A Comparison of Methods for the Isolation and Fractionation of Reticulocyte Ribosomes

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1. Polysomes, ribosomes and pH 5 enzymes were isolated from rabbit reticulocytes by acidifying the post-mitochondrial supernatant to pH 6·0 to precipitate all ribonucleoprotein particles and about half the pH 5 enzymes; the precipitate was redissolved in buffer, pH 7·6, and fractionated by zone centrifuging. 2. The isolation of polysome-rich and ribosome-rich fractions from the post-mitochondrial supernatant was also examined. 3. Studies of the stability of polysomes revealed that dissociation into sub-units occurred when both bound and free Mg^{2+} was chelated by EDTA or when the pH was increased above pH 8·8.

Several aspects of protein biosynthesis are now widely accepted (Lipmann, 1963). In bacterial cells the genetic information required for the synthesis of a protein is carried from the chromosome to the site of synthesis by a relatively unstable fraction of RNA (m-RNA*), which combines with ribosomes to form polyribosomal structures (polysomes). m-RNA, although it appears to be more stable, plays a similar role in mammalian cells.

Reticulocytes are of particular interest to studies of protein biosynthesis because they synthesize mainly one protein, haemoglobin (Schweet, Lamfrom & Allen, 1958). Polysomes from reticulocytes would be expected to be a source of homogeneous messenger. Possible methods for isolation of polysomes have therefore been explored. The isolation of polysomes and ribosome-rich fractions may be achieved by differential centrifuging (Arnstein & Cox, 1963). An alternative procedure is based on the observation that polysomes and ribosomes are precipitated from the post-mitochondrial supernatant at pH 6·0. The concentrated suspensions of ribonucleoprotein particles obtained on resuspending the precipitate may then be fractionated by zone centrifuging. The stability of polysomes isolated in this way has also been studied, primarily to find conditions favouring the dissociation of intact m-RNA from polysomes (cf. Gros *et al.* 1961).

METHODS

The post-mitochondrial supernatant from rabbit reticulocytes was prepared as described by Arnstein, Cox & Hunt (1964). Ribosome fractions were isolated either by differential centrifuging or by precipitation at pH 6 as described in the Results section.

Centrifuging. An MSE Major centrifuge was used for all low-speed centrifuging. A Spinco model L ultracentrifuge was used in all other preparative work.

Sucrose gradients were prepared by layering 15%, 20%, 25% and 30% (w/v) sucrose solutions in 10 mM-KCl–1·5 mM-$MgCl_2$–10 mM-tris–HCl buffer, pH 7·6 (cf. Warner, Knopf & Rich, 1963). A period of about 24 hr. was allowed for the formation of a linear gradient. Satisfactory results were obtained with both swing-out (Spinco SW 25) and fixed-angle (Spinco no. 30) rotors. Fractions were collected by a siphoning technique.

A Spinco model E ultracentrifuge fitted with u.v.-absorption optics was used for analytical work. The extinction of the photographic film was measured with a double-beam recording microdensitometer (Joyce–Loebl, Newcastle upon Tyne).

Amino acid incorporation by the cell-free system. Ribosomes were isolated from lysed reticulocytes either by centrifuging at 105000*g* for 1 hr. or by precipitation at pH 5. The incorporation of L-[^{14}C]phenylalanine into protein by the cell-free system described by Arnstein *et al.* (1964) was determined after incubation for 1 hr. at 37°. In Expt. 1 either pH 5 enzymes (2·4 mg.) and P_{60} ribosomes (1 mg.) (see the Results section for nomenclature of ribosomes), washed once by resuspension in medium A_1 (0·25 M-sucrose – 25 mM-KCl – 1 mM - $MgCl_2$–50 mM - tris–HCl buffer, pH 7·6) and resedimenting at 105000*g* for 1 hr., or ribosomes (1·4 mg.) isolated together with the enzyme fraction (4·2 mg.) by precipitation at pH 5 were used. Each tube contained (in 0·9 ml.) KCl (48 μmoles), $MgCl_2$ (8 μmoles), tris–HCl buffer, pH 7·6 (45 μmoles), L-[^{14}C]phenylalanine (2 μc, specific activity 10 μc/μmole) in 0·05 ml. of the amino acid mixture described by Borsook, Fischer & Keighley (1957), phosphoenolpyruvate (10 μmoles), ATP (1 μmole), GTP (0·25 μmole) and pyruvate kinase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) (0·1 mg.). In Expt. 2, ribosome fractions from a sucrose density gradient

* Abbreviations: m-RNA, messenger RNA; s-RNA, transfer RNA.

(0·5mg. of ribonucleoprotein) were incubated with pH5 enzymes (2·8mg.), L-[^{14}C]phenylalanine (0·0625μc, specific radioactivity 5μc/μmole) in 0·05ml. of the equimolar amino acid mixture described by Arnstein *et al.* (1964), glutathione (4μmoles), KCl (20μmoles), $MgCl_2$ (2μmoles), tris–HCl buffer, pH7·6 (20μmoles), phosphoenolpyruvate (2·5μmoles), ATP (0·125μmole), GTP (0·03μmole) and pyruvate kinase (40μg.) in a total volume of 0·4ml. Polyuridylic acid (50μg.) was added where shown. In this experiment, the incubation was stopped by cooling to 0° and adding N-NaOH (0·3ml.). The solution was then kept at 37° for 15min. to hydrolyse aminoacyl-s-RNA, cooled again to 0° and acidified with 5% (w/v) trichloroacetic acid (3ml.) containing unlabelled DL-phenylalanine (1mg.). The protein was isolated by filtration on 3cm. diam. Oxoid membrane filters (Oxo Ltd., London, E.C.4), and washed twice with cold 5% trichloroacetic acid (5ml.), twice with hot (90°) 5% trichloroacetic acid (5ml.) and twice with water (5ml.). The samples were counted at infinite thinness with a low-background (1·5counts/min.) counter (model 181B; Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A.) fitted with an ultra-thin window giving a counting efficiency of approx. 15%.

RESULTS

A portion (4 ml.) of the post-mitochondrial supernatant was fractionated on a sucrose gradient and centrifuged (Spinco model L centrifuge, no. 30 rotor; 150 min. at 78410*g*), and approx. 1 ml. fractions were collected. The extinctions at 260mμ and 415mμ (haemoglobin) were measured. Two peaks are apparent from the plot of extinction at 260mμ (E_{260}) against fraction number (Fig. 1) corresponding to ribosomes (R) and polysomes (P) as reported by Warner, Rich & Hall (1962). However, the difficulties of handling larger volumes of lysate led to the examination of other procedures.

Fractionation of ribosomes by differential centrifuging. Portions of the post-mitochondrial supernatant were centrifuged for 20, 60 and 240min. at 105000*g* (Spinco model L centrifuge, no. 40 rotor). The pellets (P_{20}, P_{60} and P_{240}) that were obtained were redissolved in buffer A_4 (0·25M-sucrose–1·5mM-magnesium chloride–25mM-potassium chloride–50mM-tris–hydrochloric acid buffer, pH7·6) and fractionated by zone centrifuging.

The distribution of ribonucleoprotein particles throughout the gradient was the same for pellets P_{20} and P_{60} (Fig. 2*a* and 2*b* respectively), which were appreciably richer in polysomes than ribosomes (cf. Fig. 1). The ratio of polysomes to ribosomes found for P_{240} (Fig. 2*c*) probably corresponds to the ratio of those species in the lysate.

Residual ribonucleoprotein particles were recovered from the supernatants S_{20}, S_{60} and S_{240} corresponding to pellets P_{20}, P_{60} and P_{240} by acidifying to pH5·1 with N-acetic acid and centrifuging at 1800*g* for 20min., when a precipitate of amino acid-activating enzymes and residual nucleoprotein particles was obtained. The precipitates

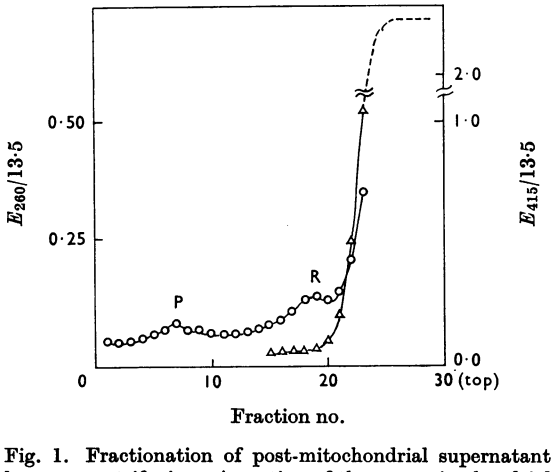

Fig. 1. Fractionation of post-mitochondrial supernatant by zone centrifuging. A portion of the post-mitochondrial supernatant (4·5ml.) was layered on a sucrose gradient (30ml.; 15–30% sucrose in 10mM-KCl–1·5mM-$MgCl_2$–10mM-tris–HCl buffer, pH7·6) for 2·5hr. at 78410*g* (Spinco model L centrifuge, no. 30 rotor). ○, E_{260}; △, E_{415} (the values indicated by the broken line are conjectural). R, Ribosome peak; P, polysome peak.

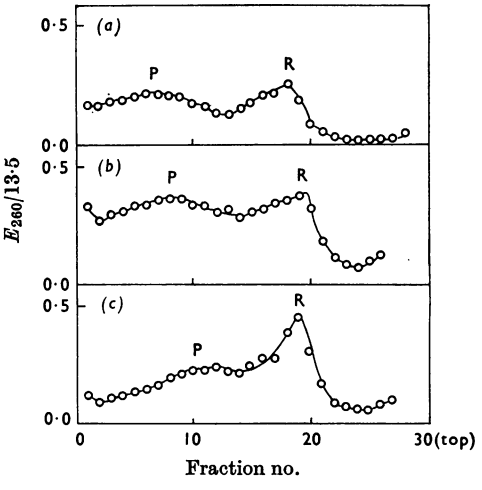

Fig. 2. Sedimentation profile of ribonucleoprotein particles obtained as a pellet from the post-mitochondrial supernatant by differential centrifuging for various periods. The pellets obtained by centrifuging the post-mitochondrial supernatant at 100000*g* for (*a*) 20min., (*b*) 60min. and (*c*) 240min. were dissolved in buffer A_1 layered on a sucrose gradient (see Fig. 1) and centrifuged at 53500*g* for 3·5hr. (Spinco model L centrifuge, SW25 rotor). The final volumes were (*a*) 28·5ml., (*b*) 29·0ml. and (*c*) 29·5ml. R, Ribosome peak; P, polysome peak.

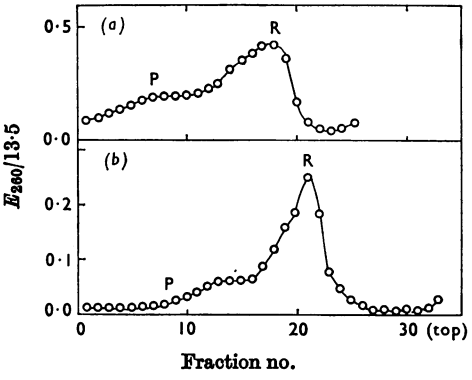

Fig. 3. Sedimentation profile of ribonucleoprotein particles remaining in the post-mitochondrial supernatant after differential centrifuging. The supernatant remaining after centrifuging at 100000*g* for (*a*) 20min. and (*b*) 60min. was brought to pH5 to precipitate ribonucleoprotein particles. After dissolution in buffer A_1 the ribonucleoprotein particles were fractionated on a sucrose gradient as described in Fig. 2. The total volume was 29·0ml. The extinction of each fraction was measured after dilution 13·5-fold. R, Ribosome peak; P, polysome peak (estimated on the basis of the assumption that the distance travelled along the gradient by any species is proportional to its sedimentation coefficient).

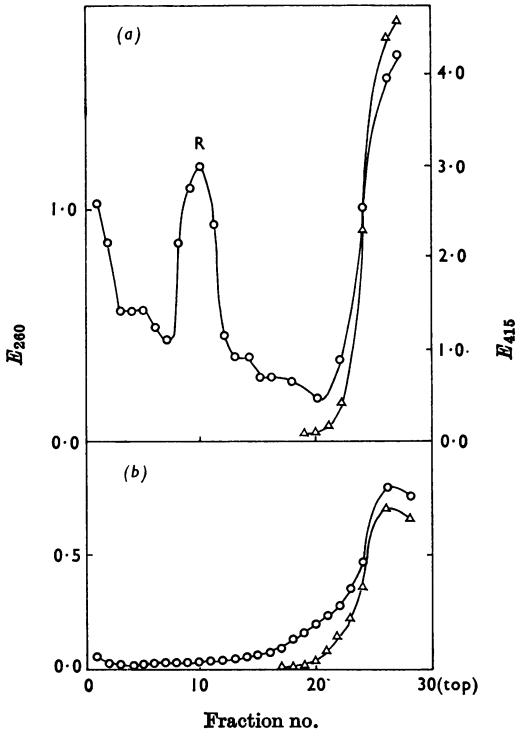

Fig. 4. Sedimentation profile of fractions obtained from the post-mitochondrial supernatant by precipitation at different pH values. The post-mitochondrial supernatant was brought to pH5·6–6·0 by titration with acetic acid. The precipitate was separated by centrifuging at low speed, dissolved in buffer A_1 and (*a*) fractionated by zone centrifuging as described in Fig. 1. The pH5·6–6·0 supernatant was then brought to pH5·0 and the precipitate so obtained was dissolved in buffer A_1 and (*b*) fractionated. ○, E_{260}; △, E_{415}. R, Ribosome peak; polysomes were obtained as a pellet (see Fig. 5).

were resuspended in buffer and fractionated on a sucrose gradient (Figs. 3*a* and 3*b*). Both polysomes and ribosomes were recovered from the S_{20} supernatant although ribosomes were the more abundant species. Principally ribosomes (amounting to 20% of the total ribonucleoprotein) were recovered from the S_{60} supernatant. No ribonucleoprotein particles were recovered from the S_{240} supernatant.

Isolation of ribonucleoprotein particles by precipitation from acidic solutions. Acidification of the post-mitochondrial supernatant to pH6·0–6·2 with N-acetic acid precipitates all the ribonucleoprotein particles and about half of the total 'pH5 enzymes', which may be isolated by centrifuging at 1800*g* for 20min. (cf. Figs. 4*a* and 4*b*). This pH range marks the upper limit, but functional polysomes have been recovered from solutions acidified to pH5 or less (Table 1). The precipitate obtained at pH6 was readily suspended in buffer (medium A_1) and the solution could be stored indefinitely at −12°. For routine preparations of polysomes and ribosomes the suspension was layered on a sucrose gradient and centrifuging (Spinco model L centrifuge, no. 30 rotor; 150min. at 78410*g*) was continued until the polysomes had sedimented.

Ribosomes were recovered from fractions 5–15 (Fig. 4*a*) by precipitation with acid at pH6, but for complete precipitation from very dilute suspensions a pH of about 5 is required. The homogeneity of the polysome and ribosome fractions was assessed by further fractionation on a sucrose gradient (Spinco SW25 rotor). Figs. 5(*a*) and 5(*b*) show that the polysome fraction is contaminated with about 10–15% of ribosomes, which may, however, have arisen by degradation during manipulation. The ribosome fraction is free from polysomes, the asymmetry of the ribosome peak being attributable to the effect of concentration on the sedimentation velocity.

The biological activity in a cell-free system of the polysome and ribosome fractions is not diminished by this isolation procedure (Expt. 1 in Table 1). The polysome fraction was found to have about 12 times the ability of ribosomes to incorporate amino acids into acid-insoluble protein (Expt. 2 in

Table 1. *Amino acid incorporation into protein by ribosomes isolated by different procedures*

Experimental details are given in the Methods section. —, Not determined.

Expt. no.	Method of preparation	Ribosome fraction	Amino acid incorporation (mμmoles of phenylalanine/mg. of ribosomes) Basal	With polyuridylic acid
1	Differential centrifuging	P_{60}	0·15	—
1	Precipitation at pH 5	Total	0·27	—
2	Zone centrifuging and precipitation at pH 6	Pellet (polysomes)	0·20	5·7
2	Zone centrifuging and precipitation at pH 6	Intermediate	0·05	3·9
2	Zone centrifuging and precipitation at pH 6	Ribosomes	0·02	6·3

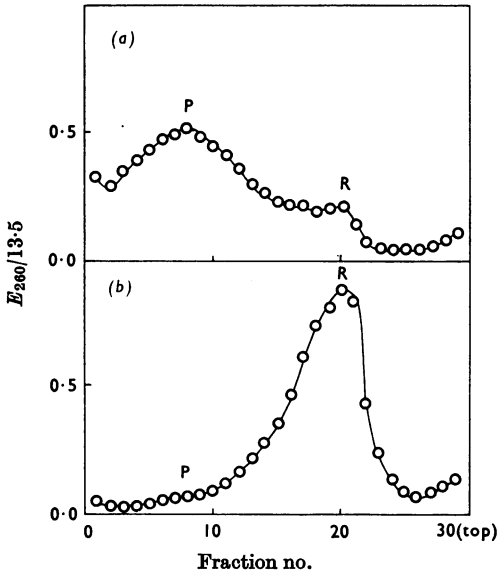

Fig. 5. Sedimentation profiles of ribonucleoprotein fractions obtained from the pH 6·0 precipitate by zone centrifuging. (*a*) The pellet obtained after centrifuging (Fig. 4*a*) was dissolved in buffer A_1 and fractionated on a sucrose gradient (Spinco model L centrifuge, SW 25 rotor) as described in Fig. 2. (*b*) The ribonucleoprotein particles were recovered from fractions 5–15 (Fig. 4*a*) by precipitation at pH 5. The precipitate was dissolved in buffer A_1 and fractionated as in (*a*). R, Ribosome peak; P, polysome peak. In both cases 1 ml. fractions were collected.

Table 1). Moreover, the addition of polyuridylic acid to the cell-free system containing ribonucleoprotein particles isolated by the pH 6 precipitation method stimulated the incorporation of phenylalanine into acid-insoluble protein (Expt. 2 in Table 1; cf. Arnstein, Cox & Hunt, 1962), both ribosomes and polysomes being stimulated to about the same extent.

Stability of ribonucleoprotein particles. The stability of the ribonucleoprotein particles was studied by examining their sedimentation properties. The sedimentation pattern at about 0° (cf. Fig. 5*a*) of polysomes (0·1 mg./l.) in 0·25 M-sucrose–1 mM-magnesium chloride–25 mM-potassium chloride–50 mM-tris–hydrochloric acid buffer, pH 7·6, was largely unchanged by incubation for 10 min. at 37° (Fig. 6*a*), i.e. conditions similar to those used for protein synthesis in a cell-free system (Arnstein *et al.* 1964), but incubation for 30 min. led to the breakdown of about 50% of the polysomes (Fig. 6*b*). Exposure to pH 4·7 at 0° did not affect the sedimentation pattern.

The stability of ribonucleoprotein particles depended on the concentration of ions in solution. Polysomes were stable in 5 μM-magnesium chloride, and no breakdown was found when sodium chloride was added to a final concentration of 40 mM (cf. Ts'o & Vinograd, 1961) (Table 2). The dilution of concentrated polysome solutions with sodium phosphate led to the formation of ribosomal sub-units (30 and 50 s), although some undissociated particles remained (cf. Lamfrom & Glowacki, 1962). The addition of EDTA led to complete dissociation into sub-units of low *S* values (20 and 40 s). The sub-units were fractionated on a sucrose gradient (10–25% sucrose in 1 mM-tris–hydrochloric acid buffer, pH 7·6) and the *S* values were then measured in the presence and absence of Mg^{2+}. Values of 27·4 and 48·1 s were obtained (Table 3) for the sub-units in the presence of 1·5 mM-magnesium chloride. Dissociation of polysomes into sub-units required sufficient EDTA to bind both free and bound Mg^{2+} (the latter was found to be roughly 1 mole of Mg^{2+}/g.atom of RNA phosphorus).

About 30% dissociation of polysomes into sub-units was observed when concentrated stock solu-

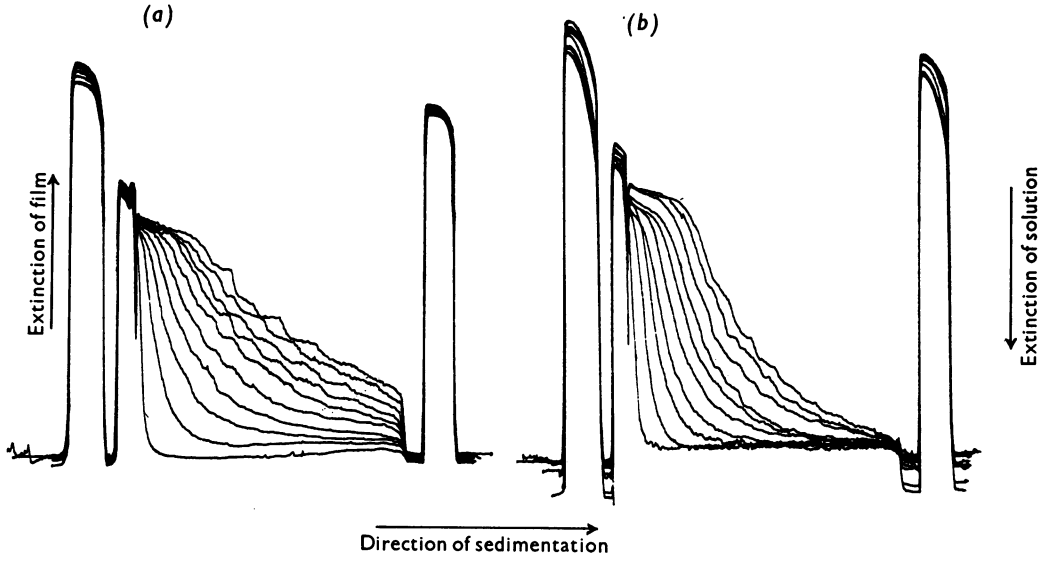

Fig. 6. Sedimentation properties of polysomes incubated for (*a*) 10min. and (*b*) 30min. at 37° in buffer A_1 adjusted to pH 7·6 at 37°. The ribonucleoprotein preparations (0·1mg./ml. of sucrose-free buffer A_1) were centrifuged at 29500rev./min. at (*a*) 4·8° and (*b*) 7·2° in the Spinco model E analytical ultracentrifuge. Photographs were taken at 4min. intervals and the extinctions of the films were estimated with a Joyce–Loebl microdensitometer. The direction of sedimentation is from left to right.

Table 2. *Effects of ionic strength and* pH *on the stability of ribosomes at about* 0°

Experimental details are given in the Methods section.

Solvent	Components	Contribution to total extinction (%)	$S_{20,w}$ (s)
10 μM-$MgCl_2$	Ribosomes	26	73
	Polysomes	74	182*
5 μM-$MgCl_2$–40mM-NaCl	Ribosomes	46	69
	Polysomes	54	162*
25mM-Phosphate buffer, pH7·6	Smaller sub-unit	13	32
	Larger sub-unit	36	48
	Ribosomes	30	71
	Polysomes	21	108*
5mM-EDTA–25mM-sodium phosphate, pH7·6	Smaller sub-unit	40	20
	Larger sub-unit	60	40
0·1mM-$MgCl_2$–10mM-tris–HCl buffer, pH7·6–8·8	Smaller sub-unit Larger sub-unit	30	35 60
	Ribosomes	10	77
	Polysomes	60	187*
0·1mM-$MgCl_2$–10mM-tris–HCl buffer, pH9·0	Smaller sub-unit		30
	Larger sub-unit		49
0·1mM-$MgCl_2$–10mM-tris–HCl buffer, pH10·0	Smaller sub-unit		18
	Larger sub-unit		43

* Mean value for species sedimenting faster than ribosomes.

Table 3. *Sedimentation coefficients of sub-units prepared by EDTA treatment*

Experimental details are given in the Methods section.

Solvent	Component	$S_{20,w}$ (s)
10mM-Potassium phosphate buffer, pH7·6	Smaller sub-unit	20·2
	Larger sub-unit	36·5
25mM-KCl–1mM-$MgCl_2$–50mM-tris–HCl buffer, pH7·6	Smaller sub-unit	27·4
	Larger sub-unit	48·1

tions (in buffer A_1) were diluted so as to give a solution 0·1mM-magnesium chloride–10mM-tris–hydrochloric acid buffer, pH 7·6–8·8 (cf. Hamilton & Petermann, 1959; Luzzatto, Banks & Marks, 1964). Complete dissociation was noticed at pH 9·0. When the pH of the tris–hydrochloric acid buffer was increased still further the sedimentation coefficients of the sub-units decreased from about 30 and 50 s to about 18 and 43 s. These results are summarized in Table 2.

DISCUSSION

Differential centrifuging. The limits of fractionation that may be achieved by differential centrifuging can be derived on the basis of the assumption that the centrifugal field is constant throughout the rotor. Let fractions $A_1, A_2, \ldots, A_n$ of the mass of the solute have sedimentation coefficients S_1, S_2, $\ldots$, S_n respectively.

After a time, t, that fraction of the slowest-sedimenting species (S_1) initially present at the meniscus will have travelled a fraction X of the total path-length and a corresponding fraction XA_1S_1 will have sedimented. By this time the corresponding fractions of other species will have travelled a distance $(S_n/S_1)X$ from the meniscus and the mass sedimented will be $(S_n/S_1)XA_n$. Hence the pellet will be a fraction y of the total mass, where

$$y = \sum_1^n (S_n/S_1)XA_n \qquad (1)$$

provided that $(S_n/S_1)X < 1$, i.e. the fastest-sedimenting species has not all sedimented. The proportion, p, of any species in the pellet is given by the equation:

$$p = (S_n/S_1)XA_n / \sum_1^n (S_n/S_1)XA_n \qquad (2)$$

although the mass of pellet increases from $t = 0$, its composition is independent of X and so also of the period of centrifuging. Thus the compositions of the ribosome fractions P_{20} and P_{60} were found to be identical (see Figs. 2*a* and 2*b*). Once the fastest-sedimenting species has all sedimented further centrifuging decreases the fractionation achieved, and ultimately when all species have sedimented the pellet has the same composition as the unfractionated mixture.

At time t the supernatant will have the composition:

$$(1-y) = \sum_1^n (1-S_nX/S_1)A_n \qquad (3)$$

and the proportion, q, of the slowest-sedimenting species will be:

$$q = (1-X)A_1 / \sum (1-S_nX/S_1)A_n$$

and will increase on further centrifuging (see Figs. 3*a* and 3*b*, and contrast them with Figs. 2*a* and 2*b*). Eventually when all of species S_2 will have sedimented ($S_2X/S_1 \geqslant 1$) only species S_1 will be present in the supernatant. Knowing that ribosomes and polysomes account for one-third and two-thirds of the mass and have S values of about 80 and 192 s respectively it may be calculated from eqn. (2) that ribosomes will be present in the pellet and from eqn. (3) that about 50% of the ribosome fraction may be obtained free of polysomes from the supernatant. The pellet obtained after centrifuging the post-mitochondrial supernatant for 1hr. at 100000*g* contains 75% of polysomes. This proportion is increased to about 85% when the pellet is resuspended in buffer and again centrifuged for the same period.

Zone centrifuging. The results (Figs. 3–6) show that the ribonucleoprotein fraction from large volumes of the post-mitochondrial supernatant may be concentrated by precipitation at pH 6·0 before zone centrifuging (cf. Wettstein, Staehelin & Noll, 1963). Both ribosomes and polysomes are stable in medium A_1 over the range pH5–8 (Table 1), and biological activity is retained so that the method is suitable for routine use.

Stability of ribosomes and polysomes. Polysomes from *Escherichia coli* can be reversibly dissociated into ribosomes and m-RNA by decreasing the concentration of Mg^{2+} (Gros *et al.* 1961). In principle, this property offers a simple method for the isolation of m-RNA free from ribosomal RNA. However, polysomes from reticulocytes do not respond in the same way to changes in the concentration of Mg^{2+}. Reticulocyte polysomes are stable for at least several hours at 0° in 5μM-magnesium chloride even if the concentration of sodium chloride is increased to 40mM (Na^+/Mg^{2+} ratio 8000:1). More drastic conditions lead to the breakdown of polysomes into sub-units, apparently without the intermediate formation of ribosomes. The sub-units found on addition of EDTA have relatively low sedimentation coefficients, which are, however, increased somewhat (to 27 and 48 s

respectively) by the addition of Mg^{2+}. So far it has not been possible to demonstrate the re-formation of metabolically active ribosomes under these conditions. It has also not yet been established whether m-RNA is released during dissociation or remains attached to one or other of the sub-units.

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