Studies on the Protein-Synthesizing Activity of the Ribosomes of Rat Liver

THE ACTIVITY OF FREE POLYSOMES

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1. The activities of microsome fractions from the liver of adult and 5-day-old rats for the incorporation of [^{14}C]phenylalanine into protein were similar in the presence and absence of polyuridylic acid. 2. The activity of a light-microsome fraction from adult liver was greater than that of a heavy-microsome fraction, and the light-microsome fraction was also more markedly stimulated by the presence of polyuridylic acid. 3. The light-microsome fraction, when analysed by density-gradient centrifugation, contained a higher ratio of free ribosomes to bound ribosomes, whereas the reverse was true for the heavy-microsome fraction. Similar results were obtained for liver from adult and 5-day-old rats. 4. When the light-microsome fraction was incubated under conditions in which amino acid was incorporated into protein there was only a small increase in the ratio of free to bound ribosomes. When such a fraction was incubated with [^{14}C]leucine and was then subjected to density-gradient centrifugation the fraction with the highest specific activity based on RNA had a density between that of the bound and free ribosomes. Treatment of the incubated fraction with ribonuclease shifted the radioactivity towards the free ribosome peak. These properties are consistent with the presence of active free polysomes. Such a component appeared also to be present when the heavy-microsome fraction was incubated under similar conditions. 5. The effect of the presence of polyuridylic acid on the incorporation of [^{14}C]phenylalanine by the light-microsome fractions from liver of adult and 5-day-old rats was greatest in the region of the free ribosomes, but it is probable that some small polysomes containing polyuridylic acid are formed. 6. Polyuridylic acid also stimulated the bound ribosomes to a small extent when the heavy-microsome fraction from the liver of young rats was incubated with [^{14}C]phenylalanine. 7. The results are discussed in terms of the various morphological constituents in liver now known to play a role in the synthesis of protein for export and for the internal activity of the cell.

The protein-synthesizing activity of the liver is characterized by production both for the internal activity of the cell and for export in the form of plasma protein. With the realization that the ribosomes were the site of protein synthesis it was suggested by a number of electron microscopists that the ribosomes bound to membranes were responsible for the synthesis of protein for secretion whereas the ribosomes unattached to membranes (free ribosomes) were involved in the synthesis of protein for intracellular purposes (Porter, 1954; Palade, 1955; Howatson & Ham, 1955; Slautterback & Fawcett, 1959; Prescott, 1960; Birbeck & Mercer, 1961). More recently Henshaw, Bojarski

& Hiatt (1963) by one method and Campbell, Cooper & Hicks (1964) by another have obtained evidence that in rat liver the free ribosomes are comparatively inactive in the absence of added polynucleotide. Since these findings were contrary to the predictions of the electron microscopists it was decided to investigate further the role of the free ribosomes in the protein-synthesizing activity of rat liver.

The quantity of free ribosomes in rat liver during maturation has been studied by Oliver, Blumer & Witham (1963). They have shown that there is a high concentration in the liver of foetal and post-natal rats but that at 5 days after birth the proportion of free ribosomes starts to fall rapidly to the adult level. For this reason we have studied not

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only liver from adult rats but also that obtained from animals 5 days after birth.

Some aspects of this work have been reported previously (Campbell & Lowe, 1964; Serck-Hanssen, Lowe & Campbell, 1965).

MATERIALS AND METHODS

Chemicals. The dipotassium salt of ATP and the sodium salt of GTP were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A., and pyruvate kinase from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The silver-barium salt of phosphoenolpyruvic acid was prepared by the method of Clark & Kirby (1963).

Solutions of free phosphoenolpyruvic acid were prepared before use and adjusted to pH 7.4 (glass electrode) with π -KOH. Tris ('specially purified') was obtained from British Drug Houses Ltd., Poole, Dorset, and polyU* from Miles Laboratories, Stoke Poges, Slough, Bucks.

Radioactive amino acids. L-[U- 14 C]Leucine (160 mc/m-mole) and L-[U- 14 C]phenylalanine (300 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks.

Animals. Adult rats (150–200 g. body wt.) were Wistar albino from a closed colony bred at this Institute. The rats were starved for about 18 hr. before being killed by a blow on the head followed by decapitation. Young rats were obtained 5 days after birth but were not starved before removal of their livers.

Preparation of microsomes and microsomal subfractions. The liver was homogenized in 2.5 times its weight of a medium (medium A) containing MgCl_2 (10 mM), KCl (25 mM) and tris buffer (35 mM) (adjusted to pH 7.8 with HCl and checked by a glass electrode at 25°), and sucrose (0.15 M) (Rendi & Hultin, 1960). The tissue suspension was centrifuged for 10 min. at 12000g to remove cell debris, nuclei and mitochondria, leaving the 'mitochondria-free supernatant'. For the preparation of unfractionated microsomes the supernatant was centrifuged in a Spinco model L preparative centrifuge (no. 40 rotor) for 60 min. at 105000g. After the supernatant had been decanted, 'washed microsomes' were prepared by gently suspending each pellet in about 4 ml. of medium A, replacing in the no. 40 cellulose tube and filling with more medium A. The suspension was then centrifuged at 105000g for 35 min. The supernatant was decanted and each pellet suspended in medium A by gentle homogenization. A 'heavy' and a 'light' subfraction were prepared by centrifuging the mitochondria-free supernatant at 25000g for 60 min. in the no. 40 rotor. The pellet was designated the 'heavy-microsome' fraction. The supernatant was then centrifuged at 105000g for 60 min. to give a pellet designated the 'light-microsome' fraction. When washed fractions were required, each subfraction was treated as described for the unfractionated microsomes.

Method of incubation. The particulate fractions were suspended by gentle homogenization in a Potter-type homogenizer in a volume of medium A such that the suspension contained approx. 10 mg. of protein/ml. Each incubation tube contained the particle suspension (0.4 ml.), pH 5 fraction (prepared as described by Decken & Campbell, 1962) (0.1–0.2 ml.), ^{14}C -labelled amino acid, 2 μ moles of

ATP, 0.25 μ mole of GTP, 15 μ moles of phosphoenolpyruvate and 50 μ g. of pyruvate kinase in a total volume of 1.0 ml. Incubation was in air for the time specified.

Separation of bound and free ribosomes by density-gradient centrifugation. To determine the ratio of bound to free ribosomes the various microsome preparations were subjected to density-gradient centrifugation in sucrose by the method of Henshaw *et al.* (1963) and modifications of it. The method recommended is to suspend the microsome pellet in a medium (medium B) containing MgCl_2 (1 mM), KCl (25 mM) and tris buffer, pH 7.8 (35 mM). Then 1 ml. of this suspension was carefully layered on 20 ml. of a linear gradient of 5–20% (w/v) sucrose in medium C (which is medium B with MgCl_2 at 0.1 mM instead of 1 mM) prepared in a tube into the bottom of which a 5 ml. layer of 50% (w/v) sucrose had already been introduced. Centrifugation was for 135 min. at 40000g (22000 rev./min.) at about 4° in the SW 25.1 rotor of the Spinco model L ultracentrifuge. Fractions (0.5 ml.) were siphoned from the bottom of the sucrose gradient with a syringe to which was attached a two-way tap and a long blunt-ended steel needle. The fractions were diluted with 1.0 ml. of water and their extinctions at 260 μ m determined.

The incubation mixtures containing ^{14}C -labelled amino acids were analysed by gently layering the mixture (1 ml.) on the gradient of the composition described above. The extinctions of the fractions at 260, 280 and 320 μ m were determined.

Extraction of protein for the assay of radioactivity. After incubation the protein was precipitated by the addition of trichloroacetic acid (5%, w/v). The precipitate was treated as follows: washed with cold 5% trichloroacetic acid, heated for 20 min. at 90° with 5% trichloroacetic acid and then washed successively with cold 5% trichloroacetic acid, ethanol, ethanol-ether-chloroform (2:2:1, by vol.), acetone and ether, and finally gently dried. When fractions from density gradients were being analysed 2 mg. of bovine serum albumin was first added to each fraction and the extraction procedure was stopped after the washing with ethanol.

Determination of specific radioactivity. The protein was dissolved in 98–100% formic acid (A.R.), transferred to 5 cm.² aluminium disks, dried under an infrared lamp and counted at infinite thinness. Radioactivity was determined in a thin-window gas-flow counter (Nuclear-Chicago Corp.). A standard planchet of 1 cm.² containing 1 μC of ^{14}C /g. gave approx. 1800 counts/min. at infinite thickness under these conditions. The efficiency of counting [^{14}C]leucine at infinite thinness under the described conditions was 25%.

The specific radioactivity of the proteins obtained from incubation mixtures that were not subfractionated by density-gradient centrifugation was determined by weighing the amount of protein on the counting disks and expressing the radioactivity as counts/min./mg. of protein. Since the radioactive fractions from density gradients contained very little protein and were diluted with albumin this direct method of determining specific radioactivity was not possible. The amount of RNA in each fraction was determined from its extinctions at 260 and 280 μ m by using the data of Warburg & Christian (1942), from which was first deducted a correction factor for the presence of ferritin, the amount of which was determined from the extinction of 320 μ m. The correction factor for the extinction at 260 μ m was obtained by multiplying the extinction at 320 μ m by

* Abbreviation: polyU, polyuridylic acid.

1.5 and that at 280 m μ by 1.4. This follows the suggestion of Munro, Jackson & Korner (1964). Provided therefore that the transfer of radioactive protein to the counting disk was quantitative this method enabled the specific radioactivity of each fraction to be based on the original amount of RNA present in each fraction from the gradient.

Estimations. Protein was determined by the Folin-Ciocalteu method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard. RNA was determined by the orcinol method of Meijbaum (1939), with hydrolysed yeast RNA as standard. These methods were used for the determination of RNA/protein ratios.

RESULTS

Comparison of incorporation of amino acid into the protein of unfractionated microsomes from the liver of adult and young rats. Campbell *et al.* (1964) have reported on the optimum conditions under which polyU stimulates the incorporation of phenylalanine into protein by the microsome fraction from rat liver. The microsome fractions from the liver of adult and young rats were incubated under these conditions and the results are shown in Table 1. These show that the activities of the microsomal fraction were similar irrespective of source and that the extents of stimulation of incorporation of [14 C]phenylalanine by the addition of polyU were also similar.

Activities and properties of the heavy- and light-microsome fractions. W. T. Oliver (personal communication) has indicated that the rough endoplasmic reticulum containing the bound ribosomes can be separated from the free ribosomes by the preparation of a 'heavy-microsome' and a 'light-microsome' fraction. The heavy-microsome fraction is obtained by centrifuging the mitochondria-free supernatant of the liver homogenate at 25 000g for 60 min., and the light-microsome fraction is obtained by further treatment of the supernatant

at 105 000g for 60 min. The activities of these fractions were compared with that of the unfractionated microsomes. The effect of the addition of polyU on the incorporation of phenylalanine by the three fractions from the liver of adult rats was also studied and the results are shown in Table 2. The activities of the unfractionated microsomes and the heavy-microsome fraction are similar, but the light-microsome fraction is rather more active. The effect of polyU was to stimulate the three microsome preparations in the increasing order: heavy microsomes; unfractionated microsomes; light microsomes.

The RNA/protein ratios of the three fractions were determined. The results showed that the unfractionated microsomes and heavy-microsome fraction had similar ratios, 0.21 and 0.20 respectively, but the light-microsome fraction had a higher proportion of RNA, with a ratio 0.33.

Determination of the ratio of bound and free

Table 2. *Effect of polyU on the incorporation of [14 C]phenylalanine into protein by various microsome fractions from adult liver*

The microsome fractions were washed and the incubation mixture contained 0.1 ml. of pH5 fraction and 0.2 μ C of [14 C]phenylalanine. The MgCl₂ concentration in the incubation mixture was 10 mM; where indicated, 50 μ g. of polyU was added. Incubation was for 30 min. Results are expressed as counts/min./mg. of protein.

Fraction	Normal liver		
	Control	PolyU added	Ratio
Unfractionated	1705	5451	3.2
Heavy fraction	1641	3831	2.3
Light fraction	2533	13447	5.3

Table 1. *Incorporation of [14 C]phenylalanine by the microsome fraction from the liver of adult and young rats in the presence and absence of polyU*

The microsome fractions from the two sources were suspended in a sufficient volume of medium A to give approximately the same protein concentration. They were then incubated for 30 min. in the presence of 0.1 ml. of pH5 fraction from the source as indicated and under the conditions given in the Materials and Methods section. Each tube contained 1 μ g. of carrier phenylalanine and 0.25 μ C of [14 C]phenylalanine and the final concentration of MgCl₂ in the incubation medium was adjusted to 10 mM by adding 0.04 ml. of 0.1 M-MgCl₂/tube. Where indicated, 50 μ g. of polyU was added. After incubation the particulate material was recovered by centrifuging at 105 000g for 60 min. and the protein precipitated with trichloroacetic acid and extracted as described in the Materials and Methods section. Radioactivity is expressed as counts/min./mg. of protein.

	Source of pH5 fraction	Adult			Young		
		Control	PolyU added	Ratio	Control	PolyU added	Ratio
Expt. 1	Adult	279	1465	5.3	214	1181	5.5
Expt. 2	Young	110	841	7.7	126	1100	8.7

ribosomes in the microsome fractions. The methods used to study the ribosome content of the microsome fractions were based on that of Henshaw *et al.* (1963). When the method described by these authors was applied to the liver of adult rats that had been starved for 2 days their results were confirmed. The results of the application of the method to the livers of 5-day-old rats are shown in Fig. 1. As expected, the liver of young rats has a higher ratio of free to bound ribosomes than that of adult rats. The RNA/protein ratios of the material isolated from two peaks with liver from young rats were 0.25 (tubes 1-14) and 0.45 (tubes 25-38); this compares with values of 0.16 and 0.50 reported by Henshaw *et al.* (1963) for liver from adult rats.

The ratios of bound and free ribosomes in the heavy-microsome and light-microsome fractions were also determined. In this case the method differed from that of Henshaw *et al.* (1963) in two ways. First, the microsome pellets were prepared in a different medium (see the Materials and Methods section); secondly, after resuspension of the pellets the aggregated material was not removed by centrifugation at low speed, the whole microsome suspension being placed on the sucrose

Fig. 1. Separation of bound and free ribosomes from the liver-microsome fraction from adult (a) and 5-day-old (b) rats. The young rats were not starved but the adults were starved for 2 days. The conditions for the sucrose density-gradient centrifugation were as described by Henshaw *et al.* (1963). Fractions (0.5 ml.) were removed from the bottom of the tube so that in this and subsequent Figures the lowest numbered fractions are the most dense. Each tube contained 25 ml.

Fig. 2. Distribution of bound and free ribosomes in microsome subfractions from the liver of adult (a) and 5-day-old (b) rats. The microsome subfractions were prepared as described in the Materials and Methods section and then subjected to density-gradient centrifugation: ○, heavy-microsome fraction; ●, light-microsome fraction. The RNA/protein ratios of the combined fractions from the liver of adult rats as indicated in the Figure were determined and the following values obtained; H_1 , 0.12; H_2 , 0.64; L_1 , 0.22; L_2 , 0.35; L_3 , 0.46.

Fig. 3. Effect of incubation of a light-microsome fraction from the liver of adult rats on the distribution of bound and free ribosomes. The light-microsome fraction was incubated for 15 min. at 37° under the conditions described in the Materials and Methods section for the incorporation of amino acids into protein. $\circ$, Control (not incubated); $\bullet$, incubated.

density gradient. The results obtained with liver from adult and young rats are shown in Fig. 2. The RNA/protein ratio of the peaks from the liver microsomes from adult rats was determined and the results are given in the legend to Fig. 2. It is clear that the RNA/protein ratio increases towards the lower density fraction and that the proportion of free ribosomes is markedly greater in the light-microsome fraction than in the heavy-microsome fraction. The precise distribution of the two types of ribosomes between the microsome fractions depends on the particular microsome preparation, but in many prepared from the liver of young rats the separation has been virtually complete.

Attempts to determine the metabolic activity of the bound and free ribosome fractions after separation. Henshaw *et al.* (1963) separated the bound and free ribosomes obtained from a rat after starvation for 5 days and determined their activities for the incorporation of [^{14}C]phenylalanine in the presence and absence of polyU. To obtain a fraction active *in vitro* they changed the Mg^{2+} concentration of the medium in which the microsomes were suspended to 10 mM. We adopted a procedure in which the microsomes were suspended in a medium containing Mg^{2+} at a concentration of 1 mM and the gradient centrifugation was also carried out at this Mg^{2+} concentration. Henshaw *et al.* (1963) stated that the separation of bound and free ribosomes was not as good when the Mg^{2+} concentration was raised above 0.1 mM and this has been our experience. However, the amount of protein recovered from the free ribosome peak is not markedly different when the Mg^{2+} concentration is changed from 0.1 to 1 mM.

The particles were recovered from the fraction

Fig. 4. Distribution of radioactivity among the different species of ribosomes after the incubation of light-microsome (a) and heavy-microsome (b) fractions from the liver of young rats with [^{14}C]leucine. Each microsome subfraction was incubated with $1\mu\text{C}$ of [^{14}C]leucine for 10 min. at 37° in the presence of 0.2 ml. of pH 5 fraction under the conditions described in the Materials and Methods section. The final concentration of MgCl_2 in the incubation medium was 6 mM. After fractionation by density-gradient centrifugation the extinctions of each fraction were determined at 260, 280 and 320 $\text{m}\mu$ and the RNA contents determined after correction for the presence of ferritin. The radioactivity of each extracted fraction was determined at infinite thinness, the specific radioactivity being calculated as counts/min./mg. of RNA in the original fraction. $\bullet$ — $\bullet$, Extinction; $\circ$ — $\circ$, total counts/min.; $\bullet$ — $\bullet$ — $\bullet$, specific radioactivity.

representing the peaks on the density gradient by centrifuging and their protein-synthesizing activities determined. The results obtained either with [^{14}C]leucine or with [^{14}C]phenylalanine in the presence and absence of polyU were erratic. We found that the extensive washing of the fractions by centrifugation through sucrose increased markedly the non-energy-dependent attachment of ^{14}C -labelled amino acids to protein when the fractions were subsequently incubated. Hence, in the absence of careful controls on each fraction incubated, the significance of the apparent incorporation of amino acids by such fractions is doubtful. Since the use of all the necessary controls was not practicable our results with this approach will not be considered further.

Fig. 5. Effect of incubation of the light-microsome fraction from the liver of young rats with pancreatic ribonuclease after incubation with [^{14}C]leucine. The conditions for the incorporation of [^{14}C]leucine into protein were as described for Fig. 4. The contents of one of the two incubation tubes were incubated without ribonuclease (a) and the other with pancreatic ribonuclease at a final concentration of $1\mu\text{g./ml.}$ (b) at 25° for 5 min. $\bullet\text{---}\bullet$, Extinction; $\bigcirc\text{---}\bigcirc$, total counts/min.; $\bullet\text{---}\bullet$, specific radioactivity.

Experiments involving the incubation of microsome fractions with ^{14}C -labelled amino acids and then subsequent fractionation by density-gradient centrifugation. The effect of incubation of the microsome fraction under conditions of protein synthesis on the distribution of bound and free ribosomes was first studied. The results obtained with the light-microsome fraction from the liver of adult rats are shown in Fig. 3. The sedimentation properties of the two peaks are not affected by incubation, although there may be a change in their relative proportions.

When the light-microsome and heavy-microsome fractions from the liver of young rats were incubated with [^{14}C]leucine and then subjected to density-gradient centrifugation and the radioactivity of each fraction was determined, the results shown in Fig. 4 were obtained. The highest specific radioactivity in each fraction was among the components that sedimented with a density between that of the free and bound ribosome peaks. The free ribosomes were noticeably inactive.

Fig. 6. Effect of incubation of the heavy-microsome fraction from the liver of young rats with pancreatic ribonuclease after incubation with [^{14}C]leucine. The conditions were exactly as described for Fig. 5(b), except that the heavy-microsome fraction was used. $\bullet\text{---}\bullet$, Extinction; $\bigcirc\text{---}\bigcirc$, total counts/min.; $\bullet\text{---}\bullet$, specific radioactivity.

To determine the nature of the component with high activity the experiment was repeated and one of the incubated fractions was treated with pancreatic ribonuclease under the conditions described by Munro *et al.* (1964) for the disruption of polysomes isolated from liver-microsome fractions treated with deoxycholate. The results obtained are shown in Fig. 5. The control again shows the high specific radioactivity in the region of the gradient which is denser than that occupied by the free ribosomes. Treatment with ribonuclease causes a sharpening of the free ribosome peak on the dense side, suggesting the removal of some denser material. The total counts and specific radioactivity peaks move towards the free ribosome peak. The fact that they are not coincident with it suggests that the disruption of the active component has not been complete. These results lend support to the idea that the active component in the light-microsome fraction consists of aggregates of ribosomes, linked together by messenger RNA, which are disrupted by treatment with ribonuclease. For the purposes of simplicity it is proposed to refer to this active component as the 'free polysomes'.

Treatment of the heavy-microsome fraction from the liver of young rats with ribonuclease produced the results shown in Fig. 6. Comparison of the results with Fig. 4 shows that there has been a marked shift both in RNA and in total radioactivity

Fig. 7. Effect of incubating the light-microsome fraction from the liver of young rats with polyU on the incorporation of [^{14}C]phenylalanine. The microsome subfraction was incubated with $1\mu\text{C}$ of [^{14}C]phenylalanine for 10 min. at 37° in the presence of 0.1 ml. of pH 5 fraction and a final Mg^{2+} concentration of 10 mM under the conditions described in the Materials and Methods section. The contents of one of the two incubation tubes were incubated without polyU (a) and the other with $50\mu\text{g}$. of polyU (b). The incubation mixture was fractionated as described for Fig. 4. ●—●, Extinction; ○—○, total counts/min.; ●—●—●, specific activity.

towards the free ribosome peak. However, much of the radioactivity remains in the region of the bound ribosomes, and no doubt this is the radioactive protein that is associated with the membrane of the rough endoplasmic reticulum whose sedimentation properties would not be expected to change markedly as a result of a short treatment with ribonuclease.

The effect on the incorporation of [^{14}C]phenylalanine of adding polyU to the incubation medium containing the light-microsome fraction from the liver of young rats is shown in Fig. 7, and on that from the liver of adult rats in Fig. 8. The effect of polyU is the same in the microsomes from both sources, being most marked in the region of the free ribosome peak. The most pronounced effect is on a component with a density a little greater

Fig. 8. Effect of incubating the light-microsome fraction from the liver of adult rats with polyU on the incorporation of [^{14}C]phenylalanine. The microsome subfraction was obtained from adult rats starved overnight. The conditions were exactly as described for Fig. 7, except that the light-microsome fraction from the liver of adult rats was used. (a) Incubated without polyU; (b) incubated with $50\mu\text{g}$. of polyU. ●—●, Extinction; ○—○, total counts/min.; ●—●—●, specific activity.

than the free ribosomes. This may indicate that small polysomes have been formed by the attachment of free ribosomes to polyU.

To determine whether polyU also stimulated the incorporation of phenylalanine into protein by the bound ribosomes the heavy-microsome fraction from the liver of young rats was also incubated with [^{14}C]phenylalanine in the presence and absence of polyU. The results shown in Fig. 9 indicate that again the most marked effect of polyU is on the free ribosomes, but all components including the bound ribosomes were stimulated.

Electron microscopy. Plate 1 shows that although the liver cell from 5-day-old rats contains a considerable amount of rough endoplasmic reticulum it also contains many ribosomes not associated with membrane. Many of these are in the form of aggregates and are probably the free polysomes the activity of which has been studied.

Fig. 9. Effect of incubating the heavy-microsome fraction from the liver of young rats with polyU on the incorporation of [¹⁴C]phenylalanine. The conditions were exactly as described for Fig. 7, except that the heavy-microsome fraction was used. (a) Incubated without polyU; (b) incubated with 50 $\mu\text{g.}$ of polyU. ●—●, Extinction; ○—○, total counts/min.; ●---●, specific activity.

DISCUSSION

Previous work from the Laboratory of Hiatt (Henshaw *et al.* 1963) and from our Laboratory (Campbell *et al.* 1964) led to the conclusion that the ribosomes associated with the rough endoplasmic reticulum were the sole site of synthesis of protein in the liver cell. The free ribosomes appeared to be inactive unless presented with a form of messenger RNA such as polyU. This thesis suggested that the proteins required for the internal activity of the liver cell as well as those for export were synthesized on the rough endoplasmic reticulum. The experience of electron microscopists was contrary to this idea, and the present work was therefore initiated with the object of resolving this paradox.

For many years it has been known that at birth the liver of many species of animals contains relatively little rough endoplasmic reticulum but that this component increases markedly within a

few days. Oliver *et al.* (1963) in particular have studied this phenomenon in the young rat. It seemed therefore that an ideal system was available in which to study the role of the free ribosomes. Electron microscopy confirmed that in the livers of the rats available in our Laboratory many of the ribosomes were not attached to recognizable membranes even at 5 days after birth. Although the ribosomes were in a sense free many of them appeared to be aggregated in an orderly manner (Plate 1). The experiments in which the activities of the microsome fraction from the liver of adult and young rats were compared (Table 1), and the fractions shown to have similar properties in the presence and absence of polyU, suggested that the free ribosomes in the liver from young rats were active. This was also indicated by the high activity of the light-microsome fraction from the liver of adult rats compared with that of the heavy-microsome fraction (Table 2).

When the ratio of bound to free ribosomes in the liver-microsome fractions from adult and young rats was analysed by the procedure of Henshaw *et al.* (1963) (Fig. 1), it was apparent that the proportion of free ribosomes was much greater in the liver from young rats than in that from adult rats, but there was little indication of the presence of aggregated ribosomes between the peaks. If instead of the whole microsome fraction a light-microsome and a heavy-microsome subfraction were used rather more material appeared to be present in the middle of the gradient (Fig. 2). The RNA/protein ratio of this material was intermediate between those of the bound and free ribosome peaks. When it proved impossible to obtain sensible results by the incubation of fractions isolated from the density-gradient centrifugation it was decided to attempt a subfractionation after incubation. Much to our surprise we found that incubation did not cause irreversible aggregation of the particulate material and the sedimentation properties of the bound and free ribosomes were not markedly changed (Fig. 3). This opened the way to a detailed study of the active components in the microsome fraction during incubation.

The results obtained with the microsome fractions from the liver of young and adult rats (Figs. 4–9) are all expressed in the same way, by using the content of RNA in the original fraction as the basis for the specific radioactivity determination. At least 35 fractions were taken from each gradient centrifugation and each was worked up separately so that the amount of protein finally remaining in each fraction, the radioactivity of which was determined, was very small and precluded any method of comparison other than that of RNA. In the determination of specific radioactivity errors due to contamination of the free ribosome

peak with ferritin were eliminated by the use of the method of Munro *et al.* (1964). The presence of ferritin was more marked in fractions from the liver of adult rats than in those of young rats, but even with the liver of adult rats the position of the free ribosome peak was not displaced when the extinction at 260 m μ was corrected for the presence of ferritin. The curves for radioactivity are seen to be uneven. It is not possible at this stage to assign any special significance to each minor peak and this is not necessary for the present discussion. The subsidiary peak arising on the dense side of the free ribosome peak is a consistent finding, and one might expect multiple peaks in the centre of the gradient if they be due to discrete aggregations of ribosomes.

The results obtained by the incubation of the light-microsome and heavy-microsome fractions from the liver of young and adult rats with either [^{14}C]leucine or [^{14}C]phenylalanine (Figs. 4–9) leave no doubt that, though the free ribosomes are comparatively inactive, a heavier fraction sedimenting between the peaks of bound and free ribosomes is very active. That this activity is shifted towards the free ribosomes by treatment with pancreatic ribonuclease is consistent with the presence of polysomes. Although these polysomes are not associated with the rough endoplasmic reticulum and may be termed free polysomes, they may be stabilized by the presence of some protein-containing membrane since the RNA/protein ratio of this fraction (Fig. 2) is less than that of the free ribosome peak.

During the course of this work Bloemendal, Bont & Benedetti (1964) showed that polysomes could be isolated from rat liver without the use of detergent and that they possessed activity for the incorporation of amino acids. Webb, Blobel & Potter (1964) have shown that the proportion of polysomes detectable in extracts of liver from adult rats prepared without the use of deoxycholate is between 25 and 50% of that when detergent is used. Henshaw *et al.* (1963) failed to detect radioactive protein in the polysome region of the density gradient after injecting rats with [^{14}C]arginine. It may be that the animals were killed too long after injection of the ^{14}C -labelled amino acid to detect such activity, for the fact that radioactive protein appeared to be associated with the bound ribosomes could have been merely because it was bound to the phospholipid membrane (see, e.g., Fig. 6, showing the effect of ribonuclease on the heavy-microsome fraction). Another reason for the failure to detect free polysomes is probably because they are unstable in the presence of low concentrations of Mg^{2+} . Hence Munro *et al.* (1964) in their experiments with polysomes isolated from liver after treatment with deoxycholate showed

that at 0.1 mM- Mg^{2+} most of the polysomes are degraded. It is difficult to know the best concentration in which to carry out the density-gradient centrifugation, for if the Mg^{2+} concentration is too high the two peaks are not well separated. This could be because the bound and free ribosomes do not separate well under these conditions or because the presence of free polysomes is obscuring the fractionation. On the other hand, with low concentrations of Mg^{2+} the apparently good separation may be due to the breakdown of the free polysomes. We believe that the conditions we have used are a satisfactory compromise.

The results that show the effect of polyU (Figs. 7, 8 and 9) point clearly to the free ribosomes as being the main site of action of this synthetic messenger RNA. In some cases the increase in incorporation of phenylalanine is about 30-fold. That the most active component is one having a slightly higher density than the free ribosomes probably indicates the formation of small polysomes by the attachment of some free ribosomes to the polyU. Similar results have been indicated from studies on the incubation of [^{14}C]polyU with microsomes (P. N. Campbell & E. Lowe, unpublished work). The results with the heavy-microsome fraction suggest that polyU has some stimulatory effect on the bound ribosomes. This could be due to contamination of this fraction with free ribosomes, but a more likely explanation is that during the course of incubation ribosomes are released from the polysomes associated with the endoplasmic reticulum and these are immediately stimulated by the presence of polyU, the radioactive protein being trapped by the phospholipid membrane.

The presence of active free polysomes in the microsome fractions explains the similar activities of the microsome preparations from the livers of adult and young rats. If the polysomes whether bound or free are equally active the relative proportion of each would not affect the total activity of the microsome fraction. The similar effect of polyU is presumably because each fraction contains the same proportion of free ribosomes.

EXPLANATION OF PLATE I

Section of a liver parenchymal cell from a rat 5 days after birth. In the field is a nucleus (n) with its limiting membrane (nm), mitochondria (m), endoplasmic reticulum (er) with ribosomes attached and other unattached ribosomes (r), some of which are aggregated to form free polysomes (arrowed). Tissue was fixed in OsO_4 in phosphate buffer, pH 7.3, and embedded in Epikote 812. Thin sections were double-stained with uranyl acetate and a lead salt and examined in a Siemens Elmiskop I electron microscope. Magnification $\times 60000$.

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(Facing p. 430)

In conclusion, it seems that the rat liver cell contains three species of ribosomes in the cytoplasm: those present in the form of polysomes attached to the rough endoplasmic reticulum, those present in the form of polysomes but not associated with the rough endoplasmic reticulum (the free polysomes), and the free ribosomes that are the products of protein synthesis and hence are not associated with messenger RNA but that are ready to perform again when a suitable messenger RNA is available. It seems probable that the bound polysomes are responsible for the synthesis of proteins for export such as albumin (see, e.g., Sargent & Campbell, 1965). The object of the activity of the free polysomes can only be surmised but, to the extent that they are not part of the rough endoplasmic reticulum, one suspects that the electron microscopists will be proved right and that the free polysomes synthesize protein for the internal activity of the liver cell.

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REFERENCES

- Birbeck, M. S. C. & Mercer, E. H. (1961). *Nature, Lond.*, **189**, 558.
Bloemendal, H., Bont, W. S. & Benedetti, E. L. (1964). *Biochim. biophys. Acta*, **87**, 177.
Campbell, P. N., Cooper, C. & Hicks, M. (1964). *Biochem. J.* **92**, 225.
Campbell, P. N. & Lowe, E. (1964). *Biochem. J.* **92**, 31P.
Clark, V. M. & Kirby, A. J. (1963). *Biochim. biophys. Acta*, **78**, 732.
Decken, A. von der & Campbell, P. N. (1962). *Biochem. J.* **84**, 449.
Henshaw, E. C., Bojarski, T. B. & Hiatt, H. H. (1963). *J. molec. Biol.* **7**, 122.
Howatson, A. F. & Ham, A. W. (1955). *Cancer Res.* **15**, 62.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Mejbaum, W. (1939). *Hoppe-Seyl. Z.* **258**, 117.
Munro, A. J., Jackson, R. J. & Korner, A. (1964). *Biochem. J.* **92**, 289.
Oliver, I. T., Blumer, W. F. C. & Witham, I. J. (1963). *Comp. Biochem. Physiol.* **10**, 33.
Palade, G. E. (1955). *J. biophys. biochem. Cytol.* **1**, 59.
Porter, K. R. (1954). *J. Histochem. Cytochem.* **2**, 346.
Prescott, D. M. (1960). *Annu. Rev. Physiol.* **22**, 17.
Rendi, R. & Hultin, T. (1960). *Exp. Cell Res.* **19**, 253.
Sargent, J. R. & Campbell, P. N. (1965). *Biochem. J.* **96**, 134.
Serck-Hanssen, G., Lowe, E. & Campbell, P. N. (1965). *Abstr. 2nd Meet. Fed. Eur. biochem. Soc., Vienna*, p. 342.
Slautterback, D. B. & Fawcett, D. W. (1959). *J. biophys. biochem. Cytol.* **5**, 441.
Warburg, O. & Christian, W. (1942). *Biochem. Z.* **310**, 384.
Webb, T. E., Blobel, G. & Potter, V. R. (1964). *Cancer Res.* **24**, 1229.