Biochimica et Biophysica Acta, 433 (1976) 75–89

BBA 77264

PREPARATION OF CHICK BRAIN SYNAPTOSOMES AND SYNAPTOSOMAL MEMBRANES

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(Received October 8th, 1975)

SUMMARY

A method is described for the preparation of synaptosomes and synaptosomal membranes from chicken brain. Procedures for isolating rat synaptosomal membranes could not be used directly; several modifications of existing procedures are reported. Purity of the subcellular and subsynaptosomal fractions was monitored by electron microscopy and measurements of ferrocytochrome *c*: oxygen oxidoreductase (EC 1.9.3.1), monoamine: oxygen oxidoreductase (deaminating) (EC 1.4.3.4), rotenone-insensitive NADH: cytochrome *c* oxidoreductase (EC 1.6.99.3), NADPH: cytochrome *c* oxidoreductase (EC 1.6.99.1), orthophosphoric monoester phosphohydrolase (EC 3.1.3.2), ATP phosphohydrolase (EC 3.6.1.4), and levels of RNA. Microsomes are the main contaminant of the synaptosomal membrane fraction. Mitochondrial and lysosomal enzymes occur in lesser amounts. No myelin contamination was observed. Marker enzymes for contaminants suggest that these synaptosomal membranes are as pure as membranes described by others, and the specific activity of a neuronal membrane marker, (Na^+-K^+)-activated ATPase, is as high as other preparations. Levels of this enzyme in the membrane fraction are enriched 13-fold over homogenate ATPase levels.

INTRODUCTION

Synaptosomes are a fortuitous artifact created by the pinching-off and self-sealing of the synaptic contact between two nerve cells during homogenization of brain tissue [1, 2]. Until recently they were the only fraction of completely neuronal origin which could be isolated in reasonable yield and with high purity. Since they are created from the axon terminals they presumably contain much of the machinery necessary for transmitting signals received from the neuronal cell body. For this reason they have been utilized in a multitude of studies designed to illuminate the mechanism of that process [3].

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Neurochemists have expended much effort to isolate purified synaptosomes and synaptosomal subfractions. The work of several groups has recently been rewarded with highly purified rat synaptosomal membranes [4–6]. However, substantial interspecies differences in the properties of these subcellular particles, as Cotman [7] has noted, prevent the direct utilization of the rat procedure on other animals.

If one goal of such preparations is the elucidation of chemical mechanisms underlying behavior, then synaptic membranes from useful behavioral models should be examined. It is questionable whether the laboratory rat is the most useful animal for such studies. Experiments analyzing the chemical basis of visual behavior of the chicken may prove to be of equal or greater utility [8]. This is because half of a chicken brain can effectively serve as control material during manipulations of the other half. The optic lobes and cerebral hemispheres are anatomically quite distinct, paired regions are symmetrical, and there is no corpus callosum. This lack of communication between halves of the chicken brain causes partial restriction of the effects of monocular visual modification to the contralateral optic lobe and cerebral hemisphere (after one or more synapses). Also, large groups of these birds can be readily maintained with relatively small intragroup variation. Thus, small differences between control and experimental brain halves may be observed. Several groups have recently described chemical changes in chick brains accompanying imprinting [9, 10].

For these reasons it would be useful to have available a procedure for the preparation of chick brain synaptosomes and synaptosomal membranes. While two reports of the preparation of chick brain synaptosomal membranes have appeared [11, 12] they were not accompanied by the thorough enzymatic and optical characterization which marked the description of some rat membrane preparations. Another recent paper has thoroughly described several fractions enriched in chick synaptosomes but not synaptosomal membranes [13].

In this paper we describe the preparation and partial characterization of highly purified chick brain synaptosomes and synaptosomal membranes. This is the first chick synaptosomal membrane preparation, at this writing, which has been characterized by enzymatic, chemical and electron microscopic data.

EXPERIMENTAL PROCEDURE

Materials

Newborn male White Leghorn Chicks were purchased from the Stillwater Hatchery, Stillwater, Oklahoma and were maintained on Purina Chick Startena until use (usually at 6–8 days of age).

NADH, NADPH, rotenone, ouabain, *p*-nitrophenyl-phosphate, ATP, and cytochrome *c* (Type III) were all obtained from Sigma Chemical Company. *m*-iodobenzylamine hydrochloride was from the Aldrich Chemical Co. and Elon was from the Eastman Kodak Co. Ficoll 400 was obtained from Pharmacia. All other chemicals were reagent grade. Water was twice deionized and then glass distilled.

Preparation of synaptosomes

Synaptosomes were prepared by modification of several existing procedures. All centrifugation times cited included acceleration but not deceleration and all steps

were carried out at 0–4 °C. 50 6–8 day old chicks were decapitated for each preparation and cerebral hemispheres and optic lobes were pooled (approx. 40 g total wet wt.). A 20 % (W/V) homogenate in medium P (0.32 M sucrose, 1 mM K_2HPO_4, 0.1 mM EDTA, pH 7.5) was prepared using 10 up-and-down strokes in a Thomas homogenizer with a motor-driven (300 rev./min) Teflon pestle. The homogenate fraction was diluted to 10 % (W/V) and centrifuged in a Sorvall RC2-B centrifuge for 5 min at $1100 \times g_{av}$ (3000 rev./min). Pellets were resuspended in medium P and recentrifuged and the supernatant thus obtained was combined with the original supernatant while the nuclear pellets were discarded. The combined supernatants were again centrifuged at $1100 \times g_{av}$ for 5 min, the pellet was discarded and the supernatant was centrifuged at $17\,300 \times g_{av}$ for 17 min to prepare the crude mitochondrial pellet. The resulting supernatant was centrifuged for 75 min at $96\,300 \times g_{av}$ (27 000 rev./min) in an SW27 rotor to prepare crude microsomes (microsomal fraction) while the pellets were washed three times by resuspending in medium P (1/3 vol. of the original homogenate) and recentrifuging. The washed crude mitochondrial pellets (crude mitochondrial fraction) were resuspended in medium P (2.5–3.0 ml/g original tissue) and 8.5 ml per gradient was centrifuged on gradients of 15 ml 14 % Ficoll 400 ($\eta_D^{20} = 1.3720$) and 15 ml 7 % Ficoll 400 ($\eta_D^{20} = 1.3607$) for 60 min at $68\,000 \times g_{av}$ (22 500 rev./min) in an SW27 rotor. To avoid packing of material at the suspension-Ficoll interface, the sample was applied to the gradient rapidly enough to mix the topmost 1 cm of Ficoll with sample. The crude myelin and synaptosomal bands were diluted with 3–4 vol. medium P and centrifuged for 30 min at $20\,000 \times g_{av}$ in the Sorvall RCB-2 centrifuge to prepare myelin and synaptosomal fractions respectively. Pellets from the Ficoll gradients were mitochondrial fraction.

The synaptosomal pellets were resuspended in medium P (2 ml/g original tissue) and recentrifuged (17 min at $17\,000 \times g_{av}$) to remove traces of Ficoll.

Preparation of synaptosomal membranes

The washed synaptosomal pellets were suspended in medium L (1 mM K_2HPO_4, 0.1 mM EDTA, pH 8.0) to 4 ml/g original tissue by homogenizing with 4 up-and-down strokes in a hand-held Thomas homogenizer. After 15 min the suspension was rehomogenized and the pH was readjusted to 8.0. The suspension was then allowed to stand in ice an additional 45 min. The resulting suspension was layered over 5 ml 1.0 M sucrose in medium L and centrifuged for 30 min at $96\,300 \times g_{av}$ in an SW27 rotor. The low speed synaptosomal pellets were discarded. All other material was mixed to homogeneity and centrifuged 14 h at $25\,000 \times g_{av}$ in the SW27 rotor. The resulting high speed synaptosomal pellets were suspended by hand homogenization in medium L (0.5–1.0 ml/g original tissue) and 6 ml were applied to each gradient of 7 ml 1.1 M sucrose, 7 ml 0.95 M, 7 ml 0.8 M, 6 ml 0.6 M, and 6 ml 0.4 M sucrose. Gradients were spun for 90 min at $68\,000 \times g_{av}$ (22 500 rev./min) in an SW27 rotor. The purest synaptosomal membrane fraction banded at the 0.8/0.95 M sucrose interface. Bands were designated by the interface at which they occur e.g., 0.4/0.6, etc. They were removed with Pasteur pipettes, diluted with 0.25 volume of medium L and centrifuged for 45 min at $106\,500 \times g_{av}$ (40 000 rev./min) in Ti50 rotor.

All pellets were suspended in medium V (50 mM histidine, 0.1 mM EDTA, 100 mM kCl, 56 mM NaCl, pH 7.5) for analyses.

Enzyme assays

Cytochrome oxidase (EC 1.9.3.1) was determined by the method of Wharton and Tzagaloff [14]. Monoamine oxidase (EC 1.4.3.4) was assayed by a modification of the procedure of Zeller, et al. [15]. 0.3 ml of suspension was diluted with 0.3 ml of 0.067 M phosphate buffer, pH 7.2 to which 50 μl/ml Triton X-100 had been added. Phosphate buffer and 0.72 mM *m*-iodobenzylamine in phosphate buffer were oxygenated for at least 10 min at room temperature immediately before use. 2.0 ml of the test and blank solutions were added to stoppered matched cuvettes in a Beckman DB-G spectrophotometer and allowed to temperature equilibrate (to 33 °C). Then 0.1 ml of suspension was added to each cuvette. Readings (at 253 nm) were begun after one min. Only *m*-iodobenzylamine was omitted from the blank. Rotenone-insensitive NADH: Cytochrome *c* oxidoreductase (EC 1.6.99.3) was assayed by the procedure of Morgan et al. [5], NADPH: Cytochrome *c* oxidoreductase (EC 1.6.99.3) was assayed by an identical procedure except that NADPH was the substrate and rotenone was omitted from the incubation medium. Oubain-sensitive (Na^+-K^+)-activated ATPase (EC 3.6.1.4) was assayed by a method combined from those of Whittaker and Barker [3], Kimelberg and Papahadjopoulis [16], and Cotman et al. [17]. 67 μl enzyme solution was added to each of 2 test tubes containing 1.2 ml of either 100 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM ouabain, 50 mM Tris, pH 7.5 or the same buffer without ouabain. After a 5 min preincubation at 37 °C the reaction was started by adding 67 μl of 60 mM ATP in buffer to each tube to give a final ATP concentration of 3 mM. 10 min later the reaction was stopped by the addition of 0.33 ml 22.5 % (W/V) trichloroacetic acid. After mixing, the suspensions were allowed to stand on ice for 15 min before removing the denatured protein by centrifugation for 5 min at $2000 \times g_{av}$. 0.4 ml of supernatant was added to 2.1 ml of color developer (1.85 ml of a mixture of 0.3 g $NaHSO_4$ and 1.1 g Elon/100 ml water plus 0.25 ml 5 % (w/v) ammonium molybdate in 1.25 N H_2SO_4). Color developer was prepared immediately before use. After a 10 min wait tubes were read within 20 min at 650 nm. *p*-Nitrophenyl phosphate acid phosphatase (EC 3.1.3.2) was assayed by the procedure of Cotman and Matthews [4]. All enzymes were assayed under conditions of linearity with enzyme concentration. All samples were kept frozen at −65 °C until assay (usually within two days of preparation).

Chemical assays

Protein was assayed by the Lowry method as described by Bruening et al. [18]. RNA was assayed by the method of Fleck and Begg [19] using a Cary Model 15 recording spectrophotometer.

Electron microscopy

Fractions were pelleted at 106 500 $\times g$ for 30 min and fixed in 2.5 % (V/V) glutaraldehyde in Caulfield's veronal acetate buffer (0.34 osM, pH 7.4). Pellets were post-fixed for 1 h in 1 % osmium tetroxide in the same buffer, rinsed and left 8–10 h in 0.5 % (W/V) uranyl acetate in Kellenberger's buffer (pH 5.1, adjusted to 0.34 osM with NaCl). Pellets were then rinsed with water, dehydrated with graded ethanol and embedded in the medium of Spurr. Blocks were trimmed so that the ultrathin (60–90 nm) sections displayed cross-sections of the pellets. Grids were viewed in a Phillips EM 300 electron microscope.

TABLE I

THE YIELD OF PROTEIN, μg RNA/mg PROTEIN, AND SPECIFIC ACTIVITIES OF SIX MARKER ENZYMES IN CHICK BRAIN PRIMARY SUBCELLULAR FRACTIONS

The fractionation scheme and fraction nomenclature are described in the Methods section. Each value is the mean of 4–5 experiments ±S.E.M. Specific activities are all expressed as μmol substrate consumed or product produced/h per mg protein.

Fraction	Protein yield mg/g wet wt.	μg RNA mg protein	Acid phosphatase	Cytochrome oxidase	Monoamine oxidase	Rotenone-insensitive NADH: cytochrome *c* reductase	NADPH: cytochrome *c* reductase	(Na^+-K^+)-activated ATPase
Homogenate	80.3±5.6	18±1	2.5±0.1	45±7	0.3±0.0	7.2±1.1	2.1±0.2	10.8±0.6
Microsomal	8.4±1.2	27±2	2.3±0.3	12±1	0.2±0.1	12.3±2.0	1.4±0.3	6.5±1.0
Crude mitochondrial	12.5±1.2	8±2	2.3±0.2	132±8	1.2±0.3	13.9±1.4	2.0±0.3	24.9±7.7
Myelin	4.5±0.6	6±1	1.7±0.2	44±12	0.5±0.2	7.1±1.2	2.1±0.3	25.2±5.0
Synaptosomal	2.5±0.3	6±1	2.0±0.1	146±19	1.0±0.2	21.3±2.0	2.6±0.1	21.7±1.9
Mitochondrial	1.6±0.1	8±1	2.6±0.2	251±50	2.3±0.5	21.6±1.7	1.8±0.1	3.4±1.2

RESULTS

Characterization of the primary fractions

Protein and RNA contents and specific activities of six enzymes were used to characterize the subcellular fractions. These data appear in Tables I and II. The yield of protein in our crude mitochondrial fraction was lower than the analogous "P2" fraction obtained by Oestreicher and van Leeuwen [13]. This is due, at least in part, to our having washed this crude mitochondrial fraction three times as suggested by Morgan et al. [5] and by Gurd et al. [6]. Oestreicher and van Leeuwen washed their crude mitochondrial fraction once. However, our yield of synaptosomes is comparable to that of Gurd et al. [6], who obtained fractions from month-old rats.

The RNA values (Table I) are in substantial agreement with those of Gurd et al. [6] but our microsomal RNA data are lower than the value reported by Morgan, et al. [5]. Though it is not entirely clear how their microsomes were prepared our microsomal fraction probably also contained myelin which may explain some of the difference.

Acid phosphatase activity is distributed throughout the Ficoll gradient again in agreement with the data of Gurd et al. [6] and of Cotman and Matthews [4]. This may represent both intact lysosomes and lysosomal enzymes adsorbed onto other membranes. Support for that idea comes from the fairly uniform distribution of acid phosphatase in the Ficoll gradient fractions (Table II). Since most brain lysosomes are fairly small and dense [20], intact lysosomes should concentrate in the pellet. Further support comes from the relative paucity of intact lysosomes seen in electron micrographs of the synaptosomal fraction (Fig. 1).

Cytochrome oxidase is a marker for the inner mitochondrial membrane [21]. Monoamine oxidase is a marker for the outer mitochondrial membrane [22]. The distribution of these two enzymes during preparation of the synaptosomes is shown in Table I. The presence of these enzymes in the myelin fraction is probably due to at

TABLE II

RELATIVE SPECIFIC ACTIVITIES (% TOTAL ACTIVITY/% TOTAL PROTEIN) OF SIX MARKER ENZYMES IN CHICK BRAIN PRIMARY SUBCELLULAR FRACTIONS

The fractionation scheme and fraction nomenclature are described in the Methods section. Each value is the mean of 4–5 experiments ± S.E.M.

Fraction	Acid phosphatase	Cytochrome oxidase	Monoamine oxidase	Rotenone-insensitive NADH: cytochrome *c* reductase	NADPH: cytochrome *c* reductase	(Na^+-K^+)-activated ATPase
Homogenate	1.0	1.0	1.0	1.0	1.0	1.0
Microsomal	0.9 ± 0.1	0.3 ± 0.1	0.6 ± 0.2	1.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
Crude mitochondrial	0.9 ± 0.1	3.2 ± 0.4	3.4 ± 0.6	1.9 ± 0.1	0.9 ± 0.2	2.4 ± 0.8
Myelin	0.7 ± 0.1	1.0 ± 0.3	1.8 ± 0.7	0.9 ± 0.1	1.0 ± 0.2	2.4 ± 0.5
Synaptosomal	0.8 ± 0.0	3.4 ± 0.7	3.3 ± 0.5	2.8 ± 0.4	1.2 ± 0.1	2.0 ± 0.1
Mitochondrial	1.0 ± 0.1	5.1 ± 1.2	7.2 ± 1.7	2.9 ± 0.5	0.8 ± 0.1	0.3 ± 0.1

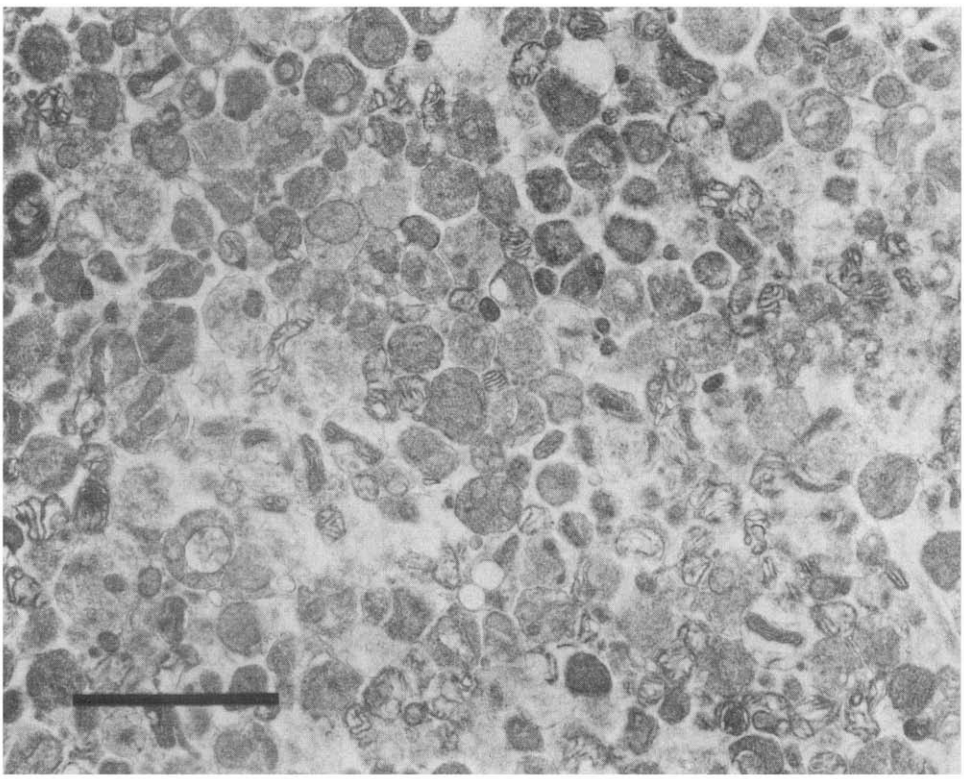

Fig. 1. Electron micrograph of material banding between 7% and 14% (Ficoll synaptosomal fraction). This material was prepared as described in the Methods section. This fraction consists primarily of synaptosomes. The major contaminant is free mitochondria. Small amounts of lysosomes and non-synaptic membrane-limited particles also occur. Myelin is not observed. The bar represents 1 μm.

least two causes. First, some small synaptosomes which contain mitochondria may not sediment into the synaptosomal fraction during the relatively short period of centrifugation. This period was chosen to minimize microsomal contamination of the synaptosomes. Second, some trapping of synaptosomes and mitochondria in the packed myelin band may have occurred.

Levels of NADPH: cytochrome *c* reductase and rotenone-insensitive NADH: cytochrome *c* reductase were also measured (Tables I and II). The distribution of NADPH: cytochrome *c* reductase in the Ficoll gradients is consistent with its designation as a synaptosomal [23] in addition to a microsomal [24] enzyme. Also, we found higher levels of this enzyme in the crude mitochondrial fraction than in the microsomal fraction. These results may, in part, be due to the occurrence of myelin in the microsomes and to the possibility that the gentle conditions we employed to homogenize the tissue may have allowed large fragments of endoplasmic reticulum to form relatively large microsomes. Similarly, this reason, combined with the fact that it is a marker for both microsomes and the outer mitochondrial membrane [25],

may explain the high levels of rotenone-insensitive NADH: cytochrome *c* reductase occurring in the synaptosomal fraction.

In agreement with previous studies, our data support the contention that (Na^+-K^+) ATPase is a marker enzyme for the neuronal membrane. The light levels of this enzyme in the myelin fraction (Table II) are probably due to the presence of pieces of neuronal membrane, synaptosomes and axons. The relatively low level of ATPase in the mitochondrial fraction suggests that few synaptosomes have left the synaptosomal fraction and sedimented into the pellet.

In general, the present data on the relative specific activities of various marker enzymes (Table II) are similar to those reported by most other workers. Levels of RNA (Table I) and acid phosphatase are somewhat lower in the synaptosomal fraction indicating decreases in the amounts of rough endoplasmic reticulum and lysosomes. The relative specific activities of a neuronal membrane marker (ATPase) [5] a mitochondrial inner membrane marker (cytochrome oxidase) [22] a mitochondrial outer membrane marker (monoamine oxidase) [23] as well as other markers for endoplasmic reticulum and mitochondria (NADPH: cytochrome *c* reductase and rotenone insensitive NADH: cytochrome *c* reductase) [6] were all higher in the synaptosomal fraction than in the brain homogenate. Except for NADPH: Cyto-

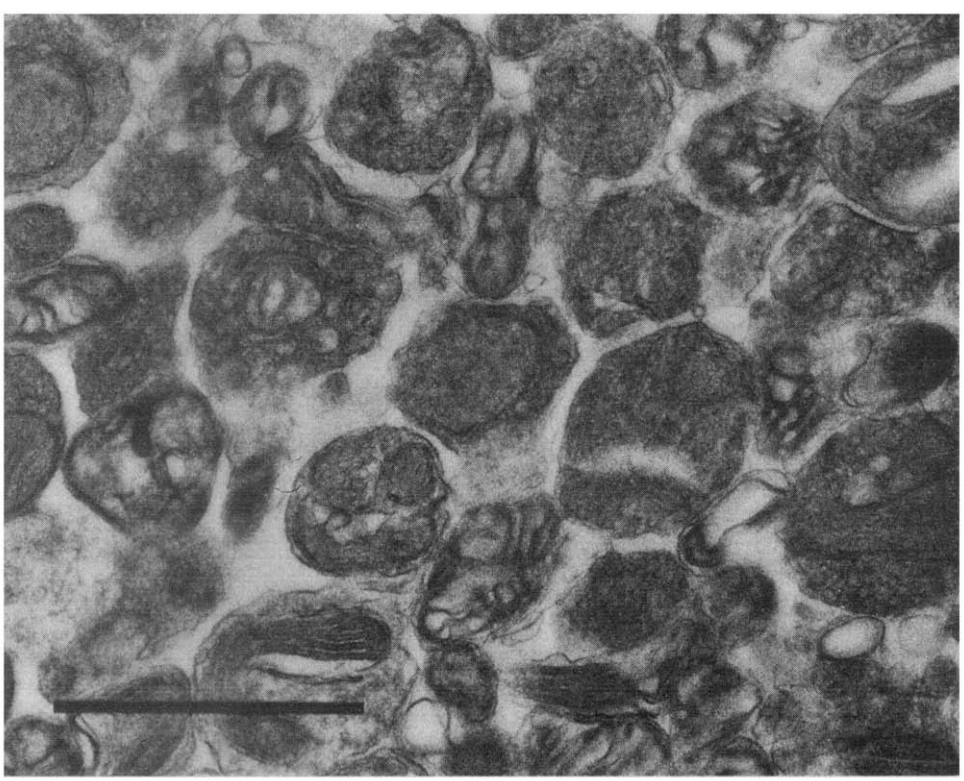

Fig. 2. Higher magnification picture of the synaptosomal fraction. Synaptosomes appear intact but adhering post-synaptic material was rarely seen. The bar represents 0.5 μm.

chrome *c* reductase our data support the utilization of these enzymes as markers for the fractions to which they have been assigned.

However, when the specific activities of our subcellular fractions were compared with those of other workers some differences were noted. The specific activities of ATPase we observed are similar to those reported by Gurd et al. [6] but they are 2–3-fold higher than values reported by Morgan et al. [5]. Also, our monoamine oxidase values are higher than values reported both by Morgan et al., and Gurd et al. This difference may be due to our using different assays. The specific activity values we obtained are similar to those reported for mitochondria by the originators of the assay [15].

Electron microscopic examination of the synaptosomal fraction revealed a picture similar to that observed by others (Figs. 1 and 2). Three washes of the crude mitochondrial fraction do appear to have helped remove small particles and to have increased the fraction of particles which are synaptosomes to over 50 % as found by Gurd et al. [6]. No myelin was observed in the synaptosomal fraction.

Characterization of synaptosomal subfractions

We are interested in the structure of the synaptosomal membrane and we are apprehensive about using the mitochondrial weighting method of Cotman [7] because the several changes of temperature in that method might alter the structure of the membrane. However, when we tried the low speed (11 500 $\times g$ for 15 min) centrifugation of the lysed synaptosomes described by Morgan et al. [5], almost all of the membrane material was discarded in the pellet. Centrifugation at lower speeds or for shorter times did not result in substantial increases in the specific activity of (Na^+-K^+)-ATPase over that of unlysed synaptosomes (Babitch, unpublished). Thus, substantive changes from the procedures for isolating rat synaptosomal membranes were necessary for our purposes to make it applicable to the preparation of chick synaptosomal membranes. To achieve separation of synaptosomal membranes from most intrasynaptosomal or other mitochondria we utilized a one-step centrifugation gradient. 5 ml of 1.0 M sucrose with 1 mM K_2HPO_4 and 0.1 mM EDTA, pH 8.0 were put in SW27 tubes and after filling the tubes with lysed synaptosomes in medium L they were centrifuged for 30 min at 96 300 $\times g_{av}$ (27 000 rev./min). Tables III and IV show that the low speed synaptosomal pellet we obtained was greatly enriched in monoamine oxidase and cytochrome oxidase suggesting the presence of more highly purified mitochondria than occurs in the pellet of the Ficoll gradient (compare with the mitochondrial fraction values in Tables I and II). After overnight centrifugation of the supernatant from the gradient step a high speed synaptosomal pellet was obtained which had 3 times the specific activity of (Na^+-K^+)-ATPase of our unlysed synaptosomes and 6 times the ATPase activity of the low speed synaptosomal pellet. This centrifugation step had one drawback, however. The large difference in densities between 1.0 M sucrose and lysis medium in the step gradient combined with the high speed of centrifugation apparently put strain on the centrifuge tubes: they occasionally collapsed. We have largely remedied this situation by using polycarbonate centrifuge tubes for this step.

It was also found that the sucrose centrifugation gradient used to purify rat synaptosomal membranes was not entirely satisfactory for the preparation of chick synaptosomal membranes. When the sucrose gradient described by Morgan et al. [5]

TABLE III

THE YIELD OF PROTEIN, μg RNA/mg PROTEIN, AND SPECIFIC ACTIVITIES OF SIX MARKER ENZYMES IN CHICK BRAIN SYNAPTOSOMAL SUBFRACTIONS

The legend for this table is the same as for Table 1.

Fraction	Protein yield (mg/g wet wt.)	μg RNA / mg protein	Acid phosphatase	Cytochrome oxidase	Monoamine oxidase	Rotenone-insensitive NADH: cytochrome *c* reductase	NADPH: cytochrome *c* reductase	(Na^+-K^+)-activated ATPase
Low speed synaptosomal pellet	0.67 ± 0.13	5 ± 1	0.8 ± 0.1	607 ± 133	2.8 ± 0.5	30 ± 2	2.2 ± 0.4	11 ± 2
High speed synaptosomal pellet	0.49 ± 0.10	3 ± 1	3.8 ± 0.7	35 ± 11	0.4 ± 0.2	24 ± 2	3.0 ± 0.8	70 ± 15
0.4/0.6	0.03 ± 0.01	0 ± 0	1.6 ± 0.4	< 2	< 0.05	16 ± 2	3.1 ± 0.2	24 ± 3
0.6/0.8	0.02 ± 0.00	2 ± 1	2.4 ± 0.2	4 ± 3	0.2 ± 0.2	30 ± 2	3.1 ± 0.4	96 ± 22
0.8/0.95	0.05 ± 0.01	2 ± 1	3.0 ± 0.7	50 ± 10	0.1 ± 0.1	25 ± 5	2.9 ± 0.7	144 ± 30
0.95/1.1	0.04 ± 0.01	1 ± 1	3.0 ± 0.6	79 ± 20	0.5 ± 0.2	18 ± 2	4.1 ± 0.6	79 ± 14
Sucrose gradient pellet	0.03 ± 0.01	0 ± 0	0.9 ± 0.8	122 ± 16	0.7 ± 0.3	20 ± 5	6.3 ± 0.4	25 ± 8

TABLE IV

RELATIVE SPECIFIC ACTIVITIES (% TOTAL ACTIVITY/% TOTAL PROTEIN) OF SIX MARKER ENZYMES IN CHICK BRAIN SYNAPTOSOMAL SUBFRACTIONS

The legend for this table is the same as or Table II.

Fraction	Acid phosphatase	Cytochrome oxidase	Monoamine oxidase	Rotenone-insensitive NADH: cytochrome *c* reductase	NADPH: cytochrome *c* reductase	(Na^+-K^+)-activated ATPase
Low speed synaptosomal pellet	0.3±0.1	13.8±2.7	8.4±1.7	3.8±0.7	1.1±0.2	1.1 ±0.2
High speed synaptosomal pellet	1.5±0.3	1.3±0.3	1.0±0.4	4.3±0.5	1.6±0.4	6.5±1.2
0.4/0.6	0.7±0.2	0.0	0.0	2.3±0.5	1.5±0.2	2.2±0.3
0.6/0.8	1.0±0.1	0.1±0.1	0.1±0.1	4.6±0.7	1.6±0.3	8.6±1.6
0.8/0.95	1.2±0.2	1.1±0.3	0.4±0.4	3.6±0.7	1.4±0.3	13.3±2.3
0.95/1.1	1.2±0.2	1.1±0.2	1.5±0.6	1.8±0.3	1.8±0.7	7.5±1.2
Sucrose gradient pellet	0.4±0.1	1.3±0.1	2.0±0.8	3.6±1.8	1.6±0.5	2.4±0.8

was used to prepare chick membranes high levels of cytochrome oxidase, monoamine oxidase and NADPH: cytochrome *c* reductase were found in the fraction with the highest specific activity of (Na^+-K^+)-ATPase. If the 0.8 M sucrose step was changed to 0.7 M most of rotenone-insensitive NADH: cytochrome *c* reductase was found in the synaptosomal membrane fraction (Babitch, unpublished). However, by changing the 1.0 M step of the sucrose gradient to 0.95 M contamination of the synaptic membranes at the 0.8 M/0.95 M sucrose interface was substantially reduced (Tables III and IV) by allowing a substantial portion of the mitochondrial contamination to pass into the 0.95/1.1 fraction.

(Na^+-K^+)-ATPase is generally regarded as the most specific enzyme marker for the synaptosomal membrane [7, 26]. Judging by that criterion alone, the 0.8/0.95 fraction is as pure a preparation of synaptic membranes as any yet described.

Partially disrupted synaptosomes band below synaptosomal membranes [5, 6]. Thus, the sucrose gradient pellet may be composed primarily of broken synaptosomes. This idea is supported by the similar ATPase, monoamine oxidase, cytochrome oxidase, and rotenone-insensitive NADH: cytochrome *c* reductase activities of the synaptosomal (Table I) and Pellet (Table III) fractions. But NADPH: cytochrome *c* reductase levels were higher in the sucrose gradient pellet than in any other fraction (Table III). This fact combined with the finding of high levels in the synaptosomal fraction reinforces the suggestion [23] that NADPH: cytochrome *c* reductase may be a synaptosomal enzyme and prevents its utilization to quantitate microsomal contamination of the synaptosomal membranes. If NADPH: cytochrome *c* reductase cannot be used as a microsomal marker then it is difficult to accurately estimate microsomal contamination. RNA is unevenly distributed in brain microsomes [6]

and rotenone-insensitive NADH: cytochrome *c* reductase is a marker for both microsomes and mitochondrial outer membranes [27]. We concluded that most of the rotenone-insensitive NADH: cytochrome *c* reductase activity in the 0.8/0.95 fraction was due to microsomal contamination by assuming that all of the rotenone-insensitive NADH: cytochrome *c* reductase in the low speed synaptosomal pellet was due to mitochondrial outer membranes: this is reasonable since its levels of monoamine oxidase were very high. But monoamine oxidase levels in 0.8/0.95 were much lower than in the low speed synaptosomal pellet while rotenone-intsensitive NADH: cytochrome *c* reductase levels were similar in both fractions. So mitochondrial outer membranes were relatively rare in 0.8/0.95. Thus, most (>90 %) of the rotenone-insensitive NADH: cytochrome *c* reductase activity of the 0.8/0.95 fraction was due to microsomal contamination. That microsomes were the major contaminant of the synaptosomal membranes was supported by the finding that the relative specific activity of rotenone-insensitive NADH: cytochrome *c* reductase was highest of all other measured contaminating enzymes (Table IV). Still, other data (RNA and ATPase levels) suggest that microsomal contamination was no greater than observed by other workers [4, 6].

Though we have not yet examined the protein patterns of these fractions on polyacrylamide gels, the contamination of the synaptic membranes by endoplasmic

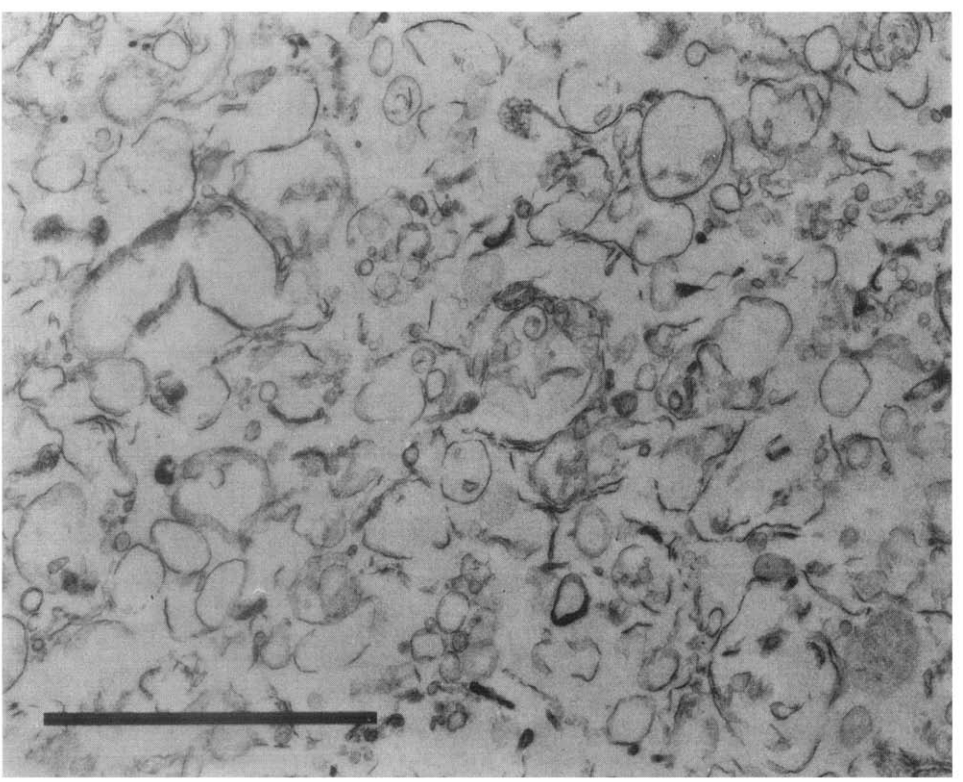

Fig. 3. Electron micrograph of material banding in the 0.8/0.95 fraction prepared as described in the Methods section. The bar represents 1 μm.

reticulum we observe suggests that we will see similarities between the microsomal and synaptic membrane patterns as have been reported by other investigators [6, 28].

Contamination of the synaptosomal membranes by other subcellular elements is probably less than by microsomes. If the low speed synaptosomal pellet is pure mitochondria, contamination of the synaptosomal membranes by mitochondria averages 8 % based on levels of cytochrome oxidase. Using monoamine oxidase as a basis results in a lower value (4 %). Lysosomes, too, do not seem a major contaminant. Though the specific activity of acid phosphatase is slightly higher in the 0.8/0.95 fraction than in the homogenate (Table III) purified preparations of lysosomes display up to 100-fold enrichments of marker enzymes [29]. Also, the synaptosomal membrane has been reported to contain intrinsic acid hydrolase activity [30].

Since we observed no myelin in the synaptosomal fraction (Figs 1 and 2) it is not surprising that none was observed in the synaptosomal membranes (Fig. 3). The membrane preparation looks quite similar to those shown by other workers [6, 26].

DISCUSSION

In preliminary experiments (Babitch, unpublished) it was found that standard methods for preparing rat synaptosomal membranes [4–6] yielded preparations with high levels of contaminating enzymes when used to prepare chick synaptosomal membranes. Here we report the adaptations necessary to utilize published procedures for preparing rat brain synaptosomal membranes for the preparation of chick brain synaptosomal membranes. As a result of those modifications our membrane preparation has been characterized as comparable to that described by other investigators.

One difference which may exist between the chick and rat membranes is that the chick membranes sediment faster. After $4.8 \cdot 10^6$ or $6.4 \cdot 10^6$ g · min of centrifugation both Morgan and Gurd found their most purified membranes at the 0.6/0.8 M interface of their sucrose gradients. After similar periods of centrifugation ($6.1 \cdot 10^6$ g · min) we find the purest synaptosomal membranes at the 0.8/0.95 M sucrose interface.

We are intrigued by the finding of modest amounts of RNA in the membrane preparation. The amounts are similar to those of Gurd et al. [6]. A report has recently appeared [31] describing the existence of both low and high molecular weight synaptosome-specific RNA species. The question of the origin of the RNA in our synaptic membrane preparations would seem to merit further consideration.

From previous experiments [6, 28] it seemed apparent at the outset that microsomes in our membrane preparation would present the most troublesome contaminant. This proved to be the case. Since we are more concerned with the purity of our preparation than the yield we have chosen not to combine the 0.6/0.8 and 0.8/0.95 fractions. To do so would have nearly doubled the total amounts of rotenone-insensitive NADH: cytochrome *c* reductase while only increasing the yield of "membrane" protein by 40 %. However, the 0.6/0.8 fraction does have very low levels of cytochrome oxidase. In performing experiments where high yields are necessary it might be appropriate to combine these two into a single "synaptosomal membrane fraction".

The standard errors of the mean for some of the values in Tables III and IV are large. The primary reason for this variability is that only occasionally were

detectable amounts of contaminating enzymes observed in the sucrose gradient fractions. For example, monoamine oxidase activity was detected in only two out of five preparations of the 0.6/0.8 fraction and in only one out of five preparations of the 0.8/0.95 fraction. Hence, the standard errors obtained from such data are large.

Though we routinely used 6–8-day-old chickens we have obtained comparable results from 2-day-old birds, though yields of synaptosomes and synaptosomal membranes were lower.

We attempted this preparation for three reasons. First, we believe learning and memory depend, in part, on the occurrence of long-term changes in the area of the synaptic cleft. Second, the chick visual system, for reasons outlined in the introduction, offers an excellent model for studying learning and memory. Third, even if the chick visual system is not a better model than the rat, it is possible that different animals learn in different ways. Thus, an alternative to the rat synaptosomal preparation may be valuable in its own right. For these reasons we feel that this preparation will play a useful role in studying the biochemical basis of learning, memory and behavior.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Institute of Neurological and Communicative Disorders and Stroke (NS12485), the Robert A. Welch Foundation (P-593) and the TCU Research Foundation (C-7452). The capable technical assistance of Ms. I. Nyström is gratefully acknowledged as is the help in assuring an adequate supply of chicks provided by Jim Marshall of the Marshall Grain Co., Fort Worth, Texas.

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