Protein Synthesis by Membrane-Bound and Free Ribosomes of Secretory and Non-Secretory Tissues

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1. Methods for the separation of membrane-bound and free ribosomes from rat brain (cortex) and skeletal muscle were described and the preparations characterized by chemical analysis and electron microscopy. The attachment of ribosomes to membranes is not an artifact of the separation procedure. 2. The rate of incorporation of L-[¹⁴C]leucine into protein *in vitro* by the membrane-bound and free ribosomes from these two predominantly non-protein-secreting tissues is compared with that by similar preparations from rat liver. With all three tissues the initial rate was higher for the membrane-bound preparations. 3. By using the technique of discharging nascent polypeptide chains by incubation with puromycin followed by treatment with sodium deoxycholate (Redman & Sabatini, 1966), a major difference was observed for the vectorial discharge of nascent protein synthesized both *in vivo* and *in vitro* on membrane-bound ribosomes from liver, on the one hand, and brain and muscle, on the other. Whereas a large part of nascent protein synthesized on membrane-bound liver ribosomes was discharged into the membranous vesicles (presumably destined for export from the cell), almost all nascent protein from membrane-bound ribosomes from brain and muscle was released directly into the supernatant. Incorporation of [³H]puromycin into peptidyl-[³H]puromycin confirmed these findings. There was thus no difference between membrane-bound and free ribosomes from brain on the one hand, and from free polyribosomes from liver on the other, as far as the vectorial release of newly synthesized protein was concerned. 4. Incubation with puromycin also showed that the nascent chains, pre-formed *in vivo* and *in vitro*, are not involved in the attachment of ribosomes to membranes of the endoplasmic reticulum. 5. The differences in vectorial discharge from membrane-bound ribosomes from liver as compared with brain and muscle are not due to the different types of messenger RNA in the different tissues. Polyphenylalanine synthesized on incubation with polyuridylic acid was handled in the same way as polypeptides synthesized with endogenous messenger. 6. It is concluded that there is a major difference in the attachment of ribosomes to the membranes of the endoplasmic reticulum of secretory and non-secretory tissues, which results in a tissue-specific difference in the vectorial discharge of nascent proteins.

In almost all animal and plant cells, a certain proportion of ribosomes are attached to cellular membranes or other structural elements, the proportion varying greatly from one type of cell to another (Fawcett, 1966; Hendler, 1968). Until now the major role assigned to this attachment is in transport of proteins for export, and indeed it is generally accepted that all proteins for secretion are synthesized exclusively on membrane-bound ribosomes or the rough endoplasmic reticulum (Palade,

1966; Campbell, 1965; Redman, Siekevitz & Palade, 1966; Redman & Sabatini, 1966; Redman, 1967, 1969; Takagi & Ogata, 1968). It is, however, virtually impossible for these studies to ascribe another role for the ribosome membrane attachment, since they have been exclusively concerned with the vectorial discharge of proteins synthesized on rough endoplasmic reticulum in predominantly protein-secreting tissues.

That there may be some other function, besides secretion, underlying the association between membranes and ribosomes is suggested by the marked proliferation of rough endoplasmic reticulum during growth and development (Tata, 1967,

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1968a,b, 1970a,b; Siekevitz, Palade, Dallner, Ohad & Omura, 1967; Ursprung & Schabtach, 1968; Pollak & Ward, 1967; Priestly, Pruyne & Malt, 1969). In both secretory and non-secretory cells, the proliferation of membrane-bound ribosomes during rapid growth and development is accompanied by the preferential synthesis of intracellular proteins. Tata (1967, 1968a,b, 1970b) has suggested that another role for ribosome-membrane attachment may be to achieve a topographical segregation of different groups of ribosomes synthesizing different classes of proteins. As a pre-requisite to testing such an idea it seemed to us essential to compare the activity of membrane-bound and free ribosomes of secretory and non-secretory tissues.

In this paper we describe methods for the isolation of free and membrane-bound ribosomes from two predominantly non-secretory tissues of the rat, cerebral cortex and skeletal muscle. Although microsomes and free polyribosomes have been prepared from these two tissues (Zomzely, Roberts & Rappoport, 1964; Adams & Lim, 1966; Dunn, 1970; Breuer, Davies & Florini, 1964; Rampersad, Zak, Rabinowitz, Wool & DeSalle, 1965; Heywood, Dowben & Rich, 1968; Chen & Young, 1968; Bullock, White & Worthington, 1968), there is no study to date which separates and compares the behaviour of membrane-bound and free polyribosomes prepared without the use of detergents. We have compared the protein-synthesizing activity of the bound and free preparations obtained from these tissues with those from liver, for which several preparatory methods have been described (Tata & Williams-Ashman, 1967; Blobel & Potter, 1967; Bloemendal, Bont, De Vries & Benedetti, 1967; Tata, 1969). In doing this we laid particular emphasis on the vectorial discharge of nascent protein from membrane-bound ribosomes from liver with those obtained from the two non-secretory tissues. This was based on the method first described by Redman & Sabatini (1966) for rat liver by which they showed that much of the newly formed protein, synthesized *in vivo* or *in vitro*, could be discharged by puromycin from membrane-bound ribosomes into the lumen of the microsomal membrane. This fraction of the protein, which is recovered by solubilization with a detergent after puromycin incubation, is thought to be that destined for secretion. It will be shown below that both brain (cerebral cortex) and muscle contain small amounts of membrane-bound ribosomes, which differ in a major way from the membrane-bound ribosomes from liver in their modes of discharge of newly synthesized polypeptide chains. A preliminary account of the differences in vectorial discharge of nascent proteins from membrane-bound ribosomes of brain and liver has been published (Andrews & Tata, 1968).

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (bred at the National Institute for Medical Research, Mill Hill), weighing 120–150 g and fed *ad libitum*, were killed by decapitation. For labelling liver and muscle RNA and protein *in vivo* radioactive precursors were injected intraperitoneally in about 0.1 ml of 0.15 M-NaCl, whereas brain constituents were labelled by intracisternal injections of 0.15 M-NaCl (0.01–0.05 ml) by using a Hamilton micro-syringe.

Subcellular fractionation. Although numerous procedures have been described for preparing microsomes, sub-microsomal fractions and ribosomes (Dallner & Ernster, 1968; Tata, 1969), no standard method was available for the preparation of membrane-bound and free polyribosomes from liver, brain and muscle for the purposes of comparing their protein-synthesizing activity. The procedures described below in which no detergents are used are considered to be important in the final evaluation of the results.

(a) Liver. The microsomal fractionation to yield light- and heavy- rough membranes and free polyribosomes was carried out through a discontinuous sucrose gradient as described earlier (Tata & Williams-Ashman, 1967; Tata, 1969) after homogenizing the tissue in medium A (0.35 M-sucrose, 0.025 M-KCl, 0.01 M-MgCl₂, 0.05 M-tris-HCl buffer, pH 7.6). The membrane fractions at the interface and the free polyribosomal pellet were gently suspended in 20 ml of medium A and sedimented at 105 000g for 1.5 h. Each pellet was suspended with three strokes in a small loose-fitting homogenizer and used within 1 h after storage at 0°C.

(b) Brain. Because of the greater morphological complexity of brain, both at the whole tissue and subcellular levels, only the cerebral cortex was studied. Several different homogenization media have been described earlier for brain microsomes and ribosomes (Zomzely *et al.* 1964; Campbell, Mahler, Moore & Tewari, 1966; Adams & Lim, 1966). Although Mahler & Brown (1968) and Merits, Cain, Rdzok & Minard (1969) have recently described detergent-free fractionation procedures, there was none described at the time we began these studies.

Cortical shells, pooled from four to eight rats, were collected in ice-chilled 'medium B' (0.25 M-sucrose, 0.1 M-KCl, 0.012 M-MgCl₂ and 0.05 M-tris-HCl buffer, pH 7.4). They were finely chopped and washed twice in 20 ml of medium B before homogenization in 3.5 vol. of medium B with three strokes of a Teflon-glass homogenizer (clearance 0.15 mm) at 800 rev./min. More than five strokes of the pestle decreased the amino acid incorporation activity of the final preparations. A mitochondria-free supernatant was obtained by centrifuging the homogenate at 10 000g for 10 min and repeating the centrifugation on the first supernatant. Removal of mitochondria at higher centrifugal forces (Zomzely *et al.* 1964) carries the risk of the membrane-bound ribosomal fraction being lost with the mitochondrial pellet. Whole microsomes were obtained from the second mitochondria-free supernatant by a 2 h centrifugation at 105 000g.

For submicrosomal fractionation, 14.0 ml of mitochondria-free supernatant was layered over 6.5 ml of 0.8 M-sucrose, containing the other components of medium A (see below) and centrifuged at 105 000g for 2 h in an MSE

50 ultracentrifuge. After the interface layer and the supernatant had been discarded, the walls of the tubes were carefully wiped with tissue paper and the pellets gently suspended in 10 ml of medium B. The suspension was layered over a discontinuous sucrose gradient consisting of 9 ml of 2.0M-sucrose and 11 ml of 1.0M-sucrose, with KCl, $MgCl_2$ and tris-HCl as in medium B, in Spinco or MSE SW 25 rotor tubes. The samples were then centrifuged to equilibrium for 16 h at 60000g at 0°C. Three submicrosomal fractions were thus obtained: a hazy layer just below the 0.25/1.0M-sucrose interface, a dense layer around the 1.0/2.0M-sucrose interface, which contained most of the membrane-bound RNA, and a pellet of free ribosomes. The interface fractions were aspirated and along with the pellet were suspended in medium B, to a total volume of 20 ml, pelleted at 105000g for 1.5 h and resuspended in 2–3 ml of medium B. These three fractions were termed 'light rough membranes', 'heavy rough membranes' and 'free polyribosomes' respectively, without, however, implying morphological identity with similar fractions obtained from liver. For amino acid-incorporation studies only the heavy rough membrane fractions, to be called membrane-bound ribosomes, and free polyribosomes were used.

(c) Muscle. Earlier methods (Breuer *et al.* 1964; Rampersad *et al.* 1965) based on homogenization of skeletal muscle in media of low ionic strength, give very low yields of muscle ribosomes because of their co-precipitation with myosin (Heywood *et al.* 1968). As the larger polyribosomes (both free and membrane-bound) are preferentially lost with the myofibrillar fraction, the final ribosomal fraction is unrepresentative of the general population of muscle polyribosomes. Chen & Young (1968) reported higher yields of polyribosomes (300–400 μ g of RNA/g of muscle) by homogenizing muscle in the medium of high ionic strength (0.25M-KCl) described by Heywood *et al.* (1968) and then sedimenting the polyribosomes by lowering the concentration of KCl to 0.06M. However, after preliminary trials, the ionic composition of media described by Earl & Morgan (1968), for obtaining detergent-treated polyribosomes from cardiac and skeletal muscle, was found to be more suitable.

Batches (50–100 g) of muscle from hind limbs were finely chopped with scissors and homogenized in 5 vol. of medium C (0.1M-KCl, 0.01M- $MgCl_2$ and 0.05M-tris-HCl buffer, pH 7.6) with an Ultra-Turrax disintegrator (operated at 80 V for a 240 V rating) for two periods of 15 s. The homogenate was centrifuged at 40000g for 30 min in an MSE 8 $\times$ 50 ml angle-head rotor. This resulted in the loss of a substantial amount of ribosomal material in the sediment, a step, however, deemed necessary for the eventual purity of microsomal fractions. A crude microsomal pellet was prepared from the supernatant by centrifugation at 105000g for 1.5 h. A suspension of the pellet in 50 ml of medium C was layered directly over 2.0M-sucrose in medium C and centrifuged at 60000g for 16 h to obtain a single 'membrane-bound' polyribosomal fraction at the interface and separated from free polyribosomes. The two fractions were suspended in medium C, centrifuged at 105000g for 1.5 h and the pellets finally suspended in 2–4 ml of medium C.

Cell sap. Homogenates of liver, brain and muscle prepared in 3.5 vol. of sucrose-free medium A, B and C, respectively, were centrifuged at 105000g for 4 h. After

aspiration of fat and surface debris, the clear post-microsomal supernatant was passed through a Sephadex G-25 column (coarse particles) with bed vol. approx. 8 times that of the supernatant, and equilibrated in the respective homogenizing medium. The effluent cell sap was clarified by centrifugation at 105000g for 2 h, the protein content was determined and the sap was then frozen in small fractions.

Incorporation of amino acids into protein by subcellular fractions in vitro. Each incubation tube (15 ml conical centrifuge tubes) contained in a final volume of 1 ml: 50 μ mol of tris-HCl (pH 7.4 for brain preparations and pH 7.6 for muscle and liver); KCl (25 μ mol for liver preparations and 100 μ mol for brain and muscle); 10 μ mol of $MgCl_2$; 10 μ mol of 2-mercaptoethanol; 3 μ mol of ATP; 1 μ mol of GTP; 5 μ mol of phosphoenolpyruvate; 10 μ g of pyruvate kinase; 0.1 μ mol of each of 20 non-radioactive L-amino acids, excluding the radioactive amino acid used; 0.1–1.0 μ Ci of the radioactive amino acid; Sephadex-treated cell sap (3.5 mg of protein). The reaction was started by adding the ribosomal preparation to tubes pre-warmed to 37°C and containing all the other components. The amounts of ribosomal material was the only rate-limiting factor and usually 50–200 μ g of RNA was added. Because of the design of the experiments on puromycin-induced discharge of nascent protein (see below), incubations were usually carried out for 16 min. The reaction was stopped (except in experiments on vectorial discharge of labelled proteins) by adding, at 0°C, 5 ml of 5% (w/v) trichloroacetic acid containing 0.1% of the unlabelled analogue of the radioactive amino acid. The precipitate was washed once with 5% trichloroacetic acid at 75–80°C (kept for 20 min), once at room temperature and twice with ethanol-ether (3:1 v/v) before determining the radioactivity.

Vectorial discharge of nascent polypeptides by puromycin. These studies were based on the differences in the distribution between the soluble and particulate fractions of the radioactive nascent polypeptide chains released by puromycin from membrane-bound and free polyribosomes from secretory tissues (Redman & Sabatini, 1966). The discharge of nascent protein labelled both *in vivo* and *in vitro* was studied in this way. The incubation conditions were the same as described above except that the amount of ribosomal preparation added was nearly doubled, i.e. 0.5–1.0 mg for liver, 0.1–0.5 mg for brain and 0.1–0.3 mg for muscle. After 8 min of incubation, 1 μ mol of puromycin in 0.05 ml of water was added to half the tubes and 0.05 ml of water to the other half, and the incubation continued for another 8 min. At this point, 0.05 ml of sodium deoxycholate, to a final concentration of 0.5% (w/v), was added to half the puromycin-treated and control samples and 0.05 ml of water to the other half. For every membrane-bound preparation there was a corresponding sample of free polyribosomes to determine any effect of the detergent *per se* on the binding of nascent protein to ribosomes. The detergent was mixed vigorously for 10 s after which 4 ml of ice-cold homogenizing medium was added to stop further incorporation. The particulate and supernatant fractions were separated by ultracentrifugation at 105000g for 2 h, the pellets suspended in 2 ml of water, and the protein in both samples was precipitated with 5% trichloroacetic acid. It was found necessary to supplement muscle preparations with 1–2 mg of bovine serum albumin before

precipitation of protein. The precipitates were then processed for radioactivity measurements as described above. The results are expressed as the percentage of radioactivity distributed between the particulate and supernatant fractions. The absolute incorporation for these experiments ranged between 1500 and 19000 c.p.m./mg of RNA, and in no case were results included based on less than 200 c.p.m. per sample.

Incorporation of [^3H]puromycin. In experiments on the incorporation of the labelled puromycin, free and membrane-bound (heavy rough membranes) ribosomes from liver were incubated in duplicate tubes for 8 min as described above, but in the absence of labelled amino acids. This was followed by the addition of $10\mu\text{M}$ -[^3H]-puromycin (final sp. radioactivity 0.5 mCi/mol) and the incubation was continued for another 8 min. At the end of this period, sodium deoxycholate (0.5%, w/v) was added to half the samples and an equivalent volume of water to the other half before dilution of the suspension with homogenizing medium and centrifugation to separate the 105000g particulate and supernatant fractions. Because of an interaction between deoxycholate, puromycin and trichloroacetic acid, duplicate controls were incubated in which microsomal material was omitted. Under the conditions of the experiments small amounts of [^3H]-puromycin, corresponding to 130 c.p.m. and 90 c.p.m. in the presence and absence of deoxycholate respectively, were precipitated by trichloroacetic acid. Deductions for these blanks were therefore made for each sample.

Determination of radioactivity. The washed precipitates were heated in 0.5 ml of 5% trichloroacetic acid at 80–85°C for 10 min and then dissolved in 10 ml of a scintillation fluid containing 180 g of nuclear-grade naphthalene, 4.0 g of 2,5-diphenyloxazole, 0.1 g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in 1 litre of dioxan. Radioactivity was measured in a Packard TriCarb scintillation spectrometer, the efficiency of counting for ^3H and ^{14}C being 32% and 75% respectively.

Chemical determinations. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin as the standard. RNA and DNA were measured by the methods of Fleck & Munro (1962) and Burton (1956) respectively. Phospholipid was extracted from a 0.7M- HClO_4 precipitate as described by Tata (1967) and the phosphorus determined

by the method of Ames & Dubin (1960). Phospholipid was estimated as $25\times$ phosphorus.

Materials. All chemicals were of analytical grade and were purchased from Sigma (London) Chemical Co., London S.W.6, U.K. or C. F. Boehringer Corp. (London) Ltd., London W.5, U.K. ATP, GTP and phosphoenolpyruvate were converted into their potassium salts before use. Polyuridylic acid (as the ammonium salt) was purchased from Miles Chemical Co., Clifton, N.J., U.S.A. and puromycin hydrochloride was obtained from Lederle Laboratories, Pearl River, N.J., U.S.A. U- ^{14}C -labelled amino acid mixture (54 mCi/mg-atom of C), L-[U- ^{14}C]-leucine (311 mCi/mmol), L-[U- ^{14}C]phenylalanine (504 mCi/mmol) and [$5\text{-}^3\text{H}$]orotic acid (6.1 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. [^3H -O-methyl]Puromycin dihydrochloride (1 mCi/ μmol) was purchased from New England Nuclear Corp., Boston, Mass., U.S.A.

Electron microscopy. All membrane-bound and free ribosomal preparations were kindly examined in the electron microscope by Dr J. A. Armstrong at the National Institute for Medical Research, Mill Hill. Pellets were fixed with glutaraldehyde–osmic acid and sectioned in different planes.

RESULTS AND DISCUSSION

Characterization of submicrosomal fractions. The relative amounts of RNA, protein and phospholipid in the different submicrosomal fractions of rat liver, brain and skeletal muscle are summarized in Table 1. The ratios presented here for liver are similar to those found earlier (Tata & Williams-Ashman, 1967; Reid, 1967; Tata, 1969). Similar ratios for membrane-bound ribosomes from brain and muscle have not been published before. Indeed the existence of membrane-bound ribosomes in skeletal muscle has been denied (Wool *et al.* 1968), although structures resembling ribosome-studded membranes have been seen in tissue sections under the electron microscope (Gustafsson, Tata, Lindberg & Ernster, 1965). Our earlier work on submicrosomal fractions from liver (Tata, 1969) had suggested that the

Table 1. *Relative RNA, protein and phospholipid content of membrane-bound and free ribosomes from liver, brain and muscle*

Microsomal subfractions were prepared as described in the text. Each value is the mean of duplicate determinations for 8–12 preparations of liver and brain, and four for muscle. The variation was within $\pm 5\%$.

Tissue	Fraction	RNA/protein ratio	Phospholipid/protein ratio	RNA/phospholipid ratio
Liver	Light rough membranes	0.15	0.20	0.75
	Heavy rough membranes	0.35	0.15	2.40
	Free polyribosomes	0.76	0.04	21.00
Brain	Membrane-bound	0.13	0.23	0.55
	Free polyribosomes	0.70	0.10	7.00
Muscle	Membrane-bound	0.35	0.38	0.91
	Free polyribosomes	0.27	0.10	2.00

RNA phospholipid ratio reflects the relative density of packing of ribosomes on microsomal membranes. Thus the 'heavy' membrane-bound ribosomes from brain and muscle resembled, in this respect, the light rather than heavy rough membranes from liver. The lower RNA phospholipid ratio of brain membrane-bound ribosomes may also be due to such membranous material as myelin-sheath fragments. The extremely low phospholipid content of the free polyribosomes indicates the absence of membranes in this fraction, unlike the experience of Murty & Hallinan (1969) in preparing free polyribosomes from liver. However, the low RNA/protein ratio of 0.27 for free polyribosomes from muscle (compared with 0.70–0.75 for brain and liver) was largely due to contamination with myosin fibrils.

Electron-microscopic examination corroborated all the above conclusions about free and membrane-bound ribosomes from the three tissues. Whereas the liver preparations were quite homogeneous in appearance, those from brain and muscle were not. The major contaminants of membrane-bound polyribosomes from the cerebrum were myelin fragments with smaller amounts of synaptosomes (see Whittaker, 1970) and possibly 'reticulosomes' (Adams & Lim, 1966). For membrane-bound ribosomes from muscle, the major contaminants were small mitochondria and sarcotubular fragments.

Whereas chemical and morphological analyses virtually ruled out the contamination of free polyribosomes with membrane-bound polyribosomes, they cannot rule out the inclusion of free ribosomes in membrane-bound fractions. This is a critical issue for vectorial discharge studies and the completeness of separation was checked in the following way. Free polyribosomes were labelled *in vivo* with a long pulse of [^3H]orotic acid and were added to the unlabelled membrane-bound fractions. After repetition of the complete fractionation procedure, not more than 3–5% of the radioactive RNA was recovered in the membrane-bound fractions. Besides confirming the extremely small contamination of membrane-bound with free polyribosomes, this experiment also ruled out the possibility that membrane-bound polyribosomes in brain and muscle may be derived artifactually as a result of the fractionation procedure.

An important feature of the fractionation procedure is that yield is sacrificed for the least mutual contamination of membrane-bound and free polyribosomes so that it is not possible to say what the relative amounts of the two fractions are in the intact cell. However, on the basis of our results for the recovery of total ribosomes, 80% for liver, 30–40% for brain and 10–15% for muscle, one can set the following minimum values for the fraction of ribosomes that may be membrane-bound *in vivo*:

Fig. 1. Rate of incorporation of [^{14}C]leucine into protein by free (○) and membrane-bound (●) ribosomes from rat liver (a), brain (b) and muscle (c). Incubation conditions are as described in the text except that only 40–150 μg of ribosomal RNA were added/ml of incubation medium. Each point gives the mean value of duplicate samples. The liver membrane-bound fraction was the 'heavy rough membrane' preparation shown in Table 1.

80% for liver; 22% for cerebral cortex; 9% for skeletal muscle. Merits *et al.* (1969) found that 14.5% of rat brain ribosomes may be membrane-bound. On the basis of the methods currently used by other workers for preparing muscle polyribosomes it is almost certain that the small population of membrane-bound ribosomes would have been discarded in the initial slow-speed centrifugation.

Amino acid incorporation by free and membrane-bound preparations in vitro. Fig. 1. summarizes the time-course of incorporation of L-[^{14}C]leucine into protein by membrane-bound and free polyribosomes from the three tissues, the amount of ribosomal RNA used being within the linear range of incorporation. In all three tissues, membrane-bound ribosomes were more active than free ribosomes, but this difference was considerably less marked for

brain and muscle preparations. (It should be noted that we have used the 'heavy rough membrane' fraction from liver for this comparison, and that the 'light rough membrane' fraction would give more comparable differences between bound and free ribosomes than those seen for brain and muscle; see Tata & Williams-Ashman, 1967.) Some workers have failed to find the difference we have observed for membrane-bound and free ribosomes from liver (Blobel & Potter, 1967; Kwan, Webb & Morris, 1968). These workers starved the rats before death, a procedure which in our hands lowers the activity of bound ribosomes. Similar comparisons have not been made for brain and muscle preparations, except that Dunn (1970) observed no difference in rate of incorporation between brain microsomes and free polyribosomes obtained by detergent treatment. It is difficult to say whether such a discrepancy arises from the method of preparation of the particles or the conditions of incubation. However, we have also observed that the incorporation ceases more rapidly with membrane-bound ribosomes than with free ribosomes; Dunn (1970) has attributed this to a limitation in the availability of usable messenger RNA.

Binding of ribosomes to endoplasmic reticulum of liver. There have been suggestions of small differences in the protein and RNA of free and bound ribosomes (Szekeley, Beney, Gaal & Vincze, 1966; Süss, Blobel & Pitot, 1966; Fridlender & Wettstein, 1970) and it seems that some factor within the membranes of rough endoplasmic reticulum, and absent in smooth endoplasmic reticulum, may be responsible for the attachment (see Pitot *et al.* 1969). By a combination of biochemical studies and electron microscopy, Sabatini, Tashiro & Palade (1966) suggested that the liver ribosome is attached to the membrane by its large subunit. In other studies in which the retention of nascent polypeptide chains was studied by treating the rough microsomes with EDTA or trypsin, it was thought that nascent protein chains may anchor the ribosome to the membrane (Sabatini *et al.* 1966; Chefurka & Hayashi, 1966). In view of the importance of the association between membrane binding and active protein synthesis for the interpretation of our studies on the puromycin-induced release of nascent proteins, it was decided to investigate this point. Experiments were therefore performed in which we measured the release of free ribosomes (as judged by their ability to sediment through 2.0 M-sucrose at 105 000g) from liver membrane-bound preparations treated with increasing doses of puromycin under conditions of active protein synthesis *in vitro*.

The results illustrated in Figs. 2 and 3 show that there is no appreciable release of ribosomes from the membrane-bound fraction treated with puro-

Fig. 2. Effect of increasing concentrations of puromycin on the incorporation of [^{14}C]leucine into polypeptides *in vitro* and the release of free ribosomes from membrane-bound ribosomes of rat liver. Incubations were carried out as described in Table 2 and the text. Radioactivity in the trichloroacetic acid-insoluble fraction was measured as an index of the extent of incorporation ($\bullet$), and the fraction of RNA (determined spectrophotometrically) pelleted through 2M-sucrose after incubation as an index of the release of ribosomes from the membrane-bound fraction ($\circ$). Each value is the average of duplicate determinations.

Fig. 3. Effect of increasing concentrations of puromycin on the incorporation of [^{14}C]leucine into polypeptides and the release of ribosomes from membrane-bound ^3H -labelled ribosomes from rat liver, by the method of double isotope determination. Ribosomes and RNA associated with them were labelled *in vivo* by the administration to each rat of 20 μCi of [^3H]orotic acid 19 h before death. Incorporation of [^{14}C]leucine into protein by the membrane-bound ribosomes *in vitro* was carried out as described in Table 2 and the text and the ^{14}C recovered in the trichloroacetic acid-insoluble fraction used as an index of [^{14}C]leucine incorporation ($\bullet$). The fraction (%) of total ^3H -labelled RNA present in the incubation suspension, recovered in the small pellet forming at the bottom of 2M-sucrose after centrifuging at 105 000g after 2 h, was used as an index of the release of ribosomes from the membrane fraction ($\circ$). Each value is the mean of duplicate determinations.

mycin. This is true even at concentrations of puromycin that effectively abolish amino acid-incorporation and also release about 50% of nascent protein from membrane-bound ribosomes (see below). A comparison of the results of Figs. 2 and 3 also shows that the failure of puromycin to release ribosomes from membranes was equally true of the total non-radioactive membrane-bound population as well as for the more recently synthesized RNA associated with ribosomes labelled after a pulse of 18 h. Thus it seems that, at least in liver, the growing polypeptide chain is not required for the attachment of ribosomes to membranes of the endoplasmic reticulum, contrary to the claims made by Chefurka & Hayashi (1966). It could be argued that, since puromycin does not release more than 50% of nascent chains, the remaining chains might prevent release of ribosomes. However, such a possibility is unlikely in view of the finding of Blobel and Potter (1967) that puromycin induces breakdown of both bound and free polyribosomes. In addition, we also confirmed, in a separate experiment, that there was little change in the rough-surfaced vesicles examined in the electron microscope before and after incubation.

Puromycin-induced vectorial release of nascent polypeptides from membrane-bound ribosomes in liver, brain and skeletal muscle. There is little doubt now that proteins for export, such as albumin and amylase, are synthesized on rough endoplasmic reticulum (Campbell, 1965; Palade, 1966; Jamieson & Palade, 1967; Takagi & Ogata, 1968). The use of puromycin, which releases 30–50% of nascent chains from microsomes, showed that much of the protein made on bound ribosomes for secretion was

discharged into the lumen of the membrane to be eventually exported via the smooth endoplasmic reticulum–Golgi system (Sabatini *et al.* 1966). Although ferritin is thought to be synthesized on free ribosomes of liver (Redman, 1969), the predominance of synthesis of secretory proteins by liver and pancreas makes it difficult to study the synthesis of intracellular proteins on membrane-bound ribosomes in these tissues. Membrane-bound ribosomes from brain and muscle therefore offered us an opportunity to see if the proteins made on membrane-bound ribosomes from such tissues were handled differently from those in secretory tissues.

In designing the experiments on puromycin-induced release of nascent protein, the main considerations are as follows. If all protein synthesized on membrane-bound ribosomes is transported through the membrane into the vesicle, then one should observe a total sequestration of radioactive nascent chains within the particulate fraction until the membrane is disrupted by deoxycholate. The same pattern of distribution should be observed for membrane-bound ribosomes from secretory and non-secretory tissues. If on the other hand, membrane-bound ribosomes from secretory and non-secretory tissues function differently, then a different pattern of distribution of radioactivity between particulate and supernatant fractions should be observed when they are treated successively with puromycin and deoxycholate. We have assumed, on the basis of earlier observations of Redman & Sabatini (1966), that only a negligible amount of incorporation occurs into structural proteins of the endoplasmic membrane.

(a) Amino acid incorporation *in vitro*. Table 2

Table 2. *Effect of puromycin and deoxycholate on the fraction of [14 C]protein, labelled in vitro with [14 C]leucine, retained with the 105 000g particulate fraction*

The incubation mixture (1 ml) contained the following amounts of free and membrane-bound ribosomes: liver, 0.5–1.0 mg of RNA; brain, 0.1–0.5 mg of RNA; muscle, 0.1–0.3 mg of RNA. Where indicated, 10 mM-puromycin was added after 8 min of incubation, which was continued for another 8 min. At the end of this period half the samples were treated with 0.5% (w/v) sodium deoxycholate and the 105 000g particulate fraction separated from the supernatant. The fraction of [14 C]protein recovered in the pellet was then measured. Each value is the mean of four to ten experiments with s.d. $\pm 5\%$.

Fraction	Tissue	No. of expts.	Range of [14 C]protein radioactivity (c.p.m./mg of RNA)	Percentage of total [14 C]protein recovered in 105 000g sediment after			
				Control	Deoxycholate	Puromycin	Puromycin then deoxycholate
Membrane-bound	Liver	10	3200–18 400	83	64	75	37
	Brain	8	1739–13 960	86	72	37	27
	Muscle	6	1550–10 400	78	71	36	32
Free ribosomes	Liver	4	9400–19 000	91	86	59	53
	Brain	8	2080–14 700	88	83	31	27
	Muscle	6	1700–9 350	83	80	47	42

Table 3. Release of [^{14}C]protein labelled in vitro from membrane-bound and free ribosomes from liver, brain and muscle, by treatment with puromycin and deoxycholate

In this table the results of the experiments described in Table 2 are expressed as the fraction (%) of the [^{14}C]protein recovered in untreated control microsomes or ribosomes that is released after treatment with 0.05% deoxycholate, 1 mM-puromycin or puromycin followed by deoxycholate.

Fraction	Tissue	Particulate [^{14}C]protein released (as % of untreated control) by		
		Deoxycholate	Puromycin	Puromycin, then deoxycholate
Membrane-bound	Liver	24	13	55
	Brain	15	56	70
	Muscle	8	54	60
Free ribosomes	Liver	9	35	44
	Brain	7	64	69
	Muscle	4	46	51

Table 4. Effect of poly(U) on the vectorial discharge of [^{14}C]protein labelled in vitro with [^{14}C]phenylalanine from membrane-bound ribosomes from liver and brain

[^{14}C]Phenylalanine was incorporated into ^{14}C -labelled protein and [^{14}C]polyphenylalanine by incubation of membrane-bound ribosomes in the presence of 200 μg of polyuridylic acid/ml. The effect of deoxycholate and puromycin on the discharge of particulate ^{14}C -labelled protein is expressed as in Table 3 and other conditions are as in Table 2. Each value is the mean of four or six determinations with S.D. $\pm 6\%$.

Tissue	No. of expts.	Incorporation of [^{14}C]phenylalanine (c.p.m./mg of RNA)	Particulate [^{14}C]protein and [^{14}C]polyphenylalanine released (as % of untreated control) by		
			Deoxycholate	Puromycin	Puromycin, then deoxycholate
Liver	6	14 970–41 550	20	15	50
Brain	4	11 350–29 500	8	58	64

shows that after 8 min of incubation, puromycin caused the release of a large fraction of radioactivity incorporated into protein *in vitro* by free polyribosomes of brain (64%), liver (35%), and muscle (46%). The differences in the extent of discharge may reflect tissue-specific differences in the access of puromycin to aminoacyl-tRNA binding on the ribosomes. With free polyribosomes from muscle there is the additional possibility of a different type of structural association in that muscle ribosomes have been found to be linked to myosin fibrils (Larson, Hudgson & Walton, 1969). Whatever the explanation, it still remains that puromycin releases a substantial part of nascent protein from free ribosomes of all three tissues directly into the supernatant (see Table 3). With membrane-bound ribosomes there is a distinct difference between preparations from the liver on the one hand, and those from brain and muscle on the other. Puromycin alone released only 13% of labelled protein from the liver preparation but treatment with puromycin

followed by deoxycholate released 55%. In other words, whereas puromycin caused the release of 13% of nascent protein from liver membrane-bound ribosomes directly to the surrounding medium, at least an additional 18% passed through the membrane to the interior of the vesicle. In contrast with this, puromycin followed by deoxycholate failed to release more protein than the sum of the individual effects of deoxycholate and puromycin on brain and muscle membrane-bound ribosomes.

The above difference between membrane-bound ribosomes from secretory and non-secretory tissues is unlikely to be due to the different types of messenger RNA associated with them, since the same pattern was obtained with the discharge of [^{14}C]polyphenylalanine synthesized in the presence of poly(U). As shown in Table 4, about half of the poly(U)-directed nascent polypeptides released by puromycin from liver membrane-bound ribosomes is discharged to the interior of the microsomes, whereas this was not the case with brain particles.

It is unlikely that an abnormal vectorial discharge of polyphenylalanine is masked by that of natural mRNA-directed proteins because poly(U) produced 100–500% stimulation in incorporation of [^{14}C]-phenylalanine; there is also the fact that polyphenylalanine would have a much higher specific radioactivity than phenylalanine incorporated into natural protein.

To verify more directly the vectorial discharge into the membrane of liver microsomes, the incorporation and disposal of [^3H]puromycin was

followed under the same conditions as described above. At concentrations of puromycin ($10\mu\text{M}$) that were 80% as effective as those used in other experiments, 91% of [^3H]puromycin-labelled protein was released into the microsomal vesicle (Table 5). About half of this labelled protein is freed to the surrounding medium by deoxycholate treatment, which has no effect on the radioactivity associated with free ribosomes. This observation further confirms the validity of the conclusions drawn from results in Tables 2 and 3.

(b) Nascent protein labelled *in vivo*. When liver and brain proteins were labelled *in vivo* results similar to those with labelling *in vitro* were obtained (Tables 6 and 7). Muscle preparations were not used for labelling *in vivo* because of the irreproducible labelling of proteins and the very low yield of membrane-bound ribosomes. The effect of deoxycholate alone on liver rough microsomes was more marked after labelling *in vivo* than *in vitro*, which may be due to the higher efficiency *in vivo* of chain elongation, termination and release (Redman & Sabatini, 1966; see Hendler, 1968).

(c) Vectorial discharge after prolonged incubation. Results obtained with prolonged incubation *in vitro* without puromycin (Figs. 4a and 4b) suggested that the antibiotic was merely accelerating a normal process and not introducing an abnormal situation. On incubation of membrane-bound ribosomes for up to 1 h rat liver microsomes incorporated more radioactive leucine than did those from brain or muscle, but the fraction of [^{14}C]protein released into the supernatant was much higher for the particles from non-secretory tissues. This difference is further emphasized when the behaviour of free polyribosomes from liver and brain was found to be

Table 5. *Effect of sodium deoxycholate on the release of peptidyl- ^3H puromycin from free and membrane-bound ribosomes of rat liver*

RNA (0.5 mg) was incubated in 1 ml of incubation medium with $10\mu\text{M}$ - ^3H puromycin as described in the text. After incubation, half the samples were treated with 0.5% deoxycholate and the other half with water. Radioactivity was measured in the 105 000g particulate and supernatant fractions and the [^3H]puromycin blank values were deducted as described in the text. Each value is the mean of two separate experiments.

Ribosomes	Deoxy- cholate	[^3H]Puromycin incorporated (c.p.m.)	Trichloroacetic acid-insoluble ^3H in parti- culate fraction (%)
Membrane-bound*	+	1080	47
	—	806	91
Free	+	438	60
	—	435	59

* Heavy rough membrane fraction used.

Table 6. *Effect of puromycin and deoxycholate on the fraction of protein labelled in vivo retained on the 105 000g particulate fraction derived from free and membrane-bound ribosomes from liver and brain*

For liver preparations, two rats were each injected with 5 μCi of a mixture of ^{14}C -labelled amino acids, 10 min before death. For brain preparations, six to ten rats (30–50 g body wt.) were injected intracisternally with 4 μCi of ^{14}C -labelled amino acids 30 min before death. Free and membrane-bound ribosomes from liver (0.5–1.0 mg of RNA) and brain (0.1–0.5 mg of RNA) were incubated in 1 ml as described for labelling *in vitro*. Conditions for incubations with puromycin and treatment with deoxycholate were also as described in Table 2 and in the text. Each value is the mean of results from four to six experiments with s.d. $\pm 6\%$.

Fraction	Tissue	No. of expts.	Range of [^{14}C]protein radioactivity (c.p.m./mg of RNA)	Percentage of total [^{14}C]protein recovered in 105 000g sediment after			
				Control	Deoxycholate	Puromycin	Puromycin, then deoxycholate
Membrane-bound	Liver	6	1000–1700	88	27	90	19
	Brain	6	560–12 600	77	59	48	33
Free polyribosomes	Liver	4	1170–1700	80	83	25	25
	Brain	4	980–13 000	80	74	48	32

Table 7. Release of [^{14}C]protein labelled *in vivo* from membrane-bound and free ribosomes from liver and brain, by treatment with puromycin and sodium deoxycholate

All experimental conditions were the same as in Table 6, except that the results are expressed here as the fraction (%) of the ^{14}C -labelled protein recovered in untreated control preparations that is released after treatment with 0.5% sodium deoxycholate alone, 1 mM puromycin alone, or incubation with puromycin followed by treatment with deoxycholate.

Fraction	Tissue	Particulate [^{14}C]protein released (as % of untreated control) by		
		Deoxycholate	Puromycin	Puromycin, then deoxycholate
Membrane-bound	Liver	70	0	76
	Brain	23	38	59
Free	Liver	0	70	70
	Brain	11	41	60

Fig. 4. Relationship between time of incubation and the vectorial discharge of nascent protein labelled *in vitro* by membrane-bound ribosomes (a) and free ribosomes (b) from rat liver (O), brain (Δ) and muscle ($\square$). The incorporation of [^{14}C]leucine into protein by the different ribosomal preparations was carried out as described for the puromycin-induced discharge experiments of Table 2, except that no puromycin or sodium deoxycholate were used. The fraction of total ^{14}C -labelled protein appearing directly in the 105000g supernatant is reported here, based on values averaged from two experiments.

identical (compare Figs. 4a and 4b); in both instances 25% of the newly synthesized polypeptides were released to the supernatant at 60 min of incubation. Free ribosomes from muscle were not used in these long-term incubation experiments

because of the possibility that their close association with myosin filaments may modify the patterns of release of the labelled polypeptides.

CONCLUSIONS

This paper describes for the first time the separation of membrane-bound and free ribosomes from brain and muscle that are active in protein synthesis. Our results show that membrane-bound ribosomes from these two tissues behave quite differently from similar preparations from liver with respect to the vectorial release of nascent protein. Puromycin was found to be a valuable tool in amplifying these differences. We believe that these differences reflect the predominantly secretory role of attachment of ribosomes to membranes of the endoplasmic reticulum in the liver. Membrane-bound ribosomes from the non-secretory tissues, brain and muscle, behave in this respect like free polyribosomes from both types of tissues.

The high amino acid-incorporation activity of bound polyribosomes from brain and muscle poses the question of the role of ribosome-membrane attachment in non-secretory tissues *in vivo*. Evidence obtained elsewhere points to some differences in function of membrane-bound and free ribosomes in non-secretory cells such as reticulocytes (Burka, Schreml & Kick, 1967; Burka & Bulova, 1970) and kidney cells (Priestley *et al.* 1969). There are also reports on the possibility that membrane-bound ribosomes function differently from free ribosomes in secretory and non-secretory bacteria (Schlessinger, 1963; Uchida & Yoneda, 1967; van Dijk-Salkinoja, Stoof & Planta, 1970). It has been suggested that the membrane to which ribosomes are attached may serve to protect messenger RNA from degradation (Campbell, Lowe

& Serck-Hanssen, 1967; Burka *et al.* 1967; Sarma, Reid & Sidransky, 1969; see Hendler, 1968). However, we do not yet know if the protection is selective or whether there is some ribosomal specificity factor involved in the attachment to sites on the membrane.

At the moment, therefore, we have no direct evidence for a selective role for membrane-ribosome attachment other than that for secretion. Indirectly, the marked proliferation of rough endoplasmic reticulum during rapid growth and development of secretory tissues, when additional or new intracellular proteins are formed (see Tata, 1970*a,b*), points to another function. We have found that the rapid fall in protein synthesis during post-natal development of rat brain is largely restricted to the fraction of ribosomes bound to membranes of the cerebral cortex (T. M. Andrews & J. R. Tata, unpublished work). Tata (1967, 1968*a,b*, 1970*a,b*) has suggested that a function of ribosome binding to the membrane may be topographically to segregate different populations of ribosomes presumably synthesizing different classes of proteins, a function which may be critical during rapid developmental or adaptational changes. This hypothesis presupposes that non-secretory proteins would also be synthesized on membrane-bound ribosomes. There is evidence of mitochondrial proteins being synthesized in the microsomal fraction (Gonzalez-Cadavid, Bravo & Campbell, 1968; Work, Coote & Ashwell, 1968; Kadenbach, 1970). Nascent serine dehydratase, an intracellular hepatic enzyme, has been almost exclusively localized by immunochemical techniques on rough endoplasmic reticulum (Pitot *et al.* 1969). Preliminary work in our laboratory (T. Wills, unpublished work) has shown by histochemical techniques the localization of ornithine and aspartate carbamoyltransferases in the rough endoplasmic reticulum. Extension of these studies to selective localization of induced enzymes in proliferating membrane-ribosome complexes should prove valuable in confirming an additional function for the attachment of ribosomes to cellular structures.

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