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ISOLATION OF SYNAPTIC PLASMA MEMBRANE FROM BRAIN BY COMBINED FLOTATION–SEDIMENTATION DENSITY GRADIENT CENTRIFUGATION

D. H. JONES and A. I. MATUS

Department of Anatomy, University College London, Gower Street, London WC1E 6BT (U.K.)

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SUMMARY

A method is described for the subcellular fractionation of brain to obtain a preparation highly enriched in synaptic plasma membranes. The enriched fraction is recovered from the interface of a two-step sucrose density gradient on which a hypotonically lysed crude mitochondrial fraction from brain has been separated by simultaneous sedimentation and flotation centrifugation. Enzyme marker activities associated with the neuronal plasma membrane are enriched in the synaptic plasma membrane-containing fraction while less than 10 % of enzyme markers associated with the major probable contaminants, myelin and mitochondria, are found in the same fraction. Morphological examination of the enriched fraction suggests that about 80 % of the profiles are recognisably synaptic in origin. Compared to previously described methods for obtaining synaptic plasma-enriched fractions of equivalent purity, the procedure reported here is simpler, shorter, and of greater capacity.

INTRODUCTION

Synaptosomes exist in brain homogenates as pinched-off axon terminals bearing a fragment of post-synaptic membrane attached to the pre-synaptic element via a morphologically intact synaptic cleft [1, 2]. When exposed to hypotonic conditions, synaptosomes undergo lysis freeing the synaptic membranes from the terminal axoplasm and its contained synaptic vesicles and mitochondria [3]. Reported methods for the isolation of synaptic membrane involve the hypotonic treatment of either a crude mitochondrial fraction from brain containing myelin fragments, synaptosomes, and mitochondria [4], or an enriched preparation of synaptosomes [5–10] made by centrifugation of the crude mitochondrial fraction on a two-step gradient of either sucrose [1] or Ficoll [11]. In both types of procedure the synaptic membrane is then isolated from the lysate by centrifugal sedimentation through a density gradient of 4 or more steps. This method, applied to enriched synaptosome preparations, can produce fractions containing over 80 % of synaptic plasma membrane as assessed by marker enzyme activities [8, 9]. However, the procedure is lengthy and although

yields of up to 0.1 % weight of membrane protein per wet weight of tissue have been reported [8], our own experience with such methods suggests that much lower yields are more usually obtained.

The separation of mitochondria, synaptosomes, synaptic plasma membrane and myelin by centrifugation depends upon differences in their equilibrium buoyant densities. In principle complete separation of these species on this basis can be achieved by isopycnic banding in density gradients [12, 13]. However, in published methods for separating brain-derived subcellular particles isopycnic conditions are rarely employed [14] presumably because to attain such conditions centrifuge runs in the order of 48–66 h are necessary [15, 16]. The density gradient centrifugation procedures more usually employed to prepare purified synaptic plasma membrane [5–9] are in the order of 1–2 h duration and in these cases sedimentation rate is a significant factor influencing the separation achieved. Unfortunately the sedimentation rates of the various species present in subcellular fractions from brain overlap greatly [17] and this limits the resolution that can be achieved in short runs, particularly in resolving mitochondria from synaptic plasma membrane. To overcome this problem chemical methods for selectively altering the sedimentation properties of mitochondria have been used [18, 19]. These have the disadvantage of further complicating and extending a complex isolation procedure involving an end-product whose stability to excessive treatment, especially the elevated temperatures that such methods entail, is in doubt.

As an alternative solution to this problem we have investigated the use of density gradients in which the hypotonically shocked crude mitochondrial fraction containing mitochondria, synaptic plasma membrane and myelin is suspended in sucrose of density intermediate between the equilibrium buoyant densities of mitochondria and synaptic plasma membrane. This suspension forms the lower step of a two-step density gradient, the upper step being chosen so that its density is between the equilibrium densities of synaptic plasma membrane and myelin. Under the influence of applied gravitational force the myelin and synaptic plasma membrane are induced to float toward the less dense upper phase of the gradient while mitochondria and any other subcellular particles of equilibrium density greater than the suspension phase are induced to sediment. As a result of such combined flotation–sedimentation centrifugation the influence of sedimentation rate upon the resolution of mitochondria from synaptic plasma membrane is eliminated and a true densitometric separation of these species may be achieved. We describe here a procedure employing a flotation–sedimentation density gradient with which highly enriched synaptic plasma membrane preparations can be recovered directly from a hypotonically shocked crude mitochondrial fraction from brain.

EXPERIMENTAL PROCEDURE

Materials

Female Wistar rats of 200–250 g body weight were used in all experiments. Sucrose was obtained from Fisons (Sandwich, Kent). Enzyme reagents and substrates used in assays were obtained from Sigma Chemicals (London). All other chemicals were BDH 'Analar' grade.

Isolation of plasma membrane

Rats were decapitated under light diethyl ether anaesthesia, brains were rapidly dissected and transsected immediately behind the occipital cortex and the rostral portion ("Forebrain") used in all experiments. All subsequent isolation procedures were performed at 4 °C except where otherwise indicated.

The isolation procedure is summarised in Fig. 1. Pooled forebrains of 6 rats were homogenised in 9 vol. of 10 % (w/w) sucrose by a motor driven Teflon–glass homogeniser. The homogenate was centrifuged at 800 $\times g$ for 20 min and the supernatant removed and centrifuged at 9000 $\times g$ for a further 20 min. This pellet was washed once with 10 % sucrose to yield the crude mitochondrial fraction. For lysis this pellet was resuspended in hypotonic buffer (5 mm Tris–HCl buffer (pH 8.1) [8]) and incubated at 0 °C for 30 min followed by 6 strokes in a hand operated glass–Teflon homogeniser. Where employed, sonication was effected by an MSE mK VII sonicator operated at optimum coupling for 15 s. The lysate was made up to 34 % (w/w) sucrose by addition of the appropriate volume of 48 % (w/w) sucrose. The upper phase of 28.5 % (w/w) sucrose was overlaid above the sample phase and a small volume of 10 % (w/w) sucrose overlaid onto this upper phase to give a gradient

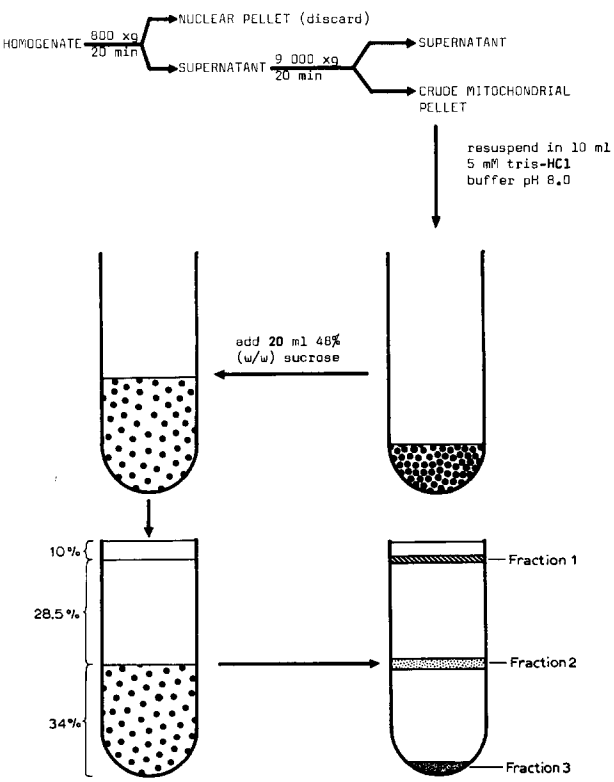

Fig. 1. Preparative precedure for isolation of synaptic plasma membrane. The enriched synaptic membranes are recovered from Fraction 2 of the gradient. *G* values are calculated at the average radius of tubes. Centrifugation times are exclusive of acceleration and deceleration.

of total volume 65 ml. These density gradients were centrifuged at 60 000 × *g* for 110 min in an MSE 65 superspeed 3 × 70 ml swing-out rotor. Three fractions were recovered, one from each interface and the pellet as indicated in Fig. 1.

Characterisation of subcellular fractions

Protein content of particulate fractions was determined by first digesting samples in 1 M NaOH for 1 h at room temperature and them employing the assay of Lowry et al. [20]. Crystalline bovine serum albumin was used as standard. (Na^+-K^+)-ATPase(ATP phosphohydrolase EC 3.6.1.4) was assayed at 37 °C by determining the difference in phosphate release by enzymic cleavage of ATP in the absence and presence of 0.2 mM ouabain. The incubation medium contained 100 mM NaCl, 30 mM KCl and 3 mM $MgCl_2$. Liberated phosphate was estimated by the method of Fiske and SubbaRow [21]. Cytochrome *c* oxidase (cytochrome *c*: O_2 oxidoreductase, EC 1.9.3.1) was assayed by a modification of the method of Duncan and Mackler [22]. The decrease in absorbance of a 30 μM solution of ferrocytochrome *c* was measured at 37 °C and 550 nm. The assay medium was buffered at pH 7.4 with 0.1 M phosphate and contained 0.1 % sodium deoxycholate. Acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) was assayed at pH 7.0 as described by Ellman et al. [23]. 2′,3′-Cyclic nucleotide 3′-phosphohydrolase was assayed by the method of Kurihara et al. [24], after activation in 50 mM Tris–HCl buffer (pH 7.4) containing 0.25 % sodium deoxycholate. Samples for electron microscopy were fixed in 2 % glutaraldehyde adjusted to pH 7.4, post-fixed in 1 % OsO_4, block-stained with aqueous 1 % uranyl acetate, dehydrated via graded alcohols and embedded in Araldite. For sectioning block faces were prepared to encompass the entire depth of the pellet.

RESULTS

The distribution of material in the sedimentation–flotation density gradient before and after centrifugation is shown diagrammatically in Fig. 1. Beforehand the lysed crude mitochondrial fraction is evenly dispersed within the lower phase of the gradient. After centrifugation particulate material is present at each of the interfaces and as a pellet. Fraction 1 is a flocculent white band appearing at the interface of the 10 % sucrose overlay and the 28.5 % sucrose upper phase. Electron microscopic examination (Fig. 4a) reveals that this fraction consists mainly of subcellular particles (0.8–2 μm diameter) bounded by multilayered membranes exhibiting the characteristic morphological features of myelin. Fraction 2 is a pale grey band of material appearing at the interface of the upper (28.5 % sucrose) and lower (34 % sucrose) layers of the gradient. It consists (Figs 2a–2c) mainly of membrane-bound vesicles of 0.5–1.2 μm diameter together with a sparse scattering of smaller dense particles (dp Fig. 2b). Many of the membrane-bound vesicles exhibit recognisable synaptic clefts (indicated in Fig. 2b by arrows at either end) with post-synaptic dense material clearly distinguishable (psd Fig. 2c). Some of these membrane vesicles bearing synaptic clefts have small clumps of synaptic vesicles trapped near what is evidently the presynaptic membrane (sv Fig. 2c) and other membrane vesicles not showing synaptic clefts within the plane of the ultrathin section also contain clumps of vesicles testifying their synaptic origin. Fraction 3 is the brown pellet which is seen in the electron

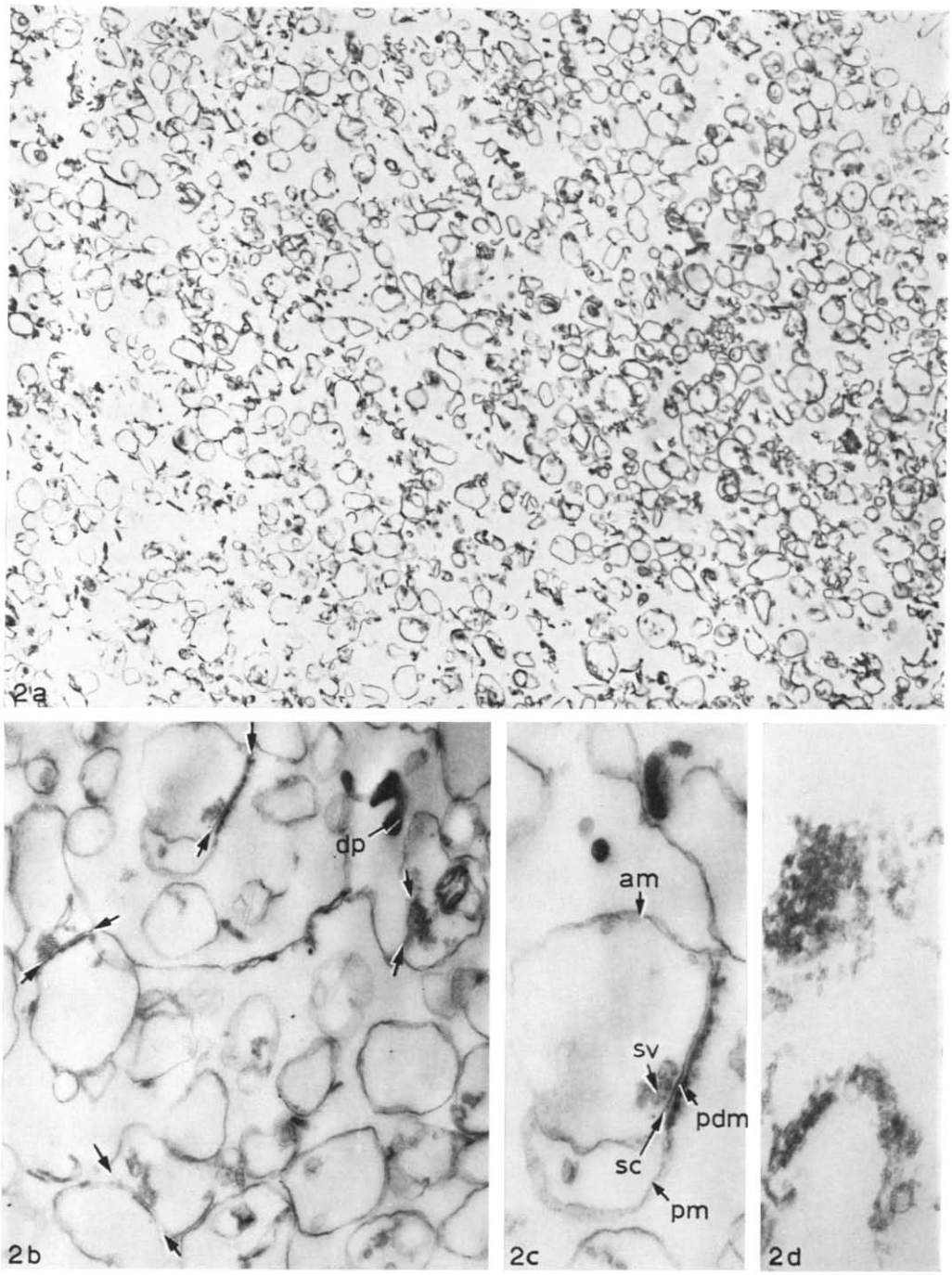

Fig. 2. Morphology of synaptic membrane-enriched fraction from density gradient. Crude mitochondrial fraction from rat forebrain, lysed by hypotonic shock without sonication was centrifuged in the density gradient as described in the text. The material is that recovered from Fraction 2 in Fig. 1. (a) Survey area of pelleted material from Fraction 2 of density gradient. Magnification:

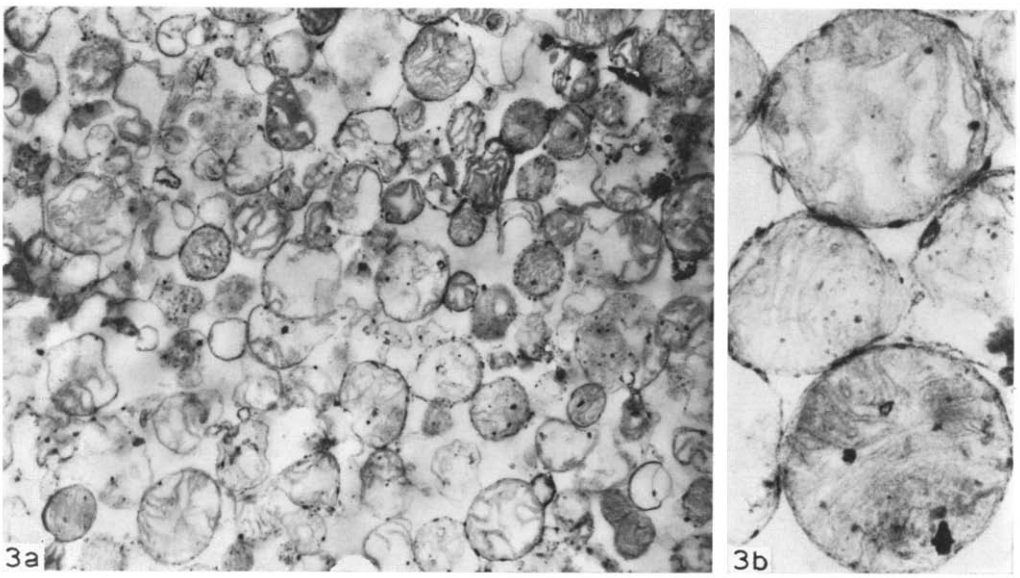

Fig. 3. Morphology of material in Fraction 3 from density gradient. (a) Typical area. Magnification: ×15 000. (b) Selected area showing particulate deposits of lead stain upon mitochondrial membranes. Magnification: ×32 0000.

microscope to consist almost exclusively of recognisable mitochondria in various states of morphological preservation (Figs 3a and 3b).

The purification of the synaptic plasma membrane has been followed through the isolation procedure by assessing at each stage the distribution of marker enzymes for the various subscellular species present. These results are summarised in Tables I and II. Acetylocholinesterase [6, 8] and $(Na^+–K^+)$-ATPase [6, 8, 9] have both been used as marker for synaptic plasma membranes. A progessive enrichment of these enzymes was found in successively purified subcellular fraction from the tissue homogenate through to Fraction 2 of the density gradient. 80 % of the acetylocholinesterase activity was associated with Fraction 2 and this was increased to nearly 90 % when sonication was employed during lysis. Sonication effected a dramatic change in the distribution of the $(Na^+–K^+)$-ATPase within the gradient. If sonication was used 90 % of this enzyme activity appeared in fraction 2 but in its absence the relatively mild hypotonic conditions employed lead to the appearance of 44 % of the activity in Fraction 2 and about 48 % of the activity in Fraction 1. A possible explanation of this observation is advanced below.

Cytochrome *c* oxidase is employed as a marker enzyme for mitochondria

×4500. (b) Selected area of pellet from Fraction 2 of gradient. Arrows indicate synaptic clefts in the plane of section. Magnification: ×15 000. (c) Lysed synaptosome recovered from Fraction 2 of density gradient to show axon terminal membrane (am) retaining characteristic bouton appearance; synaptic cleft (sc); postsynaptic membrane (pm) with postsynaptic dense material (pdm); residual synaptic vesicles (sm) trapped in presynaptic element. Magnification: ×30 000. (d) Material in Fraction 2 from density gradient visualised by staining with phosphotungstic acid to reveal synaptic membrane densities. Above cut en face, below cut in cross-section. Magnification: ×30 000.

TABLE I

ENZYME ACTIVITIES OF SUBCELLULAR FRACTIONS FROM BRAIN

The figures presented are averages from at least three separate preparations. Activities are expressed as μmoles of substrate consumed or product formed per h. Total enzyme activities in each fraction expressed as percentages of the total in the unfractionated homogenate are shown in parentheses.

Fraction	Enzyme activities			
	(Na^+-K^+)-ATPase	Cytochrome *c* oxidase	Acetylcholin-esterase	2′,3′-Cyclic nucleotide 3′-phosphohydrolase
Homogenate	486.0 (100)	5567.4 (100)	764.6 (100)	7668 (100)
Nuclear pellet	56.2 (11.5)	2783.7 (50)	311.7 (40.8)	4256.4 (55.5)
First supernatant	279.9 (57.6)	2619.4 (47)	400.9 (52.4)	3415.4 (44.5)
Mitochondrial pellet	155.8 (32.1)	2343.9 (42.1)	239.9 (31.4)	2722.4 (35.5)
Second supernatant	28.7 (5.9)	743.9 (2.5)	89.8 (11.7)	677.6 (8.8)

TABLE II

SPECIFIC ENZYME ACTIVITIES OF FRACTIONS FROM DENSITY GRADIENT

The figures presented are averages from at least three separate preparations. Activities are expressed as μmoles of substrate consumed or product formed per mg protein per h. Distribution of enzyme activities after each step expressed as percentages of the total are shown in parentheses. Series 1 are fractions derived from material lysed without sonication. Series 2 are fractions derived from material lysed by hypotonic shock and sonication. Enzyme recovery figures are the total enzyme activity in the three fractions expressed as a percentage of the total activity applied to the gradient.

Fraction	Enzyme activities			
	(Na^+-K^+)-ATPase	Cytochrome *c* oxidase	Acetyl-cholinesterase	2′,3′-Cyclic nucleotide 3′-phosphohydrolase
Series 1				
1	3.03 (48.8)	7.0 (8.3)	5.81 (18.7)	2358.0 (94.2)
2	2.73 (44.0)	7.4 (8.9)	24.85 (78.2)	130.0 (5.2)
3	0.45 (7.2)	69.1 (83.8)	0.91 (3.5)	14.9 (0.6)
Enzyme activity recovered (%)	14	46	51.6	70.7
Series 2				
1	0.00 (0.0)	16.4 (8.3)	1.31 (8.0)	1224.0 (82.1)
2	9.58 (90.6)	33.9 (17.2)	14.60 (88.6)	252.0 (16.9)
3	0.99 (9.4)	149.0 (74.5)	0.61 (3.4)	14.1 (1.0)
Enzyme activity recovered (%)	36	72.3	35.4	79.9

[ref. 25]. Its distribution parallels that of acetylcholinesterase and (Na^+-K^+)-ATPase as far as the crude mitochondrial fraction. After lysis without sonication and fractionation on the density gradient over 80 % of this enzyme activity is found in the pellet. Cyclic 2′,3′-nucleotide phosphohydrolase is known to be associated with myelin [17, 19] and less direct evidence suggests that it is generally associated with glial plasma membrane [9]. This enzyme activity is also concentrated by the prepara-

tion of the crude mitochondrial fraction but after lysis without sonication and fractionation on the density gradient about 95 % of the activity is associated with Fraction 1. If sonication is employed then about 10 % of the activity in Fraction 1 is now found in Fraction 2.

We have calculated the yield of material in the synaptic plasma membrane-enriched fraction as the weight of recovered protein determined by the assay of Lowry et al. [20] expressed as a percentage of the wet weight of brain tissue from which the fraction was derived. Using the procedure described in this report in more than twenty separate preparations the yield has been in the order of 0.1 %. The use of the two-step gradient to isolate synaptic plasma membrane from the lysed crude mitochondrial fraction increases the capacity of each complete isolation procedure to 3 times the weight of brain tissue that can be handled in methods employing a four-step gradient.

DISCUSSION

This study was undertaken to establish a reliable method of isolating synaptic plasma membranes from brain using the simplest possible procedure. Our approach was to minimise the number of steps involved in the preparation and then assess the purity and state of preservation of the synaptic plasma membrane-containing fraction thus obtained to establish a minimal procedure capable of providing acceptable enrichment.

To assess the purity of the fractions we have employed both morphological and enzyme marker criteria. Enzyme markers appear to be of greatest value in deciding the degree of contamination of synaptic plasma membrane preparations by other subcellular species, especially mitochondria and myelin membranes for which well-characterised marker enzymes exist which are known not to be associated with plasma membranes [25, 26]. Positive indicators for synaptic plasma membrane are more problematical since all of the enzyme activities that have been used for this purpose are not specifically characteristic of the synaptic membranes but are also associated with other membranes. We have employed two such markers, chosen because they are particularly associated with neuronal plasma membrane. (Na^+-K^+)-ATPase is perhaps the best defined of all plasma membrane enzymes and the particular needs of the neuronal membrane to actively transport these ions, together with the shared sensitivity of this enzyme and the neuronal Na^+ pump to inhibition by ouabain, commend its use as a neuronal plasma membrane marker. However, its presence in glial membranes cannot be excluded and it is obviously not specifically characteristic of synaptic membranes. Histochemical evidence suggests that acetylcholinesterase is predominantly associated with neuronal plasma membranes although the distribution covers both axons and dendrites and is limited to a proportion of the total neuron population of the central nervous system [27, 28]. There is also some evidence that this enzyme is associated with microsomes since it may be eluted from synaptosome preparations by washing under conditions where it is believed that synaptic material is not being removed [9].

In contrast to these inherent difficulties besetting the enzymic characterisation of the synaptic membranes, the morphological appearance of the synaptic junctional complexes (pre- and post-synaptic membranes and their associated dense-staining

material) may be confidently employed as an absolute criterion. We have established that by the use of mild conditions during lysis of synaptosomes, not only are the junctional complexes preserved, but the presynaptic membranes are minimally damaged and retain the characteristic shape of the axon terminal bouton. Thus the appearance of the synaptic plasma membrane-enriched fraction by electron microscopic examination shows that most of the profiles of recognisable morphology are empty synaptosomes, recognisable either by the presence of a synaptic cleft in the plane of section, or by minor contamination with trapped clumps of synaptic vesicles inside the otherwise empty axon terminal. Other contaminants are sparsely distributed amorphous dense bodies and occasional synaptic mitochondria trapped in the vesicularised axon terminal membrane. In practice, morphological examination can give no more than an approximate assessment of the proportion of particles to which a synaptic origin may be confidently ascribed, because the junctional region does not necessarily lie within the plane of an ultrathin section. This does not seriously hinder the identification of intact synaptosomes because they may also be recognised by their characteristic shape coupled with their content of synaptic vesicles and small mitochondria. However, a section through a lysed synaptosome which does not include the junctional region reveals only an empty membrane-bound vesicle. This difficulty cannot be overcome by serial sectioning to establish with certainty whether a given profile possesses a junction, because it is impossible to unambiguously identify empty membrane-bound profiles in adjacent sections as belonging to the same subcellular particle. Therefore in making the assessment we have counted all profiles of 0.5–1.2 μm diameter bounded by a single membrane as synaptic whether or not they show a synaptic junction or trapped synaptic vesicles within the plane of section. The assessment was made from eight randomly chosen electron micrographs each covering 500 μm^2 at 10 000 times magnification by grouping profiles into those of probable synaptic origin and contaminants. The results suggest that about 80 % of the particles present in Fraction 2 are of synaptic origin. This correlates tolerably well with the levels of contamination by mitochondria and myelin set by the enzyme marker assays, each of these being below 10 %. We have recently obtained further confirmation of this assessment by comparing the electrophoretic profiles of solubilised proteins from each of the three fractions recovered from the flotation–sedimentation gradient and these show contamination of Fraction 2 by proteins from Fractions 1 and 3 to be barely detectable (Jones, D. H. and Matus, A. I., unpublished).

The conditions employed to lyse the synaptosomes not only affected the morphology of the subcellular particles subsequently removed from the flotation–sedimentation gradient but also the distribution of enzymes between the three fractions. If vigorous conditions such as sonication are employed, then 90 % of the $(Na^+–K^+)$-ATPase activity is found in the synaptic plasma membrane-enriched fraction whereas under mild conditions this figure drops to about 44 %. Morphological examination of the fractions produced by the two methods of lysis provides a possible explanation for this difference. Without sonication the myelin present in Fraction 1 is well preserved and many of the myelin-bound profiles contain smaller single membranes which in turn sometimes contain recognisable cytoplasm (see Fig. 4b). After sonication the myelin fragments are disrupted so that they no longer enclose single membranes (Fig. 4c). It seems probable that these single membranes are axonal and are the site of the $(Na^+–K^+)$-ATPase activity present in the myelin rich

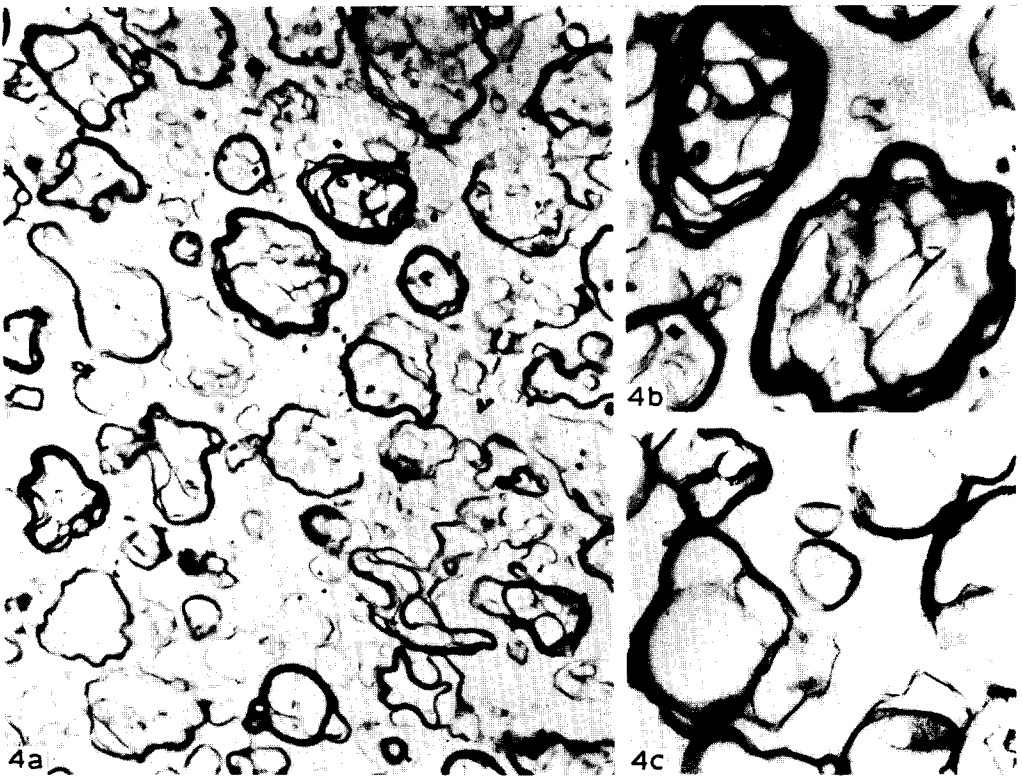

Fig. 4. Morphology of material in Fraction 1 from density gradient. (a) Typical area. Magnification: ×10 000. (b) Selected area. Magnification: ×20 000. (c) Material recovered from Fraction 1 of a density gradient on which sonicated crude mitochondrial fraction was centrifuged. Note absence of single membrane from the space enclosed by the myelin profiles. Magnification: ×20 000.

Fraction 1 from the density gradient when sonication is not used. The shift of this enzyme activity into Fraction 2 from the gradient after sonication is probably the result of these membranes being freed from the myelin fragments and banding with the synaptic plasma membrane. The morphology of all fractions is adversely affected by sonication and the increase in contamination of Fraction 2 with enzyme markers of myelin and mitochondria is probably caused by membrane fragments from these subcellular species banding in Fraction 2. The necessary precautions for obtaining optimum morphological preservation of the synaptic plasma membranes and minimal contamination by membranes of non-synaptic origin are the limitation of exposure to hypotonic conditions to the minimum time necessary for effective lysis and at minimum temperature. The use of mildly alkaline conditions to promote lysis [29] which have been shown to improve subsequent isolation of synaptic plasma membrane [8] does not appear to damage the membranes and has been routinely employed.

The preparation is obviously subject to minor contamination by synaptic vesicles as shown in Fig. 2b but the vast majority are evidently released when the synaptosomes are lysed. Synaptic vesicles were shown by Whittaker [30] to have

a buoyant density lower than that of myelin. It seems likely that they are present in Fraction 1 from the flotation–sedimentation gradient although they are not seen in the myelin-enriched pellet (Fig. 4), probably because they remain in the supernatant under the conditions of pelleting employed. This is currently under investigation.

Regarding other possible contaminants such as Golgi membrane fragments and endoplasmic reticulum we must rely on the morphological appearance of the synaptic membrane fraction which confirms that contamination from such sources is not gross. As has been previously noted [9] the possible disproportionate effects of minor contaminants upon the apparent biochemical properties of synaptic plasma membrane preparations must receive appropriate attention when such studies are undertaken.

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