DIFFERENCE IN VECTORIAL RELEASE OF NASCENT PROTEIN FROM MEMBRANE-BOUND RIBOSOMES OF SECRETORY AND NON-SECRETORY TISSUES

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The association of polyribosomes and membranes of the endoplasmic reticulum is an important factor in the control of protein biosynthesis (Campbell, 1965) and the distribution within the cell and secretion of newly synthesized protein (Palade, 1966).

In protein secreting cells, such as the pancreatic exocrine cell, Jamieson and Palade (1966; 1967a & b) have shown that the space enclosed by endoplasmic reticulum membranes acts as a channel for transport and storage of secretory protein before its release across the plasma membrane.

The vectorial discharge of nascent protein from ribosomes attached to the outside of isolated microsomal vesicles, through the membrane to the microsomal lumen has been demonstrated *in vitro* in pigeon pancreas (Redman, Siekevitz & Palade, 1966), guinea pig liver (Redman & Sabatini, 1966) and rat liver (Redman, 1967). The transfer of nascent protein from membrane-bound polysomes into the lumen was studied using the partial discharge of radioactively labelled protein by puromycin, and the subsequent disruption of microsomal membranes with detergents.

In these tissues however, most of the protein synthesized (pancreatic enzymes, serum albumin, etc.) is for export from the cell, so it was of interest to consider the role of endoplasmic reticulum when this is not the case. We therefore compared the puromycin induced vectorial release of nascent protein labelled both *in vivo* and *in vitro*, from microsomes of rat liver, a protein secreting tissue, and rat brain, predominantly non-secreting.

Preliminary work in this laboratory on microsomal subfractions from rat liver showed that the incorporation of radioactive amino acids *in vivo* by membrane-bound polysomes was very much greater than that of free polysomes prepared without the use of detergents (method of Tata & Williams-Ashman, 1967). This difference however was much smaller when the same fractions were made to incorporate amino acids *in vitro*. It was possible

that the process of isolation could alter the membrane-ribosome relationship to impair both the protein synthesizing capacity of microsomes and the mechanism for releasing nascent protein chains. For this reason the vectorial discharge of radioactive nascent protein was studied using both microsomes labelled *in vitro*, and microsomes prepared after labelling *in vivo*.

Membrane-bound ribosomes are an important site of protein biosynthesis in brain as in liver, but it will be shown that in brain virtually none of the nascent protein released from these ribosomes crosses the membrane to the interior of the microsomal vesicles. This result is compatible with the difference in secretory activity of the two tissues, and the role of endoplasmic reticulum in the intacellular transport of protein.

MATERIALS AND METHODS

Liver Fractionation: Membrane-bound and free ribosomes from livers of male Sprague-Dawley rats weighing 120-150 g. were prepared without the use of detergents according to the method of Tata & Williams-Ashman (1967). Two to four livers were pooled and homogenized in 3.5 volumes of 0.35M sucrose medium A (KCl 25mM, $MgCl_2$ 10mM, Tris pH 7.6 50mM) and a "rough membrane" preparation fractionated overnight on a discontinuous gradient of 1.5M and 2M sucrose medium A. The resultant "heavy rough membrane" fraction* and "pellet" were uzed as membrane-bound and free ribosomes respectively. After recovery from the discontinuous gradient the membrane-bound ribosomes were diluted in medium A and sedimented at 105,000 g. for 2 hrs. before being resuspended (as were the ribosomes) in 0.35M sucrose medium A.

Brain Fractionation: Cerebral hemispheres from 5-12 rats (weighing 120-150 g. for *in vitro* and 30-50 g. for *in vivo* incorporation experiments) were homogenized in 3.5 volumes of ice-cold 0.25M sucrose medium B (KCl 100mM, $MgCl_2$ 10mM, Tris pH 7.4 50mM). The mitochondria-free supernatant, obtained after twice centrifuging the homogenate at 12,000 g. for 10 min. was then layered over 6.5 ml. of 0.8M sucrose in medium B and centrifuged at 105,000 g. for 2 hours. The pelleting material was resuspended in 10 ml. of 0.25M sucrose medium B per 10 g. tissue equivalent using an all-glass homogenizer, and the suspension layered over a discontinuous sucrose gradient consisting of 9 ml. of 2M sucrose B, and 11 ml. of 1.0M sucrose B. This was centrifuged for 16 hrs. at a mean 60,000 g. in a swing out rotor (Spinco SW 25) to produce a pellet of free ribosomes and material at the 1.0-2M interface

* For the sake of convenience, the "heavy rough membrane" fraction will be referred to as membrane-bound ribosomes (or microsomes) and the "pellet" as free ribosomes.

which upon electron microscopy was found to consist predominantly of ribosomes attached to membranous vesicles (microsomes). These fractions were collected as described for liver and resuspended in 0.25M sucrose medium B. For labelling in vitro, all preparations were used for incubation within 1 hour of final resuspension.

Cell Sap was separately prepared from brain and liver by homogenizing the tissue in 3.5 volumes of medium B or A, respectively, and centrifuging the mitochondria free supernatant for 4 hours at 105,000 x g. The supernatant was then passed through a Sephadex G25 column, recentrifuged at 105,000 x g. for 2 hours and diluted to a concentration of 3 mg. protein/ml.

Protein was measured by Lowry's method, and RNA by a slight modification of the method of Fleck & Munro (1962).

Amino acid incorporation into protein in vivo.

Liver: Two rats were each given 5 μc. of ^{14}C-amino acid mixture (54 mC/mAtom C) intraperitoneally 10 minutes before death.

Brain: Six to ten rats weighing 30-50 g. were each given 4 μc. ^{14}C-amino acid mixture in 0.01 ml. water intracisternally without anaesthetic 30 min. before death and their brains pooled before processing.

Amino acid incorporation in vitro.

Eight tubes containing 1 ml. of a suspension of free or membrane-bound ribosomes were serially incubated in the amino acid incorporation medium described by Tata & Williams-Ashman (1967), together with 3 μC of ^{14}C-leucine (311 mC/mM). With brain preparations the concentration of KCl was raised to 100 μmoles per ml. of suspension. After 8 min. incubation at 37°C puromycin to a final concentration of 10^{-3}M was added to 4 tubes and an equal volume (0.05 ml.) of water to the rest. After 16 min. incubation, sodium deoxycholate (DOC) was added to a final concentration of 0.5°/o W/V to half of the puromycin and control groups, water (0.05 ml.) to the rest and, after mixing for 10 sec. 4 ml. of ice-cold medium was added to each tube to stop further incorporation. The resulting suspensions were centrifuged at 105,000 g. for 2 hours to sediment all particulate microsomal or ribosomal material. The protein in the supernatant and pellets (suspended in 1 ml. of medium A or B) was precipitated with 5°/o trichloracetic acid (TCA). The precipitates were washed once with 5°/o TCA at 75-80°C for 20 min. once with cold 5°/o TCA and twice with 2 ml. ethanol 3:1 ether, before being finally suspended in 0.5 ml. 5°/o TCA at 80-90°C, and dissolved in 10 ml. dioxane scintillation fluid for ^{14}C measurement in a Packard Tricarb liquid scintillation spectrometer.

RESULTS AND DISCUSSION

Incorporation in vitro: Fig. 1 shows the percentage of incorporated amino acid recovered in the particulate fraction when membrane-bound or free

ribosomes were sedimented after 16 min. incubation (column 1 of each histogram). The action of puromycin in releasing a large proportion of nascent protein from free ribosomes to the supernatant fraction is clearly seen in columns 3 and 4 both for liver (Fig. 1c) and brain (Fig. 1d). Of the labelled protein remaining attached to untreated free ribosomes puromycin released 64°/o from brain and 35°/o from liver ribosomes (Table 1). DOC is shown

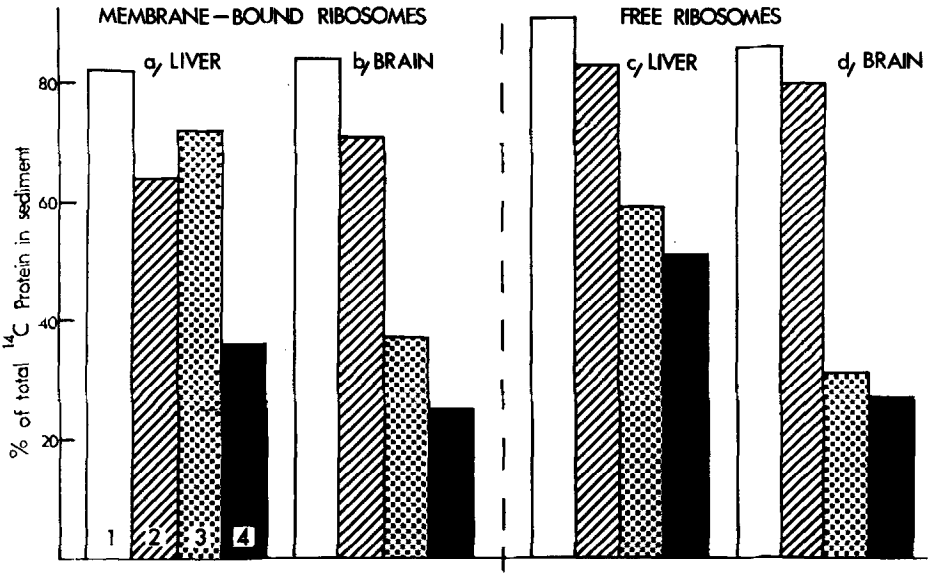

Fig. 1. Effect of puromycin and deoxycholate on the release from membrane-bound and free ribosomes of nascent protein labelled in vitro.

The release is expressed as the percentage of total ^{14}C-protein appearing in the fraction sedimented after incubation of 1 ml. of a suspension containing cell sap 0.6 mg. protein, and membrane-bound or free ribosomes 0.5-1 mg. RNA for liver preparations and 0.1-0.5 mg. RNA for brain. Other components of the cell free systems were as mentioned in the text. Each histogram shows 1. Untreated control. 2. With DOC 0.5 °/o W/V. 3. With 10^{-3}M puromycin. 4. Puromycin followed by DOC. Each value is the mean of 2-10 experiments with maximum variation $\pm$<6 °/o. The range of ^{14}C recovered as protein was 2,000-19,000 cpm/mg. RNA and all incorporations as actually measured were greater than 200 cpm.

to release little or none of this ^{14}C-protein from free ribosomes.

In contrast, DOC alone released 24 °/o of the radioactive protein sedimenting with control membrane-bound ribosomes from liver (Table 1), which is presumably that fraction of newly synthesized protein chains lying free within the microsomal lumen and unattached to ribosomes. Puromycin alone released only 13 °/o of the labelled protein to the supernatant, but combined treatment with puromycin followed by DOC released 55 °/o. This is 18 °/o more than the sum of individual DOC and puromycin effects (Table 1), suggesting

that the effect of puromycin on liver microsomes was to discharge 13 °/o of nascent protein to the surrounding medium and 18 °/o across the membrane into the microsomal lumen.

With brain however, the addition of DOC alone released only 15°/o of the ^{14}C-protein sedimenting with control membrane-bound ribosomes, whereas puromycin alone released 56 °/o (Table 1). The addition of puromycin followed by DOC released 70 °/o of the ^{14}C-protein to the supernatant, suggesting that little or none of the nascent protein discharged by puromycin was transferred to the interior of brain microsomal vesicles.

TABLE 1

RELEASE OF ^{14}C-PROTEIN FROM FREE AND MEMBRANE-BOUND RIBOSOMES BY PUROMYCIN AND DOC*

		No. of expts.	Particulate ^{14}C-protein (°/o of untreated control) released by:- DOC (a)	PURO (b)	(a+b)	DOC after PURO (c)	c-(a+b)†
^{14}C-incorporation in vitro							
Membrane-bound ribosomes	Liver	10	24	13	37	55	18
	Brain	8	15	56	71	70	0
Free ribosomes	Liver	2	9	35	44	44	0
	Brain	8	7	64	71	69	0
^{14}C-incorporation in vivo							
Membrane-bound ribosomes	Liver	6	70	0	70	76	6
	Brain	6	23	38	61	59	0
Free ribosomes	Liver	4	0	70	70	70	0
	Brain	4	11	41	52	60	8

* The range of incorporation achieved is given in the legends to Fig. 1 (in vitro) and Fig. 2 (in vivo).

† Representing ^{14}C-protein released by puromycin but appearing in the supernatant only after DOC.

Incorporation in vivo. Essentially similar differences in the pattern of release of nascent protein from membrane-bound ribosomes of brain and liver were observed when radioactive amino acids were incorporated in vivo (Fig. 2). With liver microsomes the effect of DOC alone was more marked, 70°/o of the ^{14}C-protein sedimenting with the control microsomes being released (Table 1). Puromycin alone had no effect, but puromycin followed by DOC released 76°/o into the supernatant. This suggests that puromycin discharges about 6°/o of nascent protein to the microsomal lumen under these conditions.

From brain microsomes DOC alone released 23°/o of the nascent protein

of untreated microsomes, and puromycin alone 38°/o. On addition of puromycin followed by DOC the release of 59°/o suggests that no labelled protein chains were discharged to the lumen of brain microsomes by puromycin.

In all these studies with labelled microsomes and free ribosomes it was established that in the absence of closed membranous vesicles nascent protein released by puromycin appeared in the supernatant fraction, and that DOC itself, at the concentration used, had little or no effect on the bond between ribosome and nascent chain.

It could be argued that puromycin-induced vectorial discharge is an abnormal situation unrelated to the mechanism of release by natural chain termination. However, in prolonged in vitro incubations (10-60 min.) we

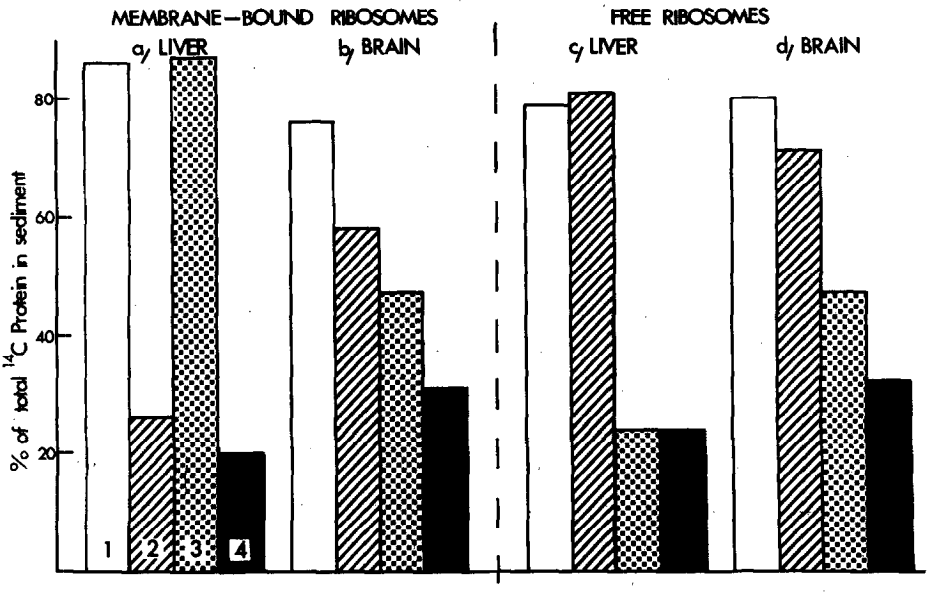

Fig. 2: Effect of puromycin and deoxycholate in vitro on the release from membrane-bound and free ribosomes of nascent protein labelled in vivo.

Incubation procedure as described in the text and conditions as in Fig.1 but omitting ^{14}C-leucine. Each histogram shows 1. Untreated control. 2. With DOC 0.5 °/o W/V. 3. With 10^{-3}M puromycin. 4. Puromycin followed by DOC. Each value is the mean of 4 to 6 experiments with maximum variation ±≤5 °/o. The range of ^{14}C recovered as protein was 200-12,000 cpm/mg.RNA.

observed (Fig. 3) a time-dependent release of nascent chains from brain microsomes to the supernatant, albeit to a much smaller extent than the puromycin-induced discharge. Prolonged incubation of liver microsomes

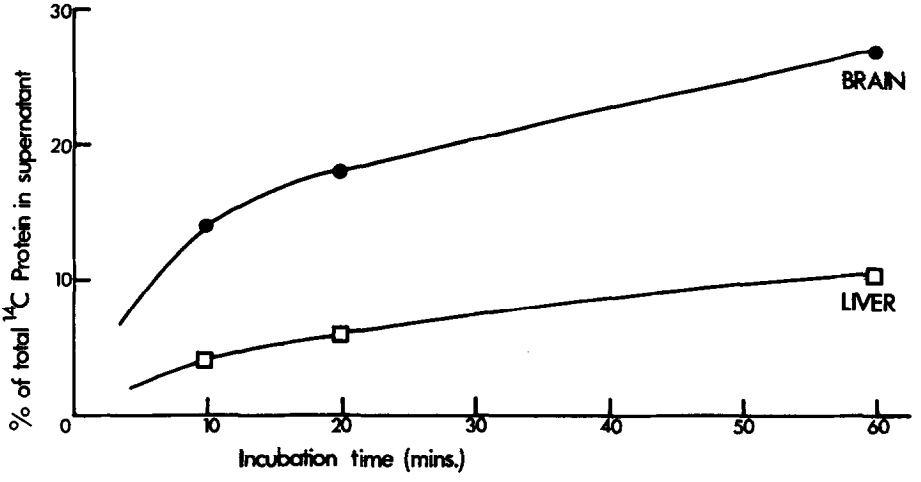

Fig. 3: Time-dependent vectorial discharge of nascent protein labelled in vitro.

Mean of 2 experiments. Membrane-bound ribosomes from brain and liver were incubated as mentioned in Fig. 1.

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on the other hand showed almost no release of chains to the supernatant. When free ribosomes were incubated under similar conditions approximately 25 % of the total ^{14}C-protein appeared in the supernatant phase at 60 min. with both brain and liver preparations. Thus, the addition of puromycin merely exaggerates the normal pattern of vectorial discharge. In conclusion, what seems to be most significant in these experiments is that nascent protein is released in different ways from ribosomes attached to endoplasmic reticulum membranes of liver and brain, the absence of any discharge of newly-formed protein into the microsomal vesicles from the latter tissue being compatible with its relative lack of secretory function.

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