

VECTORIAL DISCHARGE OF PEPTIDES RELEASED BY PUROMYCIN FROM ATTACHED RIBOSOMES*

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It is well established that upon tissue homogenization the rough endoplasmic reticulum (ER) breaks down into closed, ribosome-bearing vesicles or microsomes,¹ and it has been postulated that secretory proteins, synthesized on ribosomes attached to the ER membrane, are vectorially transferred to the cavities of the endoplasmic reticulum.² Recent work on pancreatic microsomes³ has demonstrated *in vitro* the preferential transfer of a secretory protein, i.e., amylase, from attached ribosomes to the microsomal cavity (the microsomal equivalent of the cisternal space).

The present work shows that unfinished proteins, i.e., peptides and polypeptides, labeled *in vitro* on hepatic attached ribosomes are also preferentially transferred to the interior of the microsomes upon release from the ribosomes by puromycin treatment. The results suggest that from the onset of protein synthesis the growing peptide chain is directed towards the cisternal space into which it diffuses upon its release from the attached ribosome.

Materials and Methods.—*Microsome preparation:* Guinea pig liver microsomes were isolated by differential centrifugation⁴ from a 1:5 homogenate prepared in solution A (0.25 M sucrose, 0.005 M MgCl₂, 0.025 M KCl, 0.050 M Tris-HCl buffer, pH 7.6). After one washing, the microsomes were resuspended in solution A at a concentration of 0.5-gm tissue equivalent of microsomes per 0.4 ml. Sucrose density gradient analysis of such microsomal preparations showed that they contain a small amount of free ribosomes which accounted for approximately 10% of their total ribonucleic acid (RNA).

pH 5 Enzymes: pH 5 Enzymes were prepared by the procedure of von der Decken and Campbell⁵ and resuspended in solution A (~ 0.8-gm tissue equivalent/ml).

Incubation: For the *in vitro* incorporation of amino acids into protein and polypeptides, the incubation mixture contained in 1 ml: 0.4 ml of the microsome suspension (~ 5.0 mg protein), 0.1 ml of the pH 5.0 enzymes (~ 0.7 mg protein), 1 μmole of ATP, 10 μmoles of phosphoenolpyruvate, 50 μg of pyruvate kinase, 0.25 μmole of GTP, 12.5 μmoles of KCl, 2.5 μmoles of MgCl₂, 25 μmoles of Tris-HCl, pH 7.6, and 0.15 μmole each of the 20 amino acids, including L-leucine-1-C¹⁴ (New England Nuclear Corp.) with a specific radioactivity of 23 mc per mmole.

Ten to twenty ml of the incubation mixture were incubated at 37° with constant stirring. The time course of incorporation and distribution of labeled protein was followed in 1-ml aliquots (containing microsomes from 0.5 gm of liver). For each time point, the reaction was stopped by pipetting the aliquots into 10 ml of cold (~ 4°) solution A. The ensuing suspensions were centrifuged at 105,000 × *g* for 30 min to recover the microsomes. The microsomal supernates representing the incubation media were saved for radioactivity measurements and chemical determinations.

Preparation of ribosomes from incubated microsomes: Each pellet of incubated microsomes (derived from 0.5 gm of liver) was resuspended in 4 ml of solution D (0.002 M MgCl₂, 0.050 M KCl, 0.001 M Tris-HCl buffer, pH 7.6) and treated with 0.5 ml of 5.3% sodium deoxycholate (DOC). The volume was then brought up to 10 ml with solution D, and ribosomes were sedimented by centrifugation for 2 hr at 105,000 × *g*. Both the ribosomal pellets and their supernates (DOC-soluble fractions) were saved for chemical assays and radioactivity determinations.

Separation of microsomal membranes: Ribosomes, membranes (*M*), and a soluble fraction (*S*) were isolated from each aliquot of incubated microsomes according to Ernster *et al.*⁶ The procedure (using 0.25% final DOC concentration) was applied to microsomes (0.5-gm tissue equivalent)

lent), recovered by centrifugation from the incubation medium and resuspended in 10 ml of 0.25 *M* sucrose.

Ultrasonic treatment: Pellets of incubated microsomes (0.5-gm tissue equivalent) were resuspended in 5 ml of water and sonicated for 30 sec with cooling in a Branson sonifier (at setting 8) in a plastic tube of the #40 rotor of the Spinco. The tip of the sonifier probe was immersed to the middle of the liquid column. After sonication, 6 ml of water were added to the suspension and the microsomal remnants were sedimented by centrifuging for 2 hr at $105,000 \times g$. Supernates and pellets were kept for chemical assays and radioactivity determinations.

Treatment with EDTA (Disodium ethylenediaminetetra-acetate): One hundred μ moles of EDTA were added to 0.5-gm tissue equivalent of incubated microsomes, recovered from the incubation medium, and resuspended in centrifuge tubes in 4 ml of solution D. After 10 min in the cold, the volume in each tube was brought up to 10 ml with solution E (0.050 *M* KCl, 0.001 *M* Tris-HCl buffer, pH 7.6) and the suspension centrifuged for 30 min at $105,000 \times g$. The ensuing pellet and supernate, which contained microsomes and detached, dissociated ribosomes, respectively, were saved for analysis.

Chemical determinations: The RNA and protein of the different fractions were prepared by Siekevitz' adaptation of Schneider's procedure.⁷ RNA was measured by the orcinol procedure⁸ and protein by the procedure of Lowry *et al.*⁹

Radioactivity measurements: The protein-polypeptide residues were dissolved in 88% formic acid, aliquots of which were plated on aluminum planchets and counted in a gas flow counter.

Results.—Characteristics of the in vitro incubation: During the first 10 min of incubation, C¹⁴-leucine was rapidly incorporated into TCA-precipitable protein and/or polypeptides of the microsomal system (Fig. 1). After 10 min, the rate of incorporation leveled off. At the end of 25 min of incubation, the ribosomes, obtained from incubated microsomes treated with 0.5 per cent DOC, contained ~79 per cent of the total radioactivity incorporated (Table 1), while the microsomal fraction nonsedimentable after treatment with 0.5 per cent DOC contained only 4 per cent of the radioactivity. The remaining 17 per cent was found in the incubation medium. Although the presence of ATP in the medium may be expected to result in the detachment of some ribosomes from microsomes,¹⁰ in most experiments, the distribution of RNA in the fractions showed little change during the course of incubation.

It is assumed that the radioactivity that remains in the incubation medium, after the recovery (sedimentation) of microsomes represents primarily protein newly synthesized by the small amount of free ribosomes in the system. It should be emphasized that the DOC-soluble fraction regularly contained only a very small proportion of the total radioactivity (~4%). This distribution pattern suggests that most of the label has been incorporated into incomplete polypeptides or unfinished proteins which cannot be discharged spontaneously from the attached ribosomes.

Effect of puromycin on in vitro incorporation: Inhibition of protein synthesis by puromycin was not followed by degradation of already made peptides, as indicated by the fact that the total acid-insoluble radioactivity in the system—which can be calculated at each time point in Figures 1–3 by adding the radioactivity of the different fractions—remained approximately constant, or decreased only slightly, after the addition of puromycin.

At this point, washing puromycin-treated microsomes by resuspension and centrifugation failed to produce the release into the medium of nonsedimentable radioactivity above the values released from controls.

The distribution of radioactivity within the microsomes showed, however, striking

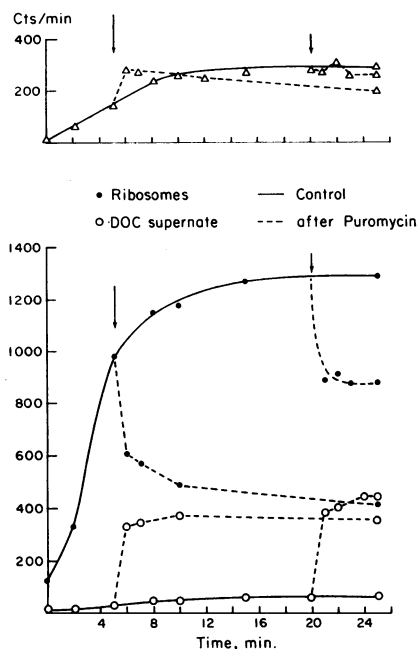


FIG. 1.—The effect of puromycin on the distribution of radioactive peptides within microsomes. Microsomes were incubated for protein synthesis *in vitro* as indicated under *Materials and Methods*. The time course of incorporation of C^{14} -leucine into proteins and polypeptides was followed in controls (—) and after $5 \times 10^{-4} M$ puromycin (---) was added at 5 and at 20 min (arrows). Radioactivity in proteins and/or polypeptides was measured in the ribosomes (●), the DOC-soluble fraction (○), and in the medium of incubation (Δ).

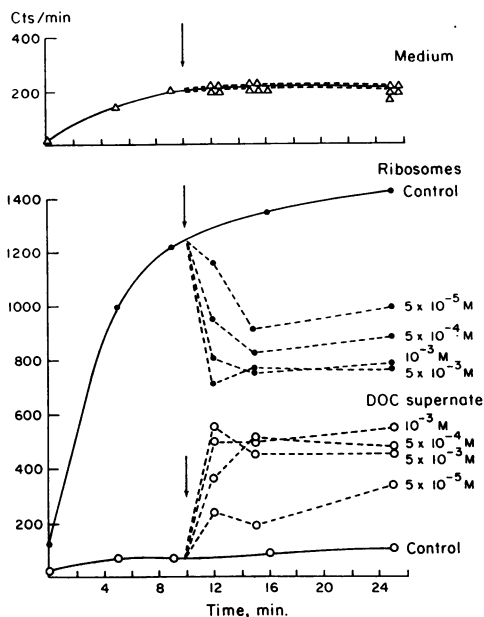


FIG. 2.—Concentration-dependent effect of puromycin on the distribution of C^{14} -leucine-labeled polypeptides within microsomes. Puromycin, at the final concentrations indicated, was added at 10 min (arrows) to microsomes incubated for protein synthesis *in vitro*. Radioactivity in proteins and/or polypeptides in ribosomes (●), the DOC-soluble fraction (○), and the medium of incubation (Δ) was measured in controls (—) and after addition of puromycin (---).

changes after puromycin. There was a rapid decrease in the radioactivity content of the ribosomes accompanied by a proportional increase in the radioactivity of the DOC-soluble fraction (Fig. 1). When puromycin was added after 5 min of incubation, the ribosomes, assayed at the end of 25 min of incubation, were found to contain only 45.7 per cent of the total TCA-precipitable radioactivity in the system (Table 1). When puromycin was added at 20 min (Table 1), the percentage of the total radioactivity of the system recovered in the ribosomes at the end of 25 min of incubation was 56.6 per cent. The effect of puromycin at 20 min occurred at a time when net incorporation had almost come to a halt in the system (Fig. 1).

TABLE 1

	Total RNA (μg)	Total cpm	Microsomes				Medium of Incubation	
			Ribosomes		Soluble in 0.5% DOC		RNA % Cpm, %	
			RNA, %	Cpm, %	RNA, %	Cpm, %	RNA %	Cpm, %
Control	1010	1642	65	78.9	8.6	3.8	26.4	17.3
Puromycin at 5 min	996	911	62.8	45.7	9.0	35.8	28.2	18.5
Puromycin at 20 min	965	1553	64.8	56.6	8.5	27.3	26.7	16.1

All samples were incubated for 25 min. RNA and radioactivity of the proteins or polypeptides in the ribosomes, DOC-soluble fraction, and the incubation medium were determined as described in the text.

The addition of puromycin also resulted in a concurrent increase of the percentage of radioactivity found in the 0.5 per cent DOC-soluble fraction. This fraction, which in the controls contained 3.8 per cent of the total radioactivity, acquired 35.8 per cent and 27.3 per cent thereof after puromycin addition at 5 and 20 min, respectively. It should be emphasized that the proportion of radioactive, acid-insoluble proteins and polypeptides found in the medium of incubation did not change significantly after addition of puromycin (Table 1).

The proportion of radioactive peptides lost by the ribosomes and recovered in the DOC-soluble fraction was, within limits, a function of the puromycin concentration (Fig. 2).

Localization within the microsomes of the peptides released by puromycin: The 0.5 per cent DOC-soluble fraction comprises both the content and the solubilized membrane of the microsomal vesicles. To find out which of these two components acquires the labeled peptides lost by ribosomes upon puromycin treatment, we used the procedure of Ernster *et al.*⁶ The results are presented in Table 2 (method II) and can be compared with those obtained by the conventional fractionation procedure with 0.5 per cent DOC (method I). By method II most of the non-ribosomal radioactivity is found after puromycin in fraction *S*, which is soluble in 0.25 per cent DOC and is assumed to represent primarily the content of the microsomal vesicles. Fraction *M*, which consists of microsomal membranes, and contains microsomal phospholipids and constitutive enzymes,⁶ shows little change in its content of newly synthesized labeled proteins and/or polypeptides after puromycin.

Effect of sonication on in vitro incubated microsomes: To check the findings just mentioned, we tried to find out whether sonication resulted in a greater loss of labeled peptides from puromycin-treated microsomes than from controls. If the peptides released by puromycin are transferred to the microsomal cavities, leakage to the medium would be expected to occur on account of rupture (possibly followed by rehealing) of microsomal vesicles caused by sonication.

Microsomes incubated for 25 min, with puromycin added at 5 min, released 23 per cent of their radioactivity when sonicated for 30 sec, while control microsomes lost only 9 per cent of their label. In both cases approximately 4 per cent of the RNA appeared in the supernatant after sonication. Thus the mild sonication applied was effective, though only partially, in releasing nonribosomal radioactivity from puromycin-treated microsomes.

Effect of streptovitacin A: Similar to puromycin, glutarimide antibiotics, such as

TABLE 2
PUROMYCIN EFFECT UPON THE DISTRIBUTION OF RNA AND PROTEIN AND/OR POLYPEPTIDE AMONG MICROSOMAL SUBFRACTIONS

	Total RNA (μg)	Total cpm	Method I*				Method II†					
			Ribosomes		DOC-Soluble fraction		Ribosomes		M Fraction		S Fraction	
			RNA, %	Cpm, %	RNA, %	Cpm, %	RNA, %	Cpm, %	RNA, %	Cpm, %	RNA, %	Cpm, %
Control	936	829	89.7	93.1	10.3	6.9	76.2	80.0	14.4	14.0	9.4	6.0
5×10^{-4} M puromycin at 5 min	896	677	89.4	66.5	10.6	33.5	76.5	53.5	13.4	13.8	10.1	32.7

Experimental details are described under *Materials and Methods*.

* Method I is fractionation with 0.5% DOC.

† Method II is fractionation according to Ernster *et al.* [*J. Cell Biol.*, 15, 541 (1962)].

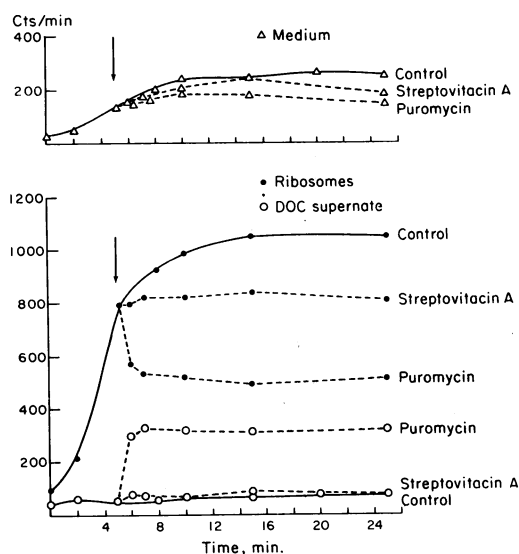


FIG. 3.—The distribution of C^{14} -leucine-labeled proteins and/or polypeptides after the inhibition of protein synthesis *in vitro* by puromycin ($10^{-4} M$) or streptovitacin A ($1.6 \times 10^{-4} M$). The inhibitors were added after 5 min of incubation. Radioactivity in ribosomes (●), in the DOC-soluble fraction (○), and in the medium of incubation (Δ) was measured in controls (—) and after the addition of puromycin or streptovitacin A (---).

protein and/or polypeptides. These results may suggest that the growing polypeptide chain contributes to the strong attachment of active ribosomes possibly by virtue of its being partially engaged in the membrane across which it will be eventually transferred. To check this possibility, microsomes incubated in the presence of puromycin were subsequently treated with EDTA.

To this intent, 0.5 gm tissue equivalent of microsomes was incubated for 25 min at 37° in the incorporation system (in the absence of puromycin), recovered by centrifugation, and subsequently treated with 100 μ moles of EDTA. The specific activity (Table 3) expressed in terms of cpm/mg RNA, of the material bound to membranes increased from 1828 to 2653 after EDTA treatment. The results suggest that those large ribosomal subunits which are less sensitive to de-

streptovitacin A, inhibit protein synthesis by acting at the level of the amino acid transfer reaction.¹¹ At variance with puromycin, however, these antibiotics fail to release the growing polypeptide from the ribosomes.¹² Figure 3 shows that streptovitacin A ($1.6 \times 10^{-4} M$) added after 5 min of incubation stopped protein synthesis in the microsomal system but produced no redistribution of label within the microsomes during the subsequent 20 min of incubation.

It has recently been shown⁴ that: (a) ribosomes are attached to the ER membrane by their large 47S subunit; (b) this subunit bears the newly synthesized polypeptide chain; (c) EDTA treatment of microsomes results in the detachment of all small subunits and ~ 50 per cent of the large subunits; (d) the subunits resistant to detachment by EDTA contain most of the newly synthesized

TABLE 3

EFFECT OF EDTA TREATMENT ON MICROSOMES AFTER *in vitro* INCORPORATION OF L-LEUCINE-1- C^{14} IN THE PRESENCE OR ABSENCE OF PUROMYCIN

	Control Microsomes			EDTA-Treated Microsomes		
	Total RNA (μ g)	Total cpm	Sp. act., cpm/mg RNA	RNA, %*	Cpm, %*	Sp. act., cpm/mg RNA
No puromycin	743	1358	1828	40	58	2653
$5 \times 10^{-4} M$ puromycin at 5 min	715	896	1253	41	90	2750
$5 \times 10^{-4} M$ puromycin at 20 min	717	1303	1817	40	70	3177

The incubation procedure, treatment of microsomes with EDTA, and the determination of RNA and radioactivity of the proteins and/or polypeptides are described in the text. All samples were incubated for 25 min.

* Amounts (expressed as percentage of controls) sedimented by centrifuging for 30 min at $105,000 \times g$ EDTA-treated microsomes.

tachment by EDTA have either a higher content of newly made polypeptides, or a higher capacity for protein synthesis *in vitro*, than large subunits less resistant to EDTA treatment.

Table 3 also shows that the proportion of RNA left on microsomes after addition of EDTA was not altered by a previous treatment with puromycin.

Since at the concentration of puromycin used ($5 \times 10^{-4} M$) the ribosomes attached to the microsomal vesicles lose a fraction of peptides which contains ~ 30 per cent of the total radioactivity (Figs. 1 and 2, Tables 1 and 2), the present experiments indicate that the release of these peptides does not affect the amount of RNA, i.e., proportion of ribosomes detached by subsequent EDTA treatment (Table 3). Hence, it can be concluded that the presence of the newly synthesized peptide is not the only factor responsible for the strong attachment of active large subunits to membranes.

The data in Table 3 also show that the fraction of radioactivity contained in the particles detached by EDTA was smaller when EDTA was applied after incubation with puromycin. While control microsomes lost 42 per cent of the radioactivity upon EDTA treatment, microsomes to which puromycin was added at 20 min of incubation lost 30 per cent, and those which received puromycin at 5 min lost only 10 per cent. This finding can be explained by the releasing effect of puromycin upon nascent peptides still bound to ribosomes; in this case, ribosomes detached by EDTA are expected to depart with less radioactivity because they have already partially discharged their radioactive peptides into the cisternal space during the previous treatment with puromycin.

Discussion.—Control experiments showed that almost no release of newly synthesized peptides from ribosomes occurs spontaneously in the *in vitro* system of guinea pig liver microsomes. This was true even when incubation was prolonged for 25 min, that is, until net incorporation almost stopped. Moreover, inhibition of protein synthesis *per se*, as caused by streptovitamin A (Fig. 3) addition during the period of most active incorporation (the first 10 min of incubation), failed to promote the release of peptides from attached ribosomes. Available evidence indicated that the release of peptides from ribosomes is related¹³—but not exclusively—to the release of terminal sRNA molecules from the ribosome-bound peptidyl-sRNA complex.^{14, 15} Experimentally, the release from sRNA and from ribosomes can be specifically accomplished by puromycin, which interferes with the amino acid transfer reaction by which polypeptides grow on ribosomes.^{16, 17} The peptidyl-puromycin molecules thus formed^{18, 19} are not firmly bound to the ribosomes, as are most of the peptidyl-sRNA molecules, and hence are released to the surrounding medium.²⁰

Quantitatively, the releasing effect of puromycin on liver microsomes is comparable to the one on bacterial¹³ and reticulocyte ribosomal systems:²¹ the peptides released by puromycin contain from 30 to 50 per cent of the total incorporated radioactivity.

Other aspects of the release process, however, are different. So far, our results bring forward two distinctive features of the microsomal system treated with puromycin: (a) The peptides lost by the ribosomes are not discharged into the medium of incubation, but are retained in the microsomes (Figs. 1–3, Table 1). (b) Within the microsomes, the puromycin-released peptides are transferred to the content

of the microsomal vesicles, which is the equivalent of the cisternal space in the intact cell. This latter effect was inferred mainly from the distribution of radioactivity in the microsomal subfractions obtained by applying the procedure of Ernster *et al.*⁶ (Table 2). Supporting evidence for transfer of the content of microsomal vesicles was obtained from the effect of sonication on puromycin-treated microsomes.

From these findings, and from what we know of the cycle of protein secretion in animal cells,^{3, 22} it appears that peptides released by puromycin and naturally released secretory proteins share a common origin in the attached ribosomes and a common fate: transfer to the intracisternal space. Such similarities suggest that the same mechanism accounts for their passage through microsomal (ER) membranes. This appears to be an indiscriminating mechanism, capable of effecting the transfer of finished proteins as well as of incomplete peptides or peptidyl-puromycin molecules. In fact, the discharge of peptides toward the cisternal space appears to be an immediate and direct consequence of their release from the ribosomes. Thus (a) transfer to the DOC-soluble fraction was practically completed during the first 2 min after addition of puromycin (Figs. 1 and 2); (b) no significant leakage of peptides to the surrounding medium of incubation was detected, nor evidences for capture of peptides in the fraction of microsomal membranes were found (Table 2). As a result, the efficiency of transfer to the cisternal space was at all times very high. Small radioactive peptides are expected to be lost during extraction by the Schneider procedure because of their acid solubility. Nonetheless, ~70–80 per cent of the radioactivity released from ribosomes was recovered in an acid-insoluble form in the DOC-soluble fraction.

On the basis of studies on the dissociation of attached ribosomes treated with EDTA,⁴ and of correlated electron microscopical observations, a model for the arrangement of ribosomes on microsomal membranes can be proposed to account for several of the observations just discussed. This model²³ provides for a central channel or space within the large subunit, separated from the cytoplasmic matrix by the 30S subunit, but continuous with the cisternal space through a permanent or intermittent discontinuity in the microsomal (ER) membrane to which the 47S subunit is attached. In the model, the vectorial (unidirectional) character of polypeptide release is explained by structural restrictions. The peptides being synthesized are assumed to grow within the central channel of the large ribosomal subunit (47S) in an environment which is (or can be made) continuous with the cisternal space through a discontinuity in the ER membrane. As visualized at present, the transfer mechanism relies primarily on release from the large subunit and on structural restrictions at the ribosome-membrane junction, and hence, it is nondiscriminatory and possibly passive.

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- ²³ Unpublished electron microscopic observations by D. Sabatini, Y. Tashiro, and G. E. Palade are the basis of this model. The existence of a channel is suggested by electron microscopy of negatively stained large subunits. Discontinuities in the membrane under the large subunits can be detected in some instances in sectioned specimens.