JCI The Journal of Clinical Investigation

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J Clin Invest. 1969;48(7):1266-1272. https://doi.org/10.1172/JCI106092.

Research Article

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RNA within the cell fluxes freely back and forth between the free and membrane-bound forms. The rate of loss of RNA in the membrane-bound compartment, both in the intact cell and in isolated membranes, is approximately threefold that found in the cell as a whole. During maturation the proportion of total cell RNA which is bound to the membrane decreases. The bulk of RNA destroyed is derived from the free compartment, but bound is also destroyed. The cell membrane participates in RNA metabolism and may be a site of RNA degradation in the erythroid cell.

Characteristics of RNA Degradation in the Erythroid Cell

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ABSTRACT The characteristics of ribonucleic acid (RNA) degradation in intact reticulocytes have been investigated. The rate of degradation during in vitro maturation at 37°C is approximately 4% per hr. RNA degradation does not proceed at 0°C, and the rate of degradation is temperature dependent with an optimum at 50°C. The process is not dependent upon glycolysis. Although all types of RNA progressively decrease during in vivo maturation, ribosomes are degraded at a rate greater than that of soluble RNA.

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INTRODUCTION

A progressive decrease in cell content of ribonucleic acid (RNA) is characteristic of maturation of the mammalian erythroid element (1). The level of RNA in erythroid cells has, in fact, proven to be a more accurate index of mean cell age than the degree of reticulocytosis in peripheral blood (2). It is unlikely that under physiologic conditions of pH and temperature RNA breakdown occurs by other than enzymatic means, and one or more specific ribonucleases have been assumed to be responsible for RNA degradation within the erythroid cell. Attempts to characterize the RNase of erythroid cells have, however, presented a confusing picture. The wide variety of conditions utilized for the extraction and assay of erythroid RNase may explain the numerous differences reported for location within the cell, pH optima, ion requirements, and substrate specificity (3–10).

The mechanism of RNA degradation in mammalian erythroid cells remains obscure. Bertles and Beck (11) showed that RNA degradation during in vitro maturation of rabbit reticulocytes proceeds rapidly to small molecular weight compounds, but few other studies of erythroid cell RNA degradation under physiologic conditions have appeared. Understanding of the mode of RNA degradation in erythroid cells, and its relationship to cell maturation, requires detailed examination of aspects of RNA degradation as it occurs in its natural environment, in intact cells, rather than in artificial systems. In the present study qualitative and quantitative aspects of RNA metabolism in intact erythroid cells have been investigated. Degradation in free and membrane-bound compartments, the relative rates of degradation of soluble and ribosomal RNA, and the source of degraded RNA and its site of destruction have been studied. The results indicate that the bulk of RNA which is degraded is derived from the free RNA compartment, but the cell membrane, which is in itself able to degrade erythroid RNA, may be a site of RNA destruction in the erythroid cell. During erythroid cell maturation ribosomal RNA is degraded at a rate greater than that of soluble RNA. The features of RNA metabolism in intact cells suggest that degradation is an enzyme-mediated process.

METHODS

Isolation of cells and preparation of cell lysates. Peripheral venous blood was collected from normal New Zealand white rabbits or from animals with a reticulocytosis induced by four daily subcutaneous injections of 1 ml of a 2.5% solution of phenylhydrazine hydrochloride. The blood was collected in heparinized syringes 3 days after the final phenylhydrazine injection and immediately placed in an ice bath. All subsequent procedures were done at 0–4°C. The cells were separated by centrifugation and washed twice

Received for publication 10 January 1969 and in revised form 28 February 1969.

with 0.9% sodium chloride which contained 1.5×10^{-3}M magnesium chloride. After each centrifugation the buffy coat was removed by aspiration.

Cell lysates were prepared by addition of 4 volumes of 7.37×10^{-3}M sodium phosphate buffer, pH 7.4, or a solution of 1.5×10^{-3}M magnesium chloride in 1×10^{-3}M Tris, pH 7.5 (solution A), to the packed cells. Cell membranes were separated by centrifugation at 17,300 *g* for 10 min. The loosely packed sedimented membranes were rewashed twice in the lysing buffer after separation by gentle agitation from the hard button of unlysed nucleated cells at the bottom of the tube. The membranes, containing less than 1% of the original cell hemoglobin (12), were suspended in a known volume of lysing buffer. Absence of nucleated elements in the membrane fraction was confirmed by phase-constrast microscopy (12).

Incubations of whole cells. Intact reticulocytes were incubated at 37°C with agitation for specified periods of time in a medium consisting of 4 volumes of a modified Krebs-Ringer bicarbonate buffer solution containing amino acids, glucose, and ferrous ions, as described previously (13). At the initiation and conclusion of the incubation period duplicate aliquots of the incubation suspension were removed, the cells washed twice, and total RNA was determined by chemical or radioactive analysis.

Preparation of RNA-^{32}P and ribosomes. Erythroid cell RNA was labeled with ^{32}P in vivo as previously described (14). Ribosomes and soluble RNA were separated by ultracentrifugation of membrane-free hemolysates prepared with solution A at 226,000 *g* for 90 min (15).

Analytical methods. Hemocytometry, ennumeration of reticulocytes, and determination of hematocrit were done by standard methods. Hemoglobin concentrations in membrane suspensions were determined by the method of Rimington (16). RNA in erythroid cells, cell lysates, and membrane suspensions was extracted as previously described (17) and quantitated by determination of RNA-ribose or ^{32}P in the hot acid extract. Nonerythroid elements of the blood have been shown not to significantly affect the results of these determinations (17).

Determination of radioactivity. ^{32}P radioactivity was determined by pipetting 0.2–0.5 ml of the RNA extracts directly into 10 ml of Bray's solution (18) in a counting vial. The vials were counted in a Packard liquid scintillation counter with an efficiency of more than 95%. In all cases sufficient counts were accumulated to reduce counting error to less than 3%.

Materials. Rabbits were obtained from commercial sources. All chemicals used were reagent grade. Carrier-free ^{32}P was obtained from Tracerlab Div., Nuclear Instruments & Radioactive Chemicals, Waltham, Mass.

RESULTS

RNA degradation in intact erythroid cells. RNA was assayed in the peripheral erythroid cells of an individual animal at intervals after induction of anemia with phenylhydrazine. During this recovery period the mean cell age of erythroid cells in the peripheral blood progressively increases as the reticulocyte count falls (19). The RNA content of this progressively older population of cells is shown in Fig. 1. The fall in reticulocytosis is closely paralleled by a progressive decrease in RNA content of the cells (Fig. 1, upper). The results

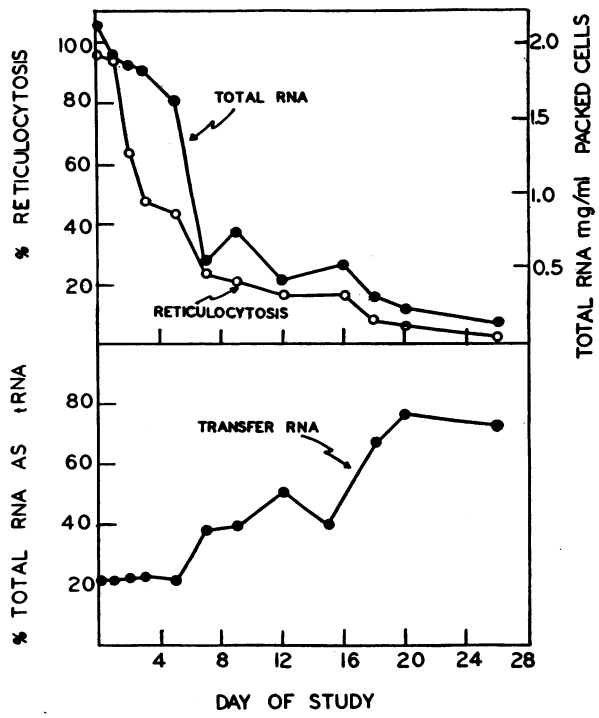

FIGURE 1 Erythroid cell RNA content during recovery from anemia. Samples of peripheral blood, obtained at intervals during recovery from a phenylhydrazine-induced anemia, were analyzed for total RNA content (upper) and the proportion of total RNA as transfer RNA (lower). On day No. 20 the animal had a normal hemoglobin, hematocrit, and reticulocyte count.

in Fig. 1 are expressed as milligrams of RNA per milliliter of packed cells, but the same relationship is found when the data are expressed per unit of hemoglobin. These findings confirm that maturation of the erythroid cell in vivo is accompanied by a progressive decrease in cell RNA content (1, 2).

Of the two major types of RNA in erythroid cells, transfer RNA and ribosomal RNA, only transfer RNA, with proven acceptor activity, has been reported to be present in mature erythrocytes (20). The relative rates of degradation of nonribosomal RNA and ribosomal RNA were therefore compared. At intervals during the recovery phase after induction of anemia, membrane-free lysates were prepared and the ribosomes separated by ultracentrifugation. The total amounts of proportions of RNA in the 225,000 *g* supernatant (soluble RNA) and in the pellet (ribosomal RNA) were determined. The adequacy of recovery and separation of soluble and ribosomal RNA under these conditions has been verified (15). Although the acceptor activity of the soluble RNA prepared in this manner was not tested, the base ratio has been shown to be identical with that of authentic reticulocyte transfer RNA (15). Immediately

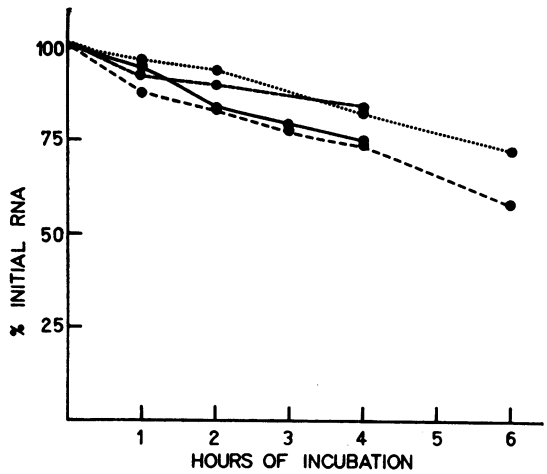

FIGURE 2 The rate of loss of RNA in intact erythroid cells during in vitro incubation. Values are expressed as per cent of RNA present at the initiation of incubation at 37°C.

after the anemic stimulus, when the mean cell age was youngest, approximately 20% of the total RNA was soluble RNA (Fig. 1, lower). When the anemia was corrected and the reticulocytosis had decreased to a normal 4%, soluble RNA represented more than 70% of the total. The increase in mean cell age which occurred during the recovery phase was thus accompanied by a progressive increase in the proportion of RNA which was soluble RNA. Since the reticulocyte does not synthesize RNA (21), and high molecular weight degradation products of ribosomal RNA do not accumulate within the cell (11, 15), these data indicate that during erythroid cell maturation soluble RNA is degraded at a rate slower than that of ribosomal RNA.

The rate of RNA degradation in erythroid cells could not be determined in vivo since maturation in the peripheral blood occurs in the presence of continued delivery of immature elements from the marrow. For this determination an in vitro system in which maturation of reticulocytes takes place was utilized (2). At intervals during incubation aliquots of the cells were assayed for RNA content. As shown in Fig. 2, RNA degradation in intact cells was linear during 6 hr of in vitro incubation at 37°C. The rate of total RNA degradation in six such studies averaged 3.9 ±2.5% per hr. Degradation of RNA in erythroid cells did not proceed at 0°C (Table I). The temperature optimum was 50°C, and the rate of degradation at 70°C was at least equal to that occurring at physiologic temperature. The lack of degradation at 0°C was apparently not due to failure of energy generation, as the presence of inhibitors of aerobic and anaerobic glycolysis, fluoride, iodoacetate, or cyanide had little or no effect on the rate of RNA degradation.

Degradation of free and membrane-bound RNA. Between 20 and 30% of erythroid cell RNA is closely bound to the cell membrane (12, 15). The characteristics of degradation in the membrane-bound compartment were compared with those of the free compartment. At the initiation and conclusion of 4 hr of incubation at 37°C intact cells were separated from the incubation medium by centrifugation, lysed, and the membranes separated from the membrane-free hemolysate. RNA determinations were done on the whole lysate, on the membrane-free hemolysate, and on the washed membranes. When examined in this manner the rate of loss of RNA in the two compartments differed (Fig. 3, left). The rate of loss in the membrane-free hemolysate averaged 3.2 ±1.6% per hr, very similar to the rate observed for total RNA in intact cells. This would be expected since free RNA represents approximately 80% of the total RNA in blood with a high degree of reticulocytosis (15). The rate of disappearance of bound RNA, in contrast, was much higher, averaging 8.9 ±1.0% per hr. On the basis of this evidence the proportion of RNA in the membrane-bound compartment would be expected to decrease with cell maturation. The data shown in Fig. 4 confirm this. The proportion of RNA bound to the membrane in intact cells decreased in every instance of in vitro reticulocyte maturation.

Rates of degradation of free and membrane-bound erythroid cell RNA were also compared during in vitro incubation of isolated cell components. Reticulocyte membranes were separated after cell lysis, washed once, and resuspended in a volume of buffer equal to

TABLE I
*RNA Loss in Intact Erythroid Cells during In Vitro Incubation**

Conditions of incubation	% initial RNA remaining after 4 hr
0°C	99.6
15°C	91.2
37°C	79.4
50°C	55.0
70°C	78.8
Plus 10^{-2}M fluoride, 37°C	74.2
Plus 10^{-2}M iodoacetate, 37°C	85.3
Plus 10^{-2}M cyanide, 37°C	78.2

* Cells were incubated as described in the Methods section for 4 hr at the indicated temperatures. At the conclusion of the incubation ice-cold trichloroacetic acid (TCA) was added to a final concentration of 5% and ribonucleic acid (RNA) determined, thus including any RNA which may have escaped from lysed cells. Hemolysis of more than 1% was noted only in the specimen incubated at 70°C, where it was 3.1%.

that of the membrane-free hemolysate. The residual hemoglobin in the isolated membranes prepared in this manner was less than 1% of that originally present. During incubation of isolated membranes the rates of loss of membrane-bound RNA, 7.3 ±2.6% per hr, and that of the membrane-free hemolysate, 2.8 ±0.8% per hr, were close to those observed in the intact cell (Fig. 3, right). In these studies, the trichloroacetic acid used to precipitate undegraded RNA (17) was added to the entire incubation suspension, thus ruling out the transfer of RNA from the membranes to the incubation fluid as a cause for the observed RNA loss. These data indicate that RNA degradation in the erythroid cell proceeds in both the free and membrane-bound compartments, that the cell membrane itself is capable of degrading RNA, and further, that RNA in the membrane-bound compartment is lost at a greater rate than is free RNA.

Exchange of RNA between free and membrane-bound compartments. The greater rate of degradation of erythroid cell RNA in the membrane-bound compartment suggested that the cell membrane might play a role in the destruction of RNA within the cell. Previous studies have shown that after pulse label with ^{32}P newly synthesized erythroid RNA appears first in the free fraction and later in the bound fraction (12). Experiments were designed, therefore, to determine the relationship between RNA degradation and the transfer of erythroid cell RNA between the free and bound compartments. An incubation mixture of membrane-free hemolysate and membranes, only one of which was labeled with ^{32}P, was reconstituted in the approximate proportions found in whole lysates. At intervals during incubation at 37°C aliquots of the reconstituted whole lysate were separated into membranes and membrane-free hemolysate

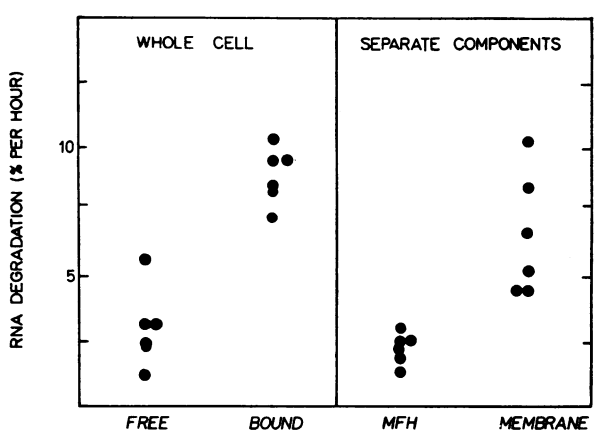

FIGURE 3 The rate of RNA degradation in isolated components of erythroid cells. Left: the cell components were separated after incubation of whole cells for 4 hr at 37°C. Right: membrane-free hemolysate (MFH) and cell membranes were first isolated and then incubated under the same conditions as were the intact cells.

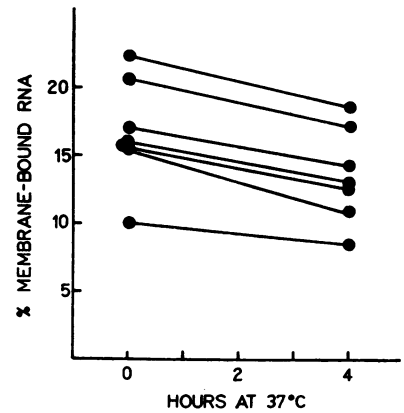

FIGURE 4 The change in the proportion of membrane-bound RNA in intact erythroid cells during 4 hr of incubation at 37°C.

for determination of total RNA and radioactivity. Analysis of the total loss of RNA, both radioactive and nonradioactive, in each compartment, and of the ultimate location of the radioactive RNA, allowed determination of the amount and source of the degraded RNA. The results are shown in Table II. RNA was degraded during incubation of whole hemolysate reconstituted from labeled, membrane-free hemolysate and unlabeled membranes (experiment A), as evidenced by a net loss of radioactivity and total RNA (column Nos. 1 and 2). The specific activity of the total RNA which was degraded during the incubation was greater than that of the total RNA present at the initiation of the incubation (column No. 3) and about equal to that of the free RNA initially present (column No. 6). This fact indicates that labeled RNA, from the free fraction, was the source of most of the material degraded.

Looking at the loss in terms of individual compartments, 24.6% of the radioactivity originally present in the free compartment was lost, although only 13% of the RNA in this compartment had disappeared (column Nos. 4 and 5). Thus, the specific activity of the free RNA decreased during incubation (column No. 6), a finding indicating that in association with degradation of free RNA there must have been a transfer of RNA from the membrane-bound compartment into the free compartment. This is confirmed by the fact that the specific activity of the RNA lost from the free compartment is higher than that originally present, explicable only by movement of unlabeled RNA into the free compartment. Total membrane-bound RNA decreased (column No. 8), but nevertheless radioactivity in this compartment increased (column No. 7). Although this increase represented only some 2% of the free RNA originally present, it can only represent a minimum figure for transfer. Since the membrane is ca-

TABLE II

The Loss and Exchange of RNA in Reconstituted Whole Hemolysates

	Column No..... RNA components		1	2	3	4	5	6	7	8	9
	Labeled	Unlabeled	Total RNA			Free RNA			Bound RNA		
			cpm ^{32}P	*mg*	*SA**	*cpm* ^{32}P	*mg*	*SA*	*cpm* ^{32}P	*mg*	*SA*
Experiment A	Free	Bound									
Start			41,200	15.3	2700	39,600	10.8	3670	1630	4.5	362
End			32,200	12.8	2500	29,900	9.4	3180	2300	3.4	677
Change			−9100	−2.5	3600	−9700	−1.4	6960	+670	−1.1	
% loss			22	16.3		24.6	13.0			24.4	
Experiment B	Bound	Free									
Start			16,400	13.6	1200	4760	10.2	467	11,600	3.4	3410
End			15,400	12.3	1260	8200	9.5	863	7240	2.8	2590
Change			−920	−1.3	708	+3440	−0.7		−4360	−0.6	7270
% loss			5.6	9.5			6.9		37.6	16.6	

* Specific activity of ribonucleic acid (RNA) in counts per minute per milligram.

pable of RNA degradation, it cannot be ruled out that some labeled RNA was transferred *and* degraded during the four hr incubation. There was, therefore, a net loss of membrane-bound RNA in association with movement of RNA from the free compartment to the bound compartment. Experiment A indicates that during erythroid cell RNA degradation there is transfer between the free and bound compartments in both directions. Although free RNA is the major source of degraded RNA it is accompanied by a shift of RNA from the bound to the free compartment. Almost half of the total net RNA loss is from the bound compartment, either by transfer out of the compartment, degradation, or both.

The conclusions from this study were confirmed when the reverse of the above experiment was done, with the reconstituted hemolysate consisting of ^{32}P-labeled, membrane-bound RNA and unlabeled, free RNA (experiment B). Radioactivity was lost, a fact indicating that RNA which was originally bound to the membrane was degraded. The smaller amount of radioactive RNA degraded in this study, in comparison to experiment A, reflects the lesser proportion of total cellular RNA normally present in the bound compartment (15). The free compartment gained radioactivity while losing RNA, a fact indicating a transfer of RNA from the bound to free compartment (column Nos. 4 and 5). Movement in the opposite direction, from the free to the bound compartment, was indicated by a loss of radioactivity in excess of loss of RNA from the membrane (column Nos. 7 and 8). The specific activity of the RNA which disappeared, being less than that originally present in the reconstituted lysate (column No. 3), confirmed that the bulk of RNA degraded was originally present in the free fraction.

These two experiments were representative of four such studies done. Analysis of the data in Table II allows the following conclusions to be drawn. There is a transfer of RNA between the free and bound compartments in both directions. The bulk of the material being destroyed was derived from RNA initially in the free compartment. RNA which was membrane bound was also destroyed. Almost 50% of the total amount of RNA degraded disappeared from the bound compartment, due to degradation and(or) transfer to the free compartment.

DISCUSSION

The objective of the present study was to characterize mammalian erythroid RNA degradation as it occurs under conditions found in the intact cell. The progressive decrease of RNA previously observed during cell maturation in vivo and in vitro (1, 11, 15, 22–24) has now been demonstrated in a linear study of an aging group of cells in an individual animal. That this decrease occurs in association with cell maturation, and does not merely reflect removal of cells from circulation, has been documented earlier (22). The present studies show that during in vitro maturation at 37°C degradation of RNA to acid-soluble products occurs at a rate of about 4% per hr. The rate of intracellular degradation is temperature dependent with an optimum at 50°C and, as the presence of glycolytic inhibitors did not significantly affect the loss of RNA, the degradative process is not energy dependent. At temperatures in excess of 50°C the rate of degradation decreases. This relationship between temperature and reaction rate is typical of a system in which a participating enzyme is inactivated by heat (25). The linear rate of degradation obseved during in vitro incubation is also consistent with an enzymatic

mode of degradation. The characteristics of RNA degradation in the intact cell thus suggest, although they do not prove, that it is an enzyme-mediated process.

The exact mechanism of RNA degradation in the erythroid cell is unknown. Degradation of polynucleotides presumably takes place by stages to oligonucleotides, nucleotides, nucleosides, and finally to ribose and free bases. RNA degradation within the erythroid cell evidently proceeds fully to the final step, since large oligonucleotide fragments do not accumulate intracellularly as the cell matures (11, 15). The enzyme or enzymes responsible for this degradation have not been isolated. Several investigators have characterized RNase activity in the erythroid cell, but the results show wide disagreement in regard to ionic requirements, pH optimum, substrate specificity, and location within the cell (3–10). These studies have not, in general, been done under physiologic conditions and thus may have little relevance to the mechanism as it occurs in vivo.

The location of ribonuclease within the erythroid cell is of particular pertinence in view of the findings of the present study. Two studies have suggested that erythroid cell RNase is primarily associated with the cell membrane (3, 4), and an RNA has been found in other mammalian cell membranes (26). The present study indicates conclusively that isolated cell membranes are in themselves capable of degrading erythroid cell RNA. The reason for the increased rate of RNA degradation by erythroid cell membranes, approximately threefold that of degradation in the cell as a whole, cannot be determined on the basis of the present studies. Possible explanations include the presence of an RNase, localized to the cell membrane, which has different properties than the enzyme in the cell interior, or the alteration of enzyme-substrate relationships due to the peculiar environmental characteristics found in the membrane. It is clear, however, that the cell membrane, associated with 20–30% of the total cell complement of RNA (12, 15), participates in RNA metabolism in the maturing erythroid cell.

RNA within the erythroid cell is not static in location. A continuous exchange between free and membrane-bound RNA in both directions has been found. This is consistent with earlier studies on the location of RNA in erythroid cells after in vivo pulse labeling indicating that newly formed RNA appears first in the free fraction and later in the bound (12). RNA has been localized to the cell membranes of mammalian cells other than the erythroid cell (27). The relationship between binding of RNA to the cell membrane and degradation of RNA is unclear. Membrane binding of RNA is known, however, to influence its rate of degradation (28, 29). Since the erythroid cell membrane is in itself capable of degradating RNA, it is unlikely that it does not participate in this process in vivo. Membrane-bound RNA in the erythroid cell is less susceptible to degradation by exogenous RNase (12, 32), but this finding may not apply to endogenous RNase. The increased rate of degradation of RNA in isolated cell membranes suggests that substrate RNA, in physiologic concentrations, is actually more susceptible to degradation when it is in contact with the membrane.

The complexity of the fluxes of RNA within the cell make it difficult to determine precisely how large a part the cell membrane plays in erythroid cell RNA degradation. Isolated membrane-free hemolysates are also capable of RNA degradation. It is possible, however, that the RNase activity of membrane-free hemolysates could result from a release of membrane RNase during cell lysis, as has been shown to occur in *Escherichia coli* (30). Net loss of RNA from the bound compartment could represent either degradation *in situ* or transfer to the free compartment. Since mature erythrocyte membranes are not capable of binding erythroid cell ribosomes in vitro, while reticulocyte membranes are (31), a change in membrane binding sites may be involved in the loss of membrane-bound RNA which accompanies cell maturation.

The more rapid rate of degradation of ribosomal RNA, as compared with soluble RNA, in the maturing erythroid cell suggests that the rate of degradation of the two types of RNA are regulated by independent mechanisms. It explains the presence of soluble RNA in the mature erythrocyte (20). Studies in this laboratory have shown that the rate of degradation of isolated ribosomes by cell lysates is significantly less than that of isolated transfer RNA.[1] Thus, there must be a special mechanism to account for the relatively rapid rate of degradation of reticulocyte ribosomes in vivo. Although ribonuclease has been directly associated with reticulocyte ribosomes (5, 6), more detailed studies have disputed this finding (7). The difference between the rates of degradation of ribosomes and soluble RNA cannot be explained by assuming that soluble RNA does not become bound to the cell membrane. Both soluble RNA and ribosomes are present in the same relative proportions in the free and the membrane-bound compartments of the erythroid cell (12, 15). Data have suggested that ribosomes are bound to the cell membrane by a specific mechanism (31, 32), and this may be related to their more rapid rate of degradation, in comparison to soluble RNA, in the maturing cell. It is of interest that whole erythroid cell lysates have been reported to degrade polyribosomes more rapidly than membrane-free lysates (33).

The close association between RNA degradation and erythroid cell maturation suggests a relationship be-

[1] Burka, E. R. Unpublished observations.

tween the two. Reticulocyte maturation is delayed by low temperature (34), as is RNA degradation. A high concentration of magnesium retards reticulocyte maturation in vitro (35) and also inhibits an RNase isolated from erythroid cells (10). Metabolic inhibitors have also been reported to slow reticulocyte maturation (36), but in the present study their presence did not prevent degradation of RNA. Thus, the precise nature of the relationship between erythroid cell maturation and RNA degradation remains unclear. Studies are now in progress to determine more clearly the role of RNA degradation in the maturation of the erythroid cell. It is clear, however, that the erythroid cell membrane, in addition to moderating numerous cellular activities, participates in the catabolism of RNA within the cell.

ACKNOWLEDGMENTS

The expert technical assistance of Mrs. Eva Hennel and Mrs. Virginia Johnston is gratefully acknowledged. These studies were supported by U. S. Public Health Service Grants HE-10473 and H-6374.

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