

BIOCHEMICAL CHARACTERIZATION OF RNA AND PROTEIN SYNTHESIS IN ERYTHROCYTE DEVELOPMENT

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ABSTRACT

Newts (*Triturus cristatus*) made anemic with acetylphenylhydrazine (APH) fail to regenerate erythrocytes (RBC's) immediately and exhibit a latent period of 1.5–2 wk during which animals lack RBC's and are aplastic. With the establishment of erythroid regeneration at 10–14 days, relatively homogeneous populations of successive erythropoietic stages occur in the blood. This feature makes possible biochemical analyses of events in early, intermediate, and late developmental stages, respectively, each of which can be obtained in vivo with minimal contamination by other stages. Previous studies have described a primitive cell population referred to as "erythroid precursor cells" (EPC's) which precedes the appearance of definitive erythroid elements. The present studies show that EPC's and early erythroid cells are engaged mainly in ribosomal production, including synthesis of rRNA and ribosomal proteins. Moreover, EPC's and early erythroid cells also synthesize tRNA and a presumed Hb-mRNA which has been identified by its sedimentation rate at 9–12 s and its content of polyadenylic acid. In intermediate stages, there occurs a fourfold decrease in the level of RNA synthesis and, while rRNA continues to be formed, there is a disproportionate accumulation of the two major cytoplasmic rRNA species in favor of the large ribosomal subunit RNA. In late developmental stages, the level of RNA synthesis is markedly diminished with little or no evidence of formation of defined RNA classes. Correlated radioautographic and biochemical studies with radioactive δ -aminolevulinic acid and leucine indicate that EPC's and other early erythroid elements synthesize not only hemoglobin but also ferritin and ribosomal proteins. It is concluded that: (a) erythroid RNA synthesis is most pronounced in the early developmental stages, being manifested predominantly by rRNA production but including tRNA and Hb-mRNA; (b) intermediate developmental stages show both "ribosomal wastage" and decreased growth rate, marking a pivotal point between the transcriptional activities of early stages and translational activities of late stages; (c) EPC's represent a cell population already committed to RBC formation and are excluded from a role as the pluripotential stem cell.

The process of erythrocyte (RBC) development occurs as a series of successive stages which are derived from an undifferentiated hemopoietic stem cell. Although functional and cytogenetic

studies have indicated the pluripotential differentiation capacity of the hemopoietic stem cell, the identity of this cell is uncertain. Presumptive pluripotential stem cells in mammals have been reported to exhibit nuclei with diffuse chromatin, prominent nucleoli, and variable ribosomal content, and, as a rule, to lack any structural specializations diagnostic of a specific blood cell line (12, 13, 42, 48). In newts (*Triturus* spp.), a cell type with similar features has been identified as an "erythroid precursor cell" (EPC) because of its role in subsequent RBC development (18). However, because of the limitations of morphological and cytochemical data, its relationship to the pluripotential stem cell could not be resolved.

Since the identity of the hemopoietic stem cell is uncertain, little is known of its metabolic activity before its commitment, or differentiation, into a specific blood cell line. It has been reported that cell renewal and proliferation are minimal in pluripotential stem cells and that these cells constitute a cell compartment existing in a prolonged G₁ or G₀ (28, 29). In contrast, the early erythroid cells, which are derived directly, or through an intermediate unipotential "erythropoietin-responsive cell" (28, 37) from pluripotential stem cells, are not only highly proliferative (22, 23, 39, 49), but also exhibit extensive RNA synthesis (19, 21, 23). Only with the completion of RNA synthesis is the bulk of hemoglobin accumulated, its production being dependent upon the prior establishment of stable RNA components in the early erythroid stages. Thus, differentiation of the pluripotential stem cell into the red cell lineage induces the synthesis of various RNA species whose production is restricted to the early erythroid stages before the major period of hemoglobin synthesis and accumulation (19, 21–23). With the use of a unique erythropoietic system in the newt (*T. cristatus*) in which relatively homogeneous erythroid stages can be obtained (20), the present studies were performed as an extension of earlier morphological and cytochemical studies. Their purpose was: (a) to analyze the kinds of RNA synthesized in the different stages of RBC development, and (b) to analyze the metabolic behavior of EPC's in an effort to clarify their relationship to RBC development and to the pluripotential hemopoietic stem cell.

MATERIALS AND METHODS

Nomenclature

The terminology and sequence of erythropoietic

stages used in these studies are: EPC → basophilic erythroblast (BE) → early polychromatophilic erythroblast (EPE) → midpolychromatophilic erythroblast (MPE) → late polychromatophilic erythroblast (LPE) → reticulocyte (RETIC) → RBC. On the basis of our studies, we have subdivided the system into early (EPC, BE, and EPE), intermediate (MPE), and late (LPE, RETIC, and RBC) developmental stages. The morphological properties of these cell types have been described elsewhere (18, 19).

Preparation and Maintenance of Animals

Newts (*T. cristatus*) were collected near Naples, Italy¹ and were splenectomized to restrict erythropoiesis to the peripheral blood. Anemia was induced by injection of 0.5–1 mg of acetylphenylhydrazine (APH) and treated animals were kept in spring water at 20°–23°C. Higher temperatures result in increased mortality while low temperatures (12°–16°C) suppress phagocytic clearance of RBC debris by macrophages, and both delay and alter the subsequent erythropoietic response.

Radioactive Labeling of Erythroid Cells and Initial Preparation

Anemic animals exhibiting the desired erythropoietic stage, or stages, were selected by microscope examination of blood smears at 9–21 days after APH injection. RNA was labeled by injection of 100–500 μCi of [³H]uridine (New England Nuclear, Boston, Mass.) (sp act = 27 Ci/mmol) or 75–200 μCi of [³H]adenosine (New England Nuclear) (sp act = 33 Ci/mmol) into the peritoneal cavity. After 1–26 h, blood cells were collected in ice-cold heparinized (0.1–1.0%) amphibian saline (1), washed 2–3 times with saline, and an aliquot was removed for determination of total cell number.

To label hemoglobin, animals were given 100–250 μCi of [³H]δ-aminolevulinic acid (New England Nuclear) ([³H]ALA; sp act = 26.4 mCi/mmol) or [³H]leucine (New England Nuclear) ([³H]Leu; sp act = 55 Ci/mmol). After 3.5–24 h, cells were collected in heparinized saline and pooled with nonradioactive mature erythrocytes from 1 to 6 nonanemic newts in order to introduce newt native hemoglobin as an internal marker. The resulting cell suspension was washed three times in ice-cold heparinized saline.

Extraction of RNA

WHOLE CELL EXTRACTION: Washed cells were suspended in 2 ml of 0.5% sodium dodecyl sulfate; 0.5 M urea; 0.01 M sodium phosphate, pH 8.5 (SUP) (50) and mixed for 10 min at room temperature with an equal volume of phenol mixture (redistilled phenol:chloro-

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form:isoamyl alcohol, 50:48:2). After centrifugation at 10,000 rpm for 10 min at 4°C, the aqueous phase was removed and the phenol phase plus interphase was extracted with 1 vol of SUP. The aqueous phases were pooled and re-extracted 2–3 times with phenol mixture. The aqueous phase was washed with chloroform:isoamyl alcohol (24:1), made 0.3 M with respect to NaCl concentration, and RNA was precipitated overnight at –20°C by the addition of 2–3 vol of 95% ethanol.

EXTRACTION OF CYTOPLASMIC RNA: Washed cells were resuspended in 1–2 ml of ice-cold 0.01 M Tris HCl, pH 7.6; 0.05 M NaCl; 0.0015 M magnesium acetate (RSB-2) and quickly lysed by the addition of 10% Nonidet P40 (NP 40, Particle Data Laboratories, Ltd., Elmhurst, Ill.) to a final concentration of 0.2–1%. After lysis, the suspension was centrifuged at 1,500 g for 5 min at 3°C to pellet nuclei and cell debris. The supernate was removed and kept on ice while the nuclear pellet was washed once with 0.5 ml of RSB-2. Both supernates were combined and SDS was added to a final concentration of 1%. Total cytoplasmic RNA was extracted at room temperature by adding an equal volume of phenol mixture and mixing vigorously for 10 min. After centrifugation at 10,000 rpm (10 min, 4°C), the phenol phase was removed and the aqueous phase and interphase re-extracted 1–2 times further with phenol mixture. After removal of traces of phenol by 2–3 washes of the aqueous phase with chloroform:isoamyl alcohol, the NaCl concentration of the aqueous phase was adjusted to a final concentration of 0.3 M and RNA was precipitated overnight at –20°C with 95% ethanol.

Satisfactory recovery of undegraded RNA from nuclear pellets was difficult to obtain despite the use of various methods that have been used with apparent success in other cell systems. Where description of total cellular RNA was desired, whole cell extraction was used to minimize nuclear RNA degradation.

RNA Analysis

SUCROSE DENSITY GRADIENTS: Precipitated RNA was collected from ethanol by centrifugation at 10,000 g for 30 min at 0°C. The RNA pellet was dried and resuspended in NETS (51) or NET buffer. 0.2–0.25 ml of the suspension was layered onto 12.4 ml of a 15–30% (wt/wt) sucrose gradient prepared in resuspension buffer and centrifuged in the Beckman SW41 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Running conditions are described in the figure legends. The gradients were fractionated using a Gilford flow cell (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) and spectrophotometer to monitor absorbance at 260 nm (A_{260}). Radioactivity in each fraction was determined by solution of an aliquot in an aqueous scintillation cocktail or by collection of TCA precipitates on Millipore filters (Millipore Corp., Bedford, Mass.).

Extraction and Identification of

Polyadenylated RNA

Polyadenylated (PolyA) RNA was isolated from poly-

some by deproteinization with Proteinase K (EM Laboratories, Inc., Elmsford, N. Y.) (34, 52), sodium dodecyl sulfate (SDS) (27), or phenol-chloroform. Polysomes were prepared from EPC's and BE's labeled with 75–200 μ Ci of [3 H]adenosine for 4–21 h. Cells were lysed in a buffer containing 0.05 M Tris-HCl (pH, 7.5), 0.04 M KCl, 0.002 M MgCl₂, dextran sulfate (50 μ g/ml), 0.2% NP-40, and rat liver postmicrosomal supernate (4). The proportion of buffer to liver supernate was 3:2. In the absence of liver supernate, polysomes were invariably degraded and yielded minimal amounts of polyA-RNA. After clarification by low-speed centrifugation, the lysate (2.0–2.5 ml) was layered onto 36 ml of a 10–40% (wt/wt) sucrose gradient prepared in lysis buffer without detergent or rat liver supernate. Gradients were centrifuged at 25,000 rpm for 2.5 h at 2°C, using the SW27 rotor. 1-ml fractions were collected, monitored for absorbance at 260 nm, and the acid-precipitable radioactivity in 0.02-ml aliquots of each fraction was determined. All steps were performed at 1°–3°C.

The pooled polysomal fractions were rendered 0.01 M in EDTA and 1% in SDS, and RNA was extracted at room temperature with phenol mixture. The aqueous phase was removed and the phenol phase plus interphase extracted once with pH 9 buffer (2). Both aqueous phases were combined and extracted 1–2 times with phenol mixture before ethanol precipitation. Alternatively, pooled polysomes were dissociated by Proteinase K (200–500 μ g/ml) in the presence of 1.5% SDS (34, 52) or by 0.5% SDS–0.5 M NaCl (27).

Polysomal RNA was dissolved in Buffer I (0.01 M Tris, pH 7.5; 0.4 M NaCl; 0.1% Sarkosyl [Geigy Chemical Corp., Ardsley, N. Y.]) and layered on a 0.2–0.5-g column of oligodeoxythymidylate-cellulose, oligo(dT)-cellulose, (Collaborative Research, Inc., Waltham, Mass.) which was equilibrated with the same buffer. The column was washed with 20–25 ml of Buffer I to elute the nonbinding RNA (Peak I), and 1-ml fractions were collected. Bound RNA (Peak II) was eluted with Buffer II (0.01 M Tris, pH 7.5; 0.1% Sarkosyl) and the relative proportion of polyA-RNA per total RNA was determined on the basis of relative radioactivity. Fractionated RNA was precipitated with ethanol before further analysis on sucrose gradients or acrylamide gels.

Hemoglobin Analysis

Washed cells, including carrier erythrocytes, were resuspended in 1 ml of 0.05 M Tris-HCl (pH 8.0) and lysed either by the addition of NP40 to 1% or by vigorous mixing on a Vortex mixer. Nuclei and cell debris were sedimented by centrifugation at 27,000 g for 15–20 min, and the supernate was collected. All steps were carried out at 1°–3°C.

Clarified lysates were converted to the cyanmet form by the addition of 0.1 vol of 5% aqueous potassium ferricyanide followed 5 min later by the addition of an equal volume of 0.05 M Tris (pH 8.0) containing 0.01% potassium cyanide (41). Following dialysis for 24 h against two changes of 2 liters of Tris-KCN buffer, the

lysate was layered onto either a Sephadex G100 column (1.6 × 90 cm) or a DEAE-Sephadex A50 column (1.6 × 30 cm) which was equilibrated exhaustively with the same buffer. The G100 column was washed with buffer at a flow rate of 16 ml/h and 3-ml fractions were collected. Hemoglobin was eluted from DEAE-Sephadex with 600 ml of a linear continuous salt gradient (0.0–0.4 M NaCl) at a flow rate of 22.8 ml/h and collection of 5.6 ml fractions. Chromatography was performed at 4°C. Fractions collected from the columns were monitored at 280 and 415 nm, and radioactivity was measured by counting an aliquot in aqueous scintillation cocktail.

Polyacrylamide Gel Electrophoresis

Protein samples were suspended in Tris-glycine buffer (pH 8.6) and run into a 2% stacking gel at 2 mA per tube for 30 min. Samples were electrophoresed on 7.5% separating gels at 4 mA per tube for 45–60 min. For analysis, duplicate gels were usually run, with one gel stained with Coomassie Brilliant Blue and the other sliced into 1-mm slices for determination of radioactivity. This procedure was necessary since the acetic acid used in the destaining procedure elutes iron from the gel.

RESULTS

Quantitative and Qualitative Aspects of RNA Synthesis

In erythropoietic stages exposed to radioactive nucleosides *in situ* (Table I) or *in vitro*, the highest amount of incorporation was observed in EPC's and BE's. Each of these early erythroid elements has been found to be extensively engaged in the synthesis of multiple RNA species (Figs. 1 and 2). In cells labeled *in situ* for 5 h, radioactivity was located mainly in ribosomal (26S, 17S) and transfer (4S) RNA (Fig. 1). Both the rRNA and tRNA components exhibited a relatively high specific activity (total radioactivity per unit of absorbance at 260 nm), indicating that most of the RNA within these fractions was recently synthesized. It

should be noted that the two major cytoplasmic rRNA species in newt erythroid cells were slightly lighter than mammalian 28S and 18S rRNAs (Fig. 6c) and are designated in this report as 26S and 17S, respectively.

By 5 h, the radioactivity in the heavier RNA fractions was heterodisperse, with a small peak in the 30S–32S region (Fig. 1). After a 1-h label, however, there was relatively little labeling of the cytoplasmic rRNA fractions (Fig. 6a and b) and most of the radioactivity was located in nuclear RNA, occurring as peaks at 60S, 40S, and 30S–34S (Fig. 2). Since the 40S and 30S–34S peaks exhibited sedimentation rates similar to rRNA precursors in other amphibian cells (16, 30, 33, 44) and were appreciably labeled in cells exposed to radioactive methyl methionine (data not shown), they were identified as rRNA precursors. Analysis of the 60S peak under denaturing conditions indicated that this fraction is probably derived from rRNA aggregation (data not shown).

With maturation of the early erythroid elements, incorporation activity began to decline (Exp. 1, Table I, BE/EPE), and, in the interval marking the transition between early and intermediate stages (Table I, Exp. 2, EPE/MPE), was decreased fourfold. In intermediate stages, radioactive labeling of the 26S rRNA species and of tRNA was observed, but labeling of 17S rRNA was inconspicuous (Fig. 3). Relative to early erythroid cells, there was a two- to fourfold decrease in the specific activities (total cpm/ A_{260} unit) of rRNA and tRNA (Fig. 3), the decrease being most pronounced in the 17S rRNA fraction. Although the total absorbance (A_{260}) in each of the two rRNA components yielded a 26S:17S ratio of 2.01 (Fig. 3), the radioactivity in the 26S rRNA fraction exceeded that in the 17S fraction by more than six times. Because the asymmetrical

TABLE I
Incorporation of [³H]Uridine into RNA of Newt Erythroid Cells

Exp	Erythroid stage	Ratio	Total cell no.	Erythroid cells	Total cpm	cpm per Erythroid cell
				% of total		
1*	EPC/BE	4:1	2.5×10^5	18	4.9×10^5	11.0
	BE/EPE	3:1	9.1×10^5	82	6.1×10^6	8.13
	MPE/LPE	1:3	23.0×10^6	84	21.0×10^6	1.09
2‡	EPC/BE	1:1	6.35×10^6	32	1.7×10^6	0.84
	EPE/MPE	2:1	6.2×10^6	80	1.1×10^6	0.22
	MPE/LPE	1:3	8.7×10^7	89	4.8×10^6	0.06

* Determined from pooled blood samples 6 h after injection of 250 μ Ci of [³H]uridine.

‡ Measured in blood sample 5 h after injection of 100 μ Ci of [³H]uridine.

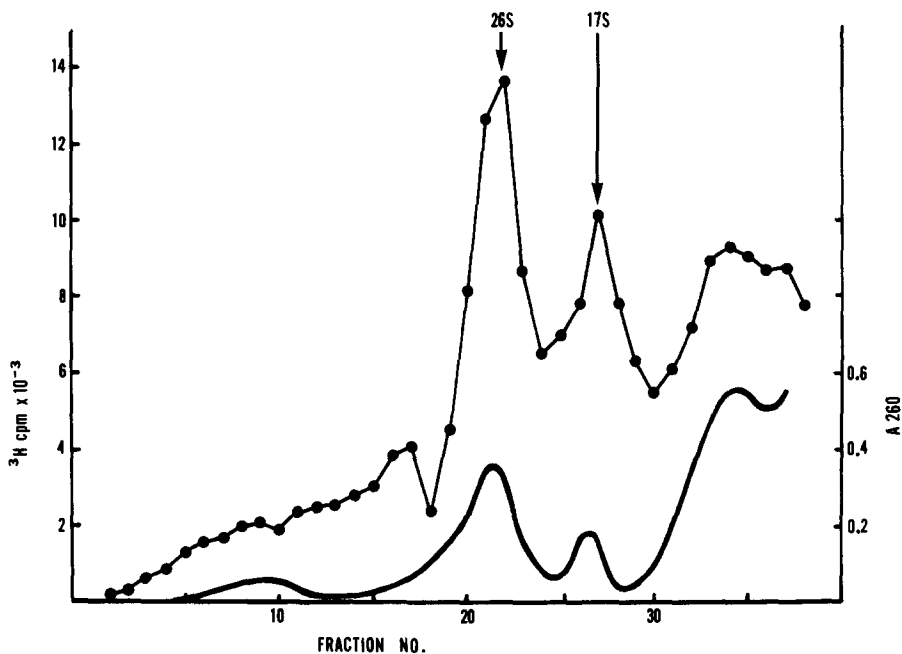


FIGURE 1 Sedimentation profile of total RNA extracted from early erythroid cells (EPC:BE = 1:1) labeled *in situ* with 100 μ Ci of [³H]uridine for 5 h. RNA was analyzed on 15-30% sucrose gradients in NETS buffer run at 18,500 rpm for 22 h at 23°C. Radioactivity (●) was determined by TCA precipitation of total sample onto Millipore filters and counting in a toluene-base scintillation cocktail. Solid line represents absorbance (A_{260}) of endogenous newt RNA.

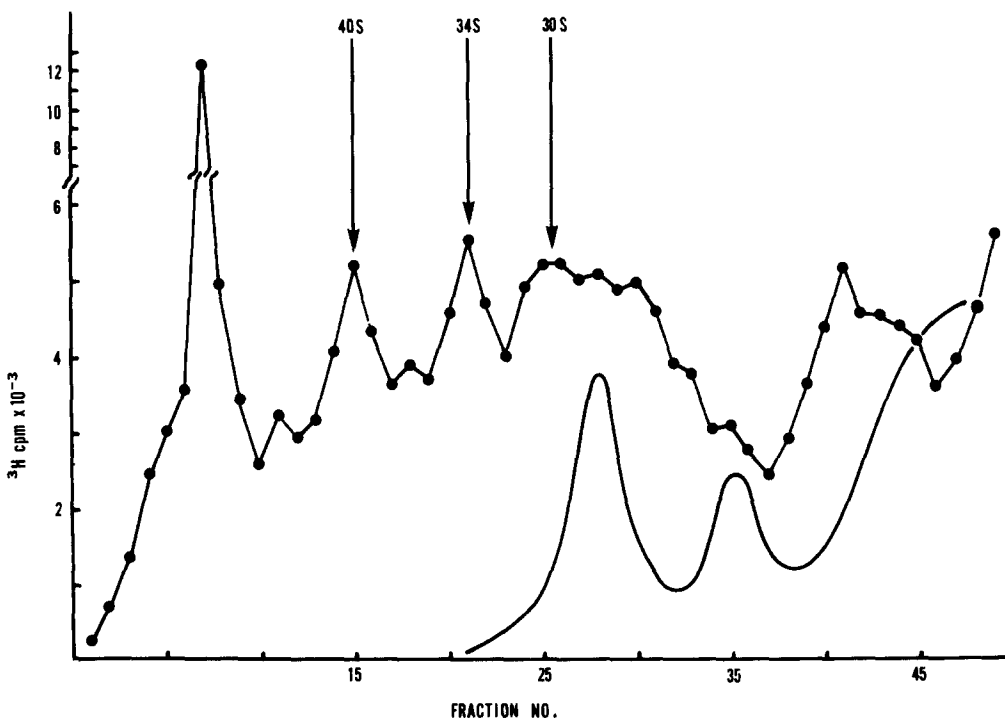


FIGURE 2 Sedimentation profile of nuclear RNA extracted from early erythroid cells labeled *in situ* with 100 μ Ci of [³H]uridine for 1 h. Running conditions identical to those in Fig. 3. Solid line represents newt 26S and 17S rRNA added as marker. ³H-radioactivity, (●).

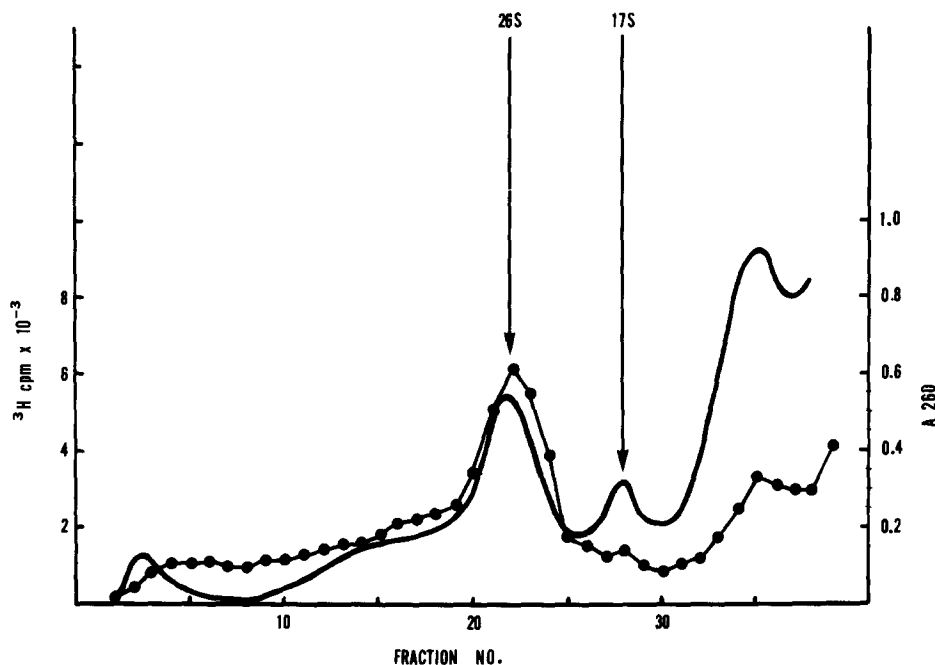


FIGURE 3 Sedimentation profile of total RNA extracted from intermediate erythroid cells (EPE:MPE = 2:1) labeled and analyzed as in Fig. 1. A_{260} , (—); ^3H -radioactivity, (●).

intracellular distribution of the 26S and 17S RNAs could affect these data, both ratios were measured in cytoplasmic fractions of intermediate cells exposed to labeled uridine or methyl methionine. While the 26S:17S A_{260} ratio was 1.8 (Fig. 4), there was five times more radioactivity in 26S RNA than in 17S RNA (Fig. 4). Thus, intermediate erythroid cells reveal a disproportionate accumulation of newly synthesized 26S rRNA relative to 17S rRNA, a phenomenon similar to "ribosomal wastage" described in inactive lymphocytes (9-11), contact-inhibited fibroblasts (14), and cultured cells exposed to inhibitors of protein synthesis (15, 53). The discrepancy observed between the labeling and A_{260} ratios of the two respective rRNA species and the decline in specific activity of rRNA can be correlated with a decreased level of rRNA synthesis in these cells and the presence in the cytoplasm of a relatively large, preexisting ribosomal population formed in and retained from the early erythroid stages.

In the late erythroid stages (Table I, MPE/LPE), relatively little radioactivity reflective of RNA synthesis was obtained, the level of incorporation being decreased 10- to 14-fold with respect to the early erythroid stages. Subsequent sedimentation analysis showed no evidence of radioactivity in any defined RNA species, except for a minor

accumulation of label in the 4S region (Fig. 5).

Similar results were obtained from corresponding stages labeled *in vitro* (data not shown). Under both conditions of labeling, RNA synthesis occurs predominantly in EPC's and BE's but is reduced or absent in intermediate and late erythroid stages, respectively.

LABELING KINETICS OF CYTOPLASMIC RNA IN EARLY ERYTHROID CELLS: Cytoplasmic RNA from EPC's or BE's labeled *in situ* for 1-2 h with uridine sedimented mainly in the 4S-5S region of sucrose density gradients (Fig. 6a and b). Although broad, ill-defined peaks were visible in the 26S and 17S regions, rRNA clearly represented a minor component of labeled cytoplasmic RNA after 1-2 h. By 5 h, the majority of newly synthesized cytoplasmic RNA was located in the 26S and 17S rRNA fractions, the labeling ratio between these fractions measuring 1.70-1.86 (Fig. 6c). In addition to rRNA and tRNA, a shoulder of radioactivity was located in the 9S-12S region (Fig. 6c). With longer labeling times (Fig. 6d), total radioactivity in all cytoplasmic RNA classes was increased but the sedimentation profile remained essentially similar to that obtained after 5 h. These results show that most of the newly synthesized RNA accumulating within the cytoplasm of EPC's and BE's is ribosomal.

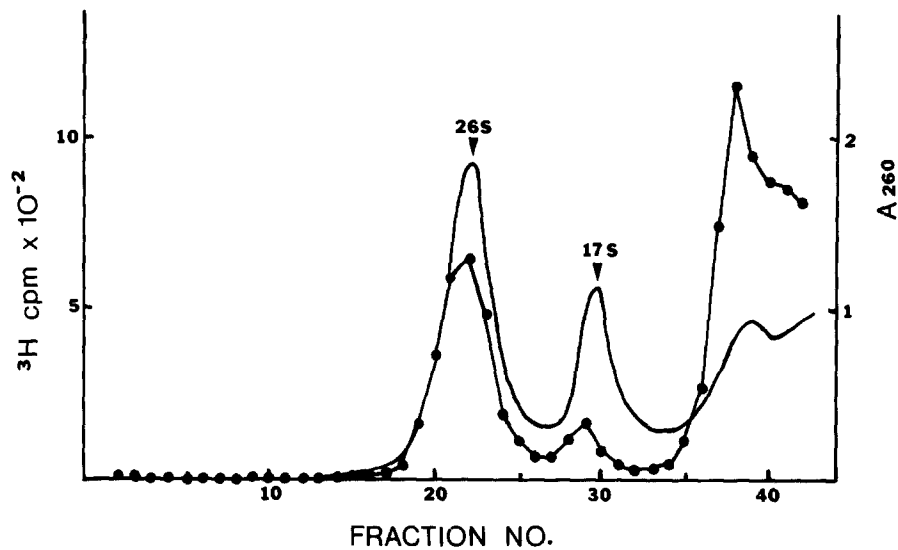


FIGURE 4 Sedimentation profile of cytoplasmic RNA from intermediate erythroid cells labeled *in situ* with 200 μ Ci of [3 H](methyl)-methionine for 5 h. A_{260} , (—); 3 H-radioactivity, (●).

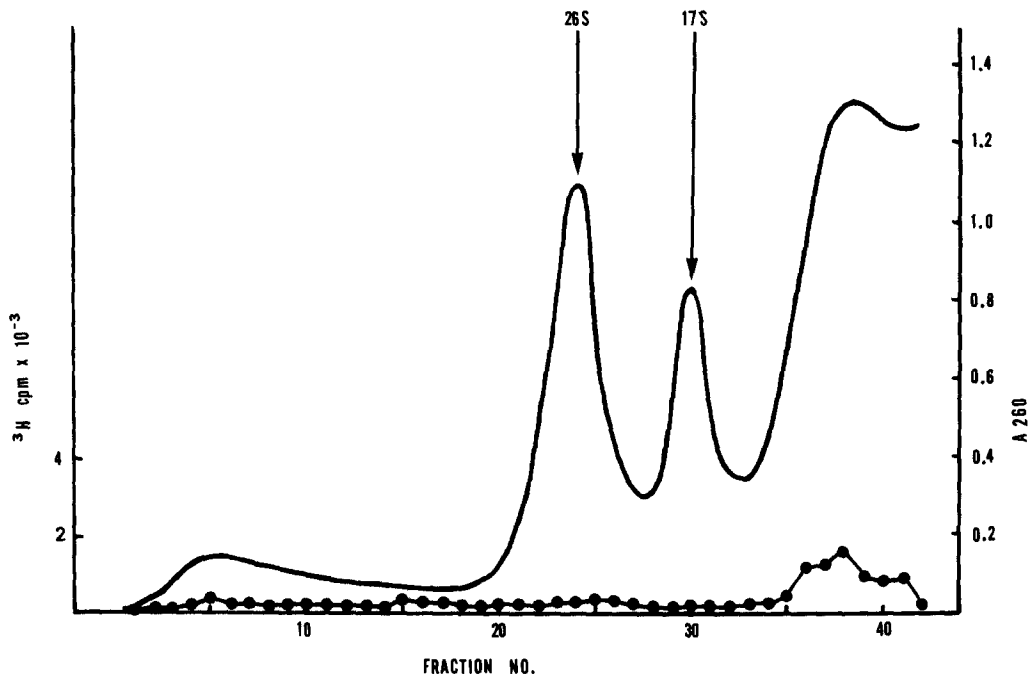


FIGURE 5 Sedimentation profile of total RNA obtained from late erythroid cells (MPE:LPE = 1:3) labeled *in situ* with [3 H]uridine. Conditions identical to those of Figs. 1 and 3. The solid line indicates absorbance (A_{260}) of endogenous new RNA. 3 H-radioactivity (●) was determined by TCA precipitation of total sample on Millipore filters and counting in a toluene-base scintillation cocktail.

POLY A-RNA IN EARLY ERYTHROID CELLS:
In EPC's and BE's labeled with radioactive adenosine, 1-5% of the total radioactivity in polyosomes represented polyA-RNA as determined by

oligo(dT)-cellulose chromatography. Upon subsequent sedimentation analysis, a major part of the polyadenylated RNA fraction sedimented as a broad peak in the 9S-12S region (Fig. 7). After

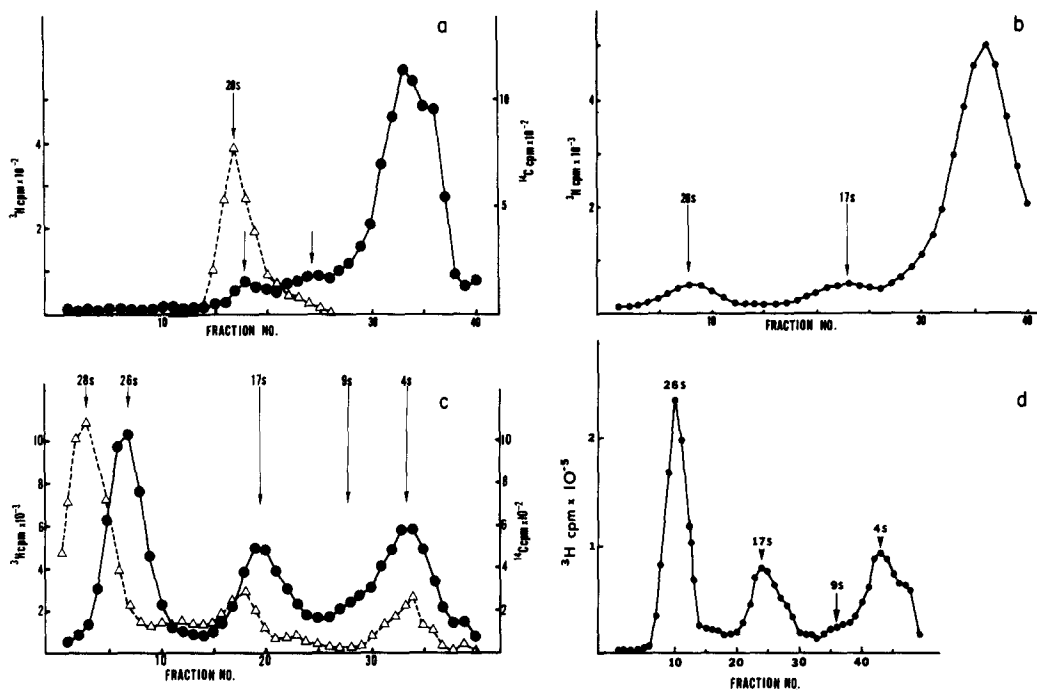


FIGURE 6 Sedimentation profile of cytoplasmic RNA from early erythroid cells labeled *in situ* with [^3H]uridine for (a) 1 h, (b) 2.5 h, (c) 5.5 h, and (d) 25.5 h. The gradient in (a) was run at 20,000 rpm for 18 h at 28°C. Remaining gradients (b, c, and d) were run at 25,000 rpm for 19 h at 28°C. ^{14}C -labeled HeLa cell cytoplasmic RNA (Δ) added as internal marker in (a) and (c). ^3H -radioactivity, (\bullet).

digestion with pancreatic and T1 ribonucleases, 14–28% of the radioactivity in the 9S–12S fraction remained acid-precipitable and, therefore, resistant to enzymic hydrolysis.

In most experiments, the sedimentation pattern of the polyA-containing RNA fraction showed several peaks. After deproteinization of polyosomes by Proteinase K (34, 52), by SDS (27), or by phenol-chloroform extraction, prominent peaks were obtained consistently at 9S–12S and 25S–26S, with more variable accumulations of radioactivity over the 15S–21S region (Fig. 8). In rebinding assays with oligo(dT)-cellulose or poly U-glass fiber filters, the polyadenylated 25S–26S and 15S–21S fractions showed approx. 500 times more binding than 26S and 17S rRNA's, respectively, ruling out the origin of these two polyA-containing species from nonspecific binding of rRNA to oligo(dT)-cellulose.

In rabbit reticulocytes, a complex consisting of Hb-mRNA (9S) associated with 28S and 18S rRNA's has been reported as a physiological phenomenon through which translation of the mRNA is facilitated and enhanced (26). On the other hand, polyA-RNA has been reported to form

nonphysiological aggregates with rRNA (3), especially upon phenol extraction (34). Analysis of polyA-RNA from newt erythroid cells with dimethyl sulfoxide (DMSO) and formamide denaturation has indicated that the 25S–26S and 15S–21S RNA fractions do not contain rRNA. While the sedimentation profiles of the 9S–12S and 15S–21S fractions were essentially coincident after denaturation, the 25S–26S RNA was dissociated into several peaks, the major peak sedimenting at 28S–29S in DMSO and formamide gradients. Significantly, the sedimentation patterns of the 26S rRNA and the 25S–26S polyA-containing RNA were different upon denaturation and analysis on denaturing gradients (unpublished results). A more detailed report on the characteristics of polyA-RNA in newt erythroid cells will be published elsewhere.

Admittedly, in the absence of demonstrated stimulation of globin synthesis by newt 9S–12S polyA-RNA in a heterologous system, the identity of this fraction as newt Hb-mRNA is unconfirmed. Nevertheless, the presence of a 9S–12S polyA-containing RNA, with a sedimentation rate similar to that for Hb-mRNA in other systems (7,

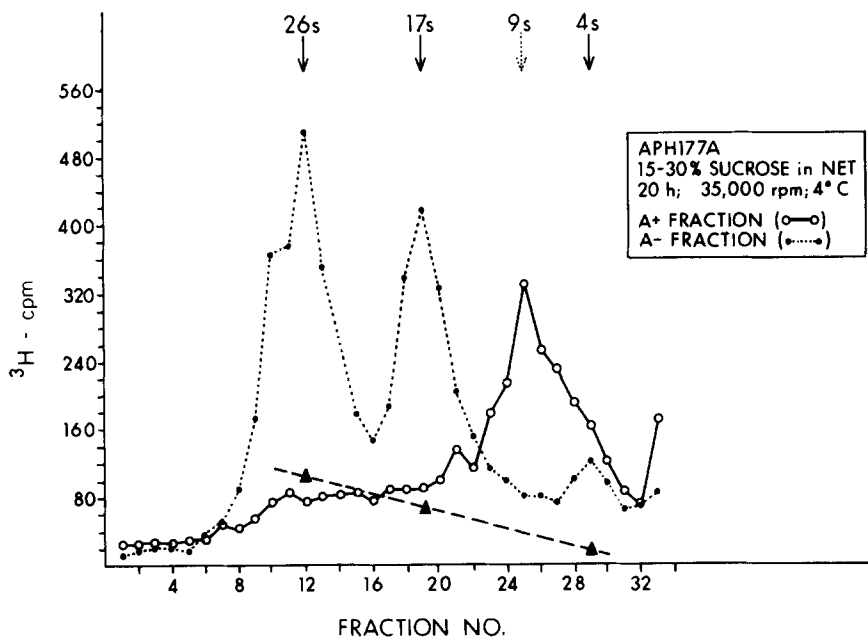


FIGURE 7 Sedimentation profile of polyadenylated (○) and nonpolyadenylated (●) RNA extracted from polysomes of newt early erythroid cells labeled for 12 h with [³H]adenosine. Pooled polysomes were treated with Proteinase K, and the suspension then was extracted with phenol:chloroform:isoamyl alcohol. The polyadenylated and nonpolyadenylated fractions were separated by oligo(dT)-cellulose chromatography and analyzed on 15–30% sucrose gradients (as indicated in box) with a SW41 rotor. Location of sedimentation markers from comparison gradient, ▲.

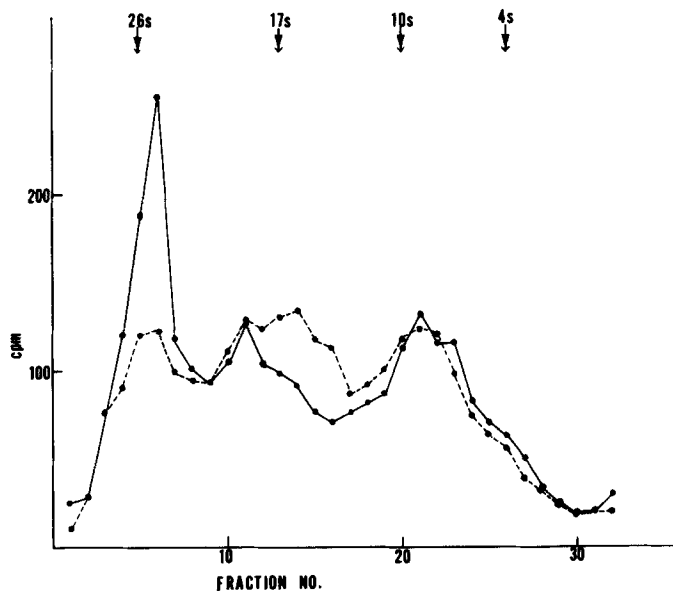


FIGURE 8 Sedimentation profile of polyadenylated RNA from polysomes of early erythroid cells labeled with [³H]adenosine for 22 h. The pooled polysomes were split into unequal portions. One portion was treated with SDS (●—●), the other was extracted with phenol:chloroform:isoamyl alcohol (●—●) before isolation of polyadenylated RNA on oligo(dT)-cellulose. RNA was analyzed on 5–30% sucrose gradients prepared in NET and centrifuged at 35,000 rpm for 14.5 h at 4°C in the SW41 rotor.

32, 40) and found in cells already synthesizing hemoglobin (see below), suggests that this fraction contains newt Hb-mRNA.

Protein Synthesis in Early Erythroid Cells

HEMOGLOBIN SYNTHESIS: In radioautographic studies, EPC's and other erythroid cells have been shown to incorporate [^3H] δ -ALA into an acid-precipitable component presumed to be hemoglobin (18, 19). *In situ* exposure of EPC's to [^3H]ALA for 3.5–20 h resulted in labeling of 40–75% of the EPC population (18, 19, and these studies). Cytoplasmic lysates prepared from these cells contained more than 80% of the total radioactivity in a peak which cochromatographed with native newt hemoglobin added as an absorbance (A_{415}) marker (Fig. 9). In undialyzed samples (Fig. 9), the remaining 10–15% of the counts eluted as a low molecular weight component corresponding to either heme or free ALA. In view of the negligible amount of radioactivity in other components, these data demonstrate that [^3H]-

ALA is incorporated largely, if not exclusively, into hemoglobin in newt EPC's.

Cytoplasmic lysates of early erythroid cells exposed to radioactive leucine showed a peak of radioactivity coincident with carrier hemoglobin (Fig. 10a), indicating incorporation of the labeled precursor into hemoglobin. Mature RBC's of adult newts (*T. cristatus*) contain four distinct hemoglobin phenotypes (unpublished data). Subsequent analysis of the hemoglobin peak in Fig. 10a showed that early erythroid cells synthesize all four Hb species and that no hemoglobin phenotype different from those observed in definitive adult RBC's is formed as a result of the induced anemia.

NONHEMOGLOBIN PROTEIN SYNTHESIS: Cytoplasmic lysates from early erythroid cells labeled with radioactive leucine exhibited a prominent peak of radioactivity (Fig. 10) which contained no significant absorbance at 415 nm and eluted in the void volume in front of the hemoglobin peak. Since radioautographic examination of the cells in these preparations showed intense labeling of EPC's and BE's but relatively little labeling

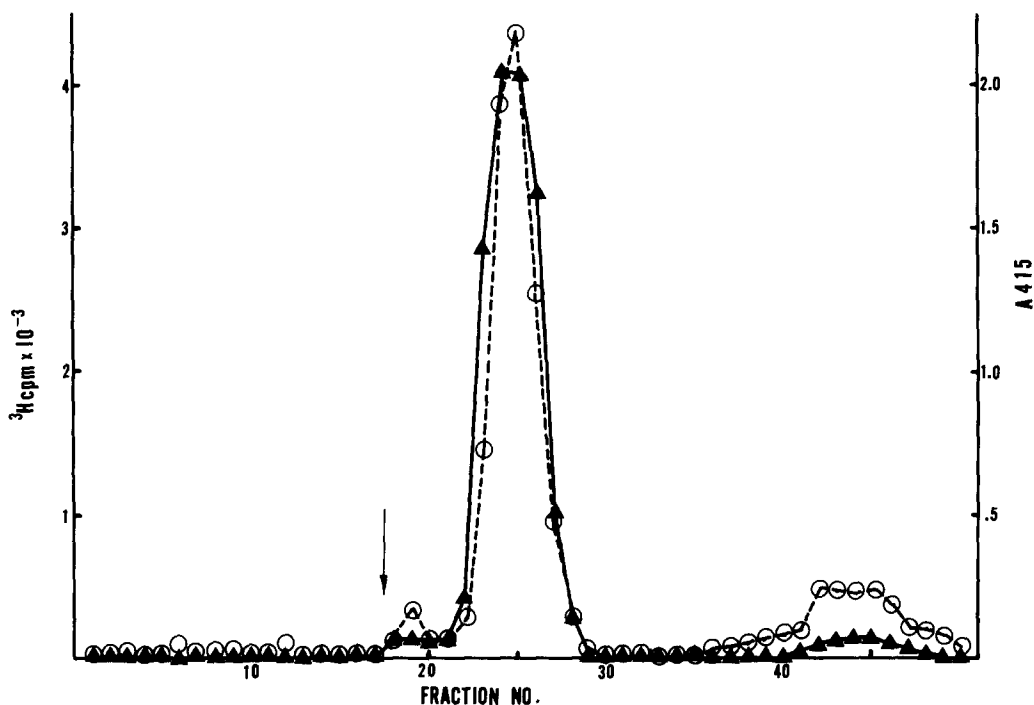


FIGURE 9 Chromatograph of cytoplasmic lysate from early erythroid cells (EPC:BE = 4:1) labeled *in situ* with [^3H]ALA for 20 h. Labeled cells were mixed with newt erythrocytes before lysis, and the lysate was clarified by centrifugation and run on Sephadex G-100. Vertical arrow designates void volume of column. A_{415} , (▲); ^3H -radioactivity, (○).

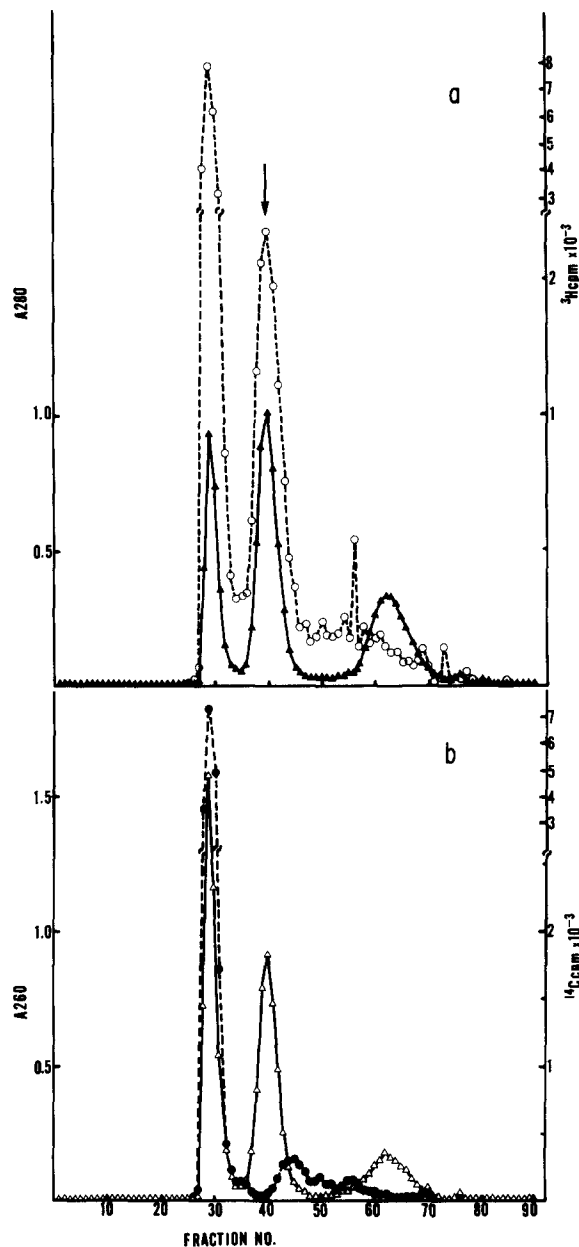


FIGURE 10 Chromatographic analysis of a cytoplasmic lysate from early erythroid cells labeled concomitantly *in situ* with [^{14}C]uridine and [^3H]leucine for 20 h. Dialyzed lysate was applied to a column of Sephadex G100. Fractions were eluted and monitored for absorbance at A_{280} , A_{260} , and A_{415} . An aliquot of each fraction was counted in aqueous scintillation cocktail to determine distribution of ^{14}C - and ^3H -radioactivity. Vertical arrow designates position of hemoglobin peak. (a) Chromatographic representation of A_{280} (\blacktriangle) and [^3H]leucine (\circ) profiles. (b) Chromatographic representation of A_{260} (\triangle) and [^{14}C]uridine (\bullet) profiles. A_{415} profile not shown.

of nonerythroid elements, this peak was considered to represent nonhemoglobin proteins synthesized within erythroid cells.

From the data presented above, it is clear that early erythroid cells are engaged in ribosomal production, a process which undoubtedly requires

synthesis of ribosomal proteins. Evidence supporting the concomitant synthesis of rRNA and of ribosomal proteins was obtained from early erythroid cells labeled simultaneously with [^{14}C]uridine and [^3H]Leu. Cochromatographic elution of ^{14}C - and ^3H -radioactivity as well as corresponding A_{260} and A_{280} profiles was seen in the nonhemoglobin peak (Fig. 10). When polysomes from these cells were dissociated by chelation with pyrophosphate (25) and analyzed on sucrose gradients, the A_{260} profile revealed two peaks, corresponding to the large and small ribosomal subunits, which sedimented somewhat more slowly than identically processed rabbit reticulocyte polysomes run on a parallel gradient (Fig. 11). Significantly, the ^{14}C - and ^3H -radioactivity profiles were coincident with the A_{260} profile (Fig. 11). The identity of the two subunit peaks was confirmed by extraction of the labeled RNA, which showed that the heavy and light peaks contained radioactivity which cosedimented with marker newt 26S and 17S rRNA's, respectively (data not shown). Thus, part of the newly synthesized protein in early erythroid cells is ribosomal protein.

Considerable radioactivity in the void volume peak was also obtained from cytoplasmic lysates of early erythroid cells labeled with ^{59}Fe (Fig. 12). The presence of hemoglobin in this peak was ruled out because of its minimal absorbance at A_{415}

content. Moreover, ribonuclease digestion in no way altered the elution characteristics, thereby ruling out the possibility of heme conjugation to nascent globin chains on polysomes (data not shown). Electrophoretic analysis of the void volume on acrylamide gels showed comigration of ^{59}Fe radioactivity with horse spleen ferritin (Fig. 13). Similarly, the void volume peak from lysates of early erythroid cells labeled with [^3H]Leu contained radioactivity in the band corresponding to ferritin (Fig. 14).

DISCUSSION

The erythropoietic process in anemic newts permits studies of cytological and biochemical events within highly homogeneous populations of successive erythroid stages in the intact animal. The degree of contamination of one developmental stage by another can be limited so that 80–90% of any erythroid cell population constitutes a single erythropoietic stage. More important, unlike most erythropoietic systems in which all stages from the most immature to the most mature are present in various proportions, the heterogeneity of developmental stages in anemic newts consists usually of the overlapping of two successive erythroid stages. In some experiments, overlapping is difficult to avoid, as, for example, in the earliest part of the response where the small number of EPC's would

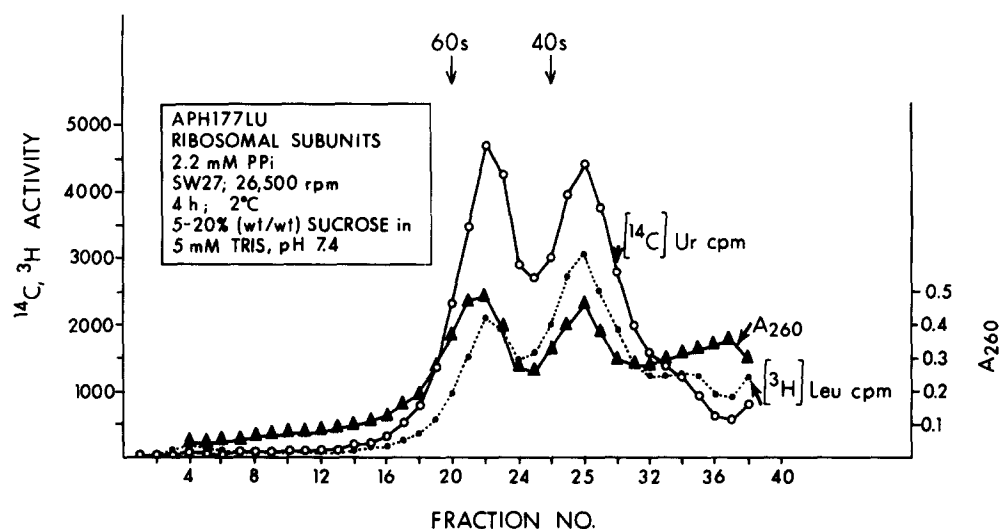


FIGURE 11 Sedimentation profile of dissociated ribosomal subunits obtained from early erythroid cells labeled concomitantly *in situ* with [^{14}C]uridine and [^3H]leucine for 24 h. Dissociation was effected by chelation with 2.2 mM sodium pyrophosphate and subunits were displayed on a 5–20% (wt/wt) sucrose gradient. Arrows (60S and 40S) represent positions of respective ribosomal subunits obtained from rabbit reticulocytes prepared and analyzed in parallel with newt cells.

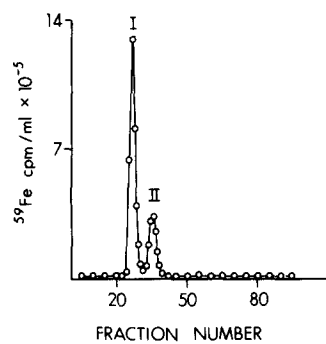


FIGURE 12 Chromatographic profile of a cytoplasmic lysate obtained from early erythroid cell (EPC:BE = 2.3) labeled *in situ* with [^{59}Fe]citrate for 20 h. Lysate was applied to a Sephadex G150 column. Peak I indicates the void volume peak while peak II represents hemoglobin.

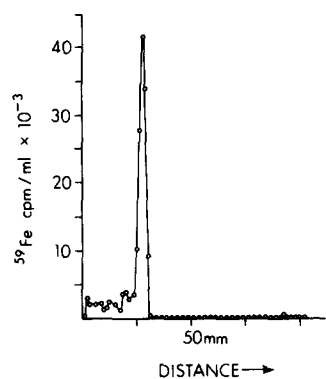


FIGURE 13 Polyacrylamide gel electrophoresis of an aliquot of peak I from Fig. 12. A single peak is observed the electrophoretic mobility of which is identical to that of horse spleen ferritin (not shown).

require the use of too many animals or during the latter part of the response where LPE's become contaminated by the appearance of new EPC's and the second erythropoietic wave. In these studies, animals were selected to maximize the proportion of a single erythroid stage and, where necessary, biochemical and radioautographic data within the same experiment were correlated.

An objective of the present study was to establish the identity of the so-called EPC in anemic newts, especially with regard to its possible role as a pluripotential hemopoietic stem cell. The new EPC exhibits morphological features that might be expected of a pluripotential stem cell. It is a structurally unspecialized cell whose features do not allow its assignment to a specific blood cell line and whose occurrence in the response to anemia precedes that of recognizable erythroid elements

(18). Furthermore, cells similar in appearance to EPC's apparently give rise to blood macrophages in response to the extensive hemolysis induced by phenylhydrazine shortly after its injection (18). The morphological similarity of the parent cells of two separate blood cell lines suggests that the parent cells represent a pluripotential stem cell population.

The results of our studies demonstrate unequivocally that newt EPC's are already committed to RBC development. Unlike pluripotential stem cells (28, 29, 37), EPC's comprise a highly proliferative cellular compartment extensively engaged in DNA synthesis and mitosis (18, 49). Moreover, EPC's are absent in hemopoietically inactive animals and appear only during periods of erythropoiesis (18, 19), a result which implies that EPC's are derived from an earlier progenitor cell. The most compelling evidence against their role as pluripotential stem cell is the finding that many EPC's are engaged in hemoglobin synthesis. Radioautographic studies have shown incorporation of radioactive ALA, a precursor in the heme biosynthetic pathway, by EPC's (18). The data obtained in the present studies have confirmed that almost all of the radioactivity in ALA-labeled EPC's is located in hemoglobin, a result which clearly establishes their already-differentiated function. Quite likely, the EPC represents a developmental stage closely related to the uncommitted stem cell and a cell type in which the events

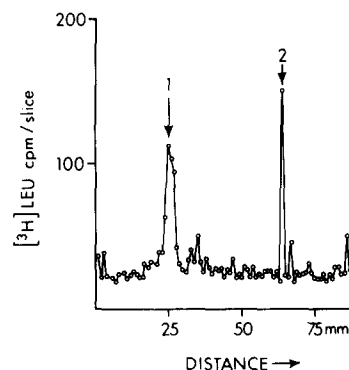


FIGURE 14 Polyacrylamide gel electrophoresis of a void volume peak from a cytoplasmic lysate obtained from early erythroid cells labeled *in situ* with [^3H]leucine for 20 h. The slow peak (arrow 1) coelectrophoreses with ferritin marker and the ^{59}Fe -peak seen in Fig. 13. A faster peak (arrow 2) is found to contain ^3H -radioactivity but, unlike peak 1, had no corresponding band on a duplicate gel stained with Coomassie Blue. This fast peak probably represents free [^3H]leucine.

characteristic of RBC development have not occurred to an extent sufficient to allow its structural recognition as an erythroid element. Despite their lack of morphological specialization, however, EPC's do not display functional behavior typical of pluripotential stem cells and must be considered one of the earliest recognizable elements in newt RBC development.

It should be noted that the newt EPC's are distinct from EPC's in mouse fetal liver, where the term designates both proerythroblasts and basophilic erythroblasts (6, 8, 35, 46). Although the newt EPC compartment may include a cell type corresponding to a proerythroblast, the majority of EPC's represent an even earlier stage in RBC development according to structural and staining criteria used to identify proerythroblasts. In the initial part of the response, the EPC population consists mainly of cells with few or no ribosomes (18, 19), but these cells subsequently give way to cells exhibiting variable but every-increasing concentrations of cytoplasmic ribosomes (18, 19). A cell type corresponding to a proerythroblast probably occurs during this latter interval (18, 19). Within a few days, the blood consists almost entirely of basophilic erythroblasts, definitive early erythroid elements derived from EPC's and distinguished from them by virtue of their strong cytoplasmic basophilia, the abundance of cytoplasmic ribosomes, and their order of appearance in the erythropoietic response. Thus, newt EPC's constitute a heterogeneous cell population whose individual members differ in size, ribosomal content, and degree of cytoplasmic basophilia and whose maturation gives rise to basophilic erythroblasts.

Collectively, EPC's, BE's, and EPE's constitute the early developmental period, a period in RBC development during which the various RNA species required for subsequent maturation are synthesized. A large proportion of the RNA produced in early erythroid cells is ribosomal, a finding consistent with the prominent, often multiple, nucleoli typical of these cells (18, 19, 47). Indeed, when viewed in conjunction with cytological data obtained in earlier studies (18, 19), the results of the present study indicate that rRNA synthesis, ribosomal production, and, possibly, synthesis of nonribosomal RNA classes occur in three phases. A phase of accumulation occurs during the interval between the initially appearing EPC's and BE's, an interval marked by a prominent increase in ribosomal concentration and RNA content in individual cells (19). Incorporation of radioactive

precursors into RNA on a per cell basis is highest where the proportion of EPC's is greater and declines subsequently as the number of EPC's diminishes. The high specific activity of the rRNA classes in the EPC-BE interval arises because most of the rRNA in these cells is newly synthesized, a result which agrees with ultrastructural data showing that an increase in ribosomal content is the major structural event that occurs between these two stages (19).

In BE's, where RNA content expressed on a per cell basis has attained the highest level observed during erythropoiesis (19), a major portion of RNA synthesis is responsible for the maintenance of the level of cellular rRNA and ribosomes. Proliferative activity, which is high in EPC's, is slightly increased in BE's (49) and serves as a means by which the number of erythroblasts can be augmented to a level sufficient to restore the circulating RBC mass. RNA synthesis in BE's, especially the production of rRNA, may be visualized as having entered a maintenance phase, with its efforts directed to sustaining the cytoplasmic RNA content characteristic of these cells in the presence of extensive proliferation.

During the latter part of the early developmental period and continuing into the intermediate stages, RNA synthesis enters a reductive phase which is characterized by a decline not only in the relative amount of RNA synthesized but also in the proportion of cells responsible for this synthesis. Whereas the vast majority of early erythroid cells is extensively engaged in RNA production, only 30-40% of the intermediate stage cells carry out this activity (19, 21). The reductive phase includes a significant decrease in total rRNA synthesis and altered maturation of rRNA precursors which results in a disproportionate accumulation of the two major cytoplasmic rRNA fractions in favor of the large subunit RNA. Although ribosomal wastage has usually been described in cells grown *in vitro* (9-11, 14, 15, 53), the results of the present studies demonstrate the occurrence of a similar phenomenon in cells within the intact organism. It is interesting that, as in stationary phase lymphocytes and contact-inhibited fibroblasts (9-11, 14), ribosomal wastage in newt erythroid cells appears to parallel a decrease in cellular growth rate (22, 49). However, the data obtained have not been sufficient to permit a determination of the precise relationship of a decreased growth rate to ribosomal wastage in newt erythroid elements.

The pattern of rRNA synthesis observed in newt erythropoiesis probably reflects a similar pattern with regard to the synthesis of other RNA classes as well. The data show a longer persistence of tRNA labeling (into late stages) but clearly at levels markedly decreased from those in early erythroid stages. Whether persistent tRNA labeling represents *de novo* synthesis or terminal addition to preexisting tRNA molecules has not been studied. It is also likely that synthesis of mRNA classes is restricted largely to the early developmental period. Clearly, the decreased level of RNA synthesis in the intermediate stages and the demonstrable absence of RNA synthesis in late erythroid elements tend to eliminate the possibility of significant levels of mRNA synthesis beyond the early erythroid stages, but further studies are necessary to resolve this issue.

It is unlikely that the pattern of RNA synthesis obtained in these studies can be attributed to changes in pool characteristics. Corresponding erythroid stages labeled either *in vitro* or *in vivo* exhibit identical results in terms of the extent of synthesis and the kinds of RNA formed. Similarly, an identical pattern of RNA synthesis relative to different stages is obtained with several precursors, including various nucleosides, purines, and radioactive phosphate. The most conclusive finding against an effect of precursor pools is that the patterns of synthesis observed correlate accordingly with significant ultrastructural phenomena. Thus, RNA production and ribosomal synthesis are most pronounced in early erythroid cells the nuclei of which contain diffuse chromatin and highly prominent nucleoli (18, 19, 47). Conversely, as RNA synthesis recedes, corresponding changes in nuclear structure occur so that, in late erythroid cells, the absence of RNA synthesis parallels the presence of extensively condensed chromatin and the virtual absence of nucleoli (19, 23, 47).

In recent studies of mammalian systems, disagreement has continued concerning the erythroid stage in which hemoglobin synthesis initially occurs, and especially, the relationship of this event to globin mRNA production (24, 45, 46). The relationship between these two events must be clarified in order to define the extent to which transcriptional and translational controls operate with respect to the onset of hemoglobin synthesis. In newt EPC's, the presence of hemoglobin cannot be detected by cytophotometric measurement (18, 19), yet correlated radioautographic and bio-

chemical data firmly establish that these highly immature erythroid cells are already involved in hemoglobin synthesis. Moreover, in radioautographs, hemoglobin synthesis can be observed in many EPC's whose cytoplasm is faintly basophilic (18). Since ribosomes account for the preponderant part of cytoplasmic RNA (and basophilia), hemoglobin synthesis is initiated before the massive accumulation of ribosomes leading to the formation of basophilic erythroblasts. This result implies that the translational machinery required to support the onset of hemoglobin synthesis is sufficiently established in EPC's. Possibly, the increase in ribosomal content following the onset of hemoglobin synthesis compensates for the high proliferation rate characteristic of early erythroid cells and ensures a sufficient quantity of ribosomes to maintain protein synthesis in an increased number of cells throughout subsequent maturation.

Demonstrable hemoglobin synthesis in EPC's constitutes functional evidence for the presence of Hb-mRNA in these cells. Although its identification is tentative at this point, the 9S-12S fraction isolated with oligo(dT)-cellulose may contain newt Hb-mRNA since: (a) its sedimentation rate is similar to that of Hb-mRNA in other erythroid cells (7, 32); (b) like many mRNA's, including Hb-mRNA (5, 17, 31, 34, 36, 38, 40, 43), it is polyadenylated; (c) it is extracted from cells already engaged in synthesis of hemoglobin.

Although hemoglobin synthesis occurs in newt early erythroid cells, the predominant activities that characterize the RBC differentiation process in the early developmental period are directed toward the establishment of various molecular components which function in the control and regulation of subsequent RBC maturation. Besides producing the various RNA classes, these cells produce ferritin, an iron-containing protein which may be involved in the regulation of hemoglobin synthesis and which certainly is an important intermediate in the process of iron transport and iron metabolism in developing erythroid cells. In addition to having a role in the synthesis of regulatory molecules, early erythroid cells show great proliferation, an activity which amplifies the number of cellular units available to complete the developmental program established with the initial commitment of the pluripotential stem cell along erythroid lines. Clearly, the expression of differentiation is more than simply the initiation of globin synthesis but includes the elaboration of other elements whose synthesis probably precedes and

occurs in parallel with the onset of hemoglobin synthesis and whose presence and stability are essential to subsequent RBC development.

Significant alterations of the metabolic behavior observed in early erythroid cells occur as the maturation process approaches the intermediate developmental period. In EPE's and MPE's, DNA synthesis and mitosis are sharply decreased compared to the early stages, resulting in marked curtailment of growth (19, 22, 49). Similarly, RNA synthesis in EPE's and MPE's drops precipitously and soon comes to a halt. From this point on, and probably indirectly related to the cessation of RNA synthesis, cellular RNA undergoes a quantitative decline (19, 21, 23). Thus, the intermediate stages appear to represent a pivotal point in RBC development where the system is transformed from transcriptional and proliferative behavior characteristic of the early stages to maturational and climactic events marked chiefly by the synthesis and accumulation of hemoglobin in the late developmental stages.

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