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ISOLATION AND STRUCTURAL STUDIES ON SYNAPTIC COMPLEXES FROM RAT BRAIN

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ABSTRACT

A fraction enriched in synaptic complexes has been isolated from rat brain. The major structural elements of synaptic complexes after isolation are a sector of pre- and postsynaptic plasma membranes joined together by a synaptic cleft and a postsynaptic density (PSD) located on the inner surface of the postsynaptic membrane. On its outer surface, the postsynaptic membrane has a series of projections which extend about halfway into the cleft and which occur along the entire length of the PSD. Proteolytic enzymes at high concentrations remove the PSD and open the synaptic cleft; at low concentrations the PSD is selectively destroyed. By contrast, the structural integrity of the PSD is resistant to treatment with NaCl, EGTA, and low concentrations of urea. Pre- and postsynaptic membranes also remain joined by the synaptic cleft after NaCl, EGTA, or mild urea treatment. High concentrations of urea cause the partial dissociation of the PSD. We conclude that polypeptides are probably one of the major components of the PSD and that the structural integrity of the PSD depends on polypeptides because disruption of the covalent or hydrophobic bonding of these polypeptides leads to a progressive loss of PSD structure.

INTRODUCTION

Synaptic transmission takes place in the region of a specialized structure called the synaptic complex. The synaptic complex consists of a highly specialized part of pre- and postsynaptic plasma membranes joined by the synaptic cleft (Palay, 1958). The presynaptic specialization consists of a hexagonal grid of dense projections extending into the cytoplasm, and the postsynaptic specialization consists of a postsynaptic density subjacent to the postsynaptic plasma membrane. These two plasma membrane specializations are commonly joined together by filaments extending into and possibly across the synaptic cleft.

The detailed role of the synaptic complex (SC)[1] in synaptic function is one of the key un-

solved problems in understanding the communication between neurons. Cytochemical studies have led to the discovery of a number of relatively selective stains for the synaptic complex, which have provided a means to obtain clues on the nature of molecules constituting the SC. Block staining with ethanolic phosphotungstic acid (E-PTA) highlights the specialized region of the synaptic complex, leaving other cellular structures only lightly stained (Bloom and Aghajanian, 1966, 1968; Jones and Brearley, 1972 a, 1972 b); bismuth iodide-uranyl lead ions also

[1] *Abbreviations used in this paper:* BI-UL, Bismuth iodide-uranyl lead; EDTA, ethylenediaminetetra-acetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N'-tetraacetic acid; E-PTA, ethanolic phosphotungstic acid; INT, p-iodonitroneotetrazolium violet; KMnO₄-L, potassium permanganate lead; PSD, postsynaptic density; SC, synaptic complex; SPM, synaptic plasma membrane.

selectively stain SC together with membrane coats (Pfenninger, 1971 *a*, 1971 *b*), and cytochemical studies with these reagents together with selective enzymatic treatment of the tissue have suggested that the synaptic specializations are primarily proteinaceous (Pfenninger, 1971 *a*, 1971 *b*; Bloom and Aghajanian, 1966, 1968). Despite the success of cytochemical studies, the approach is limited in that it is difficult, if not impossible, to evaluate the effectiveness of the enzyme or chemical and to directly identify and characterize the molecules involved.

Recently several important steps have been made toward isolating the synaptic complex in relatively pure form for further structural and chemical study in vitro. The initial breakthrough came from an observation by de Robertis and coworkers (de Robertis et al., 1967) that Triton X-100 selectively released the synaptic complex from adjoining pre- and postsynaptic membranes. This work was confirmed and extended by Davis and Bloom (1970) and Cotman and coworkers (1971) who showed that synaptic complexes could readily and unambiguously be identified by staining with E-PTA both in synaptic plasma membrane (SPM) fractions and after treatment of SPM fractions with Triton X-100. Inclusion of calcium ions was found to aid in optimal preservation of synaptic complexes. In this paper, we extend these earlier studies toward preparing a fraction more enriched in synaptic complexes and characterize the effect of various enzymes on the structure and composition of this synaptic complex fraction.

MATERIALS AND METHODS

Animals

Adult male rats (60–100 days old) of the Sprague-Dawley strain provided the brain tissue. In the initial experiments, excised cerebral cortex was used whereas in later experiments rat "forebrain" was routinely employed. Forebrain was separated from cerebellum and midbrain by transecting the brainstem ahead of the superior colliculus. The yield of brain was approximately 1 g per animal.

Reagents

Glass-distilled water was used in preparation of all solutions. Analytical grade sucrose was obtained from Mallinkrodt Chemical Works, St. Louis, Mo. All sucrose solutions were adjusted to pH 7.0 with the minimum required amount of sodium hydroxide.
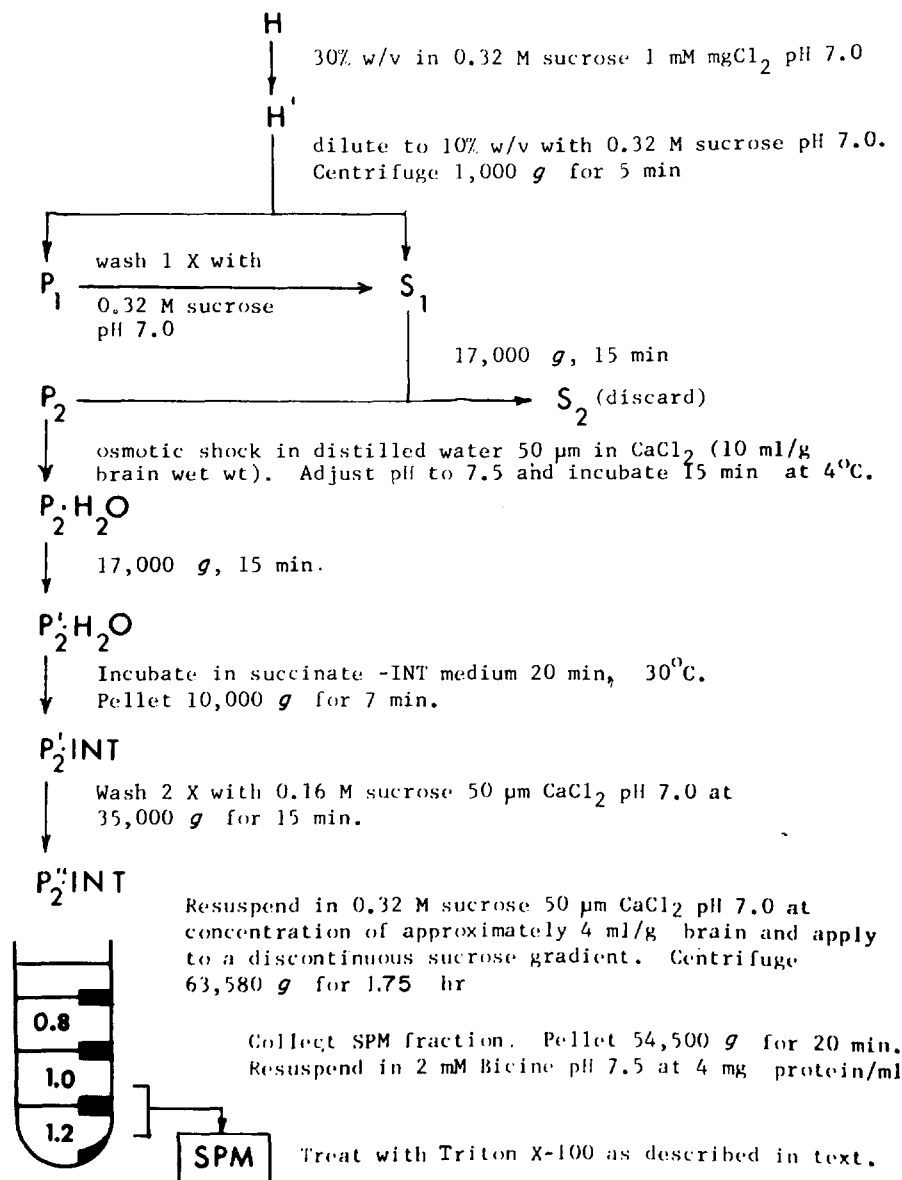
p-Iodonitroneotetrazolium violet ({(*p*-iodophenyl) 3-*p*-nitrophenyl-5-phenyl etrazolium chloride}, grade I), cytochrome *c* (type III), Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.), trypsin (bovine pancreas type I), and bromalin (type II) were obtained from Sigma Chemical Co., St. Louis, Mo. α-Chymotrypsin (bovine pancreas, 52.9 units/mg) was purchased from Worthington Biochemical Corp., Freehold, N. J. Glutaraldehyde (electron microscope grade) was obtained from Polysciences, Inc., Rydal, Pa., sodium phosphotungstic acid from Fisher Scientific Company, Fair Lawn, N. J.

Preparation of Subcellular Fractions

A synaptic plasma membrane (SPM) fraction freed from mitochondria and most nonsynaptic membrane served as source of synaptic complexes. Synaptic complex fractions were prepared from SPM by treating this membrane fraction with Triton X-100 and subfractionating the insoluble residue on a sucrose density gradient to resolve synaptic complexes from the majority of other insoluble material. The method described is based on the procedure of Davis and Bloom (1970).

Rat forebrains were rapidly removed and placed in ice cold 0.32 M sucrose, pH 7.0, containing 1 mM $MgCl_2$. Homogenates (30% w/v) were made in 0.32 M sucrose 1 mM $MgCl_2$, using a Teflon glass homogenizer (AH Thomas size C) driven at 600 rpm.

The procedure to isolate a synaptic plasma membrane fraction is illustrated in Scheme 1. The homogenate was diluted to 10% w/v with 0.32 M sucrose, pH 7.0, and centrifuged at 1000 g for 5 min in a Beckman type 30 rotor. The supernant (S_1) was saved and the pellet was washed once with 0.32 M sucrose, pH 7.0. The resulting supernants from initial centrifugations were combined and pelleted at 17,000 g for 15 min to produce a crude mitochondrial fraction (P_2). This mitochondrial fraction was osmotically shocked with 10 ml of distilled water containing 50 μm $CaCl_2$/g brain, homogenized by hand, and the pH was adjusted to 7.5 with NaOH. After 15 min the osmotically shocked crude mitochondrial fraction ($P_2' \cdot H_2O$) was pelleted at 17,000 g for 15 min. The resulting pellet ($P_2' \cdot H_2O$) was resuspended in a minimum volume of 50 μm $CaCl_2$ and 10 vol/g initial brain weight of a solution containing 60 mM succinate, 1 mM *p*-iodonitroneotetrazolium violet (INT)M, 40 mM Na phosphate buffer, pH 7.5, and 50 μm $CaCl_2$. The suspension was incubated at 30°C for 20 min, at which time it was pelleted at 10,000 g for 7 min. The pellet (P_2'-INT) was resuspended in 0.16 M sucrose 50 μm $CaCl_2$ pH 7.0 and centrifuged at 35,000 g for 15 min to remove trapped salts which interfere with the separation on the density gradient. The pellet was

H

$\downarrow$

30% w/v in 0.32 M sucrose 1 mM mgCl$_2$ pH 7.0

H'

$\downarrow$

dilute to 10% w/v with 0.32 M sucrose pH 7.0.
Centrifuge 1,000 g for 5 min

P$_1$ — wash 1 X with $\longrightarrow$ S$_1$
0.32 M sucrose
pH 7.0

17,000 g, 15 min

P$_2$ $\longrightarrow$ S$_2$ (discard)

$\downarrow$ osmotic shock in distilled water 50 µm in CaCl$_2$ (10 ml/g
brain wet wt). Adjust pH to 7.5 and incubate 15 min at 4°C.

P$_2$·H$_2$O

$\downarrow$ 17,000 g, 15 min.

P$_2'$H$_2$O

$\downarrow$ Incubate in succinate -INT medium 20 min, 30°C.
Pellet 10,000 g for 7 min.

P$_2'$INT

$\downarrow$ Wash 2 X with 0.16 M sucrose 50 µm CaCl$_2$ pH 7.0 at
35,000 g for 15 min.

P$_2''$INT

Resuspend in 0.32 M sucrose 50 µm CaCl$_2$ pH 7.0 at
concentration of approximately 4 ml/g brain and apply
to a discontinuous sucrose gradient. Centrifuge
63,580 g for 1.75 hr

0.8
1.0
1.2

Collect SPM fraction. Pellet 54,500 g for 20 min.
Resuspend in 2 mM Bicine pH 7.5 at 4 mg protein/ml

SPM   Treat with Triton X-100 as described in text.

SCHEME 1   Isolation of synaptic plasma membrane (SPM) fraction.

washed one additional time with 0.16 M sucrose, 50 µm CaCl$_2$ and resuspended in 0.32 M sucrose, 50 µm CaCl$_2$ at a concentration of about 4 ml/g brain. 5 ml of this suspension was applied to a sucrose density gradient designed for the SW 25.1 rotor which consisted of 5 ml each of 0.8 M, 1.0 M, 1.2 M sucrose, 50 µm CaCl$_2$, pH 7.0, or 9 ml of the suspension was applied to a discontinuous gradient consisting of 12 ml each of 0.8 M, 1.0 M, 1.2 M sucrose, 50 µm CaCl$_2$, pH 7.0, designed for the SW 25.2 rotor. The gradients were centrifuged at 63,580 g

for 1.75 hr (SW 25.1) or 75,465 g for 1.5 hr (SW 25.2). The SPM fraction which banded at the 1.0 M–1.2 M sucrose interface was collected, diluted with 3 vol of 50 µm CaCl$_2$, pH 7.0, and pelleted at 54,500 g for 20 min, and resuspended in 2 mM Bicine (N,N-bis{2-hydroxyethyl} glycine) buffer, pH 7.5, at a protein concentration of 4 mg/ml.

The SPM fraction was treated with Triton X-100 to achieve solubilization and disruption of non-synaptic complex membrane elements. A defined quantity of Triton X-100 was added per milligram

of membrane protein as specified in the Results section as a solution consisting of 4 mg/ml Triton X-100, 2 mM ethylenediaminetetraacetic acid (EDTA), and 2 mM Bicine, pH 7.5. The Triton X-100 solution was slowly added to the membrane suspension (4 mg protein/ml) while stirring the mixture. The temperature was maintained at either +4°C or room temperature as described in the Results section. After 10 min at the appropriate temperature, the suspension was applied to a sucrose gradient consisting of 4 ml each of 1.0 M, 1.2 M, 1.4 M, and 1.5 M sucrose 50 μm in CaCl₂, pH 7.0, and centrifuged at 63,580 g for 1.25 hr in SW 25.1 rotor. It was essential to be certain that the various gradient interfaces were not sharp because particles below 1 M sucrose aggregated at sharp discontinuities in the gradient. The bands which formed at the various interfaces were separated with a tube cutter, diluted with water, and pelleted at 100,000 g for 30 min. The time required to reach the final gradient fractions from sacrificing the animals was approximately 16 hr.

Electron Microscopy

Samples were prepared for electron microscopy by fixation with 4% glutaraldehyde in Caulfield buffer (Caulfield, 1957) in suspension for 30 min, pelleted, and postfixed with 1% osmium tetroxide in Caulfield buffer. To ensure representative sampling of fractions, small pellets consisting of 0.15–0.30 mg of particle protein were prepared, embedded flat, and cross-sectioned through the entire thickness of the pellet in a manner similar to that previously described (Cotman and Flansburg, 1970). After fixation, the samples were stained with uranyl acetate in Kellenberger buffer (Kellenberger et al., 1958) overnight, dehydrated in graded ethanols and propylene oxide, and embedded in Epon-Araldite or Maraglas. Samples to be treated with bismuth iodide or E-PTA were not postfixed with osmium tetroxide and were not stained with uranyl acetate before embedding in plastic. E-PTA staining was carried out as previously described (Cotman et al., 1971). Sections were cut on a LKB III ultramicrotome and stained in one of the following ways: lead citrate for 5 min (Venable and Coggeshall, 1965), or 1% potassium permanganate (Glauert, 1965) for 1–2 min followed by lead citrate, or bismuth iodide (Pfenninger, 1971 a) followed by uranyl acetate and lead citrate. The staining procedure used for each sample is specified in the text. E-PTA samples were examined directly without further staining. Sections were photographed in either a Zeiss EM 9S electron microscope operated at an accelerating voltage of 60 kv or Siemens 1A electron microscope at 80 kv. Survey micrographs were taken at both edges and the center of the section in

order to sample the top, bottom, and center of the pellet. Samples requiring an initial magnification of 20,000 times or greater were photographed in the Siemens microscope.

Enzymatic and Chemical Treatment of SC Fractions

For study of the effect of proteolytic enzymes, SC fractions were prepared using 1 mg of Triton to 1 mg of SPM protein. In most experiments, all particles sedimenting through 1.0 M sucrose after Triton treatment were used; in a few cases the 1.2–1.4 M fraction was used.

SC fractions were treated with proteolytic enzymes as follows: An aliquot of the SC fraction was resuspended in 1 ml of 0.05 M Tris, pH 7.0, at a protein concentration of 0.2–0.3 mg/ml. Trypsin, α-chymotrypsin, or bromalin was added at a concentration ranging from 1 to 40 μg enzyme/mg SC fraction protein as specified in the Results section. SC fractions were incubated at 37°C for 20 min, at which time the samples were cooled to +4°C, glutaraldehyde fixative was added, and the fractions were pelleted at 100,000 g for 0.5 hr. Duplicate samples were used to determine the extent of proteolytic action and treated identically except that glutaraldehyde was not added. The residual protein content in the pellet was assayed and compared to controls incubated without proteolytic enzymes. To evaluate the effect of more extensive proteolytic action, SC fractions were resuspended in 1 ml of 0.1 M Hepes buffer, pH 7.4, at a protein concentration of 0.40 mg/ml. Trypsin or chymotrypsin was added at a concentration of 65 μg/mg SC fraction protein and the sample was incubated for 3 hr at 37°C before addition of glutaraldehyde. The fractions were pelleted at 100,000 g for 1 hr.

SC fractions were treated with 1 M urea, 8 M urea, 50 mM ethylene glycol bis(β-aminoethylether) N,N'-tetraacetic acid (EGTA) and 1 M NaCl in 0.05 M Tris buffer, pH 7.3, for 2 hr at room temperature at a protein concentration of 0.3 mg/ml. Control samples were incubated in 0.05 M Tris, pH 7.3. To study the effect of sonication, samples were sonicated for 10 sec at a setting No. 3 in a Branson Instruments sonifier fitted with a microtip.

RESULTS

The Isolation of Synaptic Complex Fractions

Synaptic complexes (SC) were isolated from a fraction of synaptic plasma membranes (SPM) treated with Triton X-100 by centrifugation of the Triton-treated fraction on a sucrose density gradient. The synaptic plasma membrane fraction

which served as starting material for preparation of SC was prepared from the crude mitochondrial fraction based on the succinic dehydrogenase-iodonitroneotetrazolium procedure essentially as described by Davis and Bloom (1970). This method of preparing SPM increases the buoyant density of mitochondria so that SPM can be collected at heavier densities essentially free of mitochondrial contamination. The technique involves the succinic dehydrogenase-catalyzed deposition of an insoluble dense formazan within the interior of the mitochondrion which results in a dramatic increase in mitochondrial density. More than 95% of the mitochondria are removed from synaptic membrane fractions as determined by the low levels of cytochrome oxidase activity measured. As shown in Table I, the specific activity of cytochrome oxidase in the synaptic plasma membrane fraction used for purification of SC (1.0–1.2 M fraction) is less than $\frac{1}{50}$ that of the 1.2 M fraction, which is an impure preparation of brain mitochondria. Electron microscope analysis showed that, like fractions of SPM prepared by other methods, this fraction contained numerous SC which can be seen in OsO_4-fixed material or highlighted by E-PTA staining. In view of the relative absence of mitochondria and the presence of synaptic complexes, this

TABLE I

Distribution of Cytochrome Oxidase Activity in the Sucrose Density Gradient Used to Isolate an SPM Fraction

Fraction	Cytochrome oxidase	
	Specific activity*	% of total‡
Homogenate	0.275, 0.178	
0.8 M	0.005, ND§	0.5, 0.0
0.8–1.0 M	0.018, ND	0.6, 0.0
1.0–1.2 M	0.007, 0.024	0.6, 2.2
1.2 pellet	0.680, 0.740	98.3, 97.8

Data are from two experiments. Sucrose concentrations designate fraction banding at interface of 0.8–1.0 M sucrose, 1.0–1.2 M, or above 0.8 M sucrose. The fraction at the 1.0–1.2 M sucrose interface (SPM) was used as starting material to isolate synaptic complex fractions.

* μmoles of reduced cytochrome c oxidized/min per mg protein.

‡ % recovery from gradient input for cytochrome oxidase activity was 40–50%.

§ ND, not detectable.

SPM fraction, which can be obtained relatively quickly and in reasonable yield, provides a suitable material for further purification of SC. The yield of the 1.0–1.2 fraction is about 3 mg/g brain wet weight.

Addition of Triton X-100, at a Triton-to-protein ratio of 1 mg of Triton to 1 mg membrane protein, solubilizes 60% of the protein, and in the presence of small quantities of bound calcium leads to selective solubilization of nonjunctional membrane while preserving synaptic complexes. When the resulting suspension is applied to a sucrose density gradient and centrifuged to isopycnic conditions, the particles separate into two classes. Some distribute at heavier and some at lighter densities than membrane not treated with Triton. This effect is illustrated in Fig. 1. In the absence of Triton treatment, membrane rebands between 0.96–1.35 M sucrose with the major peak at 1.20 M sucrose; after Triton treatment of this membrane fraction, the major peak is lost and particles band between 0.52 and 1.58 M sucrose. Based on studies of continuous gradients, a discontinuous gradient consisting of four steps (1.0, 1.2, 1.4, 1.5 M sucrose 50 μm $CaCl_2$, pH 7.0) was selected and used for most subsequent studies. On the discontinuous gradient the distribution of protein is shown in Table II. Approximately 50% of the total insoluble protein is lighter than 1.0 M sucrose, 20% rebands at the same isopycnic density as before Triton treatment, and the remainder sediments to heavier densities.

Morphological examination showed that the composition of the fractions fell into two general classes. The fraction above 1.0 M sucrose contained many free membrane fragments but few synaptic complexes. All other fractions banding below 1.0 M sucrose, contained many synaptic complexes. SC can be identified by their characteristic morphology in fractions fixed with glutaraldehyde-OsO_4 and stained with uranyl lead ions (G, OsO_4-UL) or by their selective staining in glutaraldehyde-fixed fractions, stained with E-PTA. Fig. 2 illustrates the composition of a G, OsO_4-UL prepared sample collected from the pellet of a gradient consisting of 1.0 M sucrose. Many SC which appear as dense barlike structures are seen (arrows), together with fragments of membrane. The prominent darkly staining component of the SC is the postsynaptic density (PSD). As reported previously (Davis and Bloom,
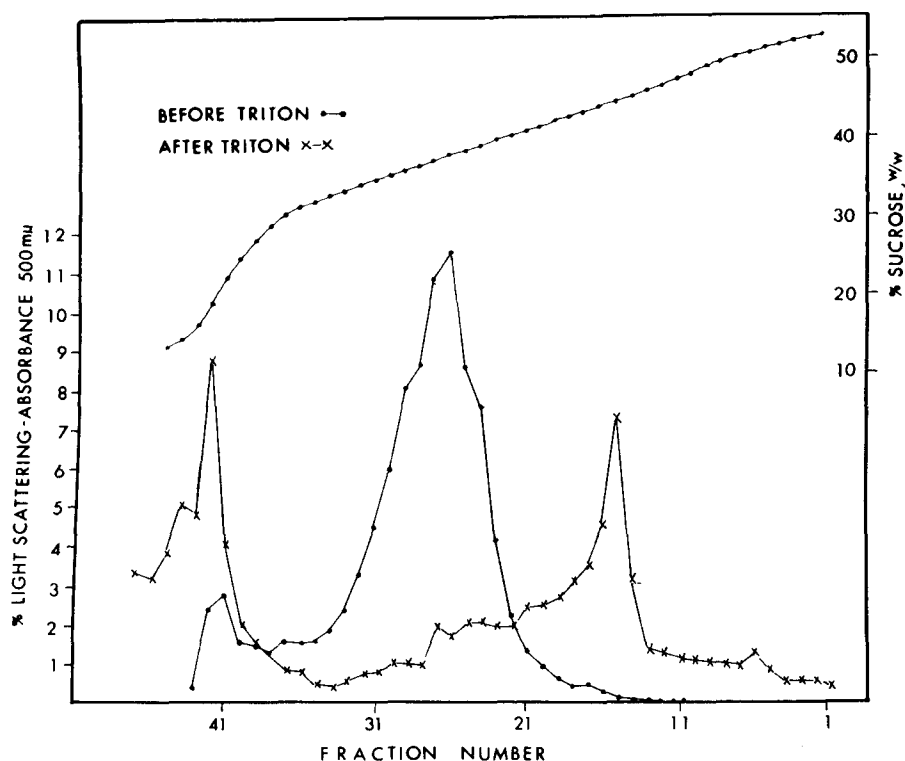
FIGURE 1 Light-scattering optical density profile of SPM fraction before and after solubilization with Triton X-100 on a 25–55% w/w continuous sucrose gradient. Before Triton treatment, 80% of the material bands between densities of 1.13 g/cc (0.96 M sucrose) and 1.18 g/cc (1.35 M sucrose) with the main peak at 1.16 g/cc (1.20 M sucrose). After Triton treatment, the peak at 1.16 g/cc is lost and the material is spread to both lighter and heavier densities. A peak is seen at 1.07 g/cc (0.52 M sucrose) and 1.21 g/cc (1.58 M sucrose).

1970; Cotman et al., 1971) and illustrated in Fig. 3, the SC can be identified by staining with E-PTA. The prominent PSD stains and appears as a barlike structure (arrows). In these preparations and as shown previously (Cotman et al., 1971), E-PTA stains a fine intercleft line characteristic of SC, together with dense projections in some SC. Bismuth iodide-uranyl lead ions, which are relatively selective stains for SC (Pfenninger, 1971 a), also highlight the SC in these fractions.

On a multi-step discontinuous gradient consisting of four steps of 1.0, 1.2, 1.4, and 1.5 M sucrose (50 μm CaCl₂, pH 7.0), fractions banding at the interfaces between 1.0 M and 1.2 M, 1.2 M and 1.4 M, 1.4 M and 1.5 M sucrose and pelleting through 1.5 M sucrose all contained numerous SC as determined by electron microscopic analysis. In general, it appeared that the quantity of free membrane decreased with increasing sucrose concentrations while, conversely, there was an

increase in number of contaminating mitochondrial fragments, although the latter contamination made up only a few per cent of the fractions. A large number of SC in the more dense fractions appeared to be detached from adjacent plasma membrane.

Fractionation on a discontinuous gradient showed that the distribution of membrane fragments and synaptic complexes depends on the precise solubilization conditions, including temperature during addition of Triton and the quantity of Triton added. Solubilization at room temperature (26°C) released essentially the same quantity of protein (60%) but shifted the distribution of insoluble protein in density gradients to heavier densities. This shift is illustrated by the data shown in Table III. When solubilization is carried out at 26°C, the two dense fractions contain 27% of the total protein, whereas when solubilization is carried out at

TABLE II

The Distribution of Protein in Different Fractions after Triton X-100 Solubilization of SPM Fraction

Fraction	% of total protein
M	
1.0	50.2 ± 2.3
1.0–1.2	20.5 ± 4.0
1.2–1.4	14.2 ± 1.3
1.4–1.5	3.5 ± 2.0
1.5	11.0 ± 1.0

Solubilization was carried out at 4°C at a Triton-to-protein ratio of 1:1. The per cent of recovery in the gradient for protein was 90–100. Data are mean ± average deviation taken from four experiments. Fractions are designated by sucrose concentrations where fractions band. The 1.0 M fraction is the particulate material sedimenting above 1.0 M sucrose; the 1.0–1.2 M, 1.2–1.4 M, and 1.4–1.5 M fraction is the material sedimenting at the interface of the sucrose layers; and the 1.5 M fraction the pellet.

+4°C these fractions contain only 14% of the total particulate protein. Electron microscope analysis showed that when solubilization is carried out at 26°C, the fractions more dense than 1.2 M sucrose contained abundant synaptic complexes, most of which showed very little adjacent plasma membrane. More synaptic complexes appear to be released from adjacent membrane and to sediment to heavier densities under these conditions.

Increasing the Triton-to-protein ratio to 2:1 increased solubilization of membrane protein to 70% when carried out at +4°C while still preserving relatively undamaged synaptic complexes. On a discontinuous sucrose gradient after solubilization at a triton to protein ratio 2:1, the distribution of protein shifted to higher densities (Table III). Whereas at a Triton-to-protein ratio of 1:1 approximately 11% of total insoluble protein pelleted through 1.5 M sucrose, at a Triton-to-protein ratio of 2:1 50% of total insoluble protein pelleted through 1.5 M sucrose. Under these conditions with increased Triton, the 1.5 M pellet contained many SC as determined by electron microscopy. Other fractions more dense than 1.0 M sucrose also contained SC.

A rapid suitable preparation of synaptic complexes can be obtained by using a Triton-protein ratio of either 1:1 or 2:1 and pelleting over 1.0 M sucrose 50 μm $CaCl_2$, pH 7.0, from 1.25 hr at

68,000 *g*. Using this procedure, the yield of the synaptic complex fraction is approximately 0.10–0.15 mg of protein/g brain wet weight. The SC fraction prepared at a Triton-to-protein ratio of 1:1 contains more contaminating membrane than that prepared at a Triton-to-protein ratio of 2:1, whereas the SC fraction isolated at a higher Triton concentration is somewhat purer but more structurally damaged.

High Resolution Structure of SC

A high magnification photograph of a cluster of isolated SC is shown in Fig. 4 *a*. The major structural elements of synaptic complexes after isolation are a prominent postsynaptic plasma membrane (Ps) with a PSD and often including an attached sector of presynaptic membrane (Pr). Commonly, the postsynaptic plasma membrane is vesicularized into a small closed vesicle just large enough in size to enclose the postsynaptic density. The PSD appears rigid in well-preserved complexes since it is rarely bent back upon itself or otherwise distorted; its barlike structure is well maintained.

The details of the synaptic cleft can be best defined where only a small sector of presynaptic membrane remains attached (Fig. 4 *c*, 4 *d*). Adjacent to such attached presynaptic membrane, the postsynaptic membrane shows a series of knobs or bristles (arrows) which project from it. These projections are regularly spaced and extend about 70–100 A from the postsynaptic membrane into the cleft; they follow quite closely the entire distance of the PSD and are not seen beyond it. A comparable structure is not evident coming from the presynaptic plasma membrane once detachment has occurred. In intact complexes, fibers extend from the presynaptic membrane up to or into the postsynaptic projections. Currently, resolution is not adequate to determine whether these structures terminate at the postsynaptic plasma membrane projection or at the postsynaptic membrane itself. The PSD is not a uniformly electron-opaque structure, but seems to consist of an array of globular and fibrous elements (Fig. 4 *b*). Small circles measuring 70–90 A in diameter with a relatively electron-transparent center can be seen as a substructure of the PSD matrix in these fractions.

Morphological examination of isolated SC also emphasizes one of their properties which is not evident from in vivo studies. In isolated
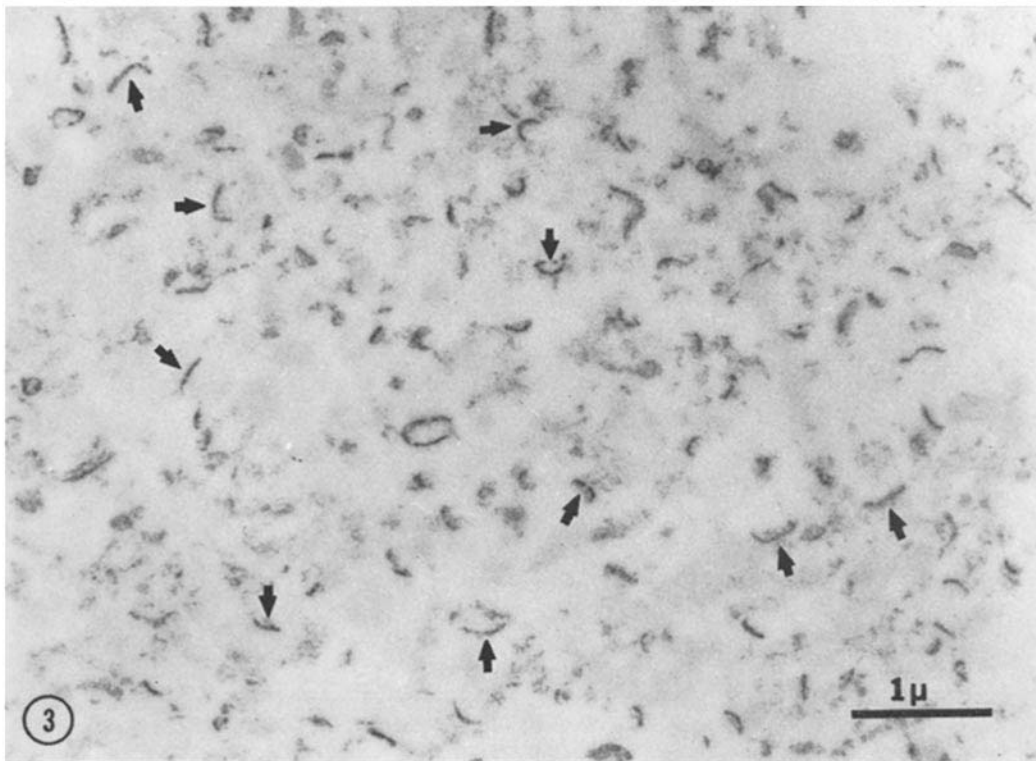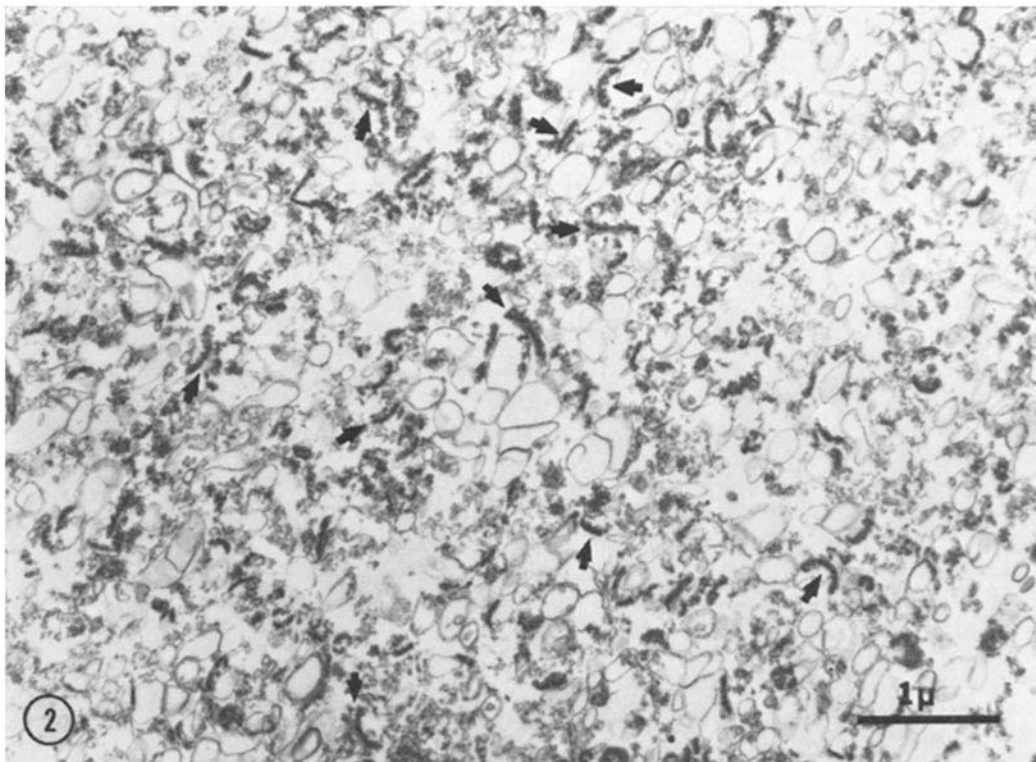
FIGURE 2   Survey electron micrograph of synaptic complex fraction collected from the pellet of a 1.0 M sucrose gradient. The dark barlike structures (arrows) are the postsynaptic density portion of a synaptic complex. × 18,400.

FIGURE 3   Survey electron micrograph of a synaptic complex fraction stained with E-PTA. Numerous synaptic complexes are seen (arrows). The prominent feature which stains is the PSD. × 18,400.

TABLE III

Distribution of Total Protein in Different Fractions after Triton Solubilization under Conditions where the Triton-to-Protein Ratio (T/P) and Temperature were Varied during the Solubilization

Fraction	% of total protein		
	T/P 1:1		T/P 2:1, + 4°C
	+ 4°C	+ 26°C	
M			
1.0	50.2 ± 2.3	46.3 ± 3.5	26.5 ± 2.4
1.0–1.2	20.5 ± 4.0	17.2 ± 0.1	8.4 ± 3.0
1.2–1.4	14.2 ± 1.3	8.6 ± 1.7	7.3 ± 1.6
1.4–1.5	3.5 ± 2.0	6.5 ± 0.5	5.0 ± 3.2
1.5	11.0 ± 1.0	21.1 ± 0.2	52.0 ± 1.5

Nomenclature for fractions is as described in Table II.

fractions SC are frequently seen in contact with one another. Often apparent arrays of SC are found such as in Fig. 4 where a number of SC are interconnected. The PSD can be found attached to presynaptic membranes of other complexes and to other PSD. Also, some PSD contain a membrane fragment (M) embedded in the PSD matrix which appears to have been adsorbed by the matrix. During isolation, SC fractions display certain adhesive properties; they stick to themselves at sharp discontinuities in a density gradient and resist easy resuspension. All these observations point to a high degree of adhesiveness in these structures.

Effect of Some Proteolytic Enzymes on SC Structure

To learn more about the structural organization and composition of SC, we incubated SC with various chemicals and enzymes and evaluated the effect of these agents on the ultrastructure of SC. We tested the effect of proteolytic enzymes (trypsin, chymotrypsin, and bromalin) on SC, particularly with regard to the postsynaptic density. On duplicate samples, we measured the amount of protein released from the pellet to determine the effectiveness of proteolytic digestion. All three proteolytic enzymes had similar general effects, but differed in details. Proteolytic enzymes at very low concentrations were quite destructive, particularly to the PSD.

Trypsin at 7 μg/mg SC protein incubated at 37°C for 20 min caused considerable damage; under these conditions, 27% of the insoluble protein is released. As shown in Fig. 5, the postsynaptic density was damaged (arrows) and it appears spread and thinned out. The PSD still retains a discrete structure and gives the impression of small circles interdispersed in a network of undefined material. Close examination of background material shows that much of it is attached to small membrane fragments. In contrast to the marked effect on the postsynaptic density, the synaptic cleft retains enough structure after trypsin treatment to hold the pre- and postsynaptic plasma membranes together. A number of synaptic clefts can be seen where a presynaptic membrane fragment is attached to a postsynaptic plasma membrane fragment (Fig. 6). When higher concentrations of trypsin are used for longer incubation times (65 μg trypsin/mg protein, 37°C 3 hr), 70% of the protein of the SC fraction was solubilized and essentially all recognizable SC were destroyed. The pellet contained only membrane fragments with a minimum of background material (Fig. 7). Centrifugation of the resulting supernatant for 12 hr at 100,000 g did not reveal any sedimentable particulate material. Identical morphological results were obtained in fractions fixed with glutaraldehyde-osmium tetroxide and stained with uranyl lead (UL) or permanganate-lead (KMnO₄-L) stains. We also examined fractions treated with trypsin and stained with bismuth iodide uranyl lead (BI-UL) as described by Pfenninger (1971 a). Whereas untreated SC fractions showed an affinity for BI-UL, only a sparse amount of background material was evident in trypsin-treated samples where 70% of the protein was solubilized.

The ultrastructural effects of chymotrypsin were less than those of trypsin when the solubilization of protein was equivalent. Chymotrypsin at 7 μg/mg SC protein incubated at 37°C for 20 min released 25% of the SC protein. The structure of SC, particularly the PSD, was damaged but to a lesser extent than with equivalent trypsin treatment (Fig. 8). PSD are not thinned to the extent seen with trypsin, and considerably more background material, probably originating from PSD, was evident. Under conditions where 70% of SC protein is removed from the pellet by chymotrypsin, recognizable SC are not found

in the pellet whether glutaraldehyde-osmium tetroxide-fixed material is stained by UL or KMnO₄-L.

Bromalin at low concentrations (7 μg/mg SC protein incubated at 37°C for 20 min) effected less solubilization of protein (9%) than an equivalent amount of trypsin or chymotrypsin. Increasing the bromalin concentration to 40 μg/mg protein and incubating for 20 min at 37°C produced the same degree of solubilization (25–27%) of the SC fraction as 7 μg/mg protein of trypsin or chymotrypsin. At an ultrastructural level, the effect of this bromalin treatment was about the same as trypsin. As shown in Fig. 9 *a*, PSD (D) essentially disappeared while some background material persisted. A number of SC retain a sector of presynaptic plasma membrane (Pr). After bromalin treatment, sections stained with KMnO₄-L in glutaraldehyde-osmium tetroxide-fixed fractions revealed particularly effectively the projections extruding from the postsynaptic membrane. These projections (J) were evident, even though most of PSD was removed (Fig. 9 *a*, *b*).

Effect of Various Chemicals on SC Structure

The effects of three chemical agents (NaCl, urea, EGTA) on SC structure were studied to evaluate the role of different stabilizing forces on the structural integrity of SC. Using these agents, it should be possible to alter ionic interactions, hydrophobic interactions, and coordination complexes involving calcium in a relatively independent manner. NaCl at concentrations of 1 M produced relatively little effect on PSD or the attachment of pre- and postsynaptic membranes. Numerous examples of intact SC were apparent. Likewise, urea at 1 M had little effect. Small structural changes may have occurred, but both intact thickenings and attached pre- and postsynaptic membranes were easily found. Sonication of SC in the presence of 1 M urea was more effective in dissociating SC. Three major structural changes were brought about by sonication in 1 M urea: (*a*) the size of each individual particle in the fraction was reduced; (*b*) pre- and postsynaptic membranes became disarrayed and examples of intact synaptic clefts were rare; (*c*) the PSD, although clearly recognizable, lost its characteristic dense barlike appearance and became partly dissociated into a weblike matrix. The appearance of the PSD after 1 M urea-

sonication treatment is shown in Fig. 10 *a*, *b*. This treatment reveals that the PSD is comprised of an interlacing network of fibers (arrows) separated by open spaces within the PSD (Fig. 10 *a*). Incubation of SC in 8 M urea had a similar effect on SC structure as sonication in 1 M urea. PSD densities were dispersed and synaptic clefts were very rare. Neither urea treatment released detectable quantities of protein. EGTA has a powerful and selective ability to sequester Ca⁺². At an ultrastructural level, incubation in 50 mM EGTA produces only minimal disruption of SC structure and the SC appeared much like the control. Occasionally the presence of fibers which measure 20–40 A could be seen spinning off intact SC.

DISCUSSION

In this paper we have described a procedure for obtaining a fraction enriched in synaptic complexes. Synaptic complexes are identified by selective staining with E-PTA or with bismuth iodide-uranyl lead staining as well as by their distinctive morphology in OsO₄-fixed material. The major contaminant is plasma membrane, most of which exists as a short extension of the complex. In addition to segments of plasma membrane, the postsynaptic densities are the most prominent component of the SC fraction. The actual amount of material in the fraction belonging to the complex itself is difficult to assess from electron micrographs.

The structure of isolated synaptic complexes closely resembles that of a Gray type I (Gray 1959) seen in intact tissue. SC have a clearly defined, relatively thick postsynaptic density with a presynaptic membrane fragment attached across the synaptic cleft measuring approximately 150 A. Examination of the cleft, particularly when the cleft is dissociated in some SC, showed that part of the cleft structure is a series of projections originating from the postsynaptic membrane and extending approximately halfway into the cleft. A comparable set of projections has not been seen extending into the cleft from the presynaptic membranes, and it is at present not clear exactly what detailed attachment system is used. Our data on the structure of the cleft agree with the observations of Van der Loos (1963) who described a set of projections of postsynaptic origin which extend to approximately the center of the synaptic cleft. Our data also provide clues on the substructure of the PSD.

In our fractions, the PSD contains fibers and, in addition, seems to also have a number of small circular structures. Sotelo (1971) has observed circular substructures of a similar size in the PSD of pleurodele cerebellar synapses, and it is possible that similar substructures of the PSD are revealed in our fractions. Also, Sandri and coworkers (1972) have reported granular particules in the PSD of freeze-etched synapses which are similiar in size to the elements observed in isolated SC. At present, however, we cannot rule out the possibility that the circular elements arise from artifacts of the isolation procedure or the electron microscopy.

The nature of the molecules constituting the PSD is of particular interest in view of the distinctive types of PSD in cerebral cortex and the potential importance of this structure in synaptogenesis and synaptic transmission. Cytochemical studies have pointed towards the PSD being proteinaceous in nature, which is compatible with their specific staining properties (Bloom and Aghajanian, 1966, 1968; Pfenninger, 1971 a). They are sensitive to proteolytic enzymes and resist treatment with RNase, DNase, collagenase,

hyaluronidase, and neuraminidase (Bloom and Aghajanian, 1966, 1968; Pfenninger, 1971 b). Our own work extends this approach to in vitro conditions where (a) the proteolytic action can be quantitated, (b) the reactants are not diffusion limited, and (c) the use of fixatives before proteolysis is avoided. Our studies strongly support the conclusion that proteins are a major structural component of the PSD. We investigated the action of trypsin, chymotrypsin, and bromalin on SC fractions. We find that SC, particularly the PSD, is damaged by proteolytic action, with the degree of structural integrity depending primarily on the extent of proteolytic action. When the extent of proteolysis is equivalent, all three enzymes studied give approximately comparable results. Minor but definite differences are observed, particularly with chymotrypsin which is less effective in catalyzing the breakdown of the PSD than trypsin or bromalin.

Our data agree in most respects with those of Bloom and Aghajanian (1966, 1968) and Pfenninger (1971 b). One major exception is in the sensitivity of the PSD to trypsin. Both Bloom and Aghajanian and Pfenninger observed that trypsin

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Figure 4 A high magnification electron micrograph of isolated synaptic complexes. (a) A prominent postsynaptic density (PSD) is seen attached to a fragment of postsynaptic plasma membrane (Ps) which in some cases is attached to a sector of presynaptic membrane (Pr) via a synaptic cleft (C). The interconnection of membranes and PSD in this cluster shows the adhesive properties of the synaptic complex. The PSD in the upper left-hand corner contains a membrane fragment (M) on both sides of the PSD. Directly below is a membrane vesicle which is attached to two other PSD. × 76,000. (b) Detail of a PSD selected to illustrate the substructure of the PSD seen in isolated synaptic complexes. Some densely staining circles measuring about 70–90 A with a relatively electron-transparent center are seen (arrows). These circular (or spherical) structures appear to be embedded in a fibrous matrix and together make up the PSD. × 224,000. (c, d) Details of the synaptic cleft. In those complexes which have a small attached sector of presynaptic membrane, or where presynaptic membrane is detached, it is common to see a series of projections (arrows) which are attached to the postsynaptic membrane adjacent to the sector of presynaptic membrane. These projections or bristles are tightly spaced and extend to about the center of the cleft. × 224,000. Glutaraldehyde-OsO4 fixation followed by uranyl lead staining as described in Methods (G, OsO4-UL).

Figure 5 Micrograph of a synaptic complex fraction after mild trypsinization where 27% of the protein from the fraction is solubilized. The PSD is disarrayed. Both pre- and postsynaptic membranes have remained attached in one of the SC by way of a synaptic cleft (C). The substructure of the PSD contains discrete electron-opaque elements, some of which take on a circular appearance (arrows). × 120,000; G, OsO4-UL.

Figure 6 A high magnification micrograph after trypsin treatment, showing a complex where pre- and postsynaptic membranes have remained attached (C), but postsynaptic density (PSD) is almost completely destroyed. × 224,000. Fixation and staining are as is described in Fig. 5.

Figure 7 Micrograph of a synaptic complex fraction after extensive trypsinization where 70% of the protein of the fraction is solubilized. Membrane fragments, but not SC, remain. × 24,000; OsO4-UL.
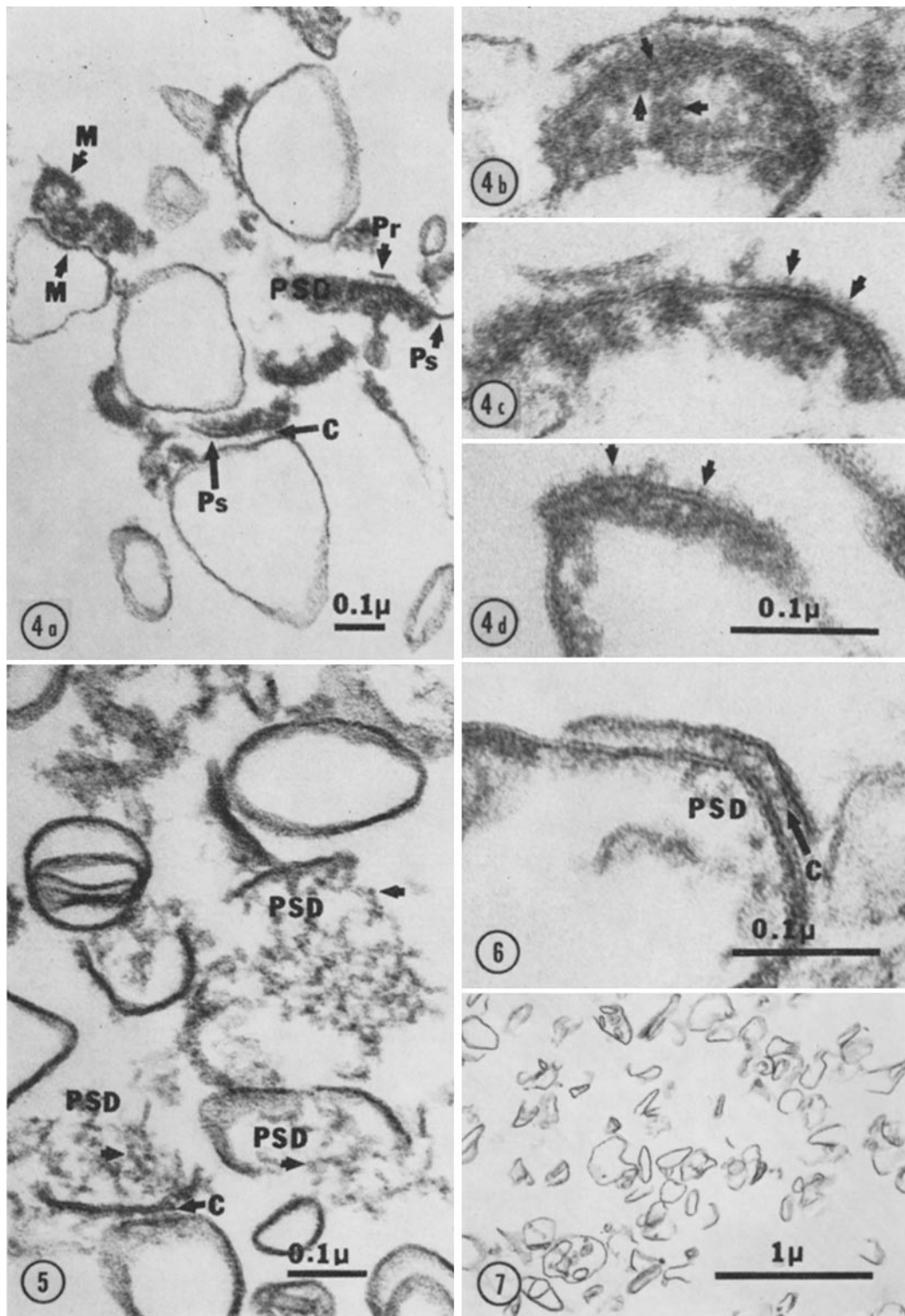
caused the loss of PSD material in glutaraldehyde-osmium tetroxide-fixed tissue stained with uranyl and lead ions. Pfenninger, however, noted that the appearance of the PSD depended on the staining technique used to visualize the PSD. PSD appeared relatively unaltered in glutaraldehyde-bismuth iodide-treated tissue except for a slight decrease in its thickness. Other proteolytic enzymes did not give results which depended on the staining technique. We observed that trypsin, like both chymotrypsin and bromalin, caused the disappearance of the PSD independent of the staining technique used. Apart from the different tissues and preparations used, the incubation conditions employed by Pfenninger for trypsin treatment may be somewhat milder than the conditions used with other enzymes studied or those employed by us. Alternately, the extensive treatment of SC during isolation may have altered their susceptibility. In any event, when trypsinization releases 70% of the protein of SC fractions, the PSD is essentially absent in bismuth iodide-uranyl lead-stained sections as well as in specimens stained by conventional methods, and the supernatant does not contain small particulate material. By contrast to the general conclusions of these studies, Barrantes and Lunt (1970) observed that the SC was quite resistant to digestion with trypsin, pronase or, bromalin. These investigators utilized material embedded and sectioned before enzyme treatment. Either the SC is made more resistant to hydrolysis or other

structures are made more sensitive due to one or more aspects of their procedure. In view of susceptibility observed on isolated fractions and fixed whole tissue, we conclude that PSD depends on polypeptides for its structural integrity and that polypeptides comprise the majority of PSD materials.

The effect of a number of chemicals on SC was studied, with the objective of further determining the stabilizing forces holding the PSD together. The PSD was not appreciably altered by incubation in 1 M NaCl, suggesting that ionic interactions are not solely responsible for its integrity. Urea, an agent which does not carry a charge and which is capable of denaturing proteins by disrupting hydrophobic bonds (Nozaki and Tanford, 1963), promotes a partial dissociation of PSD. The action of urea suggests that hydrophobic interactions play a role in maintaining SC structure. EGTA in concentrations sufficiently high to reduce the free calcium ion concentration to below 10^{-9} M (Portzehl et al., 1964) had only minimal effects on PSD. Nonetheless, it may be that some Ca^{+2} remains bound and is sufficient for structural stability. The question of whether EGTA is a sufficiently strong chelating agent to remove all bound Ca^{+2} is currently under investigation.

A number of interesting characteristics of the synaptic contact were seen. The cleft region seemed quite resistant to mild proteolytic action by all three enzymes studied. Certainly it was

FIGURE 8 Effect of chymotrypsin on the ultrastructure of a SC fraction under conditions where 25% of the protein of the fraction is solubilized. Chymotrypsin causes somewhat less extensive damage to the PSD than is seen with trypsin. Some SC with easily recognizable PSD and attached presynaptic membranes can be seen (SC). Throughout the background densely staining material (B) is seen which may originate from partly damaged PSD. More extensive treatment with chymotrypsin, until 70% of the protein is solubilized, completely removed SC from the fraction. × 56,000; G, OsO₄-UL.

FIGURE 9 Effect of bromalin, a mixture of proteolytic enzymes, on SC ultrastructure under conditions where 25% of the protein is solubilized. (a) The PSD was damaged, but residual portions can still be seen (D). Sectors of presynaptic membrane (Pr) could be found attached to some postsynaptic membranes. The postsynaptic projections (J) could be seen even after this bromalin treatment in some SC. × 57,000. (b) High magnification of SC after bromalin treatment as described above. The PSD is almost completely gone (D), yet a segment of presynaptic membrane remains (Pr). Postsynaptic projections (J) are seen within the cleft. × 129,000. Glutaraldehyde-OsO₄ fixation. Staining with uranyl acetate was carried out in the block, followed by permanganate and lead staining of the sections.

FIGURE 10 SC after incubation in 1 M urea and mild sonication. In (a) a high magnification field shows that the PSD appears to be opened up and consists of a series of interlacing fibers (arrows). × 220,000. In (b) a lower magnification field is shown. × 80,000; G, OsO₄-UL.
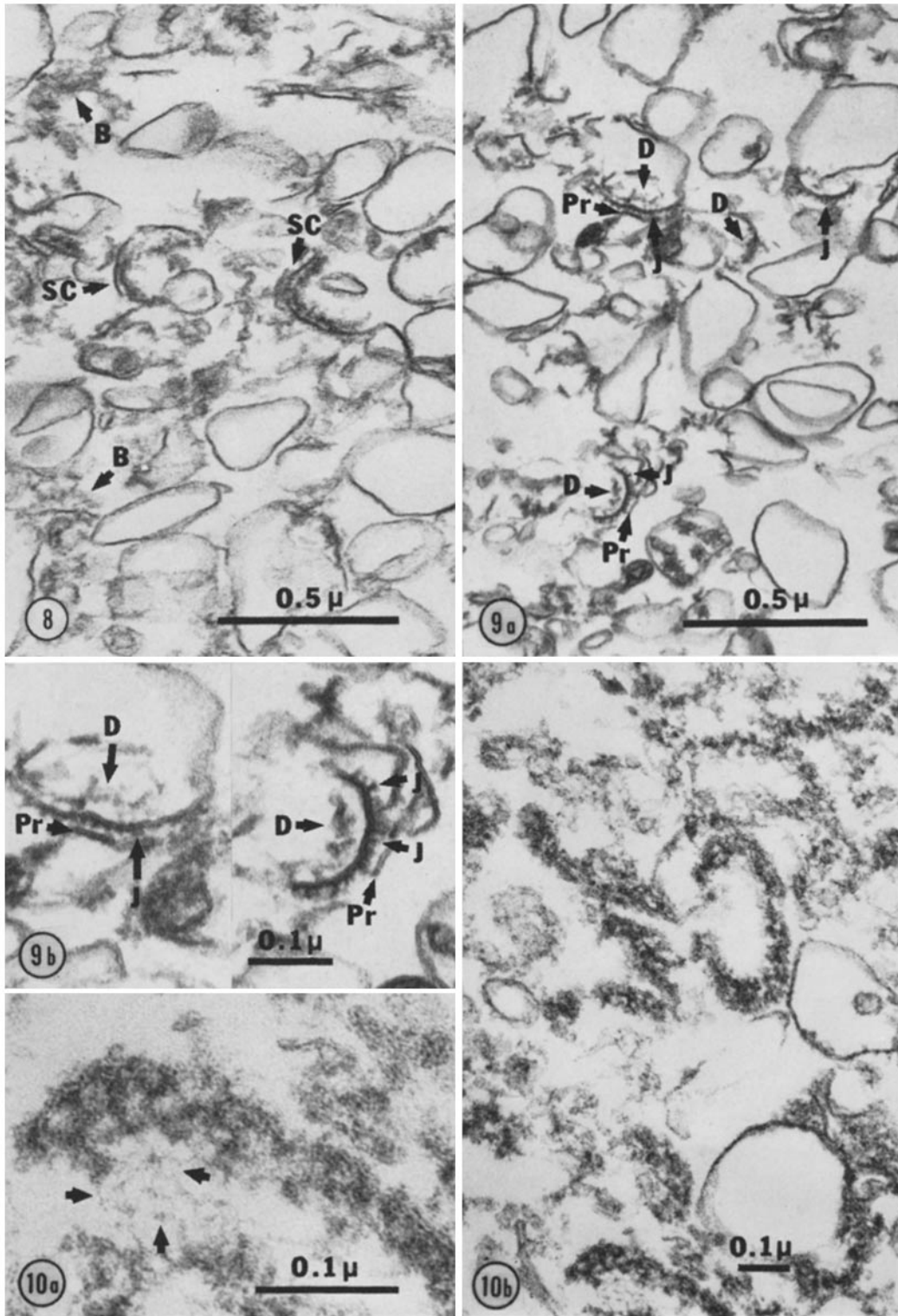
not the most sensitive element. The synaptic cleft survived trypsin treatment better than the PSD since joined pre- and postsynaptic membranes were evident even though the PSD was absent. Pfenninger (1971 *b*) also noted the resistance of synaptic contacts to trypsin in the subfornical organ. Bromalin was also harsher on the PSD than on the integrity of the synaptic contact. Treatment of SC fractions with 1 M NaCl at neutral pH did not markedly change the number of intact synaptic clefts. Extensive quantitative analysis is required, however, to determine whether the data are in disagreement with Pfenninger who reported that 20–25% of synaptic contacts are disrupted by 1 M NaCl. Incubation in presence of 1 M urea or 50 mM EGTA also did not dissociate all cleft regions. We conclude that the synaptic contact is remarkably resistant to a variety of treatments Many contacts resist dissociation throughout the isolation procedure and can withstand treatment with 1 M NaCl, 1 M urea, 50 mM EGTA, and mild proteolytic action.

In addition to possible enzymatic functions previously described (Florendo et al., 1971), our studies suggest several important properties of the postsynaptic density which may be of functional significance. The PSD is structurally very rigid. Its lattice-like structure, favorable for strength, may provide support for synapses and maintain the postsynaptic region in a relatively rigid structural relationship to the presynaptic terminal. Stabilization of the synaptic complex might serve to increase the precision of synaptic transmission by restricting membrane fluctuations. Possibly by providing continuity between the cytoplasm and specialized postsynaptic membrane, the PSD provides (*a*) a means for effective and possibly rapid turnover of molecules in the synaptic region, and (*b*) a way to preserve the specialized constituents at the synaptic membrane (Sandri et al., 1972) and prevent a fluid interaction (Singer and Nicolson, 1972) and diffusional equilibration with adjacent membrane elements. Another property which we observed in isolated fractions is the remarkably distinctive adhesive nature of the synaptic complex, particularly the PSD. These fractions adhere to the walls of centrifuge tubes, invariably aggregate in discontinuous gradients if the discontinuities are too sharp, and are extremely difficult to resuspend from firm pellets. Similarly, in electron micrographs it is common to find

examples where the PSD of one complex is attached to others. As pointed out in Fig. 4, various complexes in the aggregate are attached to others, and some of the PSD have a membrane attached to both sides of the PSD. It appears as if the PSD material of one complex has a strong affinity for that of other complexes and for membrane fragments. Detailed study of the nature of this adhesive material may provide clues on the formation and maintenance of synaptic connections.

In this study we have confirmed the involvement of proteins in the structural organization of the synaptic complex. The next problem is to isolate the proteins and determine their role in the function and organization of this highly specialized region. This method, which is an outgrowth of earlier efforts (de Robertis et al., 1967; Davis and Bloom, 1970; Cotman et al., 1971), should be an important step toward this end.

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