Microsomal Components in Relation to Amino Acid Incorporation by Preparations from the Developing Rat Brain

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1. After incorporation of [^{14}C]valine *in vitro*, cerebral microsomes were separated into membrane-bound and free ribosomes by sucrose-density-gradient centrifugation. 2. In preparations from both 4-day-old and adult rats, free and bound ribosomes incorporated [^{14}C]valine. Free ribosomes could be found as polysomes, which were highly active. 3. Microsomes labelled with [^{14}C]valine *in vitro* were fractionated after deoxycholate treatment into a preliminary sediment, sedimented at 105000g (5min.), and ribonucleoprotein particles, sedimented at 150000g (70min.), to determine the role of membrane-bound ribosomes. In the adult the ribonucleoprotein particles retained most of the radioactivity, whereas in the young the preliminary sediment was as highly labelled as the ribonucleoprotein particles. 4. The labelled preliminary sediment from young preparations was both ribonuclease- and deoxycholate-resistant, and the nature of this material is discussed in terms of a possible structural component of microsomal membranes.

Basic mechanisms of protein synthesis in the brain do not appear to differ from those of other tissues (Lajtha, 1964). After labelling of brain proteins *in vivo*, microsomes were found to have the highest specific activity (Clouet & Richter, 1957, 1959; Furst, Lajtha & Waelsch, 1958). Isolated microsomes from brain incorporate amino acids at rates comparable with those by microsomes from other tissues (Satake, Mase, Takahashi & Ogata, 1960; Acs, Neidle & Waelsch, 1961; Zomzely, Roberts & Rapaport, 1964). Hanzon & Toschi (1959, 1960, 1961) have investigated the nature of cerebral microsomes. The microsomes consisted of vesicles and particles and have been correlated with the original cell structure, the endoplasmic reticulum identified by Palay & Palade (1955) being the main contributor to this fraction.

Differences in the incorporation of [^{14}C]valine by cerebral microsomes from 4-day-old and adult rats have been reported by Adams & Lim (1966). A separation of cerebral microsomes into the various components may be useful in ascertaining the relative contributions of these components in the incorporation of [^{14}C]valine *in vitro*. Membrane-bound and free ribosomes of liver microsomes have been separated by sucrose-density-gradient centrifugation to study the incorporation of amino acids *in vivo* and *in vitro* (Henshaw, Bojarski & Hiatt, 1963; Campbell, Serck-Hanssen & Lowe, 1965). A similar method, as well as the use of deoxycholate to obtain RNP‡ particles, was employed in the present study to investigate the incorporation of [^{14}C]valine by microsomes from the developing rat brain.

MATERIALS AND METHODS

Animals. Wistar albino rats bred in this Institute were used when 3–4 days of age (weight about 7g.) or as adults (weighing 200–250g.).

Chemicals. The disodium salts of ATP and creatine phosphate were purchased from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). Sodium deoxycholate of analytical grade was obtained from British Drug Houses Ltd. (Poole, Dorset). Lubrol W was from Imperial Chemical Industries Ltd. (Wilmslow, Cheshire). Bovine pancreatic ribonuclease (five-times crystallized) was from Mann Research Laboratories Inc. (New York, N.Y., U.S.A.). DL-[1-^{14}C]-Valine was obtained from The Radiochemical Centre (Amersham, Bucks.) and used undiluted (33·9μc/μmole).

Preparation of homogenates and subcellular fractions. Rats were killed by decapitation, the brains were rapidly removed and transferred to an ice-cold dish, and the cerebral cortices were removed and dissected. All subsequent procedures were done at 0–4°. The cortices were pooled and weighed and homogenized in tris-buffered

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‡ Abbreviations: RNP, ribonucleoprotein; (Na^+,K^+)-ATPase, Na^+-plus-K^+-stimulated adenosine triphosphatase.

sucrose medium (0·25M-sucrose–4mM-$MgCl_2$–25mM-KCl–50mM-tris–HCl buffer, pH 7·4). Centrifugations were carried out in the no. 50 rotor of the Spinco model L ultracentrifuge. The homogenate was centrifuged twice at 15000g (10min.) to remove cell debris, nuclei and mitochondria. The resulting post-mitochondrial supernatant was then centrifuged at 150000g (70min.) to obtain a microsomal pellet, with the upper two-thirds of the final supernatant being used as cell sap in amino acid-incorporation experiments. The pellet was rinsed with the tris-buffered sucrose medium described above and gently resuspended in the same medium in an all-glass homogenizer. A final microsomal suspension was obtained by using the supernatant resulting from a low-speed centrifugation (1800 rev./min. in the MSE Mistral 6L refrigerated centrifuge) of the resuspended microsomes to remove aggregated material.

Preparation of ribonucleoprotein particles. RNP particles were prepared either from the post-mitochondrial supernatant to obtain RNP particles for ultracentrifugal analysis, or from microsomal suspensions, either before or after incubation with [^{14}C]valine, according to the method of Siekevitz (1962). Freshly prepared 5% (w/v) sodium deoxycholate in 0·2M-glycylglycine–NaOH buffer, pH 8·2, was added with constant stirring to the suspension of microsomes, to give a final concentration of 0·25%. The clarified suspension was immediately centrifuged at 105000g (5min.). The supernatant was carefully removed and centrifuged at 150000g (70min.) to obtain RNP particles. The pellet was washed with the tris-buffered sucrose medium described above and gently resuspended in the same medium in an all-glass homogenizer.

Ultracentrifugal measurements of ribonucleoprotein particles. RNP particles in the tris-buffered sucrose medium described above were made up to a concentration of 1–2mg./ml. The particles were centrifuged in a 30mm./4° cell in the AN-E rotor of the Spinco model E analytical ultracentrifuge. Photographs were taken with schlieren optics, at 4min. intervals after the rotor (temperature regulated at 1·5°) had reached full speed (29500 rev./min.). Exposure time was 20sec. and the bar angle was 50°. Centrifugation was carried out for 21min.

Incubation conditions. Microsomal suspensions (final concn. 1–2mg./ml.) in the tris-buffered sucrose medium described above were incubated with concentrations of cell sap designed to give maximal incorporation of 0·5μc of [^{14}C]valine, as described by Adams & Lim (1966). The additives were $MgCl_2$ (10mM), KCl (20mM), ATP (1mM), creatine phosphate (20mM) and tris–HCl buffer, pH 7·6 (20mM). The final volume was 1ml. and the mixture incubated at 37° for 30min. When microsomes were to be separated on the sucrose density gradient, 1ml. of ice-cold buffer without sucrose (mM-$MgCl_2$–25mM-KCl–35mM-tris–HCl buffer, pH 7·8), containing valine (50mM), was added to give a final sucrose concentration of less than 5% (w/v). When labelled microsomes were to be treated with deoxycholate and recentrifuged to obtain RNP particles and other subfractions, the reaction was stopped by the addition of 9ml. of the tris-buffered sucrose medium described above, containing valine (50mM).

Sucrose-density-gradient centrifugation. This was done as described by Campbell *et al.* (1965), with certain modifications. Suspensions (2ml. volumes) of microsomes or RNP particles were carefully layered on 23ml. of a linear 5–20% (w/v) sucrose gradient in mM-$MgCl_2$–25mM-KCl–35mM-tris–HCl buffer, pH 7·8. A 5ml. cushion of 50% (w/v) sucrose was initially introduced at the bottom of the tube. Microsomes were centrifuged for 2½hr. and RNP particles for 3hr. at 53000g (25000 rev./min.) at 4° in the SW 25 rotor of the Spinco model L ultracentrifuge. Fractions (0·5ml.) were collected as described by Campbell *et al.* (1965), diluted with 2ml. of water and their E_{260} values determined.

Preparation and measurement of radioactivity in protein. When microsomes were assayed for relative distribution of radioactivity in microsomes and supernatant, or, after deoxycholate treatment, in the 105000g (5min.) sediment, RNP pellet and deoxycholate-soluble fraction, the protein was precipitated and the radioactivity measured as described by Adams & Lim (1966).

Fractions derived from sucrose-density-gradient centrifugation of ribosomes were also assayed in the same way except that 0·2mg. of bovine serum albumin was added before the initial precipitation with 5% (w/v) trichloroacetic acid, containing valine (final concn. 50mM). Fractions derived from RNP particles were assayed according to the method of Munro, Jackson & Korner (1964), with slight

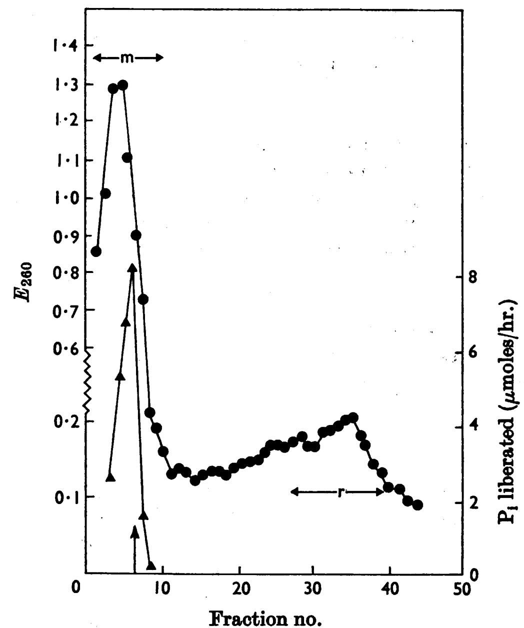

Fig. 1. Separation of bound and free ribosomes from cerebral microsomes of adult rats. The conditions for the sucrose density gradient were as described in the Materials and Methods section. The 20–50% sucrose interface is indicated by the arrow. Fractions (0·5ml.) were removed from the bottom of the tube so that in this and other Figures the lowest-numbered fractions are the most dense. The RNA/protein ratio of fractions corresponding to membrane-bound ribosomes (m) was 0·1, and that of fractions corresponding to free ribosomes (r) was 0·35. ●, E_{260}; ▲, μmoles of phosphate liberated/hr. on stimulation by Na^+ plus K^+.

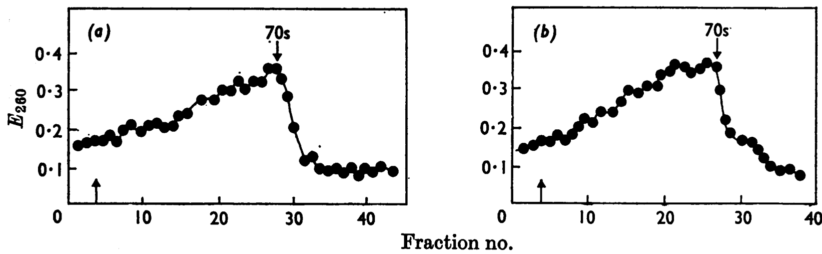

Fig. 2. Sucrose-density-gradient centrifugation of RNP particles obtained from cerebral microsomes of (*a*) adult and (*b*) 4-day-old rats. Centrifugation was for 3hr. The approximate position of monomers is indicated by the arrows.

modifications. The precipitate in 5% trichloroacetic acid was heated at 90° for 15min. and filtered on Oxoid membranes with a Millipore filter. The precipitates were washed with 5% trichloroacetic acid and ethanol and the membranes stuck to a cardboard disk. They were dried and counted in a Nuclear–Chicago continuous-gas-flow end-window counter (30% efficiency).

Estimations. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). RNA was determined by the orcinol method (Hurlbert, Schmidt, Brumm & Potter, 1954).

Adenosine-triphosphatase assay. The procedure described by Schwartz, Bachelard & McIlwain (1962) was used for determinations in the presence and absence of Na^+ and K^+.

RESULTS

Fractionation of microsomes and ribonucleoprotein particles on the sucrose density gradient. (Na^+,K^+)-ATPase of cerebral microsomes has been shown to be associated with membrane structures rather than with the RNP granules (Schwartz *et al.* 1962). This enzymic marker was used to locate the presence of membrane-containing fractions separated on the gradient. As with liver microsomes, cerebral microsomes could be separated into free ribosomes and ribosomes bound to membrane (Fig. 1). The membrane-bound fraction sedimented as a sharp peak at the region of the interface between the 50%-sucrose cushion and the 20% sucrose. Free ribosomes appeared as a polydisperse fraction with a wide range of sedimentation values. (Na^+,K^+)-ATPase could be detected in the membrane-bound fraction but not in the free ribosomal fractions.

RNP particles were obtained from cerebral microsomes after deoxycholate treatment. On sucrose-density-gradient centrifugation of RNP particles from cerebral microsomes of both 4-day-old and adult rats, the peak corresponding to membrane-bound ribosomes had disappeared and the RNP particles were distributed throughout the gradient, as was the case with the free ribosomes (Figs. 2*a* and 2*b*). Ultracentrifugation analysis of

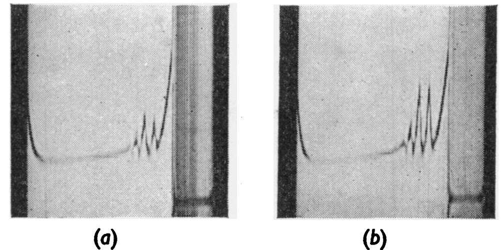

Fig. 3. Sedimentation patterns of RNP particles from cerebral cortex of (*a*) adult and (*b*) 4-day-old rats in tris-buffered sucrose medium at 1·5°, bar angle 50°. Initial concentrations were (*a*) 1mg./ml. and (*b*) 1·5mg./ml. Exposures were taken 9min. after attaining full speed at 29500 rev./min. The direction of sedimentation was from right to left. Sedimentation coefficients: (*a*) 65s, 97s, 120s and 143s; (*b*) 62s, 91s, 114s and 137s.

RNP particles gave values corresponding to monomers and dimers as well as larger aggregates (Figs. 3*a* and 3*b*).

Incorporation of [^{14}C]*valine by cerebral microsomes.* Incubation of microsomes under conditions of protein synthesis resulted in an increase of material sedimenting as free ribosomes with a corresponding loss of material in the membrane-bound region (Fig. 4). Similar results were obtained with cerebral microsomes of both young and adult rats. No obvious change in the location of the peaks was seen on incubation, a finding in agreement with that of Campbell *et al.* (1965), who used hepatic microsomes. Subsequent experiments were performed with microsomes that had incorporated [^{14}C]valine *in vitro*. Immediately after the period of incubation the microsomal–cell-sap mixture was centrifuged on the gradient. After centrifugation, fractions were collected for determination of E_{260} as well as for assay of radioactivity. The results are shown in Figs. 5(*a*) and 5(*b*). The specific activity was expressed as counts/min./E_{260} unit. This was the only basis for comparison, since the

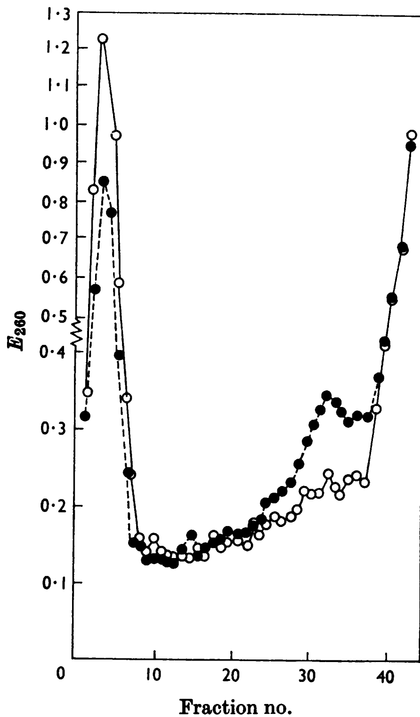

Fig. 4. Effect of incubation of cerebral microsomes of adult rats on the distribution of bound and free ribosomes. The microsomes were incubated with additives for 30min. at 37° under the conditions described for the incorporation of amino acids into protein. ○, Control (not incubated); ●, incubated.

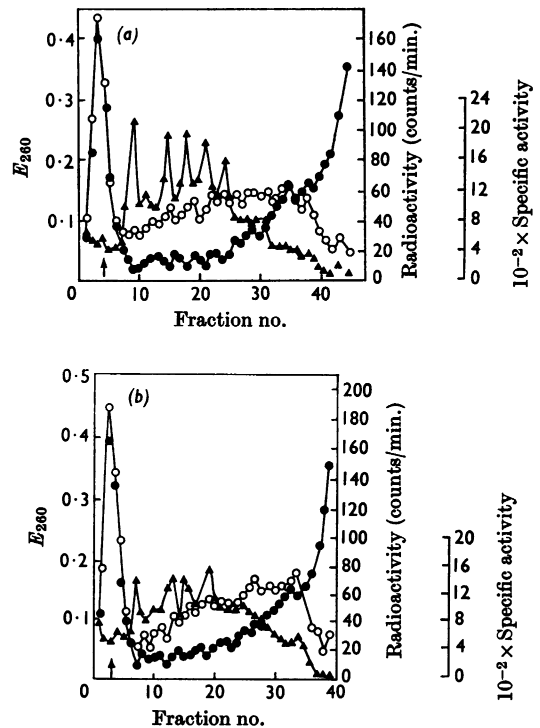

Fig. 5. Incorporation of [^{14}C]valine by cerebral microsomes of (*a*) adult and (*b*) 4-day-old rats. Microsomes were incubated with [^{14}C]valine *in vitro* under the conditions described in the Materials and Methods section. After fractionation by sucrose-density-gradient centrifugation the E_{260} of each fraction was determined and the radioactivity was determined after addition of carrier protein. ●, E_{260}; ○, radioactivity; ▲, specific activity (counts/min./E_{260} unit).

samples in the fraction were diluted with carrier protein. The amount of RNA present in each fraction was too small and could not be quantitatively isolated.

Both free ribosomes and membrane-bound ribosomes appeared to incorporate [^{14}C]valine in microsomes of 4-day-old and adult rats. In the free-ribosomal fractions, although the specific activity was highest in the polysomal region, the lighter ribosomes appeared to be active too. There was very little release of labelled protein into the soluble region.

Radioactivity of ribonucleoprotein particles from cerebral microsomes, labelled in vitro, *of adult rats.* RNP particles prepared from cerebral microsomes have been shown to incorporate amino acids *in vitro* as readily as cerebral microsomes, although initially the rates of incorporation are not as high as microsomal rates (Zomzely *et al.* 1964). The contribution of RNP particles in the incorporation of [^{14}C]valine by microsomes *in vitro* was determined by solubilizing the membranous components and recovering RNP particles after the microsomes had incorporated [^{14}C]valine. The labelled RNP particles were separated on the sucrose density gradient and fractions were collected and treated as described in the Materials and Methods section to obtain the pattern of labelling in the RNP particles. The recovery of radioactivity in these particles, when compared with the microsomal radioactivity, was quantitative, even though only 45–50% of the RNA originally present in the microsomes was accounted for by the RNP particles. A comparison of the distribution of radioactivity in the RNP particles with that of the microsomes showed that radioactivity formerly associated with the membrane-bound region appeared in the heavier RNP particles (Fig. 6). The labelling pattern in the lighter part of the gradient appeared to be similar to the microsomal pattern.

Effect of ribonuclease on the labelled ribonucleo-

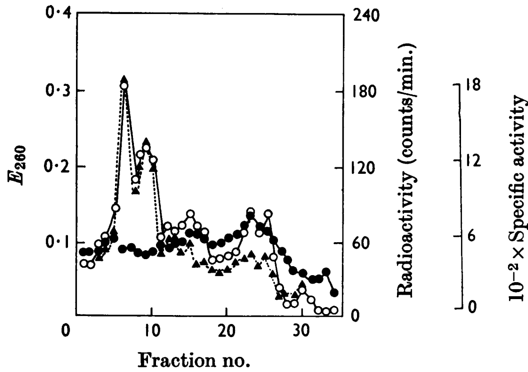

Fig. 6. Radioactivity of RNP particles obtained from cerebral microsomes, labelled with [^{14}C]valine *in vitro*, of adult rats. Microsomes were incubated with [^{14}C]valine under conditions of protein synthesis. RNP particles were obtained from microsomes after treatment with deoxycholate as described in the Materials and Methods section. After sucrose-density-gradient centrifugation for 3hr. fractions were assayed for radioactivity. ●, E_{260}; ○, radioactivity; ▲, specific activity (counts/min./E_{260} unit). Total activity in original microsomes: 2150 counts/min.; total activity in RNP particles: 2000 counts/min.

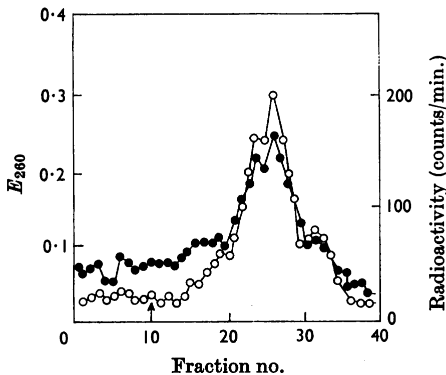

Fig. 7. Effect of ribonuclease on the labelled RNP particles of adult rats. RNP particles were obtained from microsomes labelled with [^{14}C]valine *in vitro* under conditions of protein synthesis. After incubation with ribonuclease (1 μg./ml.) at 25° for 5min. the RNP particles were centrifuged in the sucrose density gradient for 3hr. Fractions were assayed for radioactivity after determining their E_{260} values. ●, E_{260}; ○, radioactivity.

protein particles. RNP particles obtained as before from labelled cerebral microsomes of adult rats were incubated with 1 μg. of ribonuclease/ml. for 5min. at 25°. This treatment has been shown to convert polysomes into 80 s ribosomes (Munro *et al.*

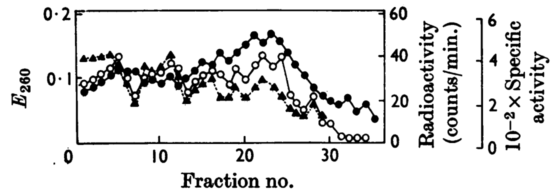

Fig. 8. Radioactivity of RNP particles obtained from cerebral microsomes, labelled with [^{14}C]valine *in vitro*, of 4-day-old rats. Microsomes were incubated with [^{14}C]valine under conditions of protein synthesis. RNP particles were obtained from microsomes after treatment with deoxycholate as described in the Materials and Methods section. After sucrose-density-gradient centrifugation for 3hr. fractions were assayed for radioactivity. ●, E_{260}; ○, radioactivity; ▲, specific activity (counts/min./E_{260} unit). Total activity in original microsomes: 1500 counts/min.; total activity in RNP particles: 780 counts/min.

1964). The results of sucrose-density-gradient centrifugation of these particles are shown in Fig. 7. Compared with the results in Fig. 6 there has been a marked shift of radioactivity to the lighter ribosomes, and this confirms the polysomal nature of the heavier RNP particles.

Radioactivity of ribonucleoprotein particles from cerebral microsomes, labelled in vitro, *of 4-day-old rats.* The situation with regard to RNP particles isolated from microsomes, labelled *in vitro*, of young rats, was rather different. Although the labelling of RNP particles resembles the adult pattern in that the larger polysomes had the highest specific activity (Fig. 8), there was a loss of radioactivity when the RNP particles were compared with the original microsomal preparation. The recovery of radioactivity was half that observed in the original microsomal preparation, even though the recovery of RNA in the RNP pellet was similar to that of the adult (45–50%). Examination of the supernatant, including solubilized membranes, showed that very little radioactivity was present here. This finding prompted the examination of the preliminary sediment obtained by centrifuging deoxycholate-treated microsomes at 105000g (5min.) before the centrifugation at 150000g (70min.) to obtain the pellet of RNP particles.

Labelling in subfractions of microsomes. Microsomes labelled *in vitro* were fractionated after deoxycholate treatment into a preliminary sediment, RNP particles and deoxycholate-soluble fraction, and the protein in these fractions was assayed for radioactivity in the normal manner. The first pellet was not well defined as was the case with the RNP pellet. It was translucent with greyish edges, as opposed to the RNP pellet, which was transparent and compact. Table 1 shows the results obtained.

Table 1. *Radioactivity of subfractions of rat cerebral microsomes labelled* in vitro *and then treated with deoxycholate*

Microsomes were labelled with [^{14}C]valine *in vitro* under conditions of protein synthesis. After incubation they were treated with sodium deoxycholate (final concn. 0·25%). Initial sediment was obtained after centrifugation of deoxycholate-treated microsomes at 105000g (5min.); RNP particles were centrifuged down at 150000g (70min.).

	Adult			Young		
Total radioactivity (counts/min.)	3153			3458		
Microsomal protein (mg.)	2·1			2·3		
Microsomal RNA (μg.)	235			247		
	Protein (μg.)	RNA (μg.)	Radioactivity (counts/min.)	Protein (μg.)	RNA (μg.)	Radioactivity (counts/min.)
Initial sediment	30	<10	93	44	<10	1553
RNP particles	223	106	2653	235	113	1584
Supernatant	—	—	312	—	—	306

Table 2. *Effect of higher concentrations of detergent on the labelling of subfractions of cerebral microsomes of 4-day-old rats*

Results are expressed as percentages of the total microsomal radioactivity. The subfractions were obtained as in the previous experiments in Table 1. The concentrations of sodium deoxycholate and Lubrol W indicated final concentrations.

	Distribution of radioactivity (%)			
	Sodium deoxycholate			Lubrol W
	(0·25%)	(0·3%)	(0·5%)	(0·4%)
Initial sediment	47	47	45	52
RNP particles	48	46	49	42
Supernatant	5	7	6	6

The preliminary sediment in preparations from 4-day-old rats was found to be heavily labelled and to contain as much radioactivity as the RNP particles. The corresponding fraction from the adult contained much less radioactivity. Virtually all the radioactivity was present in the RNP particles from adult rats. These findings would explain the quantitative recovery of radioactivity in the RNP particles obtained from cerebral microsomes from adult rats and the loss of a proportion of the radioactivity observed in RNP particles obtained from those of young rats.

Effect of higher concentrations of detergents. The possibility exists that a portion of the microsomes may not have been solubilized, and this would sediment before the RNP particles. The use of higher concentrations of deoxycholate indicated that the labelling in the preliminary sediment with cerebral microsomes of young rats was not due to unsolubilized microsomal fragments, as the distribution of radioactivity in the fraction was not affected by different concentrations of deoxycholate (Table 2). When Lubrol W was used at concentrations resulting in maximal solubilization of (Na^+,K^+)-ATPase associated with the membrane (Swanson, Bradford & McIlwain, 1964), similar results with regard to the labelling of the subfractions were obtained.

Effect of further detergent treatment on the preliminary sediment. Hendler & Tani (1964) have referred to a deoxycholate-treated membrane fraction sedimenting at 34850g (15min.) before obtaining membrane ribosomes from *Escherichia coli*. With chloroplast preparations, preliminary centrifugation at 23000g (30min.) after deoxycholate treatment resulted in the sedimentation of chloroplast protein (Eisenstadt & Brawerman, 1964). Aronson (1965) suggested that nascent protein, especially structural protein, with high affinity for membrane, might stabilize polysome complexes in sporulating bacteria. The preliminary sediment may therefore consist of insoluble fragments of membrane as well as tightly bound polysomes. Further treatment of this sediment with more deoxycholate might effect the release of polysomes by a solubilization of the remaining

membranes. The preliminary sediment was obtained from labelled cerebral microsomes of young rats as before and resuspended. The suspension was divided into two equal parts and to one part was added more deoxycholate (final concn. 0·3%). Both the original suspension and the clarified suspension were centrifuged on the gradient. Fractions were collected from the whole of the cushion as well as from the gradient for radioassay. With both treated and untreated suspensions there was a sediment at the bottom of the centrifuge tube. The sediment, opaque and rough, was also prepared for radioassay. The results are shown in Fig. 9.

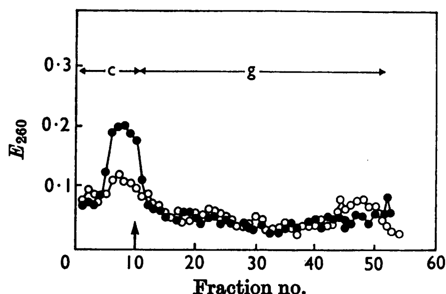

Fig. 9. Effect of further treatment with deoxycholate on the preliminary sediment. Cerebral microsomes from 4-day-old rats were treated with 0·25% deoxycholate after labelling with [^{14}C]valine *in vitro*. A preliminary sediment was obtained by centrifugation at 105000*g* (5min.). After resuspension one half was treated with additional deoxycholate (0·3%). Both the treated and untreated halves were centrifuged on the sucrose density gradient for 3hr. ○, Re-treated preliminary sediment; ●, preliminary sediment. Fractions from the cushion and interface (c) and from the gradient (g), as well as the sediment at the bottom of the tube, were assayed for their radioactivity. Total activity in cushion: 345 counts/min.; total activity in gradient: 180 counts/min.; total activity in sediment: 880 counts/min. (in both cases radioactivities in the different fractions were approximately the same).

The results suggest that there were labelled RNP particles associated with the sediment that appeared on the gradient after sucrose-density-gradient centrifugation. However, most of the radioactivity was associated with the dense material in the cushion and at the bottom of the tube. Additional deoxycholate did not release radioactivity from this material.

Radioactivity of subfractions after treatment of labelled microsomes with ribonuclease. To decrease the number of polysomes associated with membrane so that the initial sediment would contain less polysomal material, labelled microsomes were first treated with ribonuclease and then subjected to deoxycholate treatment. Table 3 shows the effects of using 1 or 2μg. of ribonuclease/ml. on the labelling of the different fractions. There appeared to be a slight shift of radioactivity from the preliminary sediment to RNP particles, although not much difference was observed with either 1 or 2μg. of ribonuclease/ml. This does suggest that polysomes could be detached by prior treatment with ribonuclease. However, most of the radioactivity present in the sediment was recovered in the same position after treatment with ribonuclease.

Effect of ribonuclease on the preliminary sediment. The preliminary sediment was obtained as before by centrifugation of deoxycholate-treated microsomes at 105000*g* (5min.). The sediment was resuspended and then incubated with 1μg. of ribonuclease/ml. After centrifugation on the gradient, fractions were collected as before. However, since there was very little ultraviolet absorption by the fractions of the gradient, 0·5ml. fractions were not diluted with water before E_{260} was determined. Results are shown in Fig. 10. Ribonuclease treatment resulted in the release of some radioactivity from the sediment as labelled monomers, which could have been present as polysomes. However, the dense labelled material retained a high proportion of the radioactivity and appeared to be both detergent- and ribonuclease-resistant.

Table 3. *Effect of ribonuclease on the labelling of subfractions of cerebral microsomes of young rats*

Microsomes labelled *in vitro* were incubated with either 1 or 2μg. of ribonuclease/ml. for 5min. at 25°. After treatment with sodium deoxycholate (final concn. 0·25%) the initial sediment was obtained by centrifugation at 105000*g* (5min.); RNP particles were centrifuged down at 150000*g* (70min.). Results are expressed as percentages of the total microsomal radioactivity.

	Distribution of radioactivity (%)		
Ribonuclease ...	None (control)	1μg./ml.	2μg./ml.
Initial sediment	46	40	41
RNP particles	48	53	52
Supernatant	6	7	7

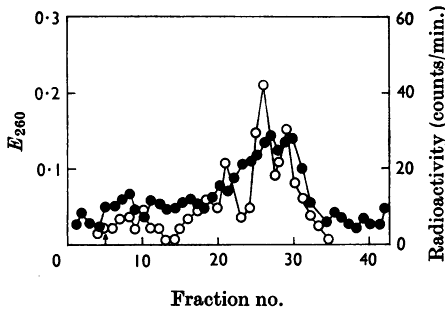

Fig. 10. Effect of ribonuclease on the preliminary sediment. Cerebral microsomes from 4-day-old rats were labelled with [^{14}C]valine *in vitro* under conditions of protein synthesis. After treatment with 0·25% deoxycholate the clarified suspension was centrifuged at 105000g (5min.). The preliminary sediment obtained was resuspended and incubated with pancreatic ribonuclease at 25° for 5min. and then centrifuged in the sucrose density gradient for 3hr. Fractions from the gradient as well as an amorphous material at the bottom of the tube were assayed for radioactivity. ●, E_{260}; ○, radioactivity. When a control (preliminary sediment) was centrifuged on the gradient much less radioactivity was released into the gradient. Total activity in gradient: 330 counts/min.; total activity in the dense material: 1200 counts/min.

DISCUSSION

The detergent deoxycholate solubilizes membrane components of cerebral microsomes (Hanzon & Toschi, 1959; Swanson *et al.* 1964) as is the case with other tissues. The RNP particles recovered had RNA/protein ratios 0·45. They also occurred in a wide range of sedimentation values (Figs. 2 and 3), corresponding to polysomal aggregates of these particles. Similar observations have been made by Murthy & Rappoport (1965) and Zomzely, Roberts, Brown & Rapaport (1965).

In the incorporation of [^{14}C]valine by free ribosomes, although radioactivity in the region of monomeric ribosomes may be explained by the release of labelled monomers during protein synthesis, it appears that the free polysomes incorporated amino acids, as in liver (Campbell *et al.* 1965). That there are polysomes present in liver which are unattached to membranes has been shown by Bloemendal, Bont & Benedetti (1964), who prepared polysomes in high yield without utilizing a detergent. The method involved centrifugation through a discontinuous sucrose density gradient. Hanzon & Toschi (1960) had previously observed the sedimentation of particles into 1·8–2M-sucrose when a post-mitochondrial supernatant from brain homogenates was centrifuged in a discontinuous sucrose density gradient. These particles had RNA/protein ratios 0·36–0·41. Electron micrographs showed the presence in the pellet of particles irregularly arranged in clusters. Analytical ultracentrifugal studies indicated that practically all the particles were in the form of aggregates in medium containing mM-magnesium chloride. These results suggest that free polysomes also occur in brain and that they can incorporate amino acids quite efficiently (Figs. 5*a* and 5*b*).

The appearance of more radioactivity associated with the membrane-bound region in microsomes from adult brain, in the polysomal region after treatment of the microsomes with deoxycholate, suggests that ribosomes bound to the membrane were polysomal in nature (Fig. 6). This bears out the observation of the increase of polysomal material after deoxycholate treatment (Fig. 2). However, a portion of the radioactivity associated with the polysomes can be attributed to free polysomes, which would be included on the gradient. In mammalian systems the importance of membrane-bound ribosomes in protein synthesis has been well documented. Campbell, Cooper & Hicks (1964) have shown that the major synthesizing activity of the liver cell was associated with membrane-attached ribosomes, and that the membrane plays a part in the control of the activity of the attached particles. Decken (1963) and Sargent & Campbell (1965) also concluded that microsomal membranes play an important part in the synthesis of serum albumin. Schlessinger (1963) has suggested that, in the bacterial cell, the bulk of protein synthesis occurs on membrane-bound ribosomes. Similar findings have been reported by Moore & Umbreit (1965). Similarly, in brain preparations, membrane-bound polysomes appear to be highly active in incorporating amino acids.

In microsomal preparations from young rats, radioactivity was associated with the membrane-bound ribosomes (Fig. 5*b*). Deoxycholate treatment released RNP particles predominantly as aggregates (Fig. 2*b*). These RNP particles recovered after deoxycholate treatment did not retain a major proportion of the radioactivity as was the case with the adult preparation (Fig. 8). An examination of the preliminary sediment recovered by centrifuging deoxycholate-treated microsomal suspensions at 105000g (5min.) showed the existence of labelled material. Although a few polysomes were associated with this sediment, most of the radioactivity in the sediment was associated with material that was both deoxycholate- and ribonuclease-resistant (Figs. 9 and 10 and Table 3).

Investigations on the early developmental stages of embryonic chick liver have implicated the presence of proteins that eventually give rise to the lipoproteins of the endoplasmic reticulum in a form that sedimented with the microsomal fraction

(Pollak & Shorey, 1964). Treatment of a rough-membrane fraction with both ribonuclease and deoxycholate followed by centrifugation at 105000g (60min.) resulted in a pellet that was free from RNA. It was suggested that the residual granules that remain after the ribonuclease–deoxycholate treatment represented a new cell component, which was termed 'reticulosomes'. Pollak, Ward & Shorey (1966) extended these studies to an investigation of the interaction between reticulosomes and micellar phospholipid. Reticulosomes were obtained from regenerating liver, which had abundant quantities of these particles. The reticulosomes were incubated with micellar phospholipid, resulting in the appearance of a protein–phospholipid complex, which behaved like the smooth membranes of the microsomal fraction when centrifuged in a sucrose–caesium chloride gradient, remaining at the interface (Dallner, 1963). A mixture of the reticulosome–phospholipid complex and free reticulosomes, when centrifuged, sedimented independently, the former remaining at the interface and the latter sedimenting to the bottom of the tube. Pollak *et al.* (1966) claim that electron micrographs of the complex resembled those of smooth microsomal membranes. These observations would tend to support the assumption that the radioactivity in the membrane-bound region in the young microsomes, after incorporation of [^{14}C]valine *in vitro*, would be due to the presence of a highly labelled protein fraction that sediments with the rough endoplasmic reticulum, and, though deoxycholate solubilizes membranous components, it would have no effect on this dense membrane precursor.

Aronson (1965, 1966) postulated that, in sporulating bacteria, membrane-bound polysomes would function in the synthesis of polypeptides capable of associating tightly with some components of the membrane. These polypeptides could be involved in the synthesis of structural proteins. Polysomes engaged in the synthesis of myofibrils have been shown to be closely associated with these polymers (Cedergren & Harary, 1964). It has been inferred that the polypeptides being synthesized specifically interact with the multistranded fibrils, and that polysomes making the appropriate peptides are found on the surface where they are needed. Thus the preliminary sediment in young brain preparations could consist of structural-type protein (reticulosomes) plus a few attached polysomes that can be released by further deoxycholate and ribonuclease treatment. Pollak & Shorey (1964) have suggested that reticulosomes were to be found in abundant quantities in tissues where rapid synthesis of the endoplasmic reticulum occurs. At 4 days the cortex of the rat would be in the 'critical' period of cerebral development (McIlwain, 1966) where extensive growth of cells, axons and dendrites is occurring. This would involve synthesis of structural components.

In the adult brain there is very little regeneration (Clemente, 1964), and the high levels of protein synthesis reported have led to hypotheses implicating protein synthesis in the functional activity of neurons (Hydén, 1960; Dingman & Sporn, 1964) as well as in the axonal flow of proteins in nerve cells (Weiss, 1961). In the developing rat brain it appears that the different requirements for protein synthesis may be reflected in the incorporation of amino acids *in vitro* by microsomes.

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