Studies on the Role of the Morphological Constituents of the Microsome Fraction from Rat Liver in Protein Synthesis

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(*Received* 4 *December* 1963)

It is established that the major protein-synthesizing activity of the liver cell resides in the microsome fraction. This has been shown both by studies in the intact animal and also by incubation of the isolated fraction. More recently evidence has been obtained for the synthesis of serum albumin by such a preparation (Campbell & Kernot, 1962; Sargent & Campbell, 1963).

Morphologically the liver microsome fraction is complex and it is necessary therefore to determine which of the various elements is responsible for the synthesis of protein. Palade & Siekevitz (1956) were the first to show that a major constituent of the microsome fraction is pieces of the rough-surfaced endoplasmic reticulum. This is thought to consist of a phospholipid membrane studded with ribosomes. Other elements are the smooth-surfaced reticulum, also associated with the Golgi zone, and free ribosomes. These three constituents are depicted in Fig. 1. It is not certain to what extent the different constituents are truly separate entities, for the free ribosomes may have been derived from the rough-surfaced endoplasmic reticulum, their detachment giving rise to the smooth-surfaced membrane. Nevertheless, elements with these characteristics may be seen in electron micrographs of the intact liver cell.

If the microsome fraction of rat liver is treated with detergents such as deoxycholate or Lubrol then the membrane components are rendered soluble and the particles may be collected by centrifugation. As shown in Fig. 1 these particles may have come from the rough-surfaced endoplasmic reticulum or from the free ribosomes. Many call these particles obtained by the use of detergents 'ribosomes', but since it is not clear in what way the ribosomes have been affected by the detergent it seems preferable to refer to them as 'RNP† particles'.

The view held at present is that the protein-synthetic activity of the cell is confined to the ribosomes and that the endoplasmic reticulum is concerned only with the segregation and transport of protein in and from the cell. The present work is an attempt to examine critically this viewpoint by studying the protein-synthetic activity of a variety of microsomal subfractions whose morphology has been checked by examination in the electron microscope.

Protein-synthetic activity may be determined in a variety of ways, but two have been employed in the present work: first, the ability of the fraction to effect the energy-dependent incorporation of ^{14}C-labelled amino acid into uncharacterized protein, and secondly, the effect of the addition of a polynucleotide, polyU, on the incorporation of [^{14}C]phenylalanine.

Some aspects of this work have been reported in a preliminary form (Campbell & Cooper, 1963).

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. Pyruvate kinase and the silver–barium salt of phosphoenolpyruvic acid were obtained from Boehringer und Soehne G.m.b.H., Mannheim, Germany.

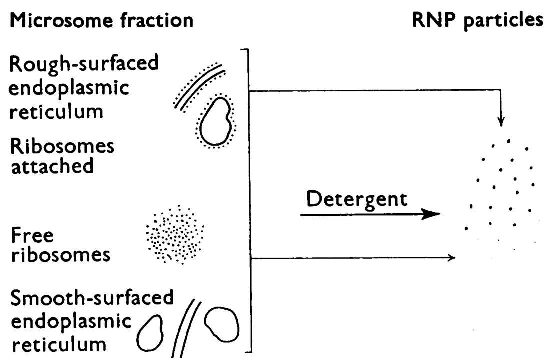

Fig. 1. Diagrammatic representation of the morphological components of the microsome fraction from rat liver and the action of a detergent such as deoxycholate on it. The RNP particles may be derived either from the free ribosomes or the ribosomes attached to the reticulum.

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† The abbreviations used are: RNP, ribonucleoprotein; polyU, polyuridylic acid.

Solutions of free phosphoenolpyruvic acid were prepared before use and adjusted to pH 7·4 (glass electrode) with N-KOH. Tris was obtained from British Drug Houses Ltd., Poole, Dorset. Sodium deoxycholate was obtained from Merck A.-G., Darmstadt, Germany, the non-ionic detergent Lubrol W from Imperial Chemical Industries Ltd., Manchester, and polyU from Miles Laboratories, Stoke Poges, Slough, Bucks.

Radioactive amino acids. Uniformly labelled L-[^{14}C]-leucine (45·6 mc/m-mole) and L-[^{14}C]phenylalanine (62 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks.

Animals. Rats (150–200 g. body wt.) were Wistar albino highly inbred at this Institute. Regenerating liver was obtained from rats 2 days after partial hepatectomy by the method of Higgins & Anderson (1931). The rats were starved for about 18 hr. before being killed by a blow on the head followed by decapitation.

Isolation of ribonucleoprotein particles. The RNP particles were isolated by the method of Rendi & Hultin (1960) as described in detail by Decken & Campbell (1962).

Preparation of microsomes. Microsomes were prepared by a method that followed closely that of Zamecnik & Keller (1954). 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 by the addition of HCl and checked by a glass electrode at 25°) and sucrose (0·15M) (Rendi & Hultin, 1960). The tissue suspension was centrifuged for 10 min. at 12000g to remove cell debris, nuclei and mitochondria. The supernatant was centrifuged in a Spinco model L preparative centrifuge (no. 40 rotor) for 50 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 1·2 ml. of medium A by gentle homogenization.

Preparation of a membrane fraction. This method involved the detachment of lipid and lipoprotein from the remaining components of the microsome fraction by homogenizing the microsomes with iso-octane according to the method of Hawtrey & Schirren (1962). A suspension of washed microsomes was homogenized with an equal volume of ice-cold iso-octane. After 30 min. at 0° the emulsion was centrifuged at 105000g for 60 min. This produced a gelatinous nucleoprotein pellet at the bottom of the centrifuge tube and a layer at the interface between the sucrose and the iso-octane. The iso-octane layer was aspirated and discarded, and the fraction at the interface was collected with a spatula.

Subfractionation of the microsomes by sucrose-density-gradient centrifugation. The method used was essentially that described by Dallner, Orrenius & Bergstrand (1963), except that the centrifugation procedures were modified for a Spinco model L ultracentrifuge rather than a Christ model Omega used by those authors. The liver was homogenized in 0·3M-sucrose and the homogenate centrifuged for 30 min. at 20000g. After the addition of $MgCl_2$ to the supernatant containing the microsomes (supernatant P) the total microsome fraction was obtained by diluting supernatant P with 0·3M-sucrose containing $MgCl_2$ (10 mM) and centrifuging at 105000g for 100 min. For the subfractions supernatant P was layered over 1·5M-sucrose containing $MgCl_2$ (10 mM) and centrifuged for 75 min. at 105000g. This provided a pellet designated the 'rough vesicles', and an upper phase. The upper phase including the fluffy layer at the interface was aspirated, diluted with 0·3M-sucrose and centrifuged at 105000g for 100 min. The pellet was collected and designated the 'smooth vesicles'.

Method of incubation. All 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.), cell sap (0·1–0·2 ml.) or pH 5 fraction (prepared as described by Decken & Campbell, 1962) (0·1–0·2 ml.), ^{14}C-labelled amino acid, 2 μmoles of ATP, 10 μmoles of phosphoenolpyruvate and 50 μg. of pyruvate kinase in a total volume of 1·0 ml. In the presence of pH 5 fraction, 0·25 μmole of GTP was also added. Incubation was for 60 min. in air at 37° for the RNP-particle preparations and 30 min. for all the other preparations.

Extraction of protein for radioactive assay. After incubation of the various preparations the protein was precipitated by the addition of 5% (w/v) trichloroacetic acid. 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.

Measurement of radioactivity. The protein was either plated on 0·28 cm.^{2} Perspex disks and counted at infinite thickness, or the protein was dissolved in 98–100% formic acid (AnalaR), transferred to 5 cm.^{2} aluminium disks, dried under an infrared lamp and counted at infinite thinness. In the latter case the weight of protein was determined by weighing and the result expressed as counts/min./mg. of protein. In either case the 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.

Estimations. Protein was determined by the Folin–Ciocalteu method of Lowry, Rosebrough, Farr & Randall (1951). RNA was determined by the orcinol method of Mejbaum (1939).

RESULTS

Chemical analysis and morphology of various microsomal subfractions

Preparations obtained with the use of iso-octane. (1) RNA and protein estimations. As explained in the Materials and Methods section homogenization of the microsome fraction with iso-octane produced two fractions: 'interface' and 'pellet'. The RNA:protein ratio for the interface fraction varied from 0·13 to 0·20 and that for the pellet from 0·22 to 0·29. Hawtrey & Schirren (1962) reported RNA:protein ratios 0·08 for the interface fraction and 0·36 for the pellet.

(2) Electron microscopy, Hawtrey & Schirren (1962) stated that the pellet fraction consisted of nucleoprotein in spite of the fact that the RNA:pro-

Table 1. *Comparison of the activities of microsomes and ribonucleoprotein particles from normal and regenerating liver for the incorporation of* [^{14}C]*leucine*

The microsomes and RNP particles were prepared from normal rats and rats 40 hr. after partial hepatectomy. The conditions for incubation of the preparations were in general as described in the Materials and Methods section. Each tube contained, in 1 ml., 0·2 ml. of cell sap or pH 5 fraction from normal liver and 0·25 μc of [^{14}C]-leucine. GTP and pyruvate kinase were included when pH 5 fraction was included. After incubation at 37° in air, 30 min. for microsomes, and 60 min. for RNP particles, the contents of the tubes were diluted to 11 ml. with medium A and centrifuged at 105000g for 60 min. The pellets were then treated with 5% (w/v) trichloroacetic acid and the radioactivity of the protein was determined at infinite thinness. The results are expressed as counts/min./mg. of protein.

Preparation	Source	Soluble fraction for incubation	Radioactivity of particulate protein
Microsomes	Normal liver	pH 5 fraction	596
	Regenerating liver	pH 5 fraction	1014
	Normal liver	Cell sap	803
	Regenerating liver	Cell sap	1393
RNP particles	Normal liver	pH 5 fraction	1718
	Regenerating liver	pH 5 fraction	1660
	Normal liver	Cell sap	2885
	Regenerating liver	Cell sap	2886

tein ratio was much lower than that usually obtained by the use of deoxycholate (0·36 compared with 0·95). The electron micrographs of this fraction (Plate 1a) showed that it contained virtually only ribosomes in apparently good morphological condition.

Hawtrey & Schirren (1962) suggested that the interface fraction consisted of smooth-surfaced endoplasmic reticulum and that the RNA present was not in the form of ribosomes but represented an integral part of a phospholipid-rich membrane, as first suggested by Chauveaux, Moule, Rouiller & Schneebeli (1962). Examination of electron micrographs of this fraction (Plate 1b) show that, though there is membrane present, there are also abundant ribosomes which could account for all the RNA in the fraction. It is not possible to tell what proportion of the total ribosomes is free or what proportion is attached to the modified membrane.

Subfractionation of the microsome fraction according to the method of Dallner et al. (1963). (1) RNA and protein estimations. As explained in the Materials and Methods section, differential centrifugation in sucrose produced two fractions, one designated 'rough' and the other 'smooth'. Although for technical reasons the centrifuge methods used had to be changed from the original in detail, the close similarity between the analytical results obtained by the two methods suggests that the fractions obtained were similar. The range of results obtained for RNA:protein ratios with many preparations were as follows: unfractionated, 0·19–0·24; 'smooth' fraction, 0·04–0·05; 'rough' fraction, 0·23–0·34.

(2) Electron microscopy. As shown in Plate 2, the 'smooth' fraction contained many smooth vesicles but little rough-surfaced reticulum. There are, however, many free ribosomes, and these particles could account for the RNA content of the fractions. Dallner *et al.* (1963) concluded that the 'smooth' fraction was free from particles, but examination of their electron micrograph reveals the presence of ribosomes.

Protein-synthetic activity

Comparison of microsomes and ribonucleoprotein particles from normal and regenerating liver. It has been repeatedly shown that the microsome fraction obtained from the liver of a rat within 48 hr. of partial hepatectomy is more active for the incorporation of amino acids than is that obtained from normal liver. The increased activity is associated both with the particulate fraction and with the soluble cytoplasm. Experiments were performed to assess the relative contribution to the greater activity of the regenerating-liver particulate fraction of the membranous and RNP components. This was achieved by comparing the activity of washed microsomes and RNP particles from normal and regenerating liver incubated in the presence of either cell sap or pH 5 fraction from normal liver. After incubation the particulate fraction was recovered and the radioactivity determined. The results shown in Table 1 indicate that in the presence of either pH 5 fraction or cell sap the microsomes from regenerating liver are more active than those from normal liver, whereas there is virtually no difference in the activity of the RNP particles.

Effect of polyuridylic acid on the incorporation of phenylalanine by unfractionated microsomes and ribonucleoprotein particles. The effect of polyU on the incorporation of [^{14}C]phenylalanine by both microsomes and RNP particles was markedly

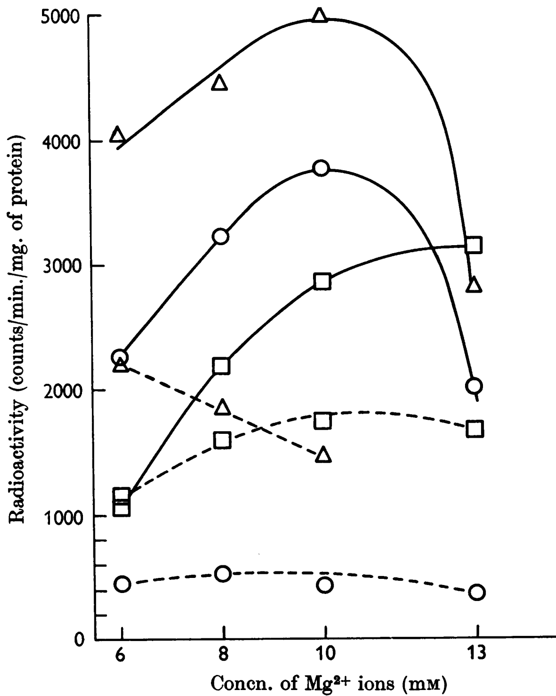


Fig. 2. Effect of polyU on the incorporation of [^{14}C]-phenylalanine by microsome and RNP-particle preparations. The concentration of Mg^{2+} ions is based on the estimation method of which details are given in the Addendum. The preparations were incubated under the conditions given in the Materials and Methods section. Where indicated, 24 μg. of polyU was added to the microsome preparation and 45 μg. to the RNP-particle preparations. ○, Microsomes from normal liver, with (——) and without (----) polyU; △, microsomes from regenerating liver, with (——) and without (----) polyU; □, RNP particles from normal liver, with (——) and without (----) polyU.

affected by the concentration of Mg^{2+} ions in the incubation medium. Fig. 2 summarizes the results obtained. Increasing the Mg^{2+} ion concentration has little effect on the activity of normal liver microsomes, decreased the activity of regenerating-liver microsomes and increased the activity of RNP particles. In the presence of polyU the activity of both microsome preparations rose to a peak at about 10 mM-Mg^{2+} ion, but the optimum concentration with the RNP particles was higher. The effect of increasing concentrations of Mg^{2+} ions on the incorporation of [^{14}C]phenylalanine by RNP particles from normal and regenerating liver is shown in Fig. 3. There is virtually no difference in the shape of the curves for the two preparations and the optimum Mg^{2+} ion concentration is about 15 mM.

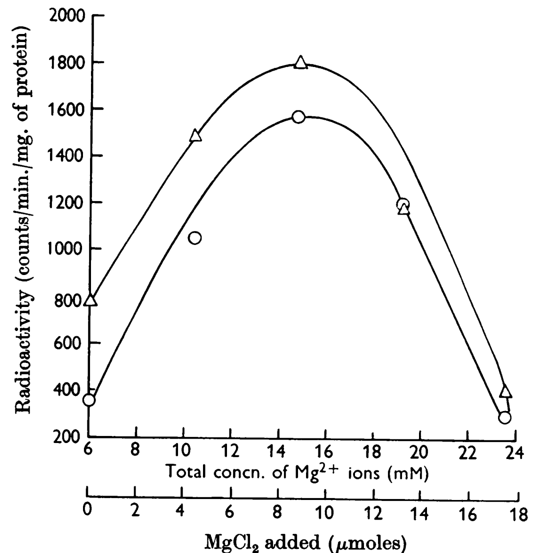


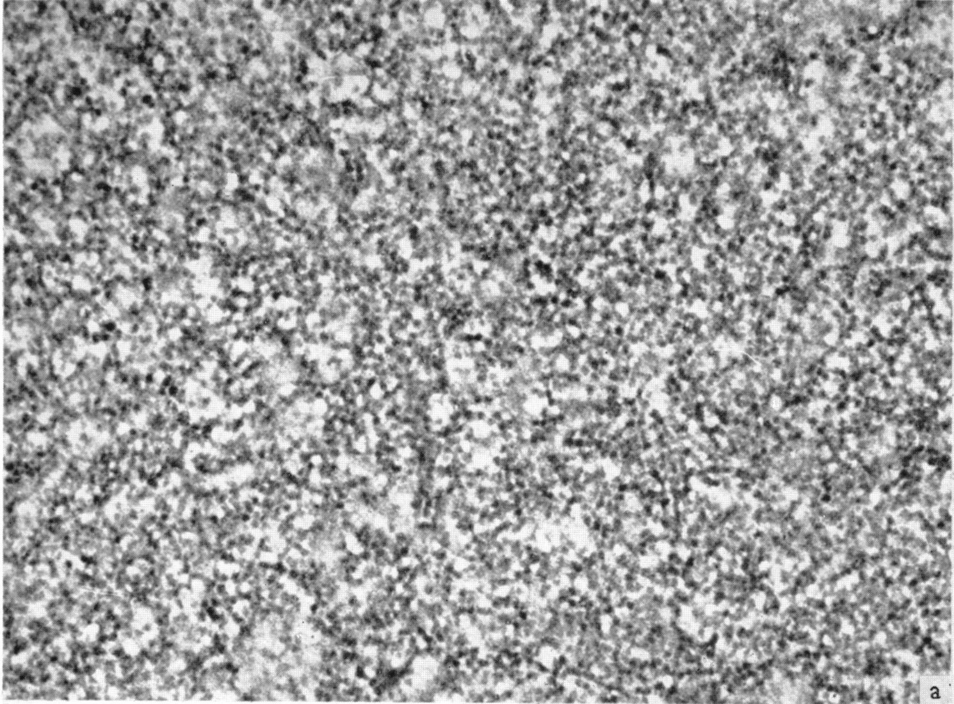
Fig. 3. Effect of polyU on the incorporation of [^{14}C]-phenylalanine by RNP particles from normal and regenerating liver and the effect of variations in the concentration of Mg^{2+} ions. Incubations were carried out as described in the Materials and Methods section in the presence of 24 μg. of polyU. pH 5 fraction was prepared from the appropriate source. ○, RNP particles from regenerating liver; △, RNP particles from normal liver.

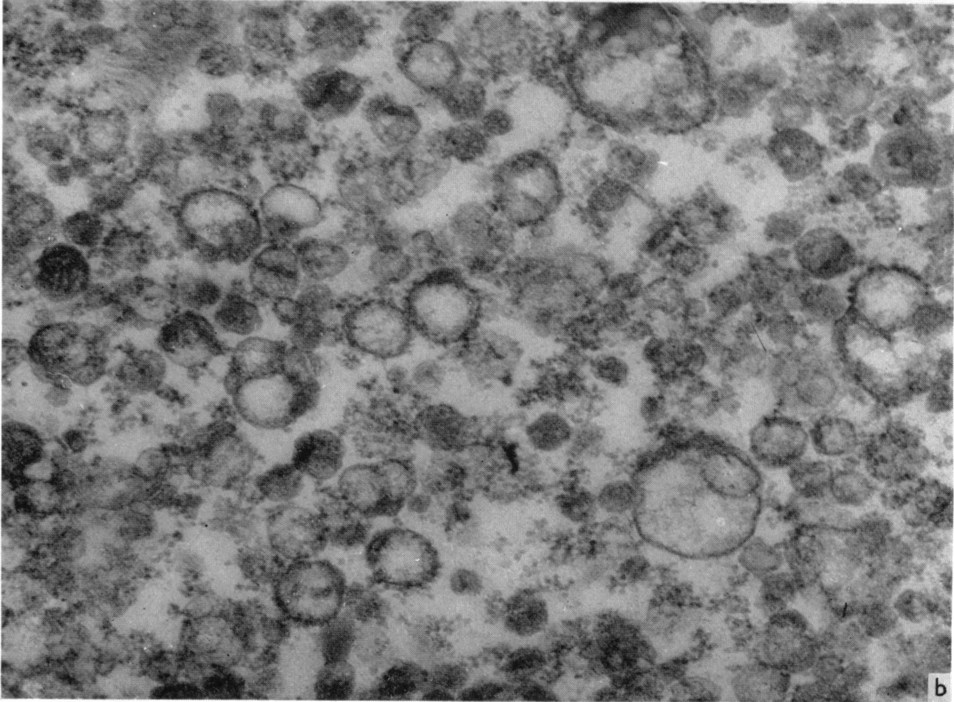
One possible reason for the difference in the effect of Mg^{2+} ion concentration on the microsomes and RNP particles could be that the RNA:protein ratio of the RNP particles is about 4 times that of the microsomes. A similar experiment was performed with much lower concentrations of the RNP-particle suspension such that the RNA concentration was similar to that used with microsome preparations. This change had no effect on the results.

The effect of increasing the concentration of polyU in the medium was to raise the activity of normal liver microsomes. The optimum concentration of polyU varied between different preparations but was usually about 100 μg./ml.

EXPLANATION OF PLATE 1

Plate 1. (*a*) Heavy fraction from a liver microsome preparation after treatment with iso-octane. The pellet contains only ribosomes. Magnification: ×60000. (*b*) Interface fraction from a liver microsomal preparation after treatment with iso-octane. Membranes together with free and attached ribosomes are present. Magnification: ×60000. Pellets were fixed in osmium tetroxide in veronal buffer, pH 7·3, and embedded in Epon 812. Thin sections were stained with uranyl acetate and examined with a Siemens Elmiskop 1 and Philips EM100 electron microscope.





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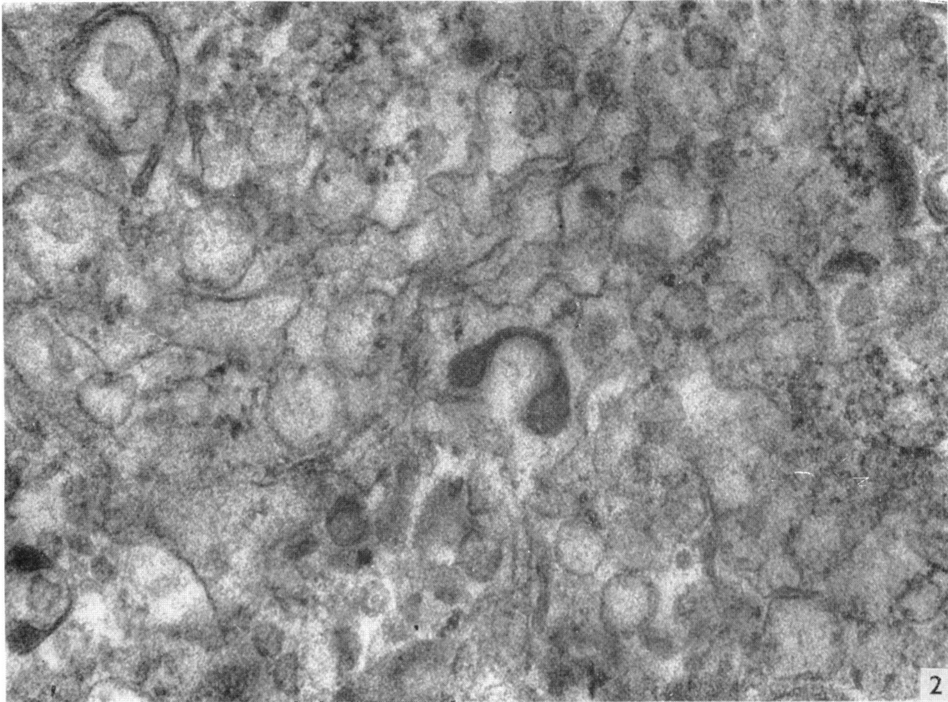

Plate 2. 'Smooth vesicle' fraction obtained by sucrose-density-gradient centrifugation from a liver microsome preparation. In addition to the smooth membranes, free ribosomes are still present in this pellet. Magnification: ×60000. Pellets were fixed in osmium tetroxide in veronal buffer, pH 7·3, and embedded in Epon 812. Thin sections were stained with uranyl acetate and examined with a Siemens Elmiskop 1 electron microscope.

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Having established that 10 mM-Mg^{2+} ion was the optimum concentration for the effect of polyU on liver microsomes it was necessary to determine the influence of changing the concentration of other constituents of the incubation medium. Table 2 shows that when the amount of pH 5 fraction is increased the ratio of the activity of the microsomes in the presence and absence of polyU tends to decrease. For this reason it has been usual to use 0·1 ml. of pH 5 fraction/ml. of incubation medium when studying the effect of polyU, even though this concentration is sub-optimum for the incorporation of amino acid in the absence of polyU.

To minimize variations between the composition of the microsome preparations these were washed with medium to remove cell sap before incubation. It seemed possible that this procedure might have an effect on the response of the microsomes to polyU. The effect of polyU on washed and unwashed preparations in the presence of pH 5 fraction was compared. The differences were not great but in general the washed preparations were more susceptible to polyU than were the unwashed ones. The effect of incubating microsomes in the presence of pH 5 fraction and cell sap was also compared. The effect of polyU was more marked in the presence of pH 5 fraction than with cell sap.

Table 2. *Effect of varying the amount of* pH 5 *fraction in the incubation medium on the effect of polyuridylic acid on the stimulation of incorporation of* [^{14}C]*phenylalanine by microsomes from normal liver*

The conditions for incubation of the microsome preparation with 0·25 μc of [^{14}C]phenylalanine were as described in the Materials and Methods section. Where indicated 25 μg. of polyU was included in the medium, the Mg^{2+} ion concentration of which was 10 mM. The results are expressed as counts/min./mg. of protein.

Amount of pH 5 fraction (ml.)	Radioactivity of protein		
	Control	polyU added	Ratio
0	270	2155	7·7
0·1	502	3827	7·3
0·2	675	3552	5·3

Because of the influence of Mg^{2+} ions on the action of polyU the effect of changing the K^{+} ion concentration was studied. The usual concentration in the incubation medium is 15 mM. When this was raised to 20 or 25 mM there was no significant effect on the degree of stimulation of [^{14}C]phenylalanine incorporation by polyU.

Since starvation has been shown to change the proportion of free and bound ribosomes in the liver cell (Henshaw, Bojarski & Hiatt, 1963), the effect of overnight starvation on the activity of the microsomes was studied. No consistent effect either on amino acid incorporation in general or on the effect of polyU was observed as the result of starvation.

The results shown in Fig. 2 indicate that, although the microsomes from regenerating liver were more active than those from normal liver in the presence of polyU, the percentage increase due to polyU was similar in the two cases. This point was examined more extensively and the results are presented in Table 3. These show that there is a tendency for the ratio of activity in the presence and absence of polyU to be higher with the preparations from regenerating liver, but the difference is of doubtful significance and was not always consistent. It seems immaterial, so far as the effect of polyU is concerned, whether the livers were taken 24 or 48 hr. after operation.

Fessenden & Moldave (1963) have shown that the pH 5 supernatant plays an important part in the synthesis of protein by RNP particles. It seemed possible therefore that the smaller stimulation of incorporation of phenylalanine by polyU in the system containing RNP particles and pH 5 fraction compared with microsomes and pH 5

Table 3. *Comparison of the effects of polyuridylic acid on the stimulation of incorporation of* [^{14}C]*phenylalanine by the microsome fraction from normal and regenerating liver*

The conditions of incubation of the preparations were in general as described in the Materials and Methods section. Each tube contained, in 1 ml., 0·1 ml. of pH 5 fraction from the appropriate source, 10 μmoles of $MgCl_2$ and 0·25 μc of [^{14}C]phenylalanine. The results obtained in the two experiments cannot be compared strictly. The ratios of the activities in the presence and absence of polyU are given in parentheses. The results are expressed as counts/min./mg. of protein.

Expt. no.	Preparation	Radioactivity of protein		
	PolyU added	None	25 μg.	50 μg.
1	Normal liver	399	2770 (6·9)	4040 (10·0)
	Regenerating liver 24 hr. after operation	570	4450 (7·8)	5670 (10·0)
2	Normal liver	476	2255 (4·7)	2924 (6·1)
	Regenerating liver 48 hr. after operation	923	5198 (5·6)	6484 (7·0)

fraction might be due to the lack of the pH 5 supernatant in the particle system (see Table 1). There was, however, no significant difference in the effect of polyU on the incorporation of phenylalanine by the particles when cell sap replaced pH 5 fraction in amounts that were either suboptimum or optimum for incorporation.

Activity of preparations after treatment with iso-octane. In agreement with the observations of Hawtrey & Schirren (1962) the membrane fraction obtained after treatment with iso-octane incorporated amino acids into protein. Under conditions that were optimum either for incorporation in the absence of polyU or in its presence the activity was only 20–50 % that of normal microsomes. The effect of polyU on this fraction was less than with normal microsomes (Table 4). The effect of Mg^{2+} ion concentration on the polyU effect showed that the optimum Mg^{2+} ion concentration was 14 mM, which is more typical of the RNP particles than whole microsomes (Fig. 4).

Hawtrey & Schirren (1962) also reported that the pellet fraction was active for amino acid incorporation and believed it to be even more active than particles prepared by the use of detergents. As shown in Table 5, the particles we have prepared with iso-octane are less active than those prepared with Lubrol and deoxycholate. The effect of polyU on the two types of particles seems to be very similar. In an attempt to raise the activity of the iso-octane-treated particles an additional washing was included in the preparation but no improvement in the activity of the preparation resulted.

Activity of subfractions of microsomes prepared by the use of sucrose-density-gradient centrifugation. Table 6 shows a comparison of the activity of the two subfractions produced by the method of Dallner *et al.* (1963) compared with that of the unfractionated pellet. The activity of the 'rough' fraction compared with the unfractionated pellet was variable, being 50–100 % of that of the pellet. The activity of the 'smooth' fraction in the absence of polyU was always very small. In the presence of polyU the increase in activity of the 'rough' fraction for the incorporation of [^{14}C]phenylalanine was at least as high as that of the unfractionated pellet. The effect of polyU on the 'smooth' fraction was very marked. When the effect of Mg^{2+} ion concentration on the activity of the 'smooth' fraction in the presence of polyU was studied the optimum concentration was 10 mM. The effect was more typical of ribosomes attached

Table 4. *Comparison of the activity of a membrane fraction produced by the action of iso-octane with that of the original microsome preparation*

The membrane fraction was prepared as described in the Materials and Methods section. The conditions of incubation were as generally described in the Materials and Methods section. Each tube contained, in 1 ml., 0·1 ml. of pH 5 fraction and 0·25 μc of [^{14}C]phenylalanine. The Mg^{2+} ion concentration of the medium was 10 mM. The results are expressed as counts/min./mg. of protein.

Preparation	Radioactivity of protein	Ratio
Unfractionated microsomes		
No polyU added	601	6·4
Plus 50 μg. of polyU	3947	
Membrane fraction		
No polyU added	294	4·1
Plus 50 μg. of polyU	1198	

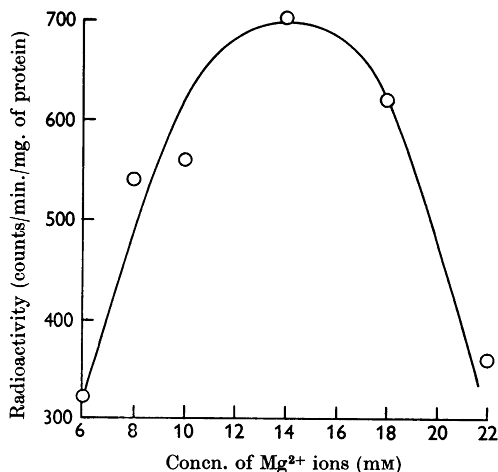

Fig. 4. Effect of Mg^{2+} ion concentration on the incorporation of [^{14}C]phenylalanine by the membrane fraction produced by the action of iso-octane. The incubations were carried out in the presence of 50 μg. of polyU and 0·25 μc of [^{14}C]phenylalanine.

Table 5. *Comparison of the activities of ribonucleoprotein particles produced by the action of iso-octane and deoxycholate*

The particles were prepared as described in the Materials and Methods section. The conditions of incubation were as generally described in the Materials and Methods section. Each tube contained, in 1 ml., 0·1 ml. of pH 5 fraction and 0·25 μc of [^{14}C]phenylalanine. The Mg^{2+} ion concentration of the medium was 10 mM. The results are expressed as counts/min./mg. of protein.

Preparation	Radioactivity of protein	Ratio
Deoxycholate-treated RNP particles		
No polyU added	933	2·4
Plus 50 μg. of polyU	2285	
Iso-octane-treated RNP particles		
No polyU added	212	2·4
Plus 50 μg. of polyU	515	

Table 6. *Comparison of the activities in the absence and presence of polyuridylic acid of subfractions of liver microsomes produced by sucrose-density-gradient centrifugation*

The fractions were prepared as described in the Materials and Methods section. The conditions of incubation were as generally described in the Materials and Methods section. Each tube contained, in 1 ml., 0·1 ml. of pH 5 fraction and 0·25 μc of [^{14}C]phenylalanine. A control series of incubations was done in the absence of ATP or phosphoenolpyruvate. The results were used to correct the radioactivity of the experimental tubes for incorporation in the absence of a source of energy. The results are expressed as counts/min./mg. of protein.

PolyU added (μg.)	Unfractionated microsomes		'Rough' fraction		'Smooth' fraction	
	Radioactivity of protein	Ratio	Radioactivity of protein	Ratio	Radioactivity of protein	Ratio
0	922	—	408	—	33	—
25	6122	6·6	3213	7·9	1157	35
50	6752	7·3	4119	10·1	1582	48

to reticulum than of RNP particles. A comparison of the activity of the 'smooth' fraction in the presence of polyU when the preparation is made from either normal or regenerating liver revealed no marked differences.

An attempt was made to remove the membrane with deoxycholate from the 'smooth' fraction to provide a sample of RNP particles derived from free ribosomes. It proved very difficult to obtain a fraction with a higher RNA:protein ratio than 0·22, compared with 0·95 under similar conditions when the unfractionated microsome pellet was used. This possibly suggests that the smooth membranes are less susceptible to treatment with deoxycholate than are the rough membranes. Some of the RNA in the smooth membrane may not be present as granules and may be soluble in the presence of deoxycholate.

An attempt was made to stimulate the incorporation of [^{14}C]leucine by the 'smooth' fraction by the addition of RNA to the incubation medium. No stimulation resulted when RNA preparations from either rat-liver microsomes or rabbit reticulocytes were used.

DISCUSSION

The morphology of the liver cell differs markedly from that of the other two types of cell that have played such a prominent role in studies on the biosynthesis of proteins by subcellular fractions. So far as the ribosomes are concerned the reticulocyte and bacterial cell are closely similar in that in neither case are the ribosomes attached to an endoplasmic reticulum. In rat liver, though most of the ribosomes appear to be attached to a membrane some also occur free in the cytoplasm. It has been generally supposed that the reason for this difference in morphology is that with the liver cell much of the protein is being synthesized for export, whereas neither the reticulocyte nor the bacterial cell synthesizes protein for this purpose. It may be therefore that the role of the membrane of the endoplasmic reticulum is almost entirely concerned with the transport of the newly synthesized protein out of the cell. In testing this hypothesis the opportunity also arises of determining whether the free ribosomes are functionally different from those attached to reticulum.

The experiments on the relative protein-synthetic activity of the preparations from normal and regenerating liver lend support to the idea that the membrane component of the microsome fraction is important in controlling the activity of the preparation. As shown in Table 1, though the microsome fraction from regenerating liver is more active than that from normal liver even when incubated in the presence of cell sap or pH 5 fraction from normal liver, the difference is lost when the RNP particles are compared. As has been shown by Decken & Campbell (1964), the activity of isolated deoxycholate-treated particles varies, but no consistent evidence has been obtained to indicate that the particles from regenerating liver are more active than those from normal liver. Since the major difference between the RNP particles and the microsomes is the existence of the membrane it appears likely that the latter plays some role in the control of the activity of the microsome fraction. Whether the membrane is effective because the ribosomes are held in some particular configuration or whether it contains some factor such as the X fraction of Hoagland & Askonas (1963) or an enzyme such as transferase II of Fessenden & Moldave (1963) must await further work. In any event it became clear that in studying the effect of synthetic polynucleotides it was essential to examine their effects on preparations containing both membranes and RNP particles.

Maxwell (1962) was the first to show that the liver microsome fraction was susceptible to polyU and that the incorporation of [^{14}C]phenylalanine could be stimulated. This observation was confirmed by Weinstein & Schechter (1962) and later by Henshaw *et al.* (1963). Fessenden, Cairncross &

Moldave (1963) showed that preparations containing RNP particles rather than microsomes were similarly affected. The results shown in Fig. 2 demonstrate that, though unfractionated microsomes from either normal or regenerating liver and RNP particles are stimulated by polyU, there are differences between the two kinds of preparation. First, the effect of polyU at a given concentration is more marked with whole microsomes than with RNP particles. Secondly, the effect of Mg^{2+} ions on the polyU stimulation is different in the two cases: thus with both the preparations of microsomes the effect is optimum at 10 mM-Mg^{2+} ion whereas with the RNP particles it is optimum at 15 mM. When the microsomes are treated with iso-octane and (according to the electron micrographs in Plate 1) the ribosomes are detached from the membrane, the polyU effect is decreased compared with normal (Table 4) and the effect of Mg^{2+} ions is now very similar to that of RNP particles (Fig. 4). It appears that a higher concentration of Mg^{2+} ions is required for the attachment of polyU to the ribosomes when they have been detached from the membrane. The fact that the free ribosomes present in the fraction containing the smooth-surfaced endoplasmic reticulum did not require a high concentration of Mg^{2+} ions may be because of the presence of the membrane component or because they are a different species of particle. Hultin & Pedersen (1963) found that with ribosomes from Ehrlich ascites-tumour cells the optimum Mg^{2+} ion concentration for stimulation by polyU was 8 mM.

There was no marked difference in the effect of polyU on the microsomes derived from normal and regenerating liver, but it is difficult to draw any firm conclusions from this finding. The fact that the activity of regenerating-liver microsomes is more susceptible to changes in the Mg^{2+} ion concentration suggests that Mg^{2+} ions may be involved in the attachment of the ribosomes to the endoplasmic reticulum. Support for this idea is provided by the work of Sachs (1958), who showed that pyrophosphate caused the detachment of ribosomes from the rough-surfaced endoplasmic reticulum of the liver microsome fraction. Griswold & Pace (1956) had previously shown that Mg^{2+} ions were concentrated in the latter fraction. Such considerations may also explain the differences in the Mg^{2+} ion concentration required for the optimum effect of polyU on the incorporation of [^{14}C]-phenylalanine by the different morphological components.

In the above experiments both types of preparations were incubated with pH 5 fraction. As has been explained this is not an adequate source of the soluble factors for the RNP particles although it is for the microsomes. Experiments were conducted therefore to determine whether the effect of polyU on the RNP particles was any greater when the pH 5 fraction was replaced by cell sap. No significant effect was detected.

Although the use of iso-octane to subfractionate the microsome fraction offered interesting possibilities of preparing a membrane fraction free of ribosomes this has not proved possible in our hands. It is clear from the electron micrograph (Plate 1*b*) that the preparation still contains many ribosomes. The fact that such a preparation retains at least some activity suggests that the precise tertiary structure for the membrane is not essential for the activity of the ribosomes normally attached to it, but further work will be necessary to confirm this conclusion.

The experiments involving the use of sucrose to subfractionate the microsome pellet indicate that the fraction which Dallner *et al.* (1963) claim to have a high proportion of the rough-surfaced endoplasmic reticulum and hence labelled 'rough' is slightly more susceptible to polyU than are the unfractionated microsomes. In contrast the so-called 'smooth' fraction, although virtually inactive for the incorporation of [^{14}C]phenylalanine in the absence of polyU, is stimulated markedly by this polynucleotide.

The electron micrograph (Plate 2) showed the 'smooth' fraction to have plenty of ribosomes present. Very few of these were attached to endoplasmic reticulum and hence it must be assumed that they were the free ribosomes of the cytoplasm. Henshaw *et al.* (1963) have subfractionated the liver microsomes by density-gradient centrifugation into two fractions containing RNA. The heavier one had a protein:RNA ratio of 6 and was assumed to be the rough-surfaced endoplasmic reticulum; the lighter one with sedimentation coefficient 83s (similar to that of the deoxycholate-treated ribosomes) had a protein:RNA ratio of 2 and was assumed to be the free ribosomes. The protein:RNA of this fraction is much higher than the 1 of the deoxycholate-treated particles, and is close to that of the particles obtained by the action of iso-octane. Henshaw *et al.* (1963) do not report on an examination of their preparation in the electron microscope. In view of the methods they used it seems probable that their light fraction contained some smooth-surfaced reticulum, which accounts in part for the high protein:RNA ratio of these fractions. They found that the free ribosome fraction has a low endogenous activity for phenylalanine incorporation but could be stimulated to at least the same activity as the other fractions in the presence of polyU. Thus the properties of their free-ribosome fraction and our 'smooth' fraction seem very similar, the difference between the two being the proportion of smooth membranes and free ribosomes. The marked stimulation of the

'smooth' fraction by polyU encouraged us to test various preparations of RNA from liver microsomes, liver and reticulocyte ribosomes and liver nuclei for their ability to stimulate the synthesis of protein by this fraction, So far no reproducible stimulatory effect has been detected.

It is difficult to be sure that the properties attributed to the 'smooth' fraction are due entirely to the free ribosomes and not to the membrane components. We have tested the effect on amino acid incorporation of adding RNP particles to the 'smooth' fraction but the particles did not thereby acquire a greater activity. The fact that the 'smooth' fraction and the free ribosome fraction obtained by Henshaw *et al.* (1963) have such very similar properties in spite of the great difference in the protein:RNA ratio in the two fractions suggests further that the membrane is having little influence on the activity of the ribosomes.

SUMMARY

1. The object was to determine the role of the different morphological constituents of the microsome fraction from rat liver in protein synthesis with particular reference to the part played by the membrane components.

2. The following microsomal and submicrosomal fractions were characterized by electron microscopy or analysis for RNA and protein or both: (*a*) The whole microsome pellet from rat liver; this is believed to contain ribosomes attached to endoplasmic reticulum, free ribosomes and reticulum which is not associated with ribosomes; the RNA:protein ratio is about 0·25. (*b*) The ribonucleoprotein particles obtained by treatment of (*a*) with deoxycholate; the RNA:protein ratio is about 1·0. (*c*) An interface fraction obtained from (*a*) by treatment with iso-octane; this appears to contain a modified phospholipid membrane intermixed with free ribosomes; the RNA:protein ratio is 0·13–0·20. (*d*) A pellet fraction also obtained from (*a*) by treatment with iso-octane which appears to contain ribosomes most of which were originally attached to endoplasmic reticulum; the RNA:protein ratio is 0·22–0·29. (*e*) A fraction obtained from (*a*) by sucrose-density-gradient centrifugation which contains endoplasmic reticulum and free ribosomes but practically no attached ribosomes; the RNA: protein ratio is about 0·05.

3. The effect of polyuridylic acid at various concentrations of Mg^{2+} ions on the incorporation of [^{14}C]phenylalanine into protein was studied by incubating each fraction in the presence of an energy source. The optimum Mg^{2+} ion concentration for the incorporation of [^{14}C]phenylalanine in the presence of polyuridylic acid for fractions containing ribosomes and endoplasmic reticulum, i.e. 2 (*a*) and 2 (*e*) above, was 10 mM. For fractions containing ribonucleoprotein particles detached from the endoplasmic reticulum by either deoxycholate or iso-octane the optimum Mg^{2+} ion concentration was 15 mM. The results were the same whether the preparations were made from normal or regenerating liver.

4. The optimum conditions for stimulation of [^{14}C]phenylalanine incorporation by microsomes (fraction 2*a*) involved incubation of the preparations washed free of cell sap in the presence of small amounts of pH 5 fraction. Under these conditions there was no significant difference in the degree of stimulation by the addition of polyuridylic acid with preparations from normal and regenerating liver. Microsomes (fraction 2*a*) were stimulated to a greater extent than ribonucleoprotein particles (fraction 2*b*).

5. Fraction 2 (*e*) had very low activity for the incorporation of [^{14}C]phenylalanine compared with fraction 2 (*a*), but was more markedly stimulated by polyuridylic acid than any of the other fractions.

6. It is concluded that the major protein-synthesizing activity of the liver cell is associated with the ribosomes attached to endoplasmic reticulum. The free ribosomes seem to be comparatively inactive but may be stimulated to synthesize protein by combination with an RNA fraction possessing messenger-like characteristics (i.e. polyuridylic acid).

We thank Professor F. Dickens, F.R.S., for his encouragement, Miss Elizabeth Lowe and Miss Eileen Walters for their conscientious assistance, and Mr Brian Waynforth for performing the partial hepatectomies. The work was made possible by a grant to the Medical School from the British Empire Cancer Campaign. Dr Cecil Cooper was a 'Research Career Development Awardee K3-GM-1602, U.S. Public Health Service' on leave of absence from the Department of Biochemistry, Western Reserve University School of Medicine.

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ADDENDUM

The Estimation of Magnesium in Preparations of Liver Microsomes

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(*Received* 4 *December* 1963)

As shown in Fig. 2 of the main paper (Campbell, Cooper & Hicks, 1964), the concentration of Mg^{2+} ions in the incubation medium has a profound effect on the stimulation of the formation of polypeptide from phenylalanine by polyU (polyuridylic acid) in the presence of microsome or ribonucleoprotein-particle preparations. The action of the synthetic polynucleotide might have been due to its effect on the distribution of Mg^{2+} ions between the particulate and soluble components of the system. For this reason, and because it was considered desirable to determine directly the concentration of such an important component of the incubation medium, the amount of Mg^{2+} ions bound to the particles under the usual conditions of incubation was determined.

METHODS

The magnesium concentrations were determined by emission flame spectrophotometry. The recorded intensities of the 2852Å Mg line for the sample and standard solutions were obtained by the short-interval wavelength-scanning method of Warren (1959) by using the improved version of the original instrument (Warren, 1962). The method gives a coefficient of variation for a single determination of 2% in the concentration range employed, namely 0·3–1·0 μg./ml. The plot of magnesium concentration against line intensity is linear in this range. Triplicate recording for each sample and the standard were used for calculating the magnesium concentration. Interference effects were absent.

The incubation tubes were prepared as described by Campbell *et al.* (1964), and contained all the components normally used for studying the effect of polyU on liver microsomes with the exception of the [^{14}C]phenylalanine. A 0·1 ml. sample from each incubation tube was diluted for the estimation of the total magnesium concentration. The remainder of the contents of the tube, approx. 0·9 ml., was ultrafiltered without dilution through a membrane con-

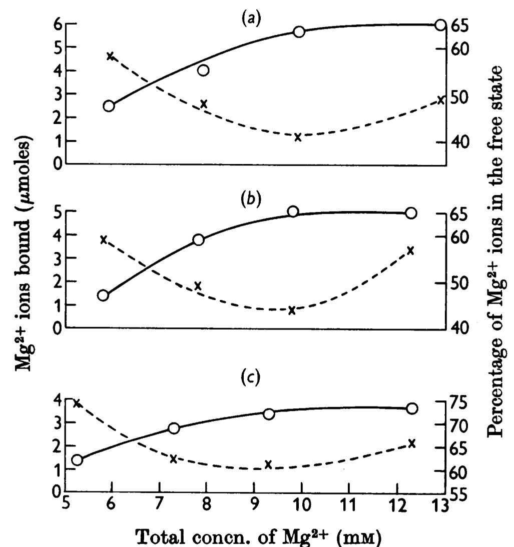

Fig. 1. Effect of varying the Mg^{2+} ion concentration on the amount of Mg^{2+} ion bound to microsomal particles. The microsome fraction was prepared from normal liver and washed as described by Campbell *et al.* (1964). Each tube contained 0·4 ml. (*a*), 0·3 ml. (*b*) or 0·2 ml. (*c*) of the standard microsome suspension, the appropriate amount of medium A being added to make up the volume. Each tube also contained, in 1 ml., 0·2 ml. of pH 5 fraction, 0·25 μmole of GTP, 50 μg. of pyruvate kinase, 15 μmoles of phosphoenolpyruvate and 2 μmoles of ATP. The Mg^{2+} ion concentration was controlled by the addition of the appropriate volume of 0·1 M-$MgCl_2$. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) on samples of the pH 5 fraction and microsome preparation used for the experiment. The protein concentrations were: (*a*) 5·5 mg./ml.; (*b*) 4·0 mg./ml.; (*c*) 2·5 mg./ml. ○, Mg^{2+} ions bound; ×, percentage of Mg^{2+} ions in the free state.

sisting of a short length of 8 mm. diam. Visking tubing knotted to form a bag, the open end being secured to a piece of glass tubing by a close-fitting rubber stopper fitted to a test tube with a side arm. The bag, after being washed with distilled water, was equilibrated with a drop of the sample solution; this drop was discarded before evacuating the tube and introducing the rest of the sample. Approx. 0·2 ml. of the filtrate was collected and diluted for the determination of the ultrafilterable magnesium. It was found necessary to perform the ultrafiltration with the undiluted sample since dilution affects the distribution between the residue and the filtrate. By equilibrating the membrane with the sample to be filtered the capacity of the membrane to adsorb Mg^{2+} ions is decreased.

RESULTS AND DISCUSSION

The results obtained when the Mg^{2+} ion concentration of the incubation medium was varied between 5 and 13 mM are shown in Fig. 1. Irrespective of the concentration of protein the maximum amount of Mg^{2+} ions bound was reached at about 10 mM, the actual quantity being bound decreasing with the protein concentration. After the optimum concentration of Mg^{2+} ions was reached the percentage of the Mg^{2+} ions in the free state naturally started to rise. As shown by Campbell *et al.* (1964) the concentration of Mg^{2+} ions at which the stimulation by polyU of phenylalanine incorporation is optimum at 10 mM. It follows that the effect of polyU is optimum when the particulate fraction is saturated with Mg^{2+} ions, excess of Mg^{2+} ions having an inhibitory effect.

When a similar experiment was carried out in which duplicate tubes contained 48 μg. of polyU, the presence of polyU had no significant effect on the percentage of Mg^{2+} ions that was bound by the particles. Under the experimental conditions used 75% of the polyU was not ultrafilterable. It is concluded that the effect of polyU on the incorporation of phenylalanine is not to be attributed to a change in the amount of Mg^{2+} ions bound to the particles.

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Stimulation and Solubilization of the Sodium Ion-Activated Adenosine Triphosphatase of Cerebral Microsomes by Surface-Active Agents, especially Polyoxyethylene Ethers: Actions of Phospholipases and a Neuraminidase

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(*Received* 14 *November* 1963)

Part of the adenosine-triphosphatase activity of cerebral tissues is firmly attached to microsomal material and, by centrifuging cerebral dispersions, can largely be separated from the differently activated adenosine triphosphatases of other subcellular fractions (Deul & McIlwain, 1961; Schwartz, Bachelard & McIlwain, 1962). For many aspects of its further study, however, release from the microsomal structure is necessary and has now been achieved.

Importance is given to the microsomal enzyme through characteristics of its activation by Na^+ and K^+ ions (McIlwain, 1962*a*, *b*, 1963; Aldridge, 1962), which connect it and similarly activated enzymes in other tissues (Skou, 1957; Dunham & Glynn, 1961; Bonting, Simon & Hawkins, 1961; Wheeler & Whittam, 1962) with a possible role in active cation movement. Some reflexion of this function might be found in relationships between the enzyme and microsomal structures, and be displayed on its release; thus a suggestion of how the enzyme performs a role in ion movement postulates particular relationships between the enzyme and membrane structures (McIlwain, 1962*a*, 1963). It was, therefore, especially interesting to find that certain surface-active agents, before they released the adenosine triphosphatase, enhanced its activity, and did so specifically in the presence of sodium