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SYNAPTIC PLASMA MEMBRANES FROM RAT BRAIN SYNAPTOSOMES: ISOLATION AND PARTIAL CHARACTERIZATION

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SUMMARY

We have isolated a highly purified plasma membrane fraction directly from synaptosomes prepared by centrifugation on a Ficoll–sucrose gradient. A critical feature of the preparation method is use of alkaline conditions to achieve effective osmotic shock so that membranes effectively resolve from mitochondria. If osmotic shock is carried out at alkaline pH, 70 % of the (Na^+–K^+)-activated-ouabain-sensitive ATPase (EC 3.6.1.4) of the synaptosomes can be recovered with only 7 % of total cytochrome oxidase (EC 1.9.3.1) in synaptosomal membrane fractions. In contrast, if osmotic shock is carried out at neutral pH, 50 % of the mitochondria overlays 85 % of the membranes. A fraction enriched in synaptic plasma membranes (SPM fraction) can be prepared on a simple discontinuous gradient in relatively good yield. The SPM fraction bands between 25–32.5 % sucrose; the yield is about 1.5 mg of protein per g tissue wet wt. An alkaline phosphatase (EC 3.1.3.1) is found in the SPM fraction. The distribution pattern of alkaline phosphatase parallels that of (Na^+–K^+)-ATPase. 5′-Nucleotidase (EC 3.1.3.5) and acetylcholinesterase (EC 3.1.1.7) sediment at lighter densities than (Na^+–K^+)-ATPase or alkaline phosphatase although there is considerable overlap. The SPM fraction contains *β-N*-acetylglucosaminidase. This enzyme is not completely washed out of the SPM fraction by salt treatment but is released by low concentrations of Triton X-100.

INTRODUCTION

The structural properties of synapse plasma membranes specify and control ionic permeabilities required for the release of transmitter and the generation of the postsynaptic potential[1]. During the development of synaptic connections in brain, the surface properties of these membranes are thought to be one of the critical features providing the required specificity[2,3]. To study the properties of these membranes at a chemical level, a method is required to prepare large quantities of plasma membrane from synapses. From ultrastructural studies of synapses it has been found that three membrane components are intimately associated in both tissue sections and isolated synaptosomes. These components are a presynaptic membrane, a postsynaptic portion

Abbreviation: SPM fraction, synaptic plasma membrane fraction.

and a synaptic thickening which joins the two plasma membrane components. When plasma membrane is prepared from synaptosomes it is usually isolated as a complex consisting of a pre- and postsynaptic plasma membrane connected by a synaptic thickening. A fraction rich in these structures has been called a synaptic plasma membrane fraction (SPM fraction)[4].

Various workers have developed methods to prepare a plasma membrane fraction of synaptosomal origin. RODRIGUEZ DE LORES ARNAIZ *et al.*[5] and COTMAN *et al.*[6] have prepared a membrane fraction enriched in synaptic plasma membranes directly from an osmotically shocked crude mitochondrial fraction. One limitation of this approach is that the preparation is somewhat contaminated by intracellular membranes[6]. Another is that there is no certainty as to the identity and homogeneity of the plasma membranes because the crude mitochondrial fraction contains a variety of vesicular elements bound by a plasma membrane and a number of free plasma membranes. These include axonal, dendritic and glial fragments. Recently, COTMAN *et al.*[7] have found that a portion of the plasma membrane from glial cells sediments in the crude mitochondrial fraction and has an isopycnic banding density very similar to that of synaptic plasma membranes and intact synaptosomes on sucrose gradients. Also LEMKEY-JOHNSTON AND DEKIRMENJAIN[8] have reported that axonal membranes constitute a major source of membranes (about 45 %) in crude mitochondrial fractions, and like glial membranes have an isopycnic density very similar to synaptic plasma membranes and synaptosomes.

In order to circumvent such difficulties and to further reduce intracellular membrane fragments it seems essential to prepare synaptic plasma membranes directly from synaptosomes. Membranes have been prepared directly from synaptosomes isolated on Ficoll–sucrose gradients[9]; however, in our experience and that of others[9] the yields have been very low. Many synaptosomes sediment with the mitochondria, probably because of ineffective lysis. Not only does this account for the low yields but it also introduces a serious limitation regarding the origin of the synaptic plasma membrane band. If only a small percentage of the total membrane in the synaptosome fraction is recovered in the SPM fraction the resulting SPM fraction may consist primarily of contaminating membranes. This is especially true of synaptosomes prepared in sucrose gradients since these contain large quantities of axonal and other membranes of unidentified origin.

In this paper we report a method to prepare a highly purified synaptic plasma membrane fraction in relatively high yield directly from synaptosomes prepared on Ficoll–sucrose gradients. Synaptosome fractions prepared on Ficoll–sucrose gradients are reported to consist from 60–80 % synaptosomes[9,10]. Two critical features of the preparation method described here are the conditions required to give optimal osmotic shock of the synaptosomes and the conditions of sucrose gradient for separation of synaptic plasma membranes following osmotic shock. We have characterized this membrane fraction by morphological and enzymatic analysis.

EXPERIMENTAL

Centrifugation procedures

A membrane fraction was prepared by differential and density gradient centrifugation from rat forebrain. 8–10 male Sprague–Dawley rats (Simonsen Labs,

Gilroy, Calif.) 20–30 days old were used for each experiment. The brains were placed in cold 0.32 M sucrose (w/w), homogenized at 20 % w/v, in a teflon glass homogenizer and diluted to 7–10 % (w/v) for centrifugation. Differential centrifugation was carried out based on conditions previously determined[11]. A crude nuclear fraction (P_1) was removed by centrifugation at 3000 rev./min (1100 × *g*) in a Spinco 30 rotor to a total applied force of $2 \cdot 10^7$ $\omega^2 t$ (about 5 min) monitored by a digital integrator. The supernatant was transferred and centrifuged at 14000 rev./min (17000 × *g*) in a 30 rotor for about 10 min to a total applied force of $100 \cdot 10^7$ $\omega^2 t$. The crude mitochondrial fraction (P_2) was resuspended in 15 ml of 10 % sucrose by hand homogenization. 5 ml of this suspension was applied to a two step discontinuous Ficoll–sucrose gradient, consisting of 10-ml layers of 13 % (w/v) Ficoll (Pharmacia) in 0.32 M sucrose and 7.5 % Ficoll (w/v) in 0.32 M sucrose. After centrifugation in a Spinco SW 25.1 rotor at 25000 rev./min (63581 × *g*) for 45 min ($17000 \cdot 10^7$ $\omega^2 t$) a synaptosomal fraction was obtained at the interface of the 7.5–13 % Ficoll–sucrose layer. The synaptosome band was removed, diluted with 4 vol. of 10 % sucrose and pelleted at 30000 rev./min for 30 min. The synaptosomal pellet was resuspended in a small volume of 10 % sucrose and osmotically shocked in 5 vol. 6 mM Tris, pH 8.1, for 1.5 h unless otherwise specified. After osmotic shock the fraction was concentrated by centrifugation for 15 min at 25000 rev./min in a 30 rotor, and the pellet resuspended in 10 % sucrose, and applied to a continuous or discontinuous sucrose gradient. The discontinuous sucrose gradient consisted of successive 5-ml layers of 25, 32.5, 35 and 38 % sucrose (w/w). Centrifugation was carried out for 1.5 h at 25000 rev./min in an SW 25 rotor. Each band was separated with a tube cutter, removed, diluted with 0.1 mM EDTA solution and pelleted (30000 rev./min for 30 min). The pellets were resuspended in 10 % sucrose for assay. Fractions from continuous gradients were collected by pushing out the gradients with heavy sucrose. We experienced some variation in different Ficoll lots, and it was necessary to recheck the resulting separation with each new lot.

Enzyme assays

Cytochrome oxidase (cytochrome *c*:O_2 oxidoreductase, EC 1.9.3.1) was assayed as described by DUNCAN AND MACKLER[12]. (Na^+–K^+)-activated ouabain-sensitive ATPase ((Na^+–K^+)-ATP phosphohydrolase, EC 3.6.1.4) was assayed as previously described[7]. Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) was assayed in 0.05 M sodium acetate, 2 mM $MgCl_2$, pH 5.0 (0.1 % Triton), in the presence of 2 mM *p*-nitrophenyl phosphate. The color reaction was developed by making the solution 0.1 M in NaOH[13] and the turbidity cleared by making the solution 1 % in Triton X-100. The absorbance was read at 412 nm. Alkaline phosphatase (EC 3.1.3.1) was measured in 0.09 M Tris buffer–2 mM $MgCl_2$, pH 9.5, with 3 mM *p*-nitrophenyl phosphate. The reaction was terminated by addition of NaOH and Triton X-100 as for acid phosphatase. 5′-Nucleotidase (EC 3.1.3.5) was assayed in 0.05 M Tris buffer–2 mM $MgCl_2$, pH 7.7, with 3 mM AMP. The reaction was terminated and color developed by addition of Fiske–SubbaRow phosphate reagent[14]. Protein was removed by centrifugation prior to reading the absorbance at 660 nm. Nucleosidediphosphatase (EC 3.6.1.6) was assayed in 0.05 M Tris buffer–0.2 mM $MgCl_2$, pH 8.0, with 3 mM ADP as substrate.

Phosphate released was determined as for 5′-nucleotidase. β-*N*-acetylglucos-

aminidase (β-2-acetylamino-2-deoxy-D-glucoside acetylaminideoxyglucohydrolase, EC 3.2.1.30) was assayed as described by SELLINGER *et al.*[15] using 0.1 % Triton X-100 in place of digitonin. Acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) was assayed as described by ELLMAN[16]. Protein was determined by the method of LOWRY *et al.*[17]. NADH cytochrome reductase (NADH:cytochrome *c* oxidoreductase, EC 1.6.2.1) was assayed as described by DALLNER *et al.*[18] in the presence of 1 μg/ml antimycin A. All enzyme reactions were linear over the range studied.

Electron microscopy

Samples were prepared as previously described[12,20].

RESULTS

Synaptosomes were isolated from a crude mitochondrial fraction (P_2) on a discontinuous Ficoll–sucrose gradient. This method yields a relatively purified population of synaptosomes (Fig. 1). Most, but not all vesicular structures, are synaptosomes. Only an estimate of purity can be made, particularly without very extensive serial section work, but approx. 60–75 % of the subcellular structures are synaptosomes. This is in agreement with results reported by other workers using a similar method[9,10]. Based on the survey of a number of micrographs we find in addition to identifiable synaptosomes quantities of free membranes, mitochondria and membrane vesicles not identifiable as synaptosomes, a few containing ribosomes and neurofilaments

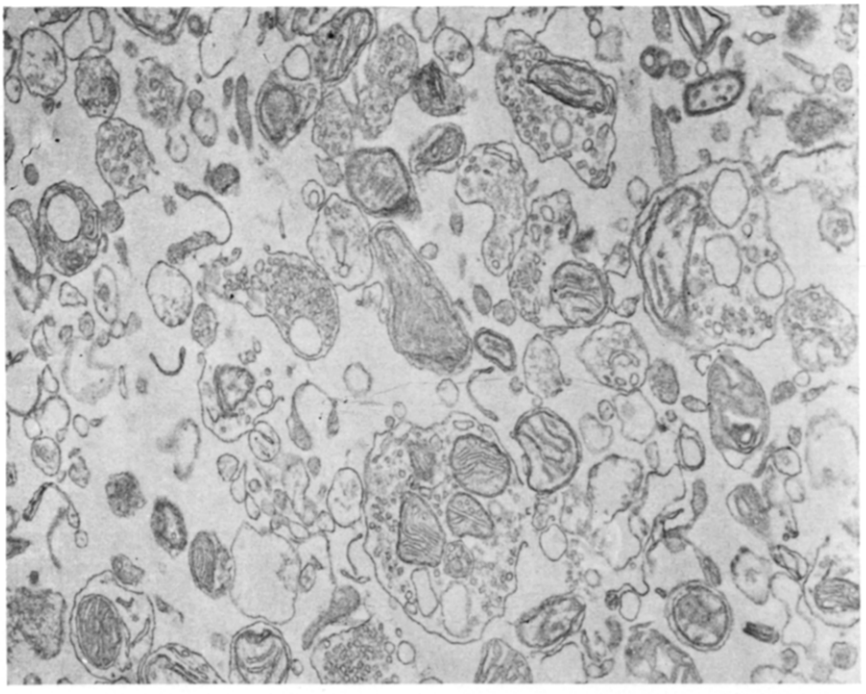

Fig. 1. Morphology of synaptosome fraction from 7.5–13 % band of Ficoll–sucrose gradient. Numerous synaptosomes are seen.

Occasionally myelin fragments are seen, particularly when older animals are used. Often free membrane can be identified as a postsynaptic membrane originating from a synaptosome; therefore, it is not contamination. Contaminating membranes arising from membrane vesicles which are not synaptosomes and existing as fragments, account for about 20% of the total fraction. Free mitochondria (those not inside synaptosomes or otherwise membrane bound) constitute approx. 10% of the fraction.

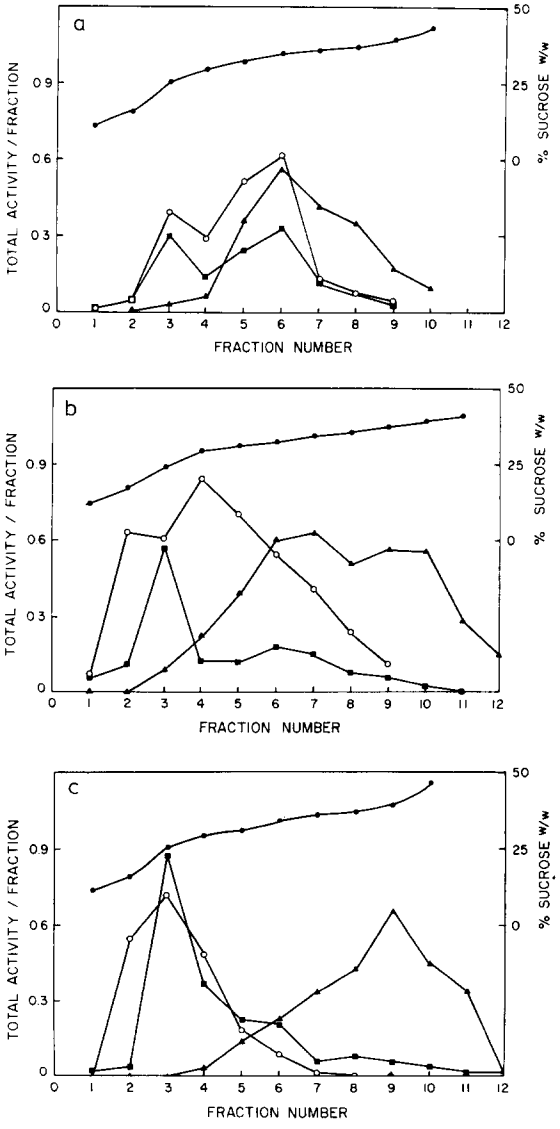

Fig. 2. Effect of pH during osmotic shock on the subsequent separation of synaptosomal components on a 25–45% continuous sucrose (w/w) gradient. ■—■, acetylcholinesterase; ○—○ (Na^+–K^+)-ATPase; ▲—▲, cytochrome oxidase. Osmotic shock was carried out at pH 7.1 in (a) at pH 7.6 in (b) at pH 8.5 in (c). The gradients were centrifuged at 25000 rev./min in an SW 25.1 rotor for 1.5 h.

Thus most of the plasma membrane in this fraction originates from synaptosomes. Starting from a purified synaptosome fraction the problem of isolating SPM fraction then becomes one, in principle, of effectively separating these membranes from mitochondria and synaptic vesicles, and of preparing them in a high enough yield so that plasma membrane must originate from synaptosomes.

The subfractionation of synaptosomes into membrane and mitochondrial fractions depends markedly on the conditions of osmotic shock, particularly the pH. Fig. 2 illustrates the effect of pH during osmotic shock on the separation. At neutral or slightly acid pH the resulting separation is not very satisfactory. Mitochondria do not effectively resolve from membranes. Some 50% of the cytochrome oxidase overlaps 89% of the (Na^+-K^+)-ATPase (Fig. 2A). At higher more alkaline pH's the separation is dramatically improved. At pH 8.5, 95% of the mitochondria separate from about 85% of the membranes (Fig. 2C). This pH effect is time dependent, requiring approx. 1.5 h of preincubation at 4°.

In order to perform routine separations quickly and prepare relatively large quantities of membrane, we developed a discontinuous sucrose gradient. The selection of this gradient was based on the concentrations of sucrose required to separate plasma membrane from mitochondria and other membranes. (Na^+-K^+)-ATPase, cytochrome oxidase and electron microscopic analyses were used to determine the appropriate conditions. On the basis of these studies we selected a gradient of 25, 32.5, 35 and 38% sucrose (w/w). 25% sucrose is effective in banding any myelin contamination. It also retains most microsomal membranes at short centrifugations times. 32.5% is the cutoff point to separate membrane from 90% of the mitochondria. At higher densities mitochondria are found in significant quantities although there are still plasma membranes. 38% sucrose effectively resolves purified mitochondria from most

TABLE I

THE PERCENT DISTRIBUTION OF DIFFERENT ENZYMES IN SUBFRACTIONS FROM OSMOTICALLY SHOCKED SYNAPTOSOMES

Fraction 1 is the band floating on 25% sucrose; Fraction 2 is at the interface of 25–32.5% sucrose; Fraction 3 is at the 32.5–35% sucrose band; Fraction 4 is at 35–38% sucrose; and Fraction 5 is the pellet through 38% sucrose. The number in brackets refers to the number of experiments. The percent recoveries are (Na^+-K^+)-ATPase, 43% alkaline phosphatase, 44%; 5′-nucleotidase, 103%; acetylcholine esterase, 64%; acid phosphatase, 84%; β-*N*-acetylglucosaminidase, 71%; cytochrome *c* oxidase, 84%; antimycin insensitive NADH cytochrome reductase, 68%; and ADPase, 84%; and protein, 80%. *N*, number of experiments.

Enzyme	*Fraction*:				
	1	*2*	*3*	*4*	*5*
Lowry protein ($N = 5$)	18.5 ± 1.4	31.8 ± 1.7	18.0 ± 2.3	20.3 ± 2.7	11.5 ± 4.9
(Na^+-K^+)-ATPase ($N = 3$)	18.0 ± 4.5	53.0 ± 3.9	13.0 ± 4.9	13.1 ± 3.0	3.0 ± 1.1
Alkaline phosphatase ($N = 2$)	21.4 ± 7.0	50.3 ± 0.2	14.3 ± 0.3	7.3 ± 3.4	6.7 ± 3.4
5′-Nucleotidase ($N = 3$)	35.2 ± 1.6	42.5 ± 3.7	13.4 ± 2.1	6.3 ± 0.6	2.6 ± 0.5
Acetylcholinesterase ($N = 3$)	39.6 ± 6.7	43.7 ± 6.9	10.5 ± 1.1	4.4 ± 1.1	1.8 ± 0.1
Acid phosphatase ($N = 2$)	16.6 ± 1.0	42.8 ± 4.8	20.1 ± 2.9	14.6 ± 3.2	5.8 ± 2.3
β-*N*-acetylglucosaminidase ($N = 4$)	24.1 ± 3.4	43.6 ± 2.9	15.8 ± 2.2	8.9 ± 3.6	7.5 ± 3.1
Cytochrome *c* oxidase ($N = 3$)	0.6 ± 0.1	6.2 ± 2.4	19.3 ± 5.5	43.1 ± 3.5	30.4 ± 7.6
Antimycin-insensitive NADH cytochrome reductase ($N = 7$)	9.3 ± 3.0	19.7 ± 4.5	29.9 ± 7.3	29.0 ± 7.0	12.1 ± 6.6
ADPase ($N = 2$)	28.2 ± 1.7	33.3 ± 1.9	14.9 ± 0.9	13.0 ± 1.6	10.7 ± 2.2

membranes. 35 % sucrose is used to subdivide the lower region of the gradient at the density region where intact synaptosomes would isopycnically band if they are present. Although not all parameters were studied on this gradient, a rate sedimentation gave the best separation (1.5 h at 60000 × *g*). This is approximately the total applied force required to achieve sedimentation of mitochondria from membranes.

The fractions at the top of this discontinuous gradient (Fractions 1 and 2) were predominately membranes. Fraction 1 banding on top of the 25 % sucrose layer contained 18 % of the total particulate protein applied to the gradient and 18 % of the total (Na^+–K^+)-ATPase activity (Table I). Fraction 2, collected at the 25 % and 32.5 % sucrose interface, contained 50 % of the (Na^+–K^+)-ATPase activity recovered in the gradient and only 7 % of total cytochrome oxidase (Table I). Fraction 2 had the highest relative specific activity for (Na^+–K^+)-ATPase in this gradient (Table II).

By morphological analysis it was found that Fraction 2 contained the richest population of synaptic plasma membranes not contaminated by mitochondria. Fig. 3 illustrates an electron micrograph of this fraction. It contains almost exclusively membrane profiles about the size of synaptosomes. Some membrane profiles contain

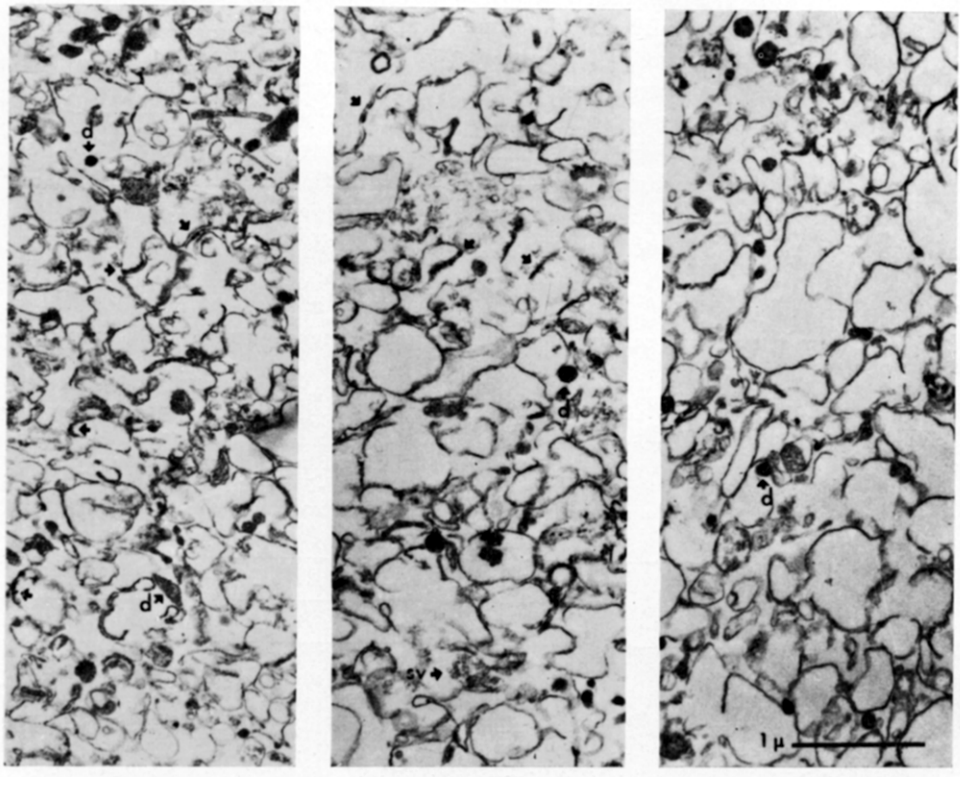

Fig. 3. Morphology of fraction sedimenting at interface between 25 and 32.5 % sucrose (w/w). The sample was prepared by centrifuging a small quantity of the preparation into a thin pellet as previously described[19]. This pellet is thin enough so that the entire thickness of the pellet can be sectioned and viewed on a single grid. (a) and (c) are from the edges of the pellet and (b) is from the center. Arrows show some of the synaptic thickenings in this preparation. Some synaptic vesicles (sv) remain adhering to the membranes. A number of small dark staining solid structures (d) are present in the fraction.

residual synaptic vesicles adhering inside the membrane. Many membranes have an attached synaptic thickening, although in some instances these appear detached from the membrane. The state of preservation of thickenings varied somewhat from preparation to preparation. The most common contaminate is a small dark staining solid structure. Some appear to be lysosome-like particles because of their size, profile and dense appearance; others, cytoplasm condensed inside of membranes. Fraction 2 in contrast to Fraction 1 contained many small membrane fragments, some myelin, and very few if any synaptic thickenings.

The fractions below Fraction 2 contained primarily mitochondria mixed to various degrees with assorted membrane fragments as determined either by electron microscopy or enzyme distribution. 93% of the total cytochrome oxidase activity and 70% of antimycin insensitive NADH cytochrome *c* reductase distribute in Fractions 3, 4, and 5 (banding from 35–38% sucrose), as well as 30% of the total (Na^+–K^+)-ATPase (Table I). The highest relative specific activity for cytochrome oxidase is in Fraction 5. The relative spec. act. for antimycin insensitive NADH cytochrome *c* reductase is approximately equal in Fractions 3, 4, and 5. The relative spec. act. of (Na^+–K^+)-ATPase progressively decreased from Fractions 3 to 5 (Table II).

5′-Nucleotidase, acetylcholinesterase, alkaline phosphatase, and ADPase, were studied as possible plasma membrane enzymes in subfractions from synaptosomes. The relative spec. act. of 5′-nucleotidase is highest in Fraction 1 and became progressively reduced as the density of the gradient increased (Table II). 78% of 5′-nucleotidase activity is found in Fractions 1 and 2 which are enriched in membranes (Table I). Since this enzyme has not been examined in membrane fractions of synaptic origin, we studied its pH optimum and magnesium requirements. The pH optimum was 9.0 and to a lesser degree between pH 7 and 8. Recently Bosman reported that 5′-nucleotidase from rat brain cerebellum is optimally active at pH 6.8. Since at alkaline pH the measurement of 5′-nucleotidase activity might include some non-specific phosphatase activity, we routinely measured the enzyme at pH 7.7 in the presence of Mg^{2+}.

TABLE II

RELATIVE SPECIFIC ACTIVITY OF VARIOUS ENZYMES OF THE FRACTIONS FROM OSMOTICALLY SHOCKED SYNAPTOSOMES

The synaptosomes were osmotically shocked at pH 8.1 in 6 mM Tris, 0.06 M sucrose for 1.5 h at 4°. Centrifugation was carried out for 1.5 h at 25000 rev./min in an SW 25.1 rotor. Relative spec. act. = % total activity/% total protein.

Enzyme	*Fraction*:				
	1	*2*	*3*	*4*	*5*
(Na^+–K^+)-ATPase	0.98 ± 0.24	1.67 ± 0.12	0.72 ± 0.27	0.65 ± 0.15	0.26 ± 0.10
Alkaline phosphatase	1.16 ± 0.38	1.58 ± 0.01	0.79 ± 0.02	0.36 ± 0.17	0.58 ± 0.30
5′-Nucleotidase	1.91 ± 0.09	1.34 ± 0.12	0.74 ± 0.12	0.31 ± 0.03	0.23 ± 0.04
Acetylcholinesterase	2.15 ± 0.36	1.38 ± 0.22	0.58 ± 0.06	0.22 ± 0.05	0.16 ± 0.01
Acid phosphatase	0.90 ± 0.05	1.35 ± 0.15	1.12 ± 0.16	0.72 ± 0.16	0.51 ± 0.20
β-*N*-Acetylglucosaminidase	1.30 ± 0.18	1.37 ± 0.09	0.88 ± 0.12	0.44 ± 0.18	0.65 ± 0.27
Cytochrome oxidase	0.03 ± 0.01	0.20 ± 0.08	1.07 ± 0.31	2.13 ± 0.17	2.64 ± 0.66
Antimycin-insensitive NADH cytochrome reductase	0.56 ± 0.16	0.65 ± 0.14	1.41 ± 0.41	1.40 ± 0.34	1.39 ± 0.57
ADPase	1.53 ± 0.09	1.05 ± 0.06	0.83 ± 0.05	0.64 ± 0.08	0.93 ± 0.19

The distribution profile, however, did not appear to depend on the pH at which 5′-nucleotidase was assayed. In one experiment we analyzed the distribution in subsynaptosomal fractions at both pH 7.7 and 9.0 and found that although the enzyme was more active at 9.0 the distribution was identical at both pH values.

Acetylcholinesterase accurately followed the distribution of 5′-nucleotidase (Tables I and II). The highest relative specific activity was in Fraction 1 and became progressively reduced as the density of the gradient increased.

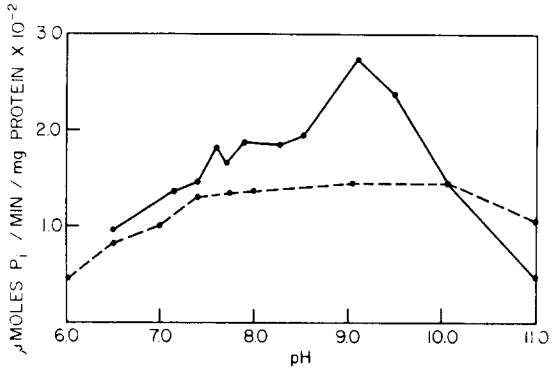

Fig. 4. The effect of pH in the absence and presence of 10 mM Mg^{2+} on 5′-nucleotidase activity. 5′-Nucleotidase is optimally active at pH 9.0 and activated by Mg^{2+}.

Alkaline phosphatase was partially resolved from 5′-nucleotidase and acetylcholinesterase. Fraction 2 had the highest relative specific activity for alkaline phosphatase (Table II) and contained 50 % of the total enzyme activity (Table I). Alkaline phosphatase and (Na^+–K^+)-ATPase showed a very similar distribution. Fraction 2 has the highest relative specific activity from both enzymes. In contrast, Fraction 1 was highest for either 5′-nucleotidase or acetylcholinesterase. Alkaline phosphatase using *p*-nitrophenylphosphate as substrate was optimally active at pH 10.0–10.5 in Fraction 2. The enzyme required 2 mM Mg^{2+} for maximal activity; it was not activated by K^+ or inhibited by ouabain.

33 % of the ADPase from synaptosomes is found in membrane fractions. However, compared to 5′-nucleotidase, alkaline phosphatase or (Na^+–K^+)-ATPase more ADPase activity is found in Fractions 4 and 5 (Table I) suggesting the presence of this enzyme in both membranes and mitochondria. Fraction 5, for example, contained 11 % of the total activity of ADPase and only 3 % of the (Na^+–K^+)-ATPase activity.

Because our morphological observations suggested the possible presence of lysosomes in the SPM fraction (Fraction 2), we analyzed acid phosphatase and *β-N*-acetylglucosaminidase. Approx. 44 % of the total enzyme activity was found in the SPM fraction. It may be that lysosomes contaminate this fraction. Alternately, lysosomal enzymes may be adsorbed onto these membranes in a manner similar to that seen for choline acetylase[20,21] or are in fact part of a membrane contained in this fraction. To evaluate these possibilities, we treated Fraction 2 with various solutions (Table III) to remove adsorbed protein or break lysosomes. Washing the SPM fraction with salt solutions achieved removal of 32–55 % of *β-N*-acetylglucosaminidase; 0.9 % NaCl in Tris buffer, pH 8.5, solubilized 36 % of this enzyme while 1 M NaCl in Tris, pH 8.0, solubilized 55 %. Dilute solutions of Triton X-100 (0.01 %) solubilized 61 %

TABLE III

THE PERCENT OF β-*N*-ACETYLGLUCOSAMINIDASE ACTIVITY SOLUBILIZED BY VARIOUS TREATMENTS

The result of these treatments on synaptic plasma membranes is compared to liver. The 32.5–35 % sucrose fraction from synaptosomes was used for brain, and a mitochondrial–lysosomal fraction was used for liver. The liver fraction was prepared by centrifugation of a liver homogenate to $100 \cdot 10^7\ \omega^2 t$ after first removing the nuclear material ($2 \cdot 10^7\ \omega^2 t$). The fraction was washed once at $80 \cdot 10^7\ \omega^2 t$. Each tissue was incubated in the designated solution for 30 min at 4° at a protein concentration of approx. 0.1 mg/ml. A sample of the total was removed and the samples pelleted at 30000 rev./min for 15 min. The supernatants were quantitatively removed and the pellets resuspended in 10 % sucrose for assay.

Treatment	*Brain*		*Liver*	
	% *solubilized*	% *recovery*	% *solubilized*	% *recovery*
Water	3	81	33	98
0.9 % NaCl–0.02 M Tris, pH 7.0	33	85	76	99
0.9 % NaCl–0.02 M Tris, pH 8.5	36	82	87	103
0.9 % NaCl–0.02 M Tris, pH 7.0, 0.01 % Triton X-100	35	82	79	95
0.9 % NaCl–0.02 M Tris, pH 8.0, 0.01 % Triton X-100	61	76	89	90
0.9 % NaCl–0.02 M Tris, pH 8.0, 0.1 % Triton X-100	96	81	100	77
0.9 % NaCl–0.02 M Tris, pH 8.0, 0.5 % Triton X-100	96	81	100	88
1 M NaCl–0.02 M Tris, pH 8.0	55	88	93	85

of β-*N*-acetylglucosaminidase, but only a few percent of the protein, while 0.1 % solubilized essentially all the glucosaminidase, but only approx. 25 % of the protein. We can conclude that glucosaminidase is not tightly integrated into the membrane of this brain fraction and is either adsorbed or in lysosomes. If the enzyme is adsorbed by brain membranes, we would expect that brain membranes would adsorb solubilized lysosomal enzymes from liver. Brain membranes did not adsorb soluble acid phosphatase or β-*N*-acetylglucosaminidase from liver. The supernatant from an osmotically shocked mitochondrial fraction from liver was added to a brain crude mitochondrial fraction and the mixture was homogenized, incubated for 1 h at room temperature and pelleted. Despite the presence of additional enzyme from the liver supernatant no additional enzyme was bound to brain particulate material. Interestingly β-*N*-acetylglucosaminidase was more readily removed from a liver mitochondrial fraction than from the SPM fraction (Table III). We can conclude the β-*N*-acetylglucosaminidase is more tightly associated with this sub-synaptosomal fraction than with lysosomes from liver.

In Table IV the specific activity of Fraction 2 (25–32.5 % sucrose) is compared to that of brain homogenates. NADH cytochrome *c* reductase in the presence of antimycin is decreased from the total homogenate, as is cytochrome oxidase. Acid phosphatase, alkaline phosphatase, β-*N*-acetylglucosaminidase are approx. 1–1.5 times that of the homogenate and (Na^+–K^+)-ATPase is 3.5 times the homogenate. Since a 3-fold enrichment is not very large, we examined the possibility that enzyme activity is lost due to one or more aspects of the procedure. In two experiments we followed the recovery of (Na^+–K^+)-ATPase throughout the entire fractionation procedure. The recovery in the Ficoll–sucrose gradient was 60 ± 10 %. Osmotic shock

activated the enzyme slightly, but pelleting and resuspension in 10 % sucrose restored the enzyme to its level prior to osmotic shock. If the specific activity of the 25–32.5 % fraction is corrected for loss of activity and we assume that all fractions undergo proportionate inactivation, the enrichment from the homogenate would be five-fold.

DISCUSSION

We describe a method to isolate a synaptic plasma membrane fraction from synaptosomes in good purity and relatively high yield. The yield of 1.5–2.0 mg/g brain wet weight (2–3 % of the particulate protein of the homogenate) is similar to the yield of plasma membrane derived from liver by various fractionation procedures[22]. (Na^+–K^+)-ATPase is enriched approx. 5-fold after correction for enzyme inactivation.

This enrichment is quite small relative to the 20–25 fold purification of marker enzymes achieved for liver. The explanation for this is probably due to a higher proportion of plasma membranes in brain homogenates. Neurons with their extensive branching must have a higher surface to volume ratio and relatively more plasma membrane than more spherical cells such as those in liver. TOUSTER *et al.*[22] calculated that plasma membranes in liver tissue constitute approx. 5 % of the total particulate protein of the homogenate on the basis of the enrichment of a plasma membrane enzyme, 5′-nucleotidase. From an estimate that neurons have 4–8 times more surface area than liver cells, the percent of total particulate protein in brain which is plasma membrane can be calculated*. The conclusion derived from such calculations is that 10–20 % of particulate material in a brain homogenate is neuronal plasma membrane. If neuronal membranes comprise 10–20 %, an enrichment of only 5–10-fold from the homogenate could be realized. While such calculations are subject to inaccuracy, the point we wish to make is that a marked purification such as seen in liver may not be possible in brain since a greater proportion of the total homogenate is plasma membrane.

To purify a constituent of neuronal plasma membrane more than 5–10-fold either it would have to be confined to certain neurons or to a minor region of the neuronal plasma membrane which can be separated from other neuronal membranes. Neurons differ morphologically from each other and clearly regions of neuronal membrane are specialized at an electrophysiological level and there are indications of

* A cell without extensive processes such as a liver cell can be approximated by a sphere. A neuron, which is almost entirely processes, can be approximated by a rod. If the volumes are equal, the differences in surface area depend on their respective radii. A liver cell has a mean radius of about 15 μm[68] and a neuronal process has a mean radius of approximately 0.5 μm. The hepatocyte surface, however, is in fact greater than the calculated for a smooth sphere because a portion of the plasmalemma facing the sinusoids and canaliculi is in the form of microvilli. This increases the surface area by about 2.5 times[69]. Based on the radii values above, the rod shaped cell would have 20 times the surface area of the sphereical cell or about 8 times that of a hepatocyte. Since 5 % of a liver cell is plasma membrane[22] and a neuron has 8 times the surface, 40 % of the particulate material of a neuron is plasma membrane. Neurons may be estimated to comprise 50 % of the mass of brain, so that 20 % of the homogenate would be neuronal plasma membrane. Altering the radius of a neuronal process to 1 μm or decreasing the mass of neurons to only 25 % of the homogenate would reduce the neuronal plasma membrane content to 10 %. These are both probably underestimated. Many cell processes in neuropile have radii of 0.25 μm or less, and brain tissue is almost certainly more than 25 % neurons. Thus 10–20 % of the particulate material in a brain homogenate is probably neuronal plasma membrane.

TABLE IV

SPECIFIC ACTIVITY OF ENZYMES

The specific activities (μmoles/min per mg protein) of various enzymes is given for the homogenate (H), Fraction 2 (25–32.5% sucrose) and Fraction 5 (38% sucrose pellet). *N* is the number of experiments.

Enzyme	*Fraction*:		
	H	*2*	*5*
$(Na^+–K^+)$-ATPase ($N = 8$)	0.079 ± 0.057	0.203 ± 0.057	
Alkaline phosphatase ($N = 4$)	0.330 ± 0.090	0.341 ± 0.054	
5′-Nucleotidase ($N = 3$)	0.029 ± 0.010	0.036 ± 0.004	
Acetylcholinesterase ($N = 4$)	0.262 ± 0.108	0.356 ± 0.150	
Acid phosphatase ($N = 3$)	0.488 ± 0.249	0.602 ± 0.023	
β-*N*-Acetyl glucosaminidase ($N = 5$)	0.022 ± 0.008	0.024 ± 0.010	0.010 ± 0.002 ($N = 3$)
Cytochrome oxidase ($N = 6$)	0.448 ± 0.156	0.177 ± 0.145	2.551 ± 0.734
Antimycin-insensitive NADH cytochrome reductase ($N = 5$)	0.032 ± 0.004	0.016 ± 0.004	
ADPase ($N = 3$)	0.066 ± 0.020	0.049 ± 0.017	0.027 ± 0.010 ($N = 2$)

specialization at a chemical level as well[23–25]. However, for functions such as Na^+ and K^+ pumping there are probably more similarities than differences among neurons and in sectors of the membrane. With the exception of acetylcholinesterase which is confined to certain neurons[26–28], the markers used for membranes are probably of a general nature. In other, presumably less specialized cells such as erythrocytes or liver cells, chemical heterogeneity has been found in plasma membranes[29,30] and heterogeneity is almost certain to exist in neuronal membranes as well. At present, however, membrane heterogeneity cannot be distinguished from cellular heterogeneity in brain. Advances will probably require work on brain regions combined with histochemistry.

A critical variable of the isolation procedure described here is the pH during osmotic shock. Alkaline conditions favor a better separation of membrane from mitochondria, probably because of a more effective lysis of synaptosomes. If synaptosomes are lysed in unbuffered solutions (distilled water), the pH of the resulting solution is slightly acidic[20] and the synaptosomal components do not effectively resolve on sucrose gradients. The effect of alkaline buffers is not known, but may be a combination of factors, including an incubation effect. Related findings have been observed in other systems. The use of moderately alkaline conditions has been described as advantageous for the lysis of red blood cells[31], for the removal of loosely bound or adsorbed proteins from membrane[20,21,32,33], for promoting membrane vesicularization[34], and for lysing zymogen granules[35,36]. It has also been noted that the separation of rat intestinal plasma membrane fragments from mitochondria is improved if the preparation is incubated ("aged") at 37° for 3 h (ref. 37). The plasma membrane fragments then sediment at lighter densities much in the same way that membranes fragments in our synaptosomal preparation do.

Alkaline phosphatase is probably a plasma membrane enzyme in liver[38–41] and possibly in other tissues[42–44]. Alkaline phosphatase from sheep brain has been solubilized and found to be a glycoprotein[45,46]. In synaptosome subfractions, alkaline

phosphatase accurately parallels the distribution of (Na^+–K^+)-ATPase and is probably a plasma membrane enzyme associated in part with synaptic plasma membranes. It is not exclusively a plasma membrane enzyme in brain, however, because histochemical studies on brain have located a very high concentration of alkaline phosphatase in capillary walls[47,48].

On the basis of an extensive review of the literature on plasma membranes, STECK AND WALLACH[49] have concluded that 5′-nucleotidase is at present the most specific and general plasma membrane activity known. 5′-Nucleotidase is associated with plasma membranes derived from liver[50,51] and other cells and there is some evidence for a similar localization in brain[52–54].

In subfractions derived from synaptosomes, 5′-nucleotidase activity is most concentrated in Fraction 1, although there is considerable overlap into Fraction 2. Fraction 1 contains a variety of relatively small membrane fragments and is characterized as having a lower (Na^+–K^+)-ATPase content than Fraction 2 and a relative absence of synaptic thickenings. The 5′-nucleotidase contained in Fraction 2 is optimally active at pH 9.0 in the presence of Mg^{2+}; Mg^{2+} stimulates the enzyme more at pH 9 than at neutral pH. 5′-Nucleotidase has recently been purified from brain homogenates and its various properties analyzed[55]. The purified enzyme shows a single relatively sharp pH optimum of 6.8 in the presence of 10 mM Mg^{2+}. The contrast between the pH profile for 5′-nucleotidase reported here for membrane preparations and that observed with purified brain enzyme[55] may result either from the presence of more than one activity in our membrane preparations or a kinetic property[56] displayed only by the membrane bound enzyme. 5′-Nucleotidase in liver plasma membranes also displays a double pH optimum (pH 7.4 and 9.5) and Mg^{2+} is required for the activity at pH 9.5 (ref. 51).

Both 5′-nucleotidase and acetylcholinesterase appear to be associated in highest concentration with small light membrane fragments and not primarily with synaptic plasma membranes. Synaptic plasma membranes are most concentrated in Fractions 2 and 3 whereas 5′-nucleotidase and acetylcholinesterase have their highest relative specific activity in Fraction 1 and their relative spec. act. progressively decreases as the density of the gradient increases. HARWOOD AND HAWTHORNE[54] also noted a similar distribution between 5′-nucleotidase and acetylcholinesterase in membrane fractions prepared from an osmotically shocked crude mitochondrial fraction. The identity of these small membrane fragments in Fraction 1 containing high concentrations of 5′-nucleotidase and acetylcholinesterase is not known but tentative conclusions can be drawn from histochemical studies on brain. By histochemical analysis acetylcholinesterase is found associated with axonal and dendritic membranes and endoplasmic reticulum[27,47,57] and to some degree with presynaptic endings[58]. KOKKO *et al.*[56] observed that acetylcholinesterase activity is concentrated around small neuronal elements in neuropile and SHUTE AND LEWIS[27] observed that most of the acetylcholinesterase is confined to thin axons. The high concentration of acetylcholinesterase in membrane fraction which sediment like small particles is compatible with the histochemical observations. Many of these acetylcholinesterase containing membranes no doubt arise from axons and/or possibly dendrites and possibly even endoplasmic reticulum. By similar reasoning 5′-nucleotidase may also be present in higher concentration in axonal, dendritic or glial membranes than in synaptic membranes. In fact, histochemical analysis has demonstrated 5′-nucleotidase activity in glial cells[59]. The

reduced activity of 5'-nucleotidase in synaptosomal membranes might be involved with their high adenyl cyclase activity[60] which requires a source of AMP.

Two enzymes generally attributed to lysosomes in brain and other tissues, acid phosphatase and β-*N*-acetylglucosaminidase[61], are found in the SPM fraction, indicating possible lysosomal contamination. Both enzymes are relatively resistant to mild washing treatments, but are removed with mild detergent treatment. Thus, they are not a structural component of the synaptic plasma membrane since the membrane itself is relatively resistant to such treatment[62]. In liver, these lysosomal enzymes are not as resistant to washing treatments, indicating a fundamental difference between brain and liver lysosomal enzymes. Recently, VIGNAIS AND NACHBAUR[63] presented evidence suggesting that lysosomal membranes retain significant quantities of certain lysosomal enzymes following osmotic shock. These membranes had banding densities very similar to the SPM fraction and to outer mitochondrial membranes from liver. If acid phosphatase and β-*N*-acetylglucosaminidase are contained within lysosomes or lysosomal membranes in the SPM fraction, they probably account for very little of the protein since purified lysosomes have a specific activity 100 times that of the homogenate[64]. Since certain enzymes previously believed to be exclusively lysosomal have been found in plasma membranes[65,66], we cannot exclude the possibility that such enzymes may be loosely associated with synaptic plasma membranes in addition to being in synaptosomal lysosomes[67]. The presence of certain hydrolytic enzymes in neuronal membrane would be appropriate for the maintenance of the neuronal surface. In the following paper we further evaluate the enzymatic activity of this SPM fraction and other subsynaptosomal fractions.

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