THE STRUCTURE OF POSTSYNAPTIC DENSITIES ISOLATED FROM DOG CEREBRAL CORTEX

I. Overall Morphology and Protein Composition

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ABSTRACT

A postsynaptic density (PSD) fraction, including some adherent subsynaptic web material, has been isolated from dog cerebral cortex by a short-procedure modification of the methods of Davis and Bloom (21, 22) and Cotman and Taylor (20), using Triton X-100. The fraction has been visualized by thin-section, replica, and negative (phosphotungstic acid) staining electron microscopy and its proteins separated by high-resolution SDS gel electrophoresis. Morphologically, the preparation seems to be quite pure, with very little membrane contamination. The density is composed of protein, no nucleic acids, and very little phospholipid being detectable. The fraction had no ATPase or GTPase activity, but it did have a very small amount of cytochrome *c* oxidase activity (of a specific activity less than 0.5% that of a mitochondrial fraction) and a small amount of 5′-nucleotidase activity (of a specific activity between 6 and 7% that of a synaptic membrane fraction). Electron micrographs reveal cup-shaped structures ~400 nm long and ~40 nm wide, made up of apparent particles 13–28 nm in diameter. However, *en face* views, and particularly micrographs of replicas and PTA-stained preparations, reveal a disk-shaped structure, outside diameter ~400 nm, in which filaments are seen to extend from the central part of the density. High resolution gel electrophoresis studies indicated some 15 major proteins and perhaps 10 more minor ones; the predominant protein had a mol wt of 51,000, followed by ones at 45,000, 40,000, 31,000, 26,000, and several at 100,000. A comparison by gel electrophoresis of density fraction proteins with those of a lysed synaptosomal membrane fraction containing some adherent densities indicated some comigrating proteins, but the major membrane fraction protein, mol wt 52,000, was not found in the density fraction. Antibodies raised against the density fraction reacted with a preparation of solubilized synaptic membrane proteins. By both these criteria, it was considered that the density and the synaptic membrane have some proteins in common. By separately mixing ^{125}I-labeled myelin, synaptic vesicle, and mitochondrial fraction proteins with synaptosomes, and then isolating the density

fraction from the mixture, it was concluded that a major 26,000 mol wt density fraction protein was common to both mitochondria and density, that none of the proteins of the density were contaminants from the mitochondrial fraction, that a minor ~150,000 band was a contaminant from the synaptic vesicle fraction, and that the moderately staining PSD fraction protein of 17,000 mol wt band was the result of contamination by the major basic protein of myelin. On the basis of the marker enzymatic assays and the mixing experiments, it is considered that the density fraction is moderately pure biochemically, and that its protein composition, aside from a few exceptions noted above, reflects its *in situ* character.

The point of contact between two neurons, termed the synaptic junction by Palay (59), consists of the highly differentiated regions of the pre- and postsynaptic plasma membranes joined together by structures within the synaptic cleft. Recent articles (1, 7, 29, 30, 40, 61, 62, 63, 64) have delineated further the morphology of the junction, including its postsynaptic part. This latter structure, lying along the cytoplasmic side of the postsynaptic membrane, has been called the postsynaptic thickening (29, 59), the postsynaptic web (23), or the postsynaptic density (1, 7) and is very prominent in the central nervous system. In the last few years attempts have been made to isolate either the whole junction or a part of it, and two fractions have resulted, the so-called synaptic junction complex and the postsynaptic density (PSD) (cf. reviews by Cotman and Banker [13] and Kornguth [46]). The former fraction consists largely of synaptic plasma membranes, both pre- and post-, still retaining the specialized aspects of the junction, including the postsynaptic part. The latter fraction is the subject of this paper, and is defined as that structure lying closely adjacent to the postsynaptic membrane plus adherent subsynaptic web material.

The first attempts at isolating synaptic-junctional complexes (16, 20, 21, 22) were based on the original observation by Fiszer and de Robertis (27) that the detergent Triton X-100 can solubilize most of the membrane components of the synaptosomes, leaving the density and its associated membranes intact. Later, Cotman et al. (14) used *N*-lauroyl sarcosinate to obtain a well-purified PSD fraction, mostly free of membranes. These studies have strengthened the concept (1, 14, 68) that the PSD is a separate subcellular structure which can be isolated free of its membrane attachment and which has a characteristic morphology. We present here a method for the isolation of a purified PSD fraction from brain by the use of Triton X-100, as well as observations on its structure and its chemical constituents. Because of the complexity of the brain, this fraction comprises a mixture of densities from neurons which respond to different chemical transmitters. Despite this, we believe that a study of this type is necessary before any successful attempt can be made to elucidate the neurological function, if any, of the density, a function unknown at present.

MATERIALS AND METHODS

Isolation of Postsynaptic Densities

The method of fractionating brain cortex and isolating PSDs was based on the original methods of Whittaker (73) and de Robertis (24), as modified by the experiences of Davis and Bloom (21, 22) and of Cotman and Taylor (20), and further changed by us. The procedure is outlined in Fig. 1.

The cerebral cortex was removed from a dog after nembutal death, or cortices from 20 rats after decapitation. They were then rinsed three times in Solution A (Fig. 1). Homogenization was performed by 12 up and down strokes with a motor-operated Teflon-glass homogenizer (0.25 nm clearance), using 10-g (wet weight) cortex aliquots per 40 ml of Solution A. The resultant homogenates were combined and diluted to 10% (wt/vol) in Solution A and filtered through four layers of cheesecloth. All the *g* values are maximum centrifugal forces. A low-speed (1,475 *g*) pellet was obtained (cf. Fig. 1) and washed by resuspending the pellet with three strokes of the homogenizer in the same 10% volume of Solution A. After centrifugation, the supernates were pooled, spun at 755 *g*, and this supernate was then centrifuged at 17,300 *g*. These pellets were washed by resuspension in Solution A and centrifugation at 17,300 *g*, and the resultant pellet, containing synaptosomes and mitochondria, was resuspended in Solution B (Fig. 1), using six strokes of the homogenizer. If the SB 110 rotor (International Centrifuge B60) is used, 24 ml of the Solution B resuspending medium is used per 10 g (wet weight) of original cortex. If the SW-25.1 rotor (Beckman-Spinco L40; Beckman Instruments, Inc. Spinco Div., Palo Alto, Calif.) is used, 15 ml of Solution B is used per 10 g of cortex. The gradients (cf. Fig. 1) contain

8 ml of the resuspended material, 10 ml each of 0.85 M, 1.0 M, and 1.2 M sucrose solutions, all containing 1.0 mM $NaHCO_3$, when the SB110 rotor mentioned above is used, and 5 ml of the material plus 9 ml each of the sucrose-$NaHCO_3$ solutions when the SW-25.1 is used.

The sucrose-density gradients were run for two h at 100,000 *g* in any one of the two rotors used. The band between 1.0 and 1.2 M sucrose containing synaptosomes was then resuspended in 4 vol of Solution B and centrifuged at 48,200 *g* for 20 min. If the long procedure was used (cf. Fig. 1), the synaptosomes were lysed by suspending in 10 ml of 6 mM Tris HCl (pH 8.1) per gram (wet weight) of original cortex, and stirring the suspension in the cold for 45 min. The synaptosomal membranes were spun down at 48,200 *g* and then purified on a second set of sucrose density gradients prepared as described above. The band between 1.0 M and 1.2 M sucrose contained the synaptosomal membrane fraction, having pre- and postsynaptic membranes, plus PSDs and web-material. This fraction was spun down at 48,200 *g*, resuspended with six strokes of a Dounce homogenizer, and the protein content of the suspension was estimated by reading absorbance at 280 and 260 nm. This suspension was diluted to 4 mg protein/ml with Solution B. An equal volume of 1% vol/vol Triton X-100 in 0.32 M sucrose-12 mM Tris HCl (pH 8.1) was added, and the resultant, somewhat clarified suspension was stirred in the cold for 15 min. This suspension was spun down at 48,200 *g* and the pellet was resuspended in 2.5 ml of Solution B per 10 g original (wet weight) cortex, and 2 ml of this material was layered on gradients composed of 3 ml of 2.0 M sucrose, 3.5 ml of 1.5 M sucrose-1 mM $NaHCO_3$, and 3.5 ml of 1.0 M sucrose-1 mM $NaHCO_3$. The gradients were spun for two h or overnight at 275,000 *g* in the SB-283 rotor (International Centrifuge). The PSDs banded between 1.5 M and 2.0 M sucrose, though on some occasions some PSD material reached into the 2.0 M sucrose solution. In some cases, the PSD band was pipetted from the gradient, distilled H_2O was added to 12 ml, and then spun down at 275,000 *g* for 1 h to give the final PSD fraction. In other cases, the pipetted band was diluted to a final volume of 5.5 ml with distilled water, and an equal volume of 1.0% Triton-150 mM KCl was added. This suspension was kept in the cold for 1 h and then respun for 1 h at 275,000 *g* to give the final PSD fraction; the latter treatment rids the fraction of most of the contaminating membranes. The band floating on the 1.0 M sucrose solution was identified as mostly synaptosomal membranes by electron microscopy, while the band between the 1.0 and 1.5 M sucrose solutions was a mixture of membranes and densities.

However, it was found that a short procedure could be used (cf. Fig. 1), since the PSD fraction which resulted was equally as intact and as virtually free of membranes as was the PSD fraction obtained via the long procedure, and gave the same gel electrophoretic pattern as did the fraction isolated by the long procedure (not shown). In the short procedure, the synaptosome fraction was not lysed and put on the second gradient but was directly treated with the detergent. The synaptosome fraction pellet was resuspended in 6 mM Tris·HCl (pH 8.1) so that the final concentration was 4 mg of protein per ml, as read at 280 and 260 nm. Triton X-100, containing sucrose and Tris·HCl as described above, was added to a final concentration of 0.5% (cf. Fig. 1), stirred for 15 min in the cold, and then centrifuged at 48,200 *g*. The resultant pellet was resuspended in 2.5 ml of Solution B per 10 g original (wet weight) cortex and put on the same density gradient as for the long procedure.

Two conditions were found necessary for a large and uniform recovery of a PSD fraction from every preparation. It was found that the PSD fraction is extremely sticky to glass and cellulose nitrate tubes, as already mentioned by Cotman et al. (14), resulting in variable and low yields of the fraction. However, we found that with the use of polyallomer tubes throughout the procedure, the yield was increased two- to three-fold, and became uniform. The other condition for good yield was found to be the volume of homogenizing and resuspending media at all stages of the procedure. With the volumes given above, good yields were obtained; if lower volumes were used, in order to cut down on the number of centrifuges used, then lower and more variable yields were obtained.

Each resultant PSD fraction pellet was usually divided into approximately equal quarters with a razor blade; one part was used for protein determination by the Lowry method (51), two parts for gel electrophoresis, and the remainder for electron microscopy studies as described below.

Gel Electrophoresis

Samples to be used for gel electrophoresis (~50 μg of PSD fraction protein and ~100 μg of synaptic membrane fraction protein) were resuspended in a solution containing 0.08 M Tris buffer, pH 6.7, 7% glycerol, and 3% β-mercaptoethanol. The suspension was briefly (~30 s) sonicated, and sodium dodecyl sulfate (SDS) was added to a final concentration of 2%. The sonication was necessary to break up the pellet so that solubilization by SDS was visibly complete; very little stained protein remained at the top of the gel after electrophoresis.

SDS gel electrophoresis was performed according to the discontinuous buffer system of Neville (57) except that a slab gel (30 cm wide × 24 cm long), containing a continuous acrylamide gradient, was used. These slab gels, containing 0.1% SDS, were made with a gradient-forming apparatus so that the concentration of total acrylamide in the separating gel varied linearly from 5 to 15%, while that of a stabilizing sucrose gradient varied from 5.6 to 17.3%. The total acrylamide in the stacking gel was kept at 4.5%. The gradient was made so that, of the total acrylamide, 2.6% was methylenebisacrylamide all throughout the separating gel and in the stacking gel. The amount of ammonium persulfate was reduced to

allow for a longer time for polymerization. The pH levels of the lower reservoir and lower, separating, gel buffer, of the upper reservoir buffer, and of the upper, stacking, gel buffer were respectively 9.18, 8.64, and 6.10. EDTA at a final concentration of 1 mM was added to the upper reservoir buffer. Electrophoresis was carried out for 12–15 h at 25 mA per gel at room temperature, until the tracking dye, bromphenol blue at a final concentration of 0.001% in the sample, reached the bottom of the slab. The gels were fixed and stained at the same time, by shaking at room temperature for 3 h in a solution of 0.25% Coomassie blue in 50% methanol-7% acetic acid. Excess dye was removed by repeated washings with 30% methanol-7% acetic acid, and finally with the latter solution containing 5% glycerol. Molecular weight markers, which gave a good straight line when R_f was plotted against log of molecular weights (cf. Fig. 8), were: β-galactosidase (130,000 daltons), bovine serum albumin (68,000 daltons), ovalbumin (43,000 daltons), liver alcohol dehydrogenase (41,000 daltons), yeast alcohol dehydrogenase (37,000 daltons), α-chymotrypsinogen (26,000 daltons), lysozyme (14,000 daltons), and cytochrome *c* (12,000 daltons).

Chemistry and Biochemistry

Extraction of the sample to determine protein, phospholipid and nucleic acids was performed by the Schneider procedure (65) as modified by Hutchinson et al. (39). Protein was determined by precipitation with cold 10% TCA, washing the precipitate with cold 5% TCA and then with cold H_2O, and determination of the protein by the method of Lowry et al. (51) using bovine serum albumin (BSA) as a standard. It was found necessary to heat the protein suspension in 1.0 N NaOH at 100°C for 30 min in order to solubilize all the protein. Phospholipid was determined by first extracting the washed protein precipitate with $CHCl_3$:CH_3OH (2:1), twice for 30 min at room temperature, centrifuging, combining the supernates, and determining lipid phosphorus by hydrolyzing the lipid extract in 10 N H_2SO_4 and estimating phosphate in the digest by the Fiske-Subbarow method (26). Proteolipid was estimated by the procedure of Folch-Pi (28). RNA was estimated by the orcinol procedure (55), and DNA by the Burton method (11).

The ATPase and GTPase assays (30 min, 30°C incubation) contained 25 mM imidazole buffer, pH 7.2, 1.0 mM ATP or 1.0 mM GTP; various concentrations of Ca^{++}, Mg^{++}, or KCl were added (cf. Results). Inorganic phosphate was measured, after 10% TCA precipitation of the proteins, by the sensitive Soyenkoff method (66) which can measure down to 2 nm of phosphate. Cytochrome c oxidase was measured according to Cooperstein and Lazarow (12) while 5′-nucleotidase was determined by the release of inorganic phosphate (P_i) from 5′-AMP, according to Widnell and Unkeless (74).

Electron Microscopy

The Hitachi HU-11B electron microscope was used for all transmission microscopy. For thin-sectioned material, pellets of the synaptosomal membrane and PSD fractions were fixed by immersing them first in 2.5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) and then in 1% osmium tetroxide in 0.03 M barbital buffer (pH 7.4). The pellets were dehydrated by standard procedures, embedded in Epon, and the Epon was polymerized at 60°F for three days. The top, middle, and bottom of the pellet were sampled in all cases. Thin sections were cut on a Porter-Blum microtome MT2B, and then stained sequentially with 8% uranyl acetate and 4% lead citrate. For staining with 1% phosphotungstic acid (PTA), the PSD fraction was suspended in water, sonicated for 10 s and then put directly on Formvar- and carbon-coated grids. For making replicas, the PSD fraction was pipetted directly from the last density gradient onto a cover glass coated with 0.1% poly-L-lysine and allowed to settle there. After several minutes, excess material was rinsed off with water and the adherent material was fixed in 2.5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4), dehydrated, and dried in a Sorvall Critical Point Drying System (Du Pont Instruments, Sorvall Operations, Newtown, Conn.). The material was coated with platinum and carbon in an Edwards 306 Vacuum Coater (Edwards High Vacuum, Inc., Grand Island, N.Y.). The replica was floated off in a dish of conc. HF, washed in water and Clorox, and transferred to 200-mesh grids for examination.

Immunology

Antisera against various rat and dog cerebral cortex subcellular fractions were raised in New Zealand Red rabbits by intramuscular injections, at bi-weekly intervals, of the antigen emulsions made by suspending the fractions in two parts of 0.9% NaCl and emulsified in one part of Freund's complete adjuvant. The total volume of the injections varied between 0.5 and 1.0 ml. Each type of antigen fraction was injected into two rabbits. Antirat synaptosomal membrane antiserum was prepared by injecting the rabbits 5–6 times with 10–15 mg of protein per injection of a synaptosomal membrane fraction (cf. Fig. 1). Antirat PSD fraction antiserum was obtained by injecting each of the rabbits four and eight times, respectively, with 0.5–1.0 mg of protein of the PSD fraction per injection. Anti-dog PSD fraction antisera were of two types: Either the fraction was treated as described above, or it was first denatured in 1% SDS-1% β-mercaptoethanol. Two rabbits producing antibodies to each of these PSD fractions were used. They received 0.5–2.0 mg of protein per injection for 4–7 times. Blood was collected by heart puncture 1–2 wk after each booster, beginning after the second booster. Pools of the sera within each group were made and the immunoglobulin fractions were enriched about three

times by $(NH_4)_2SO_4$-precipitation at 50% saturation, followed by dialysis against 50 mM sodium barbital (pH 8.6) containing 0.1 M NaCl and the protease inhibitor (50 U/ml Trasylol solution). Immunodiffusion according to Ouchterlony (58) was carried out on 3 × 2-inch glass plates layered with 8 ml of 1% (wt/vol) agarose in 50 mM sodium barbital (pH 8.6) and 1% (vol/vol) Triton X-100. Before staining, the plates were extensively washed in 0.9% NaCl and then in distilled water for 1 h, and dried with light pressure under two changes of filter papers. Staining was performed by immersing in a solution of Coomassie brilliant blue R in 30% methanol and 7% acetic acid. For reaction against the various antisera in the experiment described in Fig. 12, the dog synaptosomal membrane fraction (cf. Figs. 1 and 11) was extracted with 50 mM sodium barbital buffer (pH 8.6)-1% (vol/vol) Triton X-100. Since PSDs are insoluble in Triton, the extract was sedimented for 1 h at 100,000 *g*, and the supernate, considered to contain only membrane proteins, was used as the extract.

Chemicals

The chemicals used and their manufacturers are as follows: Sigma Chemical Co., St. Louis, Mo.: SDS, poly-L-lysine, β-mercaptoethanol, Tris, ammonium persulfate, Coomassie blue, cytochrome *c*, 5′-AMP, ATP, GTP; Worthington Biochemical Corp., Freehold, N.J.: ovalbumin, β-galactosidase, liver alcohol dehydrogenase, yeast alcohol dehydrogenase, α-chymotrypsinogen, lysozyme. Triton X-100 was obtained from Packard Instrument Co., Downer's Grove, Ill.; chloramine-T, acrylamide and methylene-bisacrylamide from the Eastman-Kodak Co., Rochester, N.Y.; BSA from Armour Co., Chicago, Ill.; osmium tetroxide from Polysciences, Inc., Warrington, Pa.; Epon from Ladd Research Industries, Burlington, Vt.; glutaraldehyde from Fisher Chemicals, Fairlawn, N.J.; PTA from J. T. Baker Chemical Co., Phillipsburg, N.J.; Trasylol from Mobay Chemical Corp., New York, N.Y.; agarose from Behring Diagnostics, Somerville, N.J. Soyenkoff's reagent (2-*p*-dimethylaminostyrylquinoline ethosulfate) was a gift of Dr. Soyenkoff, New York University. Carrier free ^{125}I-Na, 11-17 mCi/μg, was obtained from Amersham, England.

RESULTS

Fractionation and Chemistry

The short or long procedures described in Fig. 1 gave uniform preparations of PSD fractions from dog brain, as determined by electron microscopy (EM), chemistry, and gel electrophoresis of the proteins. Since the short procedure was as good as the long procedure in these regards, it was the one finally adopted. The long procedure is needed, however, if synaptosomal plasma membranes are to be obtained (cf. Fig. 1), as observed by EM,

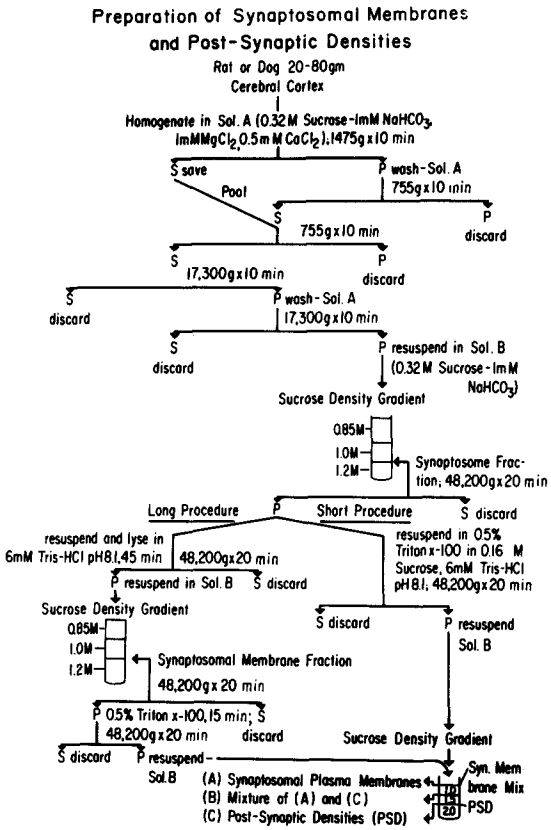


FIGURE 1 Scheme of fractionation of postsynaptic densities from dog or rat brain cortex. The fractionation is explained more fully in Materials and Methods. All the *g* values are given as maximum centrifugal forces.

since the comparable band on the density gradient obtained with the short procedure gave a mixture of membranes, including plasma, mitochondrial, endoplasmic reticulum, and synaptic vesicle membranes. Fractions from rat brain were very similar, but, for reasons of economy, dog brain was used throughout, and all the results in this paper were obtained on fractions from dog brain.

In the first sucrose density gradient (Fig. 1), myelin floated on top of the 0.85 M interface, a mixture of endoplasmic reticulum, Golgi, and plasma membranes settled at the 0.85-1.0 M interface, and the synaptosomal fraction sedimented to the 1.0-1.2 M interface, all observed by electron microscopy. In the second density gradient of the long procedure, the synaptosomal membrane fraction (cf. Fig. 11) centrifuged to the 1.0-1.2 M interface, with the synaptosomal mitochondria

forming a pellet at the bottom of the tube. In the final density gradient, the synaptosomal plasma membranes floated on top of the 1.0 M sucrose, the PSDs settled at the 1.5–2.0 M interface, while the interface at 1.0–1.5 M contained a mixture of membranes and densities, all observed by electron microscopy.

The yield of the PSD fraction was 1.0–1.5 mg protein from 10 g (wet weight) of brain cortex. The unpurified PSD sample (obtained from the short procedure before the Triton-KCl wash) gave higher values, from 1.5 to 2.0 mg of protein, but this was contaminated with membrane fragments (cf. Fig. 2). After treatment with Triton-KCl, there occurred a small loss of protein (cf. Fig. 9). The amount of phospholipid in the short-procedure unpurified PSD fraction was about 35 μg/mg protein, and this was probably due to the small amount of residual membranes in the fraction (cf. Fig. 2). After removal of most of the residual membranes by Triton-KCl treatment (cf. Fig. 3), the amount of phospholipid was lowered to approximately 10 μg/mg protein. All determinations of DNA, RNA, and proteolipid in the fraction gave negative results.

Morphology

Figs. 2, 3 and 4 show a representative view of the isolated PSD fraction. The pellet was examined from top to bottom, and was uniform throughout. The difference between Figs. 2 and 3 is that while both are derived via the short procedure, the fraction shown in Fig. 3 has been further treated by Triton-KCl to eliminate most of the membrane contamination, without much change in the structure of the density or in the protein composition (cf. Fig. 9, slots 2, 3, 4). The isolated densities are structurally similar to their appearance *in situ* (29, 30, 41, 46, 60, 61, 63, 64) if we define the density *in situ* as made up of material lying closely adjacent to the membrane plus a loose web-like material extending into the cytoplasm and sometimes showing dense bodies as a part of this web. Actually, *in situ* ethanolic-PTA staining clearly indicates that this web-like material is adherent to the dense band adjacent to the membrane (8). The appearance of the isolated densities also conforms to the preparations isolated and described by Davis and Bloom (21), Cotman et al. (14, 16), Cotman and Taylor (20), Davis and Bloom (22), while Matus and Walters (53), using 1.2% deoxycholate as a detergent, isolated densities which appeared like diffuse lattices. The isolated PSDs are revealed as densely staining structures about 360 nm long (range 160–530 nm) with a dense band being about 40 nm wide, and with the diffuse material extending ~100 nm from this band. A singular feature of the PSDs, making identification rather easy, was the semicircular profiles observed in cross-section. The outline of the density is not distinct, and there is much material in the background which sometimes is seen to be loosely attached to the central body (Fig. 3, arrows), and which can be seen more clearly at higher magnification (Fig. 4).

Fig. 4 shows other features of the isolated densities, features which identify them as PSDs, for these same features are seen in *in situ* sections of brain. These features include subsynaptic bodies about 30 nm wide (Fig. 4*b* and *f*), connected to the main mass by fine filaments. The dense mass itself is sometimes seen to consist of smaller apparent particles, from 13 nm to 28 nm in diameter (Fig. 4*a*). Sometimes a membrane fragment, obviously postsynaptic, still remains attached to the central mass (Fig. 4*f*), and sometimes even a complex is found consisting of the density and the synaptic cleft material still connected to a presynaptic membrane fragment (Fig. 4*e*). Occasionally, a profile such as that seen in Fig. 4*d* is observed; because of its staining properties and dimensions, we look upon this profile as an *en face* view of the density showing a somewhat hollow interior with some lighter-staining material still in the center (c.f. [63]). The picture obtained so far is that of a core structure composed of proteins packed together in the form of apparent particles, plus an adhering sub-ring of larger bodies held to the central mass and to one another by filamentous material. Figs. 2 and 3 also give the appearance of densities sticking to one another, as already mentioned by Cotman and Taylor (20), which is a counterpart of the stickiness observed when cellulose nitrate tubes were used. It is clear that the detergent treatment has succeeded in isolating a postsynaptic structure free of its presynaptic counterpart, and free of the membranes to which it adheres in the cell. Because of the indistinct nature of the cleft material, it is difficult to ascertain whether the PSD isolation procedure removes the material from the density in all the preparations.

Further observations were made on the isolated PSD fraction, either by other visualization methods (described in this paper) or by treating the fraction with various reagents in order to try to

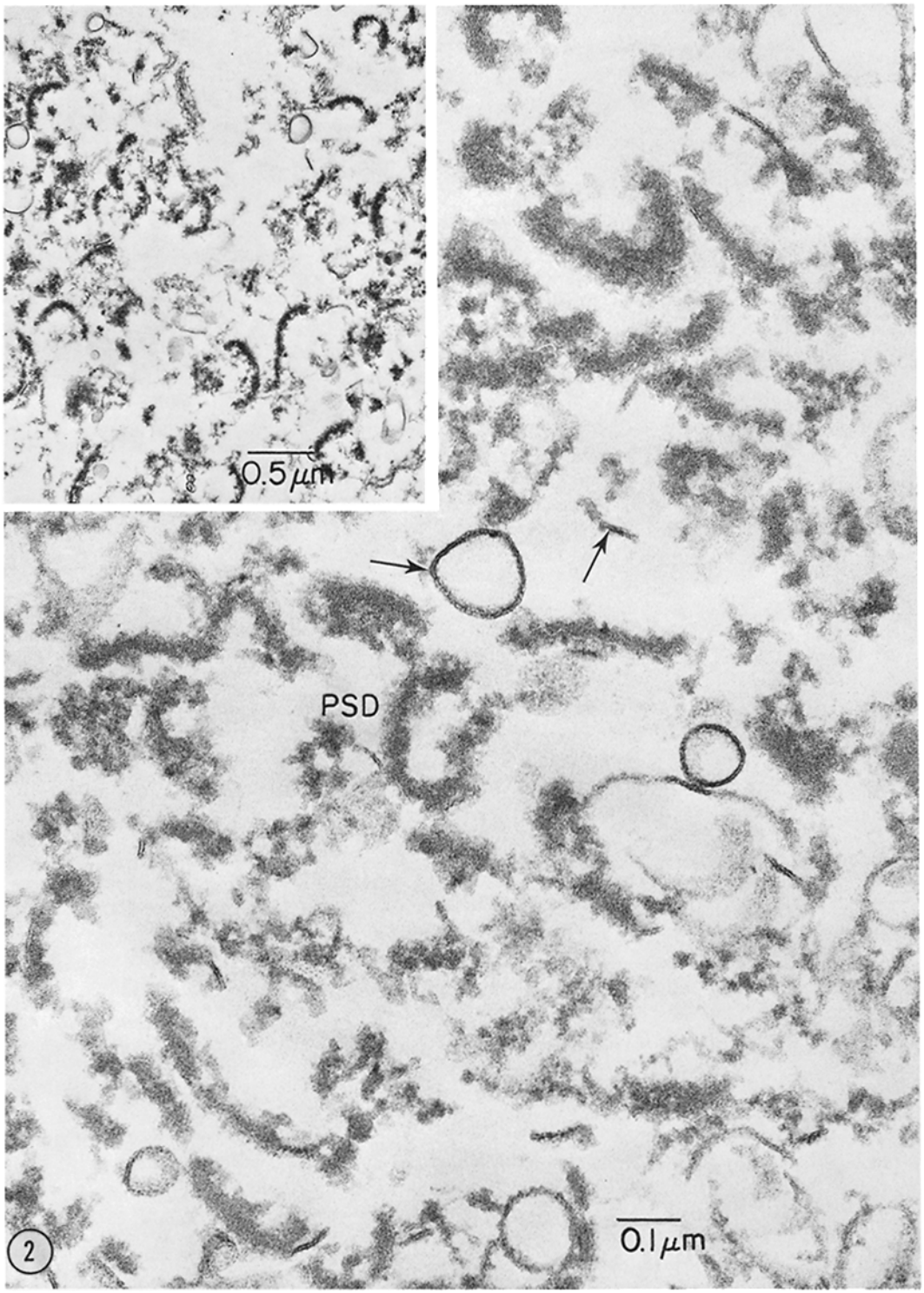


FIGURE 2 PSD preparation before treatment with 0.5% Triton X-100-75 mM KCl. Densely staining structures (PSD) about 360 nm long and about 40 nm wide are revealed. Membrane contamination appears as starkly-stained circular or rod-shaped profiles (arrows). × 100,000. Insert shows view of a typical field. × 20,000.

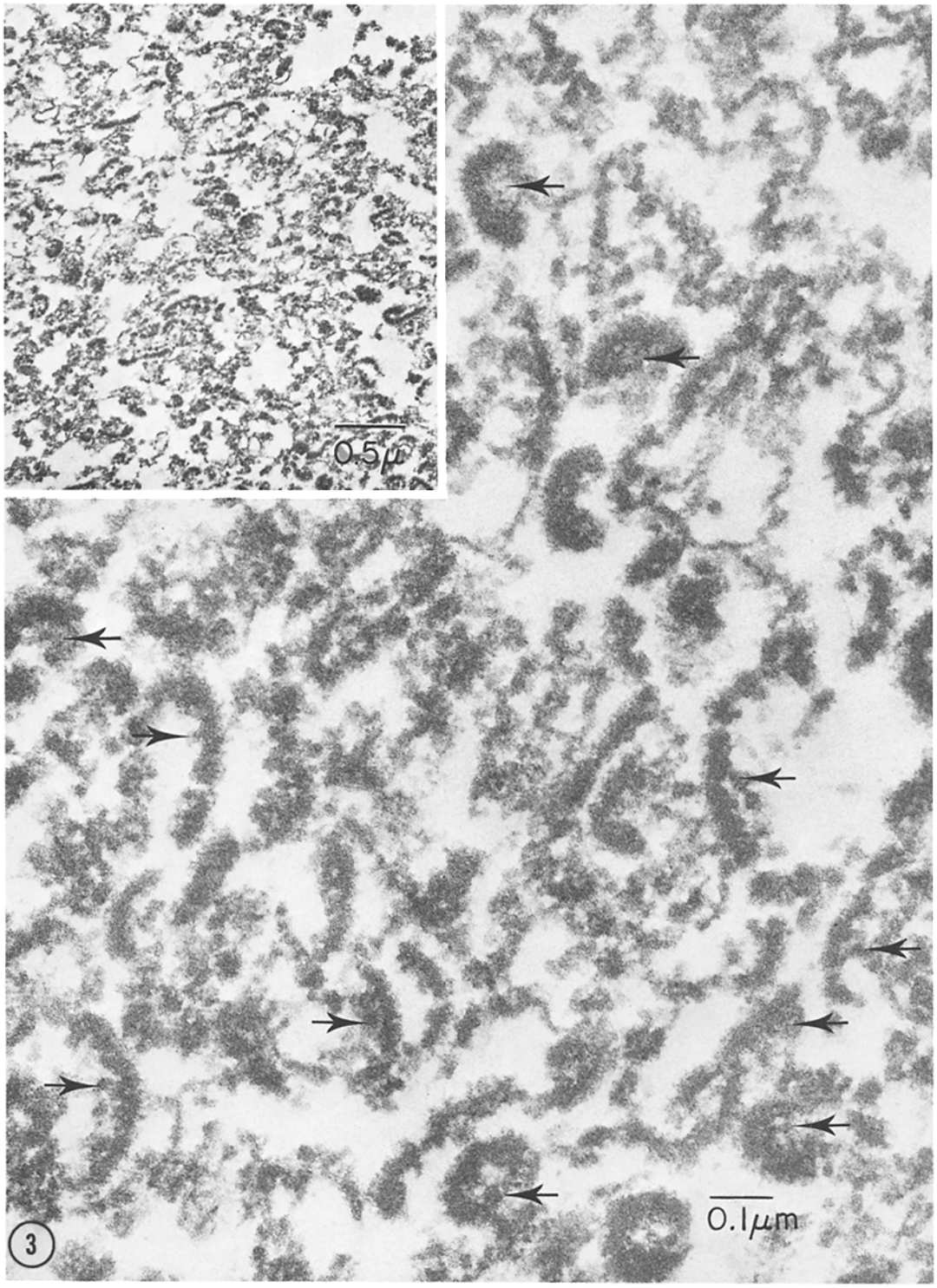


FIGURE 3 PSD preparation after treatment with 0.5% Triton X-100-75 mM KCl. The Triton-KCl treatment is described in Materials and Methods. Membrane profiles are removed by washing the final pellet with 0.5% Triton X-100-75 mM KCl. × 100,000. Insert × 20,000. Arrows indicate subsynaptic bodies or subsynaptic web material.

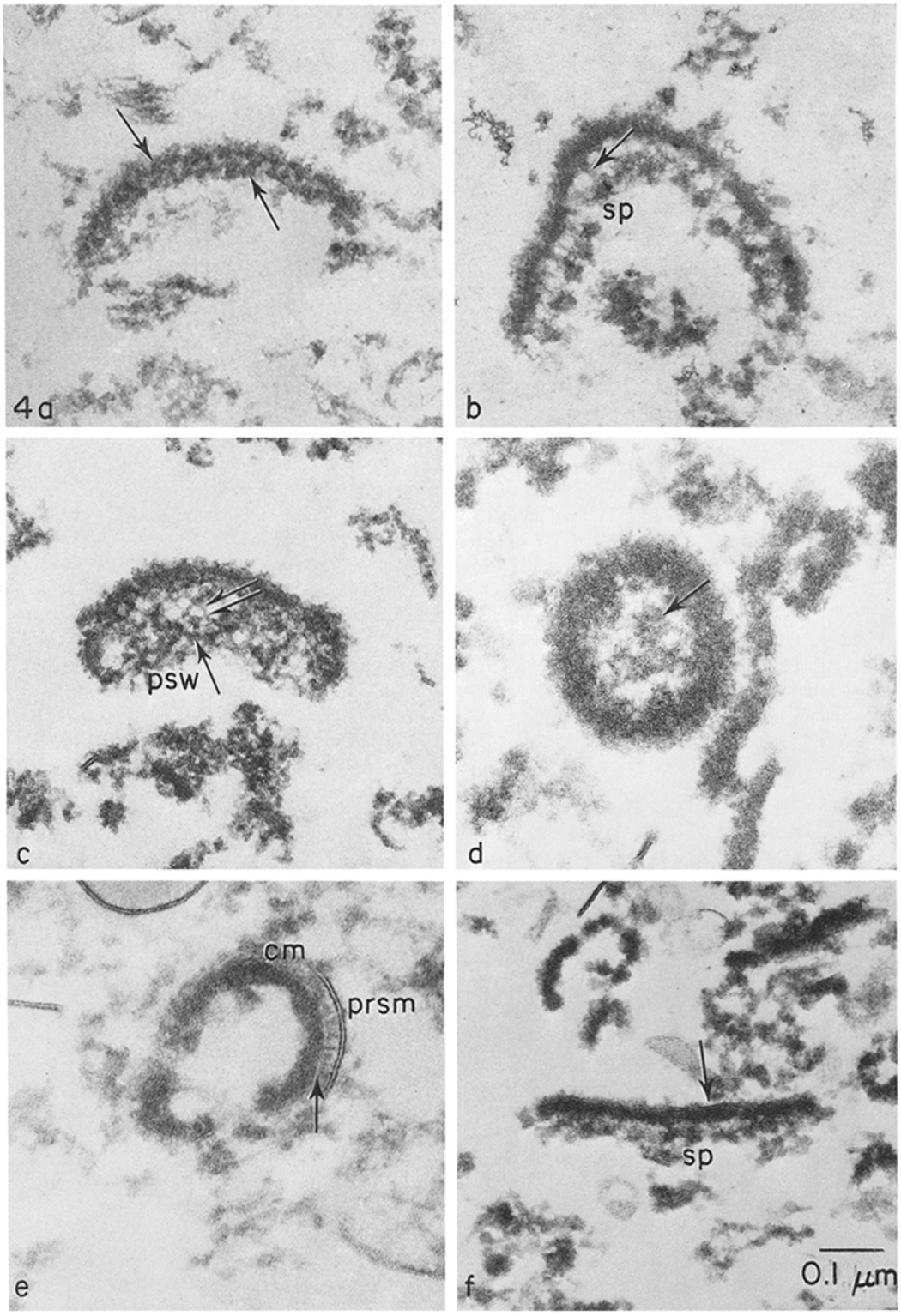
break them up (described in the following paper, reference 6). Figs. 5–7 show images of the PSD obtained by making replicas of the material and by negative (PTA) staining procedures. For the first time, direct indications are observed of the filamentous nature of the isolated density. The replica profiles (Figs. 5 and 6) show clumped-together densities, with long filaments about 10 nm in diameter in the background; sometimes, these filaments appear to connect individual densities. In Fig. 6*c* it can be noted that the long filaments seen in Fig. 5 are not superimposed upon the density, but appear to arise from the structure of the density (arrow). This structure is revealed (Fig. 6) to be filamentous as well as particulate in nature, and this point will be discussed in the following paper (6). A further indication that the structure contains filamentous material is brought out very clearly in the PTA-stained image (Fig. 7), obtained after sonication of the PSD preparation. We think that the appearance of filaments after replica formation and after PTA staining is due to a lesser (Fig. 6) or greater (Fig. 7) unravelling of the dense structures seen in Figs. 2 and 3. In Fig. 7, the profiles of the densities are quite indistinct, they being aggregated; sometimes, a few are seen (insert) which have the same shape and dimension as the *en face* one observed in Fig. 6*a*. All these views show an apparent hole in the center which can be pointed out by arrows in Figs. 5–7. The picture which emerges from these images is that of a disk-shaped structure about 400 nm in diameter, perhaps with a hole in the center. The thin-sectioned material (Figs. 2–4) gives images of cross-sections of the structure, so that the appearance of a hole is only rarely seen, but evidently the drying-down process incumbent upon the other procedures results more in top views of the PSD. Serial sections of cerebral cortex have given images of apparently perforated densities (unpublished observations), as has already been mentioned by Peters and Kaiserman-Abramof (63); the perforation could be akin to our apparent "hole". The possible nature of the filaments and of their involvement in the structure of the density will be discussed in the following paper (6). Our tentative conclusion is that the fraction as isolated represents a purified postsynaptic density fraction, based on the assumptions that the nondescript dense material represents either broken densities or densities obliquely sectioned, and that the lighter-staining, somewhat filamentous material seen in the background in Figs. 2, 3 and 4 is a part of the density, adhering to it at the cytoplasmic site.

Biochemistry

Another criterion for the purity of the PSD is the presence or absence of enzymatic activities known to be associated with other subcellular fractions. Table I gives the results for cytochrome c oxidase, a mitochondrial membrane marker, and 5′-nucleotidase, a plasma and microsomal membrane marker (74). The specific activity of the oxidase in the PSD fraction is 0.3% of that in the mitochondrial fraction, and even though this value may include a 50% inhibition of the enzyme by Triton X-100, the activity in the PSD fraction is still very low. The specific activity of the nucleotidase in the PSD fraction is 6–7% of that in the synaptosomal fraction. The results indicate that, while there may be no contamination of the PSD fraction by visible mitochondrial membranes, there could be some contamination by plasma or microsomal membranes. However, the amount of membrane contamination in the PSD fraction, as viewed by EM (Fig. 3), cannot account for the 6–7% presence of the plasma membrane or microsomal 5′-nucleotidase activity in the fraction. Therefore, we view this activity in the PSD fraction as being due to solubilized membrane proteins which have separated out with the PSD fraction during the procedure (see below). Other membrane enzyme activities, the ATPase and GTPase, were not found in the short-procedure PSD fraction. No activity for these enzymes was found when up to 3 mM Mg^{++} or 3 mM Ca^{++} or 200 mM KCl were added, singly or in various combinations.

Gel Electrophoresis and Immunology

Other criteria for the purity of the PSD fraction, as well as tentative identification of some of its proteins, are provided by gel electrophoresis and immunological studies on this fraction. Through the use of a high resolution gel electrophoresis system, described in Materials and Methods, good profiles can be obtained of the protein composition of the density fraction. Fig. 9, slot 1, gives a typical pattern, with the molecular weights of the major bands determined from the standard curve (Fig. 8). The major polypeptide has a molecular weight of 51,000 daltons, followed by bands with molecular weights of 45,000, 40,000, 31,000,


4a
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26,000, two bands at 18,000 and 17,000, a diffuse band at ~100,000, a diffuse band at 55,000 and at 59,000, bands at 63,000, 71,000, and a double band at 185,000 daltons. It can be seen that the Triton-KCl treatment used to get rid of the membrane fragments (Figs. 2 and 3) hardly changes the resultant PSD fraction gel pattern (Fig. 9, slots 2, 3, and 4), with possibly only a small amount of the 45,000 mol wt band having been removed specifically. Furthermore, the addition of the protease inhibitors, Trasylol and phenylmethylsulfonylfluoride, to the isolation media produced no change in the gel pattern (Fig. 9, slots 5, 6), indicating no proteolysis of proteins during the isolation procedure. The tentative identification of some of these proteins will be discussed in the following paper (6).

Despite the observation that the electron micrographs of the PSD fraction (Fig. 3) showed very little membrane contamination, and that the cytochrome *c* oxidase data (Table I) also indicated very little specifically mitochondrial membrane contamination, it was disturbing that the 5′-nucleotidase data (Table I) indicated a 6–7% contamination by plasma and/or microsomal membranes. A possible explanation is that the Triton X-100 treatment solubilized some membrane proteins which then separated out with the PSD fraction. But treatment of a purified PSD preparation with 500 mM KCl for 60 min in the cold did not change the electrophoretic pattern of the proteins in the PSD fraction, as compared to an untreated sample. This would indicate that whatever extraneous proteins are there in the PSD fraction are probably not held by electrostatic interaction. However, two types of experiment were further performed to more clearly distinguish true PSD proteins from possible extraneous artifacts. One was to compare the protein SDS gel electrophoretic profile of the PSD fraction with those of other subcellular fractions, specifically: (*a*) the myelin fraction (the band at the top of the 0.85 M interface of the first density gradient; Fig. 1), (*b*) the mitochondrial fraction (the pellet at the bottom of the tube in the second density gradient of the long procedure; Fig. 1), and (*c*) a synaptic vesicle fraction, containing mostly synaptic vesicles plus larger vesicles of unknown origin, obtained by taking the supernate after the lysed synaptosomes were spun at 48,200 *g* for 20 min and sedimenting this supernate at 90,000 *g* for 2 h to give a synaptic vesicle fraction pellet.

In the myelin fraction from dog brain, there are three major proteins, at ~35,000, 22,000 and 17,000 mol wt; the former two do not correspond to any bands in the PSD fraction pattern. The latter comigrates with the 17,000 mol wt band of the PSD fraction and similarly stains a light blue, rather than bluish purple, with Coomassie blue, and probably represents the major basic protein of myelin (25). If the myelin fraction is treated with 0.5% Triton X-100, a small part of this protein, and only this protein, is extracted. The protein gel electrophoretic pattern of the mitochondrial fraction showed two major bands, at 52,000 and 26,000 mol wt, and many minor ones. The former band comigrated with the major band in the synaptic membrane fraction, which contains much less mitochondria than does the mitochondrial fraction, as observed by EM; the nature of the protein comprising this band is unknown. The 26,000 mol wt band, as well as 6–8 minor ones, comigrated with some of the proteins of the PSD fraction. There were many protein bands from the mitochondrial fraction in the regions with molecular weights from 26,000 to 18,000, from 31,000 to 26,000, and below 17,000, which did not appear at all, or in very small amounts, in the pattern

FIGURE 4 Selected figures of PSD's from thin-sectioned PSD fraction. (*a*) The PSD is composed of apparent particles (arrows) 13–28 nm in diameter. × 100,000. (*b*) Subsynaptic bodies (*Sp*), about 30 nm in diameter, are attached to the dense band by filamentous material (arrow). × 100,000. (*c*) The postsynaptic web (*psw*) remains attached to the PSD and consists of dense apparent particles about 13 nm in diameter (single arrow) interconnected by filamentous material (double arrow). × 100,000. (*d*) *En face*, the PSD appears as a disk-shaped structure with an outside diameter of about 300 nm, containing structures about 50 nm (arrow) in the center. × 100,000. (*e*) In some cases, the cleft material (*cm*) remains with the PSD, and the postsynaptic membrane seems to have been removed (arrow). Part of the deeply-stained presynaptic membrane (*prsm*) remains in contact with the cleft material. × 100,000. (*f*) Sometimes, fragments of the postsynaptic membrane (arrow) remain attached to the PSD. Subsynaptic bodies (*Sp*) are also seen. × 100,000.

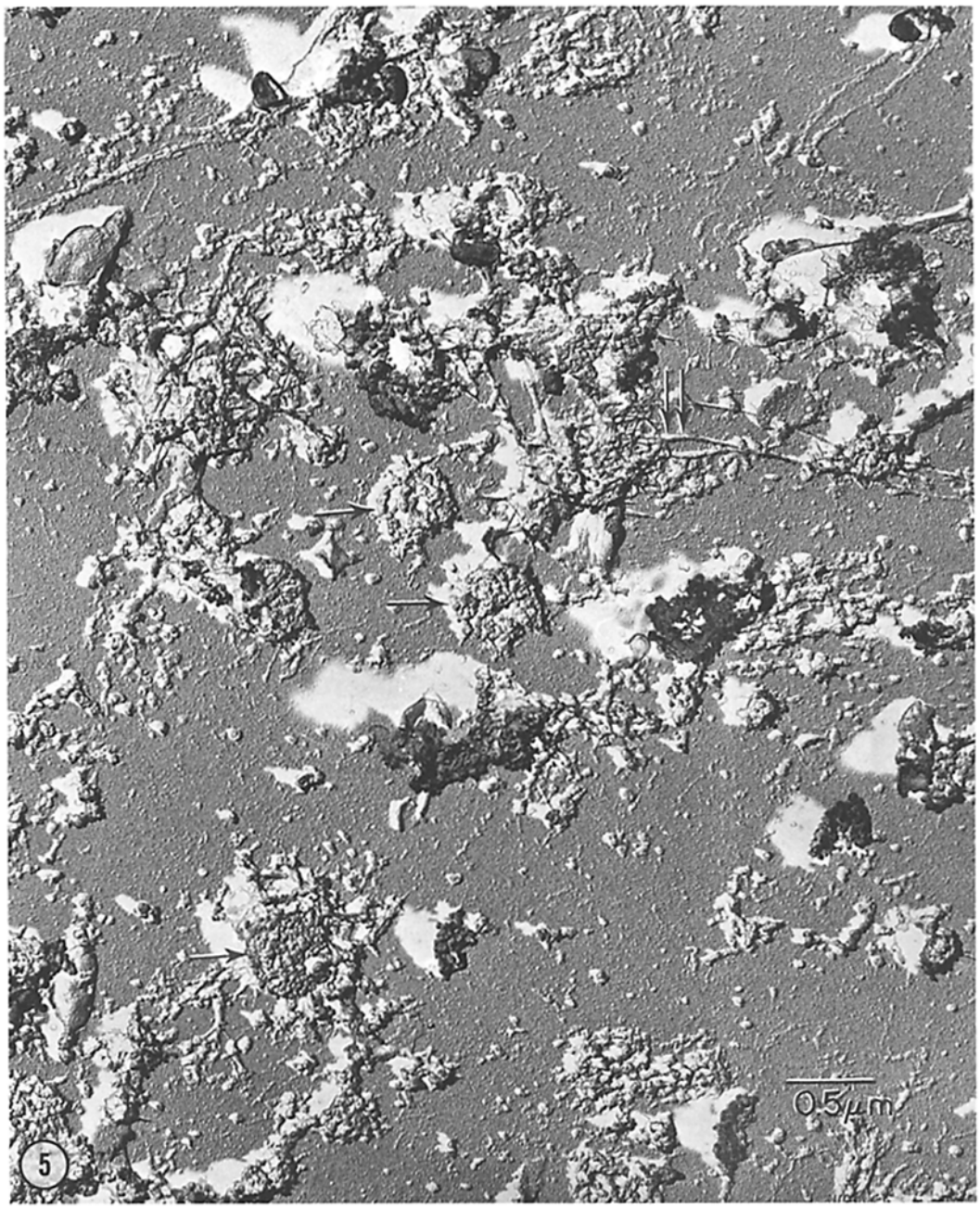

FIGURE 5 Replica of PSD preparation. Shown are filamentous bundles (double arrows) and PSDs (single arrows) which are observed as disk-shaped structures about 400 nm in diameter. × 25,000.

from the PSD fraction. The synaptic vesicle fraction protein pattern indicated two major bands, at ~100,000 and at 31,000 mol wt, with many minor ones; these two bands, as well as 8-10 of the minor ones, comigrated with bands from the PSD fraction. In summary, of the major bands seen on the gel profile from the PSD fraction, only the 17,000, the 26,000, the 31,000, and the ~100,000 mol wt ones are found as major bands in the myelin, mitochondria, and synaptic vesicle

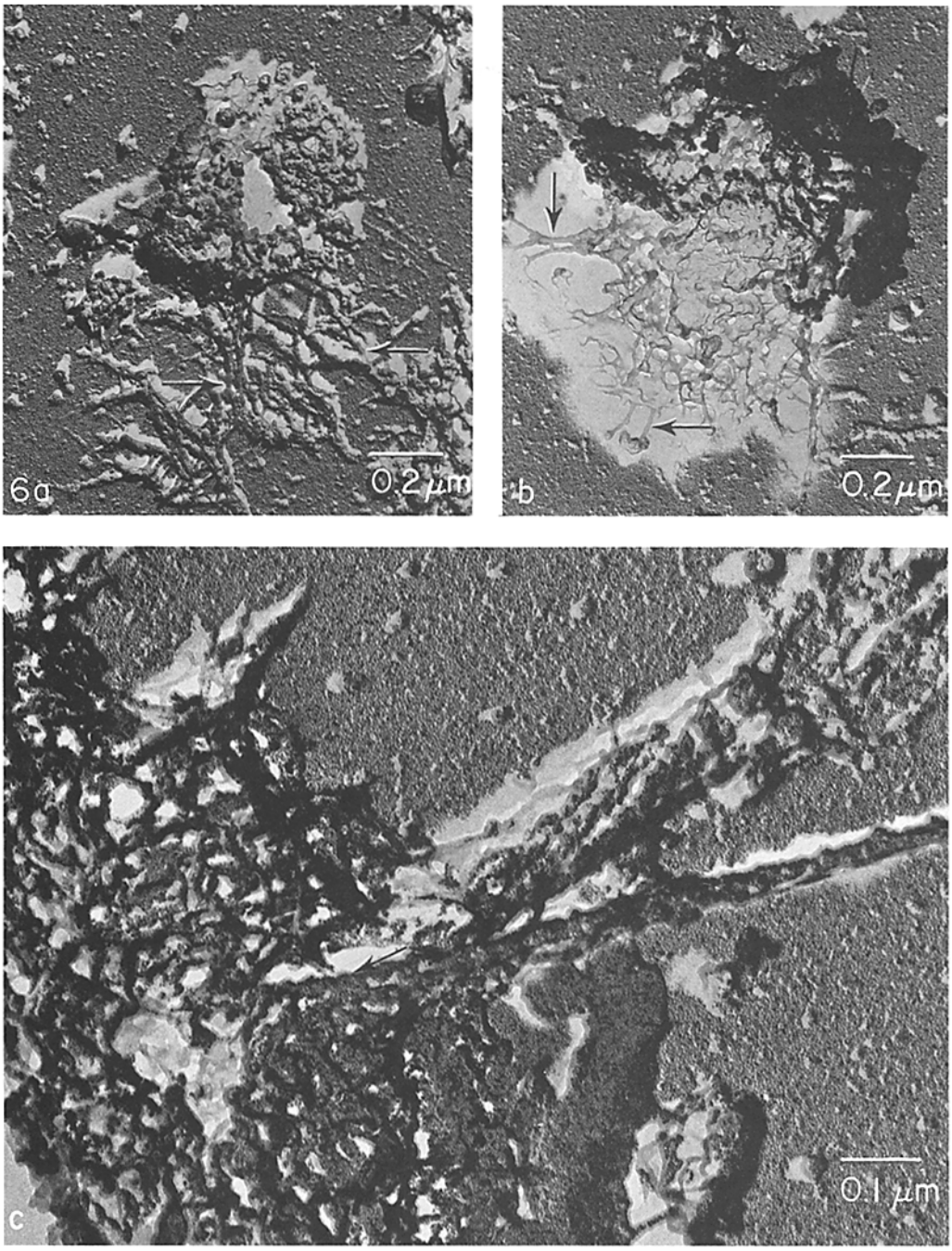

FIGURE 6 Selected views of replicas of PSDs from a PSD preparation. (*a*) Replica of a PSD shows its disk-shaped appearance with an apparent hole in the center, with filaments emanating from this structure (arrows). × 45,000. (*b*) A partially coated replica shows a PSD as it appears when critical point dried. Many small filamentous units are seen (arrows) which are approximately 10 nm in diameter. × 45,000. (*c*) The continuity (arrow) of an outreaching filament with the filamentous network within the PSD is seen. × 108,000.

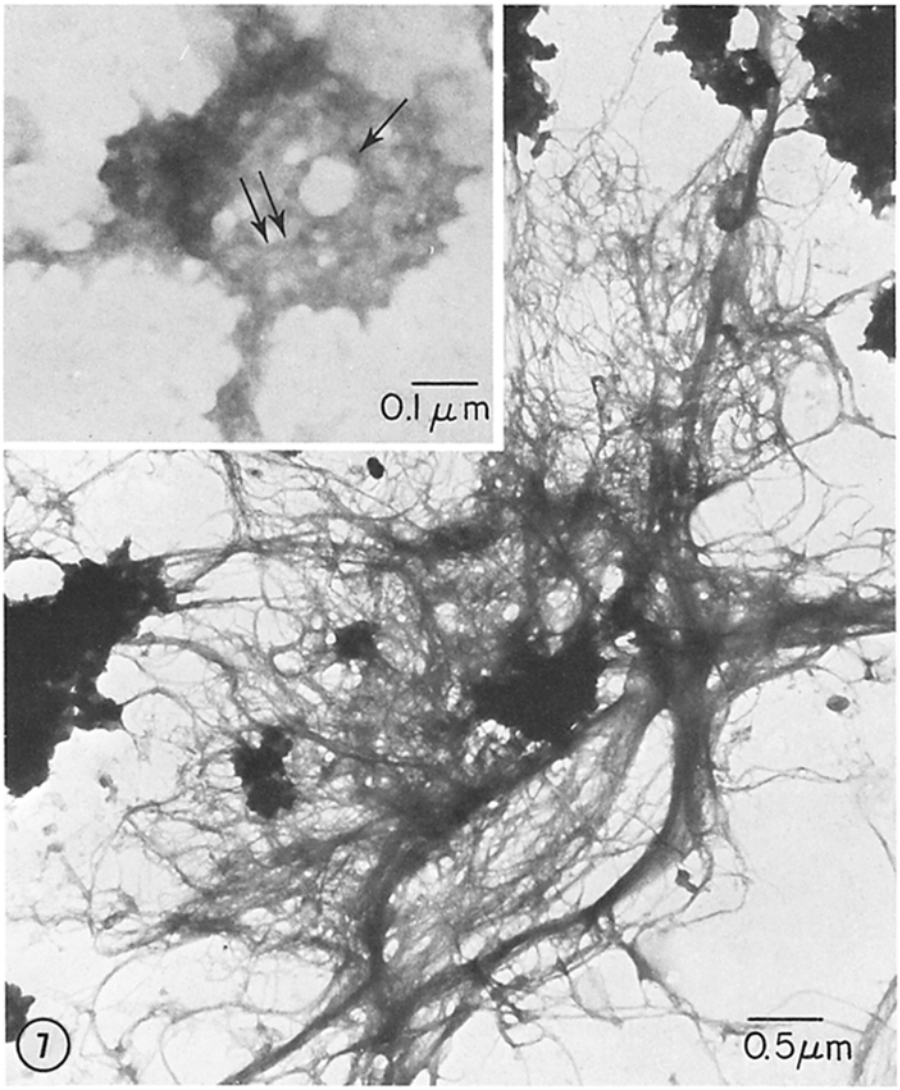

FIGURE 7 PTA-stained sonicated PSD pellet. Many interwoven filaments are seen among clumps of PSDs. × 20,000. Insert shows a PTA-stained *en face* view of a PSD appearing as a lattice structure with an apparent hole in the center, and consisting of apparent particles (single arrow) interconnected by filamentous strands (double arrow). × 75,000.

fractions, respectively, while the 51,000, 45,000, 40,000, 18,000, 55,000, 59,000, and the doublet at ~185,000 mol wt are found in the other fractions only in very small amounts.

While all of the above shows that some proteins from each of the three membrane fractions comigrated with some of the proteins in the PSD fraction, this is only partly indicative of possible contamination of the PSD fraction by these proteins. To try to resolve this question, a radioactive-mixing experiment was employed. Practically all the proteins, including all the major proteins, of the myelin, synaptic vesicle, and mitochondrial fractions were iodinated with $^{125}I^-$, as was verified by comparing a stained gel electrophoresis profile with a radioautograph of the same gel. However, the autoradiographic intensity pattern differed in some instances from the Coomassie blue staining pattern. These radioactive fractions were then mixed separately with synaptosomes, and the PSD fraction, Triton-KCl washed, was isolated from this mixture; any radioactivity in the PSD fraction

TABLE I

Cytochrome c Oxidase and 5'-Nucleotidase Activities of Various Brain Subcellular Fractions

Fraction	Cytochrome c oxidase	5'-nucleotidase Exp. 1*	5'-nucleotidase Exp. 2*
	sp. act	*sp act*	
Synaptosomes	16	0.52§	0.57
Synaptic membrane fraction	133	0.56	ND
Mitochondria‖	314‡	ND	ND
PSD-short procedure	1	0.039	0.034
PSD-long procedure	0	0.052	ND

The assays were performed as given in Methods. Specific activity of cytochrome oxidase is given as μmol cytochrome c oxidized per min per mg protein, while that of 5'-nucleotidase is given as μmol P_i released from AMP per 15 min per mg protein. ND = not determined.

* In Experiment 1, the short-procedure PSD fraction was not washed with Triton-KCl, as noted in Materials and Methods, while in Experiment 2 it was so washed.

‡ The activity was not inhibited by the presence of 0.1% Triton X-100 in the assay, but a 50% inhibition ensued when 0.2% or 0.5% was included in the assay.

§ The presence of 0.5% Triton X-100 in the assay gave a 30% activation, after subtraction of the P_i blank in the Triton itself.

‖ The mitochondrial fraction used was the pellet obtained in the second gradient of the long procedure (cf. Fig. 1) and thus represents intrasynaptosomal mitochondria. Electron micrographs show this fraction to contain mitochondria, mostly damaged, with small amounts of unknown membrane fragments and some PSD's.

was then considered to be due to protein contaminants. The protocol of the procedure and the results are shown in Table II. It can be seen that the PSD fraction was contaminated by 3.7% of synaptic vesicle membrane proteins (but the synaptic vesicle fraction does contain many membrane vesicles of unknown origin), by 0.1% of the myelin fraction proteins, and by 6.5% of the mitochondrial fraction proteins.

To further determine which proteins in the PSD fraction were probably contaminants from other fractions, the radioactive PSD samples from the above experiments were electrophoresed and autoradiographs were made of the gels. In the case of the mixing experiment using the synaptic vesicle fraction, only one band in the subsequently isolated PSD fraction was strongly radioactive, that one at ~150,000 mol wt, corresponding to a minor band in the gel of the PSD fraction. There was also very faint radioactive banding in the 51,000, 45,000, and 31,000 mol wt regions of the PSD fraction gel pattern. However, since the specific radioactivity (autoradiographic intensity/Coomassie blue stain) was much less for the 51,000 and 45,000 mol wt bands in the PSD fraction gel than for the comparable bands in the synaptic vesicle fraction gel, we conclude that most of the proteins in these bands are intrinsic to the PSD and are not contaminants from the synaptic vesicle fraction. The possibility that the 31,000 mol wt band in the PSD fraction gel pattern is a contaminant is harder to resolve. While its radioactivity in the PSD fraction gel is very faint, the relative amount of the stained band in the gel of this fraction is less than that of the comparable band in the gel of the synaptic vesicle fraction. At the present time, we conclude that only the minor ~150,000 mol wt PSD fraction band is a contaminant from the synaptic vesicle membrane fraction.

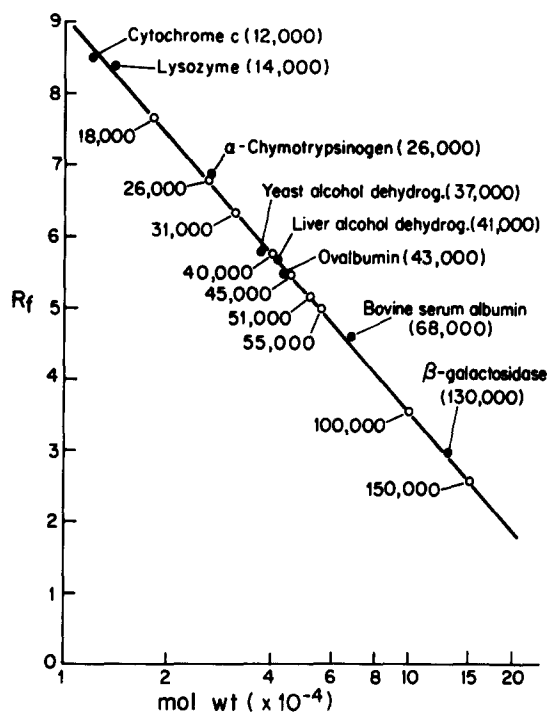

FIGURE 8 Plot of molecular weights of standard proteins versus relative movement on a 5 to 15% continuous gradient gel. Details are given in Materials and Methods. ●: standards; ○: estimated molecular weights of major bands in PSD preparation (cf. Fig. 9). It should be pointed out that the molecular weights of the bands (cf. Figs. 9 and 10) above β-galactosidase, mol wt 130,000, are an extrapolation, and their estimated molecular weights could be too low.

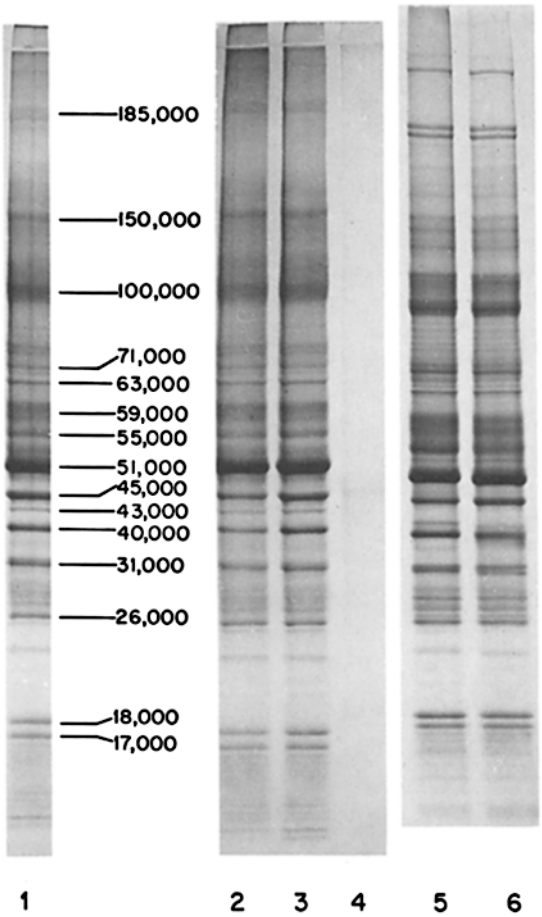

FIGURE 9 Gel electrophoresis of PSD fraction proteins and results of treatment with Triton-KCl and with protease inhibitors. Electrophoresis was performed as described in Materials and Methods. Slot 1: standard PSD gel, with molecular weight estimations of major bands, determined from the graph of Fig. 8; Slot 2: standard PSD gel; Slots 3 and 4: PSD treated with 0.5% Triton-75 mM KCl as described in Materials and Methods, and then centrifuged to give a pellet gel (3) and supernatant gel (4); the proteins in the supernate were treated with 10% TCA overnight in the cold, then centrifuged, and the precipitate was dissolved in SDS; Slot 5: standard PSD gel obtained without the use of protease inhibitors; Slot 6: gel of PSD obtained from experiment in which 1 mM phenylmethylsulfonylfluoride was added to all solutions mentioned in Fig. 1 and in which 1,000 KIU/ml of Trasylol was also added to Solution A (cf. Fig. 1).

In the case of the mixing experiment using the myelin fraction, only one radioactive band in the subsequently isolated PSD fraction was discernible, that one at 17,000 mol wt, corresponding to the myelin basic protein (25), and therefore we assume that the presence of this band in the PSD fraction is due to a contamination by this protein from the myelin fraction.

In the case of the mixing experiment using the radioactive mitochondrial fraction, there were six faintly radioactive bands in the subsequently isolated PSD fraction, at 17,000, 26,000, 45,000, 51,000, ~100,000, and above 200,000 mol wt. On the basis of the specific radioactivity of these bands in the PSD fraction as compared to the mitochondrial fraction, none of the PSD bands

TABLE II

A Measure of the Contamination of the Isolated PSD Fraction by Proteins of Various Other Subcellular Fractions

Fraction added	Specific Radioactivity: Added fraction (1)	Specific Radioactivity: Isolated PSD fraction (2)	Calculated contamination (2/1 × 100)
	cpm/mg protein		%
Myelin	5.03×10^6	5.15×10^3	0.1%
Synaptic vesicles	4.76×10^6	0.175×10^6	3.7%
Mitochondria	6.74×10^6	0.439×10^6	6.5%

The various fractions used were the following (see Fig. 1): the myelin fraction was the band at the top of the 0.85 M sucrose in the first density gradient; the synpatic vesicle fraction was obtained by taking the supernate after the lysed synaptosomal fraction was spun at 48,200 *g* for 20 min, and then sedimenting this supernate for 2 h at 90,000 *g* to give a pellet, the synaptic vesicle fraction; the mitochondria was the pellet at the bottom of the tube in the second density gradient of the long procedure. These three fractions, containing 2–6 mg of protein, were incubated in ice for 2 min in the presence of 0.01% chloramine-T and one mCi $^{125}I^-$, then adding 0.02% $Na_2S_2O_4$, and, after 10 min, 0.2% KI. The suspension was then washed three times in Solution B by alternate sedimentation and resuspension. A comparison of the Coomassie blue-stained gels of these fractions with autoradiographs of the gels showed that most of the proteins in each fraction were iodinated. The $^{125}I^-$-labeled fractions were mixed separately with the synaptosomes at a protein ratio of 1–5 parts fraction to 100 parts synaptosomes, and the mixture was carried through the short procedure to obrain the PSD fraction, which was then washed with Triton X-100-KCl as described in Methods. Specific radioactivities of the original subcellular fraction and of the PSD fractions isolated from the mixtures were determined by counting in a Packard Auto-gamma scintillation spectrometer, Model 5220.

seem to be due to mitochondrial contamination. Of these bands, only the 26,000 mol wt one is a major protein in both the mitochondrial and the PSD fractions, and it may represent a protein common to both. Finally, it should be pointed out that some of the radioactive proteins in the radioactive mitochondrial fraction are PSD proteins, since PSDs could be isolated from the mitochondrial fraction, accounting for ~4% of the proteins in this fraction. Thus, the radioactivity in the PSD fraction (6.5%, Table II) isolated in the mixing experiment could be due in part to a concentration of PSDs from the mitochondrial fraction during the re-isolation, and might not be entirely due to a contamination of the PSD fraction by mitochondrial proteins.

It was of interest to compare the isolated PSD fraction with the synaptosomal membrane fraction from which it was derived by Triton X-100 treatment (Fig. 1). Fig. 10 shows gel patterns of each of these fractions, while Fig. 11 is an electron micrograph of the synaptosomal membrane fraction. This latter fraction is composed of disparate elements, some of which can be identified as plasma membrane and as synaptic vesicles. The amount of synaptosomal complexes and PSDs (arrows) is quite small, 8–12 in the representative figure, and in profile lengths it comprises only a small percentage (~5%) of the total surface membrane profiles. It was surprising therefore to observe on gel electrophoresis the banding of many of the polypeptides of this fraction coincident with that of the density fraction (Fig. 10). While there are many polypeptides of similar movement, 15 polypeptides of the synaptosomal membrane fraction are found either exclusively or predominantly in this fraction as compared to the PSD fraction, with the by far predominant one having a molecular weight of ~52,000 (between the 51,000 and 54,000 mol wt bands marked on the figure), and others being of lower molecular weights, between 45,000 and 40,000, between 40,000 and 31,000, between 31,000 and 26,000, between 26,000 and 18,000, and smaller than 17,000. Of the density polypeptides, the doublet at 185,000 and the 51,000, 45,000, 40,000, 18,000, and 17,000 mol wt ones are predominantly density proteins. We conclude that the 15 polypeptides of the membrane fraction are of intrinsic membrane origin, that 7 polypeptides of the PSD fraction are of intrinsic PSD origin, but that there are some 15 membrane and PSD polypeptides which comigrate on the gel, such as the 100,000, 59,000,

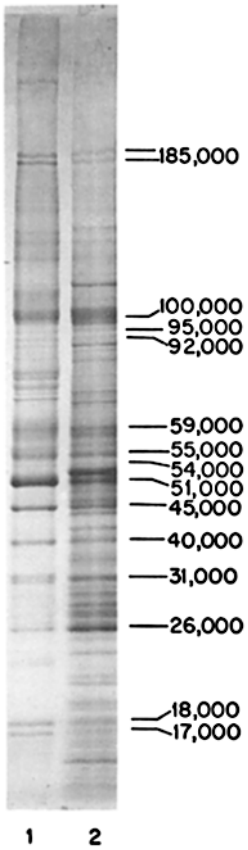

FIGURE 10 Comparison of gels of PSD and of synaptosomal membrane fractions. The synaptosomal membrane fraction was that obtained as depicted in Fig. 1; gel electrophoresis was performed as in Materials and Methods. Slot 1: standard PSD gel; Slot 2: gel of synaptosomal membrane fraction. Lines and molecular weights on right depict polypeptides which run identically in the two preparations.

55,000, 51,000, 45,000, 40,000, 31,000, 26,000, and 18,000 mol wt bands. One of the common bands, the 45,000 mol wt one, is probably actin (6). The observation (Fig. 10) that the by far predominant 52,000 mol wt membrane band is not found in the PSD fraction would indicate very little contamination of the latter by intact membranes, and furthermore would strengthen our conclusion of the electrophoretic identity between some PSD fraction and membrane fraction proteins.

Another indication of the similarity between some of the membrane fraction and PSD fraction proteins is given in Fig. 12. There it is shown that a Triton extract of the dog synaptosomal mem-

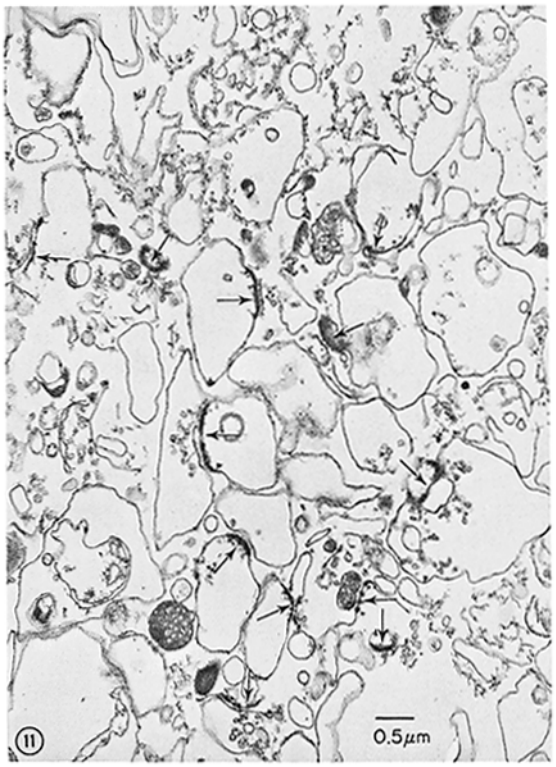

FIGURE 11 Synaptosomal membrane fraction. This fraction, obtained by the long procedure, shows synaptosomal plasma membranes, some with synapses. Arrows indicate recognizable post-synaptic densities. × 20,000.

brane fraction, from which the PSDs were removed by sedimentation, cross-reacted with antisera to rat synaptosomal membrane fraction and, significantly, with anti-sera to both dog PSD and rat PSD fractions. Several precipitin lines are visible, with confluencing of the anti-PSD fraction and antisynaptosomal membrane fraction lines, indicating the presence of immunologically similar proteins in these two fractions. On the basis of the assumption that the major proteins in the Triton X-100 extract are derived from the synaptosomal plasma membrane, we conclude that the antibodies are reacting against these proteins. The strength of the precipitin lines would also indicate that they do not represent those PSD fraction proteins which constitute only a small part of the membrane fraction (Fig. 11), but instead represent true membrane proteins.

DISCUSSION

At the outset, we should remark upon the reproducibility of our PSD preparations, as observed by EM and also by gel electrophoresis. The variations in the preparations were two-fold: the amount of membrane contamination, varying from zero to that seen in Fig. 2, even after the Triton X-100 KCl wash, though most of the preparations had much less membrane content that that seen in Fig. 2; and the amount of background material which, because of its staining properties, density, and general appearance, we take to be partially broken-up densities or sub-synaptic web material. It is also possible that this background material represents oblique sections through the density, as seen in some of the profiles in Figs. 2 and 4. The gel profiles were also greatly reproducible. The gels in Fig. 9 represent three different experiments, and that in Fig. 10 yet another experiment, and yet all

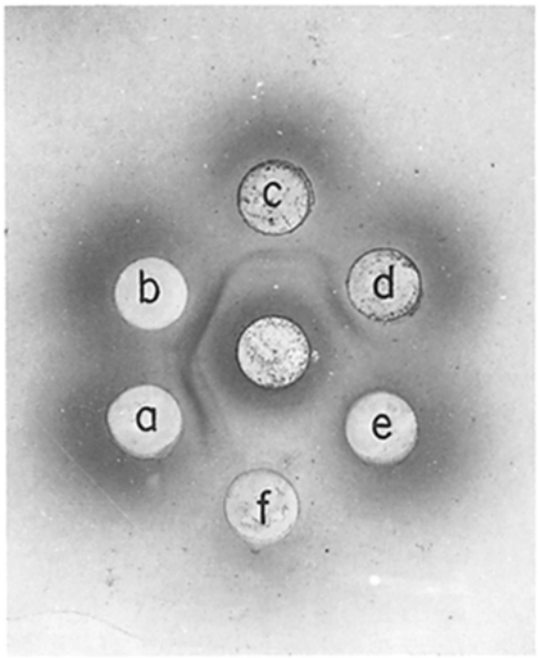

FIGURE 12 Double immunodiffusion of antisera to dog and rat PSD fractions and to rat synaptosomal membrane fraction against extract of dog synaptosomal membrane fraction. Conditions for obtaining the various fractions and the antisera to them are given in Materials and Methods. The center well contains the supernate from a 1% Triton X-100 extract of dog synaptosomal membrane fraction (cf. Fig. 1) after centrifugation at 100,000 *g* for 1 h to rid the fraction of the PSDs. *a* = antirat synaptosomal membrane fraction; *b* = antirat PSD fraction; *c* = antidog PSD fraction; *d* = antidog PSD fraction with the fraction treated with 1% SDS and 1% β-mercaptoethanol before injection; *e* = unrelated serum; *f* = preimmune serum.

the major and even many of the minor proteins are clearly discernible in all the cases. The only variations have been the inconstant appearance of bands ~120,000–150,000 mol wt, some of which are probably contaminants, and the resolution in the 100,000 mol wt region, for reasons unknown. This reproducibility of the gel profiles is even more marked when cognizance is taken of the figures in the following paper (6) where many more experiments are represented.

A morphological estimate of the purity of the fraction is beclouded by the very nature of the PSD. It has no recognizable boundary, such as a mitochondrion, or even a ribosome. It is clear, from descriptions of the synaptic junction complex (8, 40, 61, 64), that that part of the density lying adjacent to the postsynaptic membrane is attached on its cytoplasmic side by filamentous material to what has been called a subsynaptic web having sub-synaptic bodies. The attachment can be seen in some of our isolated densities (Figs. 3, 4*b*, 4*c*, 4*f*), and thus the attached material would seem to correspond to the subsynaptic bodies and subsynaptic web material seen *in situ*. This attachment was verified by making serial sections of the isolated PSD and synaptic membrane fractions, where it was revealed that in one section the material that appeared near the PSD in the cytoplasm was, in the next section, seen to be connected to it by filamentous material (unpublished results). On the basis of these observations, we assume that some of the background material seen in our PSD fraction is this subsynaptic part of the density, some apparently still attached, and some apparently not, possibly due to the plane of the sectioning, as mentioned by Cotman et al. (14). We estimate that about one-half of the material (Fig. 3) is comprised of distinct PSDs and that the remainder is this densely-staining nondescript material, corresponding to the "PSD-like" material mentioned by Cotman et al. (14) which comprised about one-half the amount in their n-lauryl sarcosinate-derived PSD fraction. If this material is part of the PSD, or derived from it, then our PSD fraction is quite pure morphologically, there being no other recognizable subcellular organelles present. At this time, our tentative conclusion is that our PSD preparation is a reflection of the same structure in the cell but that at times the isolated structure seems to be more damaged than at other times. Finally, what we call an intact density is that seen in the thin-section profiles of Fig. 4, or in the flattened disk-shaped structures seen in Figs. 5–7, since these most closely approximate the structures seen *in situ* (cf. 40, 61, 63).

A biochemical estimate of the purity of the fraction can be obtained from the data in Tables I and II, and from the results of chemical analyses, of the gel electrophoresis profiles, and of the radioactive-mixing experiments which are described in the text. Assuming 20 μg of membrane protein per total protein in the PSD fraction (based on the chemical value of 10 μg of phospholipid per mg total PSD fraction protein), then only 2% of the proteins in the fraction are due to visible membrane contamination. This value is higher than the 0.1% value derived from possible myelin membrane contamination (Table II) and the 0.3% value derived from the mitochondrial membrane cytochrome c oxidase data in Table I, but it is lower than the 6–7% value derived from the plasma and/or microsomal membrane 5′-nucleotidase contamination data (Table I), the 3.7% value derived from possible synaptic vesicle and unknown membrane contamination data (Table II), and the 6.5% derived from possible mitochondrial membrane contamination data in Table II. The simplest conclusion is that while visible membrane protein contamination is perhaps as low as 1%, some of the membrane proteins, of varied origin, which are solubilized by Triton X-100 have become separated, during the isolation procedure, into the PSD fraction and cannot easily be washed out of this fraction. Thus, some of the bands of the PSD fraction shown in the gel profile of Fig. 9 are probably not intrinsic PSD proteins. At the present moment, we can say that, of the major bands, only the 17,000 mol wt one is a myelin contaminant, as are some of the minor proteins, such as the 150,000, the inconstant 120,000, the 55,000 and the 59,000 mol wt ones. But we are almost certain that the major 51,000, 45,000, 40,000, 26,000, 18,000 mol wt bands, the bands in the 100,000 mol wt region, and probably the 31,000 mol wt band, are all intrinsic PSD proteins, at least by the experimental criteria which have been set up. The identity of some of these latter proteins will be discussed in the following paper (6). Finally, while we have no evidence as to the carbohydrate content of these proteins, it would appear from results of other laboratories (31, 45, 52) that fractions enriched in PSDs have much less glycoprotein content than synaptic junction complexes or synaptic membrane fractions.

The major band in the gel pattern of our PSD fraction is one representing a protein of 51,000

mol wt. This value is different from the 53,000 mol wt one given by Banker et al. (3) and by Walters and Matus (71), which we think represents a mixture of proteins; however, it is probably similar to the protein having a 52,000 mol wt value given by Kelly and Cotman (45) and to the protein having a 50,000 mol wt value given by Therien and Mushynski (67). A difference between out gel pattern and those of the abovementioned authors is that we found more polypeptides of lower molecular weight than 51,000, some being major bands, though a band at 45,000 mol wt has been described by Kelly and Cotman (45) and Therien and Mushynski (67), while Kelly and Cotman (45) also described a prominent band at 28,000 mol wt, probably corresponding to ours at 26,000 mol wt.

The presence of these lower molecular weight polypeptides may be explained by our solubilization of nearly all the PSD fraction proteins, as far as we could see, by the greater resolution of our discontinuous gel system, and by our use of higher concentrations of acrylamide which would tend to slow the migrations of, and help the resolution particularly of, relatively low molecular weight polypeptides. Another possibility is that the use of n-lauroyl sarcosinate (45, 67) and of 1.2% deoxycholate (53) in the isolation of the density selectively solubilized lower molecular weight components. It could be argued that the presence of low molecular weight proteins in our preparation was due to proteolysis, but Fig. 9, slots 5 and 6, shows that, even when protease inhibitors were present in the isolation media, there resulted the same gel pattern as in their absence. It could also be argued that the low molecular weight polypeptides are artifacts of our Triton X-100 isolated PSDs, with these polypeptides having been adsorbed onto the PSD during the procedure. However, the only subcellular fraction which contained a sizable number of lower molecular weight polypeptides, the mitochondrial fraction, had polypeptides of molecular weight between 26,000 and 13,000, lower than 15,000, and in the 29,000–26,000 mol wt region, and, except for the latter region, these do not appear in our PSD fraction. Furthermore, the radioactive-mixing experiment indicates that these mitochondrial polypeptides do not contaminate our PSD fraction. Tentative identifications of some of the proteins in the PSD fraction will be given in the following paper (6), as well as a tenative conclusion as to the interactions of those proteins with one another to build up the ordered structure of the density.

The synaptosomal membrane fraction from dog brain, from which the PSDs are derived, is similar to preparations obtained by others from brains of guinea pig (46), rat (15, 19, 20, 22, 32, 34, 35, 37, 42, 54, 56), rabbit (38), pig (48), and chick (2, 69). All of these preparations contain a mixture of plasma membranes plus some synaptic vesicle membranes plus membranes of unknown origin, and, in addition, a small number of synaptic complexes or junctions still connected to the neuronal plasma membrane. Some of these surface membranes are derived from glial cells, but it is impossible to tell their proportion in the preparation. In some cases (4, 5, 9, 10, 16, 17, 18, 19, 31, 33, 36, 43, 44, 49, 50, 70, 71, 75), gel electrophoresis was performed on the protein composition of the membrane fraction. In the case of SDS gel electrophoresis where molecular weight determination can be compared, Banker et al. (4) found dominating polypeptides of 41,500, 52,400, and 99,000 daltons, Levitan *et al.* (50) found ones of 42,00 and 53,000, Karlsson et al. (44) found ones of 44,000, 52,000, and 95,000, Hemminki (36) found ones of 45,000, 50,000, and 58,000, Walters and Matus (71) found a major one at 53,000, Gurd (31) found two major ones at 107,000 and 54,000, and Kornguth and Sunderland (49) found a 53,000 one which they labeled tubulin-like. These values are to be compared to our major ones (in descending staining densities) of 52,000, 26,000, 51,000, 31,000, and the bands at 100,000. Our 52,000 mol wt band, which is also the major band in the mitochondrial fraction and probably corresponds to the major protein in the 50,000–53,000 range found by others, is not the β-subunit nor the α-subunit of tubulin, since it can be resolved quite easily from the tubulin subunits on our gel system (see the following paper [6]); the identity of this major protein, in both the synaptic membrane and mitochondrial fractions, is at present unknown.

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