
| 0                     | 0.50                        | 19.8                   |
| 0                     | 0.005                       | 3.8                    |
| 6                     | 0.005                       | 11.8                   |
| 12                    | 0.005                       | 15.0                   |
| 18                    | 0.005                       | 19.1                   |
| 18                    | 0.500                       | 35.0                   |

**TABLE II**

*Goat Marrow Cells Cultured In Vitro for a Total of 96 h, Labeled 72-96 h*

**Figure 7** Schematic presentation of the experimental protocol designed to analyze the initiation of hemoglobin C synthesis in vivo in sheep. Bone marrow samples were removed 0.5 h before, and 0, 9, 18, 24, and 138 h after, an injection of ESF. Samples were grown either in suspension cultures and labeled from 0 to 9 h to determine the type of globin produced by the differentiating cells, or in clot cultures labeled from 72 to 96 h to analyze globins synthesized by CFU-E. See Materials and Methods.
Figure 8 Radiochromatogram of globins labeled in cultures of differentiating cells and colony-forming cells obtained from a sheep before and after ESF stimulation in vivo. Colonies were generated at 1.0 U ESF/ml. In each chromatogram, the position of the globin peaks was confirmed by the protein absorbance profile and comigration of ¹⁴C-labeled globin carrier. Top panel indicates globins formed before ESF injection. Middle panel indicates globins formed in cells removed 9 h after in vivo ESF injection. Bottom panel indicates globins synthesized in cells removed 139 h after ESF injection.
but substantial $\beta^c$ synthesis was not observed until 96-120 h. We have shown that differentiating cells, e.g., those making adult hemoglobin, cannot be stimulated to produce $\beta^c$ globin (references 3 and 4, and Fig. 8), and therefore it is unlikely that individual colonies making adult hemoglobin can switch to the production of hemoglobin C. Rather, it seems likely that the precursors for colonies accumulating hemoglobin early in the culture period were committed to adult hemoglobin production at the time of explantation, while other, presumably uncommitted precursors gave rise to colonies which at 4-5 days make $\beta^c$ globin. Similar considerations apply to goat marrow, although colony formation and the onset of beta $^c$ synthesis in vitro are more nearly coincident (3), rendering the heterogeneity in colony-forming cells less apparent. However, fractionation of goat marrow by unit gravity sedimentation has revealed more $\beta^c$ synthesis in cultures containing colonies derived from slowly sedimenting cells than in cultures containing colonies derived from more rapidly sedi-
menting cells. Thus, the heterogeneity with respect to the potential for hemoglobin C production was also demonstrated within the colony-forming cells of goat marrow.

A possible explanation for this heterogeneity in the colony-forming cell population is provided by consideration of the erythropoietic process. Erythroid precursor cells, thought to be derived from a pluripotent hematopoietic stem cell, are amplified by cell division as a population of erythroid-committed cells before acquiring ESF sensitivity (19, 20). Subsequently, the precursor cells become recognizable erythroid, although they undergo another series of amplifying divisions before becoming fully hemoglobinized enucleated erythrocytes. Any precursor cell in this maturation pathway which has acquired sensitivity to the proliferative effect of ESF and which is still capable of three sequential cell divisions can give rise to an erythroid colony containing at least eight cells. Axelrad and co-workers (2) and others (9, 11) have operationally defined two classes of mouse erythroid colony-forming cells. Colony-forming unit-erythroid (CFU-E) cells are larger, more rapidly sedimenting cells which give rise to 8–64 cell colonies at low ESF concentrations after 48 h in vitro. Burst-forming unit-erythroid (BFU-E) cells are smaller, less rapidly sedimenting cells which give rise to macroscopic colonies after 7–9 days but only at a high ESF concentration. Thus, from our experiments, we might infer that the colony-forming cells responsible for the dramatic increase in \( \beta^C \) synthesis between 4 and 5 days in sheep cultures may be an earlier precursor (BFU-E-like) than the colony-forming cells (CFU-E), which give rise to colonies that make only adult hemoglobin earlier in the culture period. These notions are also compatible with the observation that more \( \beta^C \) synthesis occurs in cultures derived from goat cells of intermediate sedimentation velocity than from more rapidly sedimenting cells.

Both hemoglobin A and C have been demonstrated to occur in the same red cell (8, 18). This suggests that ESF does not initiate hemoglobin switching by causing the selective proliferation of precursor cells already committed to hemoglobin C synthesis. The mode of action of erythropoietin and its role in hemoglobin switching are discussed in detail in reference 17. Erythropoietin induction of hemoglobin switching by an action on an immature precursor cell before the onset of hemoglobin synthesis seems in many ways analogous to its action during erythropoiesis in general. Thus, the hemoglobin switching model may be useful in defining the molecular mechanism of erythropoietin action.

Brief exposure of sheep (in vivo) and goat (in vitro) bone marrow to high ESF concentration results in generation of colonies in which hemoglobin C is synthesized, even when these colonies subsequently develop at low ESF concentration (Table II, Fig. 9). These results provide further evidence that the modulating effect of ESF with respect to hemoglobin phenotype is irreversible and occurs early in erythropoiesis (3). Our observations may provide the framework with which to design experiments that will more precisely define the cellular and molecular mechanism(s) of hemoglobin switching and erythropoietin action.

We wish to thank Dr. W. French Anderson for encouragement and inspired discussions of the "switch" mechanism. Our appreciation is extended to Mr. Bernard Keufner who performed all the carboxymethylcellulose column chromatography; to Dr. Thomas Musliner who performed the G-100 chromatography of ESF; to Mr. Mac McCaskill, and Mr. James Hoes who assisted in animal surgery; and to Mr. Leonard Stuart and his associates at Poolesville Animal Center and Mr. Max S. Foltz, Mr. Jesse N. Judy and Mrs. Donna Mathews at the Max Foltz Farms who cared for the animals.

Received for publication 7 May 1976, and in revised form 26 July 1976.

REFERENCES

1. Adamson, J. W., and G. Stamatoyanopoulos. 1973. Activation of hemoglobin C synthesis in sheep culture. Science (Wash. D. C.). 180:310-312.

2. Axelrad, A. A., D. L. McLeod, M. M. Shreeve, and D. A. Heath. 1974. Properties of cells that produce erythrocytic colonies in vitro. In Hemopoiesis in Culture. W. A. Robinson, editor. U. S. Government Printing Office, Washington, D. C. 226-234.

3. Barker, J. E., W. F. Anderson, and A. W. Niemhuiss. 1975. Hemoglobin switching in sheep and goats. V. Effect of erythropoietin concentration on in vitro erythroid colony growth and globin synthesis. J. Cell Biol. 64:515-527.

4. Barker, J. E., J. A. Last, S. L. Adams, A. W. Niemhuiss, and W. F. Anderson. 1973. Hemoglobin switching in sheep and goats. II. Erythropoietin-dependent synthesis of hemoglobin C in goat bone marrow cultures. Proc. Natl. Acad. Sci. U. S. A. 70:1739-1743.

5. Barker, J. E., and A. W. Niemhuiss. 1976. A method for the deionization of bovine serum album-
min. In Tissue Culture Association Manual. V. J. Evans, editor. Tissue Culture Assoc. Rockville, Md. 211-212.

6. DENTON, M. J., and H. R. V. AENSTEN. 1973. Characterization of developing adult mammalian erythroid cells separated by unit gravity sedimentation. Br. J. Haematol. 24:7-17.

7. GARBUZZA, T. G., M. A. SCHUMANN, R. K. SILVER, and H. B. LEWIS. 1968. Erythropoietic kinetics in sheep studied by means of induced changes in hemoglobin phenotype. J. Clin. Invest. 47:1895-1904.

8. GARRICK, M. D., M. REICHLIN, M. MATRIOLE, and R. MANNING. 1973. The anemia-induced reversible switch from hemoglobin A to hemoglobin C in caprine ruminants: immunohemochromics evidence that both hemoglobins are found in the same cell. Dev. Biol. 30:1-12.

9. GREGORY, C. J., E. A. McCULLOCH, and J. E. TILL. 1973. Erythropoietic progenitors capable of colony formation in culture: state of differentiation. J. Cell. Physiol. 81:411-420.

10. HUISMAN, T. H. J., J. P. LEWIS, M. H. BLUNT, H. R. ADAMS, A. MILLER, A. M. DOZY, and E. M. BOYD. 1969. Hemoglobin C in newborn sheep and goats: a possible explanation for its function and biosynthesis. Pediat. Res. 3:189-193.

11. ISCove, N. N., and F. SIEBER. 1975. Erythroid progenitors in mouse bone marrow detected by macroscopic colony formation in culture. Exp. Hematol. 3:32-43.

12. ISCove, N. N., F. SIEBER, and K. H. WINTERHALTER. 1974. Erythroid colony formation in cultures of mouse and human bone marrow: analysis of the requirement for erythropoietin by gel filtration and affinity chromatography on agarose-concanavalin A. J. Cell. Physiol. 83:309-320.

13. McKOO, D., R. J. MILLER, P. H. PAINTER, and W. R. BRUCE. 1970. Erythropoietin sensitivity of rat bone marrow cells separated by velocity sedimentation. Cell Tissue Kinet. 3:55-65.

14. McLEOD, D. L., M. M. SCHREEVE, and A. A. AXEL- RAD. 1974. Improved plasma clot culture system for production of erythrocytic colonies in vitro. Quantitative Assay for CFU-E. Blood. 44:517-534.

15. MILLER, R. G., and R. A. PHILLIPS. 1969. Separation of cells by velocity sedimentation. J. Cell. Physiol. 73:191-206.

16. NIENHUIS, A. W., and W. F. ANDERSON. 1972. Hemoglobin switching in sheep and goats. I. Change in functional globin messenger RNA in reticulocytes and bone marrow cells. Proc. Natl. Acad. Sci. U. S. A. 69:2184-2188.

17. NIENHUIS, A. W., J. E. BARKER, and W. F. AN- DERSON. 1976. The effects of erythropoietin on hemoglobin synthesis. In Kidney Hormones. Vol. II. J. W. Fisher, editor. In press.

18. NIENHUIS, A. W., and H. F. BUNN. 1974. Hemo- globin switching in sheep and goats. IV. Occurrence of hemoglobins A and C in the same red cell. Science (Wash. D.C.). 185:946-948.

19. SCHOFIELD, R., and L. G. LATHA. 1976. Cellular kinetics of erythropoiesis. In Congenital Disorders of Erythropoiesis. D. J. Weatherall, editor. CIBA Found. Symp. 37:3-15.

20. STEPHENSON, J. R., and A. A. AXELRAD. 1971. Separation of erythropoietin-sensitive cells from hemopoietic spleen colony-forming stem cells of mouse fetal liver by unit gravity sedimentation. Blood 37:417-427.

21. STEPHENSON, J. R., A. A. AXELRAD, D. L. Mc- LEOD, and M. M. SCHREEVE. 1971. Induction of colonies of hemoglobin synthesizing cells by erythropoietin in vitro. Proc. Natl. Acad. Sci. U. S. A. 68:1542-1546.

22. TEPPERMAN, A. D., J. E. CURTIS, and E. A. MCCULLOCH. 1974. Erythropoietic colonies in cultures of human marrow. Blood. 44:659-669.

23. THURMON, T. F., S. H. BOYER, E. F. CROSBY, M. K. SHEPHARD, A. N. NOYES, and F. STOHLMANN. 1970. Hemoglobin switching in non-anemic sheep. III. Evidence for presumptive identity between the A-C factor and erythropoietin. Blood. 36:593-606.