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ISOLATION OF PLASMA MEMBRANES FROM RAT BRAIN

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SUMMARY

A method is described for the isolation of plasma membranes from synaptosomal fractions prepared on isotonic Ficoll density gradients. Fractions obtained were analysed chemically and enzymatically and appear to be over 85 % plasma membrane. The major contaminant consists of outer mitochondrial membranes, presumably released from mitochondria during the osmotic shock treatment. Putative neuronal membrane markers, gangliosides and sodium, potassium-dependent ATPase, were enriched over 10-fold in these membranes in comparison to the homogenate. Low activities of 2',3'-cyclic AMP 3'-phosphohydrolase were detected in these membrane preparations, which suggests some contamination with glial cell plasma membrane.

The suitability of these preparations for chemical, enzymatic and metabolic studies is discussed.

INTRODUCTION

Since NEVILLE[1] first isolated plasma membranes from the nuclear fraction of liver, and WALLACH AND KAMAT[2] isolated Ehrlich ascites tumour cell plasma membranes from the microsomal fraction, a large number of studies have appeared on the characteristics of plasma membranes from various tissues (for review see KORN[3]). These techniques have not yet been applied to the central nervous system, in part because of the presence of low density myelin fragments which could complicate the preparative procedures. Furthermore it appears that these methods would produce mixed preparations of glial and neuronal plasma membranes, while, in view of its excitability, interest has been largely concentrated upon the neuronal membrane.

This problem appeared to have been resolved by the isolation of synaptosomal fractions enriched in neuronal material, and the subsequent isolation of membrane fragments from these fractions[4,5]. Many studies have been carried out on their lipid, protein and carbohydrate composition (for reviews see WHITTAKER[6] and DE ROBERTIS AND RODRIGUEZ DE LORES ARNAIZ[7]). However conclusions drawn from these studies as to the nature of the neuronal membrane are dubious since the contamination of these preparations with microsomal or mitochondrial material (with the exception of the work of COTMAN *et al.*[8] and MAHLER AND COTMAN[9]), or with glial plasma membrane, has not been completely analysed.

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Other approaches used for the isolation of excitable membrane by microdissection[10], or from uniquely specialised tissues such as the Electroplax[11,12], or the squid retina[13], have been developed. These techniques are not discussed here, either because of the limited yields available with some of these methods, because of the limited characterization of the preparations, or because of the specialized nature of the source. This paper reports a method for the isolation of a plasma membrane fraction from brain in quantities sufficient to allow further studies on its composition and metabolism.

METHODS

Isolation of plasma membranes

All steps in this procedure were carried out at 0–4° except for the killing of the animals. Centrifugations were performed in a Spinco L-2 refrigerated ultracentrifuge. The initial homogenisation medium (sucrose–EDTA–potassium phosphate) contained 320 mM sucrose, 0.1 mM EDTA and 1 mM EDTA–potassium phosphate buffer (pH 7.5). Medium EDTA–potassium phosphate contained 0.1 mM EDTA and 1 mM potassium phosphate buffer (pH 7.5). The method used for the isolation of synaptic plasma membranes shown in Fig. 1, is based on procedures previously published[4,5], although with several important modifications.

(a) During isolation, the crude mitochondrial pellet, from which the synaptosomes were obtained, was extensively washed to eliminate microsomal contamination. In our hands, even after the three washes which we have routinely adopted, it was still possible to wash out small amounts of RNA- and acetylcholinesterase-rich membranes which presumably come from the endoplasmic reticulum.

(b) Synaptosomes were isolated, not by sucrose density gradient centrifugation, but on isotonic Ficoll–sucrose density gradients. These synaptosomes are highly sensitive to osmotic shock whereas synaptosomes isolated in sucrose density gradients shrink under the hypertonic conditions and become less fragile.

(c) The crude mitochondrial fraction, and the synaptosomal fraction derived from it, were defined as sedimenting between 1000 $\times$ *g* for 15 min and 11500 $\times$ *g* for 25 min. After osmotic shock only material not sedimenting at 11500 $\times$ *g* for 20 min was retained as the synaptic plasma membrane preparation, although this led to a considerable loss of synaptic plasma membrane enzyme markers. However, since the aim was highly purified fractions, it was necessary to eliminate material whose sedimentation properties did not significantly change during osmotic shock.

Characterization of fractions

Enzyme assays. Lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) was assayed by following the oxidation of NADH in the presence of pyruvate at 340 nm[14]. Cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) was assayed by following the oxidation of reduced cytochrome *c* at 550 nm[15]. Succinate dehydrogenase (succinate:2,6-dichlorophenolindophenol oxidoreductase, EC 1.3.99.1) was assayed by following the reduction of dichlorophenolindophenol at 600 nm except that the concentration of phenazine methosulphate was maintained at 0.17 mg/ml[16]. Monoamine oxidase (monoamine:oxygen oxidoreductase (deaminating), EC 1.4.3.4) was estimated by following the oxidative

deamination of [^{14}C]tryptamine[17]. Rotenone-insensitive NADH:cytochrome *c* oxidoreductase (EC 1.6.99.3) was assayed by following the reduction of cytochrome *c* at 550 nm[15]. 10 μg of rotenone was added in 10 μl of ethanol. Rotenone-insensitive NADPH:cytochrome *c* oxidoreductase (EC 1.6.99.1) was assayed by following the reduction of cytochrome *c* at 550 nm[18]. 10 μg of rotenone was added in 10 μl of ethanol. Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) was estimated using β-glycerophosphate as substrate[19]. β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) and β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) were assayed by the technique of GATT AND RAPPORT[20]. 2',3'-Cyclic AMP 3'-phosphohydrolase was assayed by the technique of KURIHARA AND TSUKADA[21] after activation with deoxycholate[22]. Alternatively a more rapid method involving thin-layer chromatography was used[23]. Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) was assayed by the method of ELLMAN *et al.*[24] in the presence of $5 \cdot 10^{-4}$ M octamethylpyrophosphoramide. 5'-Nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) was assayed by the method of EMMELOT *et al.*[25] (Na^+–K^+)-ATPase, ATP phosphohydrolase EC 3.6.1.3) was assayed by measuring the ATPase activity which was inhibited by 1 mM ouabain in a medium containing 150 mM NaCl and 25 mM KCl. Preliminary experiments showed that this concentration of ouabain was sufficient for maximum inhibition.

All spectrophotometric assays were performed using a Cary Recording Spectrophotometer. Inorganic phosphate released was estimated by the technique of WEIL-MALHERBE AND GREEN[26].

Chemical assays. Proteins were estimated by the technique of LOWRY *et al.*[27] after precipitation with 1 M $HClO_4$ to remove sucrose and Ficoll, which interfere with the assay. RNA was estimated as previously described[28]. Lipids were extracted in chloroform–methanol (2:1, v/v) then with chloroform–methanol (1:2, v/v). The combined extracts were partioned by a modified Folch procedure[29]. Ganglioside sialic acid was estimated by the technique of SVENNERHOLM[30]. Cerebroside galactose was measured on portions of the lower-phase lipids by the method of SVENNERHOLM[31].

Electron microscopy. Fractions were pelleted at 100000 $\times$ *g* for 60 min and fixed in 5% glutaraldehyde in 0.1 M sodium phosphate (pH 7.4). After post-fixation with 0.1% osmic acid, the fractions were dehydrated and embedded in araldite. Sections were cut using a Reichert Ultramicrotome and stained with uranyl acetate and lead citrate. Sections were examined in a Siemens Elmiskop I.

RESULTS

Preliminary to the isolation of synaptosomes from the crude mitochondrial fraction by density gradient centrifugation, the optimal number of washes of the crude mitochondrial pellet was determined. This optimum resulted from a compromise between the need to reduce adventitious microsomal contamination to a minimum while losing neither too many synaptosomes, nor too much time. As shown in Fig. 2, lactate dehydrogenase and protein were essentially washed out after two washes, but even after three washes RNA- and acetylcholinesterase-rich material was released. It is unlikely that this material is synaptic membrane from synaptosomes lysed by the washing process, since further lactate dehydrogenase was not released, and in view of the low content of RNA in synaptic membranes (see Table II).

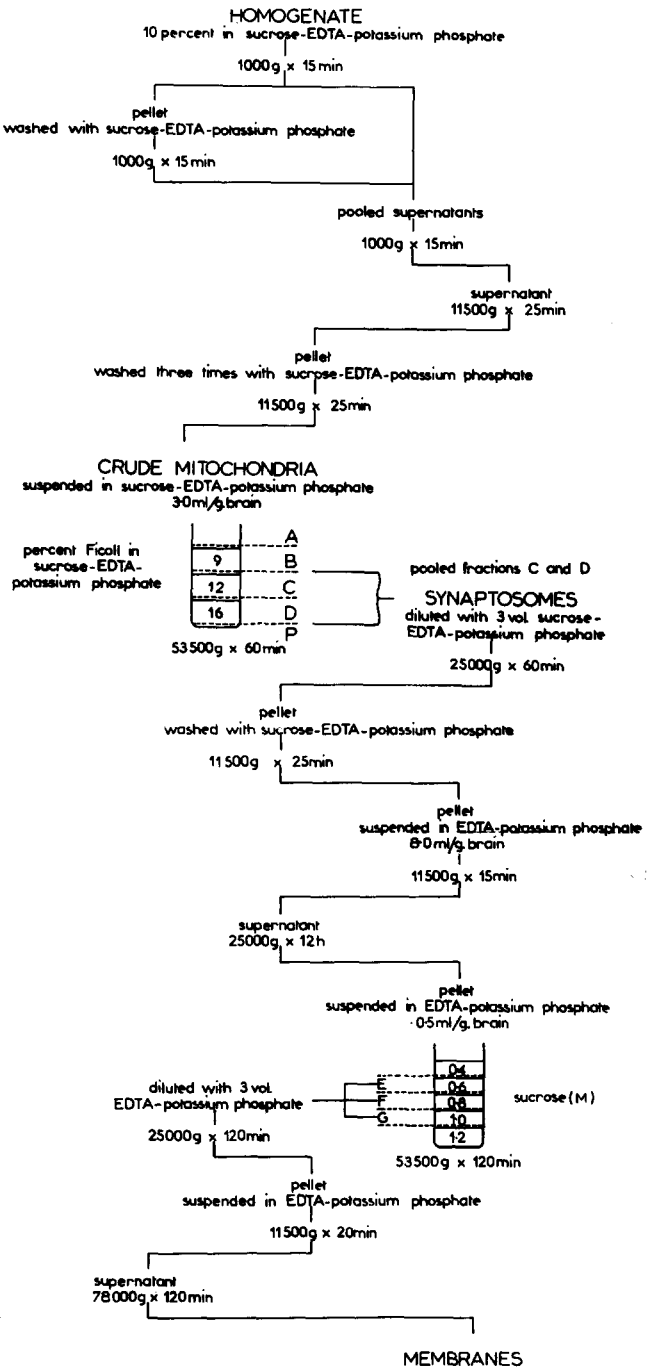

Fig. 1. Flow sheet for the preparation of synaptic plasma membranes. G values are as calculated for the centre of the centrifuge tube. Time of centrifugation from beginning of acceleration to beginning of deceleration. Sucrose–EDTA–potassium phosphate and EDTA–potassium phosphate are defined in the text.

We chose to routinely use three washes since, although further washes seemed not to destroy the synaptosomes, each wash, using the 19 rotor, added 45 min to the procedlure.

The crude mitochondrial pellet was then fractionated on a continuous isotonic Ficol gradient. The distribution of several enzymes after centrifugation at 53500 × *g* for 60 min* is shown in Fig. 3. There is a clear peak of lactate dehydrogenase between 9 and 16% Ficoll and thus this region was regarded as the synaptosome region. This peak was associated with peaks of acetylcholinesterase, $(Na^+–K^+)$-ATPase, 5′-AMPase, monoamine oxidase and cytochrome *c* oxidase. In contrast to the clear peak obtained with lactate dehydrogenase under these conditions, acetylcholinesterase, $(Na^+–K^+)$-ATPase and 5′-AMPase were also found in high specific activities in the material which did not sediment through the 6% Ficoll barrier. It seems likely that under our conditions of relatively brief centrifugation, small membrane fragments bearing the acetylcholinesterase, 5′-AMPase, $(Na^+–K^+)$-ATPase and possibly 2′,3′-cyclic AMP 3′-phosphohydrolase were not sedimented into the synaptosome band resulting in a further purification of the synaptosomes.

TABLE I

ENZYMATIC CHARACTERISATION OF FRACTIONS FROM THE CRUDE MITOCHONDRIAL PELLET

Fractions were collected from the discontinuous Ficoll gradient as shown in Fig. 1.

Enzyme	*Enzyme activity* (*μmoles substrate consumed/mg protein per h*)				
Fraction:	*A*	*B*	*C*	*D*	*P*
Lactate dehydrogenase	38.2	46.1	88.6	62.1	24.7
Cytochrome *c* oxidase	11.1	47.4	93.0	118.8	186.3
Succinate dehydrogenase	0.8	4.3	8.9	12.1	18.3
Monoamine oxidase	0.01	0.07	0.14	0.17	0.15
Acetylcholinesterase	17.2	1.4	2.3	1.6	0.8
$(Na^+–K^+)$-ATPase	17.0	5.3	8.4	9.7	2.6
5′-Nucleotidase	6.0	1.1	1.5	1.1	0.7
2′,3′-Cyclic AMP 3′-phosphohydrolase	1560	720	168.2	131.3	37.2

For routine preparation on a large scale, discontinuous gradients are easier to handle, and thus the enzyme activities were reassayed on the type of gradient shown in Fig. 1. As shown in Table I, the fractions obtained on these gradients corresponded to those expected from Fig. 2. The appearance of the material in the 9–16% Ficoll region is shown in Fig. 3. There are many synaptosomes present characterized by small agranular vesicles, and much more rarely by synaptic junctions. There were also some other membranous bodies, but membrane fragments, and myelin fragments were rare. There was however an appreciable contamination with free mitochondria. The hypothesis that the acetylcholinesterase, 5′-AMPase and $(Na^+–K^+)$-ATPase in the region above 6% Ficoll corresponded to light membrane fragments was strengthened by the observation that the hazy band above the 0.32 M

* This time was chosen on the basis of preliminary experiments which showed that the lactate dehydrogenase activity peak approached equilibrium at this time. The 15 min centrifugation used by KUROKAWA *et al.*[32] was not sufficient to sediment synaptosomes from the bulk of the myelin.

sucrose–9% Ficoll interface contained high specific activities of these enzymes, as well as particularly high levels of 2′,3′-cyclic AMP 3′-phosphohydrolase, higher than obtained in highly purified myelin. The significance of the latter observation will be discussed later.

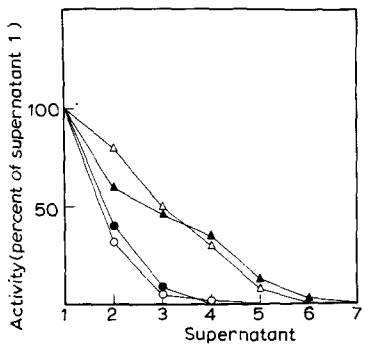

Fig. 2. Efficiency of washing the crude mitochondrial pellet. The supernatants from each centrifugation were estimated. Results are expressed as percent activity in post-mitochondrial supernatant (Supernatant 1). ○—○, protein; ●—●, lactate dehydrogenase; △—△, acetylcholinesterase; ▲—▲, RNA.

Membrane fractions from the discontinuous sucrose gradient were analysed as shown in Tables II and III. Cytochrome *c* oxidase and succinate dehydrogenase are believed to be markers for the inner mitochondrial membrane[33]. Results on cerebral mitochondria have given equivocal results[34–36], but our results are consistent with this localization. On a protein basis there is < 1% contamination of Fractions E and F with inner mitochondrial membrane whereas Fraction G was contaminated to around 3%.

TABLE II

ASSESSMENT OF CONTAMINATION OF FRACTIONS E, F, AND G USING NEGATIVE MARKERS

	E	*F*	*G*	*Mitochondria*	*Microsomes*	*Soluble*	*Myelin*	*Homogenate*
Monoamine oxidase *	0.12	0.16	0.09	0.18	—	—	—	—
Cytochrome *c* oxidase *	0.30	1.60	7.20	249.00	—	—	—	—
Succinate dehydrogenase *	0.11	0.06	0.80	24.00	—	—	—	—
Lactate dehydrogenase *	0.48	0.32	0.20	—	—	144.80	—	—
Acid phosphatase *	0.07	0.07	0.03	4.07	—	—	—	—
β-Galactosidase *	0.80	0.74	0.64	156.00	—	—	—	—
β-Glucosidase *	0.64	0.60	0.44	124.00	—	—	—	—
RNA **	<1	<1	<1	—	103.0	—	—	—
Rotenone-insensitive NADH:cytochrome *c* reductase *	0.40	0.50	0.30	3.78	10.2	—	—	—
Rotenone-insensitive NADPH:cytochrome *c* reductase *	0	0	0	0	1.1	—	—	—
2′,3′-Cyclic AMP phosphohydrolase *	258	198	174	—	—	—	758	246
Cerebrosides and sulphatides ***	<1	<1	<1	—	—	—	26	—

* μmoles substrate consumed/mg protein per h.
** μg nucleic acid/mg protein.
*** Percent lipid weight.

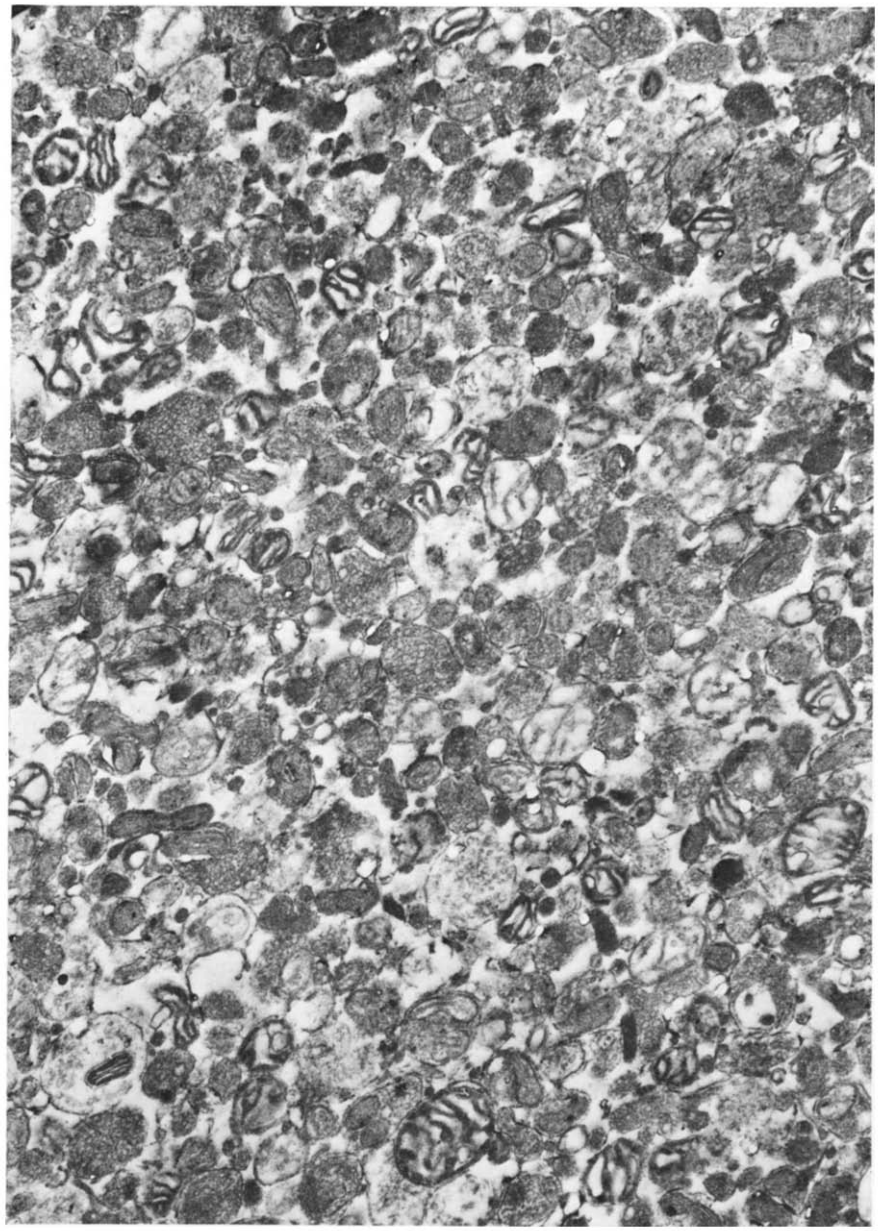

Fig. 3. Typical field of the material sedimenting in a discontinuous Ficoll gradient between 9 and 16% Ficoll. Fractions C and D (as defined in Fig. 1) were pooled. Magnification: × 23000. Myelin fragments are not observed, the major particles observable can be identified as synaptosomes from the presence of synaptic vesicles. Free mitochondria and non-synaptic membrane-limited particles can also be seen.

Monoamine oxidase is now accepted as a marker for the outer mitochondrial membrane after considerable controversy[37], although this localization has not been established for cerebral mitochondria. The specific activity of monoamine oxidase in

TABLE III

CHARACTERIZATION OF MEMBRANE FRACTIONS FOR PLASMA MEMBRANE MARKERS

	E	*F*	*G*	*Homogenate*
$(Na^+–K^+)$-ATPase *	78.90	103.10	63.50	8.90
5′-Nucleotidase *	4.64	4.76	1.47	1.14
Acetylcholinesterase *	2.28	5.34	4.07	6.30
Ganglioside **	28.60	39.80	24.00	3.60

* μmoles substrate utilised/mg protein per h.
** μg lipid *N*-acetylneuraminic acid/mg protein.

our membrane preparations is extremely high, almost as high as the specific activity of purified mitochondria. Contamination with intact mitochondria is not possible in view of the absence of inner membrane markers; the contamination would appear to be due to the presence of outer membranes of mitochondria which were presumably detached during the osmotic shock. Since the outer mitochondrial membrane is believed to account for less than 10% of the total mitochondrial protein[38], this sets a limit to the possible contamination at around 10%. A lack of parallel between monoamine oxidase and the inner membrane markers on the Ficoll gradients (*i.e.* before osmotic shock) is shown in Fig. 4 and Table I. Similar results have been obtained by HAMBERGER *et al.*[39]. This could be due either to the presence of already detached outer membranes, to the fact that synaptic mitochondria are richer in monoamine oxidase than glial and neuronal cellbody mitochondria, or to the presence of monoamine oxidase in some structure other than the outer mitochondrial membrane[40].

Rotenone insensitive NADH:cytochrome *c* reductase is believed to be a marker for the outer mitochondrial membrane[33] although the enzyme is also found in the microsomal fraction (see, however, HEIDRICH *et al.*[41]). Using this enzyme as a marker, contamination of the membranes with outer mitochondrial membrane, on the basis

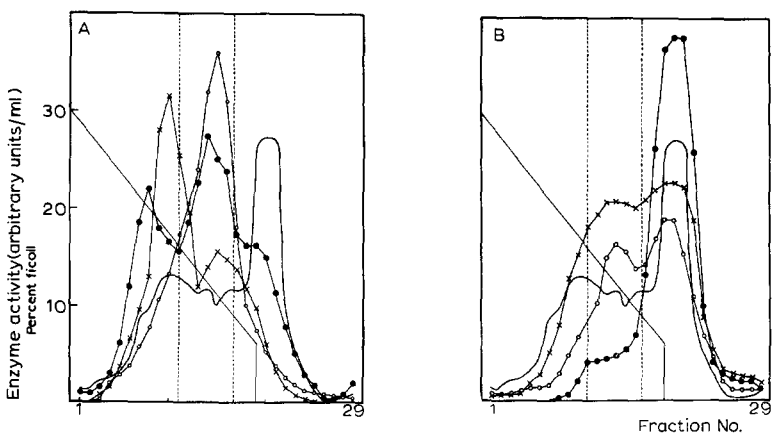

Fig. 4. Distribution of marker enzymes after continuous Ficoll density gradient centrifugation of the crude mitochondrial pellet. Fractions of 2 ml were collected dropwise. Enzyme activities and protein are expressed as arbitrary units/fraction. (A) ———, protein; ●—●, monoamine oxidase; ○—○, lactate dehydrogenase; ×—×, cytochrome *c* oxidase. (B) ———, protein; ●—●, 2′,3′-cyclic AMP 3′-phosphohydrolase; ○—○, $(Na^+–K^+)$-ATPase; ×—×, acetylcholinesterase.

of the calculation carried out for monoamine oxidase, is insignificant. The lack of parallel between the specific activities of monoamine oxidase and rotenone insensitive NADH:cytochrome *c* reductase might support the suggestion of another localization for monoamine oxidase. When compared to the microsomal fraction, contamination was less than 5%. Rotenone insensitive NADPH:cytochrome *c* reductase, believed to be more specific for microsomes than rotenone insensitive NADH:cytochrome *c* reductase[37], could not be detected. Thus contamination with either mitochondrial or microsomal membrane on the basis of these two enzymes is less than 10%. The level of RNA in the membrane ($< 1\mu g$/mg protein) is negligible when compared to the value obtained for the microsomal fraction and thus contamination with rough endoplasmic reticulum is unlikely.

The absence of several lysosomal enzymes indicated that there was little contamination with the more readily solubilized components of lysosomes. A possible contamination with lysosomal membranes, completely freed of these enzymes by the osmotic shock, could not be excluded. However it seems unlikely that release of enzyme from the lysosomes would be complete after one osmotic shock. The *β-N*-acetyl glucosamidase and acid phosphatase activities in the preparation of MAHLER AND COTMAN[9] could be due to partially disrupted lysosomes.

The soluble enzyme lactate dehydrogenase was present in very low amounts in the membrane preparations, accounting for less than 1% of the protein in the preparations. While this suggests that soluble protein bound to the membranes is not a significant contamination, this in no way excludes specific binding of the type described for choline acetyltransferase[42,43].

Myelin contamination was assessed by two methods. Synaptic membranes appear to be deficient in cerebrosides and sulphatides when compared to whole brain or to myelin[44,45], and our preparation contains very low levels of these galactolipids. On the other hand, when 2',3'-cyclic AMP 3'-phosphohydrolase was used as a myelin marker[21], high specific activities were detected in the membrane fractions which showed no correlation with the galactolipid content. This may be due to the presence of highly active glial membrane fragments, perhaps also present in the "second myelin-like fraction" of BANIK AND DAVISON[46] which would account for the high specific activity in Fraction A (Table I). In the absence of sufficiently pure preparations of glial plasma membrane, this supposition is difficult to test, and glial membrane contamination cannot be accurately estimated. But, we are inclined to believe that the presence of this enzyme indicates glial plasma membrane, when the galactolipid data rule out the presence of myelin. The possible use of this enzyme as a marker for the glial plasma membrane is at present under investigation.

Several putative markers for plasma membrane were also tested. Ouabain-inhibited (Na^+–K^+)-ATPase gave the most clear-cut results with an enrichment of 10–15 times over the total homogenate. It is generally accepted that this enzyme is a marker for the plasma membrane, but there are no reliable data as to its differential localization in glial and neuronal membranes. But it might be anticipated that the neuronal membrane, which has particular requirements for ion transport, would be enriched in this enzyme.

Gangliosides have been suggested as specific markers for the neuronal plasma membrane[47–49], although the ganglioside G_4 appears to be associated in part with myelin[50]. Gangliosides in these preparations were enriched over 10 times with respect

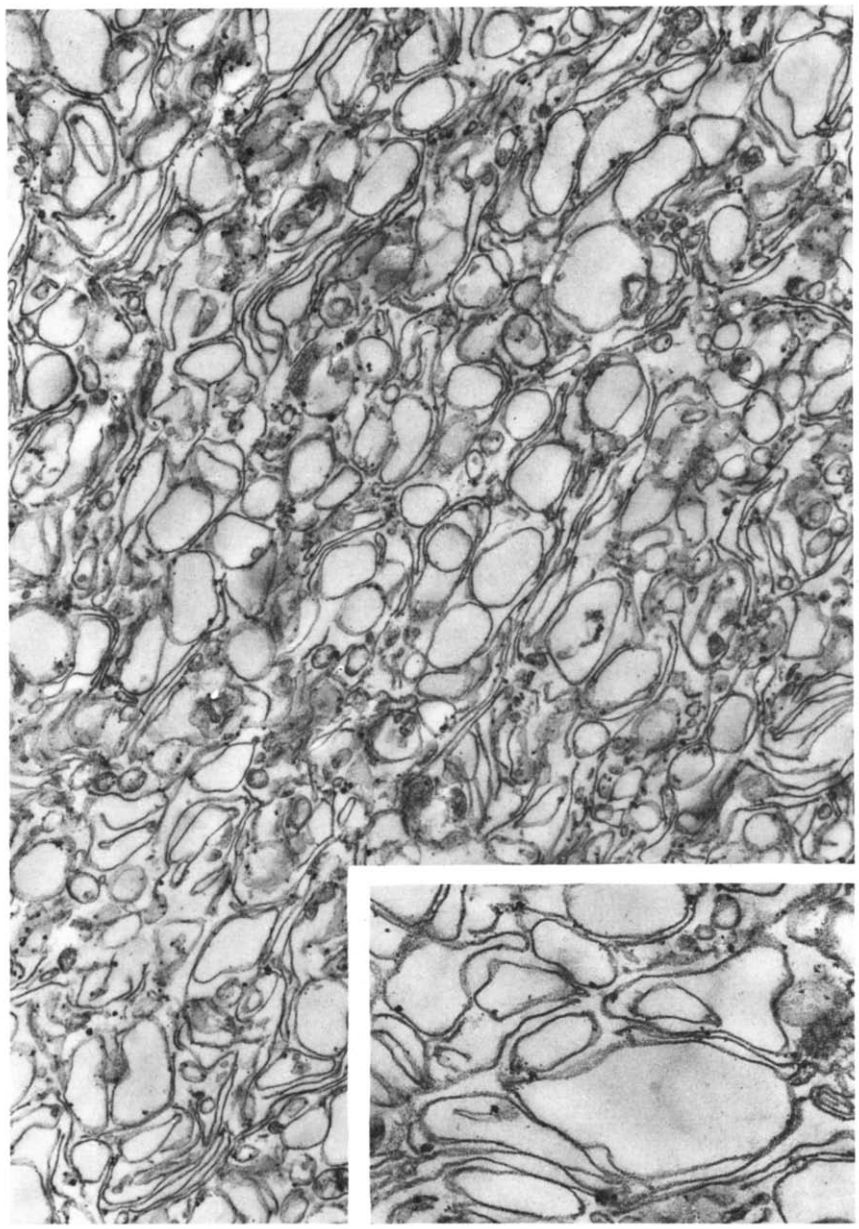

Fig. 5. Typical field of membrane Fraction F. × 30000. Insert, × 100000. The fraction consists of membranes either as extended sheets, or as membrane vesicles of synaptosomal dimension. At higher magnification, the trilaminar appearance of the membranes can be seen.

to the whole brain, thus paralleling closely the (Na^+–K^+)-ATPase enrichment (Table III). On this basis there appears to be at least a 10-fold concentration of neuronal plasma membrane in these preparations.

5′-Nucleotidase, a marker for the plasma membrane in some tissues[51] was

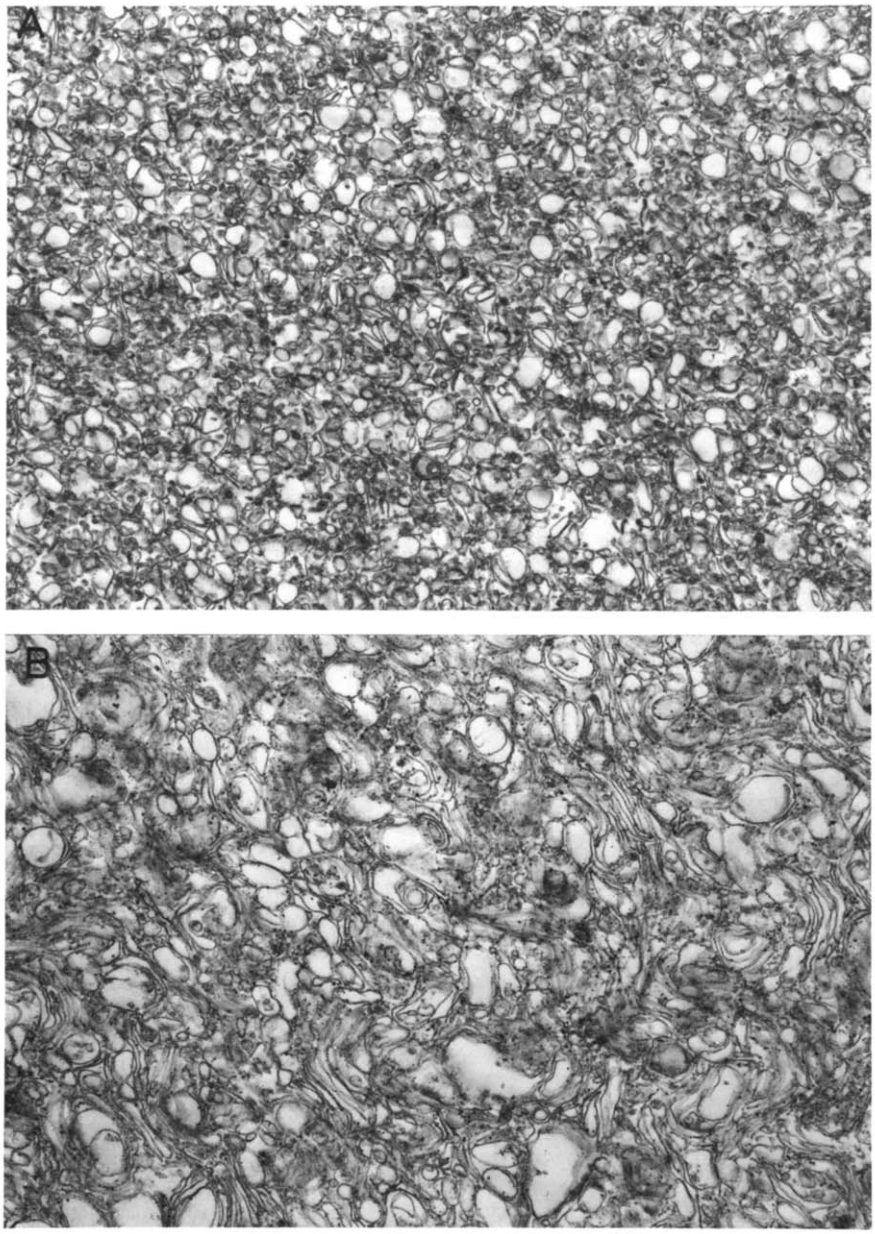

Fig. 6. (A) Typical field of membrane Fraction E. Magnification, × 14000. The fraction consists of small membrane vesicles and some synaptic vesicles. (B) Typical field of membrane Fraction G. Magnification, × 17000. The fraction consists of stacks of membrane sheets and membraneous vesicles as observed in Fraction F.

enriched in these preparations, but to a lesser degree than was (Na^+–K^+)-ATPase. Histochemical evidence suggests that this enzyme is associated with certain types of glial plasma membrane[52]. This dual localization would explain the lower enrichment of this enzyme in these preparations.

Acetylcholinesterase was found in these preparations, but was markedly less enriched than the other enzyme markers. Although this enzyme has been used as a marker for the synaptic membrane, histochemical evidence suggests that the enzyme is also found in the endoplasmic reticulum, vesicular formations and neurotubules of many neurons, and that it is not present in all neuronal plasma membranes[53]. Thus the low enrichment in these preparations is explicable, and the higher relative specific activities obtained in other studies are probably due to microsomal contamination. Considering only the acetylcholinesterase activity applied to the final sucrose gradient, this enzyme distributes similarly to the (Na^+–K^+)-ATPase and gangliosides. Thus this fraction of the acetylcholinesterase would appear to be associated with the plasma membrane, in accord with electron microscopic histochemical localization[54].

Electron microscopy of these fractions (Figs. 5 and 6) shows that there are clear differences in the morphology of the fractions. Fraction E contains small membrane fragments, as well as some synaptic vesicles, Fraction F is composed of large oval membrane profiles, and Fraction G is composed of stacks of membranes. The morphological differences in these fractions seem to have no parallel in the enzymatic and chemical assays employed as yet to characterize them. It is possible that they represent different forms derived from the same basic membrane, but until these fractions have been characterized for glial plasma membrane and Golgi apparatus membranes, definite conclusions are difficult.

The yield of membranes obtained by this preparation was around 0.12 mg protein per brain for Fraction F and slightly less for Fractions E and G. Thus, from the 120 rats routinely used, approximately 15 mg of Fraction F could be obtained. This is lower than the yields reported for other methods, but in view of the superior specific enzymic enrichments this is not important.

DISCUSSION

The preparations as obtained appear to be a plasma membrane essentially free from contamination with inner mitochondrial membranes, rough endoplasmic reticulum, myelin, lysosomes and soluble enzymes. There is some contamination with outer mitochondrial membranes (max. 10 %) and possibly with smooth endoplasmic reticulum (max. 5 %). Thus the preparations are 85–90 % pure plasma membrane, significantly purer than the preparation obtained by COTMAN *et al.*[8] and MAHLER AND COTMAN[9] on the basis of their less extensive enzymatic characterization.

While these results indicate clearly that the membrane preparation is rather pure in relation to the types of contamination for which we have assayed, it would be useful to have some sort of theoretical enrichment to aim for, when considering positive membrane markers. HARVEY AND MCILWAIN[55] quote a figure of 10^4 cm^2 of neuronal membrane per g of tissue. Accepting this figure, and assuming a density of 1.2, an average membrane thickness of 100 Å, and a protein/lipid ratio of 2:1, approximately 8 mg of neuronal membrane protein could be obtained per g of brain. Given approximately 100 mg of protein per g of brain, then enrichments on a protein basis for compounds specifically associated with the neuronal membrane would be of the order of 12. The enrichments for ganglioside and (Na^+–K^+)-ATPase are very close to this figure. While this calculation is obviously subject to an error of at least a factor

of 2, this indicates that the enrichments obtained are neither unrealistically high nor low.

A more difficult problem is that of the relative proportions of neuronal and glial plasma membranes in these preparations, a problem only complicated by the existence of two types of glial cells (astrocytes and oligodendroglia) whose membranes may behave differently during fractionation. The preceding calculation shows that the enrichment of two putative neuronal plasma membrane markers corresponds well to a theoretical value, but the presence of 2',3'-cyclic AMP 3'-phosphohydrolase activity indicates that there is some glial plasma membrane present. Isolated glial cells contain cerebrosides[49,56], which account for approximately 10% of glial cell lipids. From our figure of less than 1% cerebrosides, this would mean less than 10% contamination on a lipid basis. This calculation is also subject to many errors, not the least being the purity of the glial cells examined by these workers.

Leaving aside this problem, the preparations appear to be highly enriched in plasma membrane. The classical preparations of RODRIGUEZ DE LORES ARNAIZ *et al.*[5], as analyzed for lipids[57], contain galactolipid accounting for over 10% of the lipid weight. In view of the absence of cerebrosides and sulphatides from synaptic membranes[45] (Table II) and in general from neurons[58], this is either due to the presence of large amounts of contaminating myelin or to failure to eliminate sucrose. These workers utilize the 11500 × *g* for 20 min pellet after osmotic shock for the sucrose density gradient separation of their membranes and, in our experiments, unless the myelin fragments were rigorously removed by rejecting this pellet, myelin was readily demonstrable in these fractions by electron microscopy.

Comparison with the preparation obtained by the method of WHITTAKER *et al.*[4] is more difficult. Since these workers used the 10000 × *g* for 20 min supernatant after osmotic shock, it is likely that their preparations are much less contaminated with myelin than those of RODRIGUEZ DE LORES ARNAIZ *et al.*[5], particularly if membranes are prepared from the synaptosomes rather than from a crude mitochondrial fraction[59]. As stated by WHITTAKER, *et al.*[4] their preparations are probably contaminated with a certain amount of microsomal membranes, particularly Fraction E in view of the difference in the distribution of acetylcholinesterase and $(Na^+–K^+)$-ATPase within the gradient fractions. In our experiments, the acetylcholinesterase and $(Na^+–K^+)$-ATPase activities applied to the gradient distributed similarly, although these two enzymes had different enrichments compared to the total homogenate. Maximum specific activities were observed in Fraction F, along with 5'-nucleotidase and the gangliosides*, suggesting a more complete elimination of endoplasmic reticulum membranes than in the experiments of WHITTAKER *et al.*[4]. The enrichment of $(Na^+–K^+)$-ATPase was over 10-fold compared to less than 5-fold in the experiments of HOSIE[60]. This is probably due to a more extensive washing of the crude mitochondrial fraction, and removal of extraneous membrane at the Ficoll gradient step.

This preparation is thus purer than others reported in the literature, giving 85–90% pure plasma membrane. It should be noted that the levels of glial plasma membrane and Golgi apparatus are unknown and thus use of these preparations, either for metabolic or compositional studies, is still to be regarded critically.

* Preliminary results suggest the peak of activity in Fraction F is also observed for protein-bound sialic acid, hexosamine, and several parameters of the lipids.

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