phate, starting with the report of Felix *et al.* (1960) and extending to the last review of Laskowski (1967), are being revoked.

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On the Mechanism of Erythropoietin-Induced Differentiation. V. Characterization of the Ribonucleic Acid Formed as a Result of Erythropoietin Action*

Martin Gross† and Eugene Goldwasser‡

ABSTRACT: Erythropoietin induces the process by which primitive hematopoietic cells eventually become mature erythrocytes. The earliest action of erythropoietin so far determined is the stimulated synthesis of ribonucleic acid by rat marrow cells *in vitro*. The nature of this ribonucleic acid was reexamined. Within a few minutes of its addition to the cells erythropoietin causes the formation of a very large ribonucleic acid (150 S) that is not detectable in the unstimulated cells. This ribonucleic acid appears to be a discrete component, and not a complex of ribonucleic acid with ribonucleic acid, deoxyribonucleic acid, or protein. At later times, increased synthesis of components with sedimentation coefficients in the range 55–65, 45, 9, 6, and 4 S occurs. The 150S ribonucleic acid has a half-life of about 6 min while the other components are longer lived. Some of the properties and possible functional roles of the ribonucleic acid components are discussed.

The induction of erythrocyte formation from primitive precursor cells of the blood-forming tissues appears to be initiated by erythropoietin (Goldwasser, 1966). Even though the purification of erythropoietin is not yet completed, its availability in a partially purified form and its ability to initiate cytodifferentiation provide an attractive system for studying the biochemical mechanisms underlying this process. Previous work from this laboratory has demonstrated that erythropoietin stimulates heme synthesis (Krantz *et al.*, 1963), hemoglobin synthesis (Gallien-Lartigue and Goldwasser, 1964), glucosamine incorporation into stroma (Dukes *et al.*, 1964; Dukes and Goldwasser, 1965), and iron uptake (Hrinda and Goldwasser, 1966) by rat bone marrow cells in tissue culture, and that these effects are inhibited by actinomycin D. We have also shown that erythropoietin increases the rate of RNA synthesis in this system as early as 15 min after its addition (Krantz and Goldwasser, 1965). These findings indicate that the stimulation of RNA synthesis plays an important early role in the process of erythroid differentiation induced by erythropoietin.

Recent studies of RNA metabolism in animal cells have demonstrated two different classes of rapidly labeled, quickly sedimenting RNA (Scherrer *et al.*, 1963, 1966; Yoshikawa-Fukada *et al.*, 1965; Attardi *et al.*, 1966). One of these, sedimenting at 45 S, is the precursor of rRNA. The other component is heterodisperse, in-

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TABLE I: Test of Chase Conditions for Inhibition of RNA Synthesis.[a]

	cpm	%
Control	1355	100
Erythropoietin	1829	135
Actinomycin D + nucleosides	27	2
Erythroprotein + actinomycin D + nucleosides	28	2

[a] Twelve cultures containing 25 × 10^6 nucleated cells/ml in a 1.0-ml total volume were incubated for 5 hr; six of these had 0.10 unit of erythropoietin/ml added. After this time 8 μg of actinomycin D and 5 μmoles each of uridine and cytidine were added to three control and three stimulated cultures. All cultures were then pulsed with 1 μCi/ml of [^{3}H]uridine for 20 min and the radioactivity in the total RNA was determined by the trichloroacetic acid method.

cludes sizes greater than 50 S, and, though its function is still obscure, fulfills some of the requirements for mRNA. Lingrel (1967) has recently shown, in addition, that the rapidly labeled RNA of anemic rabbit bone marrow cells contains 45S RNA as well as two nonribosomal components.

The data in this paper show that the RNA formed as a result of a few minutes of erythropoietin action is not restricted to the 12–24S region of the density gradient as was indicated in an earlier paper (Krantz and Goldwasser, 1965). The small size of the newly formed RNA appeared to have been due to breakdown during the isolation procedure. On reexamining the problem we find that erythropoietin stimulates the synthesis of at least six types of RNA. Three of these components are in the 4–10S region, one is 45S rRNA precursor, and two others, which are distinct from rRNA, sediment at 55–65 and 150 S. Some properties of the RNAs formed as a result of erythropoietin action and their possible roles in cytodifferentiation will be discussed.

Materials and Methods

Materials. New-born calf serum was bought from the Colorado Serum Co., NCTC-109 from Microbiological Associates, and tissue culture dishes from Falcon Plastics. [^{3}H]Uridine (5000 Ci/mole), [^{14}C]uridine (51.5 Ci/mole), and [*methyl*-^{3}H]methionine (106.5 Ci/mole) were bought from New England Nuclear; [^{14}C]cytidine (20 Ci/mole) from Schwarz; and [^{3}H]cytidine (1000 Ci/mole), [^{3}H]guanosine (500 Ci/mole), and [^{3}H]adenosine (500 Ci/mole) from Nuclear-Chicago. Actinomycin D was a commercial sample. Sheep plasma erythropoietin concentrate, a gift from the National Blood Resources Branch of the National Heart Institute, was further purified in this laboratory (Goldwasser and Kung, 1968).

Conditions of Culture. Rat bone marrow cells were cultured as previously described (Krantz *et al.*, 1963), except that the medium was composed of 60% NCTC-109, 35% newborn calf serum, 5% rat serum (containing ferric nitrate at a concentration of 7.3 × 10^{-5} M). Cells in 1.0 ml of medium were cultured in 35 × 10 mm dishes, and cells in 3.0 ml of medium in 60 × 15 mm dishes. Pulsing conditions and radioactive precursors are indicated for each experiment. Cells were chased by adding actinomycin D at a final concentration of 8 μg/ml, and unlabeled precursors at a final concentration of 0.005 M.

Tests of the adequacy of chase conditions (Table I) show that addition of 8 μg/ml of actinomycin D, plus 0.005 M uridine and 0.005 M cytidine, reduces control uridine incorporation into RNA by 98% and abolishes the component of RNA synthesis that is stimulated by erythropoietin. Actinomycin D alone, at the same concentration, reduces control RNA synthesis by only 68% but still totally abolishes stimulated RNA synthesis. When the RNA from actinomycin-treated cells was examined on a sucrose gradient, we found that the synthesis of erythropoietin-stimulated RNA was inhibited at all regions of the gradient except for a small amount of RNA in the 4–10S region which may represent terminal labeling of tRNA or a small component of RNA synthesis which is not DNA dependent.

Determination of Total RNA Synthesis. Total RNA radioactivity was determined after lysing the washed cell pellets with ice-cold 5% trichloroacetic acid and washing the acid-insoluble precipitates three times with ice-cold 5% trichloroacetic acid, once with 95% ethanol, and once with ether. The precipitates were dissolved in 0.20 ml of formic acid, heated at 100° for 10 min, and then rapidly cooled to denature the DNA. The samples were counted in 10 ml of scintillation fluid containing 5 g of 2,5-diphenyloxazole/l. of a 2:1 mixture of toluene–2-ethoxyethanol, in a Packard Model 3375 liquid scintillation spectrometer.

Isolation of RNA. METHOD I. This procedure is a modification of the method previously described (Krantz and Goldwasser, 1965). Culture medium (3 ml), containing the cells, plus 1.5 ml of saline wash were added to 4.0 ml of homogenizing solution (2% sodium dodecyl sulfate, 1% naphthalenedisulfonate, 0.002% sodium dextran sulfate, 0.04% bentonite, and 0.02 M Tris (pH 7.6)). The contents were quickly mixed and then shaken with 4.0 ml of water-saturated phenol containing 0.1% 8-hydroxyquinoline for 30 sec. After centrifugation at 15,000*g* for 3 min, the aqueous phase was removed and deproteinized twice more with one-half volume of phenol, and the nucleic acid was precipitated with two volumes of 95% ethanol. The precipitate was washed twice with ethanol, dissolved in 5 ml of acetate buffer (0.10 M sodium chloride–0.01 M sodium acetate (pH 5.1)), and reprecipitated with 5 ml of 1% cetyltrimethylammonium bromide. This precipitate was washed once with acetate buffer and then reconverted into sodium nucleate by mixing with 1 ml of acetate buffer (modified to contain 1 M sodium chloride) and 2 ml of ethanol. The precipitate was dissolved in 1.5 ml of 0.01 M Tris–0.001 M magnesium chloride (pH 7.6), and DNA was hydrolyzed with 5 μg/ml of DNase (freed from RNase by

chromatography on DEAE-cellulose) at 25° for 5 min. Equal volumes of homogenizing solution and phenol were then added, the contents were mixed and centrifuged, and the nucleic acids in the final epiphase were precipitated with three volumes of absolute ethanol. All steps, except the DNase hydrolysis, were carried out at 0–5°. This procedure gives a yield of approximately 90% of the labeled RNA precipitable with trichloroacetic acid (Table II).

METHOD II. This procedure is a modification of the method of Scherrer and Darnell (1962). Culture medium (3 ml), containing the cells, plus 1.5 ml of saline wash, was added to 4.0 ml of water-saturated phenol containing 0.1% 8-hydroxyquinoline plus 4.0 ml of homogenizing solution while stirring rapidly in a water bath at 58 ± 1°. After 5-min stirring, the mixture was poured into a centrifuge tube immersed in a melting ice bath and allowed to cool for 5 min. After centrifugation at 15,000*g* for 3 min, the entire epiphase and interphase were removed, shaken with one-third volume of phenol, and centrifuged again. The epiphase was then removed and deproteinized a second time with phenol. The remainder of the procedure was identical with method I. All of the labeled RNA is found in the first supernatant but there is a small loss during the subsequent steps (Table II). In the hot phenol-extraction procedure, described by Scherrer and Darnell (1962), further deproteinization with phenol was done at the same elevated temperature as the initial extraction. We find, however, that with marrow cells more labeled RNA is lost if the second and third phenol treatments are done at 58° than at 0–5°. If the heated extract of method II is allowed to cool slowly, or is mixed during the rapid cooling, or if the interphase is not removed with the epiphase after the first centrifugation, the yield of pulse-labeled RNA is reduced to about 25%. In contrast to Morrison and McCluer's (1967) findings with rat liver, no further labeled RNA can be obtained from marrow cells by treatment at 95° for 10 min after a prior 58° extraction. We have found that although there is more label present, the yield of RNA is not affected by the presence of erythropoietin in the medium (Table II).

Density Gradient Fractionation. This procedure was as previously described (Krantz and Goldwasser, 1965) except that the rotor, speed, and time of sedimentation were as given in the figure legends, and the fractions were counted in a liquid scintillation spectrometer after the addition of 10 ml of Bray's solution (Bray, 1960).

Relative Base Composition. Relative base compositions of the RNA components were determined by pulsing cells with either [^{3}H]adenosine and [^{14}C]cytidine or with [^{3}H]guanosine and [^{14}C]uridine. The $^3H/^{14}C$ ratio was determined for each component separated on the gradients, and the relative amounts of each base were compared. This method does not allow determination of the actual base composition but does indicate relative differences in the base compositions of the several RNA components. Reversal of the labels of the same two nucleosides resulted in reversed $^3H/^{14}C$ ratios and the same relative base ratios, indicating that the different ratios did not reflect differential quenching of ^{3}H and ^{14}C at different levels in the sucrose gradient.

TABLE II: Yield of Labeled RNA with Different Methods of Isolation.[a]

Method	Erythropoietin (0.08 unit)	cpm	% Recov
Trichloroacetic acid	−	6970	100
Trichloroacetic acid	+	9540	137
I	−	6270	90
II, one phenol treatment	−	7100	102
II, one phenol treatment	+	9550	137
II, two phenol treatments	−	6410	92
II, three phenol treatments	−	5360	77
II, three phenol treatments	+	7450	107

[a] For the purpose of this table we assume that the trichloroacetic acid method yields all of the labeled marrow cell RNA. Cultures contained 25 × 10^6 nucleated cells/ml in a 3.0-ml total volume, and, where indicated, 0.08 unit of erythropoietin/ml. After 5-hr incubation, they were pulsed for 20 min with 1 μCi/ml of [^{3}H]uridine. These results are the averages of three experiments.

Results

The rapidly labeled RNA of rat bone marrow cells is rather quickly degraded after the cells are lysed in homogenizing solution and before phenol is added. The data in Figure 1 show that when compared with method II, use of method I caused a progressive shift toward smaller S with increase in time between lysis and phenol addition. We also found that the addition of phenol earlier than 20 sec after lysis resulted in marked reduction in the yield of rapidly labeled RNA. This can be prevented by using method II. To test whether the use of high temperature might cause aggregation of small-sized RNA, an aliquot from the initial emulsion of a method I preparation was kept at 58° for 5 min and then quickly cooled. This aliquot and a control kept at 0–5° were then treated identically and analyzed on a sucrose gradient. We found that both samples had closely similar radioactivity profiles, indicating that the heat treatment does not cause aggregation.

Krantz and Goldwasser (1965) originally showed that erythropoietin increases the rate of RNA synthesis of cultured rat bone marrow cells as early as 15 min after its addition. The degree of response under these conditions was small, but a significant stimulation was seen in the 12–24S region when the RNA was fractionated on a sucrose density gradient. Since we now know that rapid degradation of RNA occurred with the procedure used,

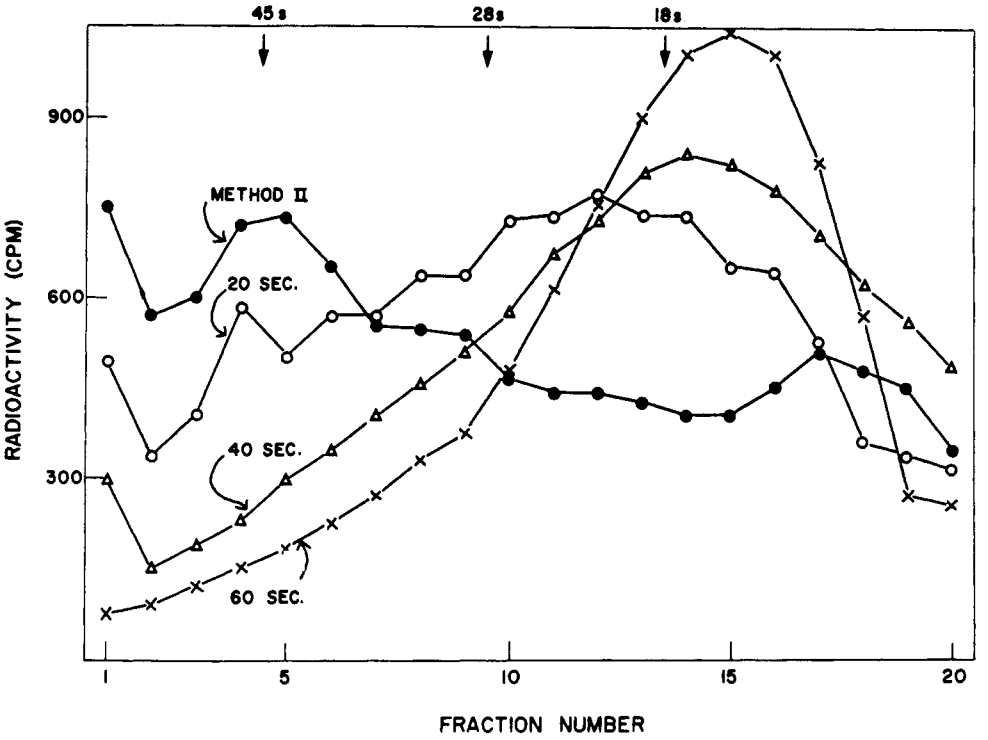

FIGURE 1: Effect of time between lysis and phenol addition on the size of rapidly labeled RNA. Cultures contained 50 × 10^6 nucleated rat marrow cells/ml in 3.0 ml of medium with 0.10 unit/ml of erythropoietin. After 30 min, the cells were pulsed for 10 min with 3 μCi/ml of [^{3}H]uridine. Each sample represents one culture dish. RNA was isolated by method I, with intervals between detergent lysis and phenol addition of 20 sec (O—O), 40 sec (▲···▲), and 60 sec (×···×); or according to method II (●···●). RNA was analyzed on a 5–20% linear sucrose gradient, centrifuged for 3 hr at 38,000 rpm in the SW39 rotor.

the experiment was repeated using method II for the extraction and isolation of the RNA, with the results shown in Figure 2. In this experiment the cells were preincubated with [^{3}H]uridine for 10 hr before both erythropoietin and [^{14}C]uridine were added in a 15-min pulse. The tritium labeling served as a measure of total RNA and, as we find with absorbancy measurement, shows no difference between control and stimulated samples throughout the gradient. The over-all stimulation of RNA synthesis (shown with [^{14}C]uridine) at this time was rather small, as was noted originally, but, in contrast to the earlier finding, it was limited to the fraction at the bottom of the tube.

The rapid, erythropoietin-induced, stimulation of uridine incorporation into RNA might reflect an increased specific activity of the UTP pool due to increased uptake of the labeled nucleoside rather than increased RNA synthesis. We consider such a possibility unlikely, because the RNA fractionated on a density gradient shows increased incorporation due to erythropoietin only at a particular region of the gradient rather than a general increase. An additional test of whether the erythropoietin effect was caused by a change in the precursor pool was done as follows: cells were prelabeled for 1 hr with [^{14}C]uridine, washed free of label, and then incubated with and without erythropoietin for 2.5 hr. Table III shows that the amount of uridine incorporated into RNA in the erythropoietin-treated cells was greater than that incorporated into control cells by about 16%, and since both sets of cells had identically labeled pools the effect cannot be ascribed to change in precursor specific activity. While the results of this experiment do not completely eliminate the possibility that the early (15 min) effect of erythropoietin is on the precursor pool, they strongly suggest that this is not the case.

The variation of the rate of stimulated total RNA synthesis (difference between control and erythropoietin-containing cultures) with time of incubation was measured, and the average of three such experiments is shown in Figure 3. The effect of erythropoietin reaches a peak at about 11 hr and persists for 17–20 hr. In some experiments, however, stimulated synthesis may last for only 10 hr with a peak at 6 hr (see, for example, Figure 8).

We also studied the effect of increasing labeling time on the distribution of radioactivity in RNA from cells incubated with erythropoietin for 3 hr (Figure 4). After 5 min (frame A) the RNA radioactivity was small and heterodisperse; the only significant difference between the control and stimulated groups was in the bottom fraction of the gradient. After 10-min labeling (frame B), the radioactivity distribution was still heterodisperse, but an increase due to erythropoietin was seen in the 35S and greater region. In frames C, D, and E the radioactivity profile changed with time until it was parallel to the absorbancy profile, and the major difference due to erythropoietin was in the 28S, 18S, and 6S peaks. These results are in accord with many reports showing a shift from heterodisperse RNA (including 45 S) to rRNA (Scherrer and Darnell, 1962). Chase experiments with stimulated marrow cells also show the con-

TABLE III: Test of Effect of Erythropoietin on Precursor Pool.[a]

Control	Stimulated	Difference
11,200 [±400]	13,100 [±500]	1,900 [±640] ($p < 0.05$)

[a] 50 × 10^6 nucleated cells/ml in a total volume of 3.0 ml were incubated with 2 μCi/ml of [^{14}C]uridine for 1 hr. The cells were then washed free of label and diluted to 8 ml. Aliquots (1.0 ml) were then transferred to culture dishes. Erythropoietin at 0.06 unit/ml was added to half of the dishes, and all were incubated 2.5 hr longer. RNA radioactivity was determined by the trichloroacetic acid method. The figure in brackets is the standard deviation of the mean. Values given in counts per minute.

version of 45S RNA to 18S and 28S RNA. Figure 4 shows, in addition, that erythropoietin markedly increased the labeling of that RNA sedimenting to the bottom of the gradient at all labeling times.

In view of the effect of erythropoietin on the bottom fraction, the RNA from the 50S and greater region was further characterized by rerunning on another gradient for a shorter time (Figure 5). In these experiments RNA was analyzed after three different conditions of culture. (A) In frames A and C there was 3-hr contact with erythropoietin, then a 30-min pulse with labeled uridine; (B) in frames B and D, there was 3-hr contact with erythropoietin, a 30-min pulse with uridine, and a 3-hr chase; and (C) in frames E and F preincubation for 24 hr was followed by 3 hr with erythropoietin and a 30-min uridine pulse. For each of these conditions, controls with no erythropoietin were also run. Frames

TABLE IV: Stability of 55–65S RNA.[a]

Control cpm after			Δ cpm Due to Erythropoietin after		
Pulse	Chase	%	Pulse	Chase	%
750	250	33	260	175	67

[a] Cultures containing 50 × 10^6 nucleated cells/ml in a total volume of 3.0 ml were incubated with and without 0.10 unit/ml of erythropoietin for 3 hr. They were then pulsed for 15 min with 1 μCi/ml of [^{3}H]-uridine, and half of the cultures was stopped immediately using method II. The other half was chased for 3 hr as in Table I, then stopped, and RNA was isolated by method II. Sucrose gradients were centrifuged at 48,000 rpm for 80 min in the SW50 rotor, and the 50S and greater region was rerun for 40 min under the same conditions. The table shows the radioactivity in the 55–65S region of the second gradient.

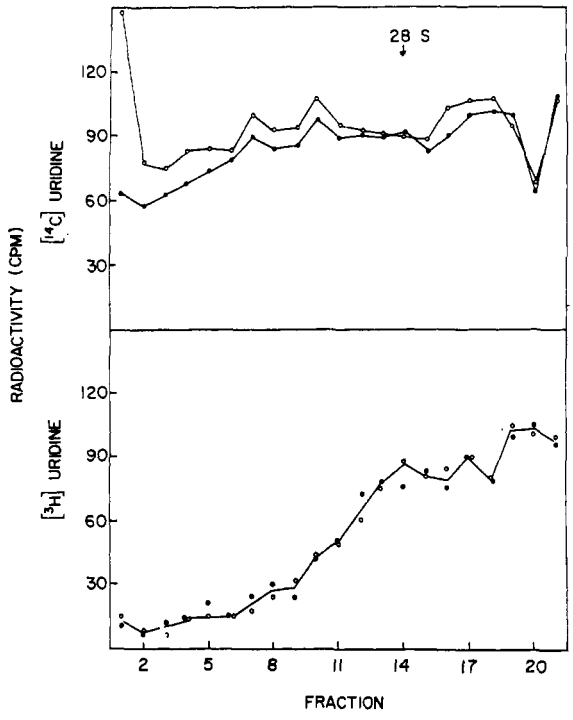

FIGURE 2: Density gradient profile of RNA after 15 min of erythropoietin action. Two cultures containing 40 × 10^6 nucleated cells/ml in 3.0 ml of medium were preincubated with [^{3}H]uridine (0.01 μCi/ml) for 10 hr, then 0.10 unit/ml of erythropoietin was added to one and [^{14}C]uridine (1 μCi/ml) to both. After 15 min both were stopped, and RNA was isolated by method II and analyzed on sucrose gradients at 48,000 rpm for 90 min in the SW50 rotor. Open circles represent erythropoietin-treated cells; closed circles, controls. The upper curve shows the distribution of RNA labeled with [^{14}C]uridine (pulse labeled); the lower curve shows the distribution of RNA labeled with [^{3}H]uridine (prelabeled).

C, D, and F represent the rerun at shorter times of the heavy fractions from A, B, and E respectively. The results in frame C show a small but definite radioactivity peak at 150 S, formed in response to erythropoietin, and no detectable amount of this component in the controls. The control cells had an appreciable peak of radioactivity in the 55–65S region which was increased in the presence of erythropoietin. When cells were chased for 3 hr after the uridine pulse, and then analyzed, the 150S peak was no longer in evidence but a broad peak centering about 45 S persisted (frame D). Cells that had been preincubated for 24 hr before the 3-hr erythropoietin treatment and 30-min pulse showed a pattern of refractionated, labeled RNA (frame F) that was very similar to the one from cells without any preincubation. A definite 150S component and a larger amount of a 55–65S component were formed in response to erythropoietin.

We studied the stability of the RNA found in the 55–65S portion of a sucrose gradient in a pulse-chase experiment (Table IV). Approximately two-thirds of the RNA (55–65 S) formed in the presence of erythropoietin persisted for 3 hr, while about one-third of the same fraction persisted in control cells. Since this region of the gradient probably consists of several different species of RNA and since the stimulated cells may represent

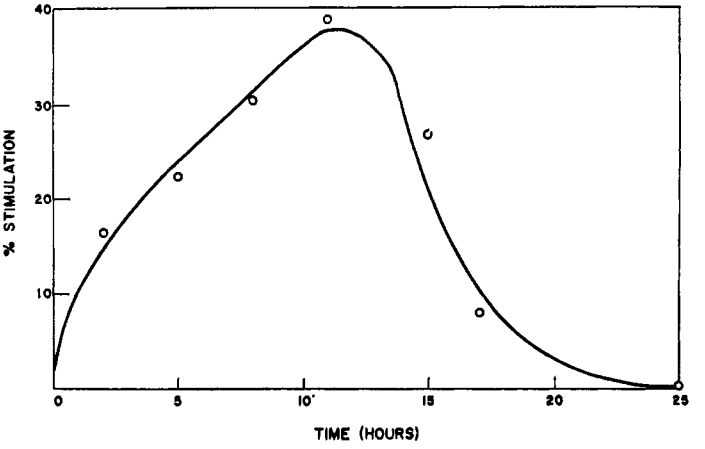

FIGURE 3: Effect of time after erythropoietin addition on stimulation of RNA synthesis. The results are a composite of three experiments. Triplicate cultures contained 15 × 10^6 nucleated cells/ml in 1.0 ml of medium. Stimulated groups received 0.05 unit/ml of erythropoietin at zero time. At the indicated times, control and stimulated groups were pulsed with 1 μCi/ml of [^{3}H]uridine for 30 min, and total radioactive RNA was determined by the trichloroacetic acid method.

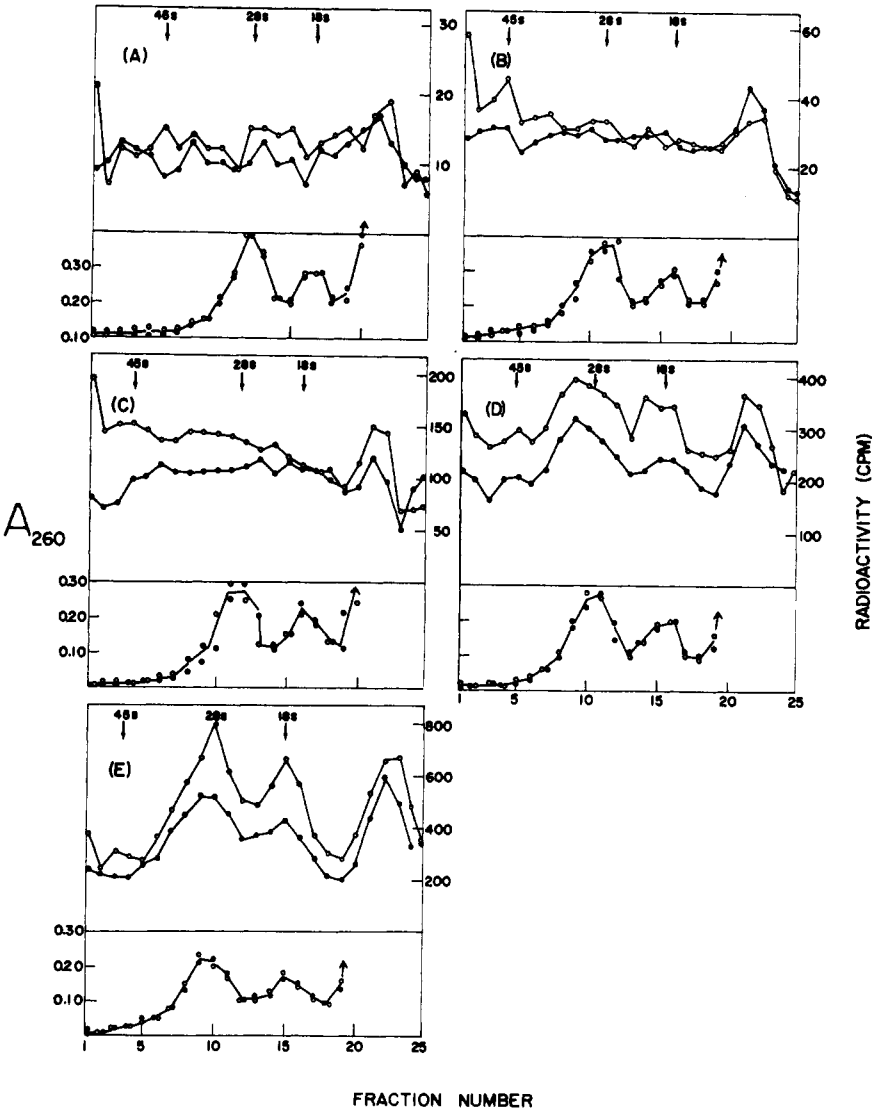

FIGURE 4: Effect of pulse time on the sedimentation profile of erythropoietin-stimulated RNA synthesis. Cultures contained 40 × 10^6 nucleated cells/ml in 3.0 ml of medium with (O···O) and without (●···●) 0.07 unit/ml of erythropoietin. After 3-hr incubation, 1 μCi/ml of [^{3}H]uridine was added. RNA was isolated by method *II* after 5 (A), 10 (B), 30 (C), 60 (D), and 120 (E) min of further incubation. Density gradient analyses were run for 120 min at 48,000 rpm in the SW50 rotor. Each RNA sample represents the cells from one culture dish.

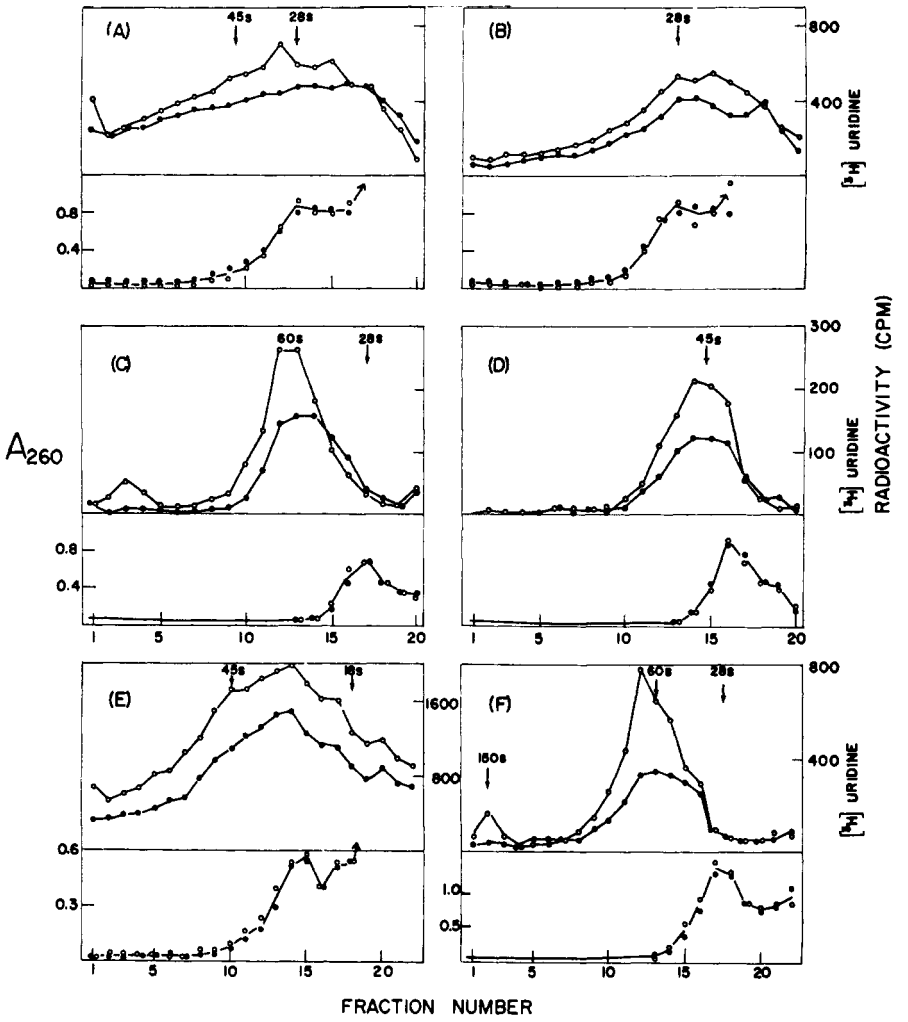

FIGURE 5: Refractionation of RNA from the 50S and greater region. (A) Cultures containing 60 × 10^6 nucleated cells/ml were incubated with (○—○) and without (●—●) 0.09 unit of erythropoietin ml for 3 hr, then pulsed for 30 min with 2 μCi/ml of [^{3}H]uridine. RNA was isolated by method II and run on a sucrose gradient for 80 min at 48,000 rpm in the SW50 rotor. (B) Cells were incubated under the same conditions as in A but chased for 3 hr by addition of 8 μg/ml of actinomycin and 5 μmoles/ml each of uridine and cytidine. (C and D) Fractions having S of 50 and greater from A and B, respectively, were pooled, precipitated with carrier RNA, and run for 35 min at 48,000 rpm. (E) Cultures containing 25 × 10^6 nucleated cells/ml were incubated for 24 hr, then 0.20 unit of erythropoietin (○—○) or an equal volume of medium (●—●) was added. After 3 hr more, all cultures were pulsed for 30 min with 2 μCi/ml of [^{3}H]uridine; RNA was isolated and analyzed as in A and B. (F) The 50S and greater region of E was rerun as in C and D. All cultures had a total volume of 3.0 ml and six replicates of each were pooled.

a population distinct from the nonstimulated cells we cannot provide a convincing explanation for this difference in rates of decay. It might be due to the presence of longer lived large RNA constituents in newly formed erythroid cells. Alternatively, the increased life span may reflect a stabilizing effect of erythropoietin on the newly formed RNA.

Under conditions of incubation where the rate of formation of 150 S is maximal (see Figure 8) a pulse-chase experiment showed its half-life to be about 6 min.

In a double-label experiment we examined the simultaneous incorporation of methyl groups from methionine and of uridine into the RNA of erythropoietin-treated marrow cells (Figure 6). The general pattern of RNA synthesis, as seen by uridine incorporation and analysis on a sucrose gradient, was heterodisperse with the only feature being a peak at the bottom of the tube. When the bottom fraction was rerun on another gradient, as described above, most of its uridine radioactivity was in a peak at 150 S. In contrast, the methyl group incorporation showed peaks at 45, 28, and 4 S as well as at the bottom, while the 55–65S region had no significant label.

We found that the sedimentation properties of the 150S, 55–65S, and 45S RNA fractions are not affected by DNase (5 μg/ml), pronase (20 μg/ml at 25° for 15 min), urea (8 M), or EDTA (0.01 M). Ribonuclease, however, causes rapid hydrolysis, showing that these three fractions represent discrete RNA species rather than complexes of RNA with DNA, protein, or other RNA.

The results of Figure 4 indicated that erythropoietin also stimulates the synthesis of RNA sedimenting in the 4–10S region. In addition, part of this RNA fraction accepts methyl groups from methionine (Figure 6), the peak of methyl-labeled RNA being consistently at 4 S,

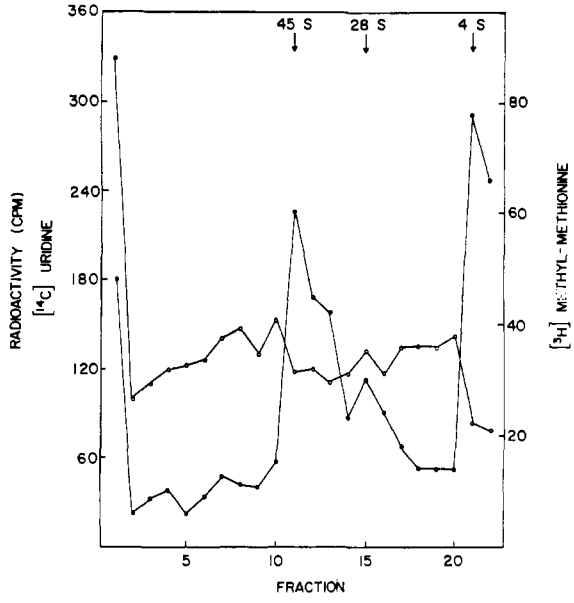

FIGURE 6: Relationship between uridine labeling and methylation of RNA synthesized in the presence of erythropoietin. Two cultures containing 40 × 10^6 nucleated cells/ml (total volume 3.0 ml) were preincubated for 10 hr, after which 0.10 unit of erythropoietin per ml of [^{14}C]uridine (○—○) (1 μCi/ml) and [^{3}H-*methyl*]methionine (●—●) (10 μCi/ml) were added to each. After a 15-min pulse RNA was isolated by method II, pooled, and analyzed on a sucrose gradient at 48,000 rpm for 90 min in the SW50 rotor.

whereas the uridine-labeled RNA sediments at 6 S. Recent work with other systems has shown that an RNA species, different from tRNA, and sedimenting at 5 or 7 S, is present in the "soluble" component of fractionated RNA (Virmaux *et al.*, 1964; Galibert *et al.*, 1967; Comb and Sarkar, 1967; Watson and Ralph, 1967). In the experiment represented in Figure 7, the 0–15S fraction of RNA, synthesized in the presence of erythropoietin, was fractionated on a second sucrose gradient for a longer time. The results show that there were peaks (uridine labeled) at 4, 6, and 9 S. The synthesis of all three is stimulated by erythropoietin at this time (3 hr after addition). Only the 4S RNA was appreciably methylated and presumably contains tRNA. Under these conditions a negligible fraction of the methyl radioactivity is found as methionyl-tRNA. The significance of the nonmethylated 6S and 9S RNA components will be considered later.

The relative base compositions of the different RNA fractions from erythropoietin-treated cells are shown in Table V. In all cases erythropoietin caused an increase in RNA synthesis of 30–40%. The relative base ratios of RNA in the 45S peak[1] are intermediate between those of 18S and 28S RNA, and 28S RNA has greater G/U and C/U ratios than 18S RNA. This agrees with the results obtained by others using ^{32}P (Scherrer and Marcaud, 1965; Hirsch, 1966). The relative base composition of the chased 55–65S RNA which is relatively free of contaminating 45S RNA shows C/U and G/U ratios that are smaller than those found for the 45S component. A greater AU and smaller GC content of the heterodisperse, high molecular weight nuclear RNA, when compared with 45S RNA, has been described in other cell systems (Yoshikawa-Fukada, 1965; Attardi *et al.*, 1966; Scherrer *et al.*, 1966; Floyd *et al.*, 1966). The relative base ratios of the 150S and 45S components are rather similar, and those of the 6S and 4S RNA components are almost identical.

TABLE V: Relative Base Compositions of RNA Components from Erythropoietin-Stimulated Cells.[a]

RNA Component	G/C	G/U	A/C	A/U	G/A	C/U
150 S	3.2	3.1	2.3	2.3	1.4	1.0
55–65 S*	3.7	2.2				0.59
45 S	2.9	2.9	2.6	2.7	1.1	1.0
28 S*	3.4	3.6				1.1
18 S*	2.5	2.1				0.83
9 S	3.3	3.4	3.9	3.9	0.87	0.97
6 S	4.8	4.8	5.0	5.0	0.95	0.97
4 S	5.4	4.9	5.4	5.0	0.99	0.95

[a] Cultures containing 50 × 10^6 nucleated cells/ml in a total volume of 3.0 ml with 0.10 unit/ml of erythropoietin were incubated for 4 hr; half of them was then pulsed for 30 min with 1 μCi/ml of [^{3}H]adenosine and [^{14}C]cytidine; the other half was similarly pulsed with [^{3}H]guanosine and [^{14}C]uridine. RNA was isolated by method II; sucrose gradient separation was for 80 min at 48,000 rpm in the SW50. The 0–15S region was rerun as described in Figure 7. The base ratios of the components sedimenting at 55–65, 28, and 18 S were determined in a similar manner but using [^{14}C]-uridine and either [^{3}H]cytidine or [^{3}H]guanosine and chasing the cultures for 3 hr. These results are an average of two experiments, except those with an asterisk which are averages of six.

To determine how stimulation of the various RNA components changes with time of exposure to erythropoietin, cultures were pulsed for 15 min with [^{3}H]uridine at different times after erythropoietin addition, and the isolated RNA was analyzed on a sucrose gradient (Figure 8). The rate of synthesis of 150S RNA was maximal in the 0–15-min period, decreased at 2–4 hr, rose to a small peak at 6 hr, and disappeared after 10 hr. In contrast, the stimulated rates of formation of RNA found in the 4–10S, 45S, and 55–65S regions gradually increased to a maximum at about 6 hr and then sharply decreased after that time.

Discussion

There is considerable evidence indicating that erythropoietin acts upon a primitive cell type to induce

[1] We are aware that each peak in a sucrose gradient may contain several species of RNA and do not imply that the base composition represents that of a single component, but use the measurements to show differences and/or similarities among the various types of RNA.

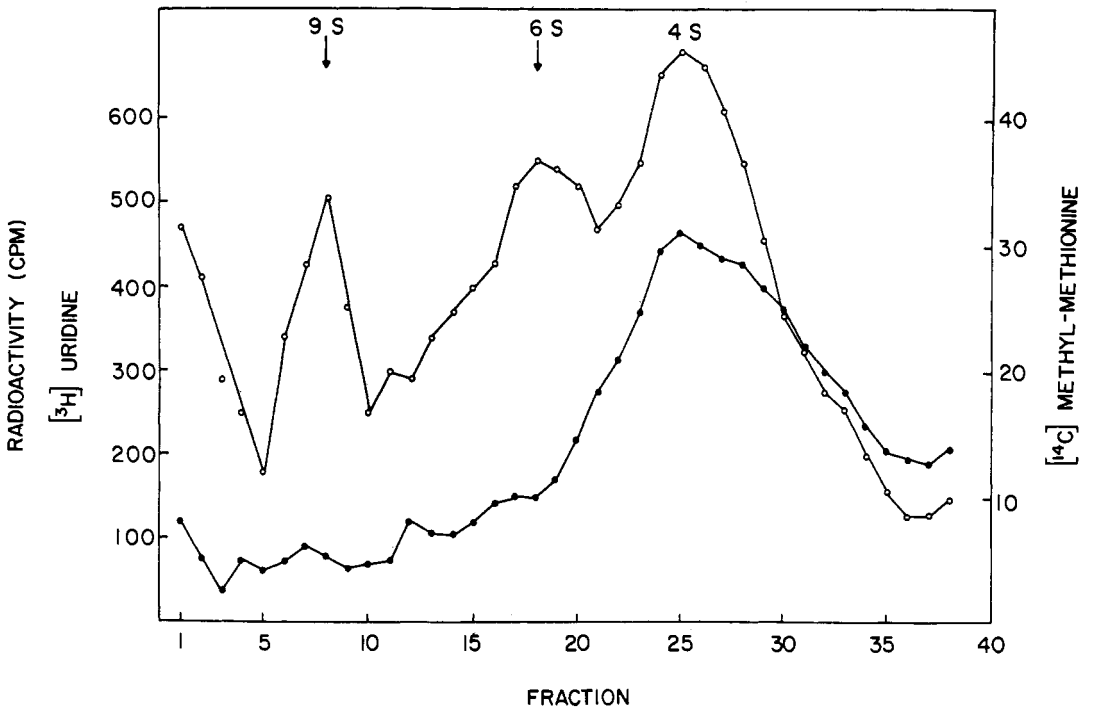

FIGURE 7: Refractionation of RNA from the 0 to 15S region. Four cultures containing 50 × 10^6 nucleated cells/ml, in 3.0 ml, were incubated in the presence of 0.08 unit of erythropoietin/ml. After 3 hr, [^{3}H]uridine (5 μCi/ml) and [^{14}C-*methyl*]methionine (1 μCi/ml) were added. After a 30-min pulse RNA was isolated by method II and fractionated on a sucrose gradient centrifuged at 48,000 rpm for 80 min in the SW50 rotor. The 0–15S portion was precipitated with carrier RNA and rerun on a sucrose gradient for 12 hr at 48,000 rpm. [^{3}H]Uridine (○—○) and [^{14}C-*methyl*]methionine (●—●).

erythroid differentiation. The morphological and chemical changes accompanying the later stages of this developmental process have been extensively studied, but induction of these changes has come under investigation only recently. Previous work from this laboratory has shown that the addition of erythropoietin to rat bone marrow cells *in vitro* causes several biochemical changes including the stimulation of RNA synthesis in as short a time as 15 min. Data presented here have shown that the earlier characterization of this RNA as sedimenting between 12 and 24 S was faulty, because of some degradation of RNA during the isolation procedure. Other investigators have isolated RNA from different cell systems using procedures similar to method I and have not found the degradation pattern we observe with bone marrow (Yoshikawa-Fukada *et al.*, 1965; Attardi *et al.*, 1966; Floyd *et al.*, 1966). Minguell and Perretta (1967), however, have recently shown that rat bone marrow cells have an unusually high activity of nuclear ribonuclease when compared with other tissues. Since we find that the rapidly labeled RNA of bone marrow is restricted almost exclusively to the nucleus, the difference between marrow and other tissues may reflect this nuclear ribonuclease activity.

In the present report we have shown that erythropoietin stimulates the synthesis of rapidly labeled RNA fractions sedimenting at 150, 55–65, 45, 9, 6, and 4 S, and that the stimulated 45S RNA is converted into 28S and 18S rRNA. Our observation that erythropoietin increases the rate of both r- and tRNA synthesis is compatible with the fact that erythroid differentiation involves extensive hemoglobin formation. The induction of specific erythroid functions by erythropoietin indicates that some types of mRNA may also be formed:

The 6S RNA component whose synthesis is stimulated by erythropoietin is unmethylated and has relative base ratios almost identical with 4S RNA. Our data also indicate that this component is synthesized in an amount approximately equimolar with 45S RNA and 28S and 18S r RNA. Many animal cell systems contain 5–7S RNA species (Virmaux *et al.*, 1964; Galibert *et al.*, 1967; Comb and Sarkar, 1967; Watson and Ralph, 1967). This class of RNA lacks methylated bases and pseudouridine, but otherwise has a base composition almost identical with tRNA (Virmaux *et al.*, 1964). It appears to bind to the 60S ribosomal subunit in a 1:1 ratio (Comb and Sarkar, 1967). Hybridization and rate study experiments have indicated that this RNA is neither tRNA precursor nor mRNA, and it is considered to be a third class of rRNA (Galibert *et al.*, 1967; Watson and Ralph, 1967). The 6S RNA component in erythropoietin-stimulated marrow cells probably represents this type of RNA. A 5S RNA component isolated from rabbit reticulocyte ribosomes (Huez *et al.*, 1967) may represent an RNA similar to the 6S RNA of rat marrow.

We think that the possibility that 55–65S and 150S RNA represent aggregates of smaller RNA molecules is minimal. Considerable evidence indicates that other similar rapidly sedimenting RNA molecules are single, linear, polynucleotide chains rather than aggregates. These classes of RNA can be treated with DNase, pronase, EDTA, or urea and refractionated without significant change in sedimentation properties (Attardi *et al.*,

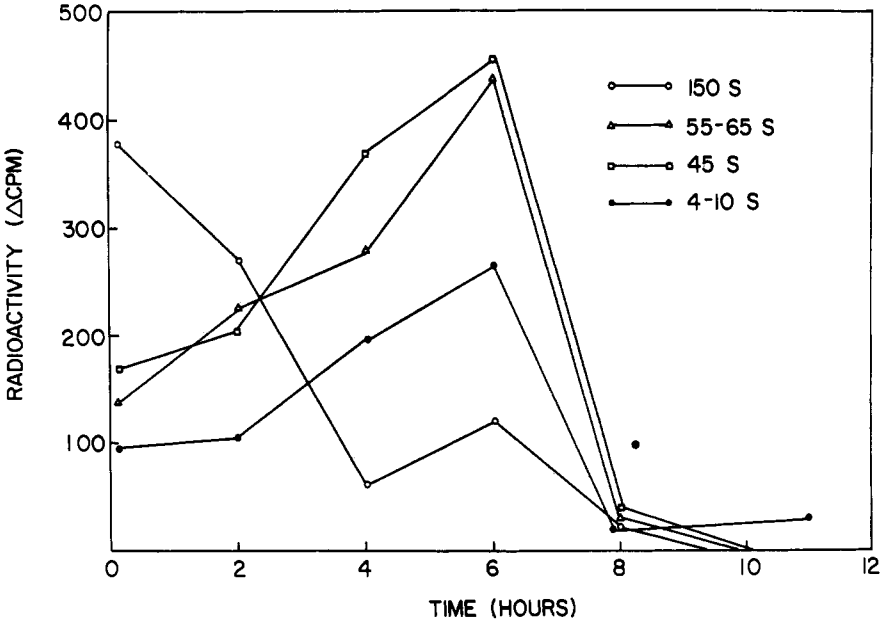

FIGURE 8: Variations in rates of erythropoietin-stimulated RNA synthesis with time. Cultures containing 30×10^6 nucleated cells/ml in a total volume of 3.0 ml were preincubated for 7 hr, and then given 0.25 unit of erythropoietin/ml or an equal volume of medium. At each indicated time one control and one stimulated culture were pulsed for 15 min with [^{3}H]uridine (2 μCi/ml) after which RNA was isolated by method II. Density gradient analyses were done at 48,000 rpm for 80 min in the SW50 rotor. The ordinate indicates the change in counts per minute due to erythropoietin for each of the components isolated.

1966; Scherrer *et al.*, 1966). Such molecules appear as single threads 4–6 μ long when visualized under the electron microscope (Scherrer *et al.*, 1966). Bramwell and Harris (1967) have recently suggested, however, that all nuclear RNA may be composed of 16S subunits. Wagner *et al.* (1967) have shown that rRNA can aggregate into larger sedimenting forms under certain conditions of high RNA concentration and hot phenol extraction. The latter results indicate that the exact chemical and physical nature of very rapidly sedimenting RNA may not be completely clear. We have found that the hot phenol extraction of method II does not cause aggregation of the smaller sized RNA isolated by the low-temperature phenol. The 150S, 55–65S, and 45S RNA components we find in marrow cells are also completely stable to treatment with EDTA, urea, pronase, or DNase, but are degraded by treatment with RNase indicating that these components represent distinct RNA species.

The role of 150S RNA in erythroid development is still completely unclear. Of all the RNA types studied, only this species, with a minimum molecular weight of about 18 million, seems to be unique to erythropoietin-stimulated cells of the marrow. In the first 15 min after erythropoietin addition, the formation of this component is stimulated to a much greater degree than that of the other RNA components. The fact that it is methylated might suggest a role for 150S RNA as a precursor of 45S RNA or of tRNA. These possibilities cannot be excluded on the basis of our present data. Since the 150S RNA component disappears rapidly during a chase it cannot be the long-lived messenger for globin synthesis. It is possible, however, that it represents what Scherrer *et al.* (1966) have termed "a unit of transcription," only an undetectably small part of which is the messenger for whatever function has been initiated by erythiopoietin. If, for example, the unit of transcription contained the messenger for globin, which was freed from all the other transcribed RNA in the 150S unit by degradation within the nucleus, less than 1% would be transferred to the cytoplasm. The rationale for transcribing a very large RNA molecule in order to obtain a very small messenger has been discussed by Scherrer (1968). Whether 150S RNA, or part of it, acts as a messenger, has a regulative function within the nucleus, or does something else must await further investigation.

Extensive study of 9S RNA isolated from rabbit reticulocytes indicates that it is long-lived, and suggests that it might be mRNA for globin synthesis (Arnstein *et al.*, 1964; Kruh *et al.*, 1964). Burny and Marbaix (1965) have demonstrated that the 9S RNA is associated with the polysomes of rabbit retriculocytes, is preferentially labeled after a 15-hr pulse *in vivo*, and is more sensitive than rRNA to degradation when reticulocyte polyribosomes are treated with a low level of RNase (Huez *et al.*, 1967). These properties are considered characteristic of mRNA. The base composition of rabbit reticulocyte 9S RNA reported by Marbaix *et al.* (1966) agrees with the relative base composition we obtain for rat bone marrow 9S RNA. It would be tempting to conclude that erythropoietin stimulation of the synthesis of 9S RNA in the nucleus of rat bone marrow cells represents the induction of globin mRNA. The demonstration of messenger activity, however, must be functional and would require showing that this specific RNA codes for globin. Considerable effort has been made to demonstrate this relationship between rabbit reticulo-

cyte 9S RNA and hemoglobin synthesis (Arnstein *et al.*, 1964; Kruh *et al.*, 1964; Schapira *et al.*, 1966). Recent work has indicated, however, that the added RNA in such experiments may affect endogenous messenger activity rather than act as a specific template (Cox and Arnstein, 1964; Schaeffer *et al.*, 1964; Drach and Lingrel, 1966; Hunt and Wilkinson, 1967). We have found that synthesis of both the 55–65S RNA and the 9S RNA are stimulated by erythropoietin and that they are rapidly labeled, relatively long lived, and distinct from the known rRNA species. Assignment of a definite functional role for these two RNA components in developing erythroid cells, however, must await further study.

We have presented evidence that erythropoietin rapidly induces a complex set of changes in synthesis of RNA by cultured rat bone marrow cells. Erythropoietin increases the production of r- and tRNA and stimulates the synthesis of other RNA components whose role in erythroid differentiation still remains to be determined. This study indicates, however, that the simplified model of erythropoietin-induced cytodifferentiation based solely on events at the transcriptional level (Goldwasser, 1966) will require modification in order to incorporate the findings reported here.

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