Effect of Sedimentation Through Sucrose Solutions on the Protein-Synthesizing Ability of Rat Liver Microsomes

By RUTH A. LESLIE* AND J. N. MANSBRIDGE

Department of Biochemistry, University of Queensland, St Lucia, Qld. 4067, Australia

(Received 2 February 1970)

1. Centrifugation of the postmitochondrial supernatant of rat liver through 1.0M-sucrose produces particles that have 85–95% less incorporating ability in a cell-free protein-synthesizing system than either ribosomes or microsomes. 2. The incorporation of [14 C]phenylalanine into protein by particles prepared by sedimentation through 1.0M-sucrose is stimulated about 20-fold by addition of poly U. 3. The content of rapidly labelled RNA of microsomes is decreased during centrifugation through 1.0M-sucrose. 4. It is suggested that degradation of mRNA occurs during the formation of the pellet in the centrifuge tube as a result of a membrane-bound alkaline ribonuclease, after removal of the ribonuclease inhibitor of the soluble fraction.

It was reported by Hunter & Korner (1966) that ribosomes prepared with a non-ionic detergent, Triton X-100, were more active in protein synthesis in a cell-free system than were ribosomes prepared with sodium deoxycholate. In further investigation of this detergent as a means for preparing ribosomes, it was found in this laboratory that concentrations of Triton X-100 from 0.1 to 1.0% gave active ribosomes, but when particles were prepared with 0.05 or 0.01% Triton X-100 or in the absence of Triton X-100 very low activities were obtained (Leslie & Mansbridge, 1967). In each case the particles were prepared by centrifugation through 1.0M-sucrose and it was evident that sedimentation of microsomes through the layer of dense sucrose solution caused a large decrease in activity. As dense sucrose solutions are now used extensively for the preparation of rat liver polyribosomes, ribosomes and microsomes and in sucrose-density-gradient centrifugation this phenomenon was further investigated. In the present paper evidence is presented that mRNA of microsomes is damaged during formation of the pellet after sedimentation through 1.0M-sucrose and possible explanations for this degradation are considered.

MATERIALS AND METHODS

Chemicals. DL-[14 C]Leucine (8.3mCi/mmol), L-[14 C]phenylalanine (44.2mCi/mmol) and [3 H]orotic acid (1000mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Poly U, the

trisodium salt of phosphoenolpyruvic acid, pyruvate kinase (EC 2.7.1.40; specific activity 500 μ mol of phosphoenolpyruvate/min per mg of enzyme at pH 7.6 and 37°C), the disodium salts of ATP and GTP and unlabelled amino acids were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. 1,4-Bis-(5-phenyloxazol-2-yl)-benzene and 2,5-diphenyloxazole were the products of Pilot Chemicals Inc., Watertown, Mass., U.S.A. Toluene was laboratory-reagent grade, sulphur-free, and Triton X-100 was obtained from Rohm and Haas, Philadelphia, Pa., U.S.A. Commercial yeast RNA was supplied by Seravac Laboratories, Maidenhead, Berks., U.K. All grade sucrose was the product of the Colonial Sugar Refining Co. Ltd., Brisbane, Qld., Australia. All other chemicals were analytical-grade reagents.

Animals. Male and female albino Wistar rats were used.

Preparation of cell sap, ribosomes and microsomes. Rat liver cell sap ('soluble fraction'), ribosomes and microsomes were prepared by the method of Munro, Jackson & Korner (1964) with the following modifications. Medium M contained 50mM-tris buffer adjusted to pH 7.4 at 4°C with HCl, 10mM-magnesium acetate or MgCl_2 , 40mM-NaCl, 100mM-KCl and 6mM-mercaptoethanol. Rat liver was homogenized in 10% (w/v) sucrose in medium M. Mitochondria, nuclei and debris were removed by centrifugation for 10 min at 11500g. The various particles were prepared by treatment of the postmitochondrial supernatant as follows.

Microsomes. The supernatant was centrifuged at 100000g for 90 min in the type 50 rotor of the Spinco model L ultracentrifuge. The pellet was resuspended in medium M and was used for the preparation of cell sap by passage through a column of Sephadex G-25.

1.0M-Sucrose-particles. The supernatant was layered over 5 ml of 1.0M-sucrose in medium M and centrifuged at 150000g for 3 h. The pellet was resuspended in medium M.

Ribosomes. The supernatant was treated with one-ninth

* Present address: School of Dental Science, University of Melbourne, Parkville N.2, Vic. 3052, Australia.

of its volume of an ice-cold solution of Triton X-100 in medium M to give a final concentration of 1.0% (v/v) Triton X-100. The detergent-treated supernatant was layered over 1.0M-sucrose and centrifuged at 150000g for 3 h. All particles were resuspended in medium M.

Protein synthesis in vitro. Protein synthesis *in vitro* was assayed by the method of Munro *et al.* (1964). The incubation mixture contained medium M adjusted to pH 7.4 at 37°C, 5mM-ATP, 0.2mM-GTP, DL-[¹⁴C]leucine or L-[¹⁴C]phenylalanine (1μCi/ml) and a mixture of unlabelled amino acids (1μg of each/ml) excluding the radioactive amino acid, approx. 6mg of soluble-fraction protein and 100μg of ribosomal protein in a final volume of 0.5ml. For 1.0M-sucrose-particles and for microsomes, which contain a higher proportion of protein than ribosomes, the ratio of protein in the soluble fraction to RNA in the particles was always greater than 60:1.

Preparation and measurement of radioactivity of labelled protein. Samples (0.1 ml) of the incubation mixture from protein-synthesis experiments were pipetted on to numbered squares of dry Whatman 3MM filter paper. These were dried and dropped into 0.5M-trichloroacetic acid. The papers were washed in three changes of trichloroacetic acid, boiled in acid for 30 min to break down aminoacyl-tRNA complexes, washed with a further change of trichloroacetic acid, then twice with ethanol-ether-chloroform (2:2:1, by vol.) and once with ether. They were dried in an oven at 60°C for 3 h. Radioactivity was measured in a Nuclear-Chicago 6068 scintillation counter, with the liquid scintillator 2,5-diphenyloxazole (4g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (50mg/l) in toluene. Results were corrected for quenching by using an external standard and by the channels-ratio method.

Determination of RNA. RNA was measured by the method of Scott, Fraccastoro & Taft (1956) as modified by Fleck & Munro (1962), by using a value of 34.2 for the extinction of a 1 mg/ml solution of hydrolysed RNA.

Determination of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used, with bovine serum albumin (fraction V; Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) as a standard.

Sucrose-density-gradient analysis. Preparations of ribosomes or microsomes were layered on a linear density gradient (4.0ml) from 0.5M- to 1.0M-sucrose with a 2.0M-sucrose 'cushion' (1.0ml) at the base of each tube. All

sucrose solutions were in medium M without mercaptoethanol. Centrifugation was carried out in the SW 39 head of the Spinco model L centrifuge at 112000g_{av} for 90 min. Fractions (0.1–0.2) were collected dropwise by piercing the base of each tube.

RNA was prepared by the method of Staehelin, Wettstein, Oura & Noll (1964). For the separation of RNA a 5–20% (w/v) sucrose gradient (5.0ml) in 0.1M-NaCl–0.01M-sodium acetate buffer, pH 5.1, was used. Gradients were centrifuged in the SW 39 head of the Spinco model L Centrifuge at 37000 rev/min for 4 h.

Radioactivity in ³H-labelled RNA was measured after precipitation with 3 vol. of ice-cold 0.5M-HClO₄. The precipitate was collected by filtration through Oxoid membrane filters, washed twice with 0.5M-HClO₄ and once with 2% acetic acid. The filters were dried in an oven at 60°C for 4 h and their radioactivity was measured in the scintillation counter with the liquid scintillator.

Assay for ribonuclease (EC 2.7.7.16). Yeast RNA was prepared as described by Utsunomiya & Roth (1966). In a final volume of 0.2 ml the incubation mixture contained 0.05 ml of the fraction for enzyme assay, 0.05 ml of 1% (w/v) purified RNA and either medium M or 0.05M-sodium acetate buffer, pH 5.8. The reaction was stopped by addition of ice-cold 0.5M-HClO₄ and the increase in acid-soluble products determined by measuring E₂₆₀.

RESULTS

When assayed in a cell-free protein-synthesizing system, the activity of microsomes on an RNA basis was not significantly different from that of ribosomes, but 1.0M-sucrose-particles had only 15% of the incorporating ability of ribosomes (Table 1). On addition of poly U, the stimulation obtained with 1.0M-sucrose-particles was about ten times that for ribosomes and microsomes. The final extent of incorporation on an RNA basis was similar for all three types of particle. The RNA/protein ratio indicated that the 1.0M-sucrose-particles had lost material rich in protein and with a low RNA content during preparation. The yields of RNA per ml of 10000g supernatant as microsomes or 1.0M-

Table 1. *Properties of ribosomes, microsomes and 1.0M-sucrose-particles*

The preparation and incubation of particles, RNA measurement and ribonuclease assay are described in the Materials and Methods section. Incubations for the measurement of protein synthesis were carried out for 10 min at 37°C. The ribonuclease assay system would measure ribonuclease activity greater than 70μg of yeast RNA hydrolysed/h per mg of particle RNA.

Type of particle	Activity in protein synthesis						Yeast RNA hydrolysed ($\mu\text{g}/\text{mg}$ of particle RNA per h)	
	Expt. I		Expt. II					
	$10^{-4} \times$ Radio- activity (d.p.m./ mg of rRNA)	Activity (%) of activity of ribosomes)	$10^{-4} \times$ Radioactivity (d.p.m./mg of RNA)		Stimulation with poly U (%)	RNA/ protein ratio		
			- poly U	+ poly U (250 $\mu\text{g}/\text{ml}$)				
Ribosomes	8.8	100	5.4	9.0	170	0.61-0.67	pH 5.8 negligible 630	pH 7.4 negligible 73
1.0 M-Sucrose particles	1.3	14.7	0.47	9.5	2020	0.25-0.33		
Microsomes	8.4	95.8	4.2	9.5	220	0.11-0.14	160	82
1.53 M-Sucrose particles	—	—	—	—	—	0.40-0.49	630	121

Fig. 1. Distribution of 1.0M-sucrose-particles and microsomes on sucrose density gradients. Rat liver microsomes were prepared 45 min after intraperitoneal injection of 0.3 mCi of [^3H]orotic acid into the rat. A 0.5–1.0M-sucrose gradient (4.0 ml) in medium M (without mercaptoethanol) with a 2.0M-sucrose 'cushion' (1.0 ml) was used. Centrifugation was at $112000g_{av.}$ for 90 min. Fractions (2.0 ml) were collected. The direction of centrifugal force was from right to left and the top of the gradient is indicated. (a) Microsomes; (b) 1.0M-sucrose-particles. —, E_{260} ;, radioactivity.

sucrose-particles agreed within 10% within any experiment. Ribonuclease activity was measured in the various preparations at pH 5.8 and 7.4 to test whether there was any change in the acid or alkaline ribonuclease activity. Alkaline ribonuclease activity was slightly higher in microsomes than in 1.0M-sucrose-particles, but the activities were at the limit of detection by the method. At pH 5.8 a relatively high activity was measured in both types of preparation, but the values were similar. Activity at this pH was decreased by 75% in particles prepared by centrifugation through 1.53M-sucrose. This method has been used by Scornik, Hoagland, Pfefferkorn & Bishop (1967) for the removal of lysosomes, and the results indicate considerable lysosomal contamination of microsomes and 1.0M-sucrose-particles. 1.53M-sucrose-particles did not show any change in ribonuclease activity at pH 7.4.

An alternative explanation for the breakdown of RNA is that protein synthesis may occur during the preparation procedures (Fessenden, Cairncross & Moldave, 1963; Fessenden & Moldave, 1963). This possibility was tested by addition of [^{14}C]leucine

Fig. 2. Distribution of RNA from 1.0M-sucrose-particles and microsomes on sucrose density gradients. RNA was prepared from liver microsomes of rats killed 45 min after intraperitoneal injection of 0.3 mCi of [^3H]orotic acid. A 5–20% (w/v) sucrose gradient (5.0 ml) in 0.1M-sodium acetate buffer, pH 5.1, was used. Centrifugation was at 37000 rev/min (SW 39 rotor) for 4 h. Fractions (0.1) were collected. (a) 1.0M-Sucrose-particles; (b) microsomes. —, E_{260} ;, radioactivity.

during preparation. No incorporation was observed.

Microsomes and 1.0M-sucrose-particles sedimented in two major peaks on a 0.5–1M-sucrose density gradient (Fig. 1); a large fast-moving peak in a position similar to that occupied by 80S ribosomes under similar conditions, and a slower one; the size of the latter peak varied in different preparations but was consistently larger for 1.0M-sucrose-particles than for ribosomes.

Analysis on sucrose density gradients of RNA prepared from microsomes and 1.0M-sucrose-particles obtained from rats injected with [^3H]orotic acid 45 min before being killed showed that considerable degradation occurred (Fig. 2). In RNA from 1.0M-sucrose-particles all the radioactivity remained at the top of the gradient, indicating extensive breakdown to low-molecular-weight products. Considerable breakdown of rRNA was also evident, although the activity of the poly U-stimulated protein-synthesizing system was not greatly affected by the treatment.

Evidence is presented that the loss of activity shown by the 1.0M-sucrose-particles occurs during formation of the pellet. The effect of varying the concentration of Triton X-100 during preparation of ribosomes is shown in Fig. 3. On examination under the electron microscope, membranes were

visible in preparations with 0.05% Triton X-100 but not with higher concentrations.

In the experiment shown in Table 2 centrifugation

Fig. 3. Effect on composition and protein-synthesizing activity of various concentrations of Triton X-100 during the preparation of ribosomes. Ribosomes and microsomes were prepared by adding Triton X-100 at the concentration shown to the 10000g supernatant of a rat liver homogenate treated as described in the Materials and Methods section. Incubation was carried out for 10 min at 37°C and incorporation measured as described. The column marked M shows the radioactivity and RNA content of microsomes.

through 0.5M-sucrose produced 83% inhibition. Sedimentation through 2.0M-sucrose caused no loss of activity. To determine whether the loss of activity with 0.5M-sucrose occurred during sedimentation or formation of a pellet, 0.5M-sucrose was layered over 2.0M-sucrose and microsomes were sedimented through the discontinuous gradient. In this experiment (Table 2) the pellet was formed after sedimentation through 2.0M-sucrose and the particles retained full activity. We conclude therefore that inactivation occurs during formation of the pellet and not during sedimentation through 0.5M-sucrose.

The inactivation of the microsomes during sedimentation through sucrose can be prevented by the addition of soluble fraction (Table 3). Particles prepared in this way retain 90% of the incorporating activity of the microsomes, and as the yield of RNA was approximately the same as for microsomes it is probable that the same population of particles is represented in each case. The proportion of the activity that could be derived from microsomes centrifuged out of the added cell sap was small (less than 9%). It has been shown (Roth, 1956, 1958; Shortman, 1961, 1962) that there is an inhibitor of alkaline ribonuclease present in the soluble fraction. It appears that during sedimentation through a discontinuous gradient a particulate ribonuclease is separated from its inhibitor in the soluble fraction and can then degrade mRNA.

Other possible explanations for the loss of activity of microsomes centrifuged through 1.0M-sucrose

Table 2. Activity of particles sedimented through various concentrations of sucrose

Incorporation of [¹⁴C]phenylalanine into protein during incubation at 37°C was measured at 0, 2.5, 5.0 and 30 min. Conditions of incubation were as described in the Materials and Methods section. Poly U (250 µg/ml) was added where indicated. The particles were prepared by centrifugation through various concentrations of sucrose. In each case the postmitochondrial supernatant was layered over the sucrose solution in medium M and centrifuged at 150000g for 3 h. For the 0.5/2.0M-sucrose-particles a lower layer of 2.0M-sucrose (2.5 ml) and an upper layer of 0.5M-sucrose (2.5 ml) were used.

Type of particle	-poly U		+poly U	
	Initial rate of incorporation (n mol/mg of RNA per min)	Total incorporation (n mol/mg of RNA)	Initial rate of incorporation (n mol/mg of RNA per min)	Total incorporation (n mol/mg of RNA)
Microsomes	0.37	2.3	0.52	3.1
0.5M-Sucrose-particles	0.09	0.4	0.26	0.95
1.0M-Sucrose-particles	0.19	1.0	0.45	2.0
1.53M-Sucrose-particles	0.47	2.8	0.93	4.5
2.0M-Sucrose-particles	0.89	4.6	0.53	4.5
0.5/2.0M-Sucrose-particles	0.92	5.5	0.88	5.9
Ribosomes	0.36	1.3	0.25	2.2

Table 3. *Protection of the microsomal protein-synthesizing system during sedimentation through 1.0M-sucrose by soluble fraction*

Incubation was for 45 min at 37°C, and the conditions of incubation were as described in the Materials and Methods section. The '1.0M-Sucrose-particles+soluble fraction' was prepared by sedimentation through 1.0M-sucrose containing soluble fraction.

Type of particle	$10^{-6} \times$ Incorporation of [14 C]leucine (d.p.m./mg of protein)	Activity (% of ribosomal activity)	Yield of RNA from 10ml of 10000g supernatant (mg)
Ribosomes	1.16	100	2.3
1.0M-Sucrose-particles	0.25	22	3.8
1.0M-Sucrose-particles+ soluble fraction	1.04	90	3.9
Microsomes	1.16	100	4.3

that were tested and shown not to apply included an inhibitor of protein synthesis in microsomes that was released in a functionally active form during centrifugation, a change in the requirement for an ATP-generating system, and mechanical damage (Smillie, 1956; Kahn & von Wettstein, 1961). No evidence could be obtained for lysosomal participation in the reaction. The acid ribonuclease of lysosomes is not active at the pH (7.4) used in these experiments.

DISCUSSION

Centrifugation of the postmitochondrial supernatant of rat liver through 1.0M-sucrose results in microsomal particles that are defective in a protein-synthesizing system *in vitro* when compared both with ribosomes and with microsomes (Table 1). An 85–95% decrease in incorporation has been observed for 1.0M-sucrose-particles relative to the incorporation by ribosomes. Addition of poly U to the incubation mixture gives a stimulation of incorporation of about 20-fold for the 1.0M-sucrose-particles, so that the amount of incorporation is raised to a value similar to that obtained when ribosomes are incubated in the presence of poly U.

We consider that this change is caused by breakdown of mRNA as a result of the activity of the ribonuclease which is inhibited by a factor in the soluble fraction of the cell. Its properties are consistent with the ribonuclease-inhibitor system described by Roth (1956, 1958) and Shortman (1961, 1962). The action of ribonuclease does not appear to take place during sedimentation of the microsomes through the sucrose but only becomes active in the pellet. These results may be compared with those of Blobel & Potter (1966) and Lawford, Langford & Schachter (1966), who showed that polyribosomes were broken down to dimers when microsomes were treated with detergent in the

absence of soluble fraction. Results obtained with microsomes sedimented in the absence of soluble fraction emphasize the importance of the membrane ribonuclease and the necessity for removing membranes thoroughly with detergents in the preparation of ribosomes. If an inadequate quantity of detergent is added and the membranes are not thoroughly removed, inactive preparations will result.

In experiments to locate the protein-synthesizing activity of microsomal fractions on a sucrose density gradient, difficulty is experienced in assaying the protein-synthesizing activity of microsomal fractions on a sucrose density gradient because of the very low concentrations of particles. As the microsomes are normally centrifuged in the absence of soluble fractions, they cannot be concentrated by centrifuging them into a pellet. It is necessary to have a sufficiently large microsome sample on the gradient so that assays of protein-synthesizing activity may be obtained from fractions without requiring concentration.

REFERENCES

- Blobel, F. & Potter, V. R. (1966). *Proc. natn. Acad. Sci. U.S.A.* **55**, 1283.
- Fessenden, J. M., Cairncross, J. & Moldave, K. (1963). *Proc. natn. Acad. Sci. U.S.A.* **49**, 82.
- Fessenden, J. M. & Moldave, K. (1963). *Nature, Lond.*, **199**, 1172.
- Fleck, A. & Munro, H. N. (1962). *Biochim. biophys. Acta*, **55**, 571.
- Hunter, A. R. & Korner, A. (1966). *Biochem. J.* **100**, 73F.
- Kahn, A. & von Wettstein, D. (1961). *J. Ultrastruct. Res.* **5**, 557.
- Lawford, G. R., Langford, P. & Schachter, H. (1966). *J. biol. Chem.* **241**, 1835.
- Leslie, R. A. & Mansbridge, J. N. (1967). *Aust. J. Sci.* **30**, 133.

- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Munro, A. J., Jackson, R. J. & Korner, A. (1964). *Biochem. J.* **92**, 289.
- Roth, J. S. (1956). *Biochim. biophys. Acta*, **21**, 34.
- Roth, J. S. (1958). *J. biol. chem.* **231**, 1085.
- Scornik, O. A., Hoagland, M. B., Pfefferkorn, L. C. & Bishop, E. A. (1967). *J. biol. Chem.* **242**, 131.
- Scott, J. F., Fraccastoro, A. P. & Taft, E. B. (1956). *J. Histochem. Cytochem.* **4**, 1.
- Shortman, K. (1961). *Biochim. biophys. Acta*, **51**, 37.
- Shortman, K. (1962). *Biochim. biophys. Acta*, **55**, 88.
- Smillie, R. M. (1956). *Aust. J. biol. Sci.* **9**, 347.
- Staehelin, T., Wettstein, F. O., Oura, H. & Noll, H. (1964). *Nature, Lond.*, **201**, 264.
- Utsunomiya, T. & Roth, J. S. (1966). *J. Cell Biol.* **29**, 395.