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OPTIMAL CONCENTRATION OF IODONITROTETRAZOLIUM FOR THE ISOLATION OF JUNCTIONAL FRACTIONS FROM RAT BRAIN

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The yield and purity of synaptic plasma membranes (SPM) and synaptic junctions (SJ) from rat brain has been examined as a function of the concentration of *p*-iodonitrotetrazolium (INT)–succinate used during their preparation. An INT concentration of 1 mg/g brain tissue (wet weight) was sufficient to obtain SPM and SJ of purity comparable to that obtained using 4–6 times that concentration of dye (1–3). At this lower level of INT the yield of SPM increased by about 100%, whereas mitochondrial contamination remained at 10–13% of the total SPM protein. At concentrations of INT below 0.5 mg/g brain tissue (wet weight) the contamination of SPM by mitochondria increased rapidly. At very low concentrations of INT (0.13 mg/g tissue) the contaminating protein of mitochondrial origin was 40–50% of the total protein in the SPM fraction. Examination by gel electrophoresis of SPM, SJ, and mitochondrial fractions with different degrees of cross-contamination allowed the assignment of marker polypeptides for mitochondrial, junctional, and nonjunctional plasma membranes. Under the conditions used to prepare SJ, a variable amount of particulate material floated over 1.0 M sucrose. It consisted of SJ and many membrane vesicles and had a protein composition similar to that of SJ contaminated by extrajunctional membrane proteins. An analogous fraction arose during in the preparation of postsynaptic densities.

INTRODUCTION

The dye 2-(*p*-iodophenyl)-3-*p*-nitrophenyl-5-phenyltetrazolium chloride (*p*-iodonitrotetrazolium violet, INT) can act as an electron acceptor in

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the mitochondrial oxidation of succinate by the intrinsic membrane enzyme succinate dehydrogenase. The reduced dye, formazan, is an intensely purple product that forms a dense, insoluble precipitate in aqueous solutions. Based on these properties, incubation of synaptosomes with INT and succinate has been used to deposit a formazan precipitate inside brain mitochondria that increases their density and thereby facilitates the separation of mitochondria from other membranous fractions, especially synaptic plasma membranes (SPM) on sucrose gradients (1–3). The concentrations of INT used conventionally range from 4–6 mg/g brain tissue (wet weight). At these concentrations INT acts as a sulfhydryl cross-linking reagent (4). Oxidation of sulfhydryl groups in turn may lead to inactivation of some neurotransmitter receptors (5, 6), inhibit enzyme activities such as protein kinases (7), affect the polymerization state of structural components like microtubules (8, 9), and modify the structure of synaptic junctions and postsynaptic densities (10, 11). From a preparative point of view, at the concentrations of INT used conventionally, the reduced formazan is also extensively incorporated in the junctional region of the plasma membranes. This gives to the synaptic junctions an additional adhesiveness that makes their handling very difficult. Furthermore, the incorporation of formazan in the SPM makes them denser and as a consequence some membranes are lost in the mitochondrial pellet. Therefore, during the purification of SPM, it would be desirable to use as low a concentration of INT–succinate as is compatible with a reasonably low contamination by mitochondria. A systematic study of the optimal concentration of INT required for that purpose has never been carried out. The results of such work are the subject of this report.

EXPERIMENTAL PROCEDURE

Subcellular Fractionation. Male Sprague-Dawley rats, 90 days old (Simonsen Labs, Gilroy, California), were killed by decapitation and the forebrain rostral to the superior colliculi used for subcellular fractionation. The tissue (54 g wet weight) was homogenized and a mitochondrial–synaptosomal P_2 pellet was obtained as previously described (1, 2). All centrifugations were performed at 4°C. Following osmotic shock to lyse synaptosomes, the P_2 pellet was divided into six equal aliquots, each corresponding to 9 g wet weight brain tissue. Each aliquot was suspended in 26 ml of 50 μM $CaCl_2$–0.2 mM HEPES buffer, pH 7.3 (buffer A); a solution of INT (2.53 mg/ml, 5 mM) containing disodium succinate hexahydrate (65 mg/ml, 0.24 M) in 0.2 M sodium phosphate buffer, pH 7.4, was added to the final concentrations indicated in Table I. The mixtures were incubated at 30°C for 25 min. All the suspensions turned different intensities of purple, the lighter shades belonging to the lower INT concentrations. The incubation mixtures were sedimented in a type-30 rotor (Beckman, Spinco) at 13,000 rpm for 10 min and washed twice with 0.16 M sucrose in buffer A. The P_2 pellet, which was dense and easily sedimented after incubation with INT–succinate,

TABLE I
CONDITIONS OF TREATMENT OF MITOCHONDRIAL PELLET P_2[a]

Exp No.	Volume INT–succinate added (ml)	Final INT conc. (mg/ml)	INT (mg/g wet brain)	Succinate (mg/g wet brain)	Approx ratio of INT concentration to conventional
1	15.0	0.92	4.2	107.8	1
2	7.0	0.43	2.1	53.1	1/2
3	3.5	0.21	1.06	26.9	1/4
4	2.0	0.12	0.53	13.5	1/8
5	1.0	0.06	0.26	6.7	1/16
6	0.5	0.03	0.13	3.4	1/32

[a] Aliquots 1–6 were each suspended in 26 ml of buffer A solution, and a solution of INT–succinate in 0.2 M sodium phosphate buffer, pH 7.4, was added to give the final concentrations indicated in the table. Where necessary 0.2 M phosphate was added to reach a final volume of 41 ml in all suspensions. Conventional INT concentration is that used by Cotman and Taylor (1).

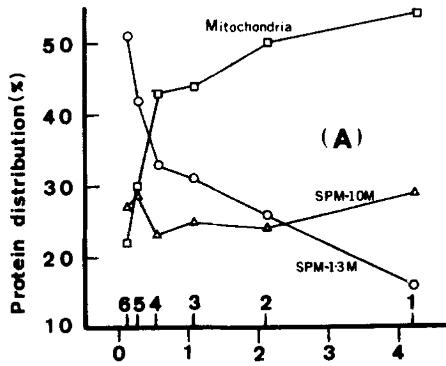
became lighter following the two washes and had to be sedimented at 21,000 rpm for 15 min in the type-30 rotor. Each washed pellet was resuspended in 10 ml of 0.32 M sucrose in buffer A and applied to the top of a discontinuous sucrose gradient consisting of successive layers (7 ml each) of 1.3 M, 1.0 M, and 0.8 M sucrose solutions (Beckman SW 25.1 rotor). After centrifugation at 21,000 rpm for 90 min, the sediment and interfaces between 1.3 M and 1.0 M sucrose and 1.0 M and 0.8 M sucrose were collected and washed with 2 vol of buffer A (30 rotor, 21,000 rpm, 20 min). The 0.8 M–0.32 M sucrose interface, containing predominantly myelin, was discarded. All other fractions were kept for determination of protein content, SDS-polyacrylamide gel electrophoresis, electron microscopy, and further subfractionation to obtain synaptic junctions (SJ).

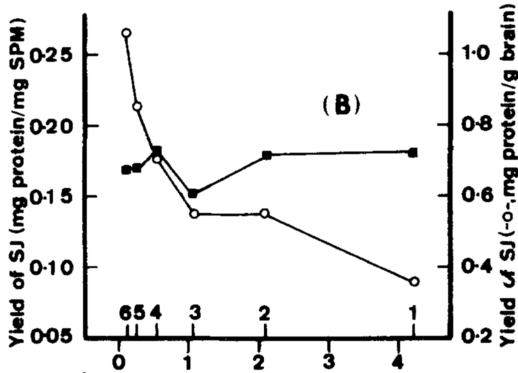
SJs were obtained from the synaptic plasma membrane (SPM) fraction that sedimented at the 1.3 M–1.0 M sucrose interface, according to Cotman and Taylor (1). Briefly, each fraction was resuspended in 0.32 M sucrose in buffer A to a final protein concentration of 4 mg/ml, cooled to 0°C in an ice bath, and two volumes of Triton X-100 solution (0.4% w/v) containing 1 mM EDTA–2 mM HEPES buffer, pH 7.4, were added dropwise, slowly, while swirling the mixture. After 10 min in the ice bath, the detergent-treated membranes were layered onto a cushion of 1.0 M sucrose (minimal volume, 5 ml) and centrifuged for 90 min in the SW 25.1 rotor at 21,000 rpm. The pellet was the SJ fraction. The interface between 1.0 M and 0.32 M sucrose always had a layer of material that was also collected separately for analysis. This will be designated as Triton–1.0 M sucrose interface or, in short, the Triton interface.

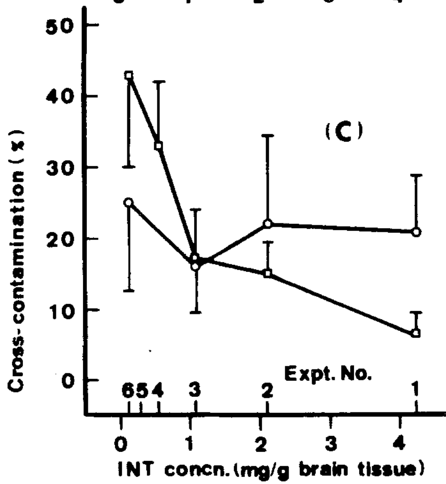
SJ from preparations 1 and 2 were intensely purple and highly adherent. SJ from preparations 3–6 were more pink and much less adhesive to each other and to the containers.

Analysis of Subcellular Fractions. Gel electrophoresis was carried out in the presence of sodium dodecyl sulfate using the discontinuous buffer system of Laemmli (12) and 7–17.5% (w/v) exponential–linear polyacrylamide gradients cast on slabs, run, and stained as described by Kelly and Luttges (13).

Fractions for electron microscopy were fixed in 4% glutaraldehyde, postfixed in 1% OsO_4,


Mitochondria
(A)
SPM-1.0M
SPM-1.3M
Protein distribution (%)
50
40
30
20
10
6 5 4 3 2 1
0 1 2 3 4


(B)
Yield of SJ (mg protein/mg SPM)
0·25
0·20
0·15
0·10
0·05
Yield of SJ (-o-, mg protein/g brain)
1·0
0·8
0·6
0·4
0·2
6 5 4 3 2 1
0 1 2 3 4


(C)
Cross-contamination (%)
50
40
30
20
10
0
Expt. No.
6 5 4 3 2 1
0 1 2 3 4
INT concn. (mg/g brain tissue)

block-stained in uranyl acetate, and counterstained with lead citrate, essentially as described by Cotman and Taylor (1). Ultrathin sections were examined in a JEOL model 100 C electron microscope.

Protein was determined by the method of Lowry et al. (14), including appropriate blanks to correct for the absorption of formazan (2).

Materials. INT, Triton X-100, disodium succinate hexahydrate, acrylamide, and *N,N,N',N'*-tetramethylethylenediamine were obtained from Sigma Chemical Co. (St. Louis, Missouri). Acrylamide was recrystallized once from hot chloroform. Glutaraldehyde (electron microscope grade) and osmium tetroxide were purchased from Polysciences Inc. (Warrington, Pennsylvania). *N,N'*-methylene-bisacrylamide and ammonium persulfate were supplied by Bio-Rad Laboratories (Richmond, California). All other chemicals were analytical reagent grade or the best grade commercially available.

RESULTS

Influence of Concentration of INT–succinate on Distribution of Protein between Mitochondrial and SPM Fractions. The morphological and biochemical properties of the subcellular fractions obtained from the crude synaptosomal–mitochondrial pellet (P_2) by centrifugation in a discontinuous sucrose gradient after incubation with 4–6 mg INT/g brain has been described by Cotman and Taylor (1). The interface at 1.0 M–1.3 M sucrose contained most of the junctional complexes, whereas the 0.8 M–1.0 M sucrose interface contained predominantly nonjunctional membrane vesicles, and the sediment consisted essentially of mitochondria (1). The distribution of protein among these fractions depended strongly on the amount of INT–succinate used (Figure 1A). Lower concentrations of INT resulted in a lower proportion of membrane protein in the mitochondrial fraction and a higher proportion in the SPM fraction at the 1.0 M–1.3 M sucrose interface. By contrast, the amount of protein at the 0.8 M–1.0 M sucrose interface remained essentially the same at all INT–succinate concentrations (Figure 1A). The amount of protein in the

FIG. 1. Influence of the concentration of INT–succinate on the yield and purity of subcellular fractions prepared from rat brain. (A) Protein distribution in a sucrose gradient among mitochondrial fraction and two SPM bands of different density. The "myelin" band, i.e., that particulate material that floated over 0.8 M sucrose, remained essentially constant and was excluded from the calculations. Total protein, obtained by adding that in the interfaces over 1.0 M sucrose, 1.3 M sucrose, and the sediment, ranged from 105 to 112 mg protein/9 g wet brain tissue. (B) Yield of SJ calculated with respect to parent SPM protein (■, left-hand scale) and with respect to starting weight of brain tissue (○, right-hand scale). (C) Cross-contamination of SPM by mitochondria (□) and of mitochondria by SPM (○) calculated by densitometry of electrophoregrams stained with Coomassie blue. Each point is the average of measurements of intensity of 2–4 different bands in two independent experiments.

mitochondrial fraction increased sharply as the concentration of INT increased from 0.13 up to 0.53 mg/g wet brain tissue; thereafter it increased more gradually. Parallel but inverse changes occurred in the protein content of the 1.0 M–1.3 M sucrose interface. That is, increasing the concentration of INT from 0.13 to 0.53 mg/g brain tissue caused a steep decline in the yield of protein in this SPM fraction, followed by a steady decrease in the amount of protein that showed no indication of leveling off (Figure 1A).

The 1.0 M–1.3 M sucrose interface was used to prepare SJs by treatment with Triton X-100 followed by centrifugation over a cushion of 1.0 M sucrose. The yield of protein in the pellet (SJ) is shown in Figure 1B as a function of the INT–succinate concentration used in the preparation of the parent SPM. The yield, expressed with respect to the protein in the SPM fraction, remained fairly constant at a value of 0.17 $\pm$ 0.01 mg of SJ protein per mg of parent SPM protein. On the other hand, the yield of SJ per g of original brain tissue increased in parallel with that of SPM as the INT concentration decreased (Figure 1B). Although a plateau seemed to be reached at 1–2 mg INT/g brain, the yield of SJ still decreased further at the concentration of INT–succinate used by Cotman and Taylor (1) (experiment 1, of Figure 1B).

Purity of Subcellular Fractions. The polypeptide composition (Figure 2) of subcellular fractions obtained using conventional concentrations of INT–succinate was compared to analogous fractions prepared at lower concentrations of INT. The simplest pattern was exhibited by mitochondria. It was characterized by the absence of bands of high molecular weight. Some bands (short arrows, Figure 2; bands f, mol wt 78,000; i, mol wt 30,000; unmarked, mol wt 33,000) were enriched in mitochondria with respect to SPM; for others (Figure 2, bands a, b, c, d, e, g, h, mol wt about 220,000, 117,000, 110,000, 96,000, 87,000, 39,000, and 36,000, respectively) the converse was true. The intensity of the mitochondrial bands of molecular weight 78,000, 33,000, and 30,000 was used to estimate the extent of contamination of SPM by mitochondria, after densitometry of the gels (Figure 1C). The intensity of the SPM bands of molecular weight 117,000, 39,000, and 36,000 was used for a semiquantitative determination of the contamination of mitochondria by nonmitochondrial membranes. The contamination of SPM by mitochondria rose sharply at concentrations of INT–succinate lower than one fourth of that used by Cotman and Taylor (1) (experiments 4–6, Figure 1C). No appreciable differences in the purity of SPM and the derived SJ fractions were observed at higher concentrations of INT (experiments 1–3, Figures 1C and 2). The contamination of mitochondria by SPM protein remained constant at approximately 20% of the total protein (Figure 1C). However, this

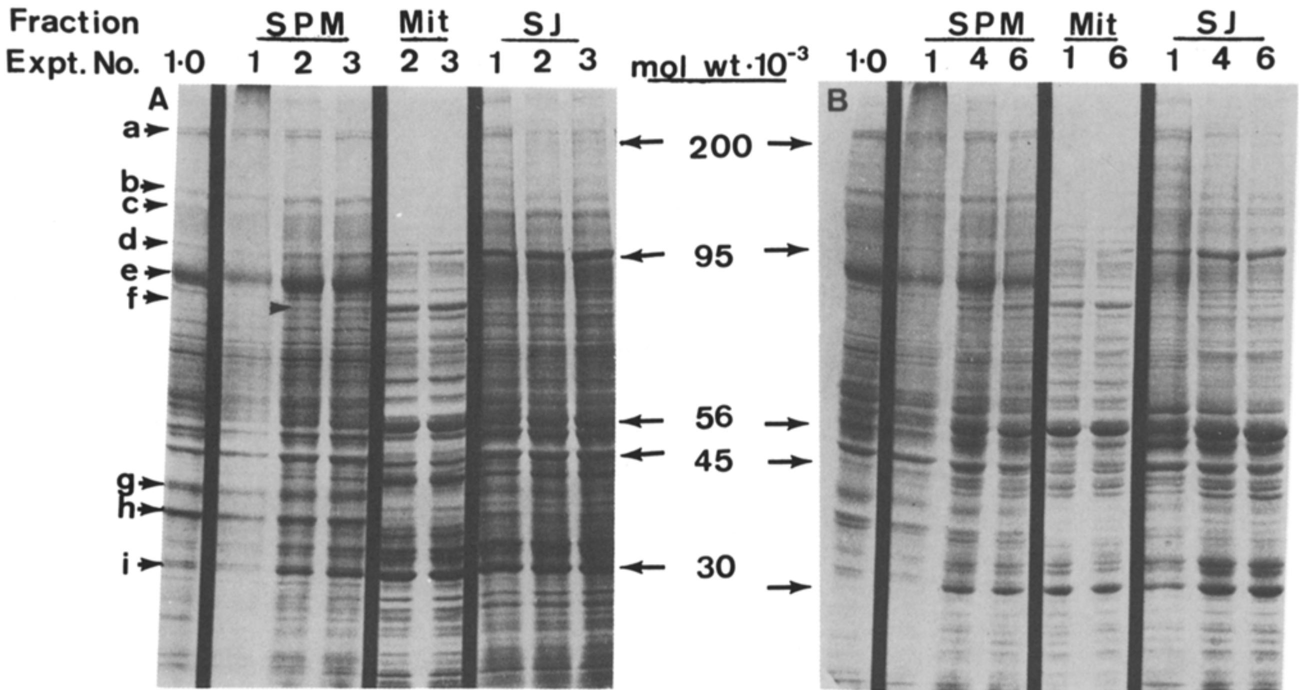


FIG. 2. SDS-polyacrylamide gel electrophoresis pattern of subcellular fractions from rat brain prepared using various concentrations of INT–succinate. Gel electrophoresis was carried out as indicated in the text. SPM, synaptic plasma membranes; SJ, synaptic junctions; Mit, mitochondria. Fraction SPM 1.0 is that material floating over 1.0 M sucrose. Other SPM fractions were the interface over 1.3 M sucrose. All samples contained 60 μg of protein. Gel slab thickness, 0.8 mm.

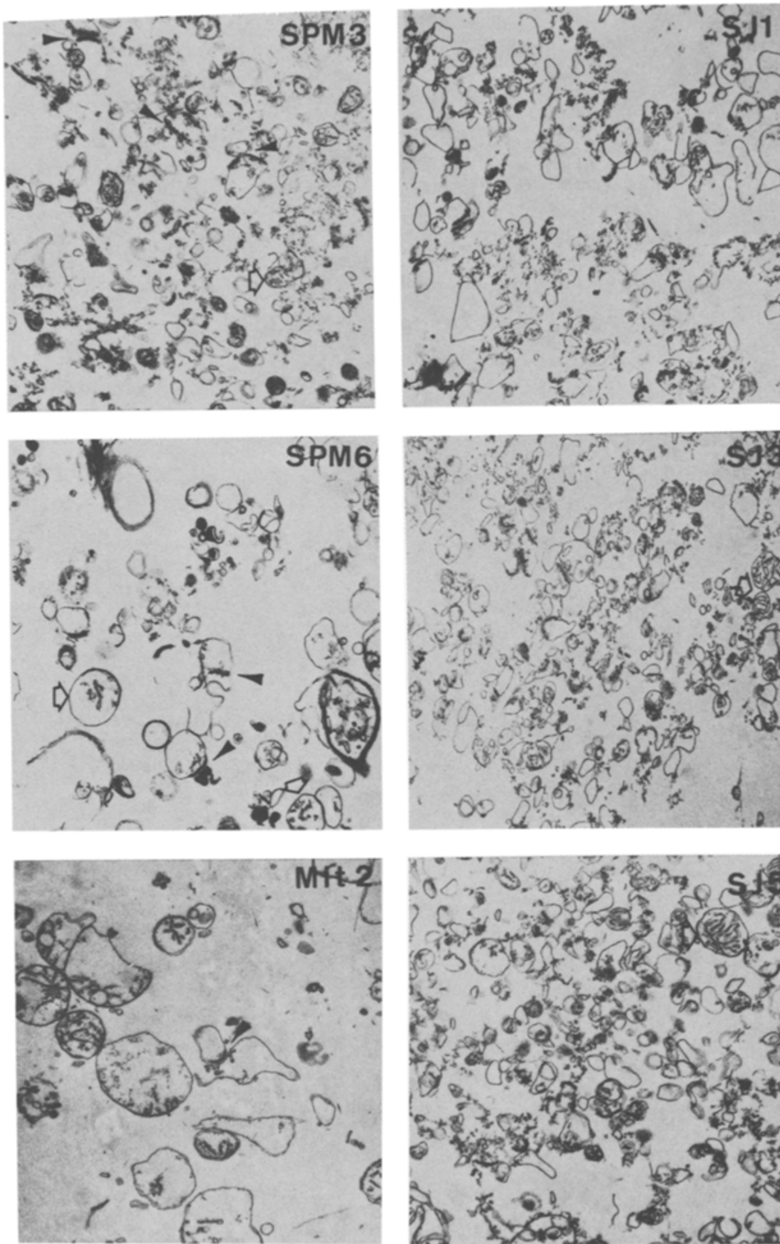


FIG. 3. Ultrastructure of subcellular fractions from rat brain prepared using various concentrations of INT–succinate. The fractions are designated by their abbreviation and the experiment number as in Table 1. Filled arrows point to junctional complexes. Open arrows point to membranes of probable mitochondrial origin. Magnification: ×11,900.

result should be taken with caution because of the large standard deviations involved (Figure 1C).

Electron microscopy of the different SPM, SJ, and mitochondrial fractions confirmed these observations (Figure 3). No differences in the appearance of SPM and SJ were observed in experiments 1–3 but, as the concentration of INT–succinate decreased, the contamination by fragments of mitochondrial origin rose noticeably (experiments 4–6; SJ5 in Figure 3). Conversely, at high concentrations of INT, junctional complexes were frequently observed in the mitochondrial fractions (Mit 2, Figure 3).

Triton X-100–1.0 M Sucrose Interface Material. The purification of synaptic junctions entails the treatment of SPM with Triton X-100, followed by sedimentation through a cushion of 1.0 M sucrose. In all preparations a variable amount of particulate material was observed floating at the interface over 1.0 M sucrose. This so-called Triton interface had a protein composition similar to that of synaptic junctions, but the proportion of some polypeptide bands was reminiscent of that found in SPMs (Figure 4B). Electron microscopic examination of this fraction in thin sections and negative staining showed it to be composed of synaptic junctions and many small vesicles (Figure 4). The yield of the Triton interface was always lower than that of SJ. However, it varied quite widely from preparation to preparation (4–10% of total SPM protein) and was higher when SJ yield was lower, as if the total SJ protein had redistributed among pellet and interface.

Electrophoretic Markers of Subcellular Fractions. Mitochondria were the only brain subfraction that had a distinct pattern of bands in SDS-gel electrophoresis (Figure 2). All other membranous subfractions, including the SPM fractions that float over 1.0 M sucrose and 1.3 M sucrose, SJ, Triton interface, and microsomal membranes (P_3 fraction, not included in this study) showed qualitatively similar patterns, differing only in the proportion of the various polypeptide components. This similarity makes it very difficult to assign particular polypeptide band markers to any given membrane subfraction. In this study we had available for comparison preparations of SPM and SJ with varying amounts of mitochondrial contamination as well as the so-called Triton interface, which may be considered an SJ preparation with varying proportions of extrajunctional membrane. Also, the SPM fraction over 1.0 M sucrose can be considered as impoverished in junctional complexes (i.e., enriched in extrajunctional membrane) and almost totally devoid of mitochondria. With these assumptions, a comparison of the patterns of the subfractions allowed the assignment of marker bands to mitochondria and junctional and extrajunctional membranes. Thus, bands f and i of Figure 2 (mol wt 78,000 and 30,000, respectively) and other unmarked bands were intense in mi-

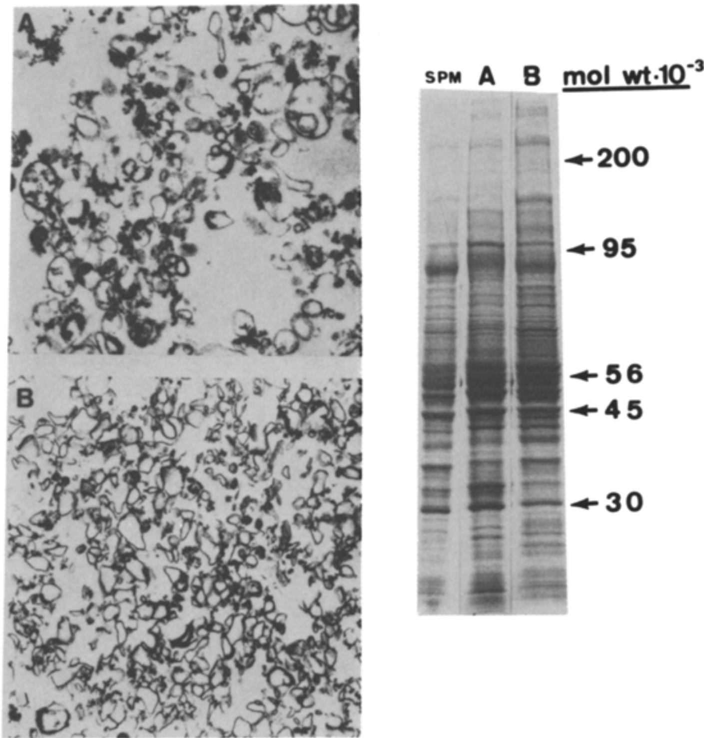


FIG. 4. Comparison of the ultrastructure and polypeptide composition of synaptic junctions and Triton interface. (A) SJ, synaptic junctional fraction; (B) Triton X 100–1.0 M sucrose interface. For comparison purposes, the electrophoretic pattern of the parent SPM fraction has also been included. Electron micrograph magnification: (A) × 16,000; (B) × 10,000. Gel electrophoresis: 60 μg of protein per sample; gel thickness, 0.8 mm.

tochondria, absent in the SPM interface over 1.0 M sucrose, and scarce in SPM over 1.3 M sucrose. Therefore, such bands were considered mitochondrial markers. Bands a, g, and h (mol wt 220,000, 39,000 and 36,000, respectively) occurred in comparable proportions in SPM, SJ, and Triton interface. Hence, they were be assumed to be uniformly distributed in junctional and extrajunctional membranes. On the other hand, bands b and e (mol wt 117,000 and 87,000, respectively) were comparatively abundant in SPM (1.0 M and 1.3 M sucrose) and the Triton interface but very scarce in SJ; hence, they were assumed to be predominantly or exclusively located in extrajunctional membrane. Conversely, bands c

and d (mol wt 110,000 and 96,000, respectively) and a diffuse set of bands located between bands d and e were enriched in SJ with respect to SPM or the Triton-interface and thus were considered markers for the synaptic junction. Probably none of these markers is exclusively located in one type of membrane. Nevertheless, the enrichment of a polypeptide in a particular subcellular fraction may help in the identification of organelles in situations, like that of the developing brain, where buoyant density does not allow an operational definition of SPM or SJ. More importantly, these bands provide a simple means to ascertain purity without resorting to electron microscopy every time.

DISCUSSION

Optimal Concentration of INT–Succinate for Preparation of SPM. The ultrastructural appearance of subcellular fractions prepared at different INT–succinate concentrations, as well as their SDS-gel electrophoretic patterns indicates that the concentrations of INT–succinate used conventionally in the preparation of SPM are about 3- to 4-fold higher than the minimal amount required. Conventional conditions (1–3) ensure a SPM preparation free of major mitochondrial contamination and in reasonable yield. However, by using a concentration of INT of only 1.0 mg/g wet weight brain tissue (16–25% of the conventional concentration) (1–3), it is possible to obtain SPM and SJ fractions of purity comparable to that previously reported (1) with a yield that is increased by about 100% in SPM and 64% in SJ (Figure 1). The lower yield using the higher concentration of INT may be due to loss of junctional complexes into the mitochondrial fraction. Although mitochondria seemed to be contaminated by about 20% nonmitochondrial material in all experiments (Figure 1C), the nature of the contaminant may be variable. Junctional complexes were frequently observed in electron micrographs of mitochondrial pellets using conventional INT concentrations, but they were much less frequent at low INT concentrations. The increased yield of SPM, and hence SJ, obtained using 1 mg INT/g brain tissue is especially significant when the fractionation of very small or very large amounts of brain tissue is envisaged. In addition, the SJs obtained are less adhesive to each other and to the walls of the containers, thus facilitating the handling of the material.

Influence of INT on Yield and Purity of SJ. At low concentrations of INT (experiments 5 and 6), the SJ prepared by Triton treatment of SPM was grossly contaminated by mitochondrial membranes (Figures 2 and 3). Junctional complexes were insoluble in Triton, but so were some mitochondrial components. Therefore, the purity of SJ preparation de-

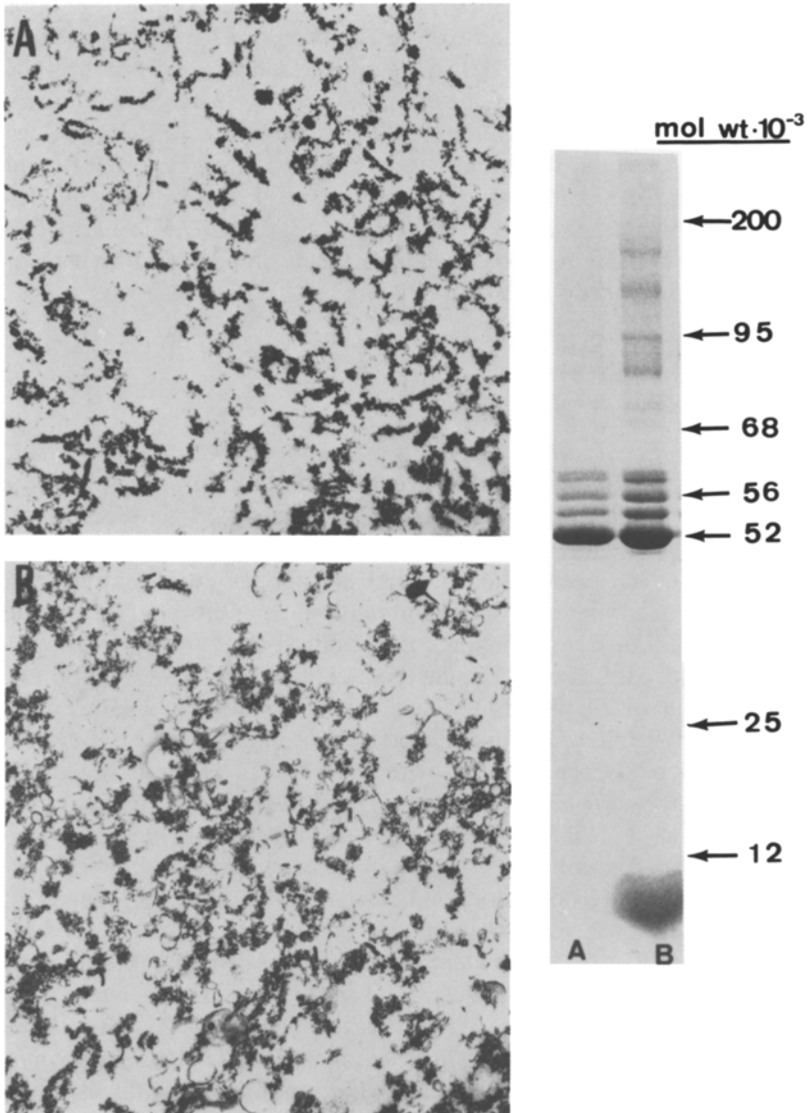


FIG. 5. Comparison of postsynaptic densities and sodium lauryl sarcosinate–1.0 M sucrose interface as regards their ultrastructural appearance and polypeptide composition. (A) Postsynaptic densities; (B) NSL–1.0 M sucrose interface. Electron micrograph magnification: ×18,000. Gel electrophoresis: 40 μg of protein per sample; gel thickness, 0.8 mm.

pends on the purity of the parent SPM. Selectivity cannot be expected from the detergent. Furthermore, the constancy in the yield of SJ protein obtained from SPM preparations contaminated by mitochondria to very different extents (17%, Figure 1C) confirms that protein solubilization under the conditions used to prepare SJ from SPM was not selective.

Nature of Triton X100–1.0 M Sucrose Interface. The electrophoretic pattern and electron microscopic appearance of this fraction indicates a great similarity to SJ. The differences in buoyant density could be accounted for by the presence in the Triton interface of a large proportion of lipid–detergent micelles solvating the SJ proteins. In the conditions used, the critical micellar concentration of Triton X-100, measured with the help of neutral red by ultraviolet difference spectroscopy, was 0.014% (w/v). Therefore, at a concentration of 0.13% (w/v), as used in the preparation of SJ, most of the detergent would be present as micelles. The yield of the Titron interface may depend on the variable concentration of detergent–lipid micelles available after maximum possible solubilization of SPMs under the conditions used.

A subfraction, similar to the Triton interface, was also found during the preparation of postsynaptic densities by treatment of SPM with sodium *N*-lauryl sarcosinate (NLS) and centrifugation on a 1.0 M sucrose cushion (2, Nieto, Kelly, and Cotman, unpublished observations). The protein composition of that fraction was similar to that of postsynaptic densities and in the electron microscope it seemed to be composed of postsynaptic density material and small vesicles (Figure 5). As with the corresponding fraction derived from Triton treatment of SPM, the yield of NSL–1.0 M sucrose interface was variable and complementary to that of postsynaptic densities. It appeared that the SPM material insoluble in NLS distributed among the sediment (operational PSDs) and the 1.0 M sucrose interface in a proportion that depended on the amount of detergent–lipid micelles associated with the particles. The critical micellar concentration of NLS under the conditions of preparation of postsynaptic densities was 0.3% (w/v), a value that, as with Triton, can account for the presence of numerous micelles in a 4% solution of that detergent.

ACKNOWLEDGMENTS

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