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Hemoglobin Messenger Ribonucleic Acid. Synthesis of 9S and Ribosomal Ribonucleic Acid during Erythroid Cell Development*

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ABSTRACT: When reticulocyte polysomes are dissociated with sodium dodecyl sulfate and analyzed by sucrose density gradient centrifugation, a ribonucleic acid of 9 S (9S ribonucleic acid) is observed. This ribonucleic acid exhibits many of the properties expected for the hemoglobin messenger ribonucleic acid, such as size, per cent of total ribonucleic acid, and involvement in polysomal structure.

Because of these unique features it was of interest to study its synthesis in relation to ribosomal ribonucleic acid synthesis during erythroid cell development. This was accomplished by injecting [^{3}H]uridine into anemic mice at various times prior to collecting the circulating reticulocytes. Cells obtained shortly after injection of the ribonucleic acid precursor are those reticulocytes originating from more mature nucleated erythroid cells while those reticulocytes obtained at longer times originate from more immature precursor cells. The data indicate that ribosomal ribonucleic acid is synthesized early in erythroid cell development while 9S ribonucleic acid synthesis is maximal in later cells.

Rabbit reticulocyte polysomes contain a 9S RNA exhibiting many of the properties expected for the hemoglobin mRNA (Marbaix and Burny, 1964; Burny and Marbaix, 1965; reviewed by Chantrenne *et al.*, 1967). Further support for this RNA being the hemoglobin mRNA has been obtained in our laboratory by showing that only one 9S RNA occurs per polysomal structure irrespective of the number of ribosomes in the polysome (Evans and Lingrel, 1969). Since polysomes are thought to be two or more ribosomes held together by a single mRNA molecule, the observation that various size polysomes contain a single 9S RNA is strong evidence in favor of this RNA being the hemoglobin mRNA.

The synthesis of 9S RNA in erythroid cells has been studied by Marbaix and Burny (1964). These workers have observed that the 9S RNA exhibits a specific activity higher than rRNA when rabbits were injected with ^{32}P 10–20 hr prior to removal of reticulocytes. This finding was interpreted to indicate that the 9S RNA was either rapidly turning over or that more 9S RNA than rRNA was synthesized after ^{32}P injection. As reticulocytes contain a stable mRNA (Marks *et al.*, 1962a,b; Burny and Chantrenne, 1964) it occurred to us that the 9S RNA was

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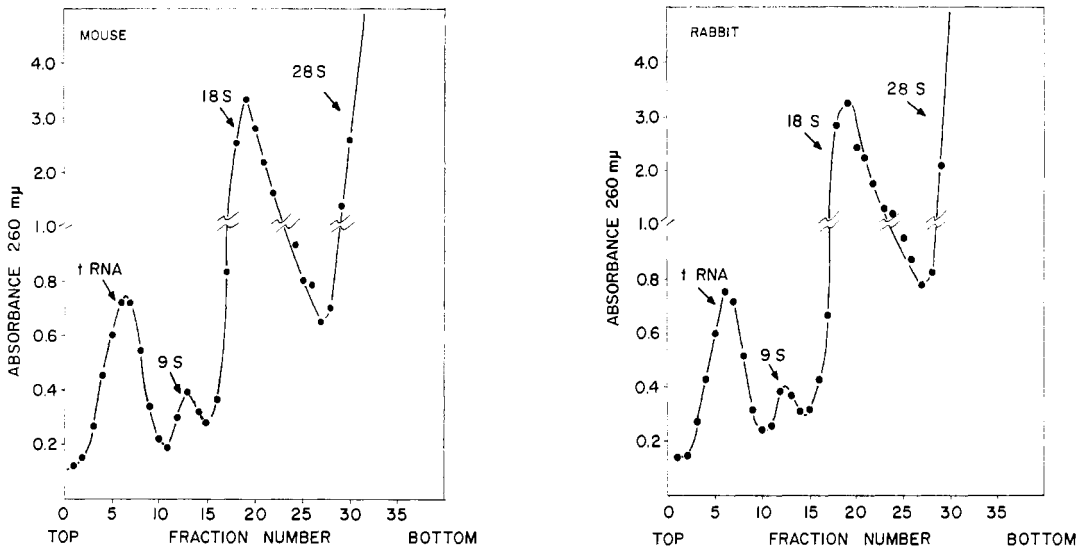

FIGURE 1: Sucrose density gradient analysis of mouse and rabbit reticulocyte polysomes. To 8 mg of polysomes in 1 ml of 5 mM Tris-HC buffer (pH 7.4) was added 0.05 ml of 10% sodium dodecyl sulfate. This solution was incubated at 37° for 5 min and layered on a 5–20% exponential sucrose gradient. Centrifugation was performed in a SW27 rotor for 25 hr at 27,000 rpm. The run was made at 1.5°. (a) Mouse polysomes and (b) rabbit polysomes.

not necessarily turning over but probably was made later in erythroid cell development than rRNA. If the foregoing consideration holds, then collection of cells soon after injection of labeled precursor, when only the more mature erythroid cells appear as reticulocytes, would result in a 9S RNA having a specific activity higher than rRNA. However, if the interval between the administration of labeled precursor and collection of reticulocytes were lengthened, the earlier erythroid cells would mature to reticulocytes; and the reverse labeling pattern would be expected, *i.e.*, rRNA would exhibit a higher specific activity than 9S RNA. Differential RNA labeling as a function of time would be strong evidence that the two RNAs are made at different stages of erythroid cell development.

This communication describes the presence of 9S RNA in mouse reticulocytes and compares its synthesis with rRNA during erythroid cell development.

Methods

Production of Anemia and Collection of Reticulocytes. Mice were made anemic by six daily intramuscular injections of 0.1 ml of 0.8% neutralized phenylhydrazine hydrochloride (Bishop *et al.*, 1961); 25-g Swiss Cox strain mice were used. On the 7th day blood was collected, heparinized, cooled to 2°, and washed three times with five volumes of cold isotonic NKM solution (0.13 M NaCl, 5 mM KCl, and 7.4 mM $MgCl_2$). The buffy coat containing white cells was removed from the packed cells after the first wash and discarded.

Preparation of Ribosomes. Cells were lysed by adding four volumes of 5 mM $MgCl_2$ followed 30 sec later by one volume of 1.5 M sucrose containing 0.15 M KCl. This procedure lyses reticulocytes, but not white cells. The lysate was centrifuged at 12,000*g* for 10 min; and the supernatant was layered over 7 ml of 36% w/w sucrose containing 0.1 M Tris-HCl (pH 7.4), 15 mM KCl, and 5 mM magnesium acetate and centrifuged at 78,000*g* for 3 hr in a Type 30 Spinco rotor (Huez *et al.*, 1967). The ribosomal pellet was resuspended in 5 mM Tris-HCl buffer (pH 7.4) with the aid of a loose-fitting homogenizer. The resuspended ribosomes were centrifuged for 10 min at 12,000*g* and the ribosome-containing supernatant was removed and adjusted to a concentration of 8 mg/ml; 10 absorbance units at 260 mμ was taken to equal 1 mg/ml of ribosome.

Labeling of Reticulocytes. To label RNA each mouse was given an intraperitoneal injection of 50 μCi of [5-^{3}H]uridine (8 Ci/mmole). The radioactivity appeared in the blood within a few minutes and disappeared with a half-life of about 10 min. Generally ten mice were used for each labeling time.

Analysis of RNA. The RNA of mouse reticulocyte ribosomes was dissociated from protein using the general procedure of Marbaix and Burny (1964). To the ribosomal suspension (8 mg/ml) in 5 mM Tris-HCl (pH 7.4) was added enough 10% sodium dodecyl sulfate to give a final concentration of 0.5.%. This solution was incubated at 37° for 5 min and immediately layered on precooled sucrose gradients.

Sucrose Density Gradient Analysis. Exponential sucrose gradients (5–20%) were prepared according to the method of Noll (1967). The mixing chamber contained 40 ml of 5% sucrose w/w in 5 mM Tris-HCl (pH 7.4) and the reservoir was filled with 30% sucrose w/w also dissolved in the Tris buffer; 15–30% sucrose gradients were prepared by placing 36 ml of 15% sucrose in the mixing chamber and 40% sucrose in the reservoir. These sucrose solutions were also w/w in 5 mM Tris-HCl buffer (pH 7.4). The tubes were filled to a volume of 37 ml, 1 ml of sample was added, and the tubes were placed in a SW27 rotor. Gradients were run at an average force of 90,000*g* at 1.5° for 28 hr or as indicated. Ribonuclease-free sucrose obtained from Mann Research Laboratories was used throughout these studies.

The gradients were analyzed by introducing 40% sucrose w/w into the bottom of the tube and continuously monitoring the solution emerging from the top at 260 mμ using a flow cell, spectrophotometer, and recorder.

Measurement of Radioactivity. As the gradient material

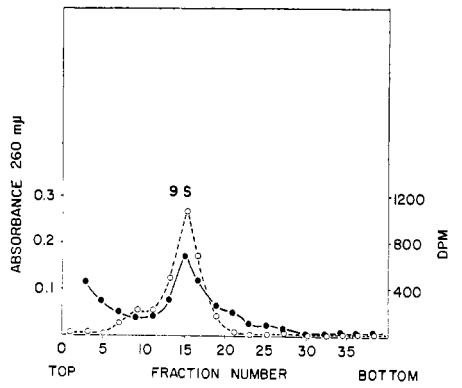

FIGURE 2: Sucrose density gradient analysis of pooled 9S RNA. Tubes 16–18 from a run similar to that depicted in Figure 4b were pooled and layered on a 15–30% exponential sucrose gradient and centrifuged at 27,000 rpm for 44 hr at 1.5° in a SW27 rotor. The solid line is absorbance at 260 mμ and the dashed line is radioactivity/1-ml fraction

emerged from the flow cell it was collected in 1-ml fractions. To each fraction was added 1 mg of carrier RNA and enough 25% trichloroacetic acid to give a final concentration of 7%. Precipitation and subsequent washing were performed at 0°. The tubes were allowed to stand for 1 hr to ensure precipitation and centrifuged for 10 min at 600*g*. The precipitate was washed with 5 ml of 5% trichloroacetic acid and the RNA pellet was dissolved in 0.4 ml of 5% trichloroacetic acid by heating at 90° for 10 min. The 0.4-ml sample was quantitatively transferred to a counting vial with the aid of 15 ml of liquid scintillation counting solution (Lingrel, 1967). All counts were corrected to disintegrations per minute.

TABLE I: Effect of Various Enzymes on Labeled 9S RNA.[a]

Enzyme Addn	% of 260-mμ-Absorbing Material Converted into Cl_3CCOOH-Soluble Material	% cpm Pptd by Cl_3CCOOH after Incubn with the Indicated Enzymes
DNase	6	98
RNase	96	1
Pronase	0	109

[a] The enzyme assays were performed as follows. To 1 ml containing 11 μg of labeled 9S RNA and 147 cpm was added 0.05 ml of 0.2 M $MgCl_2$ and 0.1 ml of one of the enzymes listed in the table. The enzymes were dissolved in 5 mM Tris-HCl (pH 7.4) and their final concentrations in the reaction mixtures were as follows: DNase, 10 μg/ml; RNase, 10 μg/ml; and Pronase, 50 μg/ml. These enzyme concentrations were found to completely convert 30 μg of DNA, RNA, and protein, respectively, into trichloroacetic acid soluble material under the conditions of the assay. The tubes were incubated at 37° for 20 min, cooled to 0°, and 0.02 ml of 2.5% serum albumin was added as carrier. This was followed by the addition of 0.3 ml of 50% trichloroacetic acid. The filtrate was read at 260 mμ using a 2-cm light-path cell and the precipitate was collected on Millipore filters. The filters were dried and added to the liquid scintillation counting fluid and counted. The counts were not corrected to disintegrations per minute.

Results

Presence of 9S RNA in Mouse Reticulocytes. For labeling studies it was considered advantageous to use small animals such as mice. This would provide the opportunity of obtaining high specific activities with the injection of minimum amounts of label and also reduce the biological variability by allowing the use of several animals for each time point. However, as 9S RNA had only been observed in rabbit reticulocyte ribosomes, it was necessary to show its presence in mouse reticulocyte ribosomes. That the 9S RNA occurs in these ribosomes is shown by the sucrose density gradient analysis of the sodium dodecyl sulfate treated mouse ribosomes (Figure 1a). This pattern is identical with that obtained from rabbit reticulocyte ribosomes (Figure 1b) (also see Burny and Marbaix, 1965). The 9S RNA from both rabbits and mice exhibit a typical RNA absorption spectrum.

Labeling of Mouse 9S RNA. When mice were injected with [^{3}H]uridine 20 hr prior to collecting reticulocytes, the labeling pattern shown in Figure 4b is obtained. Again similar to rabbits (Marbaix and Burny, 1964), the mouse 9S RNA exhibits a specific activity higher than rRNA. Figure 2 shows the recentrifugation of the 9S peak. The RNA appears as a single peak and the radioactivity coincides with the major fraction. Both the 260-mμ-absorbing material and radioactivity are sensitive to ribonuclease but not to deoxyribonuclease or Pronase, further indicating that the label is in RNA (Table I).

To exclude the possibility that the labeled RNA originates from contaminating white cells, ribosomes were isolated from preparations whose white cells had not been removed and compared with those whose white cells had been removed. No differences in the 260-mμ absorbance or labeling patterns were detected (Figure 3).

Comparison of 9S and rRNA Synthesis during Erythroid Cell Development. The RNA of reticulocytes is synthesized in the nucleated erythroid precursor cells of the bone marrow and not in reticulocytes. To observe the synthesis of RNA, at the various stages of erythroid cell development, labeled uridine was injected into the animals at various times prior to the collection of circulating reticulocytes. Those cells entering the circulation at short intervals after administration of label originate from mature nucleated cells, while those entering at later times originate from earlier or more immature cells. Labeled RNA from reticulocytes collected shortly after injection would be that RNA which was synthesized at a rather mature stage of development, such as the orthochromatic and polychromatic erythroblast stages, while labeled RNA from reticulocytes collected at longer labeling times represents RNA synthesized at a more immature stage of development, such as the pronormoblast and basophilic erythroblast.

Such labeling studies were performed as follows. Mice were made anemic over a 6-day period by daily injections of phenylhydrazine. The animals were sacrificed and reticulocytes collected on the 7th day. The injections of [^{3}H]uridine were given at various times prior to the collection of cells on the 7th day.

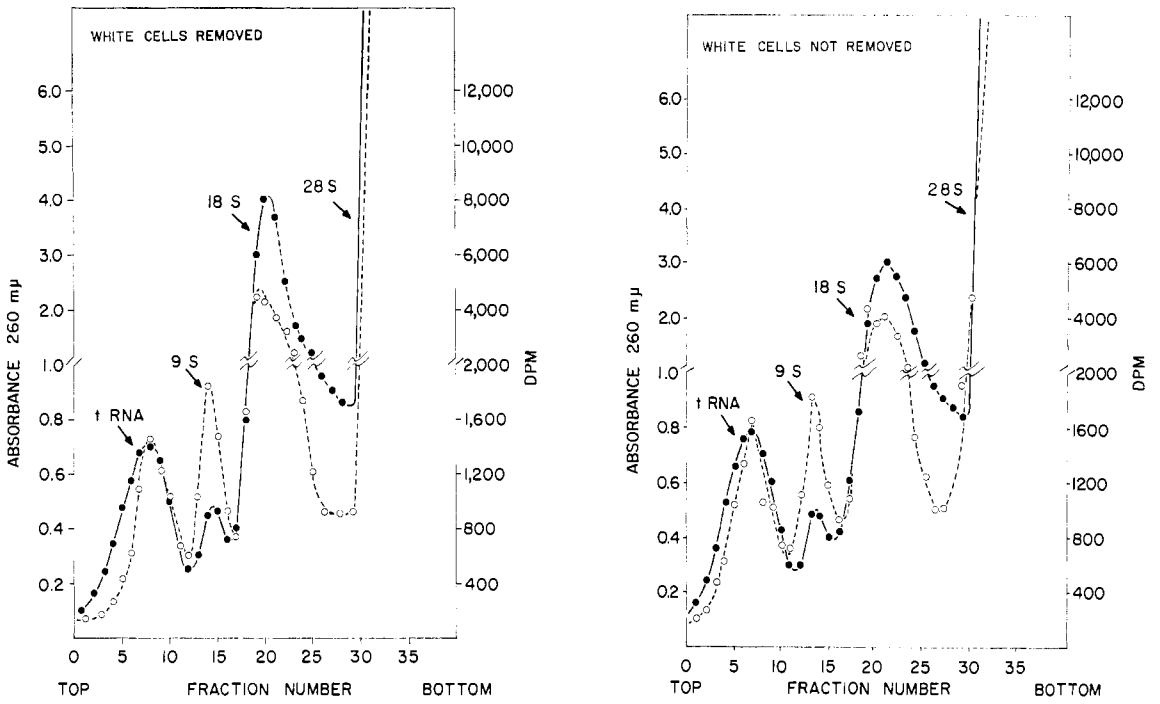

FIGURE 3: Sucrose density gradient analysis of sodium dodecyl sulfate treated mouse reticulocyte polysomes. Polysomes were isolated from (a) preparations where the white cell buffy coat had been removed and (b) preparations where the buffy coat had not been removed. The solid line is absorbance at 260 mμ and the dashed line is radioactivity.

In this way all animals exhibited the same extent of anemia when the reticulocytes were removed. The ribosomes, isolated from the cells collected at the various time points, were treated with 0.5% sodium dodecyl sulfate and subjected to sucrose density gradient analysis. The results are shown in Figure 4 and the specific activities of the various RNAs with time are summarized in Figure 5. When the label is injected into the animals 16–24 hr prior to collecting the reticulocytes, the 9S RNA exhibits a specific activity higher than either 18S or 28S rRNA and is at a maximum at 24 hr. In contrast rRNA does not reach a maximum until 36 hr. The labeling in the tRNA region was not plotted because of its contamination with 5S RNA and because of the possibility of exchange of nucleotides at the 3′ end of the tRNA.

Discussion

The presence of a 9S RNA in mouse reticulocyte ribosomes has been shown, and its properties appear similar to that of the 9S RNA of rabbit reticulocyte ribosomes. In addition the results of these studies support the idea that the 9S RNA of reticulocyte polysomes is a unique RNA. The present study also indicates that erythroid cells are capable of synthesizing 9S RNA later in their development than rRNA. Although the data indicate that somewhat less 9S RNA is synthesized in immature cells than mature ones, how much less is uncertain. This is due to the inability of evaluating the life span of reticulocytes in the circulation. Therefore, some of the reticulocytes which entered at 24 hr (containing a high specific activity 9 S) may still be present at 36 and 40 hr and the actual labeling of 9S RNA at these times may be even less. Regardless of this uncertainty, it is quite clear that early cells do not synthesize more 9S RNA than late cells. It follows from the data that 9S RNA is synthesized or still being synthesized about 12 hr after rRNA and must therefore be added to preformed ribosomes.

Recent studies in our laboratory (R. E. Lockard and J. B. Lingrel manuscript in preparation) have shown that mouse 9S RNA directs the synthesis of mouse hemoglobin when added to a rabbit reticulocyte cell-free system. This observation clearly indicates that this RNA is the hemoglobin mRNA. Schapira *et al.* (1968) and Laycock and Hunt (1969) have presented evidence for a similar RNA fraction isolated from rabbits directing the synthesis of rabbit hemoglobin in a cell-free system. It is therefore of interest to consider the results of the present study in relation to hemoglobin synthesis. It is known that very little if any hemoglobin synthesis occurs in the early erythroid cells (pronormoblasts and basophilic erythroblasts) but rather occurs in more mature cells such as the polychromatic erythroblasts, orthochromatic erythroblasts, and reticulocytes (Thorell, 1947). Although the data reported here do not give a direct assessment of the cell type in which the various RNAs are synthesized, one can make reasonable assignments. For example, it is known from radioautographic studies that the pronormoblast and basophilic erythroblast are the most active cells in RNA synthesis (Torelli *et al.*, 1964; Schmid *et al.*, 1966; Borsook, 1964a). The pronormoblasts are more active than the basophilic erythroblasts, but as there are many more basophilic erythroblasts than pronormoblasts one can assume that the majority of RNA synthesis occurs in the basophilic erythroblasts. As rRNA represents approximately 80% of the cellular RNA, it follows that the labeled rRNA appearing 36 hr after injection of [^{3}H]uridine was synthesized in basophilic erythroblasts. The 9S RNA must then be made or continue to be made in one or both of the two remaining nucleated cell types, the polychromatic or orthochromatic erythroblast. The orthochromatic stage is thought to be skipped

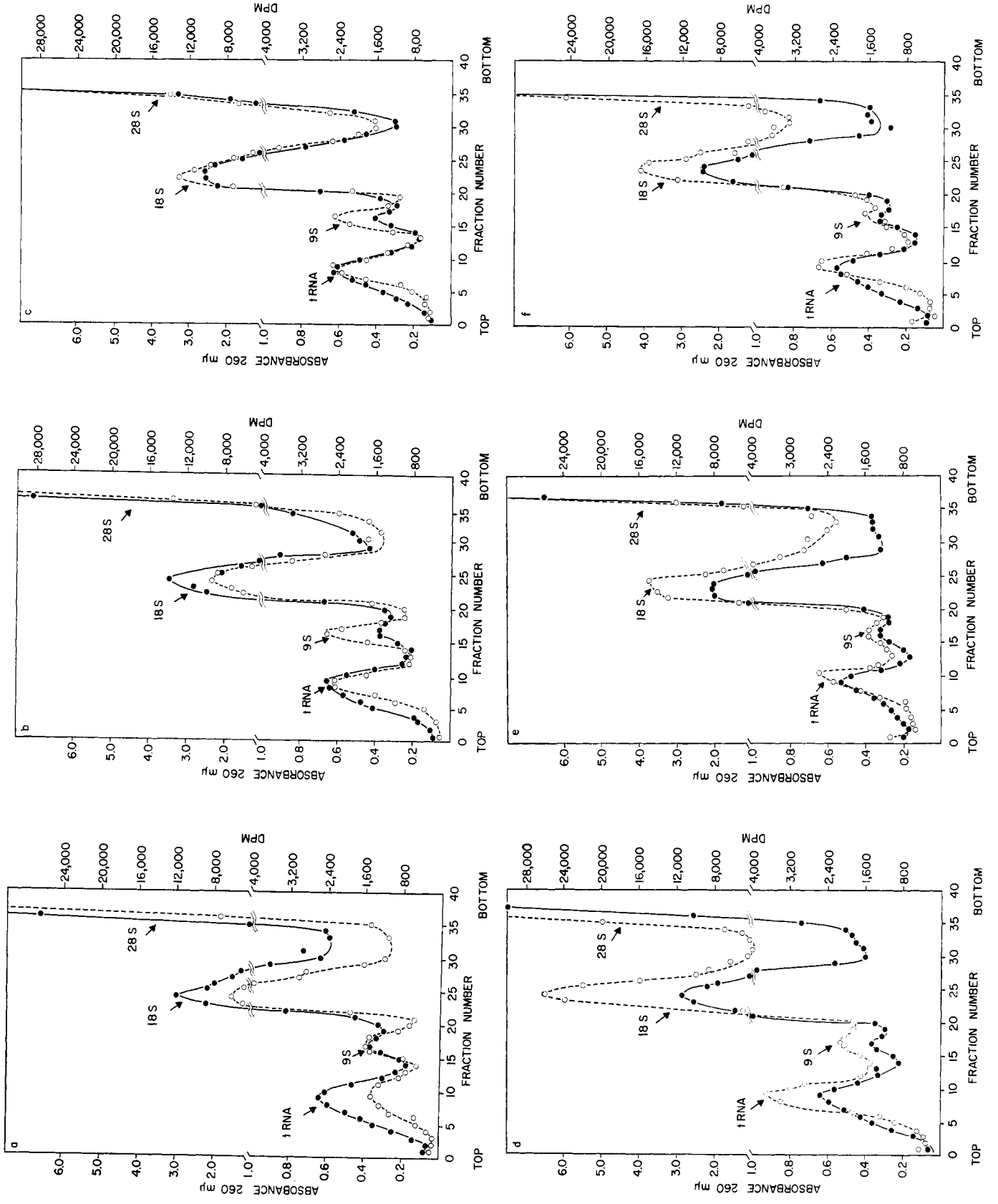
a
b
c
d
e
f
ABSORBANCE 260 mμ
DPM
FRACTION NUMBER
TOP
BOTTOM
28 S
18 S
9 S
t RNA

FIGURE 4: Sucrose density gradient analysis of labeled RNA from mouse reticulocyte polysomes. Polysomes were obtained from animals injected with 50 μCi of [^{3}H]uridine at the times indicated prior to collection of reticulocytes. (a) 16, (b) 20, (c) 24, (d) 36, (e) 40, and (f) 45 hr. The solid line is absorbance at 260 mμ and the dashed line is radioactivity/1-ml fraction.

in severe anemia (Borsook *et al.*, 1962; Borsook, 1964b), so it seems reasonable to assume that 9S RNA can be made in polychromatic erythroblasts. As mentioned above, the experimental approach does not allow an unequivocal estimate of 9S RNA synthesis in the more immature erythroid cells. Therefore the synthesis of 9S RNA in relation to hemoglobin synthesis is somewhat uncertain and two possibilities exist; first, if 9S RNA synthesis occurs in the more immature cells then it must not be used until later in development when hemoglobin synthesis is initiated (polychromatic state), or (2) if 9S RNA synthesis occurs only in the more mature cells then it may just precede hemoglobin synthesis and be the controlling factor in its production. Further investigation will be required to substantiate this relationship.

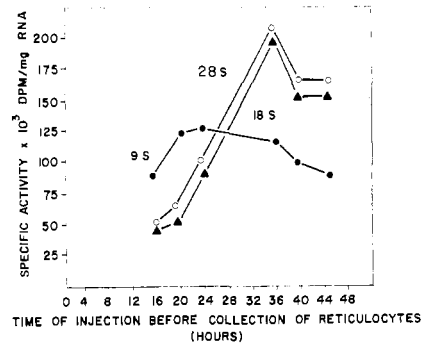

FIGURE 5: Specific activity of 9S and rRNAs isolated from animals injected with [^{3}H]uridine at various times prior to collection of reticulocytes. The specific activities of the various RNAs were obtained by averaging the values of the peak fractions.

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