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Attachment of Reticulocyte Ribosomes to Erythroid Cell Membranes *in Vitro**

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ABSTRACT: A proportion of the total ribosomes in the erythroid cell is closely bound to the cell membrane. An *in vitro* system is described in which free reticulocyte polyribosomes and monoribosomes become associated with reticulocyte cell membranes. Binding occurs at 0 and 37° and the number of receptor sites on the cell membrane appears to be limited. The number of these sites, which are not destroyed by exposure to trypsin, declines with increasing cell maturity. Free ribosomes which become associated with the cell membrane assume the sedimentation characteristics and chemical reactivity of authentic membrane-bound reticulocyte ribosomes, indicating that binding to structural components of the cell membrane is responsible for the alteration of ribosome properties. The amount of nonribosomal protein associated with membrane-bound ribosomes appears to influence their resistance to breakdown upon exposure to ethylenediaminetetraacetic acid.

Approximately 18% of ribosomes in reticulocytes of all degrees of maturity is closely bound to the cell membrane (Schreml and Burka, 1968; Burka, 1968). The significance of the binding, as well as the exact means of attachment, remains unknown (Schreml and Burka, 1968; Hendler, 1965). Both pulse-labeling experiments (Burka *et al.*, 1967) and exchange studies in an *in vitro* system (Burka, 1969) indicate that these membrane-bound erythroid cell ribosomes freely exchange with the pool of free ribosomes. The present report describes an *in vitro* system in which free erythroid cell ribosomes became attached to reticulocyte membranes, providing an opportunity to investigate the biological function and properties of membrane-bound ribosomes. The binding sites for ribosomes on the cell membrane appear to be limited in number and decrease as the cell matures. In becoming bound to the cell membrane, free ribosomes assume the physicochemical characteristics of authentic membrane-bound reticulocyte ribosomes, indicating that environmental factors related to the attachment of ribosomes are able to influence their reactivity.

* From the Cardeza Foundation for Hematologic Research, Jefferson Medical College, Philadelphia, Pennsylvania 19107. *Received May 26, 1969.* This investigation was supported by U. S. Public Health Service Grants AM HE 13431 and HE 06374. A preliminary account of these studies was presented at the Annual Meeting of the Eastern Section of the American Federation for Clinical Research, Philadelphia, Pa., Dec 8, 1967.

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Materials and Methods

The analytical and preparative methods used have been described in detail in previous communications (Schreml and Burka, 1968; Burka, 1968, 1969; Burka *et al.*, 1967).

Isolation of Cell Components and Gradient Centrifugation. Ribosomes and erythroid cell components were isolated from the blood of either normal rabbits or rabbits with a phenylhydrazine-induced reticulocytosis. Washed packed cells were lysed by addition of four volumes of a solution of 1.5×10^{-3} M $MgCl_2$ in 1×10^{-3} M Tris (pH 7.4) (solution A). Free and membrane-bound ribosomes were isolated from membrane-free hemolysates or washed cell membranes (Burka *et al.*, 1967) in the presence of 0.2% deoxycholate (Schreml and Burka, 1968). Sucrose density gradient centrifugation was carried out in the Spinco Model L-2 preparative ultracentrifuge, using either the SW-41 or SW-25 rotor, under the conditions described in the legends to the figures. The location of [^{32}P]ribosomes in the gradient was determined as previously described (Schreml and Burka, 1968).

Attachment of Free Ribosomes to Cell Membranes. The method for attaching free reticulocyte ribosomes to washed cell membranes is shown schematically in Figure 1. Free ribosomes (6–13 mg), labeled with ^{32}P *in vivo* (Burka *et al.*, 1967), were incubated at 37° for 45 min with 4–8 ml of a suspension of washed cell membranes, containing between 8 and 10 mg of protein per ml, in solution A. The concentration of ribosomes in the incubation medium approximated their concentration in intact reticulocytes (Burka, 1968). The entire incubation mix-

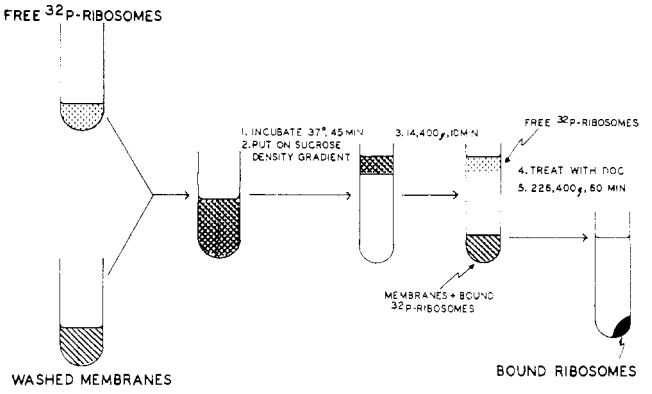

FIGURE 1: Procedure for the attachment of free erythroid ribosomes to reticulocyte membranes. All procedures other than the incubation were carried out at 0–4°.

ture was then layered over a 26-ml 10–30% linear sucrose gradient in solution A and centrifuged in the SW-25 rotor at 14,400*g* for 10 min. The dense membranes, containing any ribosomes which had become attached during the incubation, sedimented rapidly through the sucrose column to the bottom of the tube (step 3, Figure 1). Membrane recovery was quantative by this method and all unattached free [^{32}P]ribosomes remained at the top of the sucrose column, as confirmed by the absence of radioactivity at the bottom of the tube when free [^{32}P]ribosomes were centrifuged alone under similar conditions. The sedimented membranes were washed twice and then suspended in solution A, brought to a final concentration of 0.2% with sodium deoxycholate in order to release bound ribosomes from the membrane, and the bound ribosomes were recovered by ultracentrifugation (Schreml and Burka, 1968). Further studies were done on these pelleted ribosomes, which consisted of a mixture of the unlabeled ribosomes which had been originally bound to the incubated membranes and the [^{32}P]-ribosomes which had become attached to the membrane during the incubation. The proportion of labeled ribosomes derived from the membranes, referred to subsequently as bound ribosomes, was determined by comparing the radioactivity in a hot 5% trichloroacetic acid extract (Burka, 1966) of the ribosome suspension with that of a similar extract of the original free ribosomes.

Pancreatic ribonuclease (EC 2.7.7.16) was obtained from the Sigma Chemical Co., St. Louis, Mo. Trypsin (EC 3.4.4.4) was obtained from Worthington Biochemical Corp., Freehold, N. J., and freed from ribonuclease activity by chromatography on Sephadex G-50 (Tunis, 1968).

Results

Attachment of Free Ribosomes to Erythroid Cell Membranes. Membrane-bound ribosomes, as isolated from intact reticulocytes, differ from free ribosomes in sedimentation characteristics (Schreml and Burka, 1968). In order to determine if reticulocyte ribosomes became attached to washed cell membranes during *in vitro* incubation, the sedimentation characteristics of ^{32}P-labeled free ribosomes were compared with those derived from washed cell membranes after incubation (Figure 2). The sedimentation profile of free ribosomes (upper) shows a peak of 80S single ribosomes (tube 13) and a larger proportion of

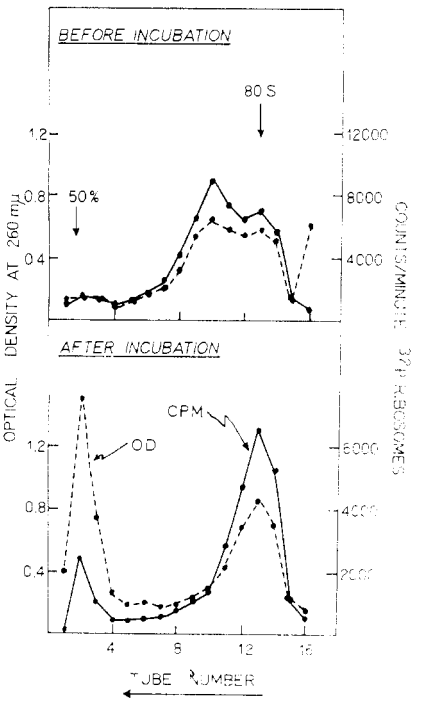

FIGURE 2: Sedimentation characteristics of free [^{32}P]ribosomes before and after incubation with washed erythroid cell membranes. Sedimentation was through an 11-ml linear gradient of 10–30% sucrose in solution A which was mixed over 1 ml of 50% sucrose in solution A (arrow). Centrifugation was in the Spinco SW-41 rotor at 38,000 rpm for 50 min. Eight drop samples were collected from the bottom of the tube and assayed for ultraviolet absorbance and for radioactivity. The direction of sedimentation is from right to left. Upper: profile of 0.78 mg (61,000 cpm) of free ribosomes prior to incubation. Recovery from the gradient was 96%. Lower: profile of ribosomes extracted by sodium deoxycholate treatment of membranes following incubation of 7.8 mg of free ribosomes (upper portion of the diagram) with 7 ml of reticulocyte membrane suspension; 1.31 mg (37,000 cpm) of the bound ribosomes, representing 10% of the total recovery, were centrifuged, with 81% recovery from the gradient. The proportion of the free ribosomes attached to the membranes during the incubation was 60.6%. See text for discussion of the origin of the 80S peak in the lower portion of the figure.

more rapidly sedimenting polyribosomes (tubes 4–12) (Marks *et al.*, 1962). Free ribosomes do not sediment to a point directly above the dense 50% sucrose cushion, indicated by the arrow, at the bottom of the tube. Following incubation of the free [^{32}P]ribosomes with washed membranes, and subsequent reisolation of ribosomes from the sodium deoxycholate treated membranes (lower), a portion of the [^{32}P]ribosomes is now located immediately above the 50% sucrose cushion (tube 2), the position in which authentic membrane-bound ribosomes, aggregated with large amounts of protein, sediment (Schreml and Burka, 1968). The ultraviolet absorbance and radioactivity in this gradient do not coincide, as the absorbance is primarily due to unlabeled ribosomes which were originally attached to the membranes. Since radioactivity in this gradient can only come from ribosomes which were previously free in the incubation mixture, the radioactivity above the 50% sucrose cushion represents free ribosomes which have become attached to the membranes during incubation, and which have assumed the sedimentation characteristics of authentic membrane-bound ribosomes. The proportion of free

TABLE I: Attachment of Free Reticulocyte [^{32}P]Ribosomes to Erythroid Cell Membranes.[a]

Conditions	% [^{32}P]Ribosomes Bound	
	Expt I	Expt II
Reticulocyte membranes, 37°[b]	63.0	60.6
Reticulocyte membranes, 0°[b]	43.0	44.2
Erythrocyte membranes, 37°[c]	0.4	0.1

[a] Free [^{32}P]ribosomes were incubated with suspensions of either reticulocyte membranes or erythrocyte membranes in solution A as described in the text. Subsequent to incubation the proportion of ribosomes bound to the isolated membranes was determined. The two experiments are duplicate studies using the same ribosome and membrane suspensions. [b] Reticulocytosis greater than 90%. [c] Reticulocytosis less than 4%.

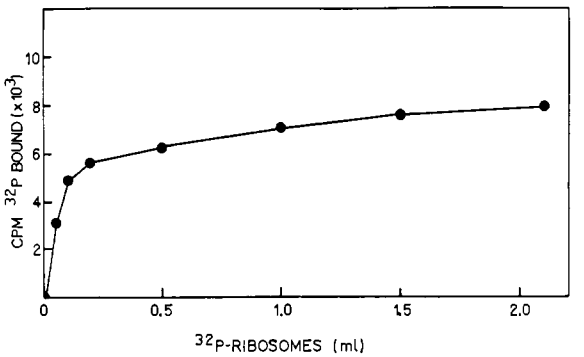

FIGURE 3: Relationship between the amount of free ribosomes in the incubation mixture and the amount of ribosomes attached to reticulocyte membranes. Increasing amounts of a suspension of reticulocyte [^{32}P]ribosomes were incubated with aliquots of reticulocyte membranes in a total incubation volume of 8 ml (see Methods). The concentration of the free ribosomes added to the incubation mixture was 6.76 mg/ml, and the specific activity, 10,844 cpm/mg. The membranes were prepared from cells with a reticulocytosis of 90%. Attachment is expressed as the total counts per minute bound to the isolated membranes after a 45-min incubation at 37°.

ribosomes which became attached during this experiment was 60.6% (only 10% of the total ribosomes derived from the membranes was put on the gradient shown in the lower portion of Figure 2).

A portion of the ribosomes derived from the sodium deoxycholate treated membranes also sedimented in the monoribosome region of the gradient (tubes 10–15), but this did not represent contamination with free ribosomes. The protein:RNA ratio of these particles varied between 3.4 and 5.2 to 1, significantly higher than the 1.7 to 1 ratio of sodium deoxycholate treated free ribosomes isolated under similar conditions (Burka, 1967), but less than that of the rapidly sedimenting particles (Schreml and Burka, 1968). In conjunction with the fact that nonbound ribosomes were excluded by the preparative procedure (steps 2 and 3, Figure 1), the high protein:RNA ratio of these particles in the monosome region suggested that they were derived from the rapidly sedimenting bound ribosomes during the homogenization which is necessary to adequately suspend these particles in solution A (Schreml and Burka, 1968). This premise was confirmed by the relationship between the degree of homogenization and the amount of these particles in the membrane-bound ribosome suspension. When only one stroke of the homogenizer was used to suspend the ribosomes the proportion of 80S membrane-bound ribosomes was 43.3%, but increased progressively to 69.8% when four strokes were used.

The rapid sedimentation of at least one-third of the total radioactivity in the ribosomes recovered from the incubated membranes is evidence in itself that ribosomes originally free in the incubation suspension had become attached to the membranes. This attachment occurred even though the reticulocyte membranes undoubtedly already had some ribosomes bound to them. In order to ensure that the sedimentation characteristics of the [^{32}P]ribosomes isolated after incubation from the membrane suspensions were due to *in vitro* attachment to the cell membranes, a mixture of isolated free [^{32}P]ribosomes and unlabeled bound ribosomes, incubated for 45 min in the presence of 0.2% sodium deoxycholate, was centrifuged through a sucrose density gradient under identical conditions. In this instance no radioactivity was associated with the rapidly sedimenting particles above the 50% sucrose cushion, indicating that radioactivity in this region of the gradient was a consequence of the incubation procedure and not due to nonspecific attraction between free and bound ribosomes.

The proportion of free ribosomes bound by reticulocyte membranes in nine incubations averaged 51.2% (range, 15–82%) of the added radioactivity. In several experiments the proportion of [^{32}P]ribosomes which became attached to the membranes exceeded the proportion of polyribosomes in the sample, indicating that both monoribosomes and polyribosomes became bound. Different membrane suspensions varied considerably in their ability to bind free ribosomes, but a single membrane suspension was relatively constant in its ability to bind ribosomes (Table I). The absolute amount of free [^{32}P]-ribosomes which became bound approached a maximum when progressively greater amounts of ribosomes were incubated with aliquots of cell membranes (Figure 3), suggesting that the number of binding sites on the membrane is limited. Incubation at 0° decreased the amount of binding to between 65 and 94% of the value at 37° (Table I). Prior exposure of reticulocyte membranes to concentrations of up to 100 μg/ml of trypsin at 37° for 1 hr, and subsequent washing, did not decrease the amount of binding. Erythrocyte membranes, in contrast to reticulocyte membranes, did not bind free ribosomes (Table I). This suggests that with maturation of the erythroid cell ribosome binding sites on the membrane disappear. The failure of ribosomes to attach to erythrocyte membranes provides additional evidence that the attachment of ribosomes to reticulocyte membranes *in vitro* is not a consequence of the preparative procedure, and cannot be ascribed to a nonspecific aggregate of ribosomes and membrane protein.

Characteristics of Newly Bound Ribosomes. Membrane-bound ribosomes differ from free ribosomes in several characteristics, among which are reduced susceptibility to breakdown by EDTA and relative resistance to the action of small amounts of pancreatic ribonuclease (Burka, 1967; Schreml and Burka, 1968). In order to determine if the free ribosomes which became attached to membranes during the incubation

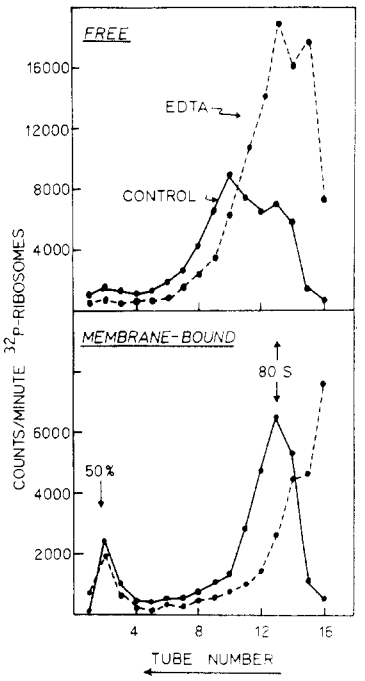

FIGURE 4: The effect of exposing free [^{32}P]ribosomes before (upper) and after incubation with washed membranes (lower) to 0.01 M EDTA. Sucrose density gradient centrifugation was carried out as described for Figure 2. The solid lines show sedimentation profiles of the ribosomes prior to exposure to EDTA, and the dotted lines show the profiles after exposure.

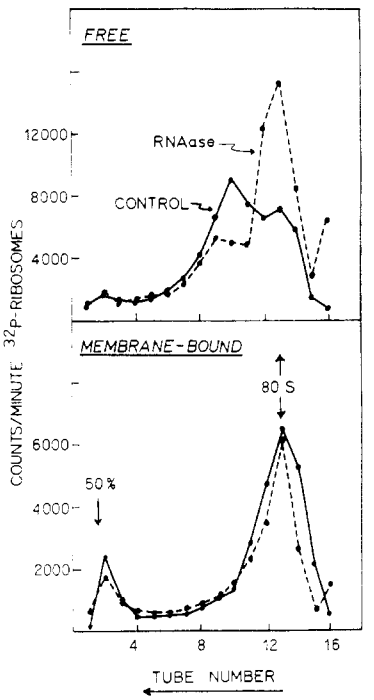

FIGURE 5: The effect of exposing free [^{32}P]ribosomes before (upper) and after incubation with washed cell membranes (lower) to 10 μg/ml of pancreatic RNase at room temperature for 30 min. No loss of total radioactivity was observed in this time interval. Conditions of centrifugation were as described for Figure 2. The solid lines show sedimentation profiles of the ribosomes prior to exposure to RNase, and the dotted lines show the profiles after exposure.

had the properties of authentic membrane-bound ribosomes, free [^{32}P]ribosomes, and the same ribosomes subsequent to their attachment to membranes, were dialyzed overnight against 1000 volumes of 0.01 M EDTA and then examined by gradient centrifugation (Figure 4). Free ribosomes were broken down to particles with a coefficient of sedimentation of 80 S or less by this procedure (upper). The lack of 40S ribosomal subparticles in this figure is due to their instability (Luzzatto *et al.*, 1965) and the poor resolution of low molecular weight particles under these particular conditions of centrifugation. After attachment to membranes *in vitro* (lower) the [^{32}P]ribosomes in rapidly sedimenting particles (tubes 1–4) are not broken down by dialysis against EDTA. In contrast to the resistance of these rapidly sedimenting particles to EDTA, the single ribosomes (tubes 10–15), derived by mechanical disruption from the membranes, did break down upon exposure to EDTA. This suggests that the association of ribosomes with large amounts of protein, presumably of membrane origin (Schreml and Burka, 1968), in the rapidly sedimenting particles is related to their resistance to EDTA.

Figure 5 shows a study in which free [^{32}P]ribosomes, and the same ribosomes following their attachment to membranes, were exposed to 10 μg/ml of pancreatic ribonuclease at room temperature for 30 min. Free polyribosomes (upper) are broken down to 80 S units by this treatment (Goodman and Rich, 1963). After *in vitro* attachment to the membrane (lower), the same ribosomes, now in rapidly sedimenting particles, were unaffected by this procedure. These studies show that following *in vitro* attachment to membranes, free ribosomes assume some of the physicochemical characteristics of authentic membrane-bound reticulocyte ribosomes.

Discussion

The existence of membrane-bound reticulocyte ribosomes *in vivo*, has been established and has been shown not to be a consequence of the experimental procedures (Schreml and Burka, 1968). The present study, by demonstrating that free erythroid cell ribosomes can attach to reticulocyte membranes *in vitro*, provides additional confirmatory evidence for the existence of membrane-bound ribosomes in erythroid cells. As the erythroid cell matures, either *in vivo* or *in vitro*, the cell content of both total and bound ribosomes decreases (Marks *et al.*, 1963, Schreml and Burka, 1968; Burka, 1969). Since erythrocyte membranes do not bind ribosomes, the present studies suggest that the decrease in the number of bound ribosomes which accompanies erythroid cell maturation may be due, at least in part, to a loss of binding sites on the membrane. Other structural and chemical changes in the cell accompany erythroid cell maturation (Danon and Perk, 1962; Westerman *et al.*, 1963) and sites which bind other macromolecules disappear during this process (Jandl *et al.*, 1959). A decrease in the number of membrane binding sites for ribosomes is consistent with the greater protein:RNA ratio found in bound ribosomes extracted from a progressively aging group of erythroid cells (Schreml and Burka, 1968), a finding which suggests that ribosome binding sites, which become less available as the cell matures, are an integral part of the protein matrix of the membrane.

The exact location of the binding sites in the membrane architecture is unknown, but presumably is protein in nature

(Schreml and Burka, 1968; Burka *et al.*, 1967). The present studies provide little further information concerning the exact mode of attachment of ribosomes to the cell membrane. The limited number of free ribosomes able to be bound by a single membrane suspension, and its relative constance, suggests that there are a limited number of binding sites on the reticulocyte membrane. The membranes used in these studies, being reticulocyte membranes, already had some ribosomes bound to them (Schreml and Burka, 1968). Variation in the number of unfilled binding sites on the membranes may explain the wide range in the amount of free ribosomes bound to membranes in the *in vitro* system.

The assumption of new physical properties by free ribosomes subsequent to their binding to the cell membrane is consistent with the finding that there is a free exchange *in vitro* between free and bound ribosomes in both directions (Burka, 1969). Free erythroid cell ribosomes and RNA are not inherently different from ribosomes and RNA which are bound to the cell membrane, either in types or proportions of molecular species (Burka, 1968; Burka *et al.*, 1967; Christman and Goldstein, 1969). The findings indicate that the sedimentation characteristics and chemical reactivity of membrane-bound ribosomes are related to the association with increased amounts of protein, and not due to any particular properties of the ribosomes *per se*. The finding that membrane-bound ribosomes which were mechanically freed from the large rapidly sedimenting ribosome–protein aggregates, with a protein: RNA ratio between that of free and the rapidly sedimenting bound ribosomes, were broken down by EDTA suggests that the resistance of the rapidly sedimenting particles to EDTA is related to the amount of protein with which they are aggregated. It is not known whether the decreased activity in protein synthesis by membrane-bound reticulocyte ribosomes, in comparison with free ribosomes (Schreml and Burka, 1968), is also related to the association with nonribosomal protein. The system described, by allowing studies of ribosomes before and subsequent to attachment to the cell membrane, provides a means for further investigation of the effect of nonribosomal protein on ribosome function.

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