ISOLATION OF POSTSYNAPTIC DENSITIES FROM RAT BRAIN

C. W. COTMAN, G. BANKER, L. CHURCHILL, and D. TAYLOR

From the Department of Psychobiology, University of California, Irvine, California 92664. Dr. Banker's present address is the Department of Anatomy, Washington University School of Medicine, St. Louis, Missouri 63110. Dr. Churchill's present address is the Department of Pharmacology, University of Wisconsin Medical Center, Madison, Wisconsin 53706.

ABSTRACT

Most synapses in the central nervous system exhibit a prominent electron-opaque specialization of the postsynaptic plasma membrane called the postsynaptic density (PSD). We have developed a procedure for the isolation of PSDs which is based on their buoyant density and their insolubility in N-lauroyl sarcosinate. Treatment of synaptic membranes with this detergent solubilizes most plasma membranes and detaches PSDs from the plasma membrane so that they can be purified on a density gradient. Isolated PSDs appear structurally intact and exhibit those properties which characterize them in tissue. The isolated PSDs are of the size, shape, and electron opacity of those seen in tissue; they stain with both ethanolic phosphotungstic acid and bismuth iodide-uranyl lead and the fraction contains cyclic 3',5'-phosphodiesterase activity. Quantitative electron microscope analysis of the PSD fraction gives an estimated purity of better than 85%. Inasmuch as the PSD is associated primarily with dendritic excitatory synapses, our PSD fraction represents the distinctive plasma membrane specialization of this specific synaptic type in isolation.

Where two neurons make synaptic contact, the specialization of their membranes is called the synaptic junction (Palay, 1958; Peters et al., 1970). It consists of highly differentiated parts of the pre- and postsynaptic plasma membranes joined by a synaptic cleft. The presynaptic specialization consists of a hexagonal array of dense projections extending into the cytoplasm (Akert et al., 1969). The postsynaptic specialization varies with the type of synapse and most often includes a prominent electron-opaque structure located on the cytoplasmic side of the postsynaptic membrane just within the synaptic area. Based on its appearance, this specialization is called a postsynaptic density (PSD)[1] (Akert et al., 1969; Bloom, 1970), a web (de Robertis, 1964), or thickening (Palay, 1956; Gray, 1959). A clear understanding of the role of the individual elements at a synapse requires the chemical and enzymatic characterization of these structures, and this depends on the isolation of a pure fraction. In this paper we

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[1] *Abbreviations used in this paper:* BIUL, bismuth iodide-uranyl lead; E-PTA, ethanolic phosphotungstic acid; INT, *p*-iodonitrotetrazolium violet; $KMnO_4$-L, potassium permanganate lead; NLS, N-lauroyl sarcosinate; OsO_4-UL, osmium tetroxide-uranyl lead; PSD, postsynaptic density; SJC, synaptic junctional complex; SM, synaptic membrane.

describe a method for the isolation of PSDs from rat brain.

In the mammalian central nervous system PSDs . are characteristically associated with certain types of synapses. In cerebral cortex, for example, two morphologically distinct classes of synapses have been recognized based primarily on the characteristics of the PSD (Gray, 1959; Colonnier, 1968). One class (asymmetric or Gray type I) is characterized by a prominent PSD which extends 200–670 Å (van der Loos, 1964) into the cytoplasm and gives this synapse its asymmetric appearance (Gray, 1959; Colonnier, 1968). The other class (symmetric or Gray type II) is distinguished by the sparseness or absence of a PSD on the cytoplasmic side, giving these synapses a symmetrical appearance (Gray, 1959; Colonnier, 1968). In the cat visual cortex, asymmetric synapses are the major type and make up 80% of the total (Colonnier, 1968).

Synapses with a prominent PSD of the asymmetric type are usually excitatory (Gray, 1959, 1969; Walberg, 1968). In the cerebellum, asymmetric excitatory synapses include the parallel fiber-Purkinje cell synapses (Gray, 1961; Larramendi and Victor, 1967), mossy fiber-granule cell synapses (Gray, 1961), climbing fiber-Purkinje cell synapses (Chan-Palay and Palay, 1970), and climbing fiber-Golgi cell synapses (Chan-Palay and Palay, 1971); in the hippocampal formation, entorhinal-granule cell synapses (Nafstad, 1967), mossy fiber-pyramidal cell synapses (Blackstad and Kjaerheim, 1961; Hamlyn, 1962); in the lateral geniculate, retinal-class 1 cell synapses (Guillery and Colonnier, 1970; Hendrickson, 1969); in the olfactory bulb, mitral cell-granule cell synapses (Rall et al., 1966; Price, 1968); and in the medial septal nucleus, pyramidal-septal cell synapses (Raisman, 1969; Raisman and Field, 1973). Most of the excitatory synases are located on dendritic shafts or dendritic spines. On the other hand, inhibitory synapses are characterized by a thin sparse PSD and are usually located on neuronal soma (Chan-Palay and Palay, 1970; Gottlieb and Cowan, 1972). Thus, in general, prominent PSDs are a distinctive structural specialization associated with excitatory dendritic synapses.

The PSD exhibits a number of distinctive chemical and enzymatic properties as revealed by cytochemical methods. It is the site of a high cyclic 3',5'-nucleotide phosphodiesterase activity (Flo-

rendo et al., 1971). The PSDs, along with the other elements of the synaptic junction, selectively stain with ethanolic phosphotungstic acid (E-PTA) (Bloom and Aghajanian, 1966). Plasma membranes, even those immediately adjoining the synaptic complex, along with most other cellular structures (mitochondria, myelin, endoplasmic reticulum, etc.), fail to stain. Bismuth iodide-uranyl lead (BIUL) stains the specializations of the synaptic junction (PSD, cleft, and dense projections) and the extracellular coats of plasma membranes outside the synaptic region while it leaves relatively unstained the membrane itself and various intracellular organelles (Pfenninger, 1971).

Taken together, these data show that PSDs are morphologically distinctive entities with special cytochemical properties. In the central nervous system they are generally associated with excitatory synapses on dendrites. Yet very little is known to indicate what their function may be.

The purification of PSD described in this paper now makes possible a direct chemical analysis of the PSD and may clarify its role in synaptic function. Our isolation method makes use of the selective solubilizing action of sodium N-lauroyl sarcosinate (NLS) which previous studies have shown is useful in the isolation of gap junctions from liver tissue (Goodenough and Stoeckenius, 1972; Evans and Gurd, 1972). The method, based on the solubilization of SMs with NLS, followed by density gradient centrifugation, yields a highly purified fraction of PSD.

MATERIALS AND METHODS

Subcellular Fractionation

A PSD fraction was prepared from a SM fraction that contained intact synaptic junctional complexes (SJCs). Most of the plasma membrane could be solubilized with sodium NLS and a fraction of PSDs obtained from the insoluble residue by density gradient centrifugation.

All subcellular fractions were prepared from the forebrains of Sprague-Dawley rats, 40–60 days of age (Simonsen Laboratory, Gilroy, Calif.). The procedure described below uses Beckman type 30, SW 25.1 and SW 25.2 rotors (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). It should be readily adaptable for other rotor combinations so long as the amount of starting material and solution volumes are adjusted proportionately.

ISOLATION OF SMs: A crude SM fraction was prepared based on a minor modification of the method described by Cotman and Taylor (1972) which was

adapted from the procedure developed by Davis and Bloom (1970, 1973). Brains (20 g wet weight) were homogenized in 60 ml of 0.32 M sucrose, 1 mM $MgCl_2$ and the homogenate was diluted to 200 ml with 0.32 M sucrose before centrifugation. This material was pelleted (type 30 rotor, 1,000 g^2 for 5 min), the supernate was saved, and the pellet was resuspended by homogenization in 0.32 M sucrose and pelleted again. The remaining pellet was discarded and the combined supernates were pelleted (type 30 rotor, 14,500 g for 10 min) to obtain a crude mitochondrial fraction. This material was resuspended by homogenization and subjected to osmotic shock by diluting with 0.05 mM $CaCl_2$, pH 7.0 to a final volume of 250 ml and was allowed to stand for 20 min at 0°C.

The osmotically shocked crude mitochondrial fraction was next incubated in a solution containing succinate and p-iodonitrotetrazolium violet (INT). When the INT is reduced (coupled with succinate oxidation by succinic dehydrogenase), it forms a dense precipitate (formazan) within the mitochondria so that they can be more completely separated from the membrane fraction. After osmotic shock, 50 ml of a solution of 5 mM INT, 400 mM succinate, 0.05 mM $CaCl_2$ in 0.2 M Na phosphate buffer, pH 7.5 were added and the suspension was then incubated at 30°C for 25 min. (The INT was best dissolved in buffer before adding succinate, and the solution was freshly prepared for each experiment.) After incubation the material was centrifuged (type 30 rotor, 8,720 g for 7 min) and the supernate discarded. The pellet was resuspended by homogenization in 0.16 M sucrose containing 0.05 mM $CaCl_2$ diluted to 300 ml with the same solution and pelleted (type 30 rotor, 34,880 g for 15 min). The washing procedure was repeated once more, and then the final pellet was resuspended in 0.32 M sucrose, 0.05 mM $CaCl_2$ to a total volume of 40–50 ml. This material was fractionated by density gradient centrifugation. 5 ml of suspension were applied to each of three gradients designed for the SW 25.1 rotor (each gradient consisted of 7 ml each of 0.8, 1.0, 1.2 M sucrose, 0.05 mM in $CaCl_2$, pH 7.0) and 8–10 ml of suspension were applied to each of three gradients designed for the SW 25.2 rotor (14 ml each of 0.8, 1.0, 1.2 M sucrose, 0.05 mM $CaCl_2$, pH 7.0). The gradients were centrifuged at 25,000 rpm for 105 min. The material at the 1.0–1.2 M interface, which is the SM fraction, was collected, diluted 1:2 with 0.05 mM $CaCl_2$, and pelleted (type 30 rotor, 78,500 g for 20 min). The pellet was resuspended in a total volume of 5–8 ml of 2 mM Bicine (pH 7.5). The yield of membrane was about 60 mg protein/20 g wet weight brain. This SM fraction served as the source for the preparation of the PSDs.

ISOLATION OF PSDs: In order to isolate a PSD fraction from the SM fraction, the latter was treated

with sodium NLS and the suspension resolved on a sucrose density gradient into PSD, membrane, and soluble fractions. We used NLS as either a 0.5% (wt/vol) or 3.9% (wt/vol) solution in 10 mM Bicine, pH 7.5. NLS was recrystallized once from 90% ethanol before use. In the case of solubilization with 0.5% NLS, 0.66 ml of NLS solution was added per milligram of SM protein (final concentration about 0.4%) and incubated for 6–10 min at 4°C before loading the suspension onto the gradient. With 3.9% NLS, 20 ml of the NLS solution were added to 5–8 ml of the membrane suspension containing 40–80 mg of SM protein, so that the final detergent concentration was approximately 3%. The mixture was incubated at 4°C for 10 min before application to the gradient. After solubilization, 8–9 ml of either suspension were applied to a discontinuous sucrose gradient for the SW 25.1 rotor which consisted of 7 ml each of 1.0, 1.4, and 2.2 M sucrose, all 0.05 mM in $CaCl_2$, pH 7.0 and the gradient was centrifuged at 63,600 g for 75 min. The material at each interface was collected, diluted 1:3 with 0.1 mM EDTA and pelleted (type 30 rotor, 78,500 g for 20 min). The PSD sedimented primarily at the 1.4–2.2 M sucrose interface, while remaining insoluble membrane fragments banded primarily on top of 1.0 M sucrose. About 0.5 mg of PSD protein was obtained from 20 g of rat forebrain.

In some experiments, to minimize handling when the material was to be used for electron microscope analysis, 2.2 M sucrose was sometimes omitted from the gradient and the PSD fraction was obtained as a pellet.

ISOLATION OF OTHER FRACTIONS: A mitochondrial fraction and a myelin membrane fraction were obtained from the same gradient used to isolate SM. The material banding at the 0.32–0.8 M sucrose interface was taken as a myelin membrane fraction. A mitochondrial fraction was isolated as the pellet on a modified gradient which included a layer of 1.4 M sucrose underlying the 1.2 M layer in the usual gradient. The SJC fraction was isolated from the SM fraction using Triton X-100 as previously described (Cotman and Taylor, 1972).[3] Triton X-100 was added at a ratio of 2 µl/mg of membrane protein and solubilization was carried out at 4°C. SJCs were then obtained by pelleting through a layer of 1.0 M sucrose. Mitochondrial, myelin membrane, and SJC fractions were treated with 3.9% NLS in the same manner as described for the plasma membrane fraction.

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[2] All g values refer to Rav.

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[3] Previously Cotman and Taylor (1972) referred to this fraction as the synaptic complex fraction. The notation is changed here to conform to that formally adopted by Davis and Bloom (1973) and to be more consistent with the nomenclature used by electron microscopists (Peters et al., 1970). This also avoids the possibility of confusion with synaptosomal fractions which have also been referred to as synaptic complex fractions by some (Kornguth et al., 1969).

Electron Microscopy

In order to assess the composition of fractions, samples were prepared for quantitative electron microscope analysis by the method of Cotman and Flansburg (1970). Samples were pelleted in small capsules in order to form pellets which were flat and sufficiently thin so that they could be completely examined in a single cross section (Cotman and Flansburg, 1970). Samples were handled as previously described, except for PSD fractions. PSD fractions adhered to the walls of these tubes, so it was necessary to prepare the micropellet by placing a small volume (10–20 μl, containing roughly 20–40 μg of protein) directly in the tip of the capsule. Optimal structural preservation of PSD fractions required omission of EDTA and a minimum of mechanical shear. After centrifugation of the capsules, pellets were fixed with 4% glutaraldehyde in Caulfield buffer (Caulfield, 1957), postfixed with osmium tetroxide in Caulfield buffer, block stained with uranyl acetate (Kellenberger et al., 1958), and embedded in Epon-Araldite. Sections were cut on an LKB III ultramicrotome and stained with lead citrate for 5 min (Venable and Coggeshall, 1965). In some cases samples were stained with E-PTA, based on the method of Bloom and Aghajanian (1966) as previously described (Cotman et al., 1971), or with BIUL as described by Pfenninger (1971). Sections from some E-PTA specimens were counterstained with 1% potassium permanganate (Glauert, 1965) for 1–2 min, followed by lead citrate. For quantitative analysis of fractions, micrographs were taken with a Zeiss microscope (EM 9S) at × 8,000 magnification and examined in prints at × 24,000 or 32,000. Micrographs for detailed structural analysis were made with a Siemens 1A electron microscope.

The composition of fractions was quantitatively evaluated from a series of micrographs taken at uniform intervals through the thickness of the disklike pellet. Electron micrographs were taken at intervals of about 20 μm thick. Within each micrograph a 2 × μm square was selected at random and all particles within it were classified, counted, and their cross-sectional areas measured. Areas were estimated from measurements of length and width for roughly rectangular objects or by using an overlying grid for objects of irregular shape. For membrane vesicles the diameter was measured and the cross-sectional area of membrane computed assuming a membrane thickness of 100 Å.

Biochemical Assays

Protein was determined by the method of Lowry et al. (1951). The method was slightly modified for fractions which contained formazan since these gave erroneously high readings. In most cases the interference was corrected for by reading the sample vs. a blank which contained sample, but to which the Folin reagent was not added. In a few instances protein was solubilized in 1 N NaOH at 37°C for 1 h and the formazan removed by centrifugation before assay of the supernate.

Cytochrome oxidase (cytochrome c:O_2 oxidoreductase, E.C. 1.9.3.1), Na$^+$,K$^+$-ATPase (Na$^+$,K$^+$-activated ATP phosphohydrolase, E.C. 3.6.14), acid phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.2), and 5'-nucleotidase (E.C. 3.1.35) were all assayed by methods which have been described elsewhere (Cotman and Matthews, 1971). Cyclic 3',5'-phosphodiesterase was assayed by the method of Breckenridge and Johnson (1969). Total phosphorus was assayed by the method of Ames (1966).

Reagents

N-Lauroyl sarcosinate, sodium salt was obtained from K and K Laboratories, Inc. (Plainview, N. Y.). It was recrystallized once from 90% ethanol before use. Cytochrome c, p-iodonitrotetrazolium violet, and all substrates for enzyme assays were purchased from Sigma Chemical Co. (St. Louis, Mo.). Glutaraldehyde (electron microscope grade) and osmium tetroxide were obtained from Polysciences, Inc. (Warrington, Pa.). Other chemicals were of analytical grade and were purchased from standard suppliers. All solutions were prepared in glass-distilled water.

RESULTS

Isolation of the PSD Fraction

The strategy we developed to isolate a PSD fraction involved the preparation of an SM fraction containing synaptic junctions, incubation of the SM fraction with NLS, and separation of the PSD, which remained insoluble, from other residual contaminants by density gradient centrifugation.

The SM fraction which served as the source of PSD contained plasma membranes of various types, some opaque bodies, and a few synaptic junctions with prominent PSDs (Fig. 1, arrows). In this fraction the length of the PSD varied between 0.1 and 0.5 μM. Treatment of the SM fraction with NLS solubilized 97% of the protein and 64% of the phosphorus. Resolution on a discontinuous sucrose density gradient produced two major fractions: a light fraction floating on 1.0 M sucrose and a heavy fraction which banded between 1.4 and 2.2 M sucrose. Protein distributed equally between the heavy and light fraction and the phosphorus remained mainly in the light fraction (Table I). The other interfaces were relatively devoid of protein. The dense fraction contained relatively large amounts of bound formazan, judging from its deep purple color. It was typically
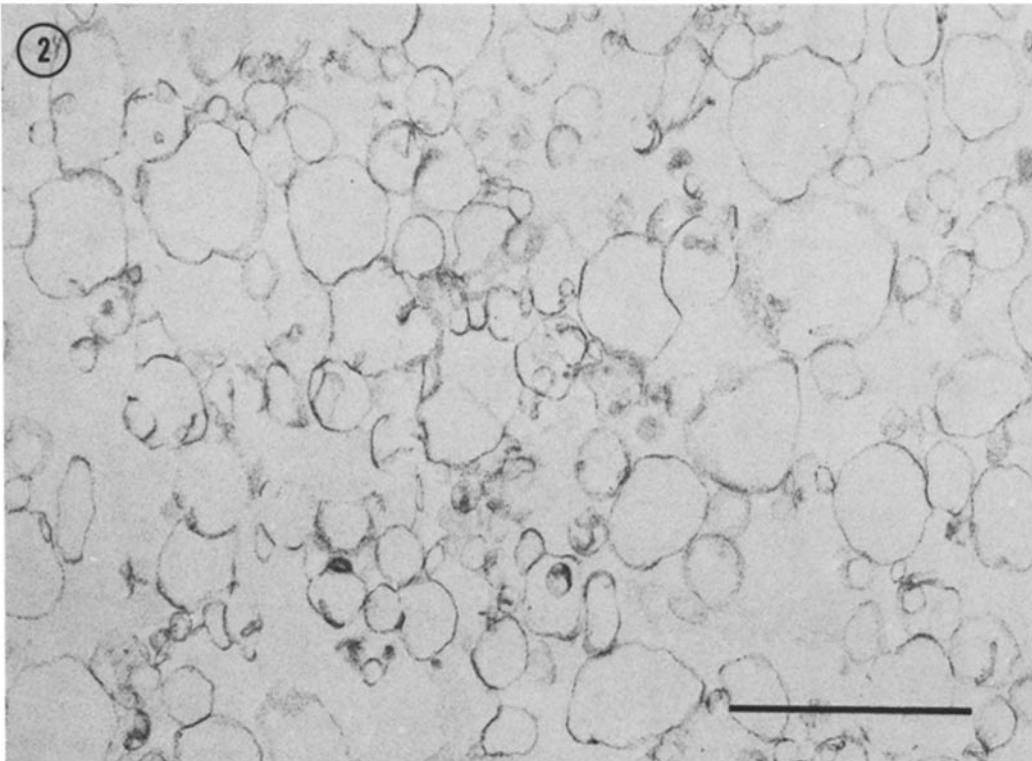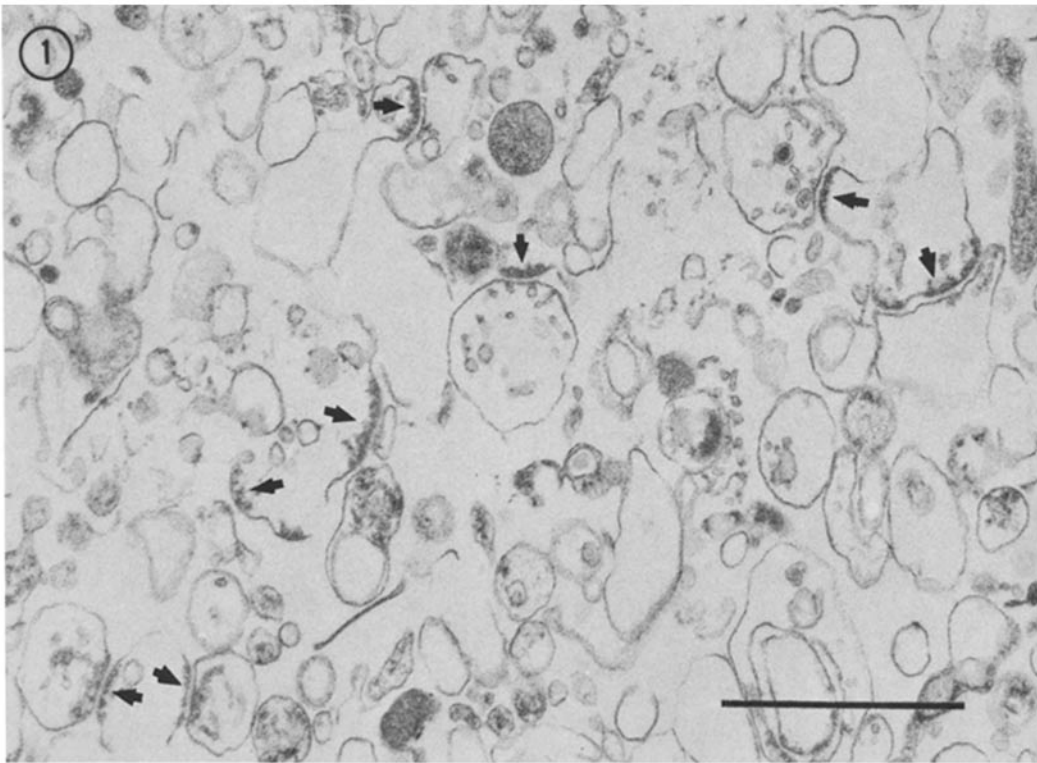
FIGURE 1 An electron micrograph of the SM fraction which was used as the source of PSDs. PSDs (arrows) are present as part of synaptic junctions. Bar is 1 μm. × 32,000.

FIGURE 2 A micrograph which illustrates the structures present in a pellet prepared from the 1.0 M sucrose fraction. Only membrane fragments are present. Bar is 1 μm. × 32,000.

TABLE I

Distribution of Protein and Total Phosphorus on the Density Gradient after Solubilization of the SM, Myelin, or Mitochondrial Fractions with 3% NLS

| | Percent insoluble* | | | |
Fraction	SM protein	SM phosphorus	Myelin, protein	Mitochondria, protein
0.0–1.0 M	46.0 ± 3.0	97.0 ± 0.2	96.0 ± 1.1	19.7 ± 5.0
1.0–1.4 M	5.4 ± 1.3	—	<1.0	7.0 ± 0.4
1.4–2.2 M	45.5 ± 1.2	2.9 ± 0.2	<1.0	3.7 ± 0.5
2.2 M pellet	3.3 ± 3.0	—	2.7 ± 0.4	69.4 ± 5.1
Total insoluble‡	2.3 ± 0.2	37.4 ± 1.6	6.1§	10.2 ± 0.7

* Data are means of two to four experiments except § which represents a single determination. Recovery of protein in gradient fractions for SM averaged 43% and for mitochondria, 80%. Recovery of phosphorus was 50%.
‡ Total insoluble refers to the percent of the protein in the starting material (SM, myelin, or mitochondria) which sediments at 44,000 g for 30 min (30 rotor) after treatment with 3% NLS.

obtained as aggregates that were quite difficult to resuspend and tended to adhere to glassware and centrifuge tubes.

Electron microscope examination of the fraction floating on 1.0 M sucrose showed that it contained exclusively membrane fragments (Fig. 2). By contrast, the 1.4–2.2 M fraction (Fig. 3) contained numerous electron-opaque barlike structures (P) which resembled PSDs seen in the SM fraction and in sections of brain tissue. These opaque barlike or platelike structures were approximately the same size as PSDs seen in the SM fraction or in tissue sections (200–600 Å wide × 0.1–0.5 μm long). Other structures were present which resembled PSDs in size and electron opacity but which were slightly more extended or amorphous in shape (P'). At high magnification (Fig. 3, inset) the barlike structures appeared to consist of a diffusely defined matrix which was similar to that seen in PSDs in the SM fraction. Few, if any, of those structures were attached to pre- or postsynaptic membranes, and only small amounts of plasma membrane were seen (M). On the basis of their morphological characteristics we tentatively identified the electron-opaque structures in the 1.4–2.2 M fraction as PSDs.

Cytochemical Characteristics of the PSD Fraction

We sought to characterize these structures further by their cytochemical staining properties. PSDs stain selectively with E-PTA in tissue sam-

ples (Bloom and Aghajanian, 1966) or in fractions (Cotman et al., 1971; Cotman and Taylor, 1972; Jones and Brearley, 1972; Davis and Bloom, 1973) and with BIUL in tissue sections (Pfenninger, 1971). As shown in Fig. 4 the barlike structures (P) stained with E-PTA and resembled those seen in the specimens fixed with osmium tetroxide and stained with uranyl and lead ions (OsO4-UL) (compare Figs. 3 and 4). In addition, the dense, irregular-shaped structures (P') stained with E-PTA. By contrast, the structures in the 1.0 M sucrose fraction did not stain. This indicates that the staining of structures in the PSD fraction was specific and further suggests that the specificity of E-PTA was not affected by NLS treatment. Structures in the PSD fraction also stained with BIUL. We found, however, that BIUL staining of subcellular fractions was somewhat less selective than has been reported for tissue sections. In the SM fraction, in addition to the synaptic junction and membrane coats, small circular structures (possibly condensed synaptosomes) stained with BIUL. Nonetheless, the stainability of the barlike structures in the PSD fraction with BIUL and E-PTA provided strong support for the identification of these structures as PSDs.

Evidence that all structures in the PSD fraction were PSDs would be gained if it could be shown that all structures, except membranes, stained with E-PTA. Qualitatively this appeared to be the case. The packing density of structures staining with E-PTA (Fig. 4) was similar to that of the structures seen in samples prepared by a general
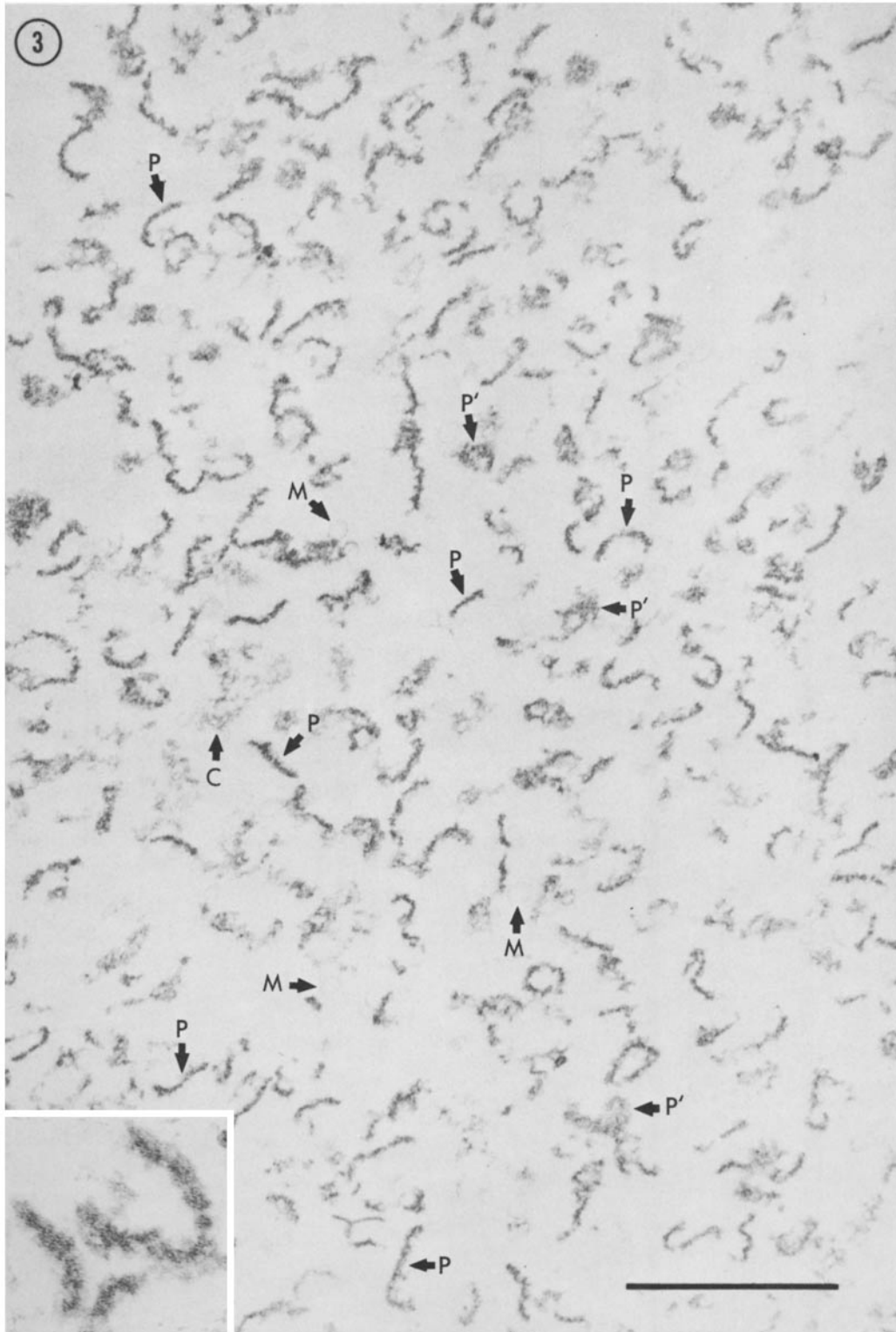
FIGURE 3   A survey micrograph of a typical field of the structures present in a pellet prepared from the 1.4–2.2 M sucrose fraction. Many electron-opaque structures which appear like PSDs are present and range in size from 200 to 600 Å wide and from 0.1 to 0.5 μm long. Other structures which appear similar in electron density and size, but which are less barlike in their overall structural appearance, are present (P′). A few fragments of membrane (M) can be found. A possible contaminant (C) is shown. The *inset* shows the predominant structure in the fraction at high magnification. The PSD consists of a relatively dense matrix. A plasma membrane is not evident along the length of the PSD. Sample was fixed with glutaraldehyde and OsO₄-UL. Bar is 1 μm. × 32,000. *Inset*, × 108,000.
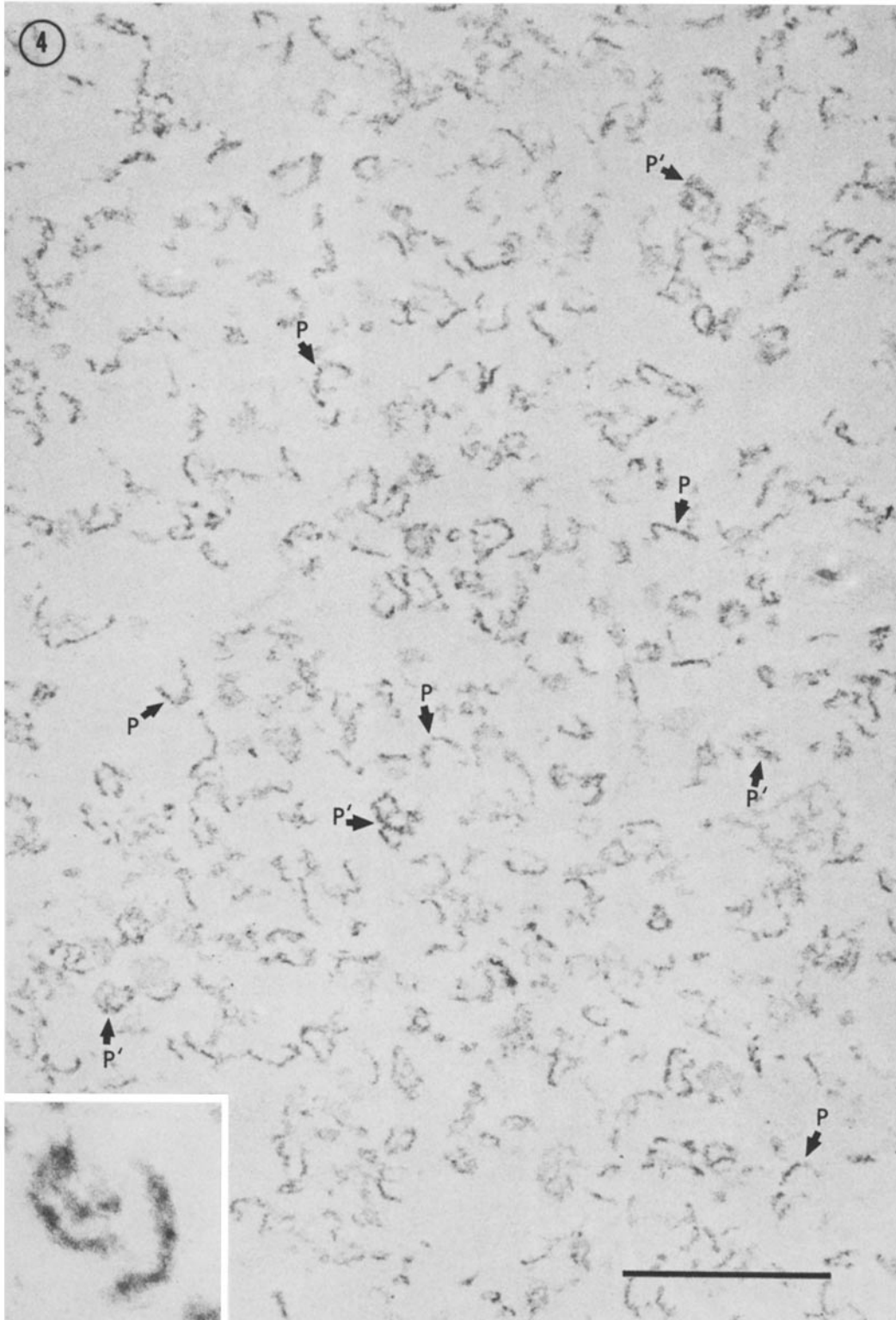
FIGURE 4 A survey micrograph of a typical field of the 1.4–2.2 M sucrose fraction after staining with E-PTA. The barlike structures are stained (P) and are very similar in appearance and size to the barlike structures seen in a preparation fixed with glutaraldehyde and OsO₄-UL (compare *insets* of Fig. 4 to Fig. 3). Besides the barlike structures, other structures which are of a similar size but more irregular in their shape also stain with E-PTA (P'). Bar is 1 μm. × 32,000. *Inset*, × 108,000.

staining procedure using OsO_4-UL (Fig. 3). Furthermore, the relative number of barlike and irregular-shapd PSDs was similar in both preparations. In order to obtain direct evidence that all structures (except membranes) stained with E-PTA, serial sections from an E-PTA block were prepared. A ribbon consisting of a few sections was divided, and one set of sections examined directly. The other set of alternate sections was counterstained with potassium permanganate and lead ions ($KMnO_4$-L) in order to reveal any E-PTA-negative structures. $KMnO_4$-L is a general stain similar to OsO_4UL, and thus reveals E-PTA-negative structures. For example, we found that if an E-PTA-stained SM fraction was counterstained with $KMnO_4$-L, membranes and other structures were revealed and the composition of the fraction appeared like that of an OsO_4-UL preparation. In the PSD fraction detailed comparison of identical areas on adjoining sections showed that the great majority of structures could be identified in both sections. The comparison of structures in the E-PTA- and E-PTA-$KMnO_4$-L-stained sections was like tracing these structures in two identically stained sections. We concluded that, except for membranes, nearly all structures in the 1.4–2.2 M sucrose fraction possessed the characteristic staining properties of PSDs. (Direct counterstaining of a pre-examined area of an E-PTA section with $KMnO_4$-L or other stains was not feasible since the damage from the beam of the electron microscope incurred during initial examination prevented subsequent staining of the area.)

Specificity of the Isolation Procedure for PSDs

The effect of NLS on fractions devoid of synaptic junctions was examined to eliminate the possibility that structures similar in appearance to PSDs might be produced by such detergent treatment. A mitochondrial fraction and a crude myelin membrane fraction were treated with NLS and resolved on a sucrose density gradient under conditions identical to those used to prepare the PSD fraction. When a mitochondrial fraction was treated with 3% NLS, protein distributed throughout the gradient, but almost no protein was obtained at the 1.4–2.2 M interface (Table I). The structures in this fraction appeared as membrane sheets in the electron microscope. No PSD-like structures were seen. Treatment of a myelin membrane fraction with 3% NLS also produced very

little protein in the fraction of a density equivalent to that of PSD (interface at 1.4–2.2 M sucrose) (Table I). Therefore, the procedure was selective: detergent treatment of other fractions that did not contain PSDs did not generate material with similar sedimentation properties or ultrastructural appearance. In addition, in view of the low levels of mitochondria or myelin in the initial SM fraction before NLS treatment (Cotman and Taylor, 1972), very little of the protein in the PSD fraction could have arisen from mitochondria or myelin.

In contrast with fractions devoid of synaptic junctions which did not produce a PSD fraction, a SJC fraction which was enriched in PSDs yielded a highly purified preparation of PSDs after treatment with NLS. SJCs were isolated by treatment of the SM fraction with Triton X-100 followed by density gradient centrifugation (Cotman and Taylor, 1972). By quantitative electron microscope analysis the fraction was composed of about 60–70% synaptic junctions including intact clefts and PSDs with small attached membrane segments and 30–40% unidentifiable membrane fragments.[4] No other structures were visible. Incubation of the SJC fraction with 3% NLS and resolution of the suspension on a sucrose density gradient produced a PSD fraction banding at 1.4–2.2 M sucrose which in the electron microscope was indistinguishable from the PSD fraction obtained directly from the SM fraction. The major difference was the increased yield of PSD per milligram of SJC protein compared to that per milligram of SM protein. Whereas 2 mg of SJC protein provided 0.5 mg of PSD, 50 mg of SM protein was required to obtain this amount of PSDs. The increased yield of PSD from the SJC fraction was in reasonable agreement with the estimated content of PSDs in the SJC fraction. Thus a PSD fraction could be directly isolated from an SJC fraction in a yield compatible with the enrichment of SJC fraction in PSDs.

Enzymatic Characterization of the PSD Fraction

We also examined fractions obtained by NLS treatment for enzymatic activities to detect possi-

[4] Churchill, L., G. Banker, and C. W. Cotman. 1974. Protein and carbohydrate composition of central nervous system synapses: Analysis of isolated synaptic junctional complexes and postsynaptic densities. Manuscript submitted for publication.

ble contamination not apparent in electron micrographs, as well as the possible presence of marker enzyme activities. Since NLS at 3% severely inactivated most enzymes studied, a lower detergent concentration was used (0.4%). Preliminary experiments indicated that this concentration produced a similar PSD fraction, but with slightly more membrane contamination. Cytochrome oxidase activity indicative of mitochondrial contamination was low in PSD fractions (Table II). Acid phosphatase, Na,K-ATPase, and 5'-nucleotidase activities were low, indicating the relative absence of membrane and lysosomal constituents. Since cytochemical studies have suggested that cyclic 3',5'-nucleotide phosphodiesterase activity may be associated with PSDs, we analyzed the phosphodiesterase activity present in these fractions. Although the levels were variable and recoveries low, the reproducible presence of phosphodiesterase in the PSD fraction was consistent with the suggestion that it is a constituent of the PSD. Of the enzymes examined, only phosphodiesterase gave any indication of association with the PSD fraction. Thus these data support the morphological data on the identification of PSDs. In themselves, however, the enzyme data must be considered with caution in view of the variable and often low recoveries and the use in these experi-

TABLE II

Enzymatic Characterization of Membrane and PSD Fractions* Obtained after Treatment of the SM Fraction with 0.4% NLS and Resolved on the Discontinuous Density Gradient

Enzyme	Fraction	Percent total activity‡			
Cytochrome oxidase	Membrane	93	97	97	
	PSD	7	3	3	
	% recovery	16	185	156	
Acid phosphatase	Membrane	98	93	93	
	PSD	2	7	7	
	% recovery	97	32	36	
Na$^+$-K$^+$—ATPase	Membrane	97	97	99	
	PSD	3	3	1	
	% recovery	11	4	11	
5'-Nucleotidase	Membrane	79	94	94	
	PSD	21	6	6	
	% recovery	20	31	44	
Cyclic nucleotide phos-	Membrane	48	74	78	42
phodiesterase	PSD	52	26	22	58
	% recovery§	0.23	0.08	3.9	0.35
Protein	Membrane	75	70		
	PSD	25	30		
	% recovery	97	—		

Each column represents data from an individual experiment.

* The membrane fraction refers in this case to the combined 1.0 M and 1.0–1.4 M fractions. The PSD fraction consists of the 1.4–2.2 M fraction and the pellet.

‡ Percent total activity refers to the distribution of particulate enzyme recovered in the gradient fractions. The percent recovery refers to the recovery of activity in the total particulate plus soluble fractions from the untreated SM fraction. The activity of cyclic 3',5'-phosphodiesterase measured in the SM fraction was 4 µmol 5'-AMP formed/mg protein/h. The activity levels of other enzymes were in the range previously reported (Cotman and Matthews, 1971).

§ The variability in the distribution of enzyme activity does not appear related to the different recoveries since the lower percentages in the PSD fraction (26 and 22%) occur in the experiments with the lowest and highest activity. Various attempts to improve the recovery were not successful.

ments of a detergent concentration which resulted
in contamination.

Quantitative Electron Microscope Analysis

Quantitative electron microscope analysis of the
PSD fraction in comparison with the SM fraction
revealed an enrichment in PSDs with a decrease in
membrane and other contaminants. As described
previously, there were two types of structures
which appeared like PSDs in the 1.4–2.2 M
sucrose fraction. One type of structure was densely
stained and barlike and was similar in length and
width to PSDs in tissue sections (0.1–0.5 μm long
$\times$ 200–600 Å wide). These were identified as
PSDs. Another type also stained densely, but
because it was thicker or branched slightly it was
classified as PSD-like. These particles might have
been damaged PSDs or PSDs sectioned so that
their barlike structure was not recognizable. As
shown in Table III, the PSDs and PSD-like
structures represented 73–93% of the identifiable
structures present in the PSD fraction compared to
only 2% of the structures in the SM fraction.
Inasmuch as estimates of composition based solely
on the number of structures present are very likely
misleading when marked size differences exist
among different structures, we also measured the
cross-sectional area occupied by each category. By
this measure, PSDs and PSD-like structures made
up 86–92% of the material in the PSD fraction
compared to 4% in the SM fraction. Thus in the
PSD fraction there was an enrichment of better
than 20-fold in PSD structures. The membrane
contamination of the PSD fraction was low
(7–25% by number, 8–12% by area) as were other
types of contaminants (0–2% by number or area).

Since the PSD-like structures stained with E-
PTA, it seemed likely that they were damaged
PSDs or PSDs sectioned so that their barlike
structure was not evident. To test the latter
possibility directly, an analysis of serial sections
was undertaken. The results established that, of
those structures that could be followed through
three serial sections, 37% of the PSD-like struc-
tures appeared as a PSD in one out of three serial
sections. Thus, variations in sectioning plane re-
sulted in the presence of PSD-like structures.
PSDs were found to be susceptible to damage.
Care had to be taken in the handling of these
fractions, otherwise more numerous extensively
damaged PSD-like structures and fewer PSDs
were observed. For example, prolonged contact

TABLE III

Quantitative Electron Analysis of the PSD Fraction and SM Fractions

| | Percent of total identified structures in fraction* | | | |
| | Number | | Area | |
Structure	PSD	SM	PSD	SM
PSD	30 34	2	30 33	4
PSD-like	43 59	<1	56 59	<1
Membrane	25 7	71	12 8	49
Other contaminants	2 0	27	2 0	46

Data are given in terms of the percent of the total number
of structures in each category and by the percent of the
area occupied by each structure. Each column represents
a separate experiment.

* The area of identifiable structures represented 95% of
the total structures in the sample of the SM fraction
analyzed. For the PSD fraction this area represented 71
and 83% of the total area, respectively. Unidentified
structures in the PSD fraction commonly appeared
within relatively large particle aggregates. In these
aggregates it was not possible to accurately distinguish
the individual structures and categorize them. However,
most of the structures seemed to be PSD or PSD-like.
The total number of structures counted were 324 and 332
for the two PSD fractions and 743 for the membrane
fraction.

with 0.1 mM EDTA (overnight at 4°C) increased
the prevalence of PSD-like structures. Thus, noni-
deal orientation of PSDs and structural damage
gave rise to PSD-like structures. Previously it was
shown that all PSD-like structures stained with
E-PTA. These three facts provide strong support
for the identification of all the PSD-like structures
as PSDs.

In conclusion, we have prepared a highly puri-
fied PSD fraction and have documented its compo-
sition in several ways. The PSD fraction contained
numerous barlike structures which closely resem-
bled PSDs seen in tissue sections. Variations from
the barlike structure appeared to arise from differ-
ences in alignment of particles with respect to the
plane of section, as well as from partial damage
due to the isolation procedure. Both damaged and
ideally sectioned and preserved PSDs stained with
E-PTA and BIUL. Contaminant particles such as
mitochondria, myelin, and membrane fragments
did not generate a PSD-type fraction when treated
in a similar manner, so that the PSD was not a

detergent artifact generated from any subcellular fraction. In addition, as shown in the following paper (Banker et al., 1974), proteins characteristic of contaminating fractions were not seen in the PSD fraction. An SJC fraction greatly enriched in clearly identifiable PSDs can be used to isolate a PSD fraction in comparably greater yield per milligram of SJC than per milligram of SM protein. Cyclic nucleotide phosphodiesterase activity was prevalent in the PSD fraction whereas enzyme markers for possible contaminating structures sedimented primarily in other gradient fractions, leaving the PSD fraction devoid of such contamination. The enrichment of PSD from the SM fraction was about 20-fold and the resulting fraction was about 85% pure.

DISCUSSION

Our results establish that PSDs can be isolated from adult rat brain in high purity. The enrichment in PSD from SM is estimated at 20-fold and that from homogenate must be substantially greater. The isolated PSDs retain those properties known to characterize them in intact tissue. They stain with both E-PTA and BIUL and they contain phosphodiesterase activity, though apparently at reduced levels. There is little indication of contamination by a number of different criteria and we conclude that our procedure produces an extremely pure fraction of PSDs. At present, however, we do not know whether isolated PSDs retain all properties represented in vivo and we have not excluded all possible types of contamination.

Isolated PSDs are different from most subcellular fractions in their physical properties. PSDs are characteristically very adhesive. PSD fractions contain aggregates which are difficult to disrupt, and the PSDs readily adhere to glass or plastic surfaces in the absence of EDTA. It is possible that these properties are a reflection of the sequestering of Ca during isolation. This is compatible with earlier observations that Ca^{2+} aided in the isolation of well-preserved SJC (Cotman et al., 1971; Davis and Bloom, 1973). In our procedure PSDs carry considerable formazan, and this phenomenon is yet to be explained. At least a part of the formazan appears bound to PSDs since we have been unable to resolve formazan entirely from PSDs under a variety of centrifugation conditions. The formazan could arise from INT reduction by other structures and a preferential binding of released formazan by PSDs, or alternatively PSDs may themselves be capable of INT reduction. We

should point out that formazan is not absolutely essential for the isolation procedure, since in one experiment we succeeded in isolating PSDs in the absence of INT, though the yield was lower than usual. The adhesive properties of the material and the presence of bound formazan are seen to a degree in SJC fractions (Cotman and Taylor, 1972), which are enriched in PSDs, but are generally less evident in the parent SM fraction.

PSDs in our fraction most often are not attached to plasma membranes, yet they closely resemble the PSDs found in intact tissue. In addition PSD structure is not greatly altered by a treatment that solubilizes most plasma membrane. Consequently the conception of the PSD as a "membrane thickening" is incorrect, as already noted (Akert et al., 1969). Rather, in agreement with van der Loos (1964), it is appropriate to consider the PSD as a separate organelle which is associated with the plasma membrane *in situ*, but which has its own distinctive existence.

The PSDs isolated with our procedure are apparently derived from the asymmetric type of synapse (type I). They are generally similar in size to the PSDs from cortical asymmetric synapses (Colonnier, 1968), and no structures in these fractions resemble the thin sparse PSDs often present at symmetric (type II) synapses. On this basis, the purified PSDs most likely arise predominantly from excitatory axodendritic synapses. Thus our PSD fraction represents the distinctive organelle of this specific synaptic type in isolation.

PSDs have not previously been isolated as a pure fraction, and the addition of this fraction to the ensemble of synaptic structures which can be isolated by subcellular fractionation further refines the "dissection" of the synapse. It is now well known that synaptic boutons, often with an attached segment of postsynaptic membrane, can be isolated free from most other contaminating structures (Gray and Whittaker, 1962; de Robertis et al., 1962). These particles, called synaptosomes (Fig. 5 a), represent a complete synaptic unit in isolation. Synaptosomes can be disrupted and the plasma membrane freed from other structures and isolated as a synaptic plasma membrane fraction (Whittaker et al., 1964; Rodriguez de Lores Arnaiz et al., 1967; Cotman and Matthews, 1971; Morgan et al., 1971) (Fig. 5 b). The synaptic membrane is still quite complex, often consisting of plasma membranes from pre- and postsynaptic neurons as well as segments from membrane outlying the synaptic junction itself. Much of the
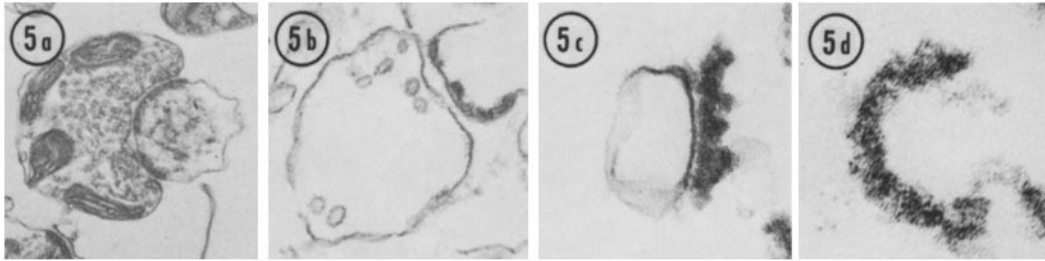
FIGURE 5   Illustration of the types of synaptic structures which can be purified from brain. Synaptosomes (Fig. 5 *a*) represent a complete synaptic unit which can be further resolved into subcomponents. PSDs (Fig. 5 *d*) are the most refined complete subcomponent of the specialized postsynaptic plasma membrane. Synaptic plasma membranes (Fig. 5 *b*) and synaptic junctions (Fig. 5 *c*) are intermediate stages.

membrane not part of the synaptic junction, as well as contaminating membrane, can be removed and SJCs prepared (de Robertis et al., 1967; Davis and Bloom, 1973; Cotman and Taylor, 1972). SJCs which are well preserved include a prominent PSD, short segments of pre- and postsynaptic membrane, and a synaptic cleft (Fig. 5 *c*). As described in this paper, PSDs can be isolated either directly from a SM fraction or from synaptic junctions (Fig. 5 *d*). These fractions, together with synaptic vesicle fractions, provide discrete components for analysis of the structure and function of central nervous system synapses.

Our PSD fraction affords a new approach to examine this part of a central nervous system synapse. In the past, the analysis of PSD has been based primarily on morphological and cytochemical techniques. The extensive enrichment and purity of our fraction provides an opporunity to analyze the properties of this structure directly. In the subsequent paper we characterize the proteins which are the major constituent of PSDs (Banker et al., 1974). The carbohydrate components of PSDs are reported elsewhere.[4]

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