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ISOLATION AND PARTIAL CHARACTERIZATION OF CHICK BRAIN SYNAPTIC PLASMA MEMBRANES*

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SUMMARY

An isolation procedure for synaptic plasma membranes from whole chick brain is reported that uses the combined flotation-sedimentation density gradient centrifugation procedure described by Jones and Matus (Jones, D. H. and Matus, A. I. (1974) Biochim. Biophys. Acta 356, 276–287) for rat brain. The particulate of the osmotically shocked and sonicated crude mitochondrial fraction was used for a flotation-sedimentation gradient step. Four fractions were recovered from the gradient after 30 min centrifugation. The fractions were identified and characterized by electron microscopy and by several markers for plasma membrane and other subcellular organelles. Fraction 2 was recovered from the 28.5–34 % (w/v) sucrose interphase and contained the major part of the activities of the neuronal plasma membrane marker enzymes. The specific activities of the $(Na^+ + K^+)$-activated ATPase (EC 3.6.1.3), acetylcholinesterase (EC 3.1.1.7) and 5'-nucleotidase (EC 3.1.3.5) were, respectively, 4.5, 2.0 and 1.2 times higher than in the homogenate. However, Fraction 2 also contained considerable amounts of activities of putative lysosomal and microsomal markers in addition to lower amounts of mitochondrial and myelin markers. Although no prepurification of synaptosomes from the crude mitochondrial fraction was performed, the synaptic plasma membranes obtained showed many properties analogous to similar preparations from rat brain described in recent years.

INTRODUCTION

In brain tissue, synapses are the elementary structures across which neurons communicate through released neurotransmitters. Biochemical information on macromolecular organization of isolated synapses and subcomponents thereof has been accumulating exponentially in recent years [2]. During the homogenization of brain tissue under isotonic conditions the synapses or nerve terminals are pinched off from the axons and the cell bodies or dendrites and reseal to form synaptosomes [3].

* A preliminary report has been presented at the Fifth International Meeting of the ISN, Barcelona, 1975, p. 174.

Synaptic plasma membrane is defined as the external, limiting membrane of the synaptosome. The greater part of the synaptosome is presynaptic in origin, but dependent on the composition of the homogenization medium; a postsynaptic attachment is present connected by the cleft material to the membrane of the nerve terminal.

In recent years a number of isolation procedures for rat brain synaptic plasma membranes have been published [4–7], based on the "classical" procedures of Whittaker et al. [8] and De Robertis et al. [9]. Some authors [4, 5] have emphasized the importance of a prepurification step of synaptosomes from the crude mitochondrial fraction on a Ficoll-sucrose gradient in order to achieve a better elimination of the major contaminating elements such as myelin and especially mitochondria. Jones and Matus [1] reported a rapid and rather simple procedure for the isolation of synaptic plasma membranes from rat forebrain from the unpelleted osmotically shocked crude mitochondrial fraction. This method, using a flotation-sedimentation technique, takes advantage of the differences in the equilibrium buoyant densities between the various subcellular organelles to be resolved. When a mixture of cell organelles is suspended in a solution of intermediate density and centrifuged, the more dense elements sediment and the less dense elements float. This principle has also been used for the isolation of synaptosomes from rat brain [7, 10].

In this paper we report the application of the flotation-sedimentation procedure to the isolation of synaptic plasma membranes from chick brain. The purification was monitored by assaying putative markers for (neuronal) plasma membranes as well as for various subcellular elements, which could presumably contaminate the synaptic plasma membrane preparation. In addition, electron microscopic analysis was used in the morphological identification of the subfractions.

MATERIALS AND PROCEDURES

Materials

Chicks were obtained as described previously [11] and were killed 6 days after hatching. One whole brain weighed approx. 1 g. Most chemicals were from J. T. Baker Chemicals B.V. (Deventer) and the biochemicals were from Sigma Chemicals (St. Louis, U.S.A.) and Boehringer (Mannheim, G.F.R.). All solutions were prepared in glass distilled water throughout.

Enzyme assays

Acetylcholinesterase (EC 3.1.1.7) was assayed by the method of Ellman et al. [12]. 5'-Nucleotidase (EC 3.1.3.5) was assayed according to Michell and Hawthorne [13]. The ATPases (EC 3.6.1.3) were assayed according to Trevor and Cummins [14]; the rate of ATP hydrolysis was measured by the rate of proton release expressed as μequiv. H^+/min per mg protein using a sensitive pH meter. The $(Na^+ + K^+)$-activated ATPase was taken as the difference of the ATPase activity measured in the absence, and the presence of 0.2 mM ouabain. The mitochondrial ATPase was defined as the difference in ATPase activity measured in the presence of ouabain and of ouabain + oligomycin (2 μg/mg protein). The cytochrome *c* reductases (EC 1.6.2.5) were measured by the method of Werner and Neupert [15]. The rotenone sensitive part of the enzyme complex was taken as the difference between the activity in the absence and the presence of 2 μM rotenone. The rotenone-insensitive activity was measured as the ac-

tivity in the presence of rotenone. Both NADH- and NADPH-dependent enzymes were assayed. Monoamine oxidase (EC 1.4.3.4) was assayed according to Weissbach et al. [16] with kynuramide dihydrogenbromide as a substrate. Glucose-6-phosphatase (EC 3.1.3.9) was determined by the method of Hübscher and West [17] in the presence of 2 mM NaF and 4 mM EDTA to prevent acid phosphatase activity. 2',3'-cyclic AMP 3'-phosphohydrolase (EC 3.1.4.1) was activated by deoxycholate and assayed according to Drummond et al. [18]. Acid phosphatase (EC 3.1.3.2) was assayed according to Gianetto and De Duve [19] with sodium β-glycerophosphate as substrate. β-Glucosidase (EC 3.2.1.21) was assayed fluorimetrically according to Robins et al. [20]. Lactate dehydrogenase (EC 1.1.1.27) was assayed according to Kornberg [21]. Glucose-6-phosphatase, 5'-nucleotidase, 2',3'-cyclic AMP 3'-phosphohydrolase and acid phosphatase were all determined through released inorganic phosphate by the method of Sumner [22]. Acetylcholinesterase, the cytochrome *c* reductases, the ATPases and lactate dehydrogenase were assayed at 25 °C, 2',3'-cyclic AMP 3'-phosphohydrolase at 30 °C and the remaining enzymes at 37 °C.

Chemical assays

Protein was solubilized in 0.5 M NaOH and assayed according to Lowry et al. [23], using crystalline bovine serum albumin as standard. Ribonucleic acid was assayed according to Littlefield et al. [24] with ultraviolet reading at 260 nm and according to Schneider [25] with the orcinol reaction.

Electron microscopy

Samples were prepared for electron microscopic examination as previously described [11].

Isolation of synaptic plasma membranes (Fig. 1)

Whole brains from chicks were homogenized in a Potter-Elvehjem [11] in a 10 % (w/v) suspension in a medium consisting of 0.32 M sucrose, 2 mM Tris, 0.1 mM EDTA and buffered with 1 mM phosphate (pH 7.5). The primary fractionation was essentially the same as described previously [11], with the major exception of the washing of the crude mitochondrial fraction, which was pelleted at 10 000 $\times g$ for 17 min to reduce microsomal contamination. The obtained pellet was osmotically shocked in 50 μM $MgCl_2$ (8 ml/g wet wt.) and homogenized in a Potter-Elvehjem and then treated with ultrasound for 3×10 s with an MSE 100 W ultrasonic disintegrator and adjusted at pH 7.5 with NaOH. After 30 min on ice the suspension was centrifuged at 48 000 $\times g$ for 30 min and the pellet P_2 (H_2O) was used for the gradient. This pellet was resuspended in 50 μM $MgCl_2$ and mixed with two volumes of 51 % (w/v) to a 34 % (w/v) final sucrose concentration and overlaid on 41 % (w/v) sucrose. Then a 28.5 % (w/v) sucrose solution was carefully pipetted on top and it was overlaid finally with 10 % (w/v) sucrose. After centrifugation for 30 min at 77 000 $\times g$ four fractions were collected from the three interphases and the pellet, respectively denoted as f1 (interphase 10–28.5 % sucrose), f2 (interphase 28.5–34 % sucrose), f3 (interphase 34–41 % sucrose) and f4 (pellet). The fractions f1, f2 and f3 were diluted to isotonic sucrose and pelleted at 48 000 $\times g$ for 30 min. All pellets were finally stored in 50 μM $MgCl_2$ adjusted to pH 8 with NaOH. When the ATPases were assayed, pellets were suspended in the same solution buffered with 5 mM Tris/phos-

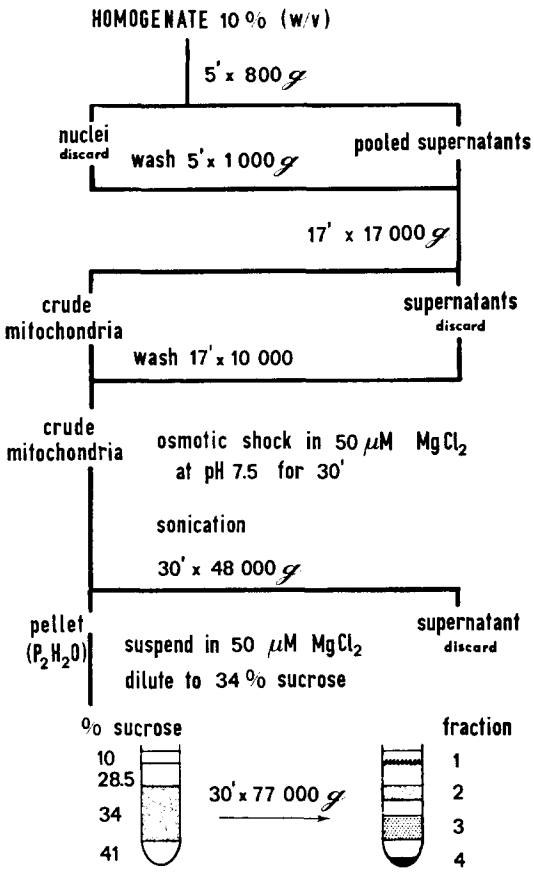


Fig. 1. Flow sheet of the isolation procedure of synaptic plasma membranes from chick brain homogenate. Time of centrifugation is given without acceleration and deceleration. g values are calculated for the centre of the centrifugation tube.

phate. ATPase and lactate dehydrogenase were assayed within 24 h after preparation. All other assays were carried out within 3 days, while the samples were stored at −20 °C.

Reference fractions

Mitochondria. Fraction 4 of the flotation gradient was used as the mitochondrial fraction without further purification.

Myelin. Fraction 1 was used as the myelin fraction. No further purification was attempted.

Microsomes. The 17 000 × g supernatant of the primary fractionation was centrifuged for 20 min at 12 000 × g. Then the supernatant was centrifuged for 60 min at 100 000 × g. The resulting pellet was the microsomal fraction.

Soluble proteins. The supernatant remaining after the pelleting of the microsomes was the soluble protein fraction.

Lysosomes. The pellet fraction from the Ficoll gradient in isotonic sucrose as previously described [11] consisting mainly of mitochondria, with no myelin or synaptosomes was used as a reference for lysosomes.

RESULTS

Preparation of synaptic plasma membranes

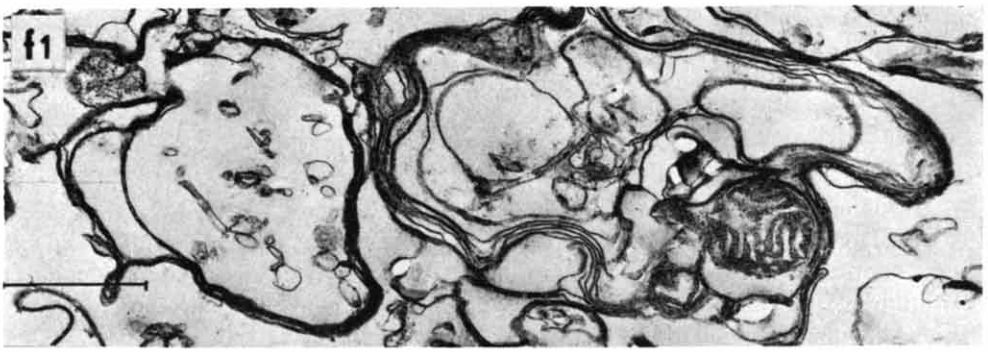
During the primary fractionation of the homogenate, the nuclear pellet and the crude mitochondrial pellet were washed once. The washing of the crude mitochondria with unbuffered sucrose is especially pertinent for the removal of microsomes, as has been shown by Raghupathy et al. [26] for the removal of nearly all membrane-bound polysomes from crude mitochondria from adult rat brain. Gurd et al. [7] reduced the specific activities of the microsomal markers in the crude mitochondrial pellet by three washes to approx. 10 % of that of the microsomal fraction, which contained also the Golgi apparatus. In preliminary experiments, we did not observe a significant decrease in microsomal markers after a second wash; therefore, we used only one wash. The subsequent osmotic shock treatment was performed at pH 7.5 for 30 min in 50 μM $MgCl_2$. Cotman and Matthews [5] performed the osmotic shock treatment on prepurified synaptosomes under more alkaline conditions during a longer period of 90 min. We observed only minor changes with their procedure applied to the crude mitochondrial fraction, resulting in a slightly greater solubilization of both soluble and membrane-bound enzymes. Some authors have emphasized the importance of prepurification of synaptosomes from the crude mitochondrial fraction [4]. In preliminary experiments we used a synaptosomal fraction prepared from a Ficoll gradient in isotonic sucrose as previously described [11] for the same osmotic shock treatment and subsequent gradient centrifugation. With regard to all assayed markers (see Methods) no significant changes were observed in the specific activities in fraction 2 except for 2-fold higher values for the mitochondrial markers in addition to a 4- to 5-fold lower yield in protein. Therefore we decided to prepare the synaptic plasma membranes directly from the adequately treated crude mitochondrial fraction.

Electron microscopic examination (Fig. 2)

Fraction 1 (f1) contained whorls of multilamellar membranes typical for myelin. A few small membrane fragments of unidentified origin and an occasional entrapped mitochondrion were observed. Fraction 2 (f2) showed many ghost-like membranes of various sizes. No myelin or mitochondria could be observed. This fraction was tentatively designated as the synaptic plasma membrane fraction. Fraction 3 (f3) was intermediate in appearance between Fractions 2 and 4, showing both synaptic ghosts and mitochondria. Finally, Fraction 4 (f4) showed a field of predominantly mitochondria with a few vesicle-like profiles.

Enzymatic and chemical characterization

A few putative neuronal plasma membrane marker enzymes in addition to a number of markers for various subcellular constituents like mitochondria, microsomes, myelin, glial plasma membrane, lysosomes and soluble proteins have been assayed in the subfractions (Table I). The enrichment over the homogenate for various markers, as shown in Table II, has been calculated from the average specific activities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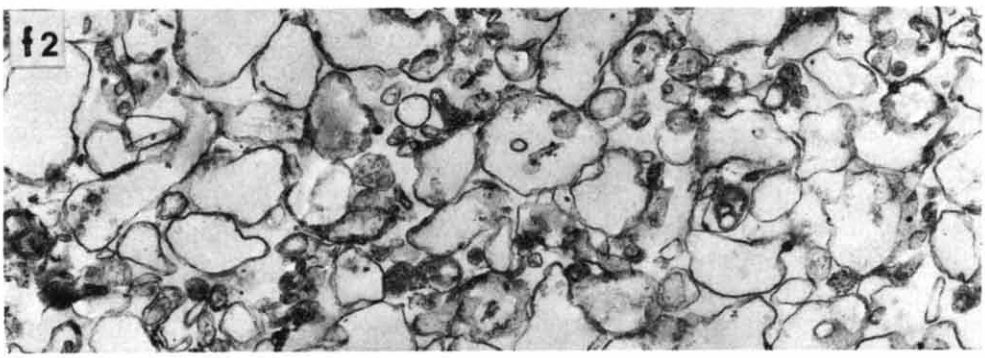
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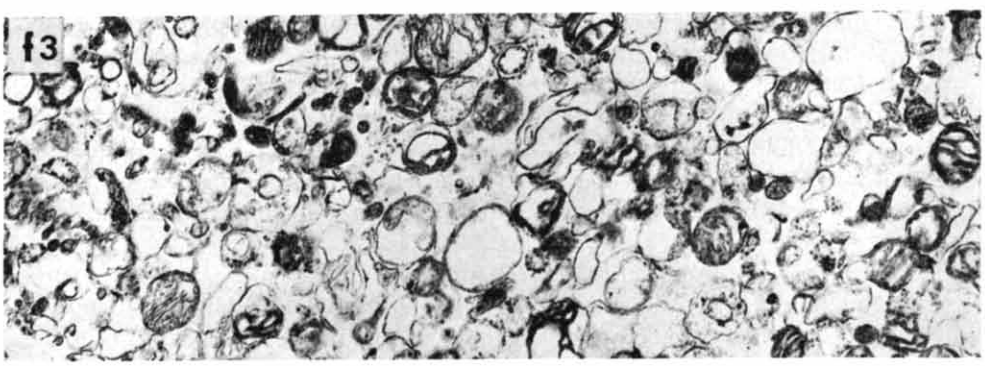
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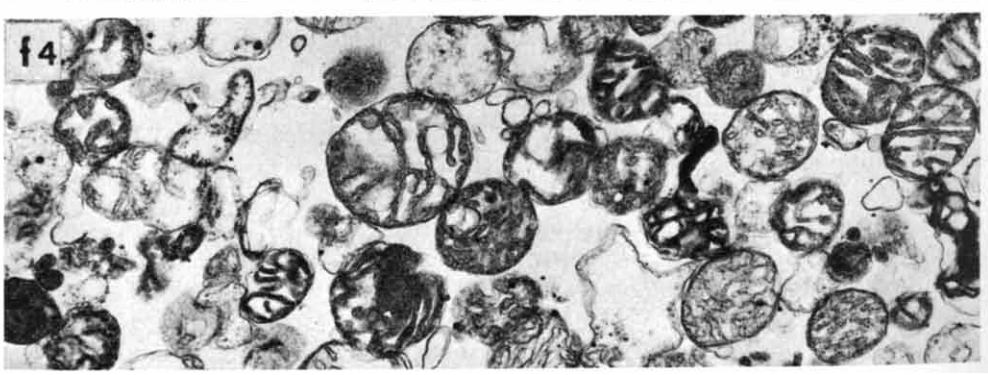
f4

TABLE I

PROTEIN CONTENT AND SPECIFIC ACTIVITIES OF MARKERS IN HOMOGENATE AND SUBFRACTIONS

Specific activities ± S.D. are expressed in nmol/min per mg protein. Protein and ribonucleic acid are expressed as indicated. HOM, homogenate of whole chick brain; $P_2(H_2O)$, the particulate of the osmotically shocked crude mitochondrial fraction. Fractions are denoted as in Fig. 1. Recovery is given in per cent content/activity recovered from the gradient; *n* represents the number of experiments.

Assay	HOM	$P_2(H_2O)$	f1
Protein (mg/g wet wt)	108.5 ± 7.5	24.0 ± 1.8	1.8 ± 0.4
ATPase	656 ± 152	1305 ± 319	169 ± 78
($Na^+ + K^+$)-ATPase ouabain sensitive	75.2 ± 44.8	120.9 ± 30.6	31.2 ± 12.7
Acetylcholinesterase	308 ± 55	311 ± 97	179 ± 104
5′-Nucleotidase	26.8 ± 9.5	26.5 ± 14.4	10.0 ± 10.3
Mitochondrial ATPase	135 ± 54	357 ± 156	28 ± 19
NADH: cytochrome *c* reductase rotenone-sensitive	16.5 ± 5.0	47.3 ± 20	6.8 ± 4.2
Monoamine oxidase	1.67 ± 0.45	3.06 ± 0.65	0.57 ± 0.23
NADH: cytochrome *c* reductase rotenone-insensitive	16.5 ± 1.3	26.5 ± 8.8	7.3 ± 2.3
Glucose-6-phosphatase	3.50 ± 0.89	6.13 ± 2.35	2.64 ± 0.96
NADPH: cytochrome *c* reductase	1.47 ± 0.46	2.33 ± 0.84	0.45 ± 0.20
Ribonucleic acid (μg/mg protein)	33.3 ± 4.5	25.3 ± 3.6	13.8 ± 4.8
2′,3′-cyclic AMP 3′-phosphohydrolase	0.80 ± 0.09	1.42 ± 0.58	10.1 ± 2.4
Lactate dehydrogenase	540 ± 149	389 ± 141	211 ± 23
β-Glucosidase	159 ± 18	183 ± 35	120 ± 34
Acid phosphatase	10.9 ± 3.1	11.5 ± 4.3	4.9 ± 1.6

Assay	f2	f3	f4	Recovery	*n*
Protein (mg/g wet wt)	3.3 ± 0.8	6.5 ± 1.3	5.6 ± 0.9	76 ± 7	11
ATPase	1372 ± 279	1667 ± 462	1910 ± 676	89 ± 10	11
($Na^+ + K^+$)-ATPase ouabain sensitive	335 ± 187	137 ± 59	75 ± 78	78 ± 23	11
Acetylcholinesterase	607 ± 152	454 ± 40	154 ± 43	77 ± 13	4
5′-Nucleotidase	31.8 ± 9.2	37.4 ± 28.6	7.7 ± 1.7	64 ± 20	9
Mitochondrial ATPase	100 ± 52	522 ± 93	2420 ± 355	145 ± 33	11
NADH:cytochrome *c* reductase rotenone-sensitive	16.4 ± 9.9	83.3 ± 44.4	285 ± 97	209 ± 36	4
Monoamine oxidase	2.09 ± 1.43	5.91 ± 1.7	7.7 ± 2.9	86 ± 23	5
NADH:cytochrome *c* reductase rotenone-insensitive	16.2 ± 5.8	25.2 ± 5.7	27.8 ± 9.0	53 ± 9	4
Glucose-6-phosphatase	4.61 ± 2.14	7.16 ± 1.82	4.14 ± 1.90	86 ± 27	4
NADPH: cytochrome *c* reductase	1.52 ± 0.40	1.71 ± 0.97	1.32 ± 0.25	53 ± 11	3
Ribonucleic acid (μg/mg protein)	22.2 ± 5.1	26.6 ± 4.8	25.0 ± 3.0	85 ± 5	8
2′,3′-cyclic AMP 3′-phosphohydrolase	0.90 ± 0.11	0.59 ± 0.12	0.19 ± 0.04	89 ± 10	5
Lactate dehydrogenase	433 ± 219	320 ± 58	159 ± 43	56 ± 12	4
β-Glucosidase	256 ± 66	259 ± 70	106 ± 17	82 ± 9	4
Acid phosphatase	14.7 ± 2.6	13.8 ± 3.3	4.0 ± 2.1	76 ± 11	4

Fig. 2. Electron micrographs of the four fractions obtained from a flotation-sedimentation gradient centrifugation. The sample introduced in the gradient was the particulate of the osmotically shocked crude mitochondrial fraction; f1, Fraction 1 collected from the interphase 10–28.5 % (w/v) sucrose; f2, Fraction 2 from the interphase 28.5–34 % (w/v) sucrose; f3, Fraction 3 from the interphase 34–41 % (w/v) sucrose; f4, Fraction 4 the pellet. The bar indicates 1 μm. Magnification is 18 800 × for all four pictures.

TABLE II

ENRICHMENT OVER THE HOMOGENATE OF MARKERS IN THE SUBFRACTIONS

Enrichment over the homogenate or relative specific activity is expressed as the ratio of the specific activity of a given fraction and that of the homogenate. Fractions are indicated as in Fig. 1. These data are calculated from the average values as given in Table I.

Assay	Fractions						
	P_2 (H_2O)	f1	f2	f3	f4	Microsomes	Soluble proteins
ATPase	2.0	0.2	2.1	2.5	2.9		
($Na^+ + K^+$)-ATPase ouabain-sensitive	1.6	0.4	4.5	1.8	1.0		
Acetylcholinesterase	1.0	0.6	2.0	1.7	0.5	1.5	
5′-Nucleotidase	1.0	0.4	1.2	1.4	0.3		
Mitochondrial ATPase	2.6	0.2	0.7	3.9	17.9		
NADH: cytochrome *c* reductase rotenone-sensitive	2.9	0.4	1.0	5.0	17.3		
Monoamine oxidase	1.8	0.3	1.2	3.5	4.6		
NADH: cytochrome *c* reductase rotenone-insensitive	1.6	0.4	1.0	1.5	1.7	1.5	
Glucose-6-phosphatase	1.7	0.7	1.3	2.0	1.2	0.9	
NADPH: cytochrome *c* reductase	1.6	0.3	1.0	1.2	0.9	3.6	
Ribonucleic acid	0.7	0.4	0.6	0.8	0.8	3.0	
2′,3′-cyclic AMP 3′-phosphohydrolase	1.8	12.7	1.1	0.7	0.2		
Lactate dehydrogenase	0.7	0.4	0.8	0.6	0.3	1.1	1.3
β-Glucosidase	1.2	0.7	1.6	1.6	0.6		
Acid phosphatase	1.0	0.4	1.3	1.2	0.4		

presented in Table I. The results will be presented and discussed mainly in terms of enrichments because the preferential localization of a given marker can be rapidly deduced from that way of representation.

Three plasma membrane marker enzymes were enriched over the homogenate in Fraction 2 (Table II). Approximately 64 % of the recovered activity of the ouabain-

TABLE III

A SURVEY OF SYNAPTIC PLASMA MEMBRANE PREPARATIONS FROM RECENT LITERATURE

Yield in mg protein/mg wet wt. original tissue. Enrichment factors for three (neuronal) plasma membrane marker enzymes are compared. ND, not determined.

Source [reference]	Enrichment over homogenate			Yield (mg/g wet wt.)
	($Na^+ + K^+$)-ATPase ouabain-sensitive	Acetyl-cholinesterase	5′-Nucleotidase	
Rat forebrain [5]	2.5–3.5	1.36	1.24	1–2
Rat brain [4]	9–14	0.5–1.0	1–4	0.1–0.2
Rat brain [6]	7.6	ND	ND	5
Rat brain cortices [7]	8–10	ND	ND	0.25
Rat forebrain [1]	1.85	1.2	ND	1
Chick brain (present work)	4.5	2.0	1.2	3.3

TABLE IV

CONTAMINATION OF VARIOUS SUBCELLULAR ELEMENTS IN THE SYNAPTIC PLASMA MEMBRANES

The average specific activities are expressed in nmol/min per mg protein. The organelle fraction of reference is indicated in Materials and Methods. Percentage contamination is calculated as the ratio of specific activity of Fraction 2 and that of the reference fraction.

Marker	Organelle	Specific activity		Contamination (%)
		Organelle	Fraction 2	
Mitochondrial ATPase	Inner membrane mitochondrion*	2420	100	1.1
NADH: cytochrome *c* reductase rotenone-sensitive	Inner membrane mitochondrion*	285	16.4	1.6
Monoamine oxidase	Outer membrane mitochondrion**	7.7	2.09	2.2
NADH: cytochrome *c* reductase rotenone-insensitive	Outer membrane mitochondrion** and microsomes	27.8	16.2	4.6
NADPH: cytochrome *c* reductase	Microsomes	5.30	1.52	28.7
Ribonucleic acid	Microsomes	98	22.2	22.6
Glucose-6-phosphatase	Microsomes	3.16	4.61	146
2′,3′-cyclic AMP 3′-phosphohydrolase	Myelin and glial membranes	10.1	0.90	8.9
Lactate dehydrogenase	Soluble proteins	702	433	61.7
Acid phosphatase	Lysosomes	38.8	14.7	38

* Correction for the mitochondrial outer membrane, 0.08 ×.

** Correction for the mitochondrial inner membrane, 0.27 ×.

sensitive $(Na^+ + K^+)$-activated ATPase was present in Fraction 2, this being 16 % of the activity of the original homogenate. Of the total ATPase activity, 24 % was accounted for by the $(Na^+ + K^+)$-activated enzyme in Fraction 2 compared to 10.5 % in the homogenate. For both homogenate and Fraction 2 the Mg^{2+}-ATPase amounted to approx. 70 % while the remainder consisted of the mitochondrial ATPase. Acetylcholinesterase was enriched 2.0-fold in Fraction 2 corresponding to 37 % of the recovered activity from the gradient. Finally 5'-nucleotidase was only slightly enriched, 1.2 times, in Fraction 2, while fraction 3 gained the highest enrichment of 1.4, corresponding to 49 % of the recovered activity from the gradient. It is relevant to compare these results for Fraction 2 with some recent preparations of synaptic plasma membranes from rat brain (Table III). Morgan et al. [4] obtained an enrichment for the $(Na^+ + K^+)$-ATPase of about 12-fold, but one has to consider that their isolation procedure is rather elaborate and gives a very low yield. The enrichment we found was 4.5-fold, which is in accordance with the results of Cotman and Matthews [5] and Gurd et al. [7]. The enrichment of 2.0 for acetylcholinesterase was slightly higher than that reported for the rat brain preparations, whereas the poor enrichment for 5'-nucleotidase was confirmed by some authors [5], but varied considerably for rat brain synaptic plasma membranes.

In addition to a positive characterization of Fraction 2 for neuronal plasma membranes, characteristic markers have been determined for various subcellular organelles that could presumably contaminate this fraction, i.e. a negative characterization. The marker enzymes for the mitochondrial inner membrane, mitochondrial ATPase and rotenone-sensitive NADH : cytochrome *c* reductase, were highly enriched in Fraction 4 (Table II). The mitochondrial outer membrane markers, monoamine oxidase and rotenone-insensitive NADH : cytochrome *c* reductase, also showed the highest specific activities and enrichments in this fraction. When Fraction 4 is taken as a reference fraction for mitochondria, a percentage contamination of only 1.1–1.6 % was observed for both inner membrane markers, whereas the outer mitochondrial membrane marker monoamine oxidase contaminated Fraction 2 with 2.2 % (Table IV). The rotenone-insensitive NADH : cytochrome *c* reductase, albeit occurring also in microsomes [27], showed a contamination of 4.6 %. For these calculations correction has been made for the assumption that the outer membrane consists of 8 % of total mitochondrial protein, and the inner membrane 27 % [28].

The microsomal contamination of Fraction 2 was assessed by ribonucleic acid and NADPH : cytochrome *c* reductase; both markers amounted to approx. 25 % with reference to isolated microsomes. An additional putative microsomal marker, glucose-6-phosphatase, was even more enriched in Fraction 2 than in microsomes (see Discussion). The myelin marker, 2', 3'-cyclic AMP 3'-phosphohydrolase, was highly enriched in Fraction 1. This fraction was used as a reference for myelin without further purification, giving a myelin contamination of approx. 9 % for Fraction 2. Lactate dehydrogenase, a marker for occluded cytoplasm [29], was present to a small extent in Fraction 2, suggesting the adherence of soluble material from the cytoplasm of the synaptosomes to the synaptic plasma membranes. The lysosomal enzymes acid phosphatase and β-glucosidase showed rather high specific activities in Fraction 2. When a purified mitochondrial fraction from a Ficoll sucrose gradient as described previously [11] was used as a reference, a maximal contamination of 38 % was calculated for acid phosphatase. One has to notice that the latter fraction con-

sisted primarily of mitochondria, suggesting that the real contamination for lysosomal material would be much lower indeed.

DISCUSSION

Most of the recent preparations of synaptic plasma membranes described in the literature are derived from adult rat brain [1, 4–7]. To our knowledge, no characterization to any substantial extent of synaptic plasma membranes from avian brain has been reported. In this study we used brains of 6-day-old chicks of which the state of development in morphological, physiological [30] and biochemical [31, 32] respect is nearly mature. The membranes from chick brain appeared to be less dense than those from adult rat brain since the major portion of the mitochondrial markers floated when we used exactly the same sucrose concentrations as Jones and Matus [1]. Lowering the sucrose concentration of the gradient solutions by changing simply % w/w to % w/v prevented flotation of mitochondria. In addition, we applied a few more modifications, a 41 % (w/v) sucrose solution was introduced on the bottom of the tube in order to obtain better purified mitochondria in the pellet (f4). Also, the time of centrifugation was reduced to 30 min because longer runs did not improve the specific activities of the plasma membrane marker enzymes in Fraction 2 (not shown). The entire procedure as scheduled in Fig. 1 can be completed within a normal working day (8 h).

Synaptic plasma membranes are specialized parts of neuronal plasma membranes, just like somatic, axonal and dendritic membranes. To date, no specific (bio)-chemical markers for either of these membranes are available [33]. Since the ($Na^+ + K^+$)-ATPase represents an essential part of, if not the entire mechanism for transporting Na^+ and K^+ across cell membranes [34], this enzyme is widely used as a synaptic plasma membrane marker enzyme. However, it has also been demonstrated in glial cell membranes [35]. Some authors have made rough calculations on the amount of neuronal plasma membrane protein in the homogenate. Morgan et al. [4] calculated a maximal content of 8 mg protein/g in rat brain cortex, which means that a specific neuronal plasma membrane marker could be enriched maximally about 12-fold. Gurd et al. [7] calculated for synaptic plasma membranes, from the same source, a protein yield of 0.5 mg/g original tissue. The yield of 3.3 mg per g brain in Fraction 2 would appear rather high (cf. Table III), but one has to consider that in preliminary experiments application of prepurified synaptosomes into the gradient yielded 4- to 5-fold less protein in Fraction 2. In our membrane preparation, ($Na^+ + K^+$)-ATPase was enriched 4.5-fold, which is an intermediate value in comparison to the results for similar rat brain preparations (Table III). Acetylcholinesterase has been used as a marker for cholinergic synaptic plasma membranes [36]. The observed enrichment of 2.0-fold was in agreement with the findings of Sun et al. [37] for a similar fraction from squirrel monkey brain, whereas the data on rat brain preparations were lower (Table III). However, the data for acetylcholinesterase must be interpreted with caution because of its ubiquitous occurrence at the subneuronal level as has been demonstrated from ultrastructural and histochemical analyses [38]. The general plasma membrane marker 5'-nucleotidase [39] was only slightly enriched in Fraction 2. Similar rat brain preparations showed variable enrichments (Table III). It is notable that Fraction 3, containing mainly synaptic ghosts and mitochondria, in the electron

micrographs, showed the highest enrichment for this enzyme, suggesting the existence of a denser membrane population richer in 5'-nucleotidase and with buoyant densities equal to the lighter mitochondria. Anyway, this enzyme has also been demonstrated in glial membranes [40].

The amount of contamination with glial membranes is difficult to estimate because specific markers are lacking [33], while both $(Na^+ + K^+)$-ATPase and 5'-nucleotidase occur in glial membranes. Besides, Cotman et al. [41] and Henn et al. [42] have shown that the membranes of cultured glial cells possess essentially the same sedimentation properties on sucrose gradients as synaptosomes. So, glial membranes might float together with the synaptic ghosts to Fraction 2 in the flotation-sedimentation gradient we used. The lack of specificity of assayed neuronal plasma membrane markers is a serious drawback to the evaluation of the membrane purity. In any case, the portion of other neuronal (i.e. somatic, axonal and dendritic) membranes cannot possibly be assessed. Also the glial membrane contamination cannot be validated.

As the crude mitochondrial fraction contains predominantly mitochondria, synaptosomes and myelin, and presumably small amounts of lysosomes, microsomal aggregates and absorbed soluble protein, markers for these cell organelles have also been determined both in the synaptic plasma membrane fraction and in the appropriate reference fractions in order to assess the amount of contamination (Table IV). The mitochondrial contamination in Fraction 2 was 1.1–1.6 % as assessed by the inner membrane markers, mitochondrial ATPase and rotenone-sensitive NADH : cytochrome *c* reductase, and 2.2–4.6 % as measured by the outer membrane markers, monoamine oxidase and rotenone-insensitive NADH : cytochrome *c* reductase. This was relatively low compared to recent synaptic plasma membrane preparations [4–7]. During the preparation of the crude mitochondrial fraction fair attention has been paid to the removal of microsomes. Still, a considerable amount, i.e. approx. 25 %, of microsomal contamination measured as ribonucleic acid and NADPH : cytochrome *c* reductase was apparent in Fraction 2 in comparison to isolated microsomes. The use of ribonucleic acid as a marker for the rough endoplasmic reticulum is limited since small amounts are present within mitochondria [43]. Moreover, some authors have reported a persistent portion of ribonucleic acid in synaptic plasma membrane preparations [7]. Cotman et al. [44] measured specific activities of microsomal enzymes in their synaptic plasma membrane preparation at one third of those of microsomes. Miller and Dawson [45] demonstrated the presence of ribonucleic acid and NADPH : cytochrome *c* reductase within synaptosomes. Hereby, we have to take into account that the microsomal fraction from brain is a rather heterogeneous population of fragments of the endoplasmic reticulum, small nerve endings, small axons and fragmented plasma membranes, both neuronal and glial [7]. Furthermore, it has been demonstrated that the protein profile for microsomes in sodium dodecyl sulphate-polyacrylamide gel electrophoresis is essentially identical to that of synaptic plasma membranes [7]. Glucose-6-phosphatase, though widely used as a microsomal marker, was found not to be a satisfactory marker for chick brain microsomes because Fractions 2 and 3 contained even higher specific activities than isolated microsomes. This observation is in agreement with the findings of Miller and Dawson [45] for rat brain synaptosomes.

The myelin contamination has been assessed by 2',3'-cyclic AMP 3'-phosphohydrolase and amounted to approx. 9 %. Recently some doubt has been raised about

the exclusive localization of this enzyme in myelin [4], because it has been detected in considerable amounts in glial membranes [46] as well as in isolated axolemma [47]. These observations are in agreement with the postulation that myelin might be derived from glial membranes [48]. The enzyme activity in Fraction 2 was about 11 % of Fraction 1, and might be derived from myelin, axonal membranes or glial membranes. Although the electron micrographs of Fraction 2 did not show multilamellar membrane sheaths, myelin might be present as single membranes.

Lysosomes are sedimenting in the crude mitochondrial fraction [49]. Since we used this fraction as starting material for the isolation of synaptic plasma membranes, the broken lysosomes or lysosomal membranes having about the same density as synaptic membranes [49] might emerge in Fraction 2. Cotman and Matthews [5] reported considerable amounts of lysosomal activities in their synaptic plasma membrane preparation. They could wash out nearly all activities with 0.1 % Triton X-100, a condition leaving the membranes unaffected and they concluded that these enzymes were not tightly integrated in the membranes. Gurd et al. [7] also demonstrated heavy lysosomal activities in their synaptic plasma membrane preparation. In addition, Verity et al. [50] reported a persistent portion of acid phosphatase, though assayed with a different substrate, in the synaptic plasma membrane fraction. These authors concluded that this activity might be intrinsic for the membranes involved.

The identification of Fraction 2 as the synaptic plasma membrane fraction was greatly hampered because of the absence of recognizable postsynaptic attachments in the electron micrographs. Morgan et al. [4] reported similar observations and as we used exactly the same homogenization medium, it is indicative that the EDTA present in the medium detached the attachments by removal of Ca^{2+}, which is unfavourable for the structural integrity of the synaptic cleft region [51]. EDTA was mainly added because of its stimulation by the same action of the $(Na^+ + K^+)$-ATPase. Nevertheless, the pictures of Fraction 2 were dominated by ghost-like profiles of about 0.6 μm in diameter and because this fraction was considerably enriched in putative neuronal plasma membrane marker enzymes, we tentatively designated this Fraction 2 as the synaptic plasma membrane fraction. The extent of contamination of this preparation is hard to establish, although upper limits for some subcellular elements are presented in Table IV. Morgan et al. [4] calculated the purity of their synaptic plasma membrane preparation to 85–90 %; however, recently they have re-assessed these figures to 50–75 % [52]. It must be stressed that the isolation procedures for synaptic plasma membranes, all being based on differential and density gradient centrifugation techniques, appear to be incapable of yielding pure synaptic plasma membrane preparations from brain tissue. Apparently, it seems to be easier to obtain over 90 % purity for different subsynaptosomal structures, like synaptic vesicles [53], synapticjunctional complexes [54] and postsynaptic densities [55].

To summarise, these studies demonstrate the preparation of an enriched synaptic plasma membrane fraction from chick brain, showing various morphological and enzymatical properties similar to such preparations obtained from adult rat brain [1, 4–7].

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