Membrane-Bound Ribonucleic Acid in Mammalian Erythroid Cells*

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ABSTRACT: It has been demonstrated that a significant proportion of mammalian erythroid cell ribonucleic acid (RNA) is closely associated with hemoglobin-free cell membranes (bound RNA). In random samples of peripheral blood, with reticulocytosis varying from 2 to 99%, 31.1 ± 8.9% of the total cellular RNA was membrane bound. The bound RNA is identical with free erythroid cell RNA in base composition and proportions of 4S, 18S, and 28S molecular species. The amount of RNA bound to erythroid cell membranes was not affected by fragmenting the membranes, destroying their selective permeability by treatment with 0.4 M butanol or 1% saponin, or by exposure to 1.5 M NaCl. Removing the negative charge on fragmented membranes with neuraminidase caused the release of 15% of the bound RNA, as did treatment with 50 μg/ml of pronase. Lecithinase "C" did not cause release of the membrane-bound RNA, but cobra venom and 0.2% desoxycholate, both of which are hemolytic, did. The precise nature of the binding was not ascertained, but it appeared to be related to the intact structural complexity of the membrane.

Newly synthesized RNA appeared in the membrane-bound fraction of erythroid cell RNA at a slower rate than in the free fraction, and was preferentially localized there with time. Erythroid RNA was less susceptible to degradation with pancreatic ribonuclease when it was membrane bound. The findings indicate that the cell membrane plays a role in nucleic acid metabolism within the erythroid cell, and suggest that binding to the membrane may influence the physiologic stability of erythroid RNA.

Certain characteristics of nucleic acid synthesis and destruction in the mammalian erythroid cell made it a useful model for the study of macromolecular metabolism. In the course of differentiation and maturation the capacity of the cell to synthesize protein, and the cell content of RNA, progressively decrease after the basophilic erythroblast stage (Bertles and Beck, 1962; Grasso *et al.*, 1963; Burka *et al.*, 1964). RNA is no longer synthesized in the reticulocyte (Marks *et al.*, 1962), but protein synthesis continues until the cell becomes a mature erythrocyte (Kruh and Borsook, 1956). Although the cell membrane actively participates in a variety of metabolic activities, no role has yet been defined for it in nucleic acid metabolism of the erythroid cell. These studies were designed to determine whether the cell membrane takes part in erythroid cell RNA metabolism. The findings show that approximately one-third of mammalian erythroid cell RNA, referred to as "bound" RNA, is closely associated with the cell membrane. The composition, metabolism, mode of attachment, and susceptibility to ribonuclease of this fraction of erythroid cell RNA have been examined. The data indicate that the cell membrane may influence the rate of degradation of nucleic acid in the mammalian erythroid cell.

Materials and Methods

Preparation and Isolation of Erythroid Cell Fractions. Peripheral venous blood, obtained from normal New Zealand white rabbits or from animals in which a reticulocytosis had been induced by daily subcutaneous injections of a 2.5% solution of phenylhydrazine hydrochloride, was collected in heparinized syringes and immediately placed in an ice bath. All subsequent procedures were done at 0–4°. The cells were separated by centrifugation and washed twice with 310 mosm[1] phosphate buffer (pH 7.4). After each washing the buffy coat was removed by aspiration. The cells were kept in 310 mosm buffer for 30 min and then lysed with eight volumes of 20 mosm phosphate buffer (pH 7.4) as described by Dodge *et al.* (1963). The cell membranes were separated by centrifugation at 17,300*g* for 10 min. The membrane-free supernatant was decanted and brought up to a known volume with 20 mosm buffer. The loosely packed sedimented cell membranes were separated from the hard button of unlysed nucleated cells at the bottom of the tube by gentle agitation and rewashed one or more times in 20 mosm buffer. Although it is difficult to remove leukocytes and nucleated red blood cells from intact cell preparation rich in reticulo-

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[1] Abbreviations used: mosm, milliosmolar; TCA, trichloroacetic acid.

cytes (Holt *et al.*, 1966), membranes prepared in this manner were devoid of nucleated elements. This was confirmed by phase-contrast microscopy.

Analytical Methods. Hemocytometry, enumeration of reticulocytes, and determination of hematocrit were done by standard methods. Hemoglobin concentrations were determined by the method of Rimington (1942).

RNA was extracted by a previously described modification (Burka, 1966) of the Schneider (1957) procedure. Extractions were done on whole lysates or cell components derived from 0.25 to 1.0 ml of packed red blood cells. Owing to the presence of large amounts of lipid material 5 ml of 50% TCA in ethanol–ether (3:1) was used for the initial precipitation of the cell membranes. RNA ribose was determined by the orcinol reaction (Schneider, 1957) using *d*-ribose as a standard. The amount of purine-bound ribose was converted to RNA by multiplying by 4.31. This factor was derived from comparison of orcinol determinations and absorbance at 260 mμ on purified reticulocyte RNA, assuming an $E_{1\text{ cm}}^{0.1\%}$ of 24. No RNA ribose was measurable following incubation of samples with 50 μg/ml of pancreatic ribonuclease at 37° for 3 hr. No correction for DNA in the samples was made since the diphenylamine reaction (Schneider, 1957) was consistently negative. Nonerythroid elements of the blood have been shown not to significantly affect the results of these determinations (Burka, 1966).

Preparation of RNA and Density Gradient Centrifugation. RNA was separated and purified by the phenol method as described previously (DeBellis *et al.*, 1964). Following dialysis for 18 hr at 4° the RNA was stored at −20° in 0.1 M sodium acetate buffer (pH 5.0) or distilled water. ^{32}P-labeled RNA was prepared by *in vivo* labeling as described by DeBellis *et al.* (1964).

Density gradient centrifugation of RNA was carried out in the Spinco SW-39 rotor, Model L-2 ultracentrifuge, through a 4.8-ml linear gradient of 5–20% sucrose in 0.1 M sodium acetate buffer (pH 5.0). Centrifugation was for 4 hr at 39,000 rpm at 4°. RNA in zones of the gradient was estimated by absorbance at 260 mμ in a Gilford Model 2000 recording spectrophotometer in a 2-mm flow-through cell. Three-drop samples were collected for determination of radioactivity.

Base Analysis. Samples of [^{32}P]RNA extracted by the phenol method were hydrolyzed in a final concentration of 0.33 M potassium hydroxide for 18 hr at 37°. Following neutralization with perchloric acid and extraction of potassium perchlorate, the nucleotides were separated by thin layer chromatography (Starr and Ramberg, 1966). The separated nucleotides were scraped off the plate and radioactivity was determined as described below in phosphor containing 4% Cab-O-Sil.

Determination of Radioactivity. Radioactivity of ^{32}P- or ^{14}C-labeled RNA, extracted by hot 5% TCA, was determined by pipetting 0.2 ml of the TCA extract directly into 10 ml of Bray's (1960) solution and counting in a Packard liquid scintillation counter. Phenol-purified RNA was coprecipitated with albumin and washed with cold 5% TCA on Millipore filters. The filters were placed in the bottom of a counting vial, air dried, and 10 ml of Bray's solution was added. Fractions collected from sucrose density gradients were dripped directly into counting vials to which 10 ml of Bray's solution was added. In all cases sufficient counts were accumulated to reduce error to less than 3%.

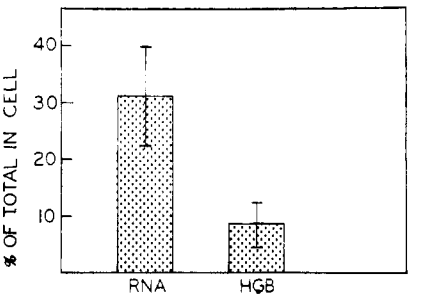

FIGURE 1: The proportion of total cellular RNA and total cellular hemoglobin associated with sedimented erythroid cell membranes following hypotonic lysis of 34 samples of peripheral blood. Vertical lines indicate standard deviation of the mean.

Materials. Rabbits were obtained from commercial sources. All chemicals used were reagent grade. Merck sucrose was used for gradients. Liquified phenol was obtained from the Fisher Scientific Co. Carrier-free ^{32}P was obtained from the International Chemical and Nuclear Corp., City of Industry, Calif. [^{14}C]Uridine was obtained from the New England Nuclear Corp., Boston, Mass. Pancreatic ribonuclease, Ringhals cobra venom, lecithinase C, and 2′, 3′ mixed nucleotides were obtained from Sigma Chemical Co., St. Louis, Mo. Pronase was obtained from Calbiochemical, Los Angeles, Calif.

Results

The Association of Erythroid RNA with the Cell Membrane. The initial studies determined whether a portion of mammalian erythroid cell RNA was associated with the cell membrane. Peripheral erythroid cells, either from normal or reticulocyte-rich blood, were lysed with eight volumes of 20 mosm phosphate buffer and the amount of RNA was determined by hot TCA extraction in crude whole lysates, in membrane-free hemolysates and in the sedimented washed membranes. The recovery of RNA from the separated cell components averaged 97.7% (range 86–109%) of that present in whole lysates. Figure 1 shows the proportion of total cellular RNA and total cellular hemoglobin remaining with sedimented membranes following lysis in hypotonic phosphate buffer. The proportion of total cellular RNA (31.1 ± 8.9%) which remained associated with the membranes was significantly greater than the 8.9 ± 3.8% residual cellular hemoglobin remaining with the membranes. Although the *absolute* amount of erythroid RNA varied directly with the degree of reticulocytosis, in random samples there was no obvious correlation between the *proportion* of total cellular RNA associated

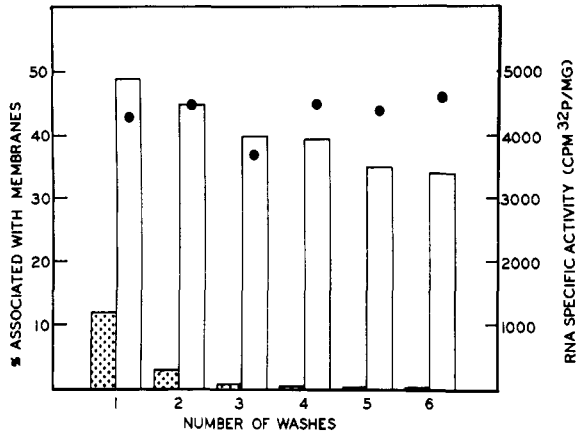

FIGURE 2: Proportion of total cellular RNA (open bars) and hemoglobin (dotted bars) associated with erythroid cell membranes following lysis and progressive washes in 20 mosm phosphate buffer. The solid circles indicate specific activity of the membrane-bound RNA, which had been previously labeled *in vivo* with ^{32}P.

TABLE I: Recovery of RNA from Erythroid Cell Components Lysed by Various Methods.[a]

Method of Lysis	Micrograms of RNA Recovered from: Cell Membranes	Micrograms of RNA Recovered from: Membrane-Free Lysates
Buffer (10 volumes, 20 mosm)	150	283
Freeze-thaw three times[b]	167	243
Butanol (0.4 M)[c]	144	243
Saponin (1%)[c]	161	230
NaCl (1.5 M)[d]	144	272

[a] RNA was determined on components of aliquots of washed cells by hot TCA extraction as described in the Methods section. Reticulocytosis of sample: 74% μg of RNA/ml of packed cells. [b] Cells were diluted in ten volumes of 20 mosm buffer after treatment. [c] Solutions made up in 20 mosm buffer. [d] Hypertonic saline treatment following lysis in 20 mosm buffer.

with the membrane and the degree of reticulocytosis in the peripheral blood. For this reason Figure 1 represents studies done on blood with a reticulocytosis which ranged from 2 to 99%. Similar results were obtained when RNA was quantitated following extraction by the phenol method, approximately one-third of the total cellular RNA being closely associated with the cell membranes.

It was necessary to determine whether RNA was associated with cell membranes which were free of hemoglobin. Multiple washes in 20 mosm phosphate buffer reduced the proportion of total cellular hemoglobin remaining with the sedimented membranes to less than 0.4% (Figure 2). The proportion of total cellular RNA remaining with the membranes (about 35% in this experiment) was not significantly affected by the washing procedure. The specific activity of the membrane-bound RNA, previously labeled *in vivo* with ^{32}P, remained constant during the multiple washes.

Manner of Association of RNA with the Cell Membrane. Studies were done in order to determine whether the association between erythroid RNA and the cell membrane was dependent upon the method of cell lysis (Table I). Since hypotonic lysis allows hemoglobin to escape from the cell while leaving the membrane grossly intact (Seeman, 1967), it was conceivable that the membrane-associated RNA remained trapped within intact red cell ghosts. When cells were lysed by repeated freezing and thawing, which fragments the membranes, the amount of membrane-bound RNA did not vary significantly from that of cells lysed in hypotonic media. The effect of agents which alter the permeability of the cell membranes and destroy their metabolic integrity was examined by lysing cells in hypotonic buffer which had been made 0.4 M with respect to butanol or 1% with respect to saponin (Hunter, 1961; Seeman, 1967). Exposure to these agents did not significantly affect the amount of RNA associated with the cell membrane. The exposure of whole hypotonic lysates to 1.5 M sodium chloride for 1 hr, a procedure which extracts both lipid and protein from the cell membrane (Mitchell and Hanahan, 1966), did not alter the amount of membrane-bound RNA. These findings indicate that the RNA which is associated with the membrane is not merely trapped within the red cell ghost, nor does the association depend upon continued selective permeability of the membrane. The data suggest that erythroid RNA is bound to the cell membrane in some specific manner.

The specific mode of binding between RNA and the cell membrane was investigated by exposing aliquots of washed fragmented membranes to enzymes which attack the protein or lipid components of the cell membrane (Table II). Control studies were done by incubating membrane-free hemolysates with the enzymes under identical conditions. All enzymes used were assayed for activity and, with the exception of pancreatic ribonuclease, were demonstrated to be free of nuclease activity against phenol-purified reticulocyte RNA. The major phospholipid component of rabbit erythroid cells is lecithin (Condrea *et al.*, 1964). Lecithinase "C," which splits phosphorylcholine from the hydrophilic end of the molecule, did not release the bound RNA. Lecithinase "A" attacks the hydrophobic end of the lecithin molecule. Ringhals cobra venom, a potent hemolytic agent which contains lecithinase "A" as well as other agents which disrupt the membrane (Condrea *et al.*, 1964) did, however, release more than one-half of the bound RNA. This action was not due to phosphodiesterase activity of cobra venom, as treatment of membrane-free hemolysates or purified reticulocyte RNA

TABLE II: Effect of Enzymes and Desoxycholate on Membrane-Bound and Free RNA.[a]

Agent	Final Concn (μg /ml)	RNA Remaining in Treated Aliquot (% of Control)	
		Membranes[b]	Membrane-Free Hemolysates
Lecithinase "C"	50	100	104
Ringhals cobra venom	50	44	103
Neuraminidase	50	84	99
Pronase	50	84	99
Pancreatic ribonuclease	50	31	6
Sodium desoxycholate	0.2%	0	100

[a] Incubation of separated components in 20 mosm phosphate buffer at room temperature for 1 hr. When incubated with whole cell suspensions *in vitro* only cobra venom and desoxycholate showed hemolytic activity. [b] The membrane suspensions were frozen and thawed three times.

under the same conditions did not degrade RNA to acid-soluble products. Not all of the bound RNA released by Ringhals cobra venom was recoverable as acid-insoluble material, however, suggesting that the venom may have released or activated a nuclease from the membrane. Treatment with a final concentration of 0.2% sodium deoxycholate completely destroyed the membrane structure and released all of the bound RNA into the wash fluid.

The two proteolytic enzymes utilized were only mildly effective in separating RNA from the cell membrane. Neuraminidase, an enzyme which splits off the group primarily responsible for the negative charge of the cell membrane, neuraminic acid, released approximately 15% of the bound RNA, which was recoverable in the membrane-free hemolysate. Pronase was chosen as a less specific peptidase since it, unlike trypsin, was free of nuclease activity. Like neuraminidase, pronase released some 15% of the bound RNA, which was recoverable in the wash fluid following treatment of isolated washed membranes, or in the membrane-free hemolysate following treatment of whole lysates. The data indicate that the negative charge on the membrane is not instrumental in the binding of RNA to the erythroid cell membrane, nor is a protein which is susceptible under these conditions to attack by pronase.

Treatment with Ribonuclease. Treatment of isolated membranes and membrane-free hemolysates with equal

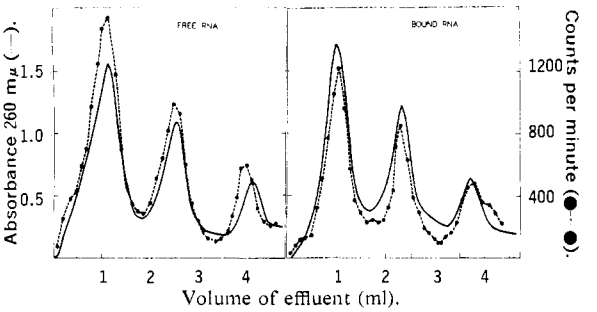

FIGURE 3: Sucrose density gradient centrifugation patterns of free and membrane-bound reticulocyte RNA extracted from peripheral blood 40 hr following *in vivo* labeling with ^{32}P. Conditions of centrifugation are described in the Methods section.

concentrations of pancreatic ribonuclease did not cause similar degrees of degradation of the RNA in these cell fractions (Table II). Membrane-bound RNA was relatively protected from the action of exogenous ribonuclease. This difference in enzyme susceptibility was related to the binding, since RNA isolated by the phenol method from either membranes or membrane-free hemolysates was degraded at equal rates by pancreatic ribonuclease. Pancreatic ribonuclease (10 μg/ml) degraded 97% of phenol-purified RNA from both cell fractions in 1 hr at 37°.

Composition of Free and Membrane-Bound RNA. The composition of the free and membrane-bound fractions of rabbit erythroid cell RNA was studied in two ways. ^{32}P-labeled RNA, extracted by the phenol method from washed membranes and membrane-free hemolysates and dialyzed overnight against acetate buffer, was examined by sucrose density gradient centrifugation. As shown in Figure 3, the three usual molecular species of mammalian RNA are present, nominally valued at sedimentation constants of 4, 18, and 28 S. In each of the fractions of erythroid RNA the proportion of these three molecular components is similar, tRNA representing approximately 18% of the total. Recovery from the gradients averaged 96.2%.

The base composition of the free and bound fractions of erythroid RNA was determined by analysis of radioactivity in nucleotides of ^{32}P-labeled RNA following alkaline hydrolysis (Table III). The base compositions of the free and bound RNA are not significantly different and are in general agreement with previous reports (DeBellis *et al.*, 1964; Burny and Chantrenne, 1964). The similarity in the composition of free and membrane-bound RNA of the erythroid cell, both in molecular species and base composition, suggests that there are no qualitative differences between the erythroid RNA fractions in either the free or bound state.

Appearance of RNA in the Membrane-Bound Fraction. The rates of turnover of the two fractions of erythroid cell RNA were investigated. At intervals following the intravenous administration of ^{32}P the specific activity of free and membrane-bound erythroid cell RNA in

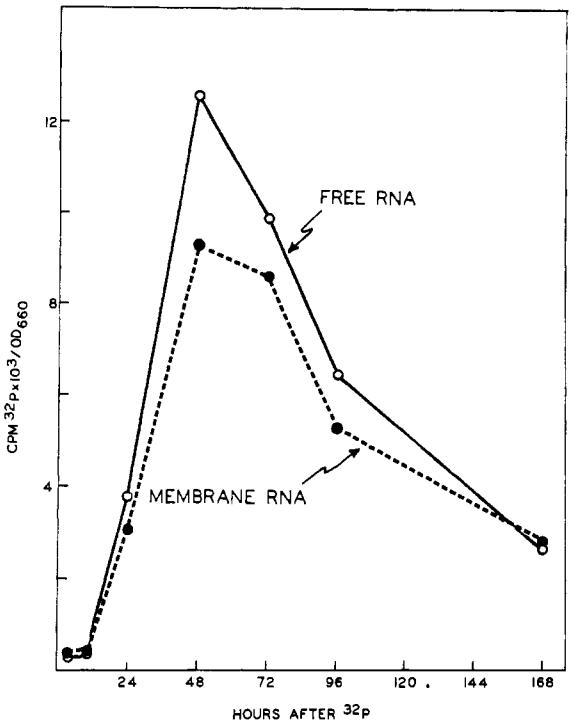

FIGURE 4: The specific activity of free and membrane-bound erythroid cell RNA at intervals following *in vivo* pulse labeling with ^{32}P. Specific activity is expressed as counts per minute of ^{32}P per OD_{660} in the orcinol reaction.

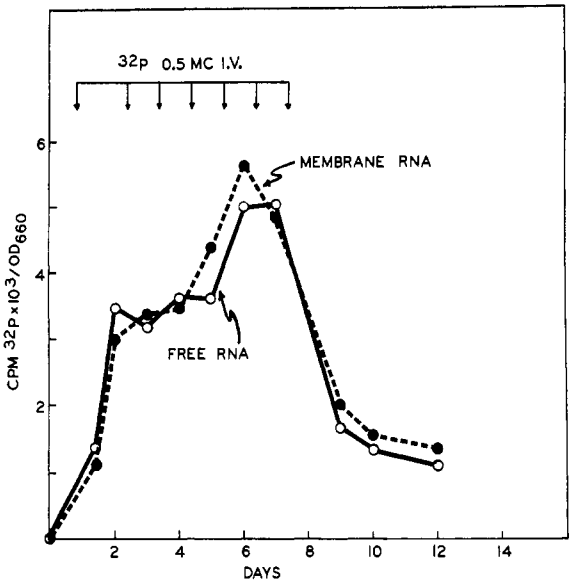

FIGURE 5: The specific activity of free and membrane-bound erythroid cell RNA during the course of repeated intravenous injections of ^{32}P. Specific activity is expressed as in Figure 4.

samples of peripheral blood were determined by the acid-extraction method. At the earliest times examined, 5 and 10 hr after pulse labeling (Figure 4), the specific activities of membrane-bound RNA and free RNA were similar. The radioactivity in the peripheral blood at this time primarily represents terminal labeling of tRNA (DeBellis *et al.*, 1964). Twenty hours after pulse labeling, when radioactive rRNA has appeared in the peripheral blood, the specific activity of membrane-bound RNA was lower than that of free RNA. This difference in specific activity was even greater at the time of peak labeling in the peripheral blood. Forty hours after pulse labeling the specific activity of membrane-bound RNA was consistently found to be approximately 75% of the value attained by the free RNA. This difference in specific activity was confirmed in samples of RNA prepared by the phenol method and can be noted by the relative positions of the lines indicating radioactivity and RNA concentrations throughout the sucrose density gradients in Figure 3. Subsequently the specific activity of the free RNA declined at a more rapid rate than that of the membrane-bound RNA, the two becoming equal 7 days following the pulse label. In order to ensure that the observed differences in specific activity were not related to the particular radioactive precursor used, ^{32}P, similar studies of specific activity were done following intravenous administration of [^{14}C]uridine. The results with this RNA precursor were identical with those observed when ^{32}P was used. These findings indicate that newly synthesized erythroid cell RNA appears in, and subsequently disappears from, the membrane-bound fraction of peripheral cells at a slower rate than it does in the free fraction.

Further studies of the specific activity of the two fractions of erythroid RNA were done following multiple injections of ^{32}P, which afford a more continuous labeling of newly synthesized RNA (Figure 5). Initially, the specific activities of the two RNA fractions behaved identically with those found following pulse label (Figure 4). With continued intermittent injection of ^{32}P

TABLE III: Base Analysis of Alkaline Hydrolysates of [^{32}P]RNA.[a]

2′, 3′ Nucleotide	% Total Cpm of ^{32}P	
	Membrane-Bound RNA	Free RNA
Adenosine	18.4 ± 0.54	18.1 ± 0.26
Cytidine	29.0 ± 1.23	28.6 ± 0.69
Guanosine	33.6 ± 1.41	31.9 ± 0.24
Uridine	19.0 ± 0.27	21.4 ± 1.13

[a] The nucleotides of [^{32}P]RNA were separated by thin layer chromatography following alkaline hydrolysis as described in the Methods section. The results are expressed as the mean percentage plus and minus standard error of the mean of four separate determinations.

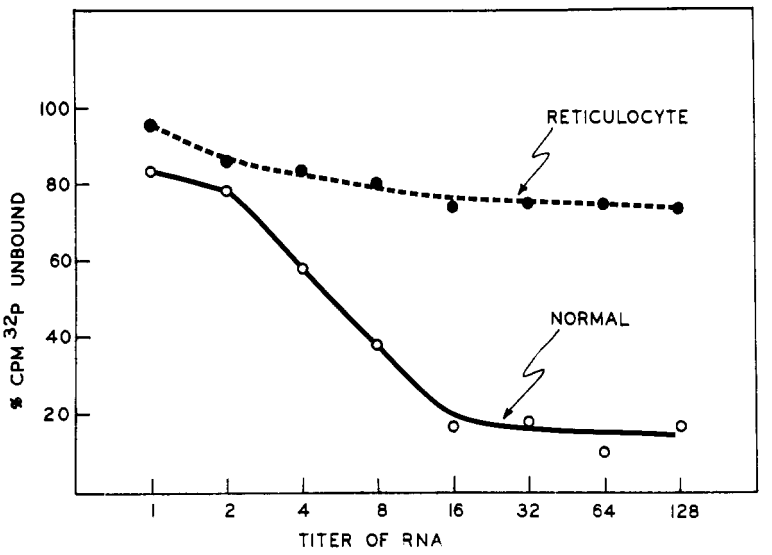

FIGURE 6: Percentage of RNA remaining unadsorbed to erythroid cell membranes obtained from normal blood or from blood with a reticulocytosis. Increasing dilutions of a solution of [^{32}P]RNA were incubated with aliquots of cell membranes containing 1.2 mg of membrane protein as described in the text. The points represent the proportion of added counts per minute remaining unadsorbed to the membranes.

the specific activity of the membrane-bound fraction became equal to or slightly higher than that of the free RNA, and remained so following the final injection of ^{32}P. The findings indicate that newly synthesized RNA becomes preferentially localized in the membrane-bound fraction of RNA with time. In conjunction with the data found following a single pulse label, the findings suggest that newly synthesized RNA initially appears in the pool of free RNA and with time accumulates in the bound fraction.

Adsorption of Reticulocyte RNA to the Cell Membrane. Rabbit reticulocyte RNA could become adsorbed to erythroid cell membranes *in vitro*. Aliquots of a membrane suspension in 20 mosm phosphate buffer were incubated with serial dilutions of ^{32}P-labeled reticulocyte RNA at 0° for 30 min. Radioactivity was determined in the twice-washed membranes and the combined supernatant and wash solutions. Total recovery of [^{32}P]RNA was always greater than 84% of the counts per minute added. Figure 6 shows results of such incubations with membranes from normal blood (reticulocytosis 2%) and from blood with a phlebotomy-induced reticulocytosis of 21%. With increasing dilution of the RNA solution a greater percentage of RNA was bound to the cell membranes obtained from normal blood. In membranes obtained from blood with a high reticulocytosis little RNA was bound at any dilution, presumably because immature cells have a greater amount of RNA already associated with the membrane. The results were similar whether the labeled RNA was derived from whole hemolysates, washed membranes, or membrane-free hemolysates. The data indicate that reticulocyte RNA can become adsorbed to erythroid cell membranes, and that the amount is limited by the quantity of membrane available.

Discussion

The principle finding of this study is the demonstration of a fraction of mammalian erythroid cell RNA which is closely associated with the cell membrane. Approximately one-third of the total cellular RNA, quantitated either by the acid-extraction method or following phenol extraction, remains associated with hemoglobin-free cell membranes. That this ribonuclease-sensitive material truly represents erythroid RNA, and not RNA derived from leukocytes, is indicated by several facts. The membrane preparations did not contain nucleated cells, as confirmed by phase-contrast microscopy, and no DNA was demonstrable in the hot TCA extracts of the membrane preparations. Previous studies have demonstrated that leukocytes do not significantly influence RNA determinations by this method (Burka, 1966). Holt *et al.* (1966) have shown that following *in vitro* labeling of reticulocyte RNA, leukocyte RNA has a *higher* specific activity than reticulocyte RNA, not a *lower* specific activity as was found in the sedimented material in this study. The base ratios, the proportions of molecular species, and the rate of enzymatic degradation of RNA purified from the membrane were identical with those from the membrane-free hemolysates. These facts are strong evidence that the RNA in the free and bound fractions are identical.

Hemoglobin is associated with the membrane due to electrostatic forces (Klipstein and Ranney, 1960) and can be removed by treatment at the appropriate pH (Dodge *et al.*, 1963). Bound RNA, unlike hemoglobin, could not be washed free from the membrane. The effect of pH on the binding of RNA to the membrane could not be directly assessed since extremes of pH destroy RNA and ribosomes. Removal of the negative

charge on the membrane with neuraminidase was only mildly effective in releasing bound RNA. This is not surprising since the over-all charge of ribosomes is negative (Ts'o, 1962) and more than 80% of the RNA within the cell is in ribosomes. For this latter reason the demonstration that reticulocyte RNA can become adsorbed to the cell membrane *in vitro* does not have great physiologic significance. It does indicate, however, that RNA can become adsorbed to the erythroid cell membrane, possibly by electrostatic attraction to positively charged groups, and that there is a limit to the amount of RNA which can be bound. This suggests the existence of binding sites.

Further studies were unsuccessful in defining the precise nature of the binding between erythroid RNA and the cell membrane. Mild proteolysis did not release RNA from its association with the cell membrane. Destruction of the protein components of the ribosomes did not permit more stringent proteolysis. Enzymatic cleavage of lecithin at the hydrophilic end of the molecule did not affect the amount of membrane-bound RNA, nor did fragmenting the membranes or altering their permeability by producing transient or permanent discontinuities. Release of membrane-bound RNA was achieved only by agents which were hemolytic, such as Ringhals cobra venom and deoxycholate. Taken together, the data indicate that RNA is progressively separated from the membrane as its internal structure is disrupted to an increasing degree. This suggests that the association of RNA with the cell membrane is in some manner dependent upon the intact structural complexity of the membrane.

The association of erythroid RNA with the cell membrane protected it from the action of pancreatic ribonuclease. Similar resistance of membrane-bound RNA to degradation has been described in bacterial systems (Aronson, 1965). This suggests that the site of action of pancreatic ribonuclease is relatively inaccessible in bound RNA, and that the cell membrane may play a role in moderating the rate of degradation of RNA which accompanies erythroid cell maturation. In random samples studied, the proportion of total cellular RNA associated with the membranes did not have any apparent relationship to the degree of reticulocytosis. This does not rule out such a relationship, however, since a sample of peripheral blood represents a cell population heterogenous with respect to age, and degree of reticulocytosis in itself is a poor index of mean cell age (Marks *et al.*, 1963). Individual longitudinal studies during reticulocyte maturation *in vivo* and *in vitro* will be necessary to determine whether the proportion of erythroid cell RNA which is membrane bound is independent of the maturity of the cell.

The kinetic studies reported here must be viewed in regard to the RNA metabolism of the mammalian erythroid cell. There is convincing evidence that the bulk of cellular RNA synthesis takes place in the nucleus (Scherrer and Darnell, 1962). In the erythroid cell, RNA synthesis is confined to nucleated precursors in the bone marrow (Grasso *et al.*, 1963; Borsook *et al.*, 1962) and does not take place in the reticulocyte (Marks *et al.*, 1962). Radioactivity appearing in the peripheral blood following pulse labeling with ^{32}P thus represents erythroid cell RNA which was synthesized previously in marrow elements. On the basis of specific activity studies reported here, it appears that newly synthesized erythroid RNA appears first in the pool of free RNA, and with time becomes localized in the pool of membrane-bound RNA. The slower rate of decline of the specific activity of bound RNA could also be explained by a slower rate of degradation of membrane-bound RNA. Further studies will be necessary to resolve this point. The observed differences in specific activity made it clear, however, that RNA binding to the membrane is not a nonspecific consequence of the method of preparation. The data further suggest that binding to the membrane may impart certain properties to the RNA which are of importance in physiologic stability.

The attachment of RNA and ribosomes to both cell wall and endoplasmic reticulum has been described in bacterial (Schlessinger, 1963) and animal cells (Palade and Siekevitz, 1956). Membrane-bound RNA has been found to differ from free RNA in function (Hendler, 1965) and rate of degradation (Aronson, 1965, 1966). The relationship of the RNA to the membranes and the significance of this relationship, however, remain not fully understood. This study provides evidence that in the mammalian erythroid cell, which does not contain an endoplasmic reticulum, a significant proportion of the cell RNA is bound to the cell membrane. This bound RNA is relatively resistant to degradation by ribonuclease. The findings suggest that the membrane may play a role in governing the decline in RNA content and capacity for protein synthesis which accompany mammalian erythroid cell maturation.

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Inhibition of Aminoacyl Transfer Ribonucleic Acid Synthetases by Modified Transfer Ribonucleic Acids*

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ABSTRACT: A series of transfer ribonucleic acids (tRNAs) which have been subjected to various modifications at their acceptor ends was prepared. The rate of esterification of lysine, valine, and phenylalanine to tRNA in the presence and absence of each of the modified tRNAs was determined. The pattern of inhibition was different in each case, suggesting that each enzyme had different requirements for tRNA binding. The rate of valine and phenylalanine esterification to tRNA was measured over a range of magnesium ion concentrations and the response to free magnesium was different in each case. Optical rotatory dispersion studies of degraded tRNAs suggested that the conformations of all of them were very similar to native tRNA. Treatment of tRNA with sodium borohydride did not affect acceptor activity for lysine, valine, or phenylalanine. Despite only very slight inhibition of phenylalanine esterification to tRNA by periodate-oxidized tRNA it was shown that periodate causes no detectable alteration of phenylalanyl-tRNA. The findings are discussed in relation to other studies employing tRNAs with modified acceptor ends.

The specificity of interactions between tRNA and aminoacyl-tRNA synthetases is of the greatest importance in maintaining accuracy in translation of the genetic message. The challenge of elucidating the role of various structural features of tRNA in achieving this high degree of specificity has attracted many investigators, and a number of approaches have been used by them in attempts to answer this challenge. Because all functional species of tRNA are assumed to terminate at the 3′ (acceptor) end in the sequence cytidylyl(3′→5′)cytidylyl(3′→5′)adenosine (CpCpA), several studies have focused on the role, if any, of this end group in recognition by aminoacyl-tRNA synthetases. Hayashi and Miura (1964) and Torres-Gallardo and Kern (1965) independently showed inhibition of valine esterification to tRNA by periodate-oxidized tRNA. The latter workers established by kinetic data that this inhibitor was competitive with tRNA and that for the esterification of both valine and tyrosine in *Escherichia coli* the inhibitory species was the oxidized homologous tRNA and not some other species. Snake venom phosphodiesterase treatment of tRNA was also reported to produce a competitive inhibitor but with a higher K_i value.

Korzhov and Sandakhchiev (1966) have reported inhibition of valine, lysine, and alanine esterification to tRNA by $tRNA_{ox}$. They further showed that the

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[1] Abbreviations used: $tRNA_{ox}$, periodate-oxidized tRNA; $tRNA_{ox-red}$, tRNA which has been oxidized with periodate and then reduced with sodium borohydride; tRNA(-A), tRNA with the terminal adenosine removed; tRNA(-pA), tRNA(-CpA), tRNA(-pCpA), tRNA(-CpCpA), and tRNA(-pCpCpA), tRNAs with the group in parentheses removed; $tRNA^{Val}$, tRNA which accepts valine; valyl-tRNA, tRNA with valine esterified to the terminal adenosine; E_{Val}, the valyl-tRNA synthetase; TCA, trichloroacetic acid; ATP, adenosine triphosphate.