ISOLATION OF SYNAPTIC JUNCTIONAL COMPLEXES OF HIGH STRUCTURAL INTEGRITY FROM RAT BRAIN

H. M. THERIEN and W. E. MUSHYNSKI

From the Department of Biochemistry, McGill University, Montreal, Quebec, Canada, H3G 1Y6

ABSTRACT

A new method has been developed for isolating synaptic junctional complexes (SJC) of high structural integrity. The major step in the isolation involves homogenization of a synaptosomal membrane (SM) fraction in a biphasic system consisting of Freon 113 and an aqueous phase containing 0.2% Triton X-100. Well-preserved SJCs, along with membrane vesicles, were recovered in the aqueous phase after low-speed centrifugation of the homogenate. The membranes were subsequently separated from the SJCs by centrifugation on a discontinuous sucrose density gradient. The purity and identity of subcellular fractions were monitored by thin sectioning electron microscopy, using specific and nonspecific staining methods. From the electron microscope studies we conclude that SJCs and their components occupy about 65% of the area covered by structures in this fraction.

The assay of enzyme activities indicates that homogenization in Triton-Freon and subsequent steps of the isolation procedure affect the activities of Na, K-ATPase, cytochrome oxidase, and acid phosphatase to different extents, but do not cause total inactivation. Electrophoresis of the SJC-enriched fraction on sodium dodecyl sulfate-polyacrylamide gels has demonstrated that a polypeptide which co-migrates with tubulin is the major component in this fraction, and that a polypeptide co-migrating with actin is also present.

The site of synaptic contact between two neurons is characterized by structural components which are collectively referred to as the synaptic junctional complex (SJC) (17). The SJC consists of pre- and postsynaptic plasma membranes bordering a synaptic cleft, a presynaptic triagonal grid of dense projections (PDP) associated with the presynaptic membrane (2), and a postsynaptic density (PSD) subjacent to the postsynaptic membrane (2, 19, 44, 46). Although various roles relating to synaptic transmission have been postulated for these elements, little biochemical data regarding their composition and enzymatic functions is presently available due to the difficulty in obtaining homogeneous preparations of SJCs.

The resistance of SJCs to solubilization by low concentrations of Triton X-100 (Triton) was first demonstrated by De Robertis and co-workers (20, 25). This observation served as a basis for the development of new isolation procedures which yield SJCs with better preserved structure and lower levels of mitochondrial contamination (13, 15, 17). However, the SJC fractions obtained still contain relatively large amounts of nonjunctional synaptic membranes and membranes of unknown origin. The presence of these contaminating membranes has impeded efforts to characterize proteins that are localized in the SJC.

The isolation of more homogeneous preparations of SJCs thus appeared to require a departure

from the conventional Triton procedure. The use of an ionic detergent, *N*-lauroyl sarcosinate (NLS), by Cotman et al. (16) yields a homogeneous fraction of PSDs. Sodium deoxycholate has also been used to prepare a fraction enriched in PSD content, but the structures are highly disrupted and the purity of the preparation has not been established (41, 55). Our major concern in developing a new isolation procedure was to obtain relatively intact SJCs in a fraction more homogeneous than could be prepared by available procedures. The method is based on the assumption that, since PDPs and the PSD are largely proteinaceous and appear to consist of aggregates of filamentous and granular material (8, 9, 15, 19, 46, 47), they would resist the shearing forces generated during homogenization in the presence of Triton and an organic solvent of low surface tension such as Freon-113 (Freon) (26). Because of the more potent action of this treatment on membranes compared to homogenization in Triton alone, a more effective disruption of nonjunctional membranes is achieved, thus facilitating their separation from SJCs by sucrose density gradient centrifugation. The data presented in this paper demonstrate that SJCs of relatively high purity and with well-preserved structure can be isolated in this way.

MATERIALS AND METHODS

Subcellular Fractionation

Synaptic plasma membranes (SM) were isolated from cerebral and cerebellar cortices of male Sprague-Dawley rats (Canadian Breeding Farms and Laboratories, Ltd., St. Constant, Quebec) weighing 160–200 g. All the solutions used for isolation were adjusted to pH 7.1 with dilute NaOH. Quoted volumes for the homogenization of brain tissue and resuspension of subcellular fractions are based on the original wet weight of the tissue. Sucrose density gradients were prepared at room temperature and allowed to stand at 4°C for 1½–2 h before use. Pellets were resuspended for washing, or before application on sucrose density gradients, by means of a Dounce-type homogenizer with a loose-fitting pestle (Kontes Co., Vineland, N. J.). Centrifugation was carried out using SS34 (Sorvall, Ivan, Inc., Norwalk, Conn.), SW27, SW27.1 and type 30 rotors (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.).

ISOLATION OF SYNAPTIC MEMBRANES: Cerebral and cerebellar cortices from 12 rats (about 13 g wet weight) were used in the isolation procedure, which is based essentially on the one described by Davis and Bloom (17). All operations were carried out at 4°C. The cortices were homogenized in 9 vol of 0.32 M sucrose containing 1 mM $MgCl_2$, 50 μM $CaCl_2$, for 2 min (12 up-and-down strokes) in a Teflon-glass homogenizer with a clearance of 0.25 mm (Kontes Glass Co.). The pestle was rotated at 1,000 rpm. The homogenate was centrifuged for 10 min at 900 *g* and the supernate stored at 0°C. The 900-*g* pellet was washed twice by resuspension in 5–7 vol of 0.32 M sucrose, followed each time by centrifugation for 10 min at 900 *g*. The resulting supernates were combined with the original one and centrifuged for 15 min at 12,000 *g*. The pellet was resuspended in 5 vol of 0.32 M sucrose, centrifuged for 10 min at 900 *g* to remove any residual large particles, and then for 20 min at 14,000 *g* to pellet the crude mitochondrial fraction.

The crude mitochondrial fraction was osmotically shocked by resuspension in 9 vol of 10 μM $CaCl_2$ with three strokes of the tight-fitting pestle of a Dounce-type homogenizer. The suspension was left on ice for 30 min, rehomogenized, and centrifuged for 25 min at 35,000 *g*. The pellet was resuspended in 54 ml of 0.32 M sucrose containing 50 μM $CaCl_2$ and applied to six discontinuous sucrose density gradients consisting of 10 ml each of 0.85, 1.0, and 1.2 M sucrose, containing 50 μM $CaCl_2$. The gradients were centrifuged for 80 min at 23,000 rpm in an SW27 rotor. The material banding at the 0.85-1.0 M and 1.0-1.2 M sucrose interphases, as well as the entire 1.0 M sucrose layer, were removed with a pasteur pipet, diluted with 2 vol of 50 μM $CaCl_2$, and centrifuged for 25 min at 35,000 *g*. The crude SM fraction was subjected to a second cycle of lysis and density gradient centrifugation under the same conditions as described above.

ISOLATION OF SYNAPTIC JUNCTIONAL COMPLEXES: The SM fraction was suspended in 10 ml of an ice-cold solution containing 0.2% (vol/vol) Triton, 0.5% (wt/vol) Dextran T-500, 1 mM EDTA, pH 7.5. The suspension was immediately added to 10 ml of cold Freon in a precooled 50-ml stainless steel homogenizer (Omni-Mixer, I. Sorvall, Inc.). Homogenization was carried out at half-maximum speed (setting 5) for two 15-s intervals separated by a 30-s pause. The stainless steel homogenizer was kept in ice during this time. This procedure is referred to in the text as Triton-Freon homogenization. Procedures following this step were carried out as rapidly as possible, with all materials being maintained at 0–4°C, because prolonged exposure to Triton appears to reduce the structural integrity of SJCs. The homogenate was centrifuged at 3,000 *g* for 10 min. The upper phase was collected with a pasteur pipet (taking special care not to withdraw any interphase material), diluted with 3–4 vol of 50 μM $CaCl_2$, and centrifuged for 25 min at 35,000 *g*. The pellet, which is referred to as fraction U, was suspended in 2 ml of 0.32 M sucrose and allowed to stand on ice for 15 min. Appropriate volumes of 1.6 M sucrose, 1% (vol/vol) Triton, and 4% (wt/vol) dextran sulfate were then added to make the suspension 1.2 M in sucrose, 0.05% in Triton and 0.02% in dextran sulfate

(final volume ≈8 ml). 4 ml of the suspension was then added to each of two sucrose density gradients. The gradients consisted of 3 ml each of 2.0, 1.5, 1.4, and 1.3 M sucrose, with every layer containing 0.05% Triton and 0.02% dextran sulfate. The gradients were topped off with 0.32 M sucrose and centrifuged for 3 h at 23,000 rpm in an SW27.1 rotor. Most of the material in the gradient was distributed between the 0.32 and 1.2 M sucrose layers. A well-defined band at the 1.5–2.0 M sucrose interphase and a barely discernible pellet were also observed. The material banding at the 1.5–2.0 M sucrose interphase was removed with a pasteur pipet, diluted with 4 vol of cold distilled water and centrifuged for 20 min at 27,000 rpm in a type 30 rotor. The pellet constitutes the SJC-enriched fraction.

ISOLATION OF OTHER SUBCELLULAR FRACTIONS: PSDs were prepared from rat forebrain according to Cotman et al. (16). Myelin was obtained from the 0.32–0.85 M sucrose interphase after centrifugation of the osmotically shocked mitochondrial fraction on the first sucrose density gradient. The myelin fraction was diluted with 50 μM $CaCl_2$ and pelleted by centrifugation at 35,000 *g* for 25 min. The myelin was homogenized in Triton-Freon and fractionated on a sucrose density gradient in a way similar to that used for the SM fraction.

A mitochondrial fraction was obtained from rat brain by including a layer of 1.4 M sucrose containing 50 μM $CaCl_2$, in the sucrose density gradient used to fractionate the osmotically shocked crude mitochondrial fraction. Material pelleting through 1.4 M sucrose was taken as the brain mitochondrial fraction. This fraction was homogenized in Triton-Freon and subsequently fractionated on a sucrose density gradient by exactly the same procedure used to isolate SJCs from the SM fraction.

Mitochondria were also prepared from rat liver by differential centrifugation. Minced liver was homogenized in 8 vol of 0.25 M sucrose containing 5 mM Tris-HCl, pH 7.4, 1 mM EDTA. The homogenate was centrifuged at 3,000 *g* for 1 min. The supernate was saved and the pellet was washed once with the same solution. The pooled supernates were centrifuged for 15 min at 27,000 *g*. The pellet was resuspended in 20 vol of 0.25 M sucrose, 5 mM Tris-HCl, pH 7.4, and centrifuged for 1 min at 3,000 *g*. The supernate was then decanted and centrifuged for 3 min at 15,000 *g*. A pinkish, loosely packed, layer of microsomes was carefully removed from the underlying brownish pellet which constituted the liver mitochondrial fraction. This fraction was subjected to osmotic shock, homogenized in Triton-Freon and centrifuged on a sucrose density gradient in exactly the same manner as the SM fraction.

Biochemical Assays

Cytochrome oxidase was assayed essentially according to Duncan and Mackler (22), with the following modifications. Sodium phosphate buffer, pH 7.4, was used at a concentration of 50 mM and samples were preincubated in 0.1% Triton for 30 min before assay. The latter step was found to increase the enzyme activity, the greatest increase being observed in fractions (synaptosomes) where mitochondria were enclosed in membrane-bound vesicles.

Acid phosphatase was assayed as described by Cotman and Matthews (14). Na,K-ATPase was assayed by a slightly modified version of the procedure of Brunette and Till (10). The sample blanks contained 1 mM ouabain but no Na^+ or K^+. The reaction was stopped by adding 2 ml of 20% TCA and inorganic phosphate was determined according to Fiske and SubbaRow (24).

Protein was determined by the method of Lowry et al. (40) using bovine serum albumin as a standard.

Electron Microscopy

Small aliquots of subcellular fractions were pelleted in bottle-necked Beem capsules (Better Equipment for Electron Microscopy, Inc. New York) according to the method of Cotman and Flansburg (12). This procedure permitted the inclusion of the entire thickness of a pellet in a single thin section. Pellets were fixed overnight at 4°C in half-strength Karnovsky fixative (34), postfixed for 1 h in 1.33% osmium buffered with collidine and block-stained for 30 min in saturated aqueous uranyl acetate (Os-UL treatment). The tissue was then dehydrated and embedded in Vestopal W as previously described (37). Samples were also fixed, stained in ethanolic phosphotungstic acid (E-PTA), and embedded in Maraglas (Marblette Co. New York.) according to Bloom and Aghajanian (8, 9).

Ultrathin sections were cut on an LKB ultrotome III using glass or diamond knives and mounted on copper grids coated with collodion and carbon. Sections of E-PTA-stained pellets were not stained any further. Sections of osmicated pellets were stained for 1 min by Reynold's lead citrate (45). All grids were examined in a Philips 300 electron microscope operated at 60 kV and using a 20- or 30-μm objective aperture.

Samples were prepared for negative staining by mixing 1 drop of an SJC fraction suspended in 0.32 M sucrose, with 10 drops of water. One drop of 0.1% bovine serum albumin and four drops of 4% phosphotungstic acid were then added to the suspension. The mixture was then sprayed by means of an all-glass nebulizer (Ted Pella Co. Tustin, Calif.) onto 400 mesh grids coated with collodion and carbon.

Quantitative analysis and serial section studies of the SJC fraction were carried out on electron micrographs at a magnification of 46,800. For purposes of quantitation, micrographs were taken at regular intervals from the top, middle, and bottom of the pellet.

SDS-Polyacrylamide Gel Electrophoresis

Electrophoresis was carried out as described by Laemmli (38). Samples were solubilized in 62.5 mM Tris-HCl,

pH 6.8, containing 2% (wt/vol) SDS, 10% (wt/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol, 1 mM phenylmethyl sulfonyl fluoride. The samples were then dialyzed overnight against the solubilization medium. Bromophenol blue was used as a tracking dye, and the samples were electrophoresed on slab gels. The separating gel was 10% in acrylamide, whereas the stacking gel was 3%. Molecular weights were estimated by reference to standard proteins. These included bovine serum albumin, gamma globulin heavy and light chains, muscle actin from rabbit, and brain tubulin from rats. The tubulin was prepared as previously described (50).

Reagents

Sodium dextran sulfate and Dextran T-500 were obtained from Pharmacia Ltd. (Montreal, Canada). Freon TF-113 was obtained from Dupont of Canada, Ltd. (Montreal, Canada). Acrylamide (electrophoresis grade) and *N,N'*-methylene bisacrylamide were from Eastman Kodak Co. (Rochester, N. Y.). SDS, specially pure, was obtained from BDH Chemicals Ltd. (Poole, England). Triton X-100 and *N*-lauroylsarcosinate (NLS), sodium salt, were obtained from Sigma Chemical Co. (St. Louis, Mo). The NLS was recrystallized once from 90% ethanol before use. Uranyl acetate, phosphotungstic acid, and osmic acid anhydride were all from Fisher Scientific Co. (Montreal, Canada). Vestopal W was obtained from M. Jaeger (Geneva, Switzerland). The Maraglas embedding kit was from Ladd Research Industries, Inc. (Burlington,Vermont).

RESULTS

Isolation of the SJC Fraction

The most important step in the method described here for isolating SJCs is the homogenization of SMs in a biphasic system consisting of Triton (0.2%) and Freon. All other treatments of the SM fraction that were attempted, including homogenization in Triton alone, did not yield comparable results. The SMs which served as starting material were prepared by a procedure which follows essentially that described by Davis and Bloom (17), except that the crude mitochondrial pellet was not incubated with *p*-iodonitrotetrazolium violet (INT). Instead, the SM fraction was subjected to a second cycle of lysis and density gradient centrifugation, which further decreases mitochondrial contamination by about 35% (see Table III) and also results in a significant decrease in the contamination of the SJC fraction by bundles of neurofilaments, as demonstrated by electron microscopy (data not shown). These probably originate from axonal fragments that are still present in the SM fraction obtained after the first cycle of lysis and centrifugation.

The emulsion formed during homogenization of SM in Triton and Freon breaks easily, due to the low surface tension of the organic solvent. After separation of the phases by low-speed centrifugation, most of the protein is recovered at the interphase (Table I) with only trace amounts in the organic phase. The upper, aqueous phase, which consists mainly of membranes and SJCs, retains about 25% of the SM protein. About one-half of the protein present in the aqueous phase is pelleted at 35,000 *g* and subsequently applied to the final sucrose density gradient. Approximately 0.5% of the original SM protein is recovered at the 1.5–2.0 M sucrose interphase as the SJC fraction.

The material banding between 0.32 and 1.2 M sucrose after centrifugation of fraction U on the final sucrose density gradient is shown in Fig. 1. The predominant structures are membrane vesicles measuring between 0.05 and 0.5 μm in diameter. Very few structures resembling SJCs can be seen throughout this fraction.

The SJC fraction recovered at the 1.5–2.0 M sucrose interphase of the final gradient is shown in Fig. 2. The fraction contains a high proportion of

TABLE I

Distribution of Proteins in Fractions Obtained after Homogenization of Synaptic Membranes in Triton-Freon

Fraction*	mg Protein/g wet wt tissue‡
SM	2.75 ± 0.20
T-F interphase	1.49 ± 0.23
T-F aqueous phase	0.47 ± 0.04
Fraction U	0.24 ± 0.02
FG 0.32–1.3 M	0.11 ± 0.01
FG 1.5–2.0 M	0.012 ± 0.004

* SM is the synaptic membrane fraction obtained after two cycles of lysis and density gradient centrifugation. T-F interphase and T-F aqueous phase are the interphase and upper phase, respectively, obtained after low-speed centrifugation of the Triton-Freon homogenate. Fraction U is obtained by pelleting the T-F aqueous phase at 35,000 *g* for 25 min. FG 0.32–1.3 M and FG 1.5–2.0 M refer to the fractions obtained between 0.32 and 1.3 M sucrose, and at the 1.5–2.0 M sucrose interphase, respectively, after centrifugation of fraction U on the final density gradient (Materials and Methods). FG 1.5–2.0 M represents the SJC fraction.

‡ Data are the means of three experiments ± SEM. Recovery of protein in subfractions of the Triton-Freon homogenate averaged 71% while recovery in the final density gradient averaged 56%.

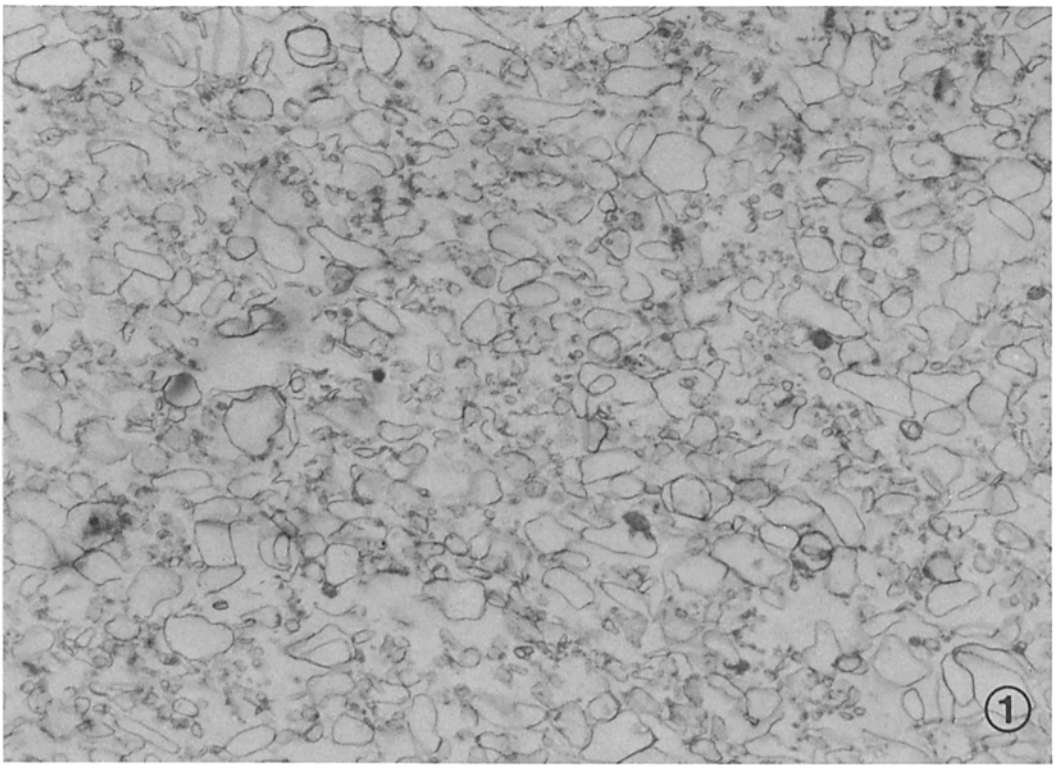
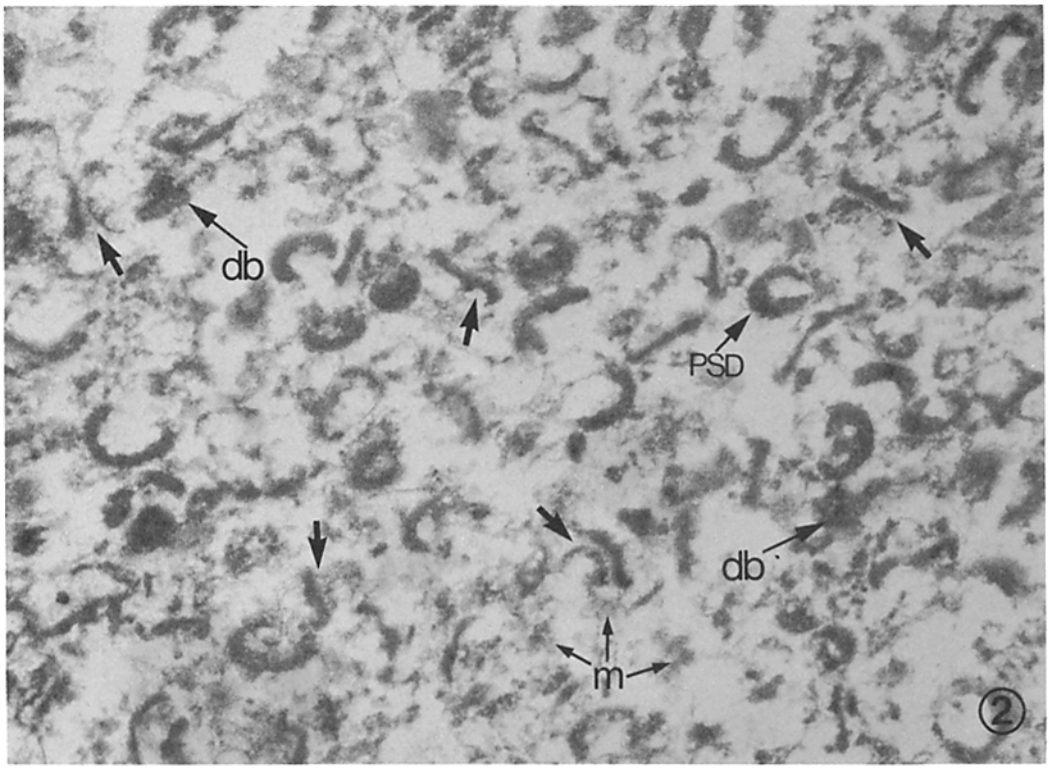


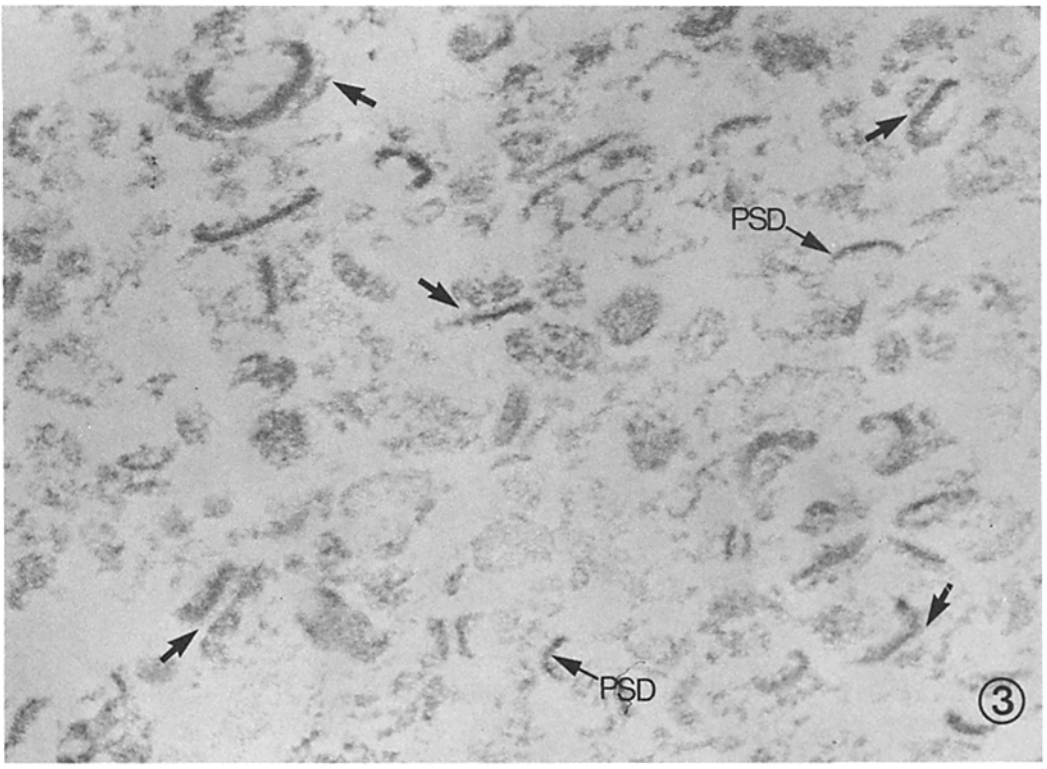
FIGURE 1 Low magnification electron micrograph of the major band (0.32–1.2 M sucrose) obtained from the sucrose density gradient used as the final step in the isolation of SJCs. The fraction is predominantly made up of membrane fragments. The fraction was postfixed with OsO_4 and stained with uranyl acetate and lead citrate (Os-UL). × 20,000.

SJCs with associated PDPs and PSD (arrows) as well as structures resembling PSDs. Little or no adjacent SM is attached to the junctional components, and only small amounts of free membrane vesicles can be seen. Since much of the area is occupied by amorphous material, which exhibits an electron density similar to that of SJCs, the enrichment in the latter structures is not as strikingly apparent as it is in an SJC fraction in which membranes are the major contaminant (15, 17).

The removal of adjacent membrane from SJCs increases their density and thus favors a better separation from contaminating membranes on sucrose density gradients. However, it also increases the tendency of SJCs to form large aggregates with one another and with membranes, as could be seen by phase-contrast microscopy (data not shown). This problem was overcome by adding 0.05% Triton and 0.02% dextran sulfate to all solutions of the final sucrose density gradient. Dextran sulfate was used as an analogue of polyethylene sulfonate, which has been shown to prevent the aggregation of mitochondria with other subcellular particles (1). The combined effects of these two agents results in a dramatic decrease in the amount of contaminating membranes in the SJC fraction.

The synaptic origin of structures assumed to be SJCs and PSDs was verified by staining the pellets with ethanolic phosphotungstic acid (E-PTA). This procedure has been shown to selectively stain SJCs *in situ* (8, 9) as well as in subcellular fractions (13, 15–17), although a decrease in specificity is observed when it is applied to isolated particulates (17). An electron micrograph of the SJC fraction stained with E-PTA is shown in Fig. 3. Intact SJCs are readily apparent (arrows) as are PSDs. Some of the background material behaves similarly to synaptic structures in that it is also visible in E-PTA sections. This provides suggestive evidence that at least some of the background material is of synaptic origin and represents SJCs that are dam-


db
PSD
db
m
②


PSD
PSD
③

aged or cut at unfavorable angles, as was demonstrated by analysis of serial sections (see below).

The possibility that mitochondria or myelin could give rise to some of the unidentifiable material in the SJC fraction, or to structures resembling SJCs, was determined by homogenizing these fractions in Triton-Freon under the same conditions used to treat the SM fraction. Because of the heavy contamination of brain mitochondria by material of synaptosomal origin, liver mitochondria were used for this purpose. The material derived from mitochondria did not give a clearly defined band at the 1.5–2.0 M sucrose interphase. Rather, the layer of 2.0 M sucrose was uniformly cloudy and a prominent pellet was obtained. The mitochondrial subfraction recovered from the 1.5–2.0 M sucrose region is shown in Fig. 4. A matrix such as that which forms the background in the SJC fraction cannot be seen. In addition, the beaded structures in Fig. 4 are rarely encountered in the SJC fraction. Myelin yielded fractions, consisting of large sheets of denatured

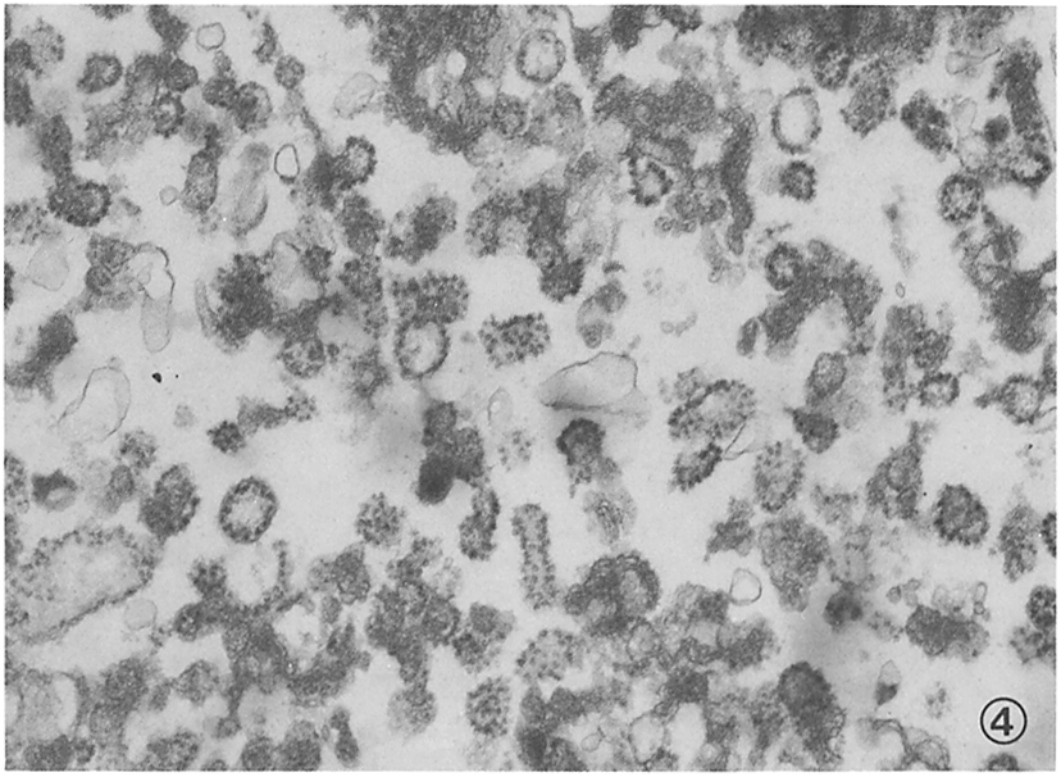


FIGURE 4 Typical electron micrograph of the mitochondrial subfraction recovered from the 1.5–2.0 M sucrose region of the density gradient after homogenization of liver mitochondria in Triton-Freon. × 46,800.

FIGURE 2 Electron micrograph of the SJC fraction obtained at the 1.5–2.0 M sucrose interphase of the final density gradient. SJCs with visible pre- and postsynaptic densities can be seen (arrows). Postsynaptic densities are also present (*PSD*). Much of the area is covered by dense bodies (*db*) and by a diffuse matrix (*m*). Os-UL. × 46,800.

FIGURE 3 Electron micrograph of the SJC fraction stained with ethanolic phosphotungstic acid (E-PTA). Well-preserved SJCs (arrows) as well as PSDs can be seen. Note the affinity of the background material for E-PTA. × 46,800.

membranes showing characteristic multilaminar patterns, in which structures resembling SJCs could not be seen.

Quantitative Electron Microscope Analysis

The high level of structural integrity retained by SJCs throughout the isolation procedure facilitated analysis of the composition of this fraction. Quantitative analysis was carried out using electron micrographs taken at uniform intervals along the top, middle, and bottom of pellets from two different SJC preparations (Materials and Methods). Quantitation was based on area occupied, and for this purpose the various elements were divided into five different categories: SJCs with recognizable pre- and postsynaptic densities, PSDs, dense bodies of uncertain origin, matrix consisting of amorphous and filamentous material, and membrane vesicles. In the latter structures, only the area covered by the bilaminar membrane was counted.

The areas occupied by 1,375 structures equally distributed between the two preparations were determined by counting squares on transparent millimeter grid sheets. This number does not include the matrix material, the area of which, however, was computed in the same way. The results are summarized in Table II. SJCs cover 20% of the total occupied area, the range for this mean value being ±5%, depending on the region of the pellet analyzed. PSDs with no apparent PDPs attached to them occupy about 1% of the area, membranes about 0.2%, while the remaining 79% is covered by dense bodies and matrix.

Since much of the material designated as dense bodies and matrix was stained by E-PTA (Fig. 3) we suspected that some of it might be of junctional origin. This was verified by analyzing serial sections of Os-UL-treated samples of the SJC fraction. Micrographs were taken of five successive sections in each of five separate sets of serial sections derived from two different SJC preparations. Each set of five serial micrographs was from a different region of the SJC pellet. The areas occupied by different structures were determined only in the central micrograph of each set. In this central micrograph, we first determined the area occupied by structures which could readily be identified as SJCs by means of their PDPs, PSD, and intersynaptic cleft. Recognizable SJCs found in the four other micrographs were then followed through the series. It was found that many of the dense bodies of the central picture are derived from SJCs identified in the adjacent sections while much of the matrix material comes from SJCs recognized as such in the first and the last pictures of each set. A total of 506 SJCs were followed in this way through the five sets of serial sections. The central picture therefore constitutes a composite since not only the area covered by the recognizable SJCs was measured, but also the area occupied by the material (dense bodies, matrix) which was identified as being junctional in origin by analysis of serial sections.

From these data we calculated a factor which could correct in part for the undetermined material in a micrograph of the SJC fraction. This correction factor was obtained by dividing the area covered by all the material determined to be of junctional origin in the composite picture by the area covered by the recognizable SJCs in the same picture. A mean value of 3.2 (SEM ± 0.2) was obtained for this correction factor. Thus, if on an average 20% of the covered area of an electron micrograph contains recognizable SJCs, multiplication by the correction factor yields a value of about 65% representing all of the material which is junctional in origin. The purity of the fraction

TABLE II

Quantitative Electron Microscopy of the SJC Fraction

Category	Percent of total area*	Corrected data‡ percent of total area
SJC	20	65
PSD	1.3	
Dense bodies	21	35
Matrix	58	
Membrane vesicles	0.2	0.2

* The area covered by different structures was determined from electron micrographs, at a final magnification of × 46,800, taken from the top, middle, and bottom of the pelleted SJC fraction obtained from two distinct preparations (Materials and Methods). SJCs contained visible pre- and postsynaptic densities while PSDs were identified by their characteristic curved appearance. Dense bodies consisted of electron-dense profiles of uncertain origin while the matrix is the diffuse background material that extends between the distinct profiles. The data represents mean values derived by determining areas occupied by 1,375 structures as well as the matrix equally distributed between the two preparations.
‡ The data was corrected by multiplying the SJC value by a factor of 3.2. This correction factor was determined by analyzing electron micrographs, at a magnification of × 46,800, of serial sections as described in Results.

may be even higher than this estimate, since unequivocal identification of an SJC requires at least one section where it is cut at a favorable angle so that the PDPs and PSD can be seen.

High Resolution Electron Microscopy

An analysis of high magnification electron micrographs demonstrates that, despite the relatively harsh conditions generated during homogenization, the SJCs retain a high level of structural integrity. This is especially apparent in the electron micrograph shown in Fig. 5, where a number of SJCs are favorably oriented so that all of their structural components are included in the plane of the section. In addition to PDPs (arrows) and PSD, junctional plasma membrane and a well-defined synaptic cleft can be seen in some of the structures. Also to be noted is the virtual absence of plasma membrane adjacent to the synaptic region.

In the SJC shown in Fig. 6, the bilaminar structure of the synaptic membranes is conserved. The presynaptic membrane appears to be damaged in the space separating the two PDPs, while the postsynaptic membrane, which in most SJCs appears to be the better preserved of the two membranes, runs along the length of the PSD.

The high resolution electron micrograph shown in Fig. 7 illustrates two characteristics seen in a large number of SJCs in the fraction. Presynaptic membrane cannot be seen in this SJC, with the possible exception of a small fragment embedded in the PDP at the far right (white arrow). The PDPs thus appear to be directly linked to the postsynaptic membrane, which runs along the length of the PSD. The PDPs also appear to form an extensive network, which is even more evident in the electron micrograph of a negatively stained SJC shown in Fig. 8. The extensive arborization of PDPs suggest either that they unravel during isolation or that they are part of a filamentous network that extends throughout the nerve ending (28).

Enzymatic Characterization of the SJC Fraction

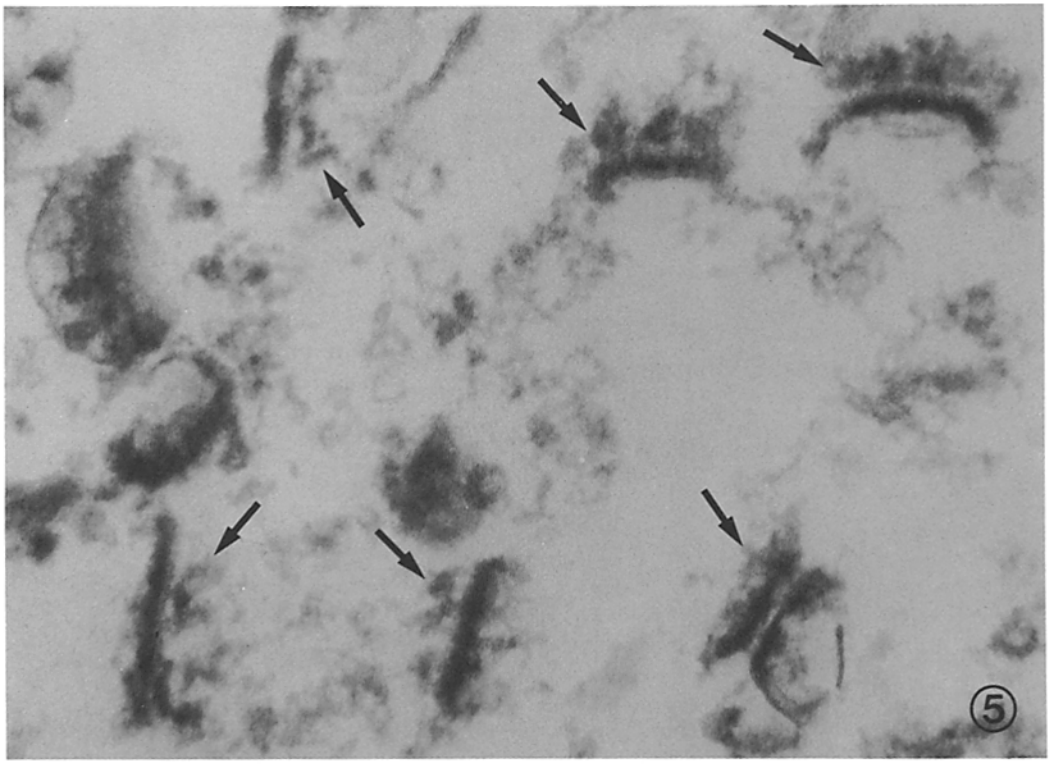
Three marker enzymes were followed throughout the isolation to determine the effect of the procedure on enzyme activity and to estimate the level of contamination of the SJC-enriched fraction. Na,K-ATPase, cytochrome oxidase, and acid phosphatase were chosen as markers for plasma membrane (32), mitochondrial inner membrane (22), and lysosomes (21), respectively. The data on enzyme inactivation are summarized in Table III. Homogenization in Triton-Freon was the first step in the isolation procedure to cause a significant reduction in the recovery of enzyme activity. The inactivation was probably due to the combined effects of partial delipidation and disruptive shearing forces generated during homogenization, and varied in magnitude for each of the enzymes tested. The enzyme most severely affected was Na,K-ATPase which lost 70% of its activity. About 40% of the cytochrome oxidase activity of the SM fraction and 25% of acid phosphatase activity were lost at this stage.

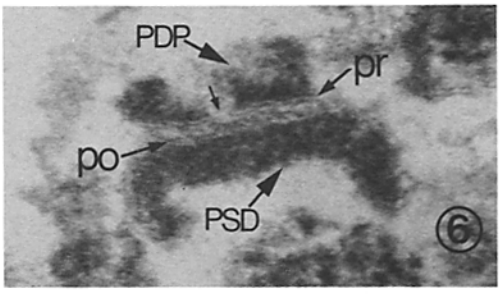
The enzymes were further inactivated during the 3 h of centrifugation on the final sucrose density gradient. This was probably due to the presence of Triton and/or dextran sulfate throughout the gradient. Cytochrome oxidase and Na,K-ATPase, the two membrane-bound enzymes, were again the most drastically affected. Only 30% of the remaining cytochrome oxidase activity was recovered, compared to 50% for Na,K-ATPase and 76% for acid phosphatase. Overall recoveries after the isolation procedure were therefore 13% for Na,K-ATPase, 14% for cytochrome oxidase, and 51% for acid phosphatase.

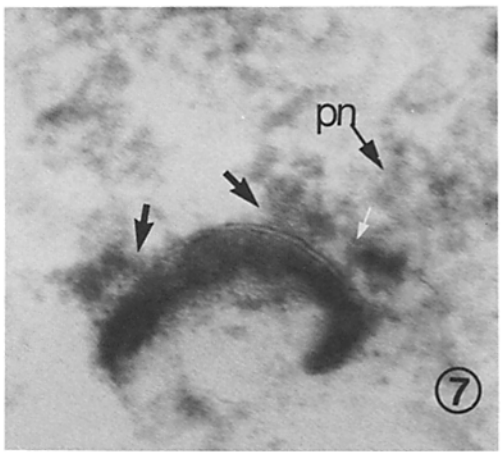
Contamination of the SJC fraction by the different subcellular components was estimated by using the enzyme inactivation data to correct the specific activities of the enzymes. Na,K-ATPase could not be detected in the SJC fraction, due to the low sensitivity of the assay method. After correction for inactivation, the specific activities of acid phosphatase and cytochrome oxidase were decreased 2- and 2.5-fold, respectively, relative to the SM fraction (Table III).

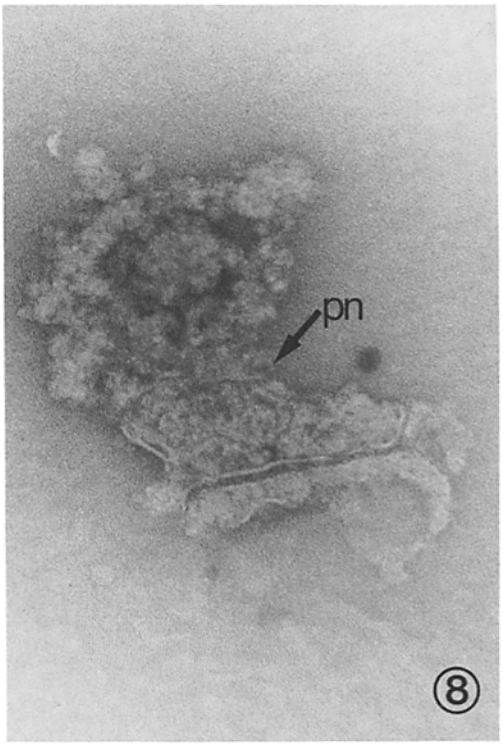
The specific activity of acid phosphatase in our SM fraction was about twice that of the homogenate. Since lysosomes isolated from liver, a tissue several times richer in this enzyme than brain (54), have a specific activity 67 times higher than the liver homogenate (49), we estimate contamination of the SJC fraction by lysosomes to be about 1%.

The SM_1 fraction obtained after the first cycle of lysis and density gradient centrifugation was the same as the SM fraction isolated by Davis and Bloom (17) in the absence of INT. They estimated the mitochondrial contamination in this fraction to be about 30%. Our inclusion of a second cycle of lysis and centrifugation yielded an SM fraction in which mitochondrial contamination was reduced


⑤


PDP
pr
po
PSD
⑥


pn
⑦


pn
⑧

1.7-fold. A further 2.5-fold decrease was achieved after Triton-Freon homogenization and centrifugation on the final density gradient. We therefore estimate a mitochondrial contamination of between 5 and 10% in the SJC fraction.

The most important conclusion derived from these results is that the isolation procedure only partially inactivates enzymes. The SJC fraction is therefore suitable for studying enzymes thought to be involved in synaptic function.

Polyacrylamide Gel Electrophoresis

The SJC fraction was electrophoresed on SDS-polyacrylamide gels to compare its polypeptide distribution pattern with that of SMs, and of PSDs prepared by the method of Cotman et al. (16). The results are shown in Fig. 9. The arrows point to the regions of the gel where tubulin (upper arrow) and actin migrate. The major bands on the gels were assigned the notation P97, P55, etc., where the "P" stands for polypeptide and the number refers to the molecular weight of the polypeptide species in thousands of daltons.

The major component of the SJC fraction is P55, which co-migrates with tubulin. The width of this band indicates that more than one polypeptide species may be present in this region. A polypeptide co-migrating with the major component of PSDs (P50) and another which co-migrates with muscle actin (P45) also represent major elements. An almost complete disappearance of components below P45 and a significant decrease in the amount of P97 differentiates the SJC fraction from the SM fraction.

In their analysis of the polypeptide composition of PSDs, Banker et al. (4) reported the presence of two major components, with molecular weights of 98,000 (polypeptide A) and 53,000 (polypeptide B). Polypeptide A, which corresponds to P97 in Fig. 9, is more prominent in PSDs than in SJCs.

TABLE III

Enzyme Characterization of Subcellular Fractions

Enzyme	Specific activity*			
	SM_1‡	SM	Fraction U	SJC
Na,K-ATPase	25 ± 4	25 ± 3 (89)§	3.5 ± 2.5 (26)	ND (13)
Cytochrome oxidase	90 ± 11	47 ± 12 (75)	11.4 ± 3.5 (46)	3.0 ± 1.2 (14)
Acid phosphatase	2.1 ± 0.2	2.9 ± 0.2 (97)	1.8 ± 0.3 (74)	0.82 ± 0.36 (51)

* Data are the means of three experiments ± SEM. Specific activity is expressed in micromoles per hour per milligram protein.

‡ SM_1 is the synaptic membrane fraction obtained after the first cycle of lysis and density gradient centrifugation. SM and fraction U are defined in the legend to Table I. SJC is the fraction recovered at the 1.5–2.0 M sucrose interphase of the final sucrose density gradient.

§ The number in parentheses refers to the total enzyme activity recovered after the isolation step that yields the fraction. This is expressed as (percent of enzyme activity recovered/percent of protein recovered) × 100, relative to the SM_1 fraction.

FIGURE 5 Electron micrograph illustrating the high level of structural integrity retained by SJCs. Note the presence of presynaptic dense projections (arrows) and the absence of membranes adjacent to the synaptic region. Os-UL. × 73,800.

FIGURE 6 High magnification electron micrograph of a SJC showing in greater detail the characteristic features, including presynaptic dense projections (*PDP*), the presynaptic membrane (*pr*), the postsynaptic membrane (*po*), and the postsynaptic density (*PSD*). The presynaptic membrane appears to be damaged in the space between the PDPs (small arrow). Os-UL. × 118,000.

FIGURE 7 High magnification electron micrograph illustrating the apparent direct linkage of PDPs to the postsynaptic membrane (arrows) and the extensive network (*pn*) associated with the PDPs. Only a small segment of presynaptic membrane can be seen in the PDP at the right (white arrow). Os-UL. × 118,000.

FIGURE 8 Electron micrograph of a negatively stained SJC illustrating in greater detail the extensive arborization on the presynaptic side (*pn*). × 183,000.

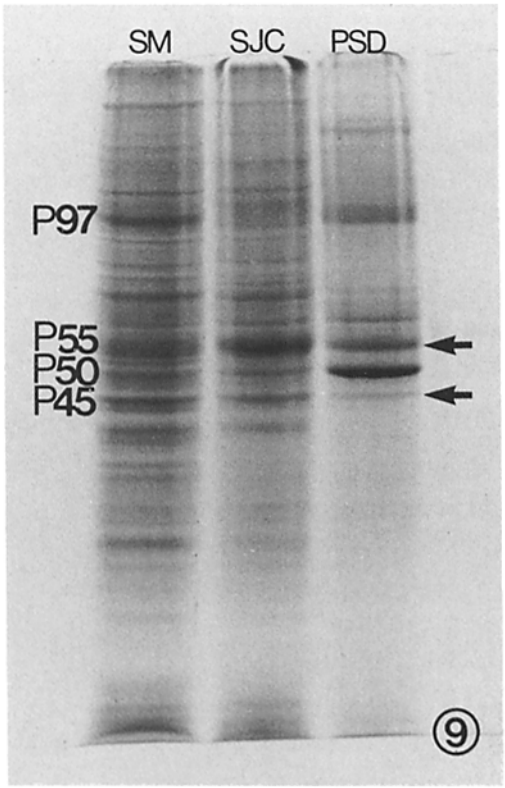


FIGURE 9 Electrophoretogram comparing the polypeptide composition of synaptic membranes (*SM*), synaptic junctional complexes (*SJC*), and postsynaptic densities (*PSD*). Polypeptides were fractionated on an SDS-10% polyacrylamide slab gel using a 3% stacking gel. The arrows point to the regions where tubulin subunits (upper arrow) and actin (lower arrow) migrate. Some of the polypeptides (*P97, P55, P50, P45*) are designated according to their molecular weight in thousands of daltons.

Polypeptide B is resolved into two bands in our electrophoresis system, with P55, the major component of SJCs, being present in much lower amount than P50.

DISCUSSION

The content of synaptic structures in the SJC fraction has been estimated at about 65%. This estimate, which is based on the area occupied by different structures in electron micrographs, is probably a conservative one because of the criteria applied in scoring structures as SJCs or PSDs (see Results). Some of the remaining background material may well represent disrupted SJCs that do not retain morphological landmarks to allow identification in serial sections.

The SJCs isolated by the Triton-Freon homogenization technique differ from the ones obtained by other methods (13, 15, 17, 20) in two major respects. Most of the SJCs are free of plasma membrane adjacent to the synaptic region and a large proportion of the structures still have PDPs associated with the PSD. Most of the SJCs resemble the Gray type I synapse (27) in that they contain a prominent PSD.

The preservation of PDPs is a surprising aspect of the procedure, since they are known to be the more labile components of SJCs (17, 20). Our results support the idea (17) that the lability of PDPs may be due to the presence of degradative enzymes trapped in the presynaptic bag formed by the residual SM which remains attached to SJCs prepared by other methods, but which is removed by our procedure. In accordance with the findings of other laboratories (13, 15, 17), we observed that calcium must be present in the media used to isolate SMs. SMs prepared in the absence of calcium provide a reduced yield of SJCs with poorly preserved structure.

The constituent membranes of SJCs are also quite well preserved despite the use of an organic solvent in the isolation. However, the presynaptic membrane appears to be more susceptible to the action of Freon than the postsynaptic membrane. It seems that the PDPs and the PSD are acting as protective agents for their associated membranes. In their studies on lipid extracted neuromuscular junctions, Rash and Ellisman (48) demonstrated that the postsynaptic membranes retain their bilaminar structure in regions of high protein content. The occurrence of the PSD as a continuous plaque, in which the constituent proteins may be arranged in a regular two-dimensional lattice (51), could also explain the greater stability of the postsynaptic membrane. An analogous situation appears to apply to the presynaptic side, since portions of the presynaptic membrane not associated with a PDP are in general more severely damaged.

In some cases, PDPs seem to remain attached to the PSD in spite of the apparent absence of a presynaptic membrane (Fig. 7). This would suggest that PDPs are directly linked through filamentous projections either to the postsynaptic membrane or to the PSD itself, as has already been proposed (35). On the other hand, the possibility that this apparent continuity is an artifact of the isolation procedure or is due to problems inherent in the assessment of thin-sectioned material, cannot be ruled out at present.

The SJCs are also characterized by the presence of an extensive network which in many cases appears to be continuous with the PDPs. This observation is in agreement with electron microscope studies of synaptic junctions *in situ* (28, 33, 39, 42). The presynaptic network, and associated structures such as PDPs, have been considered either as actual entities (28) or as artifacts of the fixation and processing of tissue for electron microscopy (29). Our results indicate that these components are not complete artifacts, since they are present in isolated SJCs. However, their organization in vivo may differ from what is observed in electron micrographs. From our observations made on several preparations of the SJC-enriched fraction, we suspect that the arborizations associated with PDPs may arise due to an unravelling of the filamentous material making up these structures. Our supposition is based on the fact that, although the SJC content is quite constant from preparation to preparation, the morphology of the fraction differs with respect to the prominence of the matrix which appears for the most part to be continuous with PDPs. These differences may be due to slight variations in the conditions of homogenization or in the processing of the material for electron microscopy.

Despite the fact that the SMs are subjected to relatively harsh conditions during the isolation procedure, significant levels of different enzyme activities remain associated with the fractions obtained on the final density gradient. Experiments in progress have so far indicated the presence of a protein kinase in the SJC fraction (unpublished results). The lipid-requiring enzymes, Na,K-ATPase (52) and cytochrome oxidase (3), are affected to the greatest extent by the isolation procedure. Since the effect of Triton on enzyme activity is generally not detrimental (31), the added delipidating action of Freon is probably responsible for partially inactivating the above enzymes.

The polypeptide distribution patterns obtained after SDS-polyacrylamide gel electrophoresis of SJCs and PSDs demonstrate that the two fractions differ from one another in the number of polypeptides present as well as in their relative amounts. This may in part reflect differences between PSDs and PDPs, since only the SJCs contain these two structural elements. We realize that a comparison of structures isolated using different solubilizing systems may in fact give rise to ambiguities. NLS (16) might extract different proteins than Triton-Freon, in which case the polypeptide composition of SJCs and PSDs would be related to the different isolation procedures rather than reflecting their actual in vivo composition. In addition, degradation by endogenous proteases or possible contributions by contaminating structures cannot be totally disregarded.

Our results indicate that P45, which co-migrates with muscle actin, may be localized predominantly in the presynaptic region, since it is one of the major components of the SJC fraction but is only present in trace amounts in PSDs. Assumptions regarding the localization of tubulin-like protein are more difficult to rationalize on the basis of available data (this paper and references 4, and 55). The electrophoretic pattern that we obtain for PSDs differs from that of Banker et al. (4). They detected only one polypeptide in the molecular weight range of 53,000 daltons (polypeptide B) while we observe two (P50 and P55). This discrepancy is probably the result of our using a better resolving electrophoresis system, which employs a stacking gel and a discontinuous buffer system (38). The major polypeptide in the SJC fraction, P55, co-migrates with tubulin, while the major component of PSDs, P50, has a molecular weight similar to that of a neurofilament subunit (18). Our results therefore indicate that the tubulin-like polypeptide (P55) is predominantly associated with PDPs while P50 occurs mainly in the PSD. The assignments are in agreement with the observed intimate association and possible continuity of microtubules with PDPs (30) and of neurofilaments with PSDs (11, 39, 42).

Walters and Matus (55) have presented immunohistochemical evidence suggesting that tubulin-like protein is almost exclusively associated with the PSD component of synaptic junctions. Since microtubules are closely associated with PDPs (30), the low levels of diffuse, positively reacting material seen in axon terminals (Fig. 3 in reference 55) indicate that the procedures used by these investigators do not provide equivalent specificity for pre- and postsynaptic structures. This may be due to a differential permeability of dendritic and nerve terminal membranes to immunohistochemical reagents.

The identification in the SJC fraction of polypeptides which co-migrate with actin and tubulin correlates with morphological (30, 42) and chemical evidence (references 7, 36, 55, 56, and unpublished results) that actin and tubulin are present in the synaptic region. These observations raise interesting questions about the possible role of actin

and tubulin in synaptic function. A proposal has been made for the involvement of an actomyosin-like system in neurotransmitter release (5) and tubulin has also been implicated in the latter process (43, 53). Colchicine-binding protein(s), presumably related to tubulin, also appears to be involved in controlling the mobility and distribution of surface receptors and other membrane proteins (6, 23). The anchorage of transmitter receptors on the postsynaptic membrane may occur by an analogous process, as has already been suggested (4, 55).

In conclusion, our results indicate that the SJC-enriched fraction prepared by the present procedure is suitable for identifying some of the structural and enzymatic proteins localized in the synapse. In conjunction with the PSD fraction of Cotman et al. (16), it may serve to differentiate between the pre- and postsynaptic localization of these proteins.

The technical assistance of Mr. L. Guluzian and Ms. S. Glen is gratefully acknowledged. The rabbit muscle actin was kindly donated by Dr. J. Gruda. We thank Ms. Monique Lagacé and Ms. Irene Cach for secreterial aid.

This investigation was supported by grant MA-5159 from the Medical Research Council of Canada. H. M. Therien is the recipient of a 1967 Science Scholarship from the National Research Council of Canada.

Received for publication 8 December 1975, and in revised form 23 August 1976.

REFERENCES

1. ACHEE, F. M., G. TOGULGA, and S. GABAY. 1974. Studies of monoamine oxidases: properties of the enzyme in bovine and rabbit brain mitochondria. *J. Neurochem.* **22:**651–661.
2. AKERT, K., K. PFENNINGER, C. SANDRI, and H. MOOR. 1972. Freeze etching and cytochemistry of vesicles and membrane complexes in synapses of the central nervous system. *In* Structure and Function of Synapses. G. D. Pappas and D. P. Purpura, editors. Raven Press, New York. 67–86.
3. AWASTHI, Y. C., T. F. CHUANG, T. W. KEENAN, and F. L. CRANE. 1971. Tightly bound cardiolipin in cytochrome oxidase. *Biochim. Biophys. Acta.* **226:**42–52.
4. BANKER, G., L. CHURCHILL and C. W. COTMAN. 1974. Proteins of the postsynaptic density. *J. Cell Biol.* **63:**456–465.
5. BERL, S., S. PUSZKIN, and W. J. NICKLAS. 1973. Actomyosin-like protein in brain. *Science (Wash. D. C.).* **179:**441–446.
6. BERLIN, R. D., J. M. OLIVER, T. E. UKENA, and H. H. YIN. 1974. Control of cell surface topography. *Nature (Lond.).* **247:**45–46.
7. BLITZ, A. L., and R. E. FINE. 1974. Muscle-like contractile proteins and tubulin in synaptosomes. *Proc. Natl. Acad. Sci. U. S. A.* **71:**4472–4476.
8. BLOOM, F. E., and G. K. AGHAJANIAN. 1966. Cytochemistry of synapses: selective staining for electron microscopy. *Science (Wash. D. C.).* **154:**1575–1577.
9. BLOOM, F. E., and G. K. AGHAJANIAN. 1968. Fine structural and cytochemical analysis of the staining of synaptic junctions with phosphotungstic acid. *J. Ultrastruct. Res.* **22:**361–375.
10. BRUNETTE, D. M., and J. E. TILL. 1971. A rapid method for the isolation of L-cell surface membranes using an aqueous two-phase polymer system. *J. Membr. Biol.* **5:**215–224.
11. COLONNIER, M., and R. W. GUILLERY. 1964. Synaptic organization in the lateral geniculate nucleus of the monkey. *Z. Zellforsch. Mikrosk. Anat.* **62:**333–355.
12. COTMAN, C. W., and D. A. FLANSBURG. 1970. An analytical micro-method for electron microscopic study of the composition and sedimentation properties of subcellular fractions. *Brain Res.* **22:**152–156.
13. COTMAN, C. W., W. LEVY, G. BANKER, and D. TAYLOR. 1971. An ultrastructural and chemical analysis of the effect of Triton X-100 on synaptic plasma membranes. *Biochim. Biophys. Acta.* **249:**406–418.
14. COTMAN, C. W., and D. A. MATTHEWS. 1971. Synaptic plasma membranes from rat brain synaptosomes: isolation and partial characterization. *Biochim. Biophys. Acta.* **249:**380–394.
15. COTMAN, C. W., and D. TAYLOR. 1972. Isolation and structural studies on synaptic complexes from rat brain. *J. Cell Biol.* **55:**696–711.
16. COTMAN, C. W., G. BANKER, L. CHURCHILL, and D. TAYLOR. 1974. Isolation of postsynaptic densities from rat brain. *J. Cell Biol.* **63:**441–455.
17. DAVIS, G. A., and F. E. BLOOM. 1973. Isolation of synaptic junctional complexes from rat brain. *Brain Res.* **62:**135–153.
18. DAVISON, P. F., and B. WINSLOW. 1974. The protein subunit of calf brain neurofilament. *J. Neurobiol.* **5:**119–133.
19. DE ROBERTIS, E., A. PELLEGRINO DE IRALDI, G. RODRIGUEZ DE LORES ARNAIZ, and L. SALGANICOFF. 1961. Electron microscope observations on nerve endings isolated from rat brain. *Anat. Rec.* **139:**220–221.
20. DE ROBERTIS, E., J. M. AZCURRA, and S. FISZER. 1967. Ultrastructure and cholinergic binding capacity of junctional complexes isolated from rat brain. *Brain Res.* **5:**45–56.
21. DI PIETRO, D. L., and F. S. ZENGERLE. 1967. Separation and properties of three acid phosphatases from human placenta. *J. Biol. Chem.*

242:3391–3396.

22. DUNCAN, H. M., and B. MACKLER. 1966. Electron transport systems of yeast. III. Preparation and properties of cytochrome oxidase. *J. Biol. Chem.* **241**:1694–1697.
23. EDELMAN, G. M., I. YAHARA, and J. L. WANG. 1973. Receptor mobility and receptor-cytoplasmic interactions in lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* **70**:1442–1446.
24. FISKE, C. H., and Y. SUBBAROW. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**:375–400.
25. FISZER, S., and E. DE ROBERTIS. 1967. Action of Triton X-100 on ultrastructure and membrane-bound enzymes of isolated nerve endings from rat brain. *Brain Res.* **5**:31–44.
26. GESSLER, A. E., C. E. BENDER, and M. C. PARKINSON. 1956. A new method for isolating viruses by selective fluorocarbon deproteinization. *Trans. Natl. Acad. Sci. U. S. A.* **18**:701–703.
27. GRAY, E. G. 1959. Axosomatic and axodendritic synapses of the cerebral cortex: an electron microscopic study. *J. Anat.* **93**:420–433.
28. GRAY, E. G. 1973. The cytonet, plain and coated vesicles, reticulosomes, multivesicular bodies and nuclear pores. *Brain Res.* **62**:329–335.
29. GRAY, E. G. 1975. Synaptic fine structure and nuclear, cytoplasmic and extracellular networks. *J. Neurocytol.* **4**:315–339.
30. GRAY, E. G. 1975. Presynaptic microtubules and their association with synaptic vesicles. *Proc. R. Soc. Lond. B. Biol. Sci.* **B190**:369–372.
31. HELENIUS, A., and K. SIMONS. 1975. Solubilization of membranes by detergents. *Biochim. Biophys. Acta.* **415**:29–79.
32. HOSIE, R. J. A. 1965. The localization of adenosine triphosphatase in morphologically characterized fractions of guinea pig brain. *Biochem. J.* **96**:404–412.
33. KADOTA, K., and T. KADOTA. 1973. Isolation of coated vesicles, plain synaptic vesicles and fine particles from synaptosomes of guinea pig whole brain. *J. Electron Microsc.* **22**:91–98.
34. KARNOVSKY, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.* **27**:137. (Abstr.)
35. KORNGUTH, S. E. 1974. The synapse: A perspective from *in situ* and *in vitro* studies. *In* Reviews of Neuroscience. S. Ehrenpreis and I. Kopin, editors. Raven Press, New York. **1**:63–114.
36. KORNGUTH, S. E., and E. SUNDERLAND. 1975. Isolation and partial characterization of a tubulin-like protein from human and swine synaptosomal membranes. *Biochim. Biophys. Acta.* **393**:100–114.
37. KURTZ, S. M. 1961. A new method for embedding tissues in Vestopal W. *J. Ultrastruct. Res.* **5**:468–477.
38. LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* **227**:680–685.
39. LE BEUX, Y. J. 1973. An ultrastructural study of the synaptic densities, nematosomes, neurotubules, neurofilaments and of a further three-dimensional filamentous network as disclosed by the E-PTA staining procedure. *Z. Zellforsch. Mikrosk. Anat.* **143**:239–272.
40. LOWRY, H. J., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurements with the folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
41. MATUS, A. I., and B. B. WALTERS. 1975. Ultrastructure of the synaptic junctional lattice isolated from mammalian brain. *J. Neurocytol.* **4**:369–375.
42. METUZALS, J., and W. E. MUSHYNSKI. 1974. Electron microscope and experimental investigations of the neurofilamentous network in Deiters' neurons. Relationship with the cell surface and nuclear pores. *J. Cell Biol.* **61**:701–722.
43. NICKLAS, W. J., S. PUSZKIN, and S. BERL. 1973. Effect of vinblastine and colchicine on uptake and release of putative neurotransmitters by synaptosomes and on brain actomyosin-like protein. *J. Neurochem.* **20**:109–121.
44. PALAY, S. L. 1958. The morphology of synapses in the central nervous system. *Exp. Cell Res.* **5**(Suppl.):275–293.
45. PEASE, D. C. 1964. Histological Techniques for Electron Microscopy. Academic Press, Inc., New York. 381 pp.
46. PFENNINGER, K. H. 1971. The cytochemistry of synaptic densities. I. An analysis of the bismuth iodide impregnation method. *J. Ultrastruct. Res.* **34**:103–122.
47. PFENNINGER, K. H. 1971. The cytochemistry of synaptic densities. II. Proteinaceous components and mechanism of synaptic connectivity. *J. Ultrastruct. Res.* **35**:451–475.
48. RASH, J. E., and M. H. ELLISMAN. 1974. Studies of excitable membranes. I. Macromolecular specializations of the neuromuscular junction and the nonjunctional sarcolemma. *J. Cell Biol.* **63**:567–586.
49. SAWANT, P. L., S. SHIBKO, V. S. KUMTA, and A. L. TAPPEL. 1964. Isolation of rat-liver lysosomes and their general properties. *Biochim. Biophys. Acta.* **85**:82–92.
50. SHELANSKI, M. L., F. GASKIN, and C. R. CANTOR. 1973. Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. U. S. A.* **70**:765–768.
51. SINGER, S. J. 1974. The molecular organization of membranes. *Annu. Rev. Biochem.* **43**:805–833.
52. TANAKA, R., and L. G. ABOOD. 1964. Phospholipid requirement of Na^+,K^+-activated adenosine triphosphatase from rat brain. *Arch. Biochem. Biophys.* **108**:47–52.
53. THAO, N. B., G. F. WOOTEN, J. AXELROD and I. J.

KOPIN. 1972. Inhibition of release of dopamine-β-hydroxylase and norepinephrine from sympathetic nerves by colchicine, vinblastine, or cytochalasin-B. *Proc. Natl. Acad. Sci. U. S. A.* **69**:520–522.

54. VERITY, M. A., and W. J. BROWN. 1967. Murine hepatic and cerebral lysosomal acid phosphomonoesterases. *Exp. Cell Res.* **35**:84–99.

55. WALTERS, B. B., and A. I. MATUS. 1975. Tubulin in postsynaptic junctional lattice. *Nature (Lond.).* **257**:496–498.

56. WALTERS, B. B., and A. I. MATUS. 1975. Proteins of the synaptic junction. *Biochem. Soc. Trans.* **3**:109–112.