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# Isolation and Properties of Polyribosomes and Fragments of the Endoplasmic Reticulum from Rat Liver

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1. A centrifugation method for the fractionation of the postmitochondrial fraction from rat-liver homogenates is described. The technique, in which no detergent is used, may be used as a tool to discriminate between two classes of ribosomes. One class is firmly bound to membranes and the other consists either of free polysomes or of ribosomes attached by weaker forces to the membranes of the endoplasmic reticulum. 2. Electron-micrograph studies revealed that the polysomes were not contaminated with bound ribosomes or with membranous fragments. 3. The separated fractions were characterized by their RNA, protein, ribonuclease and phospholipid content. 4. The influence of starvation on the RNA and protein contents of the different fractions was investigated. 5. Labelling of the various centrifugal fractions *in vivo* revealed no difference in uptake of radioactive amino acid between the two classes of ribosomes. 6. Incorporation of radioactive leucine *in vitro* and the polyuridylic acid-directed phenylalanine incorporation were similar for both classes of ribosomes.

A number of investigations have demonstrated that aggregates of ribosomes (polyribosomes) are the functional units of protein synthesis in bacterial systems as well as in mammalian tissues and in plants.

Many investigators isolate polyribosomes from rat liver after treatment of the mitochondrial supernatant with deoxycholate to remove the membranes. This method was first described by Wettstein, Staehelin & Noll (1963).

In a preliminary paper Bloemendal, Bont & Benedetti (1964) reported that polyribosomes from rat liver could be prepared in high yield without utilization of detergents. Further, Bloemendal, Bont & Feltkamp (1966) demonstrated that this procedure may be used to isolate polyribosomes from various other tissues, e.g. kidney, hypophysis and a number of tumours. Webb, Blobel & Potter (1964) used a similar method to demonstrate that polyribosomes from normal and neoplastic liver differ in the proportion of polyribosomes associated with the endoplasmic reticulum.

This method is of interest not only from a technical point of view but may contribute to a better understanding of the situation *in situ*, particularly the occurrence of loose and membrane-bound ribosomes *in vivo*. Campbell, Serck-Hanssen & Lowe (1965) provided evidence for the presence of free polysomes in the microsome fraction.

Further, it has been suggested that polysomes isolated from the postmitochondrial supernatant are derived not from the endoplasmic reticulum but from the free polysomes present in this fraction (Campbell, Lowe & Sharp, 1966).

In the present paper we give details of conditions for the optimum separation of the particles and a number of their properties.

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## MATERIALS AND METHODS

*Preparation of the polyribosomes.* Three-month-old male rats of the R-strain Amsterdam were used. Starved animals received water only. The rats were decapitated, and the livers were collected, rinsed and homogenized in 2·5ml. of ice-cold 0·35M-sucrose solution in medium B/g. wet wt. of tissue. [Medium B consists of tris–HCl buffer, pH7·6 (50mM), KCl (25mM) and $MgCl_2$ (10mM).] A Teflon–glass homogenizer cooled to 0° was used, four strokes at 500rev./min. being applied. The clearance between pestle and tube was 0·2mm. The diameter of the tube was 25mm. Nuclei, mitochondria and cell debris were removed by centrifugation at 15000$g_{av.}$ for 10min. in rotor no. 30 of a Spinco L50 preparative ultracentrifuge. Then 15ml. of the 15000$g$ supernatant was carefully layered on top of a discontinuous gradient consisting of 10ml. of 2M-sucrose in medium B and 10ml. of 1·5M-sucrose in medium B. Centrifugation at 75000$g_{av}$ was for 17hr. in rotor no. 30. The

0·35M-sucrose top layer, down to 1cm. from the 1·5M-sucrose interface and referred to as supernatant, was used as a source of soluble enzymes. In the lower part of the 1·5M-sucrose layer three bands could be distinguished and were separated carefully with the aid of a bent Pasteur pipette. At the bottom of the centrifuge tube a slightly coloured pellet was found which was collected after removal of all supernatant fluid. This is a modification of our original procedure with 0·5M-sucrose for the middle layer and a centrifugation time of 6hr. The pellet could easily be suspended in medium B.

When only small amounts of polyribosomes are required the preparative run may be made in rotor no. 50. The gradient then consists of the following layers: 2·5ml. of 2M-sucrose in medium B, 2·5ml. of 1·5M-sucrose in medium B and 5ml. of 15000*g* supernatant. The centrifugation time in this case is reduced to 3hr. at 50000rev./min. (Bont, Rezelman & Bloemendal, 1965).

*Amino acid incorporation.* Either DL-[1-$^{14}$C]leucine or U-$^{14}$C-labelled algal-protein hydrolysate was administered by intravenous injection and the incorporation *in vivo* into the proteins of the isolated fractions was followed over a period of 2hr. For incorporation *in vitro* DL-[1-$^{14}$C]leucine or DL-[1-$^{14}$C]phenylalanine was used. The techniques for plating and counting in a windowless gas-flow counter were as described by Bloemendal, Huizinga, de Vries & Bosch (1962); counting efficiency was 20%. Exact conditions are given in the appropriate Tables and Figures.

*Morphological studies.* The preparation for samples for electron-microscope studies has been described elsewhere (Benedetti, Bloemendal & Bont, 1964; Benedetti, Bont & Bloemendal, 1966*a*). The micrographs were taken in a Philips EM200 microscope operated at 80kv. The double-condenser system was used as a routine with an average spot size of 15$\mu$; to prevent specimen contamination a cooling device was used.

*Estimations.* Protein content was estimated according to the Folin–Ciocalteu method of Lowry, Rosebrough, Farr & Randall (1951). Bovine albumin was used as standard.

RNA was determined after hydrolysis of samples with 5% (v/v) $HClO_4$ by measuring the $E_{260}$, assuming that 1mg./ml. corresponds to $E_{260}$ 22.

The method of Folch, Lees & Sloane-Stanley (1957) was used for the estimation of phospholipid.

Ribonuclease was determined according to the method of McFadyen (1934).

## RESULTS

*Fractionation of the postmitochondrial fraction.* By starting from the postmitochondrial supernatant a number of other cell components could be isolated with the polyribosomes.

After centrifugation (see the Materials and Methods section) a pellet and three coloured bands were obtained. These are shown schematically in Fig. 1. The pellet at the bottom consists of polyribosomes, particles occurring in clusters and strands. Frequently a helical configuration is observed (Plate 1). The lower layer (band 3) consists of fragments of rough endoplasmic reticulum, membranous profiles dotted with ribosomes (Plate 2*a*). The ribosomes in this fraction could not be detached from the membranes by repeating the isolation procedure (Benedetti *et al.* 1966*a*). The upper layers (bands 1 and 2) contain mainly smooth membranes (Plate 2*b*).

Detailed morphological aspects of pellet and layers have been presented elsewhere (Benedetti *et al.* 1964, 1966*a*,*b*).

*Composition of the centrifugal fractions.* The distribution of RNA and protein in the separated fractions is shown in Table 1. The RNA/protein ratio in the pellet is 1.

From Table 2 it can be concluded that variation of time of starvation from 12 to 65hr. does not affect significantly the total amount of RNA and protein in the pellet. In the combined fractions isolated from the layers there is no variation in RNA content from 12hr. up to 48hr.; with starvation from 24hr. onwards a gradual decrease in protein content was observed. Without starvation a large pellet consisting of glycogen was found at the bottom of the tube.

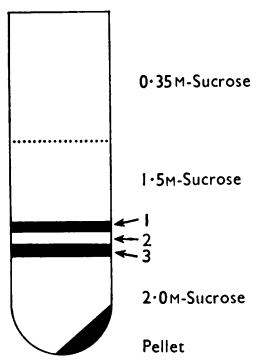
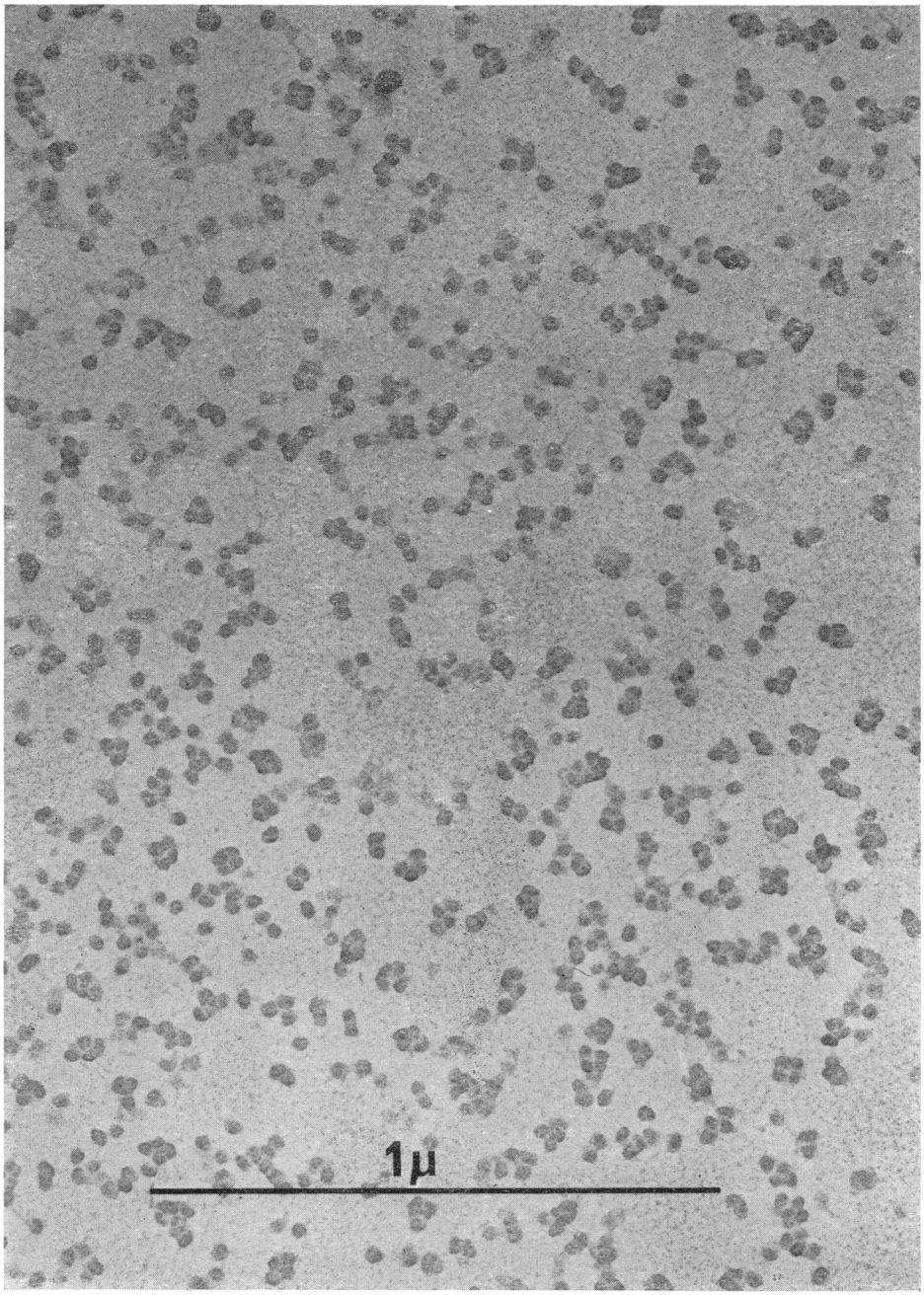



Fig. 1. Schematic representation of the result of sedimentation of the postmitochondrial supernatant after centrifugation for 16hr. at 75000*g* in rotor no. 30 of a Spinco model L50 ultracentrifuge.

Table 1. *RNA and protein contents in centrifugal fractions obtained from* 10*g. of fresh rat liver*

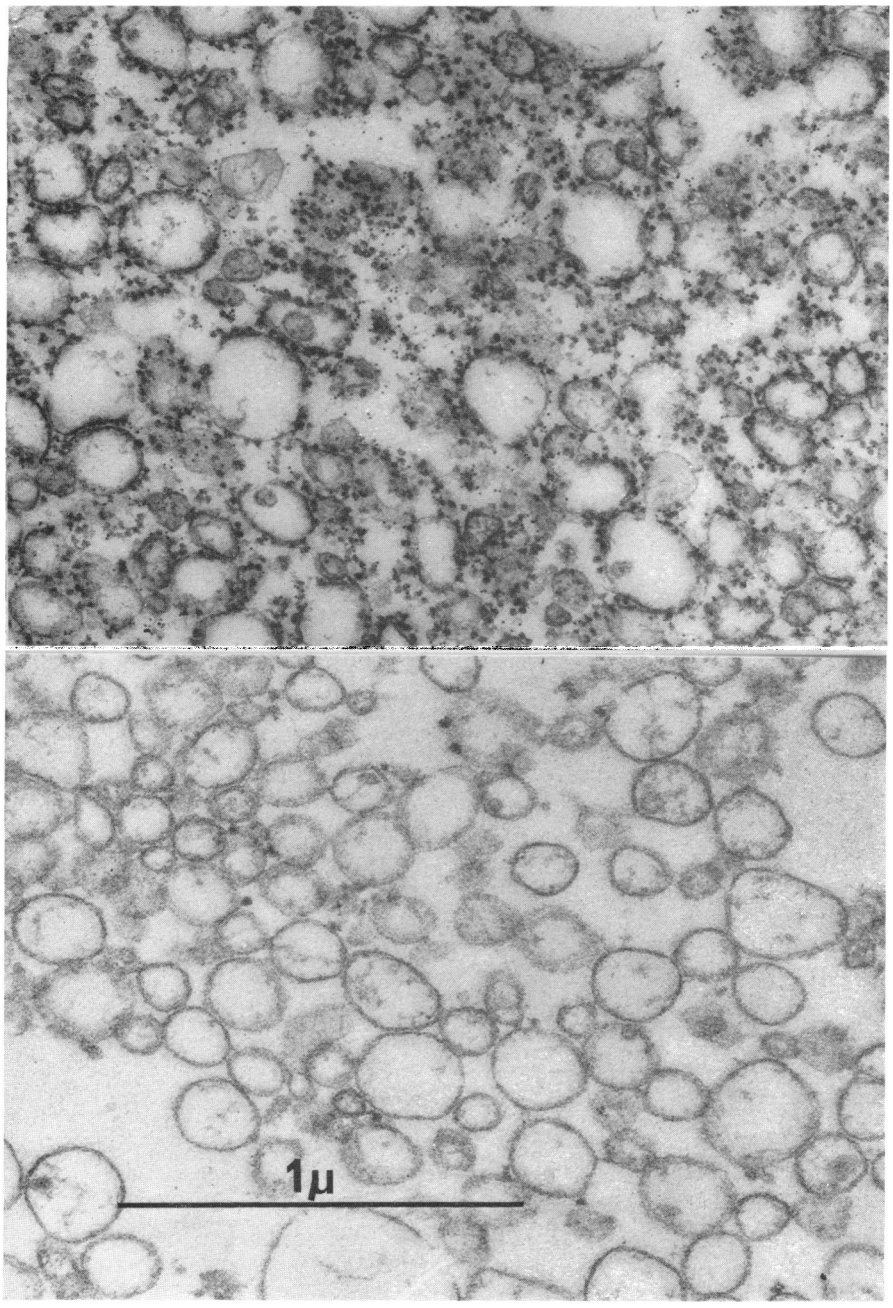
After washing bands 1, 2 and 3 total amounts of 8mg. of RNA and 10·3mg. of protein were found in the washing medium.

| Fraction | RNA (mg.) | Protein (mg.) | RNA/protein ratio |
|---|---|---|---|
| Supernatant (15000*g*) | 62·0 | 687·5 | 0·09 |
| Supernatant (79000*g*) | 21·3 | 496·0 | 0·04 |
| Band 1 | 7·0 | 65·1 | 0·11 |
| Band 2 | 1·1 | 4·0 | 0·28 |
| Band 3 | 4·8 | 8·4 | 0·57 |
| 2M-Sucrose layer | 5·4 | 4·0 | 1·35 |
| Pellet | 9·4 | 9·1 | 1·03 |




EXPLANATION OF PLATE 1

Polyribosomes spread on carbon film and shadowed while rotating. Some clusters show a helical arrangement.




EXPLANATION OF PLATE 2

(*a*) Micrograph of band 3 (thin section stained with uranyl acetate), showing membranes dotted with ribosomes. (*b*) Micrograph of band 2 (thin section stained with uranyl acetate), showing 'smooth' membranes. Only very few ribosomes are present.

After the observations of strands in micrographs of the pellet, it was necessary to prove that these structures were not linked together by phospholipid. Evidence was obtained by incubation of the pellet with phospholipase C, followed by density-gradient analysis. The pattern shown in Fig. 2(*a*) was not changed after such a treatment provided that the supernatant fraction was present (Fig. 2*b*). The necessity of the supernatant fraction for the stability of the polyribosomes during incubation at 37° has been demonstrated previously (Bont *et al.* 1965).

Further, we determined the phospholipid content in the various fractions (Table 3). In the same Table ribonuclease activity, as estimated by the McFadyen (1934) method, is summarized. Values in parentheses represent the corresponding values after treatment of the various fractions with sodium deoxycholate. From both values it is clear that phospholipid content and ribonuclease activity in the polysome pellet are low compared with the corresponding values in the layers. It should be noticed that the small amount of phospholipid cannot be removed by sodium deoxycholate treatment of the polyribosomes.

*Incorporation of* $^{14}$C*-labelled amino acid in vivo into the protein of bands and pellet.* Fig. 3 shows the specific radioactivities of protein isolated from bands and pellet at different times after injection of the radioactive amino acid. A maximum is reached in the protein of the polysomal pellet after 5 min. and then declines, crossing the curves that represent the specific activities of protein from the bands. These curves all reach a maximum after 1 hr., the highest specific activity being found in the second band, which represents labelled protein isolated from smooth-surfaced membranes.

After deoxycholate treatment of the fractions,

Table 2. *Influence of time of starvation on RNA and protein contents in bands and pellets*

All bands are equivalent to 10 g. of fresh rat liver.

| Time of starvation (hr.) | Combined bands RNA (mg.) | Combined bands Protein (mg.) | Pellet RNA (mg.) | Pellet Protein (mg.) |
|---|---|---|---|---|
| 65 | 6·9 | 69 | 11·1 | 11·8 |
| 48 | 9·4 | 76·7 | 10·0 | 10·7 |
| 34 | 9·8 | 85·9 | 10·5 | 12·7 |
| 24 | 9·7 | 104·2 | 9·6 | 11 |
| 12 | 9·8 | 101·9 | 10·5 | 10·9 |
| 0 | 10·6 | 102·5 | —* | —* |

* No pellets were isolated because of a large amount of glycogen that settles at the bottom of the centrifuge tubes.

Table 3. *Ribonuclease activity and phospholipid content in bands and pellet*

Values obtained after treatment in 1% (w/v) sodium deoxycholate of the corresponding fractions are given in parentheses.

| Fraction | Ribonuclease activity (total $\Delta E_{260}$) | Ribonuclease activity ($\Delta E_{260}$/mg. of RNA) | Phospolipid ($\mu$g. of P/mg. of RNA) |
|---|---|---|---|
| Band 1 | 33·4 (16·6) | 3 (3) | 152 (52) |
| Band 2 | 9 (3·35) | 2·7 (2·1) | 59 (18) |
| Band 3 | 11·1 (3·15) | 2·5 (1·0) | 23 (38) |
| 2 M-Sucrose layer | 10·6 (3·15) | 1·23 (0·42) | — (15) |
| Pellet | 1·2 (0·9) | 0·13 (0·06) | 0·85 (0·8) |

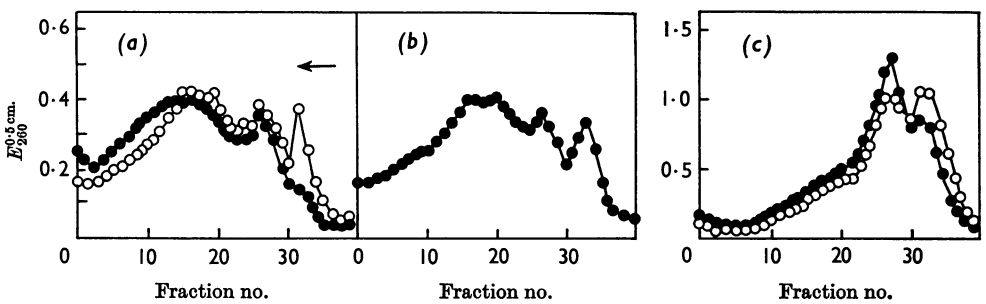



Fig. 2. Sucrose-density-gradient analysis of rat-liver polyribosomes after different incubation conditions. Polyribosomes containing 1 mg. of RNA in a final volume of 1 ml. were layered on a linear 15–35% (w/v) sucrose gradient. Centrifugation was in a Spinco model L50 centrifuge at 25000 rev./min. for 3·5 hr., rotor no. SW 25/1. (*a*) ●, Polyribosomes kept at 0°; ○, polyribosomes incubated at 37° for 30 min. in the presence of supernatant fraction (100 $\mu$g. of protein). (*b*) Polyribosomes incubated at 37° for 30 min. with 200 $\mu$g. of phospholipase C in the presence of supernatant fraction. (*c*) ●, Polyribosomes incubated at 37° for 30 min. in medium B only; ○, polyribosomes incubated with 5 $\mu$g. of ribonuclease at 25° for 10 min. The arrow indicates the direction of sedimentation.

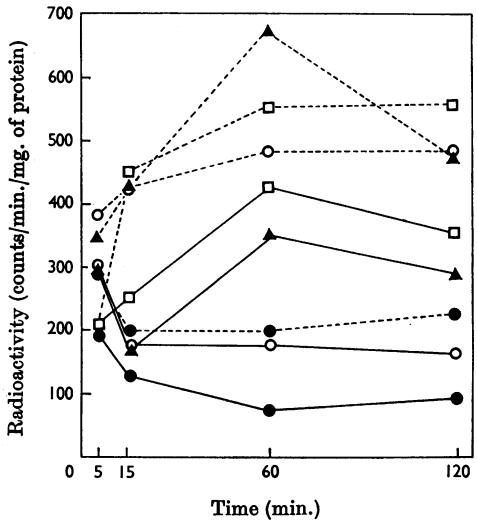



Fig. 3. Incorporation of [$^{14}$C]leucine *in vivo* into the protein of pellet and bands. Rats were starved for 2 days. At 30 min. before injection they were fed and 0·5 ml. (8 $\mu$c, 1·5 $\mu$moles) of DL-[$^{14}$C]leucine was injected intravenously per rat. The rats were killed after 5, 15, 60 and 120 min. after injection. The different liver fractions were isolated as described in the Materials and Methods section. When $^{14}$C-labelled algal-protein hydrolysate (10 $\mu$c) was used essentially the same curves were obtained. Four groups of three rats were used per experiment and their livers pooled. ●, Polysomal pellet; ○, band 3; ▲, band 2; □, band 1. ----, Fractions not treated with deoxycholate; ——, fractions after deoxycholate treatment.

Table 4. [$^{14}$C]*Leucine incorporation* in vitro *into the protein of bands and pellet*

The incubation mixture contained 0·05 $\mu$mole of DL-[$^{14}$C]leucine (7 mc/m-mole), 0·5 $\mu$mole of ATP, 0·25 $\mu$mole of GTP, 5 $\mu$moles of phosphoenolpyruvate, 25 $\mu$g. of pyruvate kinase, 0·05 $\mu$mole of 20 amino acids (minus leucine), 0·2 ml. of cell sap (8 mg. of protein) and a sample of the thrice-washed cytoplasmic subfraction corresponding to 500 $\mu$g. of RNA. The whole mixture was suspended in medium B; the final volume was 1 ml. Incubation was for 30 min. at 37°. The reaction was stopped by addition of $HClO_4$ to a final concentration of 5% (v/v). Plating and counting were as described by Bloemendal *et al.* (1962). Values in parentheses were obtained when incubation was performed after treatment with deoxycholate.

| Fraction | Radioactivity (counts/min./mg. of RNA) Complete system | Minus energy and regenerating system |
|---|---|---|
| Band 1 | 292 (—) | 101 (—) |
| Band 2 | 340 (—) | 122 (—) |
| Band 3 | 580 (1197) | 130 (89) |
| Pellet | 1572 (1384) | 75 (68) |

Table 5. *Stimulatory effect of polyuridylic acid on the incorporation of* [$^{14}$C]*phenylalanine in bands and polysomal pellet*

The incubation mixture contained 18 m$\mu$moles of DL-[$^{14}$C]phenylalanine (4 mc/m-mole), 0·27 $\mu$mole of ATP, 0·145 $\mu$mole of GTP, 1·14 $\mu$moles of phosphoenolpyruvate, 9·1 $\mu$g. of pyruvate kinase, 0·1 ml. of cell sap (about 4 mg. of protein), 12·5 $\mu$moles of $NH_4Cl$ and a sample of the thrice-washed cytoplasmic subfraction, corresponding to 400 $\mu$g. of RNA. Where indicated, 300 $\mu$g. of polyuridylic acid (polyU) was added. The mixture was dissolved in medium B; the final volume was 0·5 ml. Incubation was for 30 min. at 37°. Plating and counting were as described by Bloemendal *et al.* (1962). Values in parentheses were obtained when incubation was performed after treatment with deoxycholate.

| Fraction | Radioactivity (counts/min./mg. of RNA) −PolyU | +PolyU | Relative stimulation |
|---|---|---|---|
| Band 1 | 670 (595) | 2360 (8980) | 3·5 (15·1) |
| Band 2 | 540 (413) | 9000 (10000) | 16·7 (24·2) |
| Band 3 | 530 (591) | 10640 (8250) | 20·1 (14·0) |
| Pellet | 760 (881) | 12000 (16300) | 15·8 (18·5) |

the curve representing the specific activities in band 3 acquires the shape of the polysomal curve.

*Incorporation of* $^{14}$C*-labelled amino acid* in vitro *into protein of bands and pellet.* In Table 4 [$^{14}$C]-leucine uptake in the different fragments of endoplasmic reticulum is represented. The difference in incorporating ability between band 3 and pellet disappears after treatment with deoxycholate of these fractions (see values in parentheses). In Table 5 the polyuridylic acid-directed phenylalanine incorporation into bands and pellet is shown. The relative stimulation in band 1 is considerably lower than in the other fractions. (This difference disappears after deoxycholate treatment of the fractions: then about 20-fold stimulation is also observed in band 1).

## DISCUSSION

The basic feature of the polyribosome concept is that monomers are attached to a strand of messenger RNA.

Various methods are available for the isolation of rat-liver polyribosomes. Treatment of the post-mitochondrial fraction with deoxycholate has been applied most frequently (Wettstein *et al.* 1963; Jackson, Munro & Korner, 1963; Munro, Jackson & Korner, 1964).

In previous papers we reported a method of isolation of polyribosomes in which only a discontinuous sucrose gradient is employed (Bloemendal *et al.* 1964; Benedetti *et al.* 1964; Bont *et al.* 1965).

A slightly different method has been described independently by Webb *et al.* (1964).

We use the following nomenclature: (*a*) 'bound' ribosomes (for ribosomes bound to membranes); (*b*) 'free' ribosomes (for ribosomes not bound to membranes); (*c*) 'vacant' ribosomes (for ribosomes that can accept messenger RNA). According to Campbell, Cooper & Hicks (1964) 'free' ribosomes are identical with 'vacant' ribosomes. Moreover, Campbell *et al.* (1965) confirmed our observation of the presence of free polyribosomes in rat liver. This differs from the nomenclature chosen by Siekevitz & Palade (1958), who designated the ribonucleoprotein present in the postmicrosomal fraction as 'free' ribosomes.

We have stressed previously (Bloemendal *et al.* 1964) that, in addition to the polyribosomes found in the pellet, a certain number remain firmly attached to membranes located in the 1·5–2M-sucrose interface. These 'bound' ribosomes cannot be detached by repeating the whole isolation procedure. It remains, however, questionable whether two classes of ribosomes exist *in situ*. If they do, the question arises whether or not there exists a metabolic difference between the two classes of protein-synthesizing particles.

The following conclusions can be drawn from the results of our experiments *in vivo*. For all preparations that, judged by their RNA/protein ratio, consisted of ribosomes, the shapes of the curves representing the specific activity as a function of time were in agreement with results of Littlefield, Keller, Gross & Zamecnik (1955) (Fig. 3). After 5min. the specific activity of the ribosomes only reached a maximum. Siekevitz & Palade (1958), working with guinea-pig pancreas, observed a maximum for the specific activity of protein from the whole microsomal fraction after about 10min., whereas in other centrifugal fractions the maximum was found after about 1hr. To compare our results with the experiments by these workers and Littlefield *et al.* (1955) we expressed the specific activity for experiments *in vivo* in counts/min./mg. of protein.

Treatment of the different fractions (bands 1, 2 and 3 and pellet) with deoxycholate did not affect the shape of the curves belonging to bands 1 and 2. Band 3 acquired the shape of the ribosome curve (Fig. 3). This treatment, however, affected the magnitude of the specific activities. Free polyribosomes, for example, have a higher specific activity before than after treatment. This decrease in specific activity must be due to the preferential removal of proteins with relatively high specific activity. Bound polyribosomes (ribonucleoprotein particles isolated by treatment of band 3 with deoxycholate) show a specific activity in between those of free polyribosomes before and after deoxycholate treatment. This might be due to contamination of band 3 with band 2.

The experiments *in vitro* show that leucine incorporation/mg. of RNA is higher in the free ribosomes than in bound ribosomes. However, similar leucine uptake is observed when the bound ribosomes are detached from the membranes before incubation (Table 4). From this observation it may be concluded that there is no difference between free and bound ribosomes as such *in vitro*.

Another point of investigation was the relative stimulation of phenylalanine incorporation by polyuridylic acid. In this case a difference was observed between the relative stimulation in band 1 and that in the other fractions. After deoxycholate treatment this difference disappeared, all fractions showing the same relative stimulation. It may be concluded that no fraction in particular had a higher amount of vacant ribosomes. It must be emphasized, however, that the latter result may depend on the isolation procedure followed. Other workers (Henshaw, Bojarski & Hiatt 1963; Campbell *et al.* 1964) did find a difference.

We have investigated the effect of starvation in relation to the polyuridylic acid-directed phenylalanine incorporation and on the relative yields of free and bound ribosomes. Protein from rats starved for 65hr. did not reveal a higher content of vacant ribosomes when compared with preparations from animals starved for 24hr. Nor did the ratio of free and bound ribosomes change.

The phospholipid content in preparations of free polyribosomes is low. Nevertheless, the possibility that monomers in this preparation are linked together, not only by messenger RNA, but also by phospholipids, cannot entirely be excluded. To prove this, Tsukada & Lieberman (1965) incubated the polyribosomes with phospholipase A and with phospholipase C. In both cases an extensive breakdown of polyribosomes occurred. However, we demonstrated that the structural stability of polyribosomes as judged by sucrose-density-gradient analysis depends on a supernatant factor that we supposed to be identical with the ribonuclease inhibitor described by Roth (1957) and Shortman (1961). Evidence that the stabilizing factor is indeed a ribonuclease inhibitor was brought forward by Blobel & Potter (1966) and Lawford, Langford & Schachter (1966). Since Tsukada & Lieberman (1965) had not added supernatant in their phospholipase tests, the possibility remains that the breakdown was due to contamination of the phospholipase with ribonuclease. We have repeated the experiments with phospholipase C in the presence of supernatant and no breakdown was observed (Fig. 2*b*). However, it remains possible that the supernatant contains also a lipase inhibitor.

In conclusion it can be stated that no difference

between free and bound ribosomes could be demonstrated by the following criteria. (1) Incorporation of amino acid *in vivo*. In this connexion, experiments by Manganiello & Phillips (1965) have to be mentioned. Their studies of amino acid incorporation into free and membrane-attached ribosomes in intact liver slices revealed an equal activity in protein synthesis of both fractions. (2) Incorporation of amino acid *in vitro*. After treatment with deoxycholate no difference in incorporating ability between free and bound ribosomes was detected. (3) Electron microscopy. Micrographs identical with the pattern shown in Plate 1 have always been found when the pellet obtained after treatment with deoxycholate was examined in the electron microscope.

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