

RIBOSOME-MEMBRANE INTERACTION

Nondestructive Disassembly of Rat Liver Rough Microsomes into Ribosomal and Membranous Components

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ABSTRACT

In a medium of high ionic strength, rat liver rough microsomes can be nondestructively disassembled into ribosomes and stripped membranes if nascent polypeptides are discharged from the bound ribosomes by reaction with puromycin. At 750 mM KCl, 5 mM MgCl₂, 50 mM Tris·HCl, pH 7.5, up to 85% of all bound ribosomes are released from the membranes after incubation at room temperature with 1 mM puromycin. The ribosomes are released as subunits which are active in peptide synthesis if programmed with polyuridylic acid. The ribosome-denuded, or stripped, rough microsomes (RM) can be recovered as intact, essentially unaltered membranous vesicles. Judging from the incorporation of [³H]puromycin into hot acid-insoluble material and from the release of [³H]leucine-labeled nascent polypeptide chains from bound ribosomes, puromycin coupling occurs almost as well at low (25–100 mM) as at high (500–1000 mM) KCl concentrations. Since puromycin-dependent ribosome release only occurs at high ionic strength, it appears that ribosomes are bound to membranes via two types of interactions: a direct one between the membrane and the large ribosomal subunit (labile at high KCl concentration) and an indirect one in which the nascent chain anchors the ribosome to the membrane (puromycin labile). The nascent chains of ribosomes specifically released by puromycin remain tightly associated with the stripped membranes. Some membrane-bound ribosomes (up to 40%) can be nondestructively released in high ionic strength media without puromycin; these appear to consist of a mixture of inactive ribosomes and ribosomes containing relatively short nascent chains. A fraction (~15%) of the bound ribosomes can only be released from membranes by exposure of RM to ionic conditions which cause extensive unfolding of ribosomal subunits, the nature and significance of these ribosomes is not clear.

INTRODUCTION

Ever since it was recognized that the rough endoplasmic reticulum (RER)¹ is particularly well-

developed in mammalian tissues with extensive secretory activity (1, 2), evidence has accumulated in support of the concept that free ribosomes syn-

¹ *Abbreviations used in this paper:* NaDOC, sodium deoxycholate; poly A, polyadenylic acid; PLP, phospholipid; RER, rough endoplasmic reticulum, RM, rough microsomes; SDS, sodium dodecyl sulfate;

SM, smooth microsomes; STKM, 250 mM sucrose, 750 mM KCl, 5 mM MgCl₂, 50 mM Tris·HCl, pH 7.5; TCA, trichloroacetic acid.

thesize proteins which remain in the cell sap, while membrane-bound ribosomes manufacture products for export from the cell (3-6). Rough microsomes (RM), which are isolated by cell fractionation and represent the vesiculated remains of the RER (1, 7-9), can be made to function as miniature secretory units *in vitro*; the ribosomes bound to the outer surfaces of the RM are active in amino acid incorporation and, after reaction with the aminoacyl-tRNA analogue puromycin (10-13), release their nascent polypeptide chains vectorially to the microsomal vesicle proper (14, 15).

It is known that ribosomes interact with microsomal membranes via the large (60S) subunit (16, 17) and that the nascent polypeptide chain, which grows within a protected region in this subunit (18, 19), enters into close relationship with the membrane immediately upon emerging from the ribosome (20). Nevertheless, both *in vivo* and *in vitro* studies with puromycin (14, 21, 22) have failed to demonstrate a role for the nascent chain in binding the ribosomes to the membranes. Similarly, there is no indication that in rat liver RM ribosome-membrane interaction is mediated by messenger RNA (21, also extensive unpublished studies by T. Morimoto and D. Sabatini involving RNase digestions), although recent publications have suggested such a binding mechanism exists in tissue culture cells (23, 24).

Detailed knowledge of the nature of ribosome-membrane interaction has been difficult to accumulate because of the extreme stability of the binding; it has not been possible nondestructively to disassemble the rough microsome into its component parts, *viz.*, ribosome and membrane. Until now, the only techniques available for separation of these components were partially destructive ones. On the one hand, chelating agents (16) and concentrated salt solutions (25) release some or most of the bound ribosomes, but produce a mixture of intact membranes and damaged or denatured ribosomes. Detergents, on the other hand, release functional ribosomes (26) but only by extensively altering or destroying membrane structure. In this paper we describe a simple, nondestructive means of separating RM into ribosomal and membranous components. Our studies of the mechanism of release provide some information as to the nature of ribosome-membrane interaction. A preliminary report of this work has appeared (27).

MATERIALS AND METHODS

General

Puromycin dihydrochloride and cycloheximide were obtained from ICN Nutritional Biochemicals Div. (International Chemical and Nuclear Corp., Cleveland, Ohio), [^3H]puromycin (1.1 mCi/ μmol) was obtained from New England Nuclear (Boston, Mass.). The sources of all other reagents, the general and analytical procedures used here, and a detailed description of the preparation of rough microsomes are presented elsewhere (28). RM were either used immediately for experiments or stored frozen (-20°C) as pellets for up to 1 mo before use. RM pellets were resuspended in the appropriate buffer, using a Vortex mixing apparatus (Lab-Line Instruments, Inc., Melrose Park, Ill.), followed by gentle homogenization with a hand-operated, Teflon-pestle tissue grinder (Arthur H. Thomas Co., Philadelphia, Pa., sizes AA or A). Throughout this paper, use is made of the notation "S 250, K 750, Mg 5, T 50" to indicate, e.g., a solution containing 250 mM sucrose, 750 mM KCl, 5 mM MgCl_2 , and 50 mM Tris-HCl, pH 7.5 (at room temperature). In those cases where only the KCl concentration is given, e.g., 750 mM KCl, it is to be understood that the solution also contained 250 mM sucrose, 5 mM MgCl_2 , and 50 mM Tris-HCl, pH 7.5 (at room temperature), unless otherwise specified. It should be noted that S 250, K 25, Mg 5, T 50 is a commonly used cell fractionation buffer often referred to as 0.25 M STKM (19).

Centrifugation

General centrifugation techniques have been described elsewhere (28), including the use of the notation "1 h-41K-SB283 (283,000)" to indicate centrifugation for 1 h at 41,000 rpm in the SB283 rotor under which conditions g_{max} is $\sim 283,000$. The IEC (International Equipment Co., Needham Heights, Mass.) rotor SB283 is roughly comparable to the Spinco SW41 (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.). Linear sucrose density gradients (~ 12 ml, usually 15-40% in sucrose and always containing the same KCl, MgCl_2 and Tris-HCl concentrations as the applied samples) were formed by standard techniques (29) in either cellulose nitrate (Beckman Instruments, Inc., Fullerton, Calif.) or polyallomer tubes (IEC), and were centrifuged in the SB283 rotor which was allowed to coast to a stop. Gradients were monitored and fractionated either using an ISCO model D gradient fractionator and UV analyzer (with a 5-mm light path) (Instrumentation Specialties Co., Lincoln, Neb.) or using a Buchler Auto Densi-Flow probe (Buchler Instrument Div., Nuclear-Chicago Corp., Fort Lee, N. J.), the effluent of which was led through an LKB Uvicord (3-mm

light path) (LKB Instruments, Inc., Rockville, Md.). In each case, $A_{254\text{nm}}$ was recorded. The UV absorbance profiles are reproduced here such that in all figures the direction of sedimentation is from left to right. Fractions, usually ~ 0.5 ml of the effluent, were collected and processed for liquid scintillation counting as follows.

(a) Fractions containing [^3H]leucine- or [^3H]puromycin-labeled polypeptides were mixed with an equal volume of ice-cold 20% trichloroacetic acid (TCA) and stored overnight at 0°C . An equal volume of H_2O was then added (to final TCA concentration of 5%) and the tubes were heated for 30 min at 90°C . The tubes were cooled and the contents collected by filtration onto Whatman 3 MM disks. 5% TCA was used to facilitate transfer to the disks which were then washed with 5% TCA and alcohol-ether (vol/vol), and air-dried.

(b) Fractions containing ^3H -labeled RNA were processed as in *a*, except that the incubation at 90°C was omitted and care was taken that all processing was done at or near 0°C . The dried filter paper disks were placed in glass vials containing ~ 10 ml toluene-Liquifluor and counted in a Beckman LS-250 with an efficiency between 5 and 10%.

Other

In vitro amino acid incorporations were carried out as previously described (28). Incorporation of [^3H]puromycin into hot acid-insoluble material was assayed by the same filter paper disk method used in the amino acid incorporation studies. Counting efficiencies were 15–20%. The concentration of puromycin stock solutions was determined from UV absorbance measurements (30).

RESULTS

Since results from our laboratory had shown that puromycin treatment in solutions of high ionic strength led to disassembly of free polysomes into functionally viable ribosomal subunits (31), we examined, over a range of ionic conditions, the effect of puromycin on the stability of ribosome-membrane interaction. Our analyses clearly demonstrated that the combined action of puromycin and appropriate high KCl conditions led to efficient release of almost all the bound ribosomes from RM. Fig. 1 serves as an overall display of this KCl-puromycin release² phenomenon.

² For convenience we define the following terms.

(a) Salt-release: the release of ribosomes from RM brought about by modifications of the KCl and/or MgCl_2 concentrations in the suspension medium. Modifications are defined relative to the common fractionation buffer S 250, K 25, Mg 5, T 50. Phrases

In this experiment, rough microsomes were suspended in S 250, K 25, Mg 5, T 50 and brought by 1:1 dilution with appropriate solutions to various final KCl concentrations (in the presence of S 250, Mg 5, T 50) with or without 1 mM puromycin. After incubation (see legend to Fig. 1), equal samples were analyzed by zone sedimentation on linear sucrose density gradients made up with the same final KCl, MgCl_2 , and Tris-HCl concentrations as in the applied sample. Under these conditions of centrifugation, the ribosome monomer sedimented slightly less than halfway down the gradient (*M* in Fig. 1), and subunits, where present, were well-resolved from the material remaining near the top of the gradient. (The high UV absorbance at the top of each gradient in the lower row in Fig. 1 is due to the puromycin in the applied samples.) The absorption near the bottom of these gradients (indicated by cross-hatching in Fig. 1, and by *Mb* in other figures) coincided with the presence of a band of membranous material detectable, because of turbidity, by the naked eye. The sedimentation profiles in the upper row of Fig. 1 show that in 25 mM KCl, all ribosomes sedimented into a pellet with the RM; i.e., these RM were uncontaminated with "free" ribosomes. However, when RM were exposed to KCl concentrations higher than 25 mM, ribosomes were released from the membranes and the extent of release was a function of the salt concentration. Treatment of RM at 25 mM KCl with puromycin did not lead to ribosome release (compare upper and lower 25 mM KCl profiles in Fig. 1), which verified previous observations (14, 21, 22). However, at higher KCl concentrations (compare upper and lower profiles), there was, in addition to the salt release,² a pronounced puromycin-dependent² ribosome release which, at a given KCl concentration, was manifested as an increase in absorbance in the ribosomal region of the gradients of samples treated with puromycin, and most

such as "low KCl" or "high KCl" refer to S 250, Mg 5, T 50 plus various KCl concentrations.

(b) KCl-puromycin release: the release of ribosomes from RM brought about by the combined action of puromycin and high levels of KCl.

(c) Puromycin-dependent or puromycin-specific release: the additional release of ribosomes under a particular set of ionic conditions due specifically to the action of puromycin. This is defined as $b - a$ and may range from very little release (e.g., at low KCl), to fairly extensive release (at high KCl).

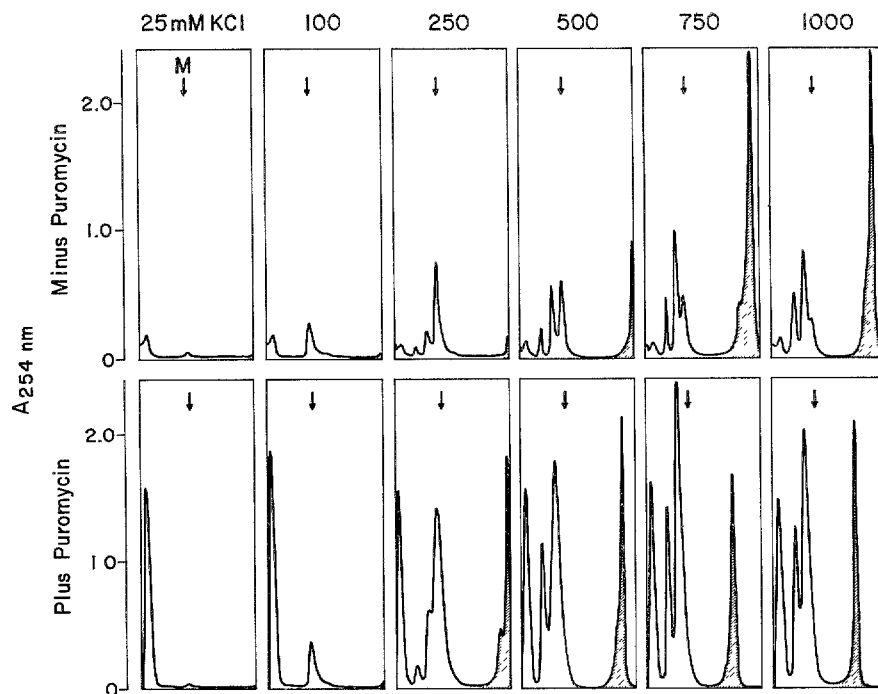


FIGURE 1 Release of ribosomes from rough microsomes by combined action of high KCl and puromycin. Equal amounts of RM were incubated in S 250, Mg 5, T 50 plus 25, 100, 250, 500, 750, or 1000 mM KCl in the absence (upper panels) or presence (lower panels) of 0.79×10^{-3} M puromycin. Samples were incubated for 70 min at 0°C, 10 min at 37°C, and then 10 min at room temperature. Samples (0.45 ml, 0.37 mg RNA) were applied to 15–40% sucrose gradients containing the appropriate K, Mg, T buffer. Sedimentation was at 20°C $1\frac{1}{4}$ h-40K-SB283 ($\sim 270,000$). All profiles are presented with top of gradient at left, and the direction of sedimentation is from left to right. The small arrow (*M*) indicates the position of the ribosomal monomer (80S). Gradients run at $[KCl] \geq 250$ mM also show small (40S) and large (60S) subunit peaks. The shading in the lower portions of these gradients is added to indicate that the UV absorption corresponded to a region in which membranes were visible (turbidity) but no attempt is made to indicate the exact extent of the turbidity. In all subsequent figures, such membranous bands are designated with the symbol *Mb*.

strikingly by a shift of the membranous components to a position corresponding to a lower isopycnic density. This shift would be expected to occur upon release of high density ribonucleoproteins from less dense lipoprotein membranes.

The membrane bands in the gradients of Fig. 1 were at or near their isopycnic positions, as shown by the fact that they did not sediment further during more prolonged centrifugation. In fact, overnight centrifugation resulted in membrane bands, located at a slightly *lower* density position (nearer the top of the gradient) than after 1–2 h centrifugation. This change in density presumably reflected the very slow phase of salt-induced ribosome release described below. The sharpness of the membrane peaks in Fig. 1 was in part due to the

tendency of microsomes to aggregate under these ionic conditions. With preparations of RM which had been stored for several weeks at -20°C , aggregation was more pronounced, the equilibrium position was reached after as little as 30 min of centrifugation, and frequently, multiple $A_{254\text{ nm}}$ peaks were detected reflecting the presence of two or more adjacent membrane bands. Except with regard to this tendency to aggregate, we have noticed no major differences between fresh and stored RM, neither have we found any differences between RM prepared by our new technique and RM prepared by more conventional procedures (19, 20).

Analysis on density gradients (data not included) of the supernatants of RM samples which,

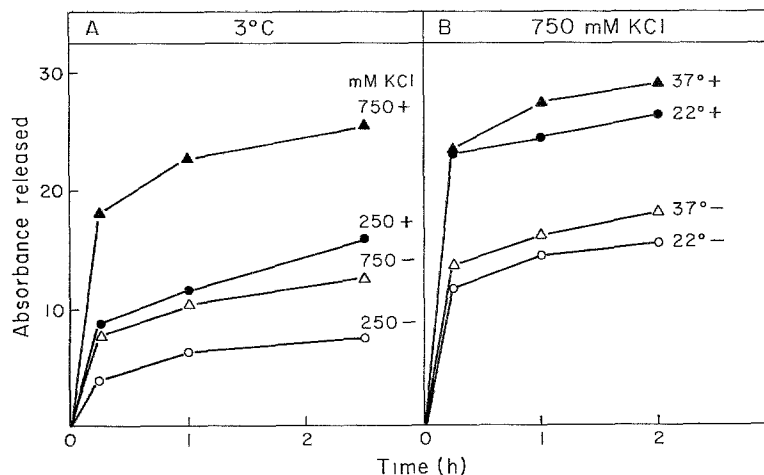


FIGURE 2 Time-course of release of ribosomes from RM. Equal samples of RM were incubated in S 250, Mg 5, T 50 at the indicated KCl concentrations for various times in the absence (open symbols) or presence (solid symbols) of 0.79×10^{-3} M puromycin. Samples were applied to 15–40% sucrose gradients in the appropriate K, Mg, T buffer and centrifuged. The absorbance profiles were reproduced and the areas corresponding to ribosomal particles were cut out and weighed. Absorbance released is presented in arbitrary units. (A) Incubation at 3°C in 250 mM KCl (circles) or 750 mM KCl (triangles). Sedimentation at 3°C: 3 h-40K-SB283 (270,000). (B) Incubation was in 750 mM KCl for all samples at either room temperature (approximately 22°C, circles) or at 37°C (triangles). Sedimentation at 20°C: 2 h-40K-SB283 (270,000). The times indicated are those for which samples were incubated before layering on the sucrose gradients. Since the “zero” time is uncertain, the data are arbitrarily extrapolated to zero release because such RM show little contamination with free ribosomes in S 250, K 25, Mg 5, T 50.

after incubation (as in Fig. 1 legend), were centrifuged at low speed (10 min-10K-SW39 [11,000]) to sediment microsomal membranes, revealed ribosomal profiles virtually identical with those obtained when the total RM were applied directly to the gradients. We therefore concluded that ribosome release occurred before density gradient centrifugation and was neither due to pressure effects within the gradients, nor to dilution during sedimentation. A distinct tendency of the released ribosomes to exist as monomers and/or higher S forms (rather than as subunits) was observed in gradients such as those in Fig. 1 when the analysis was carried out at a lower temperature (3°C). As has been discussed in greater detail elsewhere (31), much less dissociation of inactive ribosomes into subunits occurs at lower temperatures than at 20°C. In the following sections we present data to further define KCl-puromycin release both qualitatively and quantitatively, and to elucidate the mechanism of release in some detail as an approach to understanding the nature of ribosome-membrane interaction.

(a) Rate and Extent of Ribosome Release

At different KCl concentrations and temperatures of incubation, the rate of ribosome release was higher in the presence of puromycin (Fig. 2); however, in the presence or absence of puromycin, this rate was an increasing function of both the ionic strength and the temperature. The temperature dependence was more pronounced at moderate (100–250 mM) than at high (500–1000 mM) KCl concentrations. The extent of ribosome release was determined by chemical assay of the RNA³ in membrane and ribosome fractions separated by centrifugation on discontinuous sucrose density gradients (Fig. 3). With no added puromycin, as the KCl concentration was raised, increasing amounts of RNA were released from the membranes and recovered in the ribosomal fraction until a plateau value of ~40% release was reached.

³ Since greater than 95% of all microsomal RNA is ribosomal (32, 33), we assume that an estimate of percent RNA release is equivalent to an estimate of percent ribosome release.

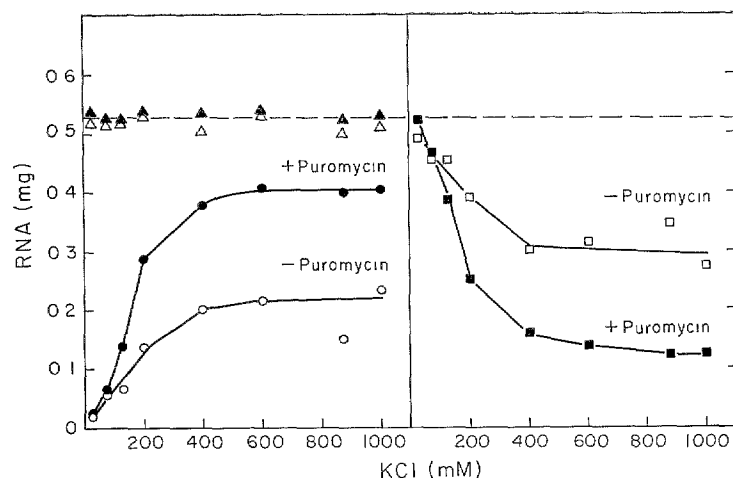


FIGURE 3 Separation of rough microsomes into ribosomal and membranous components. Rough microsomes were incubated in S 250, Mg 5, T 50 at various KCl concentrations in the absence (open symbols) or presence (solid symbols) of 0.95×10^{-3} M puromycin. Samples (1 ml, containing 0.526 mg RNA) were incubated in plastic centrifuge tubes for 60 min at 3°C, 15 min at 37°C, and then chilled. Each sample was underlaid with 1 ml of 1.8 M sucrose containing the appropriate K, Mg, T buffer. Sedimentation was at 3°C: 24 h-59K-A321 (315,000). The membranes packed at the interface were removed, along with the clear sample zone and a minimal amount of the 1.8 M sucrose layer. The ribosomal pellets were resuspended in the 1.8 M sucrose layer to which was added 1–2 ml of water. Membrane and ribosome fractions were then analyzed for RNA content. Circles indicate ribosome RNA, squares indicate membrane RNA, triangles are the sum of the respective ribosome and membrane RNA contents. The dashed line indicates the total input RNA or 100% recovery level.

at ~500 mM KCl. At KCl concentrations greater than 100 mM, puromycin treatment resulted in additional release of ribosomes until at >500 mM KCl a maximum of 75–80% of all bound ribosomes was released. In other experiments, microsomal membranes were separated from released ribosomes by differential centrifugation and repeated washing. Chemical analysis indicated that release of ribosomes by KCl-puromycin reached a maximum of ~85% when the incubations were carried out at room temperature or 37°C as long as the samples were not subsequently cooled. If samples were cooled before analysis, a small amount of “rebinding” appeared to occur, since slightly more RNA (up to 25% of the total) was associated with the membranes.

The effect of various KCl and MgCl_2 concentrations on the extent of ribosome release was evaluated on RM incubated for 2 h at room temperature in the presence of puromycin (Fig. 4). The release was strongly enhanced by elevated KCl concentrations at all levels of MgCl_2 , but, at a given KCl concentration, lower MgCl_2 concentrations only slightly favored ribosome release.

Maximum release (Fig. 4) was achieved at 1 mM MgCl_2 with KCl in excess of 500 mM; chemical determinations (data not included) showed that essentially 100% of the microsomal RNA was released from the membranes under these conditions. However, high ratios of $\text{K}^+:\text{Mg}^{2+}$ not only dissociate ribosomes, but also cause changes in the sedimentation rate which have been attributed to unfolding of ribosomal subunits (34). In particular, the 60S (large) ribosomal subunit undergoes a transition to a slower-sedimenting species which is inactive by virtue of having lost 5S RNA and several ribosomal proteins (35). The arrows in Fig. 4 indicate the approximate transition regions (between 125 and 200 mM KCl at 1 mM Mg, between 750 and 1000 mM KCl at 5 mM Mg) over which this subunit unfolding was detected in the sucrose gradients.⁴ It must be reemphasized that

⁴ We cannot, of course, distinguish between actual conformational changes due to ionic conditions, per se, and pressure effects due to the high centrifugal fields (36), the data simply indicate that functional subunits are not recovered from gradients run under excessively high $\text{K}^+:\text{Mg}^{2+}$ ionic conditions.

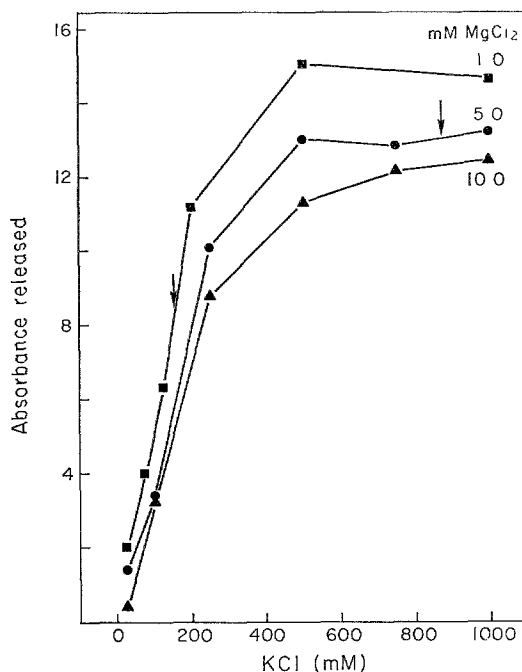


FIGURE 4 Release of ribosomes from RM at various KCl and MgCl_2 concentrations. Rough microsomes were suspended in a large volume (25 ml; approximately 0.2 mg RNA/ml) of S 250, T 50 and either 1 mM MgCl_2 (■), 5 mM MgCl_2 (●), or 10 mM MgCl_2 (▲). After ~10 min incubation on ice, the RM were centrifuged 15 min-30K-A211 (~90,000). (These equilibration washes were shown to result in negligible release of ribosomes.) The RM were then resuspended in ~2 ml of fresh buffer and brought by dilution to final conditions of S 250, T 50, 0.79×10^{-3} M puromycin, KCl as indicated, and either 1, 5, or 10 mM MgCl_2 . Samples were incubated 2 h at room temperature, and equal samples were applied to 15–40% sucrose gradients made in the appropriate K, Mg, T buffer. Sedimentation at 20°C: 2 h-40K-SB283 (270,000). Quantitation of absorbance released was as in Fig 2. The arrows next to the 1 mM Mg (■—■) and 5 mM Mg (●—●) curves indicate the approximate transition region for “unfolding” of the large ribosomal subunits (see text).

numerous experiments indicated that 80–85% of all RNA was released from RM incubated with puromycin in the presence of 750 mM KCl and 5 mM MgCl_2 , conditions which did not lead to gross conformational changes in the subunits. On the other hand, release of ribosomes in excess of 85% was only achieved under conditions which greatly altered the sedimentation of large ribosomal subunits. In fact, if the subunits are unfolded, com-

plete ribosome release can be effected in the absence of puromycin. For example, virtually all ribosomes can be stripped from RM by exposure to 1.0 M KCl, if MgCl_2 is omitted (D. Borgese, unpublished observations). Based on the above data, we adopted the following conditions for routine disassembly of rough microsomes which were chosen to maximize release of ribosomes and separation of ribosomal subunits, yet minimize deleterious effects on either ribosomes or membranes. RM were incubated at a concentration of ~1 mg RNA/ml with ~1 mM puromycin in S 250, K 750, Mg 5, T 50. The temperature was usually maintained at 3°C for 30–60 min and then raised to room temperature or 37°C for a time which depended on the particular experiment. Since we have recently found that when RM are incubated at 37°C in S 250, K 25, Mg 5, T 50 subsequent treatment with KCl-puromycin leads to the release of partially aggregated large subunits, we prefer to complete the treatment by incubating the RM for 30–60 min at room temperature. Separation was usually carried out by gradient centrifugation (as in Fig 1) at 20°C, or, in those cases where only recovery of the stripped microsomes was desired, by differential centrifugation.

(b) Characterization of the Separated Ribosomes and Membranes

To assay the functional capacity of the ribosomal subunits released from RM by KCl-puromycin treatment (750 mM KCl, 1 mM puromycin) the separated subunits were recovered from the sucrose gradients, diluted with K25, Mg 5, T 50, and sedimented. When recombined in an *in vitro* amino acid incorporation system (data not shown), these subunits were $\frac{1}{4}$ – $\frac{1}{2}$ as active as the original RM (per milligram RNA) in polyuridylic acid-dependent [^{14}C]polyphenylalanine synthesis. While the 40S subunit assayed separately showed little activity, the samples of large subunit were ~20% as active as the recombined subunits, presumably because of cross-contamination.

The appearance of the RM membranes recovered after high KCl-puromycin incubation (stripped RM) and the extent of ribosome detachment were assessed by electron microscopy. Fig. 5 *a* presents a field of untreated RM to be compared with a similar field of stripped membranes (Fig. 5 *b*). Most of the ribosome-denuded microsomal membranes were recovered as intact vesicles,

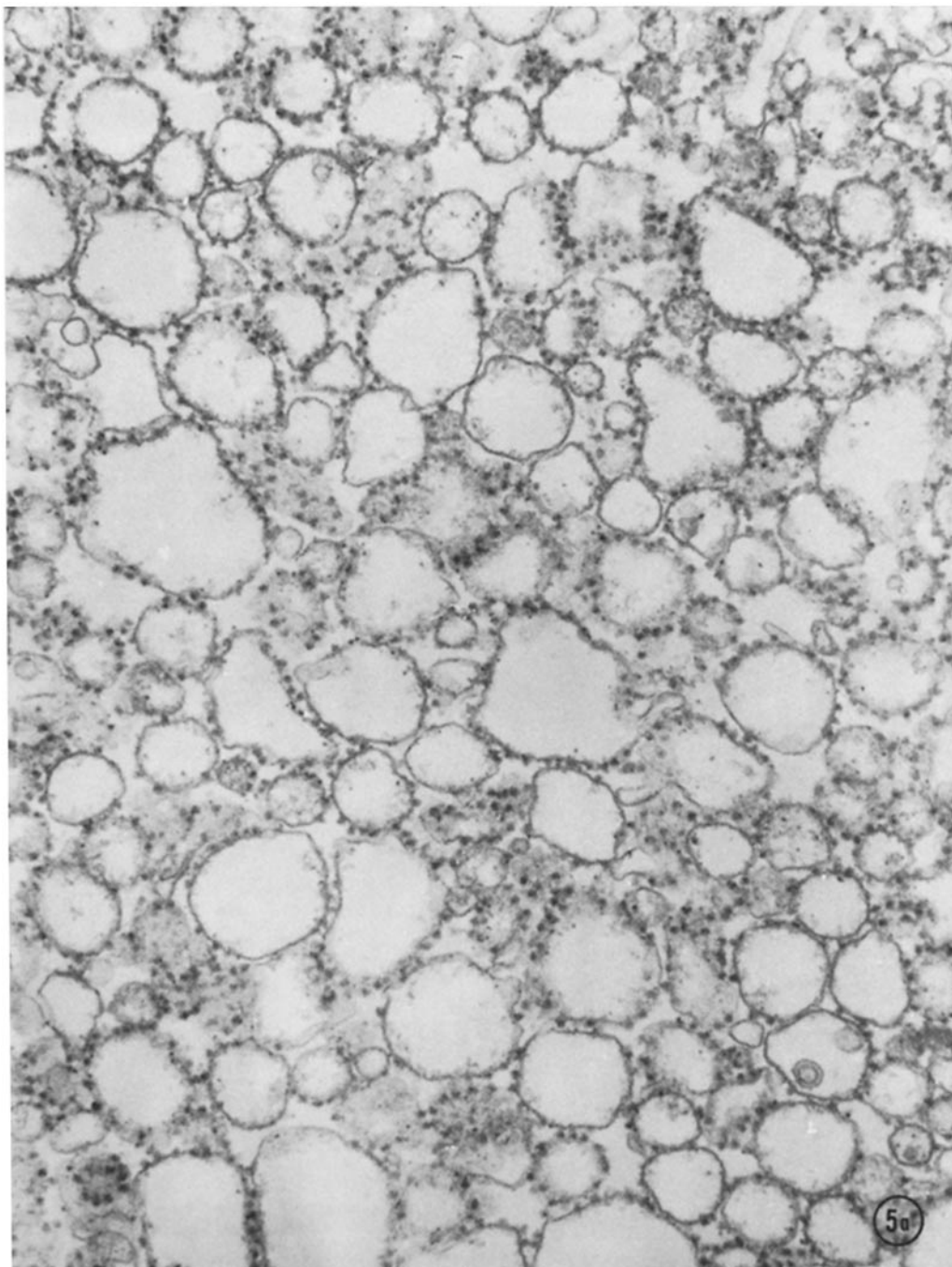


FIGURE 5 Electron micrographs of RM before and after removal of ribosomes with KCl and puromycin. Both pellets were resuspended in S 250 and processed for electron microscopy. Representative fields are shown at a final magnification of $\times 50,000$.

FIGURE 5 *a* Rough microsomes were suspended in an excess of S 250 and centrifuged at 3°C. 30 min-27K-No. 30 ($\sim 85,000$).

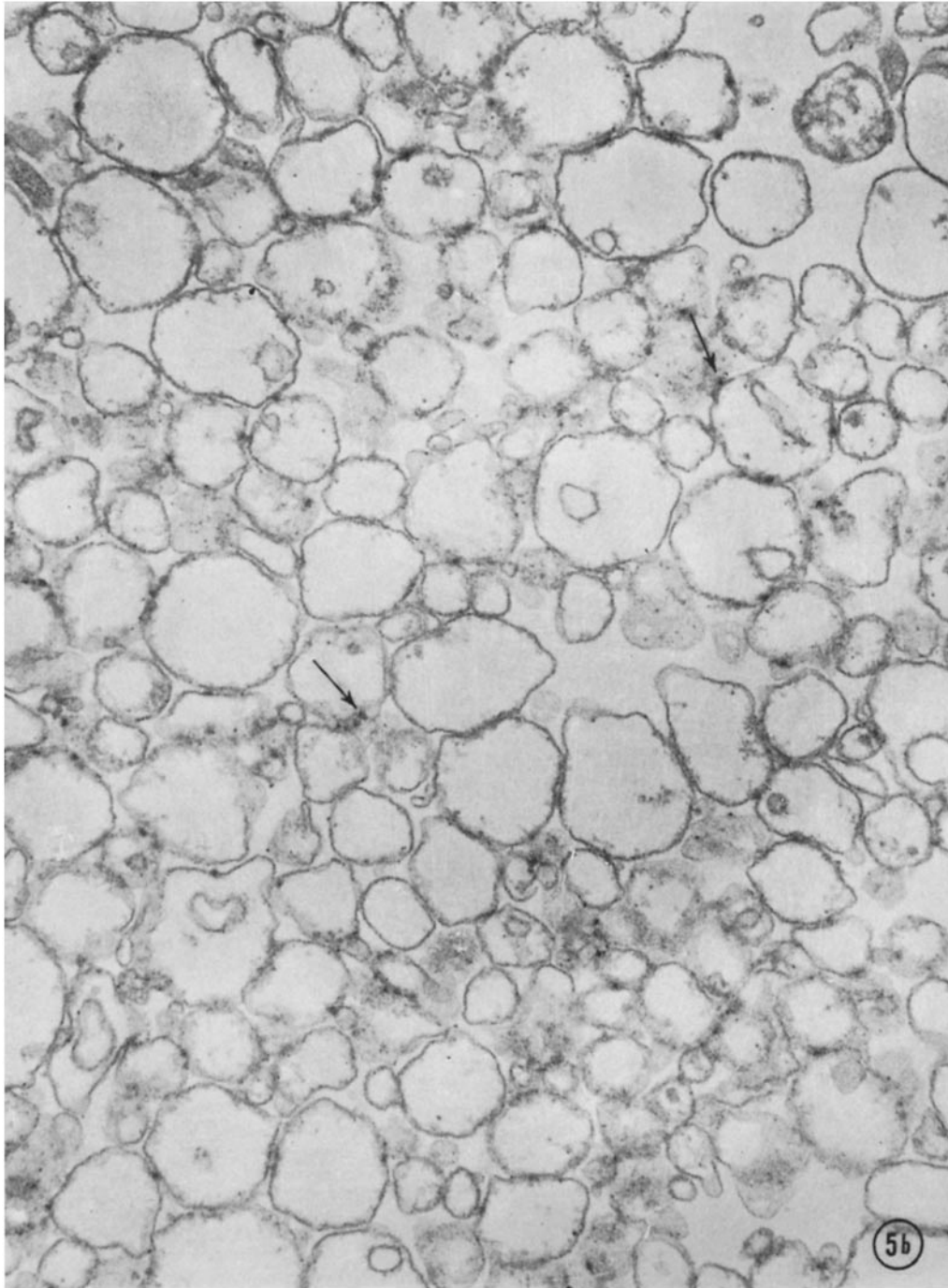


FIGURE 5 *b* Rough microsomes suspended in S 250, K 750, Mg 5, T 50 plus 0.95×10^{-3} M puromycin were incubated 60 min at 3°C and 15 min at 37°C. Samples were applied to 15–40% sucrose gradients in the same buffer. Sedimentation was at 20°C 90 min-40K-SB283 (270,000). The membrane band was collected, diluted with ice-cold K 750, Mg 5, T 50, and centrifuged 20 min-27K-No. 30 (85,000). The pellet was resuspended in S 250 and resedimented as in Fig. 5 *a*. These microsomes are referred to as stripped RM. Arrows indicate presumptive residual ribosomes (see text).

TABLE I
Chemical Analysis of Rough and Smooth Microsomes before and after Stripping of
Ribosomes with KCl and Puromycin

Sample	Protein	RNA	PLP	[³ H]RNA	RNA/Protein	RNA/PLP	PLP/Protein
	mg/ml	mg/ml	mg/ml	cpm/mg RNA	mg/mg	mg/mg	mg/mg
Rough microsomes	9.66	2.07	5.31	25,400	0.214	0.389	0.550
Stripped RM	6.57	0.358	5.20	21,600	0.054	0.069	0.791
Smooth microsomes	12.2	0.498	7.10	24,500	0.041	0.070	0.581
Stripped SM	7.95	0.063	5.46	12,600	0.008	0.012	0.687

Both rough microsomes and smooth microsomes were prepared at the same time from rats which had received an injection of [³H]orotic acid (200 μ Ci, 2.5 mCi/mol) ~40 h before sacrifice. RM and SM pellets were resuspended in S 250, K 25, Mg 5, T 50. Samples of each were incubated for 2 h at room temperature in the presence of (final concentrations) S 250, K 750, Mg 5, T 50, and 0.68×10^{-3} M puromycin. The samples were diluted with an excess of room temperature K 750, Mg 5, T 50 and centrifuged at room temperature 15 min-30K-A211 (~90,000). The pellets were resuspended in cold buffer and recentrifuged. Each pellet was then resuspended in S 250, K 25, Mg 5, T 50 to the same volume as the original sample. Samples of the untreated RM and SM, as well as the KCl-puromycin-treated microsomes, were analyzed for protein, phospholipid phosphorus, RNA, and ³H cpm in the RNA hydrolyzate.

although the mean vesicle diameter appeared to have been reduced slightly by the stripping procedure. Residual ribosomes, when detectable (arrow, Fig. 5 b), most often appeared where two vesicles abutted on one another. The results of chemical analysis of typical stripped RM which were, in this case, prepared from [³H]orotic acid-injected animals, are presented in Table I. Included for comparison are data on the original RM, as well as on smooth microsomes before and after KCl-puromycin treatment. The phospholipid (PLP) determinations indicate that the recovery of RM membranes after stripping was nearly total, while that of smooth microsomes (SM) treated for stripping was somewhat lower (probably because of incomplete sedimentation of the membranes). The recovered stripped RM contained $\sim \frac{2}{3}$ of the initial protein, and ~15% of the RNA. While the untreated rough and smooth microsomes contained RNA labeled to a specific activity of ~25,000 cpm/mg, the specific activity of the residual RNA in each stripped membrane fraction was lower. It should be noted, however, that the extremely small amount of RNA present in the stripped SM was at the lower limit of reliability of the chemical assay, while the amount of RNA remaining in the stripped RM was more significant, an error of 5-10% in the chemical assay could not be ruled out. In other experiments also using RM containing tritium-labeled RNA (same batch of RM used for the experiment in Fig. 3), with an initial spe-

cific activity of 23,000 cpm/mg RNA, all residual membranes had specific activities of $22,000 \pm 2000$ cpm/mg RNA except for those membranes recovered after puromycin treatment at 400 mM KCl or higher, which had lower values ranging from 18,500 to 19,800 cpm/mg. The significance of this lower specific activity is not clear, but it has been reported that microsomes contain polyadenylic acid (poly A) (37, 38). Computations based on the published data suggest that a minimum of 0.1-0.2% of the microsomal RNA is poly A, and since no estimate of poly A recovery was available, the poly A content of microsomes might be significantly higher. Since we used [³H]orotic acid for RNA labeling and since orotic acid is not taken up into adenine (39), it is not unreasonable to postulate the existence in the residual RNA of unlabeled poly A amounting to 1-2% (15% residual, 10-15% of this unlabeled) of the total microsomal RNA.

(c) The Specific Nature of Puromycin-Dependent Ribosome Release

The data presented up to now demonstrate a puromycin-dependent release of ribosomes which has been defined as the difference between the KCl-puromycin release and the salt release. By prewashing RM to remove those ribosomes released by salt alone, and then resuspending the RM in high KCl buffer, it was possible to study

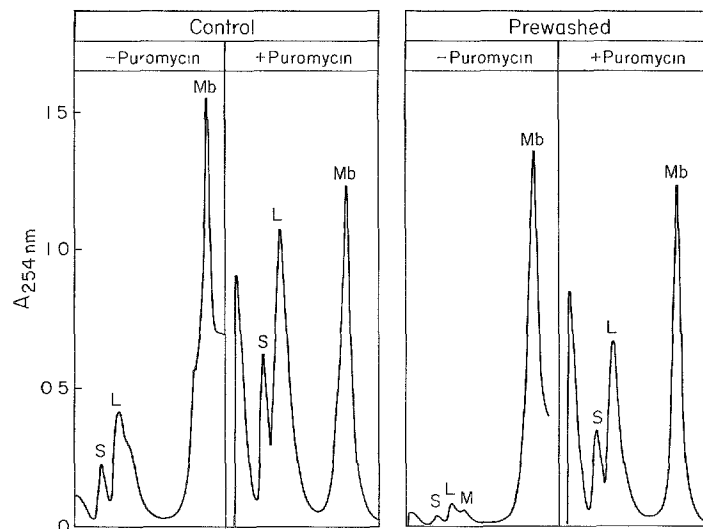


FIGURE 6 Effect of prewashing on the extent and specificity of puromycin-dependent ribosome release. RM were suspended in S 250, Mg 5, T 50 plus 25 mM KCl (control) or 750 mM KCl (prewashed). Manipulations are described in the footnote to Table II. The microsomes were resuspended in S 250, K 750, Mg 5, T 50 in the absence or presence of 0.79×10^{-3} M puromycin. Incubation and sedimentation as in footnote, Table II. S = small ribosomal subunit; L = large subunit; Mb = membrane band

puromycin-dependent release under conditions where virtually no salt release occurred (Fig 6). Thus, RM samples could be prepared in which the specificity of puromycin action (defined in percent as $100 \times \{[\text{KCl-puromycin release}] - [\text{salt release}]\} / [\text{KCl-puromycin release}]$) at all ionic strengths had been strikingly enhanced. The data in Table II indicate, for example, that RM prewashed in 750 mM KCl showed ~84% puromycin-specific release (treatment at 750 mM KCl) as compared with 53% for the control sample. It should be noted that prewashing the RM did somewhat reduce the absolute magnitude of puromycin-dependent release. Reexamination of data such as those in Fig 2 suggested that salt release proceeded in two stages, one rapid, the other slow, and it seemed reasonable to conclude that the slow phase of salt release reflected the gradual removal of ribosomes which, during shorter incubations, would be released only in a puromycin-dependent fashion.

Using RM which had been prewashed at 750 mM KCl (as for Fig 6 and Table II), several aspects of puromycin specificity were studied. It was found (data not shown) that puromycin-dependent release proceeded more slowly at low KCl, in the cold, and at low (10^{-6} – 10^{-4} M) concentrations of puromycin. The dependence of release on

puromycin concentration was most pronounced when incubations were carried out in the cold, or at moderate KCl concentrations (250 mM), or both. At room temperature and in the presence of 750 mM KCl, rapid release occurred even with 10^{-5} M puromycin. Neither puromycin aminonucleoside (0.83×10^{-3} M) nor cycloheximide (4.2×10^{-3} M) was capable of releasing ribosomes from rough microsomes. Under conditions favoring puromycin action (750 mM KCl, room temperature, 10^{-3} M puromycin), neither cycloheximide nor puromycin aminonucleoside interfered with puromycin-dependent ribosome release. However, under conditions resulting in slow puromycin-dependent release (short incubation, 10^{-5} M puromycin, 3°C, 750 mM KCl), cycloheximide (but not puromycin aminonucleoside) strongly inhibited the effect.

(d) KCl Dependence of Puromycin-Induced Ribosome Release

When RM prewashed at high KCl (750 mM) were resuspended in low KCl (25 mM), no puromycin-dependent release was observed (data not shown); thus, the puromycin-dependent ribosome release only occurred in the presence of high con-

TABLE II
Effect of Prewashing RM on the Extent and Specificity of Puromycin-Dependent Ribosome Release

Prewash	Treatment	Release in Arbitrary Absorbance Units			% Specificity puromycin
		- Puromycin	+ Puromycin	Δ Puromycin	
Control					
(25 mM KCl)	250 mM KCl	36	82	46	56
250 mM KCl	" " "	10	49	39	79
750 mM KCl	" " "	7	43	36	84
Control					
(25 mM KCl)	750 mM KCl	56	119	63	53
250 mM KCl	" " "	22	80	58	72
750 mM KCl	" " "	9	57	48	84

Equal amounts of rough microsomes were suspended in a large volume of S 250, Mg 5, T 50 and either 25 (control), 250, or 750 mM KCl. After incubation for 30 min at room temperature, the microsomes were centrifuged at room temperature, 10 min-20K-A211 ($\sim 40,000$). Each pellet was resuspended in a large volume of cold S 250, K 25, Mg 5, T 50 and recentrifuged at 3°C, 15 min-25K-A211 ($\sim 65,000$). The pellets were then resuspended in small volumes of appropriate buffers and equivalent amounts were incubated in S 250, Mg 5, T 50 and either 250 or 750 mM KCl. Incubation, in the absence or presence of 0.79×10^{-8} M puromycin, was for 60 min at 3°C, then 30 min at room temperature. Equal samples were applied to 15-40% sucrose gradients in the appropriate K, Mg, T buffer. Sedimentation at 20°C, 2 h-40K-SB283 (270,000). The extent of release was evaluated as in Fig. 2. The profiles shown in Fig. 6 formed part of these data. Extent of release in the absence (- puromycin) and presence (+ puromycin) is given. The difference or release due to puromycin alone is indicated as Δ puromycin. Δ Puromycin divided by total release in the presence of puromycin is taken as a measure of the puromycin specificity of release expressed in percent.

centrations of KCl. The nature of this salt dependence of the puromycin effect was investigated, considering the following possibilities: (i) puromycin coupling to ribosome-bound nascent chains might not proceed at low KCl concentration, (ii) puromycin coupling might occur at low KCl concentration, but the nascent chains might not be released from ribosomes; or (iii) puromycin might both be coupled to and release nascent chains at low KCl concentration, but ribosomes might remain attached to the microsomal membranes through other bonds which are affected by the KCl concentration. The results to be presented favor possibility (iii).

To determine whether the coupling of puromycin was KCl dependent, the rate at which [3 H]puromycin was incorporated into hot acid-insoluble material by RM under various conditions was measured. Fig. 7 shows that incorporation was favored by high KCl concentrations and by elevated temperatures and reached a maximum of ~ 0.6 mol puromycin/mol of ribosome at 37°C in the presence of 500-1000 mM KCl. Incorporation was only $\sim 1/4$ of this value at 25 mM KCl. However, in this experiment, labeled puromycin of high

specific activity (1 l mCi/ μ mol) was used, and the free concentration of puromycin was very low ($\sim 10^{-5}$ M; molar ratio of puromycin to ribosomes, $\sim 10:1$). This is in contrast to the experiments on puromycin-dependent ribosome release described in previous sections in which concentrations of puromycin ~ 100 -fold greater were used at puromycin:ribosome ratios of $\sim 2000:1$. We therefore tested whether [3 H]puromycin coupling would be as strongly KCl dependent as in Fig. 7 if higher puromycin concentrations were used. By diluting the [3 H]puromycin approximately 10-fold with unlabeled puromycin, we were able to work at final puromycin concentrations of 10^{-4} M; using slightly lower concentrations of RM, we achieved puromycin:ribosome ratios of $\sim 200:1$.

The initial experiments in this series showed that higher concentrations of puromycin increased coupling and that the dependence on puromycin concentration was most pronounced at low KCl concentrations. Thus, increased concentrations of puromycin tended to minimize the differences in reactivity between samples incubated at low and high KCl. We carried out an extensive investigation of the puromycin-coupling reaction as a func-

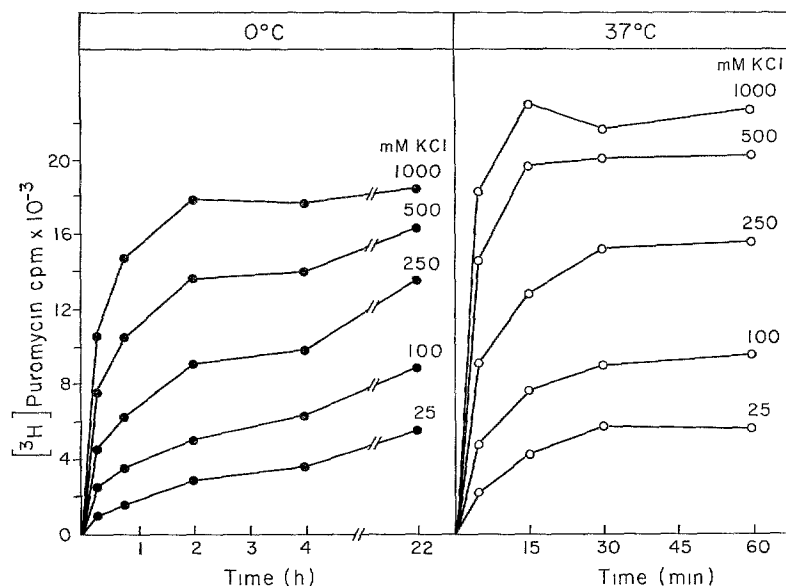


FIGURE 7 Coupling of [^3H]puromycin by rough microsomes. RM were incubated at 0°C (left) or 37°C (right) in S 250, Mg 5, T 50 plus the indicated KCl in the presence of [^3H]puromycin ($1.1 \text{ mCi}/\mu\text{mol}$; final concentration of $0.91 \times 10^{-5} \text{ M}$). At the indicated times, $100\text{-}\mu\text{l}$ samples containing 0.172 mg RNA were taken and assayed for hot acid-insoluble counts per minute (see Materials and Methods). The ^3H counting efficiency was approximately 20%. Assuming that all microsomal RNA is ribosomal (32, 33) and that 1 mol of liver ribosomes = $2.4 \times 10^6 \text{ g RNA}$ (40–42), incorporation of 0.5 mol puromycin/mol of ribosome would give rise to $\sim 17,500$ ^3H cpm incorporated/ $100 \mu\text{l}$ sample.

tion of KCl, Mg^{2+} , and/or puromycin concentrations, as well as of the time and temperature of incubation. In the course of these studies we became aware of an anomalous (i.e., proceeding nearly linearly with time and reaching levels higher than 1 mol puromycin/mol ribosome), non-ribosomal incorporation of [^3H]puromycin into a hot acid-insoluble product. Fig. 8 shows that this phenomenon was only detectable at relatively high concentrations of puromycin. When RM were incubated at the low puromycin concentration of 10^{-5} M (Fig. 8 A), coupling was strongly salt dependent and plateaued after 1–2 h; however, when the same RM were incubated with isotopically diluted puromycin at 10^{-4} M (Fig. 8 B), the coupling was less dependent on salt concentration, continued well beyond 2 h (in a nearly linear fashion), and reached values in excess of 1 mol/mol ribosome. These results were most striking when incubations were carried out at room temperature. Coupling at 3°C was, as might be expected, much lower. At 37°C , incorporation of [^3H]puromycin proceeded rapidly, but leveled off after 1–2 h; hence, the incorporation usually did not exceed 1

mol/mol ribosome and the anomalous phase of puromycin coupling was not observed. If the same low specific activity solution used at 10^{-4} M in Fig. 8 B was employed at a final puromycin concentration of 10^{-5} M , the results were virtually identical with those of Fig. 8 A. By extending the duration of the room temperature incubation it was possible to observe coupling considerably in excess of 1 mol of [^3H]puromycin/mol of ribosome. This “excess” or anomalous coupling was probably not due to microbial contamination since it was not enhanced by preincubating the microsomes at room temperature or at 37°C , and it was not noticeably inhibited by cycloheximide (10^{-4} M), chloramphenicol (10^{-4} M), dinitrophenol (10^{-4} M), or a mixture of penicillin (400 U/ml) and streptomycin sulfate (0.05 mg/ml). The excess [^3H]puromycin coupling was not reduced by additional washes of the RM in S 250, K 25, Mg 5, T 50, nor by addition of the analogue, puromycin aminonucleoside (10^{-3} M). Variable effects ranging from slight inhibition to slight enhancement were observed with ethylenediaminetetraacetate (EDTA) ($\sim 0.02 \text{ M}$), sodium deoxycholate (Na-

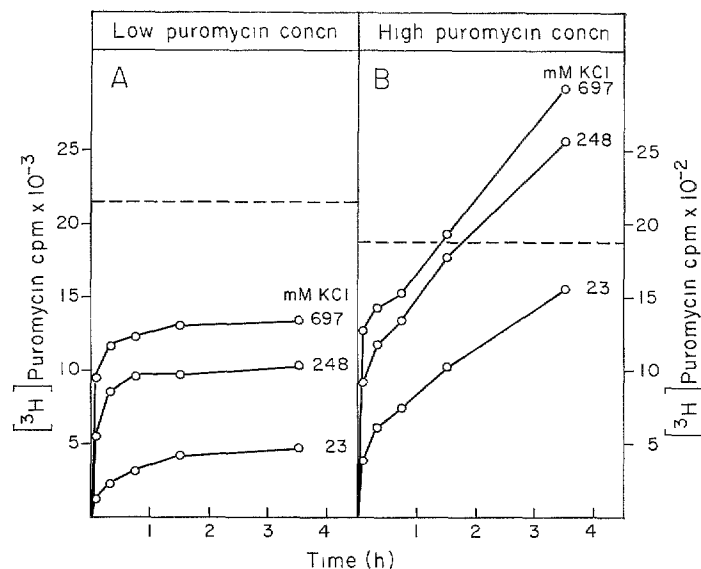


FIGURE 8 Coupling of [^3H]puromycin by rough microsomes. RM were incubated at room temperature ($\sim 22^\circ\text{C}$), in S 230, Mg 4.5, T 45 plus the indicated KCl. Incubations were carried out at a puromycin: ribosome ratio of 16 mol/mol (left; 0.824×10^{-5} M; $1.1 \text{ mCi}/\mu\text{mol}$) or at a ratio of 182 mol/mol (right; 0.932×10^{-4} M; $0.097 \text{ mCi}/\mu\text{mol}$). At the indicated times, 100- μl samples containing 0.103 mg RNA were taken for determination of counts per minute incorporated. In each panel, the counts per minute corresponding to incorporation of 1 mol puromycin/mol ribosome is indicated by the dashed line. Note that the counts per minute scales differ by a factor of 10.

DOC) (0.5%), or Triton X-100 (1–2%). Both smooth microsomes and stripped RM showed the excess coupling reaction. By first reacting SM or RM briefly with 10^{-8} M cold puromycin, sedimenting the microsomes, and then resuspending them in labeled puromycin, it was possible to label primarily the excess coupling component. The incorporated tritiated puromycin banded in sucrose density gradients coincident with the membranes. Bound ribosomes isolated from detergent-solubilized RM in such a way (see legend to Fig. 9) as to minimize contamination of the isolated ribosomes with solubilized membrane components showed no traces of excess puromycin coupling. We concluded, therefore, that microsomal membranes contained some component, distinct from the attached ribosomes, which was capable of coupling puromycin to hot acid-insoluble material. The enzyme or enzymes involved required fairly high concentrations of puromycin and survived detergent solubilization; this phenomenon is being explored further.

Using detergent-derived bound ribosomes, puromycin coupling was studied without the complications introduced by the anomalous coupling

reaction in RM. The results verified our initial conclusion that increased concentrations of puromycin and prolonged incubations (at room temperature) tended to minimize differences in reactivity between low and high KCl incubations. The data in Fig. 9 show that bound ribosomes coupled about the same amount of puromycin over a broad range of KCl and MgCl_2 concentrations and that at very low KCl (less than 25 mM), extensive reaction occurred. Because it is reasonable to assume that microsomal ribosomes react with puromycin essentially as well before as after detergent treatment, we concluded that membrane-bound ribosomes reacted as extensively with puromycin at low KCl (100 mM) as at high KCl (750 mM), even though such reaction only led to release of ribosomes at high ionic strength. Further support for this conclusion was obtained from experiments in which we estimated the extent to which ribosome release at high ionic strength was facilitated by previous incubation of the RM with puromycin at low ionic strength. RM which were incubated with puromycin in 25 mM KCl and washed (lower profiles in Fig. 10; sample B) no longer required the presence of puromycin to release ribosomes in

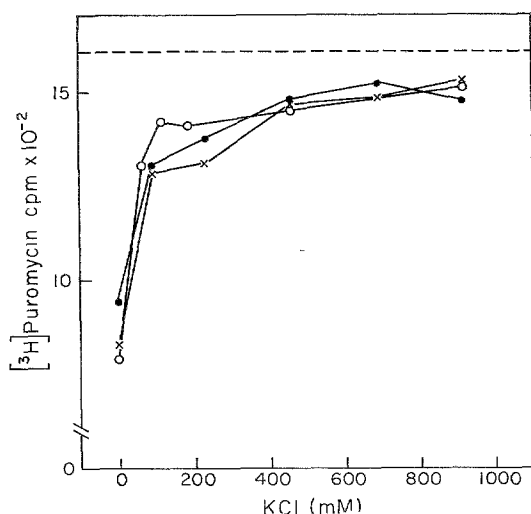


FIGURE 9 Coupling of $[^3\text{H}]$ puromycin by bound ribosomes. RM were resuspended in S 250, K 25, Mg 5, T 50 and diluted by 10% with a solution of 5% DOC and 10% Triton X-100. The suspension (~ 5 ml/tube) was placed in plastic centrifuge tubes and underlaid with 2 ml of (9 parts S 1350, K 25, Mg 5, T 50 + 1 part 5% DOC - 10% Triton) and with 2 ml of S 1600, K 25, Mg 5, T 50. Sedimentation was at 3°C : 13 h-58K-A321 ($\sim 300,000$). The supernatant was discarded, the inside of the tubes was rinsed with a small amount of water, and the pellets were resuspended in appropriate buffer. These "bound" ribosomes were incubated for 2 h at room temperature in S 230, T 45, the indicated KCl concentrations, and either 0.91 (O), 4.5 (●), or 9.1 (X) mM MgCl_2 . $[^3\text{H}]$ Puromycin (0.097 mCi/ μmol) was present at a final concentration of 0.94×10^{-4} M; the puromycin/ribosome mole ratio was ~ 146 . Duplicate 100- μl samples, containing 0.128 mg RNA were assayed for puromycin coupled to hot acid-insoluble material. The dashed line at 1610 cpm indicates coupling of ~ 0.7 mol puromycin/mol ribosome.

750 mM KCl. RM preincubated without puromycin in 25 mM KCl (upper profiles in Fig. 10; sample A) behaved identically with a nonpreincubated control (sample C, profiles not shown), and required puromycin for release of ribosomes at high ionic strength. Quantitation of the areas under the ribosomal peaks in Fig. 10 indicated that 70-80% of the ribosomes specifically released by puromycin from sample A were released in the absence of added puromycin from sample B. The extent to which puromycin coupling occurs at low ionic strength was also inferred from the effect of preincubation with puromycin (nonlabeled) at low ionic strength. Samples of A, B, and C (preincu-

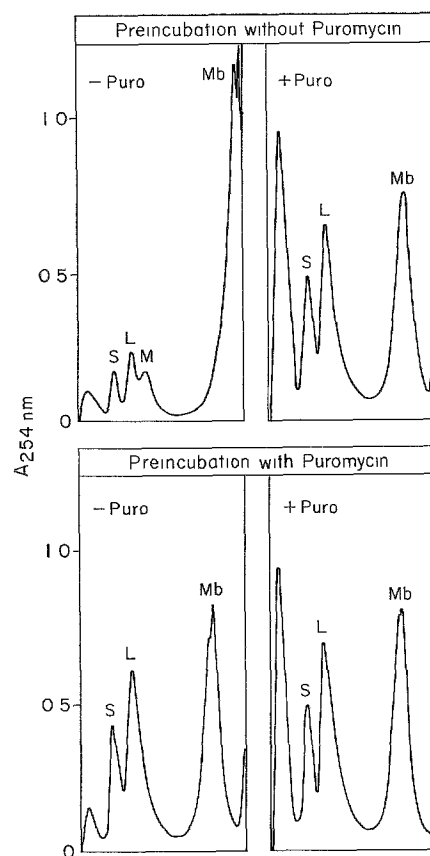


FIGURE 10 Reaction of rough microsomes with puromycin at low KCl. RM were suspended in S 250, K 25, Mg 5, T 50 and separated into three equal samples, A, B, and C. A and B were incubated in the absence (A), and presence (B) of 0.86×10^{-3} M puromycin for 60 min at 3°C and 60 min at room temperature. All three samples (C serving as a control for the room temperature incubation) were diluted with cold S 250, K 25, Mg 5, T 50 and centrifuged at 3°C : 15 min-27K-no. 30 (85,000). The pellets were resuspended in S 250, K 25, Mg 5, T 50 and rewashed by zone sedimentation (at 3°C) through 15-40% sucrose gradients made up in K 25, Mg 5, T 50; 30 min-40K-SB283 (270,000). The pellets were resuspended and incubated in S 250, K 750, Mg 5, T 50 in the absence and presence of 0.79×10^{-3} M puromycin. Incubation was for 60 min at 3°C and 15 min at 37°C . Sedimentation analysis at 20°C : 2 h-40K-SB283 (270,000). The upper panels show the results of analyzing sample A; virtually identical results were obtained with the control sample C. The lower panels represent sample B (preincubation with puromycin).

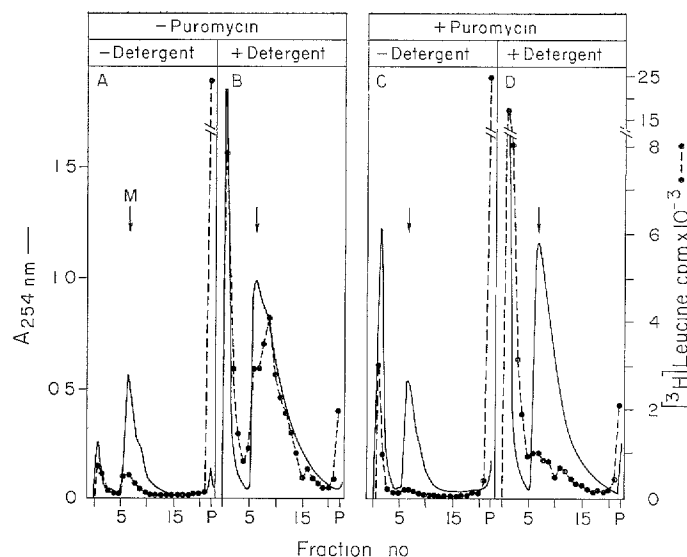


FIGURE 11 Release of ribosomal nascent chains by treatment of rough microsomes with puromycin at low KCl. The RM were prepared from rats which received an injection (portal vein) of [^3H]leucine 2 min before excision of the liver. The specific activity of the isolated RM was $\sim 2.2 \times 10^5$ cpm/mg RNA. Equal amounts of RM were incubated in S 250, K 100, Mg 5, T 50 in the absence (A and B) or presence (C and D) of 0.79×10^{-3} M puromycin. Incubation was for 60 min at 3°C and 15 min at room temperature. Samples B and D were then diluted 8% with a solution of 5% DOC, 20% Triton X-100 (A and C received water). Samples were applied to 15–40% sucrose gradients made up in the K, Mg, T buffer. Sedimentation was at 20°C : 1 h-40K-SB283 (270,000). Fractions were collected and processed for scintillation counting; the pellet was also counted (P). A_{254} , —; [^3H]leucine counts per min, ●—●.

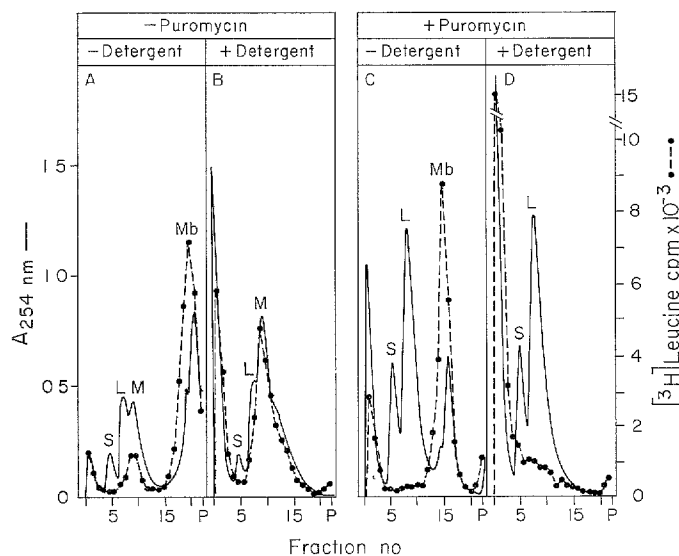


FIGURE 12 Release of ribosomal nascent chains by treatment of rough microsomes with puromycin at high KCl. The experimental protocol was identical with that of Fig. 11, with the following exceptions: (a) the concentration of RM was slightly lower than in Fig. 11; (b) all solutions contained 750 mM KCl, rather than 100 mM KCl; (c) sedimentation was for 2 h.

bated and washed as in the legend to Fig. 10) were mixed with labeled puromycin at low KCl and after 15 min at room temperature, the samples were diluted to final concentrations of S 250, K 750, Mg 5, T 50 and 0.90×10^{-5} M [3 H]puromycin (1.1 mCi/mmol, at this low puromycin concentration excess coupling was negligible). After incubation for 60 min at room temperature, assays of equal samples (100 μ l) of A and C indicated incorporation of 10,519 and 10,767 cpm respectively, while the corresponding figure for B was 3028 cpm. These results and the data in Fig. 10 showed that RM exposed to puromycin at low ionic strength reacted to at least 70% of their maximum coupling capability, yet the ribosomes were not released until the salt concentration was raised.

Having demonstrated that nascent chains in membrane-bound ribosomes do react extensively with puromycin at low KCl, we studied whether the peptidyl-puromycin molecules formed were released from the ribosomes under such conditions. For these experiments we used RM prepared from animals injected 2 min before sacrifice with [3 H]-leucine so as to label nascent polypeptide chains. The analyses (Figs. 11 and 12) were carried out using zone sedimentation in continuous sucrose density gradients since we had found that, by differential centrifugation, it was not possible to distinguish ribosome-associated radioactivity from labeled material which simply cosedimented with ribosomes. In fact, as the data in Figs. 11 and 12 show, if microsomal membranes are dissolved with detergents, the puromycin-released polypeptides which they contain have a striking tendency to aggregate and sediment heterogeneously throughout the gradient. The analysis in Fig. 11 was carried out at 100 mM KCl, an ionic strength which somewhat reduced the chain aggregation (the aggregation is particularly troublesome in K 25, Mg 5, T 50), but was sufficiently low that no puromycin-dependent ribosome release occurred (cf Fig. 1). At this low KCl concentration only a small amount of salt-released ribosomes containing some nascent polypeptide chains was present in the gradient (Fig. 11 A), while the microsomes sedimented to the bottom carrying with them most of the ribosomes and [3 H]leucine label. The use of detergent to dissolve the membranes and display all ribosomes within the gradient (Fig. 11 B) revealed that only 50% of the radioactivity was actually associated with ribosomes (i.e., in a radioactivity peak coincident with the absorbance profile in the ribosome region of the gradient). The

remainder, presumably in chains which had been completed and released from ribosomes during the 2-min *in vivo* pulse, was found near the top of the gradient. Addition of puromycin (Fig. 11 C) released to the supernatant only the radioactivity in those nascent chains which were attached to the salt-released ribosomes (cf. Fig. 11 A) and did not cause additional release of ribosomes from the microsomes. Detergent treatment of RM which had been incubated with puromycin (Fig. 11 D) revealed a radioactivity distribution quite distinct from that seen in Fig. 11 B. A larger fraction of the radioactivity was found near the top; while the rest sedimented heterogeneously throughout the gradient, there was no sign of a peak of radioactivity coincident with the ribosomal absorbance peak, thus demonstrating that the peptidyl-puromycin molecules had been discharged from the ribosomes. Summation of the radioactivity in fractions 4 through P in Fig. 11 D gave an estimate of labeled material which, by differential centrifugation, would have been sedimented into a pellet with the ribosomes. This was found to be $\sim 45\%$ of the corresponding total in Fig. 11 B, an estimate which is in good agreement with data (not shown) we have obtained using differential centrifugation.

When a similar experiment as that in Fig. 11 was carried out with RM at high ionic strength (Fig. 12) (750 mM KCl), the addition of puromycin (Fig. 12 C) resulted in release of the bulk of the ribosomes as subunits, which were virtually devoid of associated nascent chains (cf Fig. 12 B). The stripped microsomal membranes banded isopycally within the gradient and retained almost all of the radioactivity. This radioactivity was released upon detergent dissolution of the stripped membranes (Fig. 12 D) and appeared both at the top of the gradient and sedimenting heterogeneously as in Fig. 11 D. We therefore concluded that membrane-bound ribosomes not only reacted with puromycin at low KCl, but that this reaction resulted in release of nascent polypeptide chains from the ribosomes which was as efficient at low as at high KCl. We could not directly rule out the possibility that the detergent treatment itself caused release of puromycin-coupled chains at 100 mM KCl, but experiments with leucine-labeled free ribosomes not treated with detergents gave results (not shown) similar to those in Fig. 11, and therefore rendered this latter possibility unlikely.

The experiments just described showed that when ribosomes were released from RM specifically by puromycin, most of the labeled nascent

chains remained firmly associated with the residual or stripped RM (Fig. 12 C), thus providing a demonstration of vectorial discharge (14). However, because much of the radioactivity in Fig. 12 C represented completed chains not actually released from ribosomes by puromycin, we used [^3H]-puromycin to label specifically the nascent chains and demonstrate directly their vectorial discharge to the microsomes. As shown in Fig. 13 B, incubation of RM in high KCl with [^3H]-puromycin resulted in release of ribosomes and the coupling of [^3H]-puromycin to hot acid-insoluble material which banded isopycally with the stripped membranes. The small amount of radioactivity in the supernatant fractions probably represented puromycin coupled to chains from the salt-released ribosomes (see Fig. 12 A), since when RM were prewashed at high KCl and then treated and analyzed as in Fig. 13 the amount of acid-insoluble [^3H]-puromycin found in the supernatant fractions was reduced.

(e) The Nature of Salt-Released Ribosomes

To investigate if those ribosomes released by salt alone were functionally different from the bulk of the bound ribosome population, we compared the [^3H]-puromycin coupling ability in 750 mM KCl of (i) total RM, (ii) salt-released ribosomes prepared from RM by preincubation at 750 mM KCl, and (iii) RM which had been depleted of salt-releasable ribosomes by washing in 750 mM KCl. The [^3H]-puromycin radioactivity coupled was found to be 94,000, 48,000, and 119,000 cpm/mg of RNA for i, ii, and iii respectively (puromycin 10^{-5} M, 1.1 mCi/ μmol). Thus, the ribosomes released by salt alone were roughly 50% as "active" as those which could only be released by incubation with puromycin. Examination of Figs. 11 and 12 also indicates that, after *in vivo* pulse labeling, salt-released ribosomes contained less radioactivity in nascent chains (per absorbance unit) than did those ribosomes which remained attached to the RM and were released by detergent (compare Fig. 11 B with Fig. 11 A, and Fig. 12 B with Fig. 12 A). Moreover, to the extent that dissociation at high ionic strength into subunits in the absence of puromycin can be taken as an indication of inactivity (31), comparison of Figs. 12 A and B also suggested that salt-released ribosomes were relatively inactive.

So as to determine whether the specific activity ([^3H]-leucine counts per minute per milligram

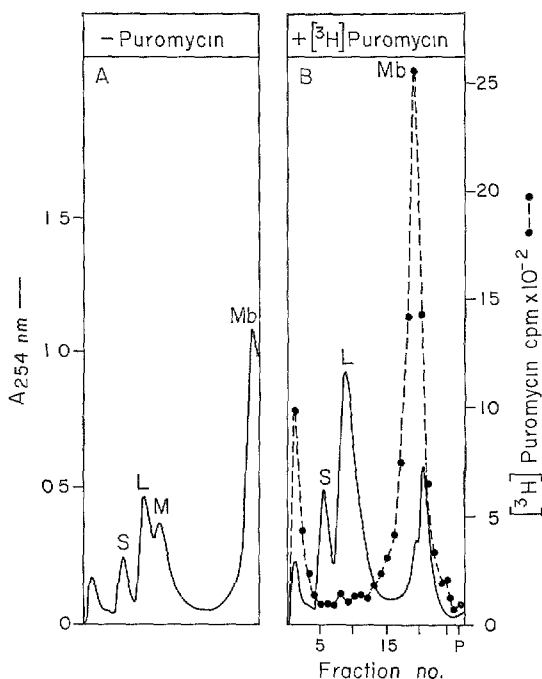


FIGURE 13 Distribution in sucrose gradient of [^3H] puromycin incorporated by rough microsomes. Rough microsomes were incubated in S 250, K 750, Mg 5, T 50 in the absence (A) and presence (B) of 0.82×10^{-5} M [^3H]puromycin (1.1 mCi/ μmol ; excess coupling was negligible under these conditions). Incubation was for 15 min at 3°C , then 30 min at 37°C . Samples were applied to 15–40% sucrose gradients in the K, Mg, T buffer. Sedimentation at 20°C : 2 h-40K-SB283 (270,000). Fractions were collected from B and, along with the pellet, were assayed for hot acid-insoluble [^3H]puromycin counts per minute, ●—●.

RNA) of salt-released ribosomes was a function of the KCl concentration at which they had been released, we analyzed pulse-labeled RM by sedimentation in continuous sucrose gradients (Fig. 14). As expected, increasing amounts of ribosomal material were released at increasing levels of KCl. In addition, it was found that increasing amounts of ribosome-associated radioactivity (i.e., coincident with the ribosomal peaks) were released as a function of the KCl concentration. Evaluation of the data in several ways gave the same conclusion: the ribosomes released at low KCl had lower specific activity than those released at high KCl. The exact magnitude of this effect depended on the means of computing specific activity, defined in terms of counts in a particular region of the gradient divided by absorbance (in arbitrary units as

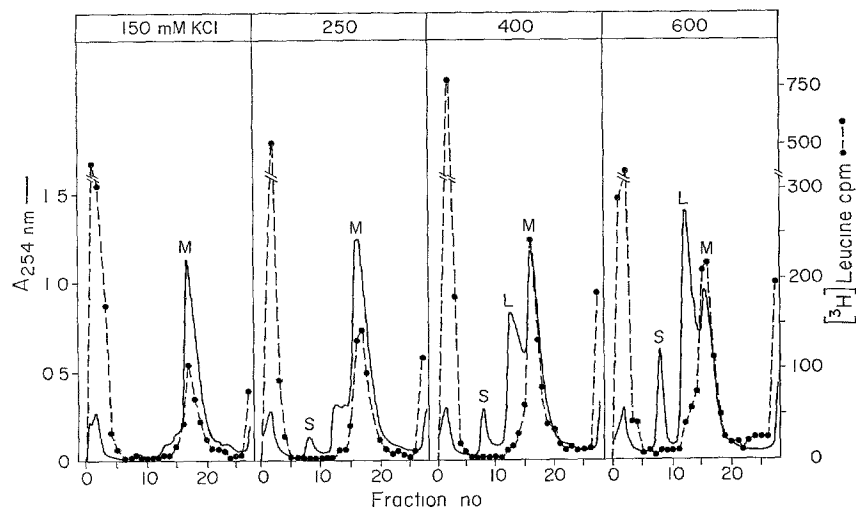


FIGURE 14 Distribution of [^3H]leucine-labeled nascent polypeptides on ribosomes released from rough microsomes at various KCl concentrations. The RM were prepared from rats which received an injection (portal vein) of [^3H]leucine 2 min before excision of the liver; specific activity of the isolated RM was 0.59×10^5 cpm/mg RNA. The RM were incubated in S 250, Mg 5, T 50 plus 150, 250, 400, or 600 mM KCl. Incubation was for 90 min at 3°C , then 60 min at room temperature. Equal samples were applied to 15–32% sucrose gradients made up in the appropriate K, Mg, T buffer. Sedimentation was at 20°C : $2\frac{1}{2}$ h-40K-SB283 (270,000). Under these conditions, membranes sedimented into a pellet; they were not resuspended for analysis. Fractions were collected and processed for scintillation counting. $A_{254\text{ nm}}$ —; [^3H]leucine counts per minute, ●—●.

evaluated in Fig. 2) under the UV profile in that region. The specific activities of “total” ribosomes released ranged from 3.2 (at low KCl) to 5.2 (high KCl), while the specific activities of the “monomer” ribosomes ranged from 3.7 up to 9.9.

While these data indicated that ribosomes released at low KCl were less active than those released at high KCl, they did not allow us to decide if the low specific activity of the former were primarily due to (i) the presence of a large percentage of inactive ribosomes (i.e., bearing no nascent chains), or (ii) the presence of a fairly uniform ribosomal population carrying relatively short nascent chains. Since the extent to which inactive ribosomes are dissociated into subunits is a strong function of the salt concentration, possibility *i* seemed likely. This was tested using salt-released ribosomes which were prepared by exposing pulse-labeled RM to various levels of KCl, removing the membranes by differential centrifugation, and sedimenting the ribosomes from the supernatants. The ribosomal pellets were all resuspended at the same KCl concentration (750 mM) and analyzed on sucrose gradients run at the same high ionic strength. The results (not shown) indicated that a

larger percentage of those ribosomes released from RM at low KCl dissociated into subunits than was the case for those released at high KCl. Parallel gradients of samples treated with puromycin demonstrated that the monomers present in 750 mM KCl were active, i.e., dissociated upon puromycin treatment. It was found that the active monomers released at low KCl had only slightly lower specific activity ([^3H]leucine per A_{254}) than did those released at high KCl. Thus, possibility *i*, above, was favored, but a small contribution of the nature of *ii* could not be excluded.

(f) *The Residual, or Unreleased, Ribosomes*

As was discussed above, total release of ribosomes from RM was only achieved under conditions leading to unfolding of ribosomal subunits. Under the high KCl-puromycin conditions which were considered to be nondestructive, a maximum of ~85% of all membrane-bound ribosomes was released. We have investigated the nature of the residual ribosomes, i.e., those resistant to release. We subjected [^3H]RNA-labeled RM to zone sedimentation in sucrose gradients and found that the

residual labeled RNA banded isopycally coincident with the membrane peak. Using the residual radioactivity in this band as a measure of the extent of nonreleased ribosomes, we found that the release was not increased by (i) addition of an excess of unlabeled free ribosomes to the RM before KCl-puromycin treatment, as a test for nonspecific trapping, (ii) incubation of RM with puromycin at low salt and/or at moderate salt concentrations before bringing the KCl concentration to 750 mM, as a test for rapid loss of activity of a special class of ribosomes due to exposure to high KCl; or (iii) incubation of RM under conditions of amino acid incorporation with puromycin and KCl added in various sequences and at various times during the incorporation, as a test for failure of some ribosomes to react with puromycin because of a particular carboxyterminal aminoacyl residue or because of stoppage of some ribosomes with peptidyl-tRNA in the acceptor, rather than the donor site (43). Attempts to examine the specific activity ($[^3\text{H}]$ leucine counts per minute per milligram RNA) of the residual ribosomes, using pulse-labeled RM, gave inconclusive results because detergent treatment of the stripped RM released aggregated ribosomes whose sedimentation profile was overlapped by the aggregated labeled material released from the membranes.

In confirmation of published reports (32, 33), we found, using sodium dodecyl sulfate (SDS)-sucrose gradients, that essentially all RNA in the RM was ribosomal. SDS gradient analyses of the residual RNA were complicated because the ribosomal RNAs had been cleaved into smaller fragments. However, when equal amounts of RNA were analyzed, all RNA absorbance peaks in the profiles from stripped RM were also present in comparable amounts in total RM similarly treated. Gradient analyses of preparations containing ^3H -labeled RNA demonstrated no differences in specific activities of the various RNA peaks between total RM-RNA and residual RM-RNA.

DISCUSSION

The data presented here demonstrate that it is possible by treatment with puromycin at appropriate ionic conditions nondestructively to disassemble rough microsomes into the component parts, viz., ribosomes and membranes. Incubation of RM with 1 mM puromycin in 0.25 M sucrose, 750 mM KCl, 5 mM MgCl_2 , 50 mM Tris·HCl, pH 7.5, reproducibly results in release of ~85% of all bound ribosomes. The ribosomal subunits released under

the above conditions may be recovered, and when recombined and programmed with polyuridylic acid, they are active in *in vitro* amino acid incorporation. We presume that the partial loss of activity (Results, section *b*), can be avoided by lowering the $\text{K}^+:\text{Mg}^{2+}$ concentration ratio in the media used for separating subunits, since 750/5 is close to the $\text{K}^+:\text{Mg}^{2+}$ value at which large subunits unfold. As defined electron microscopically and by chemical assay, the microsomal membranes are not greatly altered by the separation procedure. Exposure of microsomes to similar ionic conditions by other workers (44) has not been found to markedly reduce characteristic enzyme levels.

Our investigations into the mechanism of the KCl-puromycin release phenomenon are best discussed in terms of a schematic model of ribosome-membrane interaction in rough microsomes (Fig. 15). It is clear that some membrane-bound ribosomes are released from RM simply by raising the KCl concentration, while others are only released by the combined action of puromycin and high KCl. The existence of a class of ribosomes which can only be released by puromycin strongly suggests that the nascent polypeptide chain plays a role in anchoring ribosomes to membranes. The fact, however, that some active ribosomes bearing nascent chains can be released without addition of puromycin indicates that ribosome-membrane interaction is not always guaranteed by the presence of a nascent chain. Our studies with $[^3\text{H}]$ puromycin- and with $[^3\text{H}]$ leucine-labeled RM strongly suggest that bound ribosomes can react extensively with puromycin at low KCl, coupling the aminoacyl-tRNA analogue to hot acid-insoluble material and releasing their nascent chains. The fact that such ribosomes are released only when the KCl concentration is subsequently elevated indicates that the nascent chain is not the only factor maintaining the ribosome-membrane interaction. At low KCl, some other factor or factors are capable of maintaining ribosome-membrane binding, and only when this direct interaction is modified by the presence of high KCl is it possible to identify the nascent chain role in binding.

We conclude, therefore, that ribosomes are attached to microsomal membranes via two types of interactions: a direct one, presumably involving interaction of the large ribosomal subunit with the membrane proper, and an indirect one in which the nascent chain anchors the ribosome to the membrane. The releasing effect of high KCl suggests that the direct interaction is an electrostatic

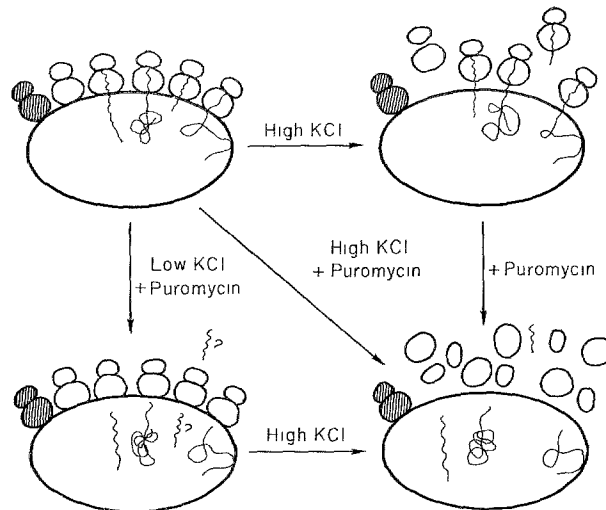


FIGURE 15 Schematic model of ribosome-membrane interaction in rat liver rough microsomes and of the mode of action of puromycin and/or high KCl.

one, but because salt-induced conformational changes in ribosomes or membranes are by no means unreasonable, other bonding types cannot be ruled out. It should be noted that in contrast to the unfolding of the large ribosomal subunit which is more sensitive to the $K^+ : Mg^{2+}$ ratio than to the absolute KCl concentration (Results, section *a*, reference 35, and unpublished data), the direct ribosome-membrane interaction is more sensitive to the KCl level than to the $K^+ : Mg^{2+}$ ratio. Thus, despite the fact that puromycin reaction (and therefore presumably chain release) is complete at low KCl and low Mg, ribosome release is very much less complete in K 100, Mg 1, than in K 500, Mg 5. The conclusion that divalent cations are relatively unimportant in the direct interaction was supported by experiments (not included) which showed that the addition of 5 mM Ca^{2+} or 5 mM Mn^{2+} to RM incubations in K 750, Mg 5 (1 mM puromycin) had no more than the small inhibitory effect on ribosome release which might be predicted from comparison of the data (Fig. 3) on release in 10 mM $MgCl_2$ vs. 5 mM $MgCl_2$. In this context, the fact that EDTA is not effective in releasing all membrane-bound ribosomes (16) is relevant as is our observation (data not included) that 1 mM [ethylenbis(oxyethylenitrilo)]tetraacetic acid (EGTA) did not increase the release observed when RM were incubated in ~ 1 mM puromycin, S 250, Mg 5, T 50 plus 100, 200, or 300 mM KCl. Whatever the exact nature of the direct ribosome-membrane interaction, it is clear that the

bonding force(s) involved is (are) sufficiently weakened by elevated KCl to create a situation in which ribosomes are only attached to the microsomal membrane by their nascent chains. Those ribosomes which are released by treatment with KCl alone consist of a mixture of inactive ribosomes (containing no nascent polypeptide chains and dissociating into subunits if the K^+ and Mg^{2+} concentrations are appropriate) and of ribosomes which are active (as judged by the presence of labeled nascent chain, coupling of [3H]puromycin, and dissociation under appropriate ionic conditions only after puromycin reaction), but which have nascent chains of lower specific activity than the bulk of the tightly bound ribosomes. It is tempting to postulate that this lower specific activity reflects the presence of relatively short chains. We would presume, therefore, that at fairly low KCl (ca. 100–200 mM) most inactive ribosomes and those with very “short” chains are released. As the KCl concentration is raised (in the absence of puromycin), increasing amounts of ribosomes are released; the additional release probably involves some inactive ribosomes, but the major effect would appear to be released of active ribosomes with slightly “longer” chains. At fairly high KCl (but under $K^+ : Mg^{2+}$ conditions such that large subunit integrity is maintained), release plateaus at ca. 40% of the total bound ribosomes. Another 40–50% of the ribosomes can only be released by the action of puromycin; we presume that their nascent chains anchor the ribosomes tightly to the

membrane. Whether the nascent chains protrude through the membrane and serve as an anchor for steric reasons (folding of long polypeptides into partial tertiary structure), or because of interaction with membrane enzymes (such as the disulfide rearranging enzyme [45] or various glycosylating enzymes [46]), remains to be investigated. The facts that release at high KCl alone proceeds in two stages, one fairly rapid, the other rather slow, and that extensive prewashing of RM at high KCl can release some of the ribosomes which, at short time intervals, respond specifically to puromycin, suggest that the distinction between chains which are tightly attached to the membrane and those which can be readily released is not an absolute one, there probably exists a gradation of chain-membrane affinities. This distinction is a convenient first approximation, however, and it is quite clear that while active ribosomes stripped from RM by KCl alone respond to puromycin by discharging their chains into the surrounding medium, ribosomes which can only be released by puromycin discharge their nascent chains vectorially, i.e., in such a way that the nascent chains remain tightly associated with the stripped RM. The nature of the interaction between these polypeptide chains and the stripped membranes is currently under investigation.

Our data do not explain the fact that approximately 15% of the membrane-bound ribosomes can only be released with detergents or by using KCl levels and $K^+ : Mg^{2+}$ ratios which cause complete unfolding of ribosomal subunits. We have not obtained satisfactory data as to whether these residual ribosomes are more or less active than the bulk of the bound ribosomes. It must be emphasized, however, that the bulk of the residual RNA is ribosomal. Although (after orotic acid labeling) the specific activity of the residual RNA may be ca. 10–15% lower than the total RNA (see Results, section *b*), we have no evidence for either a difference in the specific activities of the ribosomal RNAs or for the existence of any large, unlabeled, nonribosomal RNA. Whatever the nature of the resistant ribosomes, it is quite clear that the procedures described here allow the nondestructive release of most of the membrane-bound ribosomes, and that such separation is a necessary prelude to studies of the ribosome-membrane interaction site. We have already achieved partial success in functional reconstruction of the ribosome-membrane interaction and are now engaged in further studies of the interaction site.

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