Effect of a Modified Separation Procedure on the Size and Protein-Synthetic Activity of Membrane-Bound Liver Polyribosomes

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1. Various subcellular fractions containing ribosomes were isolated from rat liver. 2. In the presence of [^{14}C]leucine and Sephadex-treated cell sap the radioactivity incorporated into the synthesized protein resulting from the incubation of microsomal preparations or deoxycholate-treated polyribosomes was dependent on the amount of rRNA incubated. In contrast, when Sephadex-treated post-mitochondrial supernatant was incubated, the radioactivity incorporated into the synthesized protein was independent of the amount of rRNA incubated. 3. Microsomal preparations and membrane-bound ribosomes, prepared by the standard procedure, incorporated less [^{14}C]leucine into protein, per mg of rRNA incubated, than free or deoxycholate-treated polyribosomes; accordingly, polyribosomes associated with the former fractions were found mainly as monomers. 4. If microsomal fractions or membrane-bound ribosomes were prepared by a simple modification of the standard procedure, i.e. by centrifugation on to a 'cushion' of 2 M-sucrose, their protein-synthesizing activity was of the same order as that of the original post-mitochondrial supernatant, and membrane-free and deoxycholate-treated polyribosomes; in this case polyribosome profiles showed that very little degradation had occurred and compared well with those obtained for post-mitochondrial supernatant and isolated polyribosomes. 5. A method is described (Appendix) that provides a rapid and reliable assessment of the concentration of rRNA in subcellular fractions.

If the active unit for protein biosynthesis is the polyribosome one might expect that the relative synthetic activity of isolated subcellular fractions from a given tissue would depend on their polyribosome content. However, many authors have found that on the basis of RNA content the protein-synthesizing activity of the microsomal or membrane-bound ribosomal fractions is lower than that shown by membrane-free or detergent-treated polyribosomes (Campbell, Cooper & Hicks, 1964; Campbell, Serck-Hanssen & Lowe, 1965; Campbell, Lowe & Serck-Hanssen, 1967; Bloemendal, Bont, de Vries & Benedetti, 1967; Ragnotti, Lawford & Campbell, 1969; Ragnotti, Cajone & Bernelli-Zazzera, 1970; Ganosa & Williams, 1969; Macdonald & Korner, 1971). It is possible therefore, that membrane-associated polyribosomes are less active in protein synthesis than would be expected on the basis of their polyribosomal content; alternatively, polyribosomes associated with the membranes could have been disaggregated during the isolation procedure. It is important to clarify this point before reaching any conclusion concerning the effect of membranes on the activity of polyribosomes.

In the present work it is confirmed that the microsomal fractions and membrane-bound ribosomes isolated under the usual conditions are less active, per mg of rRNA, than are polyribosomes isolated from the same source. However, this difference could be abolished if microsomal fractions and membrane-bound ribosomes were prepared by a simple modification of the usual procedure, i.e. by centrifugation onto a 2 M-sucrose 'cushion'.

MATERIALS AND METHODS

Chemicals. ATP, GTP, phosphoenolpyruvate and pyruvate kinase (EC 2.7.1.40; specific activity approx. 150 units/mg) were purchased from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, West Germany). Cycloheximide (Actidione) was purchased from Nutritional Biochemical Corp. (Cleveland, Ohio, U.S.A.). Other chemicals were of A.R. grade and were obtained from either E. Merck A.-G. (Darmstadt, West Germany) or British Drug Houses Ltd. (Poole, Dorset, U.K.). Organic solvents were obtained from Carlo Erba S.p.A. (Milano, Italy). L-[U^{14}C]Leucine (specific radioactivity 311 mCi/mmol), was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). The scintillator (Omnifluor) was a product of NEN Chemicals G.m.b.H. (Frankfurt am Main, West Germany).

Animals. Male albino rats (Wistar strain) weighing approx. 200g were used. They were maintained on a diet of laboratory chow (Piccioni, Brescia, Italy) and water *ad libitum* and were starved for 16–18h before experimentation. The rats were stunned by a blow to the head, decapitated and their livers quickly removed and transferred to ice-cold medium A. All subsequent operations were performed at a temperature near 0°C.

Cellular fractionation. The following media were used: medium A: 0.25M-sucrose in TKM1 buffer (50mM-tris–HCl, pH7.8 at 20°C; 25mM-KCl; 5mM-MgSO$_4$); medium B: 0.15M-sucrose in TKM2 buffer (35mM-tris–HCl, pH7.8 at 20°C; 25mM-KCl; 10mM-MgSO$_4$). The livers were passed through a tissue press (made to the design of Porterfield, 1960). The liver mince was homogenized in 2.0vol. of medium A for the preparation of the post-mitochondrial supernatant, microsomal fraction, and membrane-free and membrane-bound ribosomes and in 2.5vol. of medium A for the preparation of the detergent-treated polyribosomes (Wettstein, Staehelin & Noll, 1963). The preparation of the different fractions is described below.

(*a*) Post-mitochondrial supernatant was prepared by centrifuging the homogenate for 20min at 12000*g*. The supernatant, recentrifuged as above, was collected and a sample (5ml) was passed through a Sephadex column (1cm × 20cm) equilibrated with medium A, the first 2.5ml being collected.

(*b*) The microsomal fraction was prepared by two different procedures: in procedure A it was obtained by centrifuging the post-mitochondrial supernatant at 270000$g_{av.}$ for 150min in a Spinco 65 rotor and in procedure B the post-mitochondrial supernatant was layered over a 1ml 'cushion' of 2M-sucrose in TKM2 buffer and then centrifuged as above. After removal of the clear supernatant with a Pasteur pipette (see below), particles that had sedimented to the bottom of the tube (procedure A) and particles at the interface of the 2M-sucrose, as well as the sucrose 'cushion' itself, were resuspended with medium B directly in the centrifuge tube, by gentle homogenization with a loose-fitting Teflon pestle, to half the original volume of the post-mitochondrial supernatant. The suspensions were centrifuged in a refrigerated centrifuge. A portion of the supernatant was diluted to an RNA concentration of 1mg/ml (see Appendix) for the determination of the protein-synthesizing activity.

(*c*) The cell-sap fraction (5ml) that had been removed by Pasteur pipette was passed through a column (1cm × 20cm) of Sephadex G-25 equilibrated with medium A, the first 2.5ml being collected. When [^{14}C]leucine was added to cell sap before Sephadex treatment and the radioactivity determined in the effluent, the first 3ml of cell sap collected was virtually free from radioactive leucine (0.02% of the total).

(*d*) Membrane-bound, membrane-free and detergent-treated polyribosomes were isolated as described by Ragnotti *et al.* (1969), with minor modifications. The post-mitochondrial supernatant was centrifuged in a Spinco 65 rotor for 150min at 270000$g_{av.}$ on a discontinuous gradient of 0.5–1.6M-sucrose, both in TKM1 buffer. Membrane-bound ribosomes, which separated at the interface, were collected in their own cell sap, then were diluted with cold cell sap and sedimented in the absence (procedure A) and in the presence (procedure B)

of a 1ml 'cushion' of 2M-sucrose in TKM2 buffer by centrifugation at 270000$g_{av.}$ for 60min in a Spinco 65 rotor. Membrane-bound ribosomes were then resuspended with medium B to half the original volume of the post-mitochondrial supernatant. Detergent-treated polyribosomes were obtained by centrifugation of the deoxycholate-treated post-mitochondrial supernatant (1.3% final concentration) on a discontinuous gradient of 0.5–2·0M-sucrose (in TKM1 buffer) for 150min at 270000$g_{av.}$ in a Spinco 65 rotor. Membrane-free and detergent-treated polyribosomes, after removal of the sucrose and careful wiping of the tube, were resuspended in medium B. Membrane-bound, membrane-free and detergent-treated polyribosome suspensions were centrifuged in a refrigerated centrifuge and a portion of the supernatant was diluted with medium B to an RNA concentration of 1mg/ml (see the Appendix for the determination of the protein-synthesizing activity.

Amino acid incorporation by subcellular fractions. Samples (0.01 or 0.02ml) of post-mitochondrial supernatant, before and after Sephadex G-25 treatment, were incubated in a final volume of 0.1ml containing: 2mM-ATP; 0.25mM-GTP; 10mM-phosphoenolpyruvate; 0.31μCi of [^{14}C]leucine; 50μg of pyruvate kinase; 6.4mM-MgSO$_4$; 131mM-sucrose; 18.5mM-KCl; 28.9mM-tris–HCl buffer, pH7.8 at 20°C. A 0.01ml sample of resuspended microsomal fraction, membrane-free and membrane-bound ribosomes or detergent-treated polyribosomes (corresponding to 0.01mg of rRNA) was incubated as specified above; cell sap was added at a concentration of 0.5mg of cell sap protein/0.1ml of incubation mixture. In the experiments where different amounts of RNA were incubated, different volumes from different dilutions (5.0, 2.0 or 0.2mg/ml) of the same microsomal or ribosomal suspensions were used. Final incubation volumes were kept constant by addition of appropriate amounts of medium B. At the end of the incubation time (30min), samples were diluted to 1.5ml with ice-cold solution, containing 10mM unlabelled leucine and 10mM-EDTA (disodium salt), adjusted to pH7.0 with 4M-NaOH (Ragnotti *et al.* 1970). The radioactivity incorporated into the samples was determined as described by Ragnotti *et al.* (1969). Radioactivity was counted to an efficiency of 82% (background 16c.p.m.) in a Tri-Carb liquid-scintillation spectrometer (model 3365, Packard Instrument Co. Inc.) to a standard error of 5% or better.

Density-gradient analysis of polyribosome profiles. Post-mitochondrial supernatant was treated with sodium deoxycholate (1.3% final concentration) and 1ml was used for polyribosome profile determination. Polyribosome profiles of the resuspended microsomal fraction or membrane-bound ribosomes were obtained by adding an equal volume of concentrated cell sap to the microsomal fraction or the membrane-bound ribosomal suspensions. The concentrated cell sap was obtained from a 50% (w/v) homogenate. Sodium deoxycholate (1.3% final concentration) was then added to the suspension to release polyribosomes from the membranes and a portion (1ml) was used for the determination of the polyribosome profiles. Samples of membrane-free or detergent-treated polyribosomes were also analysed for polyribosome profiles, but in this latter case sodium deoxycholate was omitted. Polyribosome patterns were analysed by layering the samples over a 12ml linear density gradient, consisting

of sucrose, 15–50% (w/v) in TKM1 buffer. Centrifugation was performed at 40000rev./min for 180min, in a SW40 rotor of a Spinco L2-65B ultracentrifuge. The E_{260} was recorded in a Unicam SP.800 recording spectrophotometer, by pumping the gradient through a flow cell (0.2cm light-path) at a constant rate of 1ml/min. Parallel gradients read at 320nm to evaluate ferritin interference did not alter the polyribosome profiles. Recorded patterns have not been corrected to a 1cm light-path flow cell.

Measurement of RNA and protein in the subcellular fractions. The RNA content of the subcellular fractions for the determination of the specific radioactivity was determined on separate samples by the orcinol method (Mejbaum, 1939), with hydrolysed yeast RNA as standard. Cell-sap protein was determined by the biuret method (Layne, 1957), with purified albumin as standard.

Statistical treatment of the results. Statistical significance was assessed by the analysis of variance with the program GZC 116 for the Olivetti Programma 101 desk-top computer kindly supplied by the Institute of Biometrics and Medical Statistics, University of Milan. When the protein-synthesizing activity of liver subcellular fraction was compared, the probability values are specified in the text; in other cases (Table 1) they are given in the Table.

RESULTS

Relationship between the amount of ribosomal RNA incubated and the incorporation of [¹⁴C]leucine into proteins. (a) Microsomal fraction and polyribosomes. The effect of the amount of RNA incubated on the radioactivity incorporated into proteins was determined by incubating increasing amounts of microsomal (microsomal fraction A) or polyribosomal RNA in a mixture in which the amount of cell-sap protein and all other components were kept constant. The results obtained show that the radioactivity of the synthesized protein per mg of rRNA incubated decreases as the amount of RNA incubated increases, as shown in Fig. 1, where the radioactivity of the protein is plotted against the log of RNA concentration. It is therefore evident that in the comparison of the protein synthesizing activity of isolated subcellular fractions it is imperative that similar amounts of rRNA should be incubated. The curves (Fig. 1) show the importance of the cell-sap protein/rRNA ratio; it is evident that a decrease of the ratio causes the incorporation of less radioactivity into protein. In accordance with Munro, Jackson & Korner (1964), the ratio of 50 was used in subsequent experiments, i.e. 5mg of cell-sap protein and 0.1mg of rRNA/ml.

(b) Post-mitochondrial supernatant and the influence of treatment with Sephadex G-25. Post-mitochondrial supernatant was prepared by homogenizing 1g of liver mince either in 2vol. (post-mitochondrial supernatant 1) or in 8vol. (post-mitochondrial supernatant 2) of medium A. Both preparations were then assayed for protein synthesis
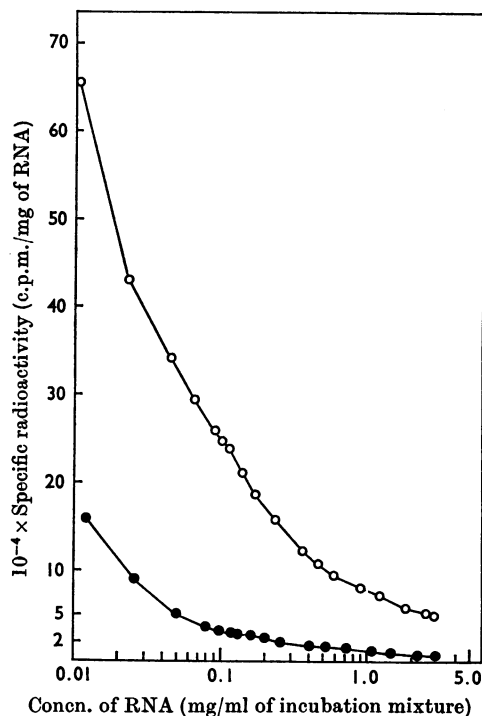

Fig. 1. Relationship between the amount of microsomal or polyribosomal RNA incubated (note logarithmic scale) and the specific radioactivity of the synthesized protein. Microsomal fraction (procedure A) and detergent-treated polyribosomes were isolated and incubated as specified in the Materials and Methods section. Cell sap was treated with Sephadex G-25 and its protein concentration was 5.3mg/ml of incubation mixture. ●, Microsomal fraction; ○, detergent-treated polyribosomes. The points represent the mean of five parallel independent experiments. The S.E.M. was 7–15%.

before and after treatment with Sephadex G-25. The results (Table 1) show that before Sephadex treatment the two preparations incorporate [¹⁴C]-leucine to a significantly different extent, i.e. the more diluted sample shows the highest specific radioactivity. After Sephadex treatment this difference is absent, i.e. the specific radioactivities of the proteins synthesized by post-mitochondrial supernatants 1 and 2 are not significantly different, regardless of their different RNA content. This could be caused by the fixed cell-sap protein/rRNA ratio of the post-mitochondrial supernatant. The ratio was 12 and Sephadex treatment had no influence on it.

Relative activity for the incorporation of [¹⁴C]-leucine into protein by various subcellular fractions. The ability of liver subcellular fractions to incorporate [¹⁴C]leucine *in vitro* is shown in Table 2.

Table 1. *Relationship between the amount of post-mitochondrial supernatant rRNA incubated and the specific radioactivity of the synthesized protein*

Effect of Sephadex G-25 treatment. Post-mitochondrial supernatant 1 and post-mitochondrial super-natant 2, obtained by homogenizing 1 g of liver mince in 2 and in 8 ml of medium A respectively, were pre-pared and 0.02 ml was incubated (see the Materials and Methods section). Radioactivity incorporated into protein represents the mean±S.E.M. of five independent experiments. RNA incubated (mean±S.E.M.) is shown under the corresponding fractions. The significance of the difference between corresponding treat-ments was assessed by the analysis of variance (see the Materials and Methods section). * $P<0.01$; † $P>0.05$.

Treatment ...	Post-mitochondrial supernatant 1		Post-mitochondrial supernatant 2	
	before Sephadex	after Sephadex	before Sephadex	after Sephadex
Radioactivity incorporated (c.p.m./mg of RNA)	21273 ± 3237	164967 ± 17829	63441 ± 5098*	178606 ± 15190†
Concn. of RNA (mg/ml)	0.530 ± 0.029	0.428 ± 0.019	0.133 ± 0.004*	0.107 ± 0.005*

Control experiments demonstrated that the in-corporation measured was energy- and temper-ature-dependent. Except in the case of post-mitochondrial supernatant, similar amounts of RNA (0.1 mg/ml per incubation) were incubated. The microsomal fraction and the membrane-bound ribosomes were prepared by procedure A (see the Materials and Methods section). The post-mitochondrial supernatant, membrane-free and detergent-treated polyribosomes were markedly more active than the microsomal fraction and the membrane-bound ribosomes. The lower protein-synthesizing activity shown by the microsomal fraction and the membrane-bound ribosomes could be due to (a) the presence of an inhibitor, (b) ribo-nuclease-induced degradation of mRNA and (c) polyribosome breakdown as the result of protein synthesis during the isolation procedure. To test these possibilities the following experiments were done. (a) First, membrane-bound and membrane-free ribosomes were separated and resuspended as described. Samples were then taken and mixed in different proportions, similar to those possibly occurring *in vivo* or in the unfractionated micro-somal preparation. Protein-synthesizing activity of membrane-free and membrane-bound ribosomes before and after mixing was then determined (Table 3). The specific radioactivities of the frac-tions, before mixing, enabled us to predict what their activity should have been when incubated to-gether. The calculated specific radioactivities are in close agreement with those found experiment-ally, showing that no interaction between the fractions occurred. A different explanation is then needed for the diminished protein-synthetic acti-vity of the unfractionated microsomal preparation. Secondly, a suspension of detergent-treated poly-ribosomes was incubated in the cold with different amounts of the supernatant obtained after sedi-mentation of the medium-resuspended microsomal preparation, before and after sonication. Samples

were then taken and assayed for protein synthesis and polyribosome profiles. Both parameters were unaffected when compared with the appropriate controls. These results seem to indicate that no inhibitor, acting on protein synthesis directly or through damage of polyribosomes, is associated or co-sediments with the microsomal fraction. (b) Polyribosomes, in the absence of cell sap, are very sensitive to ribonuclease (Blobel & Potter, 1966; Lawford, Langford & Schachter, 1966). As the study of the protein-synthetic activity of the microsomal fraction required a resuspension in medium, it seemed possible that polyribosome de-gradation occurred at this stage. Thus the in-fluence of the cell sap during resuspension was tested by determining the protein-synthetic activity and polyribosome profiles of the reconstituted post-mitochondrial supernatant. Two microsomal pel-lets were prepared by procedure A: one was resuspended with medium A and one with twice the normal concentration of cell sap, obtained from a 50% homogenate. Both microsomal suspensions were then made up to the original volume with, respectively, concentrated cell sap and medium A, so as to restore the conditions present in the original post-mitochondrial supernatant. Protein-synthetic activity and polyribosome profiles of these fractions and of the post-mitochondrial super-natant from which they had been prepared, were determined (Fig. 2). Polyribosomes from micro-somal fractions resuspended in medium or cell-sap were found to be degraded to practically the same extent; it is noteworthy that the post-mitochondrial supernatant retains an undegraded polyribosome profile compared with the isolated microsomal fraction, and hence a superimposed effect due to deoxycholate-activated ribonuclease can be ex-cluded. This result shows that, at least with our conditions, limitation of protein synthesis in microsomal preparations probably occurs because of polyribosome breakdown. Its occurrence even in

Table 2. *Protein-synthesizing activity of rat liver subcellular fractions*

Fractions from the same pooled livers were prepared and 0.01 ml of the suspension was incubated (see the Materials and Methods section). The microsomal fraction and the membrane-bound ribosomes were isolated by procedure A. Post-mitochondrial supernatant and cell sap were treated with Sephadex. Radioactivity incorporated into protein represents the mean ±S.E.M. of six parallel independent experiments. The amount of RNA incubated (mean ± S.E.M.) is shown under the corresponding fractions.

	Post-mitochondrial supernatant	Unfractionated microsomal preparation	Membrane-bound ribosomes	Membrane-free ribosomes	Detergent-treated polyribosomes
Radioactivity incorporated (c.p.m./mg of RNA)	180212±8001	57676±10918	68321±5860	198323±27000	233532±25981
Concn. of RNA (mg/ml)	0.1990±0.0150	0.1030±0.0114	0.0900±0.0045	0.0960±0.0032	0.0940±0.0063

Table 3. *Protein-synthesizing activity of microsomal preparations and of membrane-bound and membrane-free ribosomes, before and after mixing* (see the text)

Fractions were prepared and incubated as specified in the Materials and Methods section. The microsomal fraction and membrane-bound ribosomes were prepared by procedure A. Cell sap was treated with Sephadex. Radioactivity incorporated into protein represents the mean ±S.E.M. of four parallel independent experiments. The amount of RNA incubated (mean ±S.E.M.) is shown under the corresponding fractions. Theoretical values were obtained by mathematical combination, in the same proportions as for the experimental, of the specific radioactivities of membrane-bound and membrane-free ribosomes.

	Unfractionated microsomal preparation	Membrane-bound ribosomes	Membrane-free ribosomes	3:1 Experimental	3:1 Theoretical	2:2 Experimental	2:2 Theoretical	1:3 Experimental	1:3 Theoretical
Radioactivity incorporated (c.p.m./mg of RNA)	35014±4580	80170±4510	152591±17220	93785±4780	98275±6900	112993±4160	116381±10100	127622±5430	127536±16570
Concn. of RNA (mg/ml)	0.116±0.0251	0.097±0.0100	0.101±0.0063	0.097±0.0055	—	0.096±0.0082	—	0.096±0.0026	—

the presence of cell sap requires an explanation other than that of a ribonuclease-induced degradation of mRNA. (c) Polyribosome degradation could be the consequence of protein synthesis occurring during the isolation procedure. To test this possibility the microsomal fraction was isolated in the absence and in the presence of cycloheximide (0.25mg/ml; Korner, 1966). To ensure the constant presence of the inhibitor throughout the preparative procedure, in some instances the post-mitochondrial supernatant, with or without the inhibitor, was layered over a 1ml 2M-sucrose 'cushion' in TKM1 buffer, which, according to the post-mitochondrial
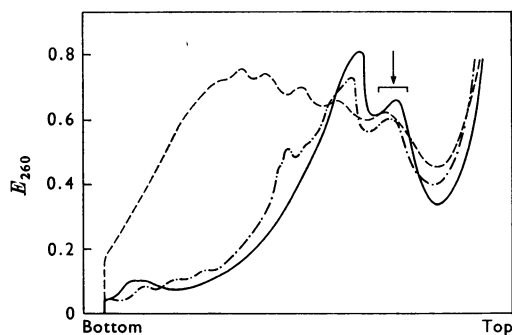

Fig. 2. Polyribosome profile of the resuspended microsomal fraction; influence of cell sap. A 1ml sample of post-mitochondrial supernatant or of the resuspended microsomal fraction was treated with deoxycholate in the presence of cell sap (see the Results section) and centrifuged on a 12ml linear gradient of sucrose (15–50%) in TKM1 buffer, for 180min at 40000rev./min in a Spinco SW40 rotor at 0°C. Polyribosome profiles of (————), post-mitochondrial supernatant (2.1mg of RNA), (———), medium (0.9mg of RNA), and ($\cdot$—$\cdot$—$\cdot$—$\cdot$) microsomal fraction resuspended in cell sap (0.9mg of RNA). The arrow indicates the position of monomers. The specific radioactivities (c.p.m./mg of RNA) of proteins synthesized by these fractions after Sephadex treatment were: 153089, post-mitochondrial supernatant; 36461 and 58525, medium and cell-sap-resuspended microsomal fraction respectively. For other experimental details, see the Results section.

supernatant used, contained or did not contain the inhibitor. After separation, the microsomal fraction was resuspended to its original volume with its own cell sap and polyribosome profiles were determined after deoxycholate treatment. Degradation of polyribosomes occurred to the same extent in the presence as in the absence of the inhibitor but, unexpectedly, regardless of the presence of the inhibitor, when the microsomal fraction was prepared in the presence of the 2M-sucrose 'cushion' their polyribosome profiles were identical with that of the original post-mitochondrial supernatant. Accordingly, protein-synthesizing activity of the microsomal fraction and membrane-bound ribosomes, isolated in the absence (procedure A) and in the presence (procedure B) of the 2M-sucrose 'cushion', was studied. In a preliminary experiment it was ascertained that the sucrose 'cushion' did not alter the ribosomal composition of the resuspended microsomal preparation. The recovery of rRNA, measured by the orcinol method, was almost identical in the presence and in the absence of the sucrose 'cushion'. The protein-synthesizing activity of the microsomal preparation and membrane-bound ribosomes, prepared from the same pooled livers, and isolated according to procedure A and B, is shown in Table 4. It is evident that when the microsomal preparation and membrane-bound ribosomes are prepared by procedure B, their protein-synthesizing activity is greatly enhanced (three times) over that obtained when the same fractions are prepared by procedure A. Polyribosome patterns of these preparations and of post-mitochondrial supernatant, membrane-free and detergent-treated polyribosomes, are shown in Fig. 3. Whereas polyribosomes isolated from A fractions are mostly found as monomers and dimers, polyribosomes from B fractions retain an undegraded profile, that compares well with that obtained from post-mitochondrial supernatant, membrane-free and detergent-treated polyribosomes. The kinetics of incorporation of [^{14}C]leucine into proteins by the various fractions was also studied. Fig. 4(a) shows that there is a marked difference between

Table 4. *Protein-synthesizing activity of rat liver microsomal and membrane-bound ribosomal preparation*

Effect of the sucrose 'cushion'. Fractions, prepared in the absence (A) and in the presence (B) of a 1ml 2M-sucrose 'cushion' in medium B, were incubated as specified in the Materials and Methods section. Within the fractions, the same pooled livers were used. Cell sap was treated with Sephadex. Radioactivity incorporated into protein is the mean±S.E.M. of six experiments. The amount of RNA incubated(mea n±S.E.M.) is shown under the corresponding fractions.

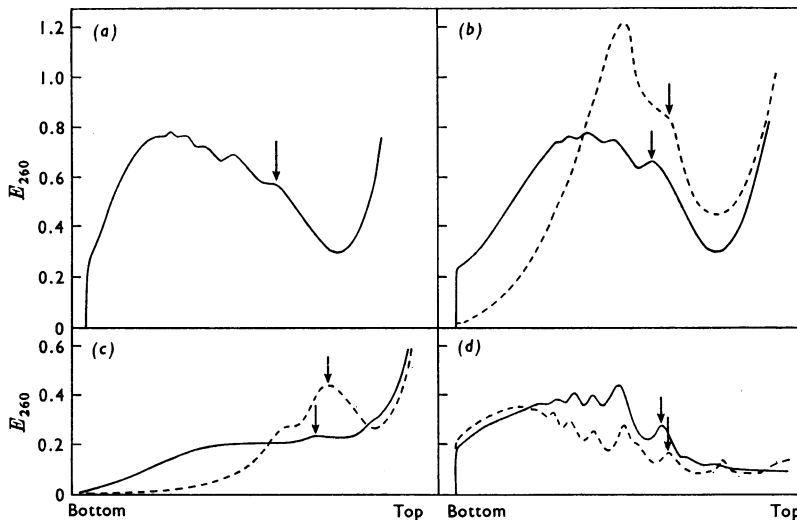|	Microsomal fraction		Membrane-bound ribosomes	
	A	B	A	B
Radioactivity incorporated (c.p.m./mg of RNA)	72783±15338	200042±18382	63203±7720	188370±5908
Concn. of RNA (mg/ml)	0.0950±0.0095	0.1010±0.010	0.10000±0.0032	0.0900±0.0022

Fig. 3. Polyribosome profiles of the fractions assayed for protein biosynthesis (Tables 2 and 4). Suspensions (1 ml), prepared as specified in the Materials and Methods section, were layered over a 12 ml linear gradient of sucrose (15–50%) in TKM1 buffer and centrifuged for 180 min at 40000 rev./min in a Spinco SW40 rotor at 0°C. Arrows indicate the position of monomers. (a) Post-mitochondrial supernatant (2.3 mg of RNA); (b) microsomal fraction prepared by procedure A (----) and procedure B (——), (1.5 and 2.1 mg of RNA respectively); (c) membrane-bound ribosomes prepared by procedure A (----) and procedure B (——), (0.6 and 0.6 mg of RNA respectively); (d) membrane-free (——) and detergent-treated (----) polyribosomes (1.1 and 1.0 mg of RNA respectively).

the kinetics of incorporation by the microsomal preparation and the membrane-bound ribosomes prepared by procedure A and those of membrane-free and detergent-treated polyribosomes. In contrast, the microsomal fraction and the membrane-bound ribosomes prepared by procedure B had the same activity for protein synthesis as detergent-treated polyribosomes. Fig. 4(b) shows the results for the post-mitochondrial supernatant. Sephadex treatment increased the amount of radioactivity incorporated, mostly as an increase of the initial rate of incorporation. Moreover, the rate of incorporation of [^{14}C]leucine into protein is very similar to that of the microsomal fraction and membrane-bound ribosomes (procedure B) and membrane-free and detergent-treated polyribosomes. Thus it is possible to conclude that when the microsomal fraction and the membrane-bound ribosomes are prepared by the modified procedure, their protein-synthetic activity is not significantly different from that obtained with Sephadex-treated post-mitochondrial supernatant and membrane-free or detergent-treated polyribosomes ($P > 0.05$).

DISCUSSION

The results will be discussed in two parts: (i), the influence of the amount of microsomal or ribosomal RNA incubated on the specific radio-activity of the synthesized protein and the protein-synthesizing activity of rat liver post-mitochondrial supernatant; (ii), the relevance of the results obtained when the protein-synthesizing activity of the membrane-associated polyribosomes was studied to the problem of the control of protein biosynthesis by the membranes of the endoplasmic reticulum.

The specific radioactivity of the synthesized protein decreased as the amount of ribosomal RNA incubated was increased if the microsomal fraction or isolated polyribosomes were incubated. In contrast with Sephadex-treated post-mitochondrial supernatant, at least in the range examined, the specific radioactivity of the synthesized protein was independent of the amount of rRNA incubated. It is notable that similar amounts of [^{14}C]leucine are incorporated with a cell-sap protein/rRNA ratio of 50 for the isolated microsomal fraction (B) or polyribosomes and a ratio of 12 with the post-mitochondrial supernatant. The post-mitochondrial supernatant would then appear to contain a factor that is required for protein-synthetic activity which is either not found or is not adequately provided in systems that contain microsomal fraction or polyribosomes plus cell sap.

However, the dependence of specific radioactivity on the amount of rRNA incubated, in the case of isolated subcellular fractions clearly indicates that
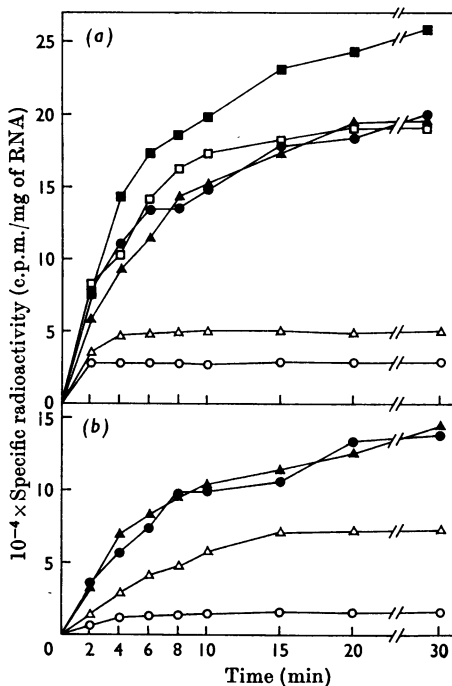
Fig. 4. Kinetics of the incorporation of [14C]leucine into protein by rat subcellular fractions. Fractions were prepared and 0.01 ml was incubated as specified in the Materials and Methods section. (a) ○, Microsomal fraction, △, membrane-bound ribosomes prepared by procedure A; ●, microsomal fraction; ▲, membrane-bound ribosomes prepared by procedure B; □, membrane-free, and ■, detergent-treated polyribosomes. (b) Post-mitochondrial supernatant prepared by homogenizing 1 g of liver in 2 ml and in 8 ml (post-mitochondrial supernatants 1 and 2 respectively) of medium A; 0.02 ml was incubated. Post-mitochondrial supernatant 1 (○) before and (●) after Sephadex G-25 treatment; post-mitochondrial supernatant 2 (△) before and (▲) after Sephadex G-25 treatment. Each curve represents a typical experiment.

to obtain comparable and constant results it is important to incubate similar amounts of rRNA. In this respect the method presented in the Appendix for a quick assessment of the concentration of rRNA in the subcellular fractions may be valuable.

The microsomal fraction isolated after sedimentation and resuspension by the usual technique had markedly less protein-synthesizing activity on the basis of rRNA, than had polyribosomal preparations. The evidence appeared to implicate the process of sedimentation and/or resuspension in the loss of activity. This view was confirmed when the microsomal preparation was centrifuged with a small 'cushion' of sucrose at the bottom of the centrifuge tube. Under these conditions the microsomal fraction had a protein-synthesizing activity similar to that of the polyribosomes. When the microsomal fraction or membrane-bound ribosomes prepared under the two sets of conditions were treated with deoxycholate in the presence of cell sap and the degree of aggregation of the ribosomes was analysed, marked differences between the fractions were found (Fig. 3). The microsomal fraction and the membrane-bound ribosomes prepared in the presence of the sucrose 'cushion' has a sucrose-density-gradient profile identical with that of the post-mitochondrial supernatant, whereas in the absence of the 'cushion' the ribosomes were found as monomers and dimers. It is therefore concluded that the difference in the protein-synthesizing activity of the fractions prepared under the two sets of conditions is attributable to degradation of the polyribosomes during centrifugation and/or resuspension in the absence of a sucrose 'cushion'. The implication from the above work is that polyribosomes, either attached to membranes (as in the rough surfaced endoplasmic reticulum) or free from membranes, have the same protein synthesizing activity. Hence the presence of membrane seems to have no effect on the activity of the polyribosomes.

From work on the activity of microsomal and polyribosomal preparations from regenerating or nephrotic rat liver it was shown that whereas polyribosomes from normal and experimentally treated animals have a similar activity there was a considerable difference in the activity of the microsomal fractions: those from regenerating (Campbell et al. 1964, 1965, 1967; Campbell & Lowe, 1967) or from nephrotic rat liver (Marsh, Drabkin, Braun & Parks, 1966) being more active than those from normal liver. From these results it was suggested that the membranes controlled the rate of protein synthesis. From the present experiments an alternative explanation may be that the microsomal preparation from regenerating or nephrotic livers possesses the same activity as the isolated polyribosomes but is less susceptible than the normal preparation to damage by centrifugation and/or resuspension in the absence of a sucrose 'cushion'.

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APPENDIX

A Rapid Method for the Determination of Ribonucleic Acid in Subcellular Fractions

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The methods available for RNA determination (Mejbaum, 1939; Schneider, 1957; Scott, Fraccastoro & Taft, 1956; Fleck & Munro, 1962), though very accurate, are time-consuming because of the many steps involved. They are not suitable for the rapid determination of RNA in the samples to be assayed for protein-synthesizing activity. The present method has therefore been developed.

Table 1. *RNA content of liver subcellular fractions determined by methods A and B (see the text)*

Values, represent the means±S.E.M. of the number of experiments given in parentheses. The A/B ratio was calculated for every pair of RNA values and is given as the mean±S.E.M. Regression equations were calculated by the program GZC 122 for the Olivetti Programma 101 desk-top computer. The range of RNA amounts (mg/ml) for which the methods were compared is given in brackets. In the regression equations ($y = a + bx$) x = mg of RNA/ml (method A) and y = mg of RNA/ml (method B).

	RNA content (mg/ml)				
	Method A	$\dfrac{\text{Mean}}{\text{S.E.M.}} \times 100$	Method B	$\dfrac{\text{Mean}}{\text{S.E.M.}} \times 100$	Ratio A/B
Post-mitochondrial supernatant (22)	4.57±0.491 [0.94–9.16]	11	2.26±0.240 [0.48–4.76]	11	2.03±0.044
Unfractionated microsomes (40)	1.82±0.307 [0.33–9.90]	17	1.35±0.223 [0.25–7.57]	17	1.35±0.022
Membrane-bound ribosomes (31)	2.14±0.419 [0.44–9.85]	20	1.28±0.234 [0.25–5.44]	18	1.67±0.026
Membrane-free ribosomes (23)	2.26±0.782 [0.29–16.36]	35	2.40±0.820 [0.31–17.24]	34	0.93±0.010
Detergent-treated polyribosomes (25)	4.07±1.200 [0.18–19.48]	29	4.51±1.352 [0.27–23.55]	30	0.90±0.025

Fraction	Regression equation	F
Post-mitochondrial supernatant	$y = 0.07081 + 0.4780\,x$	334
Unfractionated microsomes	$y = 0.01008 + 0.73615\,x$	5180
Membrane-bound ribosomes	$y = 0.08800 + 0.55848\,x$	3116
Membrane-free ribosomes	$y = 0.03536 + 1.04762\,x$	32038
Detergent-treated polyribosomes	$y = -0.04440 + 1.12080\,x$	2342