MEMBRANE-BOUND RIBOSOMES OF MYELOMA CELLS

III. The Role of the Messenger RNA and the Nascent Polypeptide Chain in the Binding of Ribosomes to Membranes

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ABSTRACT

Mild ribonuclease treatment of the membrane fraction of P3K cells released three types of membrane-bound ribosomal particles: (*a*) all the newly made native 40S subunits detected after 2 h of [^{3}H]uridine pulse. Since after a 3-min pulse with [^{35}S]methionine these membrane native subunits appear to contain at least sevenfold more Met-tRNA per particle than the free native subunits, they may all be initiation complexes with mRNA molecules which have just become associated with the membranes; (*b*) about 50% of the ribosomes present in polyribosomes. Evidence is presented that the released ribosomes carry nascent chains about two and a half to three times shorter than those present on the ribosomes remaining bound to the membranes. It is proposed that in the membrane-bound polyribosomes of P3K cells, only the ribosomes closer to the 3′ end of the mRNA molecules are *directly* bound, while the latest ribosomes to enter the polyribosomal structures are *indirectly* bound through the mRNA molecules; (*c*) a small number of 40S subunits of polyribosomal origin, presumably initiation complexes attached at the 5′ end of mRNA molecules of polyribosomes.

When the P3K cells were incubated with inhibitors acting at different steps of protein synthesis, it was found that puromycin and pactamycin decreased by about 40% the proportion of ribosomes in the membrane fraction, while cycloheximide and anisomycin had no such effect. The ribosomes remaining on the membrane fraction of puromycin-treated cells consisted of a few polyribosomes, and of an accumulation of 80S and 60S particles, which were almost entirely released by high salt treatment of the membranes. The membrane-bound ribosomes found after pactamycin treatment consisted of a few polyribosomes, with a striking accumulation of native 60S subunits and an increased number of native 40S subunits.

On the basis of the observations made in this and the preceding papers, a model for the binding of ribosomes to membranes and for the ribosomal cycle on the membranes is proposed. It is suggested that ribosomal subunits exchange between free and membrane-bound polyribosomes through the cytoplasmic pool of free native subunits, and that their entry into membrane-bound ribosomes is mediated by mRNA molecules associated with membranes.

In HeLa cells and other cells growing in culture, some membrane-bound ribosomes can be released by treatment of the membrane fraction with RNase under conditions known to degrade the mRNA preferentially (18, 28). This suggests that some ribosomes are bound to the membranes through mRNA molecules and has led, with other observations such as the release of some membrane-bound ribosomes after treatment of the cells with puromycin (28), to the concept that there exist two classes of membrane-bound ribosomes, loosely and tightly bound (28, 29). In this concept, the loosely bound ribosomes are those which are dissociated from the membranes by RNase or puromycin treatment.

Binding through mRNA might also account for the presence in the membrane fraction of P3K cells of a small amount of newly made, native 40S subunits (22), since the only direct ribosomal binding site on the membranes appears to be for the large, and not for the small subunits (31). Thus, these native 40S subunits might be initiation complexes formed on mRNA bound to the membranes; in this case, they should be released from the membranes following RNase treatment and they should have some of the characteristics of initiation complexes, e.g., carry methionine-$tRNA_F$.

In this paper, the nature of the membrane-bound ribosomal particles released by or resistant to RNase treatment has been studied and related to the length of the nascent polypeptide chains or to the presence of methionine-tRNA. In addition, the effects on the membrane-bound ribosomes of in vivo treatment with various inhibitors of protein synthesis have been explored, especially those of puromycin, which releases the nascent polypeptide chains from the ribosomes, and of pactamycin, which primarily inhibits the initiation of protein synthesis (19). On the basis of these observations and those made in the preceding papers (21, 22), a hypothesis is presented to account for the attachment of ribosomes to membranes and for the ribosome cycle on the membranes during protein synthesis.

MATERIALS AND METHODS

All procedures for cell culture, labeling, and fractionation, as well as for preparation and analysis of free and membrane-bound ribosomal fractions, have been described previously (21, 22).

Pactamycin was obtained from Upjohn Co., and anisomycin was a gift of Pfizer Co. Bovine pancreatic ribonuclease A was supplied by Worthington Biochemical Corp., Freehold, N.J., [^{35}S]methionine, 144 Ci/mmol, and [^{3}H]leucine, 40 Ci/mmol, were purchased from Amersham Radiochemical Centre.

RESULTS

Properties of Membrane-Bound Native 40S Ribosomal Subunits

EFFECT OF MILD RNASE TREATMENT ON THE MEMBRANE FRACTION: The free ribosomal and membrane fractions of cell, uniformly labeled with [^{14}C]uridine and pulsed 2 h with [^{3}H]uridine, were separated as described earlier (22). The membrane fraction was divided into two samples: one was incubated at 37°C for 90 s in the presence of 1 μg/ml of RNase, and the other (control) kept in the same conditions without RNase. The two aliquots were then placed on discontinuous sucrose density gradients to reisolate the membrane fraction. In the control sample, essentially all the radioactivity was recovered in the membrane fraction, indicating that no ribosomal particles had been released from the membranes. In the RNase-treated aliquot, about half of the total ribosomal ^{3}H- and ^{14}C-radioactivity sedimented in the free ribosomal fraction and 50% in the membrane fraction, indicating that about half of the newly made and of the uniformly labeled ribosomal particles had been detached. The released ribosomal particles, as well as the detergent-solubilized components of both RNase-treated and control membrane fractions, were then analyzed by sedimentation on 15–30% sucrose density gradients over a cushion of 69% sucrose.

The ^{3}H-labeled, newly made 40S subunits, clearly seen in the control membrane fraction (Fig. 1 *a*), have entirely disappeared after RNase treatment (Fig. 1 *b*). On the other hand, the RNase treatment did not modify the labeled 60S subunits, nor the ^{14}C-radioactivity sedimenting in the 40S region, which has been shown to correspond mostly to mitochondrial ribosomal subunits. Analysis of the material released from the membranes by RNase treatment (Fig. 1 *c*) showed the presence of ^{3}H-labeled 40S subunits (with a small amount of uniformly labeled 40S subunits, corresponding to 2–3% of the originally membrane-bound ^{14}C-ribosomes), and of 80S ribosomes representing about 50% of the ribosomes originally bound to the membranes. The specific radioactivity of the released 80S ribosomes was similar to that of the 80S ribosomes remaining associated with mem-

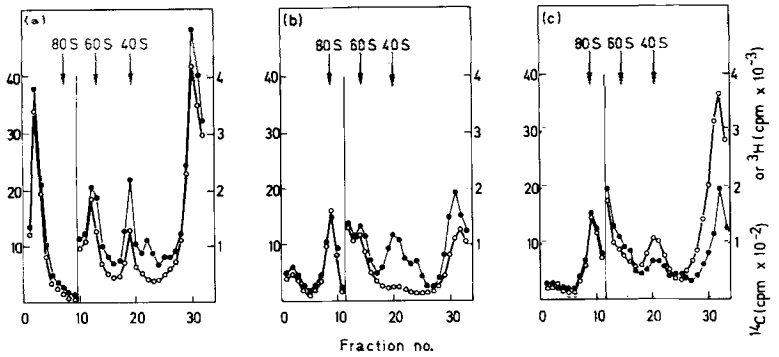

FIGURE 1 Effect of mild RNase treatment on the ribosome particles associated with the membranes. 3×10^7 P3K cells were uniformly labeled with [^{14}C]uridine (0.05 μCi/ml) and then exposed to [^{3}H]uridine (65 μCi/ml) for 2 h in the presence of ethidium bromide (1 μmol/ml). The membrane fraction was isolated in the presence of 0.15 M KCl as described (see Materials and Methods) and divided into two portions. One was incubated at 37°C for 90 s in the presence of 1 μg/ml of bovine pancreatic RNase, and the other (control) kept in the same conditions without RNase. The RNase-treated and the control membrane fractions were then placed on a discontinuous sucrose density gradient to reisolate the membrane fractions. The control experiment showed that all the ^{14}C-labeled ribosomal RNA was recovered with the membrane fraction after this second purification. On the other hand, in the RNase-treated fraction, about half of the radioactivity sedimented in the free ribosome region, and half in the membrane region. The reisolated membrane fractions were dissolved with 0.5% sodium deoxycholate and 0.5% Brij 58. These samples, as well as the RNase-released ribosomes, were applied to TK_{80} M sucrose gradients 15–30% containing a 4-ml cushion of 79% sucrose and centrifuged 8.5 h at 27,000 rpm in a Spinco SW 27 rotor. —●—●—, ^{14}C-labeled RNA; —○—○—, ^{3}H-labeled RNA. (*a*) Control, total membrane-bound ribosomes; (*b*) RNase-resistant ribosomes reisolated with the membranes; (*c*) RNase-released ribosomes recovered in the free ribosome region of the discontinuous sucrose density gradient. Note scale change for left-hand part of the gradient.

branes after RNase treatment (^{3}H to ^{14}C ratios, respectively, 15.3 and 15.7) indicating that the two categories of ribosomes do not differ by their kinetics of appearance on the membranes. Some slowly sedimenting radioactive material was also observed in the released fraction; this presumably represents degraded mRNA and low molecular weight RNA.

PRESENCE OF MET-tRNA ON THE NATIVE 40S SUBUNITS BOUND TO THE MEMBRANES: Cells were labeled for 120 min with [^{3}H]uridine in the presence of ethidium bromide, then washed and pulsed for 3 min with [^{35}S]methionine. Protein synthesis was then stopped by the addition of cycloheximide (3.6×10^{-3} M), the cells were homogenized, and the postnuclear supernate was divided on a discontinuous sucrose density gradient into free ribosomal and membrane fractions. Two types of sucrose gradients were used to analyze the ribosomes in each cell fraction, one to explore the whole range of sedimentation of ribosomal particles (Fig. 2 *a*, *b*), and the other to resolve the native 60S and 40S subunit regions more clearly (Fig. 2 *c*, *d*). Each fraction collected from the sucrose gradients was divided in two aliquots: one was precipitated in cold trichloroacetic acid to determine total ^{3}H and ^{35}S-counts; the other was used to determine the ^{35}S-radioactivity resistant to 20 min of trichloroacetic acid hydrolysis at 90°C, which represents the [^{35}S]methionine contained in nascent polypeptide chains. In both the free (Fig. 2 *a* and *c*) and the membrane-bound (Fig. 2 *b* and *d*) ribosomal fractions, the distribution of total ^{35}S-radioactivity coincided with the polyribosome and native 40S subunit ^{3}H-radioactivity, while there was no peak of ^{35}S-radioactivity in the native 60S subunit regions. Acid hydrolysis released only part of the ^{35}S-radioactivity of the polyribosome region, indicating that part of this radioactivity was in nascent chains and part in Met-tRNA. In contrast, the ^{35}S-peak coinciding with the ^{3}H-labeled native 40S subunits disappeared after acid hydrolysis, suggesting that it consists exclusively of Met-tRNA, presumably Met-$tRNA_F$ in initiation complexes. Furthermore, the ^{35}S-peak of the membrane-bound native newly made 40S subunits (Fig. 2 *d*) was higher than the ^{35}S-peak coinciding with the native newly made

40S subunits free in the cytoplasm (Fig. 2 c). If the amount of [^{3}H]uridine present in the free and in the membrane-bound newly made native 40S subunits is an indication of the respective proportions of these two types of native 40S subunits, one can estimate that initiation complexes are at least seven times more numerous amont the membrane-bound than among the free native 40S subunits.

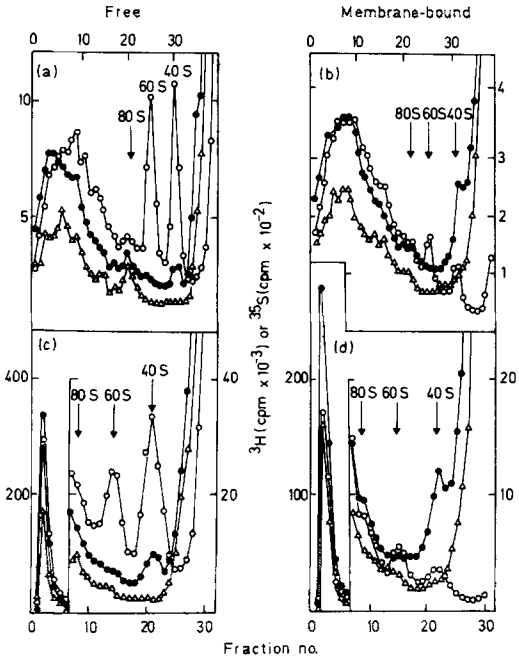

FIGURE 2 Labeling of ribosomal particles with [^{35}S]methionine and [^{3}H]uridine. 5×10^7 exponentially growing P3K cells concentrated to 2.0×10^6 cells/ml were exposed to 40 μCi/ml [^{3}H]uridine for 2 h in the presence of ethidium bromide (1 μmol/ml). At the end of the incubation, the cells were centrifuged for 30 s at room temperature, resuspended in 1 ml of the incubation medium (Dulbecco modified Eagle's medium), and diluted with 9 ml of fresh, prewarmed minimum essential medium without methionine. The cells were then labeled for 3 min with [^{35}S]methionine (57 μCi/ml, 144 Ci/mmol). The incubation was stopped by adding cycloheximide to a final concentration of $1.8\ 10^{-3}$ M and diluting the cells in 40 ml of cold Earle's saline solution. The free ribosomal and the membrane fractions were separated in the presence of 0.15 M KCl on a discontinuous sucrose density gradient. Samples of free ribosomes and membrane-bound ribosomes released from the microsomes by 0.5% sodium deoxycholate and 0.5% Brij 58 were layered on TK_{80} M sucrose density gradients either (*a*, *b*) 15–55% sucrose or (*c*, *d*) 15–30% sucrose containing a 4-ml cushion of 69% sucrose for 8.5 h of centrifugation at (*a*, *b*) 23,000 rpm or (*c*, *d*) 27,000 rpm in a Spinco SW 27 rotor. Gradients were collected and aliquots of 0.4 ml were taken to determine the total radioactivity precipitable in 10% cold trichloroacetic acid. (—O—O—, ^{3}H; —●—●—, ^{35}S), or the ^{35}S-radioactivity remaining insoluble after 30 min at 90°C in 10% trichloroacetic acid (—Δ—Δ—). Note scale change for left-hand part of the gradient in (*c*) and (*d*).

Nascent Chain Lengths of RNase Released and Resistant Membrane-Bound Ribosomes

The fact that RNase treatment releases about half the ribosomes bound to the membranes was interpreted by Rosbash and Penman (28, 29) as indicating the existence of two structurally different classes of membrane-bound polyribosomes. An alternative interpretation is that the released and resistant ribosomes belong to the same class of polyribosomes, in which the ribosomes do not all bear the same relationship to the membrane. The ribosomes carrying the longer nascent polypeptide chains may be the only ones in direct contact with the membranes; the ribosomes carrying the shorter nascent polypeptide chains may be bound to the membranes indirectly through the mRNA which links the ribosomes within the polyribosomal structure. These ribosomes should be easily detached from the membranes by RNase treatment. This would imply that the ribosomes detached from the membranes by RNase differ from those which remain attached by the length of the nascent polypeptide chains they carry.

To explore this point, a membrane fraction was isolated from cells which had been labeled for 120 min with [^{3}H]uridine and for 3 min with [^{35}S]methionine. An aliquot of the membrane-bound ribosomes was directly analyzed by sucrose density gradient sedimentation (Fig. 3 *a*). Most of the ribosomes were in polyribosomes containing, on the average, 8–12 ribosomes. The other part of the membrane fraction was incubated briefly with pancreatic RNase and divided into two fractions. One fraction was treated with detergent and the ribosomes were analyzed on a sucrose density gradient (results not shown). The other fraction was placed on a discontinuous sucrose density gradient to separate the ribosomes remaining on the membranes and the released particles, which were then analyzed by sedimentation on 15–55% sucrose density gradients (Fig. 3 *b*, *c*). 53% of the polyribosomal membrane-bound ribosomes (^{3}H-counts) were released by RNase, as well as all of

the native 40S subunits originally present on the membranes (Fig. 3 *c*). The ribosomes remaining on the RNase-treated membranes consisted of 80S ribosomes and 60S native subunits (Fig. 3 *b*). Table I shows that the specific radioactivity of the nascent peptides is 2.8-fold greater in the RNase-resistant than in the RNase-sensitive ribosomes. The average specific radioactivity computed from the addition of the values observed in these two varieties of ribosomes (Table I) was very close to the specific radioactivity found in the unseparated ribosomes of the RNase-treated membrane fraction (Table I*). The same experiment was repeated with cells labeled for 24 h with [^{14}C]uridine and pulsed for 6 min with [^{3}H]leucine, except that there was no analysis on a sucrose density gradient of the membrane-bound polyribosomes before RNase treatment, as in Fig. 3 *a*. The results were very similar to those obtained with [^{35}S]methionine, with a ^{3}H to ^{14}C specific radioactivity 2.5 times greater in the RNase-resistant than in the RNase-sensitive ribosomes. It can be concluded that the ribosomes released from the membranes by RNase treatment have nascent chains 2.5–2.8-fold shorter than those carried by the RNase-resistant ribosomes.

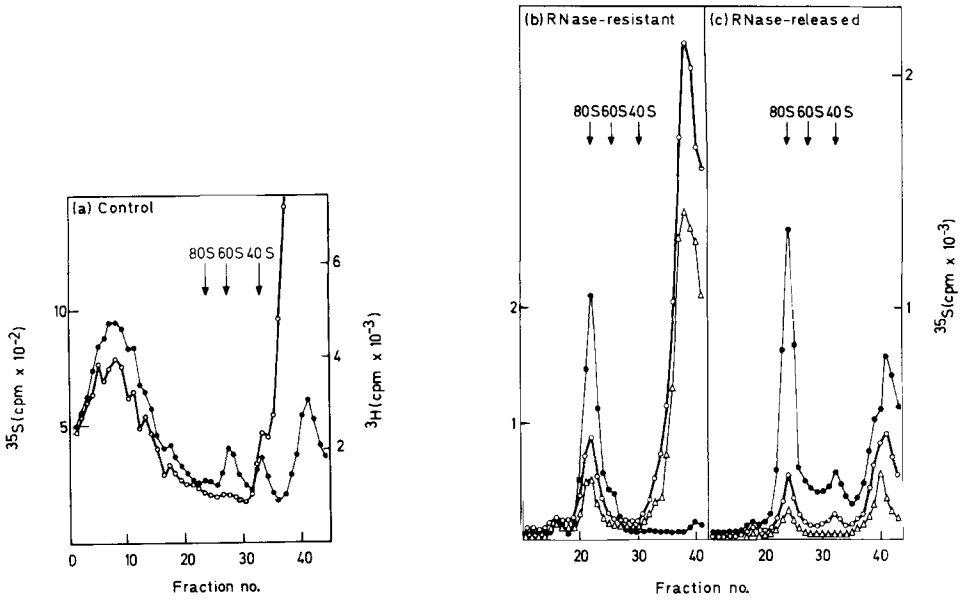

FIGURE 3 Analysis of the length of the nascent polypeptide chains present on the RNase-released and RNase-resistant membrane-bound ribosomes. 2.5 × 10^{7} exponentially growing P3K cells were incubated and homogenized as indicated in the legend of Fig. 2. The membrane fraction, isolated on a discontinuous sucrose density gradient, was divided into three portions. Two were incubated at 37°C for 90 s in the presence of 1 μg/ml of RNase, and the other kept at 4° C (control). The untreated fraction and one of the RNase-treated fractions ("RNase-control") were then dissolved with 0.5% sodium deoxycholate and 0.5% Brij 58, layered on 15–55% sucrose gradients in TK_{80} M medium, and centrifuged for 8.5 h at 27,000 rpm in a Spinco SW 27 rotor. The other RNase-treated fraction was refractionated on a discontinuous sucrose gradient into a free ribosomal (RNase-released membrane ribosomes) and a membrane fraction (RNase-resistant membrane-bound ribosomes). These last two fractions were then similarly analyzed on 15–55% sucrose gradients. Gradients were collected and aliquots of 0.3 ml were taken to determine the total radioactivity precipitable in 10% cold trichloroacetic acid (—●—●—, ^{3}H; —O—O—, ^{35}S), or the ^{35}S-radioactivity remaining insoluble after 30 min at 90°C in 10% trichloroacetic acid (—Δ—Δ—). (*a*) Control, total membrane-bound ribosomes. (*b*) RNase-resistant ribosomes associated with the membranes. (*c*) RNase-released ribosomes recovered in the free ribosome region of the discontinuous sucrose density gradient. The [^{35}S]methionine to [^{3}H]uridine ratio in the 80S ribosome region of the gradient was estimated for the RNase-control membrane fraction (gradient profile not shown) and for the RNase-resistant (*b*) and RNase-released (*c*) ribosomes of the membrane fraction. These values are indicated in Table I.

TABLE I

[^{35}S]Methionine to [^{3}H]Uridine Ratio in RNase-Released and RNase-Resistant 80S Ribosomes of the Membrane Fraction

Specific activities	Before separation*	After separation		Resistant	Mean of resistant and released‖
		resistant‡	released§	Released	
$^{35}S/^{3}H$ cold TCA-insoluble counts	0.170	0.253	0.098	2.58	0.171
^{35}S-hot/^{3}H cold TCA-insoluble counts	0.082	0.120	0.042	2.84	0.079

For experimental details, see Fig. 3 and text.

* Ratio calculated from 80S ribosomes arising from the disaggregation of the entire population of membrane-bound polyribosomes after RNase treatment ("RNase control" ribosomes in legend of Fig. 3).

‡ Ratio determined on the 80S ribosomes of Fig. 3 *b*.

§ Ratio determined on the 80S ribosomes of Fig. 3 *c*.

‖ These values represent the mean specific activity of the membrane-bound ribosomes calculated from the specific activities and proportion (47% and 53%, respectively) of the RNase-released and RNase-resistant ribosomes of this fraction.

Effects of Inhibitors of Protein Synthesis on the Association of Ribosomes with Membranes

Rosbash and Penman (28) found that incubation of HeLa cells with puromycin led to the release of almost half of the membrane-bound ribosomes. The released ribosomes were considered to represnet the postulated population of "loose" ribosomes. To explore the effects of various types of interference with protein synthesis on the membrane-bound ribosomes, P3K cells were incubated in the presence of the following antibiotics: puromycin, which causes a rapid release of almost all nascent polypeptides from ribosomes (3, 25) and a consequent progressive disaggregation of polyribosomes (7); pactamycin, which primarily inhibits the initiation of protein synthesis (19); and cycloheximide (10) and anisomycin (11), which stop the process of chain elongation. Puromycin and pactamycin were found to decrease significantly the proportion of ribosomes in the membrane fraction, while cycloheximide and anisomycin had no such effects (Table II). The sedimentation profiles of the ribosomes recovered in the free ribosomal and membrane fractions of cells incubated with puromycin and pactamycin were therefore explored (Fig. 4).

PUROMYCIN: After 15 min of puromycin treatment, the amount and size of free and membrane-bound polyribosomes have markedly decreased (Fig. 4 *e*, *f*) with a concomitant accumulation of 80S free ribosomes (Fig. 4 *f*). When the membrane fraction of the puromycin-treated cells was exposed to 0.5 M KCl and refractionated on a discontinuous sucrose gradient, 80–90% of the ribosomal RNA was recovered in the free ribosomal fraction. It has been previously shown (21) that 0.5 M KCl treatment of the membrane fraction of control P3K cells releases only about 10% of the ribosomal RNA, consisting of the monoribosomes and native 60S subunits, i.e. ribosomal particles lacking nascent chains.

TABLE II

Proportion of Membrane-Bound Ribosomes after Incubation of P3K Cells with Inhibitors of Protein Synthesis

Control	100
Pactamycin (10^{-6}M)	55
Puromycin ($4.5\ 10^{-4}$M)	63
Cycloheximide ($3.6\ 10^{-3}$M)	100
Anisomycin (10^{-3}M)	100

P3K cells uniformly labeled with [^{14}C]uridine were incubated at a density of 2×10^{6} cells/ml for 15 min at 37°C with the indicated concentrations of inhibitors. Then free ribosomal and membrane fractions were separated on discontinuous sucrose density gradients made in TK_{150} M buffer. In order to eliminate the soluble RNA counts, samples of free ribosomes and membrane-bound ribosomes, released from the microsomes by 0.5% sodium deoxycholate and 0.5% Brij 58, were analyzed by sedimentation on 15–55% sucrose density gradients made in TK_{80} M buffer. The ^{14}C-radioactivity present in the ribosomes could thus be determined for each fraction. The measured values were then compared to control values expressed as 100 (membrane-bound ribosomes represent 18% of the total cytoplasmic ribosomes [21]).

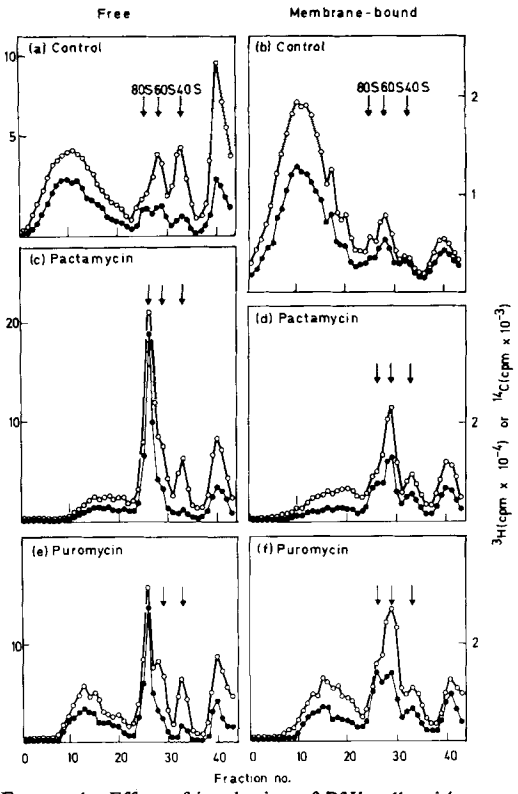

FIGURE 4 Effect of incubation of P3K cells with puromycin and pactamycin on the ribosomal composition of the free ribosomal and membrane fractions. 10^8 P3K exponentially growing cells were uniformly labeled for 24 h with [^{14}C]uridine (0.04 μCi/ml), concentrated to the density of 2.3×10^6 cells/ml, and exposed to [^{3}H]uridine (36 μCi/ml) for 2 h in the presence of ethidium bromide (1 μmol/ml). At the end of the incubation, the cells were divided into three portions. To the first, pactamycin was added at a final concentration of 10^{-6} M, to the second, puromycin at a concentration of 4.5×10^{-4} M, and the third was kept as a control. After a further 15 min, the incubations were stopped by diluting each portion of the cells with 40 ml of cold Earle's saline solution. Three corresponding free ribosomal and membrane fractions were isolated (21). Samples of free ribosomes and membrane-bound ribosomes released from the microsomes by 0.5% sodium deoxycholate and 0.5% Brij 58 were layered on 15–55% sucrose density gradients made in TK_{80} M buffer and centrifuged 8.5 h at 23,000 rpm in a Spinco SW 27 rotor. Fractions were collected for each gradient and radioactivity was measured: —●—●—, ^{14}C uniformly labeled RNA; —O—O—, ^{3}H pulse-labeled RNA. Free ribosome fractions: (*a*) control cells, (*c*) pactamycin-, and (*e*) puromycin-treated cells. Membrane-bound ribosome fractions: (*b*) control cells, (*d*) pactamycin-, and (*f*) puromycin-treated cells.

The following conclusions can be drawn from these experiments: (*a*) the attachment of ribosomes to membranes does not require the presence of their nascent polypeptide chains, but ribosomes lacking nascent chains are not firmly attached, since they can be released by high salt treatment. This finding is in agreement with the experiments performed in vitro with puromycin and high salt by Adelman et al. (1) and Harrison et al. (13); (*b*) the detachment of membrane-bound ribosomes observed under the influence of puromycin may be correlated with the observed breakdown of polyribosomes, since the ribosomes indirectly attached to the membranes through mRNA would be released after polyribosome disaggregation. The situation appears therefore quite similar to that observed after RNase treatment of the membrane fraction, and indeed Rosbash and Penman (28) have found that the ribosomes remaining attached to the membranes of cells incubated with puromycin and resistant to RNase treatment. It must be pointed out that the relationship of ribosomes and mRNA in the presence of puromycin are complex, since it is likely that ribosomes continute to read the mRNA for some time, synthesizing small peptides with C-terminal puromycin (15, 34).

PACTAMYCIN: After incubation with pactamycin, the membrane fraction should contain the products of run-off of the bound polyribosomes and blocked initiation complexes. The analysis of the ribosomal particles present in the free ribosomal (Fig. 4 *c*) and membrane fractions (Fig. 4 *d*) of P3K cells incubated for 15 min with pactamycin (at the end of a 2-h [^{3}H]uridine pulse in the presence of ethidium bromide) showed the persistence, in both fractions, of a few small polyribosomes. Most of the free ribosomes were found as 80S ribosomes, with a slight increase in the 60S and 40S native subunits. In the membrane fraction, by contrast, there was a striking accumulation of 60S subunits. In addition, in the native 40S subunit region, there was 50% increase in the ^{14}C-counts (uniform labeling), and the ^{3}H-counts more than doubled. Although the precise level where pactamycin blocks initiation is not entirely clear (33), it seems probable that this increased number of native 40S subunits which appears to be labeled with [^{14}C]- and [^{3}H]uridine in a ^{14}C to ^{3}H ratio close to that found in the polyribosomal 40S subunits of the control cells, represents initiation complexes blocked on mRNA molecules which

were part, before chain completion, of polyribosomal structures. The membrane-bound native 60S subunits are considered to be the run-off product of the most recently terminated polyribosomes (see Discussion). The 45% of the ribosomes detached from the membranes by pactamycin must represent the run-off products of the bulk of the bound polyribosomes, which are released in the cytoplasm primarily as 60S and 40S native subunits or as 80S ribosomes.

DISCUSSION

About half of the membrane-bound ribosomes of P3K cells detach from the membranes after RNase treatment, as has been observed in the case of other types of cell in culture (9, 18, 28). The present experiments characterized the ribosomal particles whose binding is RNase sensitive (presumably indirectly bound to the membranes through molecules of mRNA), and those whose binding is RNase resistant (directly bound by their 60S subunits to the membranes). It seems that about half the ribosomes in polyribosomes are directly bound to membranes, whereas the other half are attached via an RNase-sensitive binding. By contrast, none of the native 40S subunits are directly bound, whereas all the native 60S subunits are directly bound to the membranes. It was not possible to study the type of binding of the monoribosomes, since they could no longer be identified among the 80S particles resulting from the disaggregation of the polyribosomes by RNase. However, it is most likely that they are bound directly, since they are released after suspension of the membrane fraction in 0.5 M KCl (21); this treatment releases only those ribosomal particles inactive in protein synthesis, bearing neither mRNA nor nascent chains.

The two different types of binding of ribosomes to the membranes have been interpreted by Rosbash and Penman (28, 29) as suggesting the existence of two classes of membrane-bound polyribosomes, which might differ structurally and functionally. In the present series of experiments, it has in no case been possible to detect subpopulations of ribosomes which differ in buoyant density (21) or kinetics of labeling (22); after 2 h of [^{3}H]uridine labeling, the specific radioactivities of the directly and indirectly bound ribosomes were identical. However, the difference observed in the type of ribosome binding to membranes correlates with a marked difference in the amount of [^{35}S]methionine or [^{3}H]leucine incorporated into the nascent polypeptide chains after a 3–6-min pulse. The indirectly bound ribosomes contained in their nascent chains 2.5–3 times less [^{35}S]methionine or [^{3}H]leucine per ribosome than the directly bound ribosomes. It therefore appears that the two classes of ribosomes differ as to the length of the nascent chains they carry. Assuming that they are two classes of polyribosomes, one directly bound and the other indirectly bound, they probably should differ markedly in size in order to account for the average difference in length of their polypeptide chains (16, 26) (although there are examples indicating that polyribosomal size may be related, in some instances at least, to factors independent from the size of the mRNA (35); a bimodal distribution in polyribosome size which might correspond to this situation was never observed. It seems much more likely that the two classes of ribosomes belong to a single type of membrane-bound polyribosomal structure, in which not all the ribosomes bear the same relationship to the membranes. Thus, within a given polyribosome, only the ribosomes closer to the 3′ end of the mRNA molecule and carrying the longest peptide chains may be directly attached; the latest ribosomes to enter this polyribosomal structure, i.e. those closer to the 5′ end of the mRNA and carrying the shortest peptide chains, may not have yet reached direct contact with the membranes, and thus be indirectly bound through the mRNA molecule. The ribosomal particles released from the membranes by RNase might consist not only of 80S ribosomes and of the membrane native 40S subunits, but also of some 40S subunits of polyribosomal origin, these representing initiation complexes attached at the 5′ end of mRNA molecules of polyribosomes.

On the basis of the various observations made in the present series of experiments, it is possible to propose a model for the binding of ribosomes to membranes and for the ribosomal cycle on the membranes (shown in Fig. 5). Two situations should be distinguished: the entry of new ribosomes into membrane-bound polyribosomes (Fig. 5 *a*) whose mRNA is already attached to the membranes; the association of new mRNA to the membranes, leading to the formation of new polyribosomes (Fig. 5 *f* and *g*).

The mode of entry of ribosomal subunits into polyribosomes is, in this model, similar for both free and membrane-bound polyribosomes, as indicated by the kinetics of labeling of the polyribosomal 40S and 60S subunits (22). When an initiation

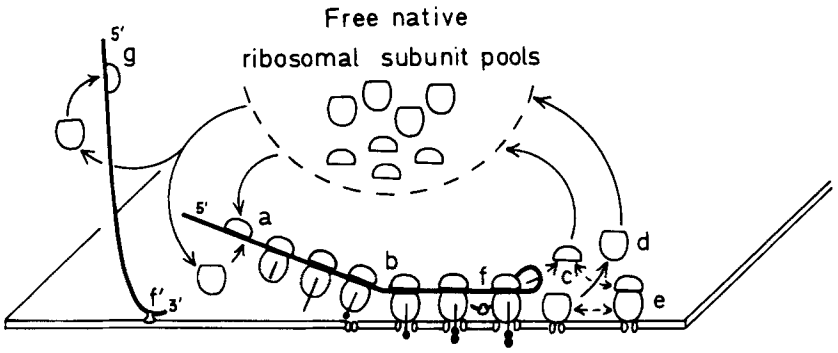

FIGURE 5 Schematic representation of the ribosome cycle on membrane-bound ribosomes. (*a*) Formation of an initiation complex on the 5′ end of an mRNA present within a membrane-bound polyribosome. The ribosomes between (*a*) and (*b*) are indirectly bound, and released by mild RNase treatment. (*b*) Direct binding of a ribosome through its 60S subunit to a specific membrane-binding site. The proper folding of the *N*-terminal segment of the nascent polypeptide chain emerging from the 60S subunit allows its immediate penetration through the membranes, resulting in a tight anchorage. Ribosomes from (*b*) to the mRNA 3′ end of the polyribosome are tightly anchored through their nascent chains, and therefore not released by high KCl treatment. It can be seen that when the mRNA is intact, high salt treatment will not release any polyribosomes. (*c*) Polypeptide chain termination, ribosome dissociation, and release of the 40S subunit. The binding of the 60S subunit is sensitive to high salt treatment. (*d*) Detachment of the large ribosomal subunit, probably resulting from the competition for membrane-binding sites by the ribosomes approaching the 3′ end of the mRNA molecules. (*e*) Membrane-bound monoribosome, lacking a nascent chain (i.e. high salt sensitive), and probably representing a side product of the ribosome cycle. (*f*) Postulated attachment of the 3′ end of the mRNA molecules to the membrane. (f′) represents the attachment site of an mRNA newly arrived on the membranes, and not yet integrated into a polyribosome. (*g*) Formation of an initiation complex on a newly arrived mRNA molecule.

site becomes available on the mRNA of a polyribosome, it binds a 40S, then a 60S subunit, derived from the pool of free native subunits. If the messenger involved belongs to a membrane-bound polyribosome, the newly added ribosome is first bound to the membranes only through the mRNA (Fig. 5, *a*); then, at some time during its movement towards the 3′ end of the mRNA molecule, it becomes directly bound (Fig. 5, *b*). Direct attachment involves two types of interaction between the ribosome and the membrane (1, 13): binding of the large ribosomal subunit to a specific receptor site of the endoplasmic reticulum, and protrusion of the nascent polypeptide chain into the membrane, resulting in a firm anchorage. The binding, but not the anchorage, is sensitive to 0.5 M KCl. Direct attachment of a ribosome to the endoplasmic reticulum does not require the presence of a nascent chain, as was observed with puromycin-treated cells, where the ribosomal structures remaining on the membranes are apparently directly attached, since they are not released by RNase (28), and almost completely released by 0.5 M KCl treatment. Thus, the loss of membrane-bound ribosomes observed in puromycin-treated cells by Rosbash and Penman (28) and by us appears to be correlated with the breakdown of polyribosomes, releasing indirectly bound ribosomes, and not with the lack of nascent chains. In vitro studies have also shown that 60S subunits lacking nascent chains can attach to stripped endoplasmic reticulum membranes at specific sites, which do not discriminate between subunits derived from free or membrane-bound ribosomes (6, 27). Furthermore, it is clear that in P3K cells the nascent chains do not enter into close association with the membranes immediately after they emerge on the surface of the large ribosomal subunits, i.e. when they have reached 35–40 amino acids in length (5, 19), as has been postulated by Sabatini and Blobel (30) in the case of the ribosomes bound on liver microsomes. If this were the case, fewer ribosomes would have been released by RNase treatment than was actually observed. Indeed, the high proportion of indirectly bound ribosomes suggests that direct binding of ribosomes to the membranes occurs in P3K cells at a time when a nascent chain of considerable length has emerged from the

ribosomal surface. It is interesting to note that in the experiments of Sabatini and Blobel (30), in vitro incubation with trypsin of liver rough microsomes carrying labeled nascent chains degraded about 20% of the radioactivity, indicating the existence of chain segments protected neither by the 60S subunit nor by the association with the membranes. Since in the liver all the ribosomes appear to be directly bound (see below), this may correspond to peptide segments emerged on the surface of attached ribosomes which have not yet penetrated into the membranes (Fig. 6 *b*). Thus, it is possible that, whether the binding of the ribosome to the membrane occurs before or after it bears a protruding nascent chain, this protruding segment has to be folded in the proper tertiary conformation to make possible its penetration through the membranes. This might happen with the help of an additional, hydrophobic *N*-terminal segment destined to be cleaved later (24, 32), should the observations made on the precursor of the immunoglobulin light chains be extended to other secretory proteins.

After completion of the polypeptide chain, the ribosome leaving the mRNA dissociates and its 40S subunit joins the native free subunit pool (Fig. 5 *c*). The large subunit remains for some time attached to its membrane binding site, from which it has to be released (Fig. 5 *d*), since it apparently is not reutilized except by joining first the native free subunit pool, as is suggested by experiments on labeling kinetics (22). The likeliest cause for the release of "run-off" 60S subunits from the membranes is the competition for membrane-binding sites due to the progression of indirectly bound ribosomes towards the 3′ end of the mRNA. After binding to membranes, these ribosomes become tightly anchored through their nascent chains; this association is stronger than that of the native 60S subunits, and therefore results in the displacement of these subunits. This mechanism for the release of 60S subunits would account for the effect of pactamycin, which leads to both a loss of membrane-bound ribosomes and an accumulation of bound native 60S subunits (see Fig. 4). Pactamycin allows chain completion but, by blocking initiation, prevents the reconstitution of polyribosomes. Thus, the ribosomes approaching chain completion at the time of antibiotic action would leave the membranes after chain termination; in contrast, the large subunits of the ribosomes bound near the 5′ end of the mRNA may stay on the membranes after polypeptide release, since they are not followed by further ribosomes competing for membrane-binding sites.

Finally, the bound monoribosomes may be the equivalent on the membranes of the monoribosomes free in the cytoplasm. Both types of ribosomes have slow kinetics of RNA labeling and may represent the product of a side reaction not involved in the ribosomal cycle accompanying protein synthesis (Fig. 5 *e*).

It may be asked why direct attachment of the ribosome to the membranes does not take place as soon as a new ribosome has been formed on the mRNA of a membrane-bound polyribosome, since it is then in the close vicinity of a membrane-binding site. The situation appears to differ in liver cells and in cells rapidly growing in culture, since RNase treatment of liver rough microsomes does not release appreciable amounts of ribosomes. It may be that the respective amounts of membrane-bound polyribosomes and of membrane-binding sites specific for ribosomes play a critical role in this respect (Fig. 6). In conditions of rapid growth, a number of ribosomal binding sites on the membrane may be too limited to bind simultaneously all the eligible ribosomes (Fig. 6 *a*). This would retard direct binding of some ribosomes, and would allow the detection of two classes of membrane-bound ribosomes, with a different sensitivity to mRNA integrity in their attachment to membranes. In other conditions, such as in liver cells, there may be no inbalance between the membrane-binding sites and the polyribosome number and size, and all the ribosomes are therefore directly attached (Fig. 6 *b*). In this case, damage to the mRNA will have no detectable effect, except if the membranes are also treated with high molarity KCl, which should lead to the detachment of the ribosomes which are not anchored by penetration of their nascent chains into the membranes, and which are no longer linked by the mRNA. This is consistent with the observation that, when liver rough microsomes whose mRNA has been degraded during the fractionation procedure (1) are exposed to high molarity KCl, up to 40% of the ribosomes become detached (2); they consist of inactive ribosomes and ribosomes with short chains. Thus, it must be emphasized that the most specific feature of the model discussed here is not the existence of directly bound and indirectly bound ribosomes, which may vary according to the tissue; rather, it is that the binding of ribosomes to the membranes can be mediated by mRNA molecules associated with the membranes.

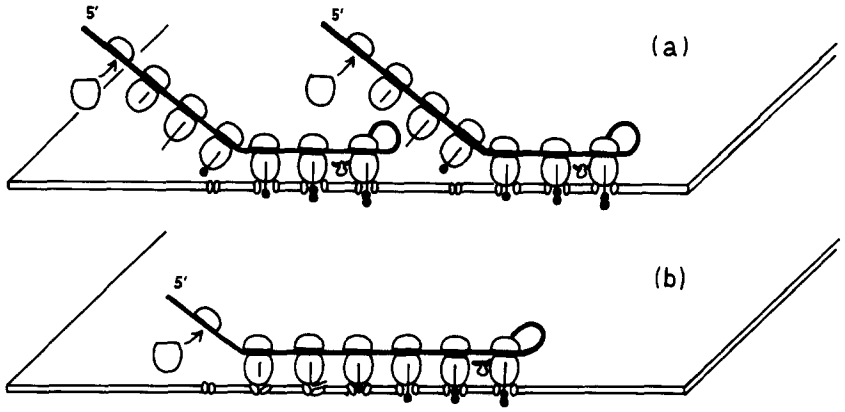

FIGURE 6 Scheme showing the possible association of ribosomes to membranes in: (*a*) rapidly growing cells, e.g., P3K cells, in which the number of ribosomal binding sites on the membranes may be too limited to bind simultaneously all the ribosomes present in polyribosomes; (*b*) liver cells, in which all the ribosomes may be directly attached because of an adequate balance between the membrane-binding sites and the polyribosome number and size. The second bound ribosome from the left carries an emerged nascent chain not yet properly folded, so that the ribosome is not tightly anchored; this unprotected nascent chain might be digested during trypsin treatment of the microsomes. RNase treatment would release no ribosomes, except for a small number of 40S native subunits. On the other hand, high salt treatment after mRNA degradation would release all the ribosomes not tightly anchored by their nascent chains.

The native 40S subunits detected on the membrane fraction have the characteristics required for initiation complexes linked to the membranes by mRNA: these are released by RNase treatment and they bear Met-tRNA, presumably Met-$tRNA_F$, with a specific radioactivity several-fold higher than that of the free native subunits. This suggests that these membrane-bound native 40S subunits may all represent 40S/Met-$tRNA_F$/mRNA initiation complexes, while among the free native subunits, the majority of the 40S particles carrying Met-$tRNA_F$ are probably not yet stabilized by association with mRNA (8). Thus, the native 40S subunits of the membrane fraction might represent initiation complexes of previously untranslated mRNA molecules which have just reached the membranes. Their number would be expected to be very small, and their detection possible only because of their very high specific radioactivity. In cells treated with pactamycin, there was an increased number of native 40S subunits detectable on the membranes. This increase appeared to consist of 40S subunits with a specific radioactivity indicating that they were initially present in polyribosomes; thus, although the nature of the blocked initiation complexes resulting from the action of pactamycin may vary depending upon a number of factors (33), it seems likely that these additional 40S subunits detected on the membranes following pactamycin treatment indeed represent initiation complexes blocked on mRNA which was previously linking polyribosomal structures. This observation adds further evidence for the existence of mRNA molecules linked to the membranes, presumably by their 3' end (Fig. 5 *f*), as is also suggested by recent observations of others (17, 23). The present experiments provide no clue as to the possible nature of the link between mRNA and the membranes. The linkage may be mediated by proteins associated with mRNA molecules (4, 13).

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REFERENCES

1. ADELMAN, M. R., G. BLOBEL, and D. D. SABATINI. 1973. An improved cell fractionation procedure for

the preparation of rat-liver membrane-bound ribosomes. *J. Cell Biol.* **56**:191–205.
2. ADELMAN, M. R., D. D. SABATINI, and G. BLOBEL. 1973. Ribosome-membrane interaction. Nondestructive disassembly of rat liver rough microsomes into ribosomal and membranous components. *J. Cell Biol.* **56**:206–229.
3. ALLEN, D. W., and P. C. ZAMECNIK. 1962. The effect of puromycin on rabbit reticulocyte ribosome. *Biochim. Biophys. Acta.* **55**:865–874.
4. BLOBEL, G. 1973. A protein of molecular weight 78,000 bound to the polyadenylate region of eurkaryotic messenger RNAs. *Proc. Natl. Acad. Sci. U.S.A.* **70**:924–928.
5. BLOBEL, G., and D. D. SABATINI. 1970. Controlled proteolysis of nascent polypeptides in rat liver cell fractions. I. Location of the polypeptides within ribosomes. *J. Cell Biol.* **45**:130–145.
6. BORGESE, N., W. MOK, G. KREIBICH, and D. D. SABATINI. 1974. Ribosomal-membrane interaction: *In vitro* binding of ribosomes to microsomal membranes. *J. Mol. Biol.* **88**:559–580.
7. BURKA, E. R., and P. A. MARKS. 1964. Protein synthesis in erythroid cells. II. Polyribosome function in intact reticulocytes. *J. Mol. Biol.* **9**:439–451.
8. DARNBROUGH, C., S. LEGON, T. HUNT, and R. J. JACKSON. 1973. Initiation of protein synthesis: Evidence for messenger RNA-independent binding of methionyl-transfer RNA to the 40S ribosomal subunit. *J. Mol. Biol.* **76**:379–403.
9. FAIFERMAN, I., A. O. POGO, J. SCHWARTZ, and M. E. KAIGHN. 1973. Isolation and characterization of membrane-bound polysomes from ascites tumor cells. *Biochim. Biophys. Acta.* **312**:492–501.
10. GODCHAUX, W., III., S. D. ADAMSON, and E. HERBERT. 1967. Effects of cycloheximide on polyribosome function in reticulocytes. *J. Mol. Biol.* **27**:57–72.
11. GROLLMAN, A. P. 1967. Inhibitors of protein synthesis. II. Mode of action of anisomycin. *J. Biol. Chem.* **242**:3226–3233.
12. HARDESTY, B., R. MILLER, and R. SCHWEET. 1963. Polyribosome breakdown and hemoglobin synthesis. *Proc. Natl. Acad. Sci. U. S. A.* **50**:924–931.
13. HARRISON, T. M., G. G. BROWNLEE, and C. MILSTEIN. 1974. Studies on polysome-membrane interactions in mouse myeloma cells. *Eur. J. Biochem.* **47**:613–620.
14. HARRISON, T. M., G. G. BROWNLEE, and C. MILSTEIN. 1974. Preparation of immunoglobulin light-chain mRNA from microsomes without the use of detergent. *Eur. J. Biochem.* **47**:621–627.
15. HOGAN, B. L. M. 1969. The effects of inhibitors of protein synthesis on the level of ribosomal subunits in ascites cells. *Biochim. Biophys. Acta.* **182**:264–266.
16. KUFF, E. L., and N. E. ROBERTS. 1967. *In vivo* labeling patterns of free polyribosomes: relationship to tape theory of messenger ribonucleic acid function. *J. Mol. Biol.* **26**:211–225.
17. LANDE, M., M. SUMIDA, Y. TASHIRO, M. ADESNIK, and D. SABATINI. 1975. Direct association of mRNA with microsomal membranes in human diploid fibroblasts. *J. Cell Biol.* **65**:513–528.
18. LEE, S. E., V. KRSMANOVIC, and G. BRAWERMAN. 1971. Attachment of ribosomes to membranes during polysome formation in mouse sarcoma 180 cells. *J. Cell Biol.* **49**:683–691.
19. MACDONALD, J. S., and I. H. GOLDBERG. 1970. An effect of pactamycin on the initiation of protein synthesis in reticulocytes. *Biochem. Biophys. Res. Commun.* **41**:1–8.
20. MALKIN, L. I., and A. RICH. 1967. Partial resistance of nascent polypeptide chains to proteolytic digestion due to ribosomal shielding. *J. Mol. Biol.* **26**:329–346.
21. MECHLER, B., and P. VASSALLI. 1975. Membrane-bound ribosomes of myeloma cells. I. Preparation of free and membrane-bound ribosomal fractions. Assessment of the methods and properties of the ribosomes. *J. Cell Biol.* **66**:000–000.
22. MECHLER, B., and P. VASSALLI. 1975. Membrane-bound ribosomes of myeloma cells. II. Kinetic studies on the entry of newly made ribosomal subunits into the free and the membrane-bound ribosomal particles. *J. Cell Biol.* **66**:000–000.
23. MILCAREK, C., and S. PENMAN. 1974. Membrane-bound polyribosomes in HeLa cells: Association of polyadenylic acid with membranes. *J. Mol. Biol.* **89**:327–338.
24. MILSTEIN, C., G. G. BROWNLEE, T. M. HARRISON, and M. B. MATHEWS. 1972. A possible precursor of immunoglobulin light chains. *Nat. New Biol.* **239**:117–120.
25. NATHANS, D. 1964. Puromycin inhibition of protein synthesis: Incorporation of puromycin into peptide chains. *Proc. Natl. Acad. Sci. U. S. A.* **51**:585–592.
26. PETERSEN, N. S., and C. S. MCLAUGHLIN. 1973. Monocistronic messenger RNA in yeast. *J. Mol. Biol.* **81**:33–45.
27. ROLLESTON, F. S. 1972. The binding of ribosomal subunits to endoplasmic reticulum membranes. *Biochem. J.* **129**:721–731.
28. ROSBASH, M., and S. PENMAN. 1971. Membrane-associated protein synthesis of mammalian cells. I. The two classes of membrane-associated ribosomes. *J. Mol. Biol.* **59**:227–241.
29. ROSBASH, M., and S. PENMAN. 1971. Membrane-associated protein synthesis of mammalian cells. II. Isopycnic separation of membrane-bound polyribosomes. *J. Mol. Biol.* **59**:243–253.
30. SABATINI, D. D., and G. BLOBEL. 1970. Controlled proteolysis of nascent polypeptides in rat liver cell fractions. II. Location of the polypeptides in rough microsomes. *J. Cell Biol.* **45**:146–157.
31. SABATINI, D. D., Y. TASHIRO, and G. E. PALADE.

1966. On the attachment of ribosomes to microsomal membranes. *J. Mol. Biol.* **19**:503–524.

32. SCHECHTER, I., D. J. MCKEAN, R. GUYER, and W. TERRY. 1975. Partial amino acid sequence of the precursor of immunoglobulin light chain programmed by messenger RNA in vitro. *Science (Wash. D.C.).* **188**:160–162.
33. SUZUKI, H., and I. H. GOLDBERG. 1974. Reversal of pactamycin inhibition of methionyl-puromycin synthesis and 80s initiation complex formation by a ribosomal joining factor. *Proc. Natl. Acad. Sci. U. S. A.* **71**:4259–4263.
34. WILLIAMSON, A. R., and R. SCHWEET. 1965. Role of the genetic message in polyribosome function *J. Mol. Biol.* **11**:358–372.
35. HUNT, R. T., A. R. HUNTER, and A. J. MUNRO. 1968. Control of haemoglobin synthesis: a difference in the size of the polysomes making α and β chains. *Nature (Lond.).* **220**:481–483.