

Synapsin I (Protein I), a Nerve Terminal-Specific Phosphoprotein. II. Its Specific Association with Synaptic Vesicles Demonstrated by Immunocytochemistry in Agarose-embedded Synaptosomes

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ABSTRACT Synapsin I (protein I) is a major neuron-specific endogenous substrate for cAMP-dependent and Ca/calmodulin-dependent protein kinases that is widely distributed in synapses of the central and peripheral nervous system (De Camilli, P., R. Cameron, and P. Greengard, 1983, *J. Cell Biol.* 96:1337-1354). We have now carried out a detailed analysis of the ultrastructural localization of synapsin I in the synapse. For this purpose we have developed a novel immunocytochemical technique that involves the labeling of isolated synaptosomes immobilized in a thin agarose gel. Special fixation conditions were designed to maximize accessibility of synapsin I to marker molecules. Immunoferritin and immunoperoxidase studies of this preparation indicated that synapsin I is localized in the presynaptic compartment and that it is present in close to 100% of all nerve endings. Immunoferritin labeling also indicated that, inside the nerve ending, synapsin I is specifically associated with the cytoplasmic surface of synaptic vesicles. In agreement with these immunoferritin results, the labeling produced by immunoperoxidase was compatible with a specific association of synapsin I with synaptic vesicle membranes. However, at variance with the very specific distribution of immunoferritin, immunoperoxidase reaction product was also found on other membranes of the terminals, presumably as a result of its diffusion over a short distance from the synaptic vesicles.

Anti-synapsin I immunoperoxidase staining of tissue sections for electron microscopy produced an uneven labeling of terminals of the neuropile, in agreement with results of a previous study (Bloom, F. E., T. Ueda, E. Battenberg, and P. Greengard, 1979, *Proc. Natl. Acad. Sci. USA.* 76:5982-5986). A comparison with results obtained in isolated synapses indicates that the limited labeling of nerve endings in tissue sections results from limited and uneven penetration by marker molecules.

The specific association of synapsin I with synaptic vesicle membranes in the great majority of nerve terminals suggests a prominent role for this phosphoprotein in the regulation of synaptic vesicle function.

Synapsin I, previously referred to as protein I (8), is a major neuron-specific substrate for cAMP-dependent and Ca/calmodulin-dependent protein kinases (18, 19, 21, 23, 25, 35, 38). It is present in high concentration in the mammalian brain

(13). Immunocytochemical studies carried out by an immunoperoxidase technique (2, 9), and more recently by immunofluorescence (8), have indicated that synapsin I is a nerve cell-specific protein that is highly concentrated at synapses and has

a very widespread distribution throughout the central and peripheral nervous systems. In fact, it seems to be present at the great majority of synapses.

The present study was undertaken to confirm at the electron microscopic level this general distribution of synapsin I at synapses, and to identify, at the ultrastructural level, the localization of protein I in the synapse. For this purpose, we have developed a novel immunocytochemical technique that involves the immunolabeling, by immunoferritin or by immunoperoxidase, of synaptosomes embedded in agarose. This procedure has been specifically designed to overcome those technical problems that affect immunocytochemical studies in fixed intact tissues. Our results indicate that synapsin I is present in the presynaptic compartment, where it is primarily associated with the cytoplasmic side of synaptic vesicle membranes. These studies also demonstrate conclusively that synapsin I is present at the great majority of synapses.

MATERIALS AND METHODS

Sprague-Dawley rats weighing 150–200 gm were obtained from Charles River Breeding Laboratories, Wilmington, MA; cyanogen-bromide-activated Sepharose 4B and agarose C were from Pharmacia (Uppsala, Sweden); Triton X-100, saponin, chicken egg albumin, bovine serum albumin (fraction V), ferritin (from horse spleen), and 3,3'-diaminobenzidine tetra-HCl were from Sigma Chemical Co. (St. Louis, MO); paraformaldehyde, glutaraldehyde (10% aqueous solution) and OsO₄ (4% aqueous solution) were from Electron Microscopy Sciences (Fort Washington, PA); sucrose was from Schwartz Mann (Orangeburg, NY); H₂O₂ (30% vol/vol aqueous solution) was from Eastman Kodak Co. (Rochester, NY).

Purified synapsin I, used for affinity purification of anti-synapsin I antibodies, was the gift of Dr. Louis J. DeGennaro.

ANTIBODIES: An antiserum directed against synapsin I was prepared as described (8) by injecting rabbits with synapsin I (synapsin Ia and synapsin Ib) that had been purified from bovine brain by a procedure involving SDS gel electrophoresis as the final step. The specificity of the antiserum has been extensively characterized (8). Rabbit antibodies specific for synapsin I (anti-synapsin I IgG [Rb])¹ were purified from the serum by affinity-chromatography (7, 11) on a synapsin I-Sepharose-4B column. Horseradish peroxidase-conjugated goat antibodies specific for rabbit IgGs (HRP-anti-IgG [G vs. Rb]) were purified from a commercial (lot 14394; N. L. Cappel Laboratories, Inc., Cochranville, PA) peroxidase-conjugated IgG fraction on a rabbit IgG-Sepharose-4B column. In both cases, specific antibodies were eluted from insolubilized antigens by low pH buffer (glycine-HCl buffer, pH 2.3). Nonimmune rabbit and goat IgGs (control IgG [Rb] and control IgG [G], respectively) and ferritin-conjugated goat IgGs directed against rabbit IgGs (F-anti-IgG [G vs. Rb]) were from Cappel.

To establish the number of ferritin particles present on each IgG-ferritin conjugate, a drop of very dilute solution of conjugated IgGs in phosphate-buffered saline containing 10⁻⁶ M bovine serum albumin was deposited on a Formvar-coated grid. After 30 s, the grid was blotted and dried. Subsequent analysis of the grid at the electron microscope revealed that the large majority of ferritin particles occurred as individual particles clearly separated from each other. This indicated that the large majority of IgG conjugates contained only one ferritin/molecule.

Preparation of a Crude Synaptosomal Fraction

A crude synaptosomal fraction was prepared by minor modifications of the method of Gray and Whittaker (15). Three rats were killed by decapitation. Brains (including brain stems and cerebella) were quickly removed and homogenized in 10 vol of ice-cold 0.3 M sucrose by nine strokes at ~900 rpm in a glass/Teflon homogenizer (0.10–0.15 mm clearance). The homogenate was then divided among four tubes and centrifuged at 1,000 *g*_{av} for 10 min at 4°C in a Sorvall centrifuge (SS-34 rotor; DuPont Co., Newtown, CT). The supernatants were

gently removed, transferred to four new tubes and centrifuged at 12,500 *g*_{av} for 20 min at 4°C in the same rotor. The resulting pellets were resuspended in a small volume of 0.3 M sucrose, pooled, and referred to as the crude synaptosomal fraction. This contained mostly synaptosomes, free mitochondria, and myelin fragments. Synaptosomes were not further purified because contaminating organelles provided an internal control for evaluating the specificity of the immunocytochemical staining procedure.

Extraction of Synapsin I from the Synaptosomes

A 200- μ l aliquot of the resuspended crude synaptosomal fraction was diluted at 0°C in 9 ml of a solution containing 0.15 M KCl and 0.01% (wt/vol) saponin (31). After a 5-min incubation on ice, the sample was centrifuged at 12,500 *g*_{av} for 20 min in a 50Ti rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) at 4°C. The supernatant was discarded and the pellet was resuspended in 100 μ l of 0.3 M sucrose with a Teflon rod. The suspension obtained was then fixed by adding 25 μ l of fivefold-concentrated fixative as described for the type A fixation (see below).

Fixation of the Crude Synaptosomal Fraction

In all cases the final fixative solution contained 3% formaldehyde (freshly prepared from paraformaldehyde [12]), 0.5% glutaraldehyde, 5 mM sodium phosphate buffer (pH 7.4) (PB). Sucrose was present at the concentration indicated for the different fixation protocols. This fixative solution was selected to optimize labeling of synapsin I and to preserve its subcellular localization. Thus, the higher concentration of formaldehyde than of glutaraldehyde was aimed at reducing the extent of cross-linking of cytoplasmic proteins (which would reduce accessibility of synapsin I) as well as minimizing loss of antigenicity of synapsin I. The low ionic strength buffer was used to prevent solubilization of synapsin I from its subcellular binding sites (20).

FIXATION IN NONLYTIC CONDITIONS: Type A fixation: Aliquots of the crude synaptosomal fraction were rapidly mixed with 0.25 vol of ice-cold, fivefold-concentrated fixative. Synaptosomes were then kept in the fixative on ice for 30 min, after which they were agarose-embedded. Type B fixation: Aliquots of the crude synaptosomal fraction were fixed by addition of a large volume (20 vol) of ice-cold (1 \times) fixative solution, containing 0.3 M sucrose. Subcellular particles were then recovered from the suspension by centrifugation (12,500 *g*_{av} for 20 min) in a Beckman 50Ti rotor at 4°C. Finally, the pelleted particles were resuspended in 0.12 M PB by scraping the compact pellet from the bottom of the tube with a Teflon rod, homogenizing the fragments of the pellet in a small glass-Teflon homogenizer, and finally passing this suspension several times through a 25-gauge needle. These resuspended particles were then used for agarose embedding. The majority of nerve endings of synaptosomal preparations fixed under these conditions appeared to have a continuous, sealed plasma membrane. Postfixation treatments were, therefore, performed in some experiments in an effort to make all plasma membranes permeable to macromolecules. These treatments included: (a) cycles of freeze-thawing of the fixed synaptosomal suspension (aliquots of the suspension were frozen by immersion in a dry-ice/isopropanol mixture and rapidly thawed in water at room temperature [RT]) and (b) detergent treatments of the subcellular particles after agarose embedding (see below).

FIXATION IN LYTIC CONDITIONS: Aliquots of the crude synaptosomal fraction were mixed with 20 vol of ice-cold (1 \times) fixative solution, which did not contain sucrose. The diluted subcellular particles were then recovered by centrifugation, and resuspended in PB, as described above for the type B fixation.

Embedding in Agarose

Aliquots (180 μ l) of fixed synaptosomes were added to tubes immersed in a 54°C water bath. After a 15-s interval, which allowed the suspension to warm up, 180 μ l of a solution (at 54°C) containing 3% agarose in 5 mM PB was added to the synaptosomal suspension. The suspension obtained was quickly mixed, while still immersed in the warm water bath, by forcing it up and down through a pasteur pipette prewarmed in a 60°C oven. Care was taken to prevent foaming. Immediately afterwards, the agarose-synaptosome suspension was transferred by pipetting into a frame made from two glass slides separated at each end by two coverslips (0.17 mm thick) and held together by binding clips as shown in Fig. 1a. The frame had also been prewarmed in a 60°C oven. The agarose mixture was then allowed to cool and solidify. At this point, the two glass slides were separated and the agarose gel, attached to one of the glass slides, was cut into 2-mm squares with a razorblade. The gel squares were then washed off the glass slide into a petri dish (Fig. 1b) by a stream from a pasteur pipette filled with 0.12 M PB. These agarose squares (agarose blocks) were then transferred to glass tubes and used for immunolabeling.

¹ *Abbreviations used in this paper:* F-anti-IgG (G vs. Rb), ferritin-conjugated goat IgGs directed against rabbit IgGs; HRP-anti-IgG (G vs. Rb), horseradish peroxidase-conjugated goat IgGs directed against rabbit IgGs; control IgG (G) and control IgG (Rb), goat and rabbit nonimmune IgGs, respectively; anti-synapsin I IgG (Rb), rabbit IgGs directed against synapsin I; EM, electron microscopy; PB, sodium phosphate buffer, pH 7.4; RT, room temperature.

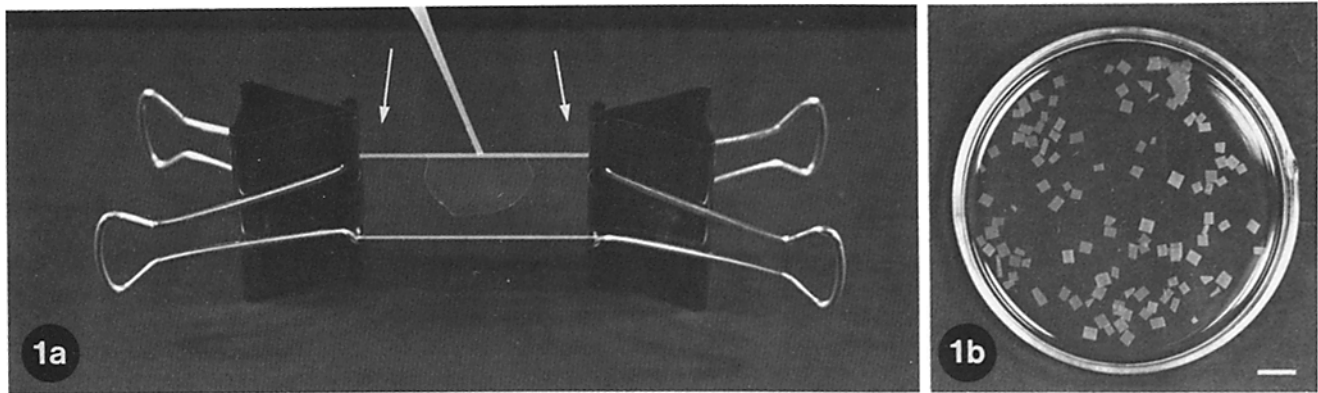


FIGURE 1 Preparation of agarose blocks containing synaptosomes. (a) Synaptosome-agarose suspension being poured from a Pasteur pipette into a frame made of two glass slides separated by two coverslips. The two coverslips are indicated by arrows. (b) Agarose blocks containing synaptosomes suspended in buffer in a petri dish. The blocks were prepared by cutting into small squares the agarose gel obtained from the frame shown in a. Bar, 1 cm.

Immunoferritin and Immunoperoxidase Labeling of Agarose-embedded Subcellular Particles

Agarose blocks containing fixed synaptosomes were sequentially processed through the steps described below. All steps were carried out at RT, except where indicated, in 12 × 75 mm glass tubes that contained six agarose blocks each. To remove possible protein aggregates, all protein-containing solutions were spun for 4 min in an Eppendorf microfuge (model 5412 Brinkmann instr., Westbury, NY.) before use. Step 1: Incubation (30 min) in Tris-HCl buffer (pH 7.4) to quench unreacted aldehyde groups. Step 2: Incubation (30 min) in solution A (0.5 M NaCl, 0.02 M PB, 50 mg/ml chicken egg albumin, and 1 mg/ml control IgG (G)). The purpose of this incubation was to saturate the nonspecific binding sites for proteins and in particular for IgGs. Step 3: Incubation (overnight at 4°C) in 200 μl of solution A containing anti-synapsin I IgG (Rb) (70–120 μg/ml) or control IgG (Rb) (70–120 μg/ml). Step 4: Washing by incubation in solution B (0.5 M NaCl, 0.02 M PB) with five changes of ~2 ml each over a 90-min period. Step 5: Incubation (120 min) in 200 μl of solution A containing F- or HRP-anti-IgG (G vs. Rb). The commercial F-anti-IgG (G vs. Rb) was used at a 1:15 dilution. The affinity purified HRP-anti-IgG (G vs. Rb) was used at an IgG concentration of ~10–50 μg/ml. Step 6: Washing in solution B as in step 4. Step 7: Fixation of the bound antibodies with 1% glutaraldehyde in 0.12 M PB for 30 min. Step 8: washing in 0.12 M PB. Agarose blocks labeled by immunoferritin were then processed for electron microscopy (EM) as described below. Blocks labeled by immunoperoxidase were rinsed in 0.12 M NH₄-phosphate buffer (Dr. J. Boyles, personal communication) with two changes of 2 ml, 5 min each and then immersed in 1 ml of the same buffer (ice-cold) containing 0.5 mg/ml 3,3'-diaminobenzidine tetra-HCl. The latter solution was passed through a Millipore filter (0.22-μm mesh) just before use. The peroxidase enzymatic reaction was carried out on ice and started by the addition of concentrated H₂O₂ to a final concentration of 0.003% (vol/vol). After 10–15 min, the reaction was terminated by dilution with 4 ml of ammonium phosphate buffer followed by several rapid changes of the same buffer. Blocks were then processed for EM.

In some experiments, a detergent treatment (45 min at room temperature in solution B containing either saponin [31] [0.01–0.5% (wt/vol)] or Triton X-100 [0.3–1% (wt/vol)]), aimed at permeabilizing plasma membranes of sealed nerve endings, was performed between steps 1 and 2.

Test for Accessibility of Synaptosomes to Ferritin Particles

To test for the ability of unconjugated ferritin to penetrate into fixed agarose-embedded nerve endings, the following procedure was used. Between step 1 and step 2 of the immunolabeling procedure, agarose blocks containing synaptosomes were incubated for 90 min at RT in solution B containing 100 mg/ml unconjugated ferritin. This incubation was directly followed by fixation in 1% glutaraldehyde in 0.12 M PB for 30 min at room temperature. Blocks were then washed in PB and processed for EM.

Immunoperoxidase Labeling of Frozen Sections of Rat Brain

Rat brains were fixed by perfusion (3% formaldehyde, 0.5% glutaraldehyde, 0.12 M PB) and frozen as described (8). Thick frozen sections (20 μm thick) were cut in a Damon IEC Minotome cryostat (International Equipment Corp., Damon Corp., Needham Heights, MA) and the sections were immediately thawed by floating them on 0.12 M PB at room temperature in a Petri dish. These sections were then processed for synapsin I immunoreactivity by the same immunoperoxidase procedure used for the agarose blocks, and subsequently processed for EM. In some cases a 45-min incubation in solution B containing 0.3% (wt/vol) Triton X-100 or saponin (0.01% or 0.5% [wt/vol]) preceded the immunostaining.

Electron Microscopy

Agarose blocks were washed with 0.1 M veronal/acetate buffer (pH 7.4) and postfixed for 1 h in 1% OsO₄ in the same buffer at 0°C. Then, after a quick rinse in veronal/acetate buffer, they were dehydrated in a graded series of ethanols and propylene oxide and embedded in Epon 812 (26).

Thin sections (silver) were cut on a Sorvall Porter-Blum MT2 ultra-microtome, stained with uranyl acetate and lead citrate and examined in a Philips 301 electron microscope operated at 80 kV.

Morphometric Analysis

Thin ultramicrotome sections of similar thickness (as assessed by their interference color) were selected for the various comparisons.

COUNTS OF SYNAPTIC VESICLES AND FERRITIN PARTICLES IN NERVE ENDINGS: Electron micrographs were taken at random until a sample of 100 nerve endings was reached for each experimental condition. The magnification of the EM negatives was × 19,000 or 25,000 and of the final printed micrographs was × 65,000. The nerve ending of each synaptosome was examined for the total number of vesicles, the total number of ferritin particles, and, in the case of synaptosomes fixed in nonlytic conditions, the cross-sectional area, which was corrected for the fractional area occupied by mitochondria or by vesicular compartments other than synaptic vesicles. Areas were measured with a Leitz planimeter (E. Leitz, Inc., Rockleigh, NJ). To determine areas when interruptions were present in the plasma membrane, the free edges of the membrane were joined with the shortest line that included in the nerve ending space all the vesicles that appeared to be part of that ending.

The following ratios were calculated for each terminal: (a) total number of ferritin particles/total number of synaptic vesicles, and (b) total number of vesicles/cross-sectional area in μm² of the terminal.

COUNTS OF FERRITIN PARTICLES ON POSTSYNAPTIC DENSITIES (PSDs): Electron micrographs of synaptosomes fixed in nonlytic conditions and labeled with control IgG (Rb) or anti-synapsin I IgG (Rb) were taken at random (magnifications as above). Only PSDs clearly not enclosed by a sealed postsynaptic plasma membrane were chosen. A line was drawn parallel to the junctional postsynaptic plasma membrane at its cytoplasmic side at a distance of ~900 Å from its cytoplasmic leaflet. 900 Å was found to be a sufficient distance to include

the thickest PSDs plus an allowance for the immunoferritin complex. Then, for each PSD we determined: (a) the length of the junctional postsynaptic plasma membrane visible in the plane of the section, and (b) the number of ferritin particles enclosed between the plasma membrane and the parallel line we had drawn.

We scored PSDs until we reached a total PSD length of 16.5 μm for both groups of samples (~50 PSDs for each sample).

PERCENTAGES OF NERVE ENDINGS ACCESSIBLE TO FREE FERRITIN OR LABELED BY ANTI-SYNAPSIN I IMMUNOFERRITIN: Electron micrographs of synaptosomal preparations labeled by immunoferritin and of synaptosomal preparations used for testing accessibility to free ferritin were taken at random (magnifications as above), until a sample size of 200 nerve endings was reached for each of the two experimental conditions. Nerve endings were then divided into two groups, as indicated in the legend to Table I.

RESULTS

Synaptosomes Fixed in Nonlytic Conditions

MORPHOLOGY: Electron microscopic examination of the crude synaptosomal fraction, fixed under nonlytic conditions and embedded in agarose before processing for electron microscopy, revealed a satisfactory retention of ultrastructural morphology. The state of preservation of individual synaptosomes, however, was highly variable. In the majority of synaptosomes the nerve ending showed a well-preserved structure ("well-preserved nerve endings"): vesicles appeared tightly packed in the terminal, coated vesicles were occasionally visible, the cytoplasm had a dense appearance, and the plasma membrane appeared continuous and intact (Fig. 2*a*). In other synaptosomes the nerve ending was severely disrupted ("disrupted nerve endings"): the synaptic vesicles were quite dispersed, the space between vesicles appeared clear (empty) with only a few interconnecting filaments, coated vesicles were in general not visible (see Discussion), and the plasma membrane was torn (often with one or more large interruptions visible in the plane of the section) (Fig. 2*b*). The morphology of the disrupted nerve endings suggested that their plasma membranes either had not resealed during homogenization, or that they had become leaky at some time before fixation, thereby allowing elution of cytosolic proteins in the solutions of low ionic strength used.

The synaptic cleft was sometimes visible in the plane of the section. In such cases, the remnant of the postsynaptic compartment consisted of a fragment of plasma membrane with the associated PSD. This plasma membrane fragment generally had free ends (e.g., Fig. 2*a*) but in some cases appeared to have resealed to form a closed compartment (Fig. 2*b*). No relationship was observed between the state of preservation of the nerve ending and the morphology of the postsynaptic compartment.

IMMUNOFERRITIN LABELING: When synaptosomes fixed in nonlytic conditions were immunolabeled by an indirect (36) immunoferritin procedure using control IgG (Rb) followed by F-anti-IgG (G vs. Rb), only scattered ferritin particles, isolated or in small clusters,² were observed on the subcellular particles present in the preparation (Fig. 2*a* and *b*). These ferritin particles were in general localized on the cytoplasmic faces of membranes, and, in particular, where these membrane

² Given the observation that only one ferritin particle seemed to be associated with each goat anti-rabbit IgG molecule (see Materials and Methods), the occurrence of background labeling by small clusters of ferritin particles may be attributable to the indirect immunoferritin procedure used. Thus, the small clusters of ferritin particles may reflect microcomplexes of rabbit IgGs and goat anti-rabbit IgGs.

TABLE I
Immunolabeling and Ferritin Penetration

	Immunolabeling %	Test for ferritin penetration %
Immunolabeled	34	
Nonimmunolabeled	66	
Penetrated by ferritin		29
Nonpenetrated by ferritin		71

Comparison between percentages of nerve endings immunolabeled by anti-synapsin I immunoferritin and nerve endings accessible to free ferritin in the same synaptosomal preparation fixed in nonlytic conditions. Agarose blocks used for the two experimental conditions were obtained from the same agarose gel. A total of 200 nerve endings were counted for each of the two conditions. Nerve endings defined as nonimmunolabeled or as nonpenetrated by ferritin were those containing ≤ 10 ferritin particles. In almost all cases, nerve endings of these two groups did not contain any ferritin particles (e.g., see Figs. 2*c*, 4, 6*d* and 7*a*). Immunolabeled endings and endings penetrated by ferritin contained, in general, a very high number of ferritin particles (up to >1,000 in large terminals; see Figs. 2*c*, 3, 4, and 6*d*).

faces appeared to be lined by a microfilamentous web of cytoplasmic proteins (e.g., Fig. 2*a*). This web probably non-specifically traps the probes used for immunolabeling.

When anti-synapsin I IgG (Rb) was used at the first antibody step of the immunostaining procedure (step 3), heavy labeling of ferritin particles occurred inside disrupted nerve endings. Such heavy labeling was specific for disrupted nerve terminals (Fig. 2*c*). Figs. 3 and 4 show several examples of heavily labeled disrupted synaptosomes. As is clearly visible in these micrographs, the overwhelming majority of ferritin particles were associated with the cytoplasmic face of synaptic vesicles, while other organelles, including larger vesicular structures, mitochondria, and plasma membranes of the terminals, were unlabeled. The qualitative and quantitative distribution of ferritin particles on membranes other than those of synaptic vesicles was very similar to the background labeling observed in samples reacted with control IgG (Rb).

Labeling seemed to occur equally on all vesicles and did not appear to correlate with their topological distribution within the terminal, in relation to the synaptic cleft. In those instances in which vesicle labeling was not homogeneous in different portions of the terminal, labeling intensity appeared to be determined by the accessibility of the vesicle membrane to ferritin particles (see e.g., Fig. 4*d*). In some cases, when space was available around a vesicle, ferritin particles formed a "corona" around it (Fig. 3, circle). Such orderly decoration of the vesicle profile was observed in a particularly clearcut fashion on those occasional vesicles that appeared to have been moved to an ectopic site as a result of the manipulations involved in preparing the fixed synaptosomal suspension (Fig. 5). Presumably labeled antibodies have relatively free access to the surface of such ectopic synaptic vesicles, with minimal steric hindrance by neighboring vesicles and/or connecting bridges of cytoskeletal proteins. When the corona was composed of a single "chain" of ferritin particles around the vesicle, the ferritin particles were at a distance of ~ 115 Å from the outer leaflet of the vesicle membrane. This distance of 115 Å could be accounted for by two IgG molecules and the apoferritin shell around the electron-opaque iron core of the ferritin molecule (1, 37). Thus, the location of the ferritin particles is compatible with the synapsin I antigenic sites being either

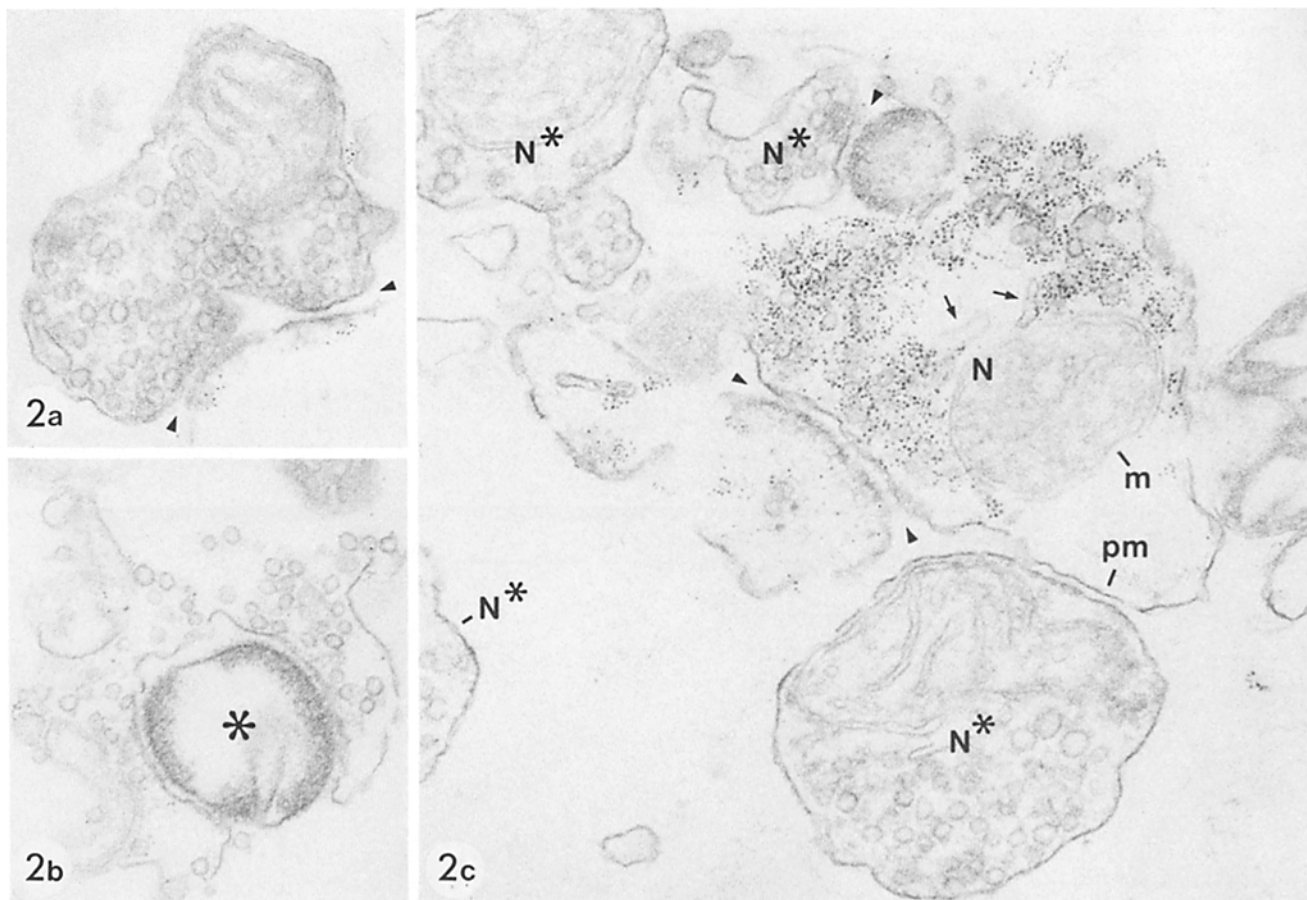


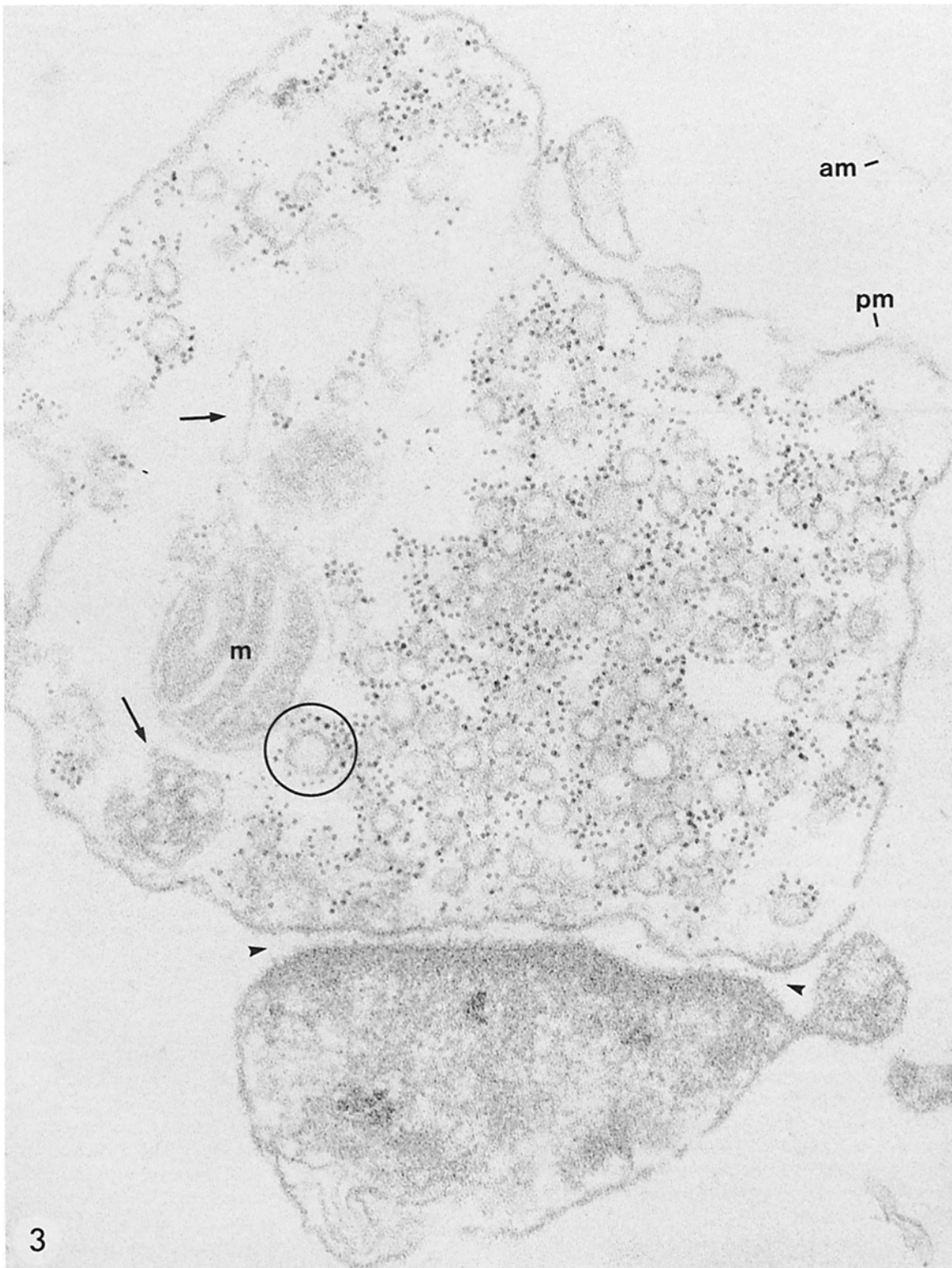
FIGURE 2 Electron micrographs illustrating morphology and immunoferritin labeling of synaptosomes fixed in nonlytic conditions and embedded in agarose. (*a* and *b*) Synaptosomes from an agarose block that was incubated at the primary antibody step with control IgG (Rb). A well-preserved nerve ending is shown in *a*. Note the continuous plasma membrane, the tightly packed vesicles, and the dense cytoplasmic matrix between vesicles. Arrowheads indicate the synaptic cleft. A disrupted nerve ending is shown in *b*. Large interruptions are visible in the plasma membrane, and synaptic vesicles are dispersed in a light (empty) matrix. The postsynaptic compartment is marked by an asterisk. (*c*) Synaptosomes from an agarose block that had been incubated at the primary antibody step with anti-synapsin I IgG (Rb). Prominent labeling by ferritin particles is present on synaptic vesicles of a disrupted nerve ending (*N*). Note in this terminal the absence of labeling on mitochondria (*m*), on the plasma membrane (*pm*) of the nerve ending, and on vesicular structures different from synaptic vesicles (arrows). Adjacent well-preserved nerve endings (*N**), which have an apparently sealed plasma membrane, are unlabeled. Synaptic clefts are indicated by arrowheads. *a*, $\times 72,500$; *b*, $\times 58,500$; *c*, $\times 77,500$.

immediately adjacent to the vesicle membrane or at some distance from the outer leaflet of the membrane. A small percentage of ferritin particles were visible at a considerable distance from synaptic vesicles. This distance could be explained either by the formation of small clusters of antibody and antibody conjugates, given the indirect nature of our immunolabeling procedure, or by the presence of vesicles out of the plane of the section.

A morphometric analysis of anti-synapsin I immunoferritin labeling in synaptosomes fixed in nonlytic conditions, and not permeabilized with detergent, is shown in Fig. 6, columns *a* and *d*. The ratio of the total number of ferritin particles to the total number of vesicle profiles in random samples of 100 nerve endings is presented. The figure shows a remarkable difference between the labeling of disrupted nerve endings in samples reacted with control IgG (Rb) or anti-synapsin I IgG (Rb) respectively. The great variability in intensity of ferritin labeling in disrupted nerve endings (Fig. 6, column *d*) is probably due to variations in their accessibility to the label. Thus, in

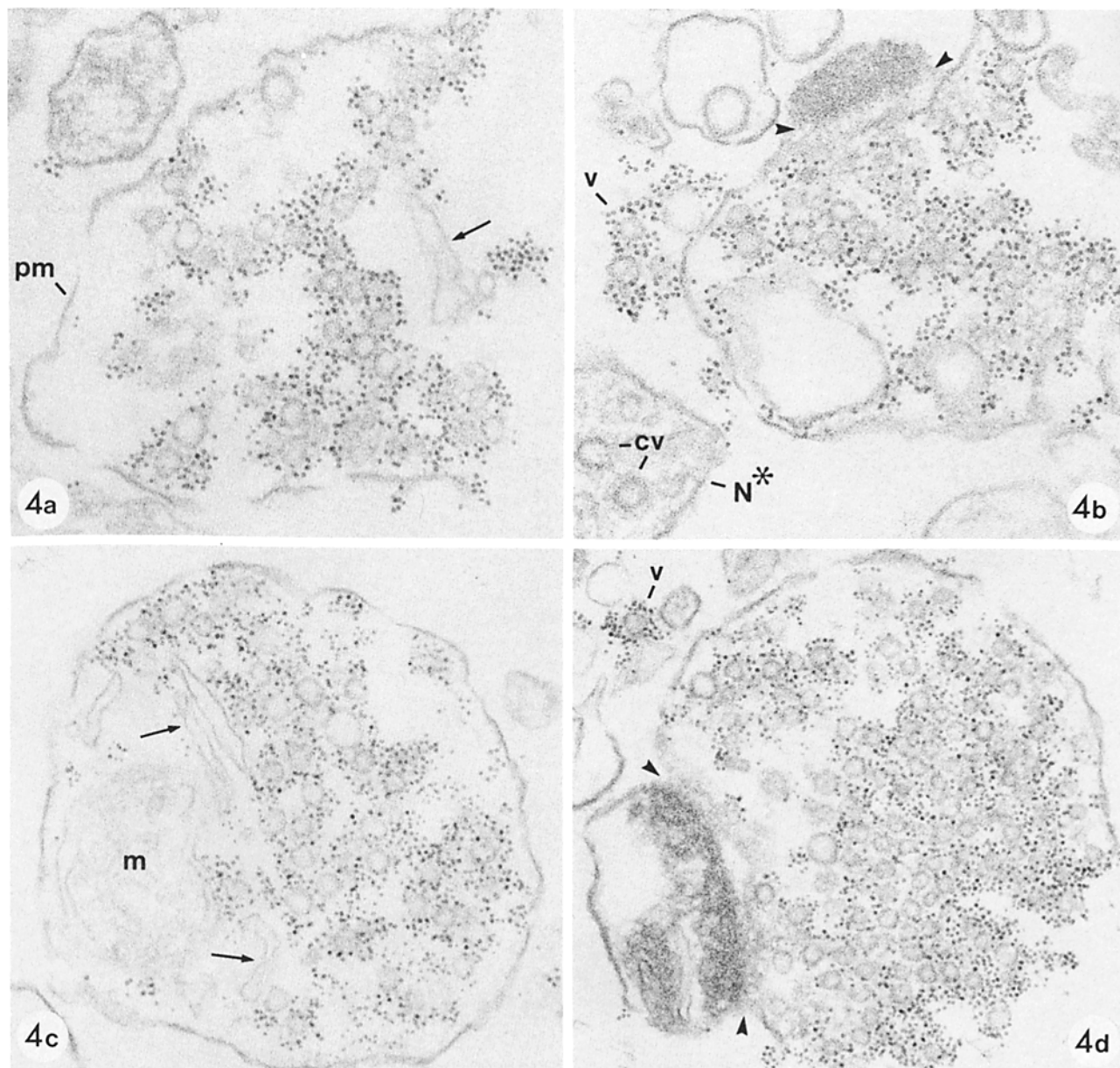
general, the highest ferritin/vesicle ratios were found in those nerve endings with the smallest vesicle/area ratio (see Materials and Methods), i.e., in those nerve endings in which synaptic vesicles were most highly dispersed (not shown). When the whole profile of a vesicle in the plane of the section appeared to be freely accessible to marker proteins, as many as 40 ferritin particles were sometimes counted around the vesicle. It is also evident from Fig. 6 (columns *a* and *d*) that well-preserved nerve endings were not labeled. This raised the possibility that this subpopulation of nerve endings was not accessible to antibodies and antibody-conjugates due to the presence of a sealed plasma membrane.

To determine if this was the case, we carried out an experiment to test for the accessibility of synaptosomes to ferritin particles (see Materials and Methods). Agarose blocks, containing synaptosomes fixed in nonlytic conditions, were incubated in the presence of a high concentration of unconjugated ferritin and then subjected to a second fixation with glutaraldehyde while still in the ferritin-containing buffer. We found that while



the disrupted nerve endings contained many ferritin particles (not shown), no ferritin was present inside the well-preserved nerve endings (Fig. 7). A comparison of percentages of nerve endings (*a*) not accessible to ferritin and (*b*) unlabeled after anti-synapsin I immunolabeling in two agarose blocks obtained from the same agarose gel indicated that the unlabeled terminals in the immunocytochemical experiments could be

accounted for by the inaccessibility of the synaptosomes to the marker molecules (Table I). This result was highly suggestive of an ubiquitous presence of synapsin I in nerve terminals, but did not exclude the possibility that the inaccessible terminals constituted a different population of nerve endings not containing synapsin I. To establish whether the inaccessible nerve endings, too, contained synapsin I, we next studied the effect



FIGURES 3 and 4 Gallery of disrupted nerve endings from synaptosomal preparations fixed in nonlytic conditions, agarose-embedded, and labeled for synapsin I by immunoferritin. The large majority of ferritin particles are associated with synaptic vesicles. In Fig. 3, ferritin particles can be seen forming a "corona" around a vesicle (black circle). Mitochondria (*m*), plasma membranes (*pm*), and vesicular structures other than synaptic vesicles (arrows) are unlabeled by the ferritin particles. At least some of the ferritin particles apparently not contiguous to synaptic vesicles might be associated with vesicles out of the plane of the section. Note in Fig. 4 *d* the uneven distribution of ferritin particles. They are more abundant on those vesicles close to the large plasma membrane discontinuity and, therefore, more easily (directly) accessible to the incubation medium. *v*, Synaptic vesicles artifactually relocated outside the nerve endings. *N**, Well-preserved nerve ending, not labeled by ferritin. *cv*, Coated vesicles. *am*, Agarose matrix. Synaptic clefts are indicated by arrowheads. Fig. 3, $\times 140,000$. Fig. 4 *a*, $\times 104,000$; *b*, $\times 104,000$; *c*, $\times 108,000$; *d*, $\times 104,000$.

of procedures aimed at damaging the integrity of the plasma membranes on subsequent immunolabeling.

IMMUNOFERRITIN LABELING AFTER PERMEABILIZATION TREATMENTS: Procedures designed to permeabilize the synaptosomal plasma membrane were carried out either on the fixed synaptosomal suspension before agarose embedding (freeze-thawing cycles) or on the synaptosome-containing agarose blocks (nonionic detergents). Well-preserved nerve endings were still recognizable after such manipulations by the

dense appearance of their cytoplasm. Some of them showed a moderate degree of labeling, but the majority, even after incubation with high concentrations of detergents, were still poorly labeled or not labeled at all. Some examples of synaptosomes immunolabeled after pretreatment with saponin or with Triton X-100 are shown in Fig. 8. Fig. 6, columns *b* and *e* show the morphometric analysis of one of these experiments. Parallel experiments carried out to test the accessibility of the cytoplasm of well-preserved nerve endings to free ferritin after

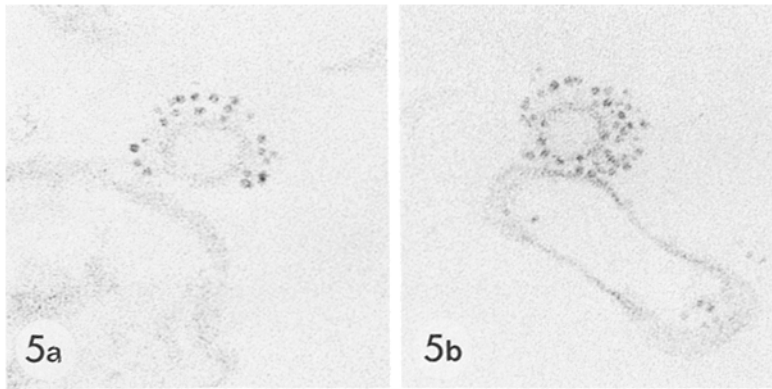


FIGURE 5 Specific decoration of synaptic vesicles by anti-synapsin I immunoferritin. The micrographs were obtained from agarose blocks containing synaptosomes fixed in nonlytic conditions. The two synaptic vesicles shown have leaked out of nerve endings and appear to have been cross-linked by the fixation to other vesicular structures. Free synaptic vesicles (not cross-linked to other structures) were never observed in our preparations. We assume that, due to their small size, they were not retained by the agarose meshwork. *a* and *b*, $\times 220,000$.

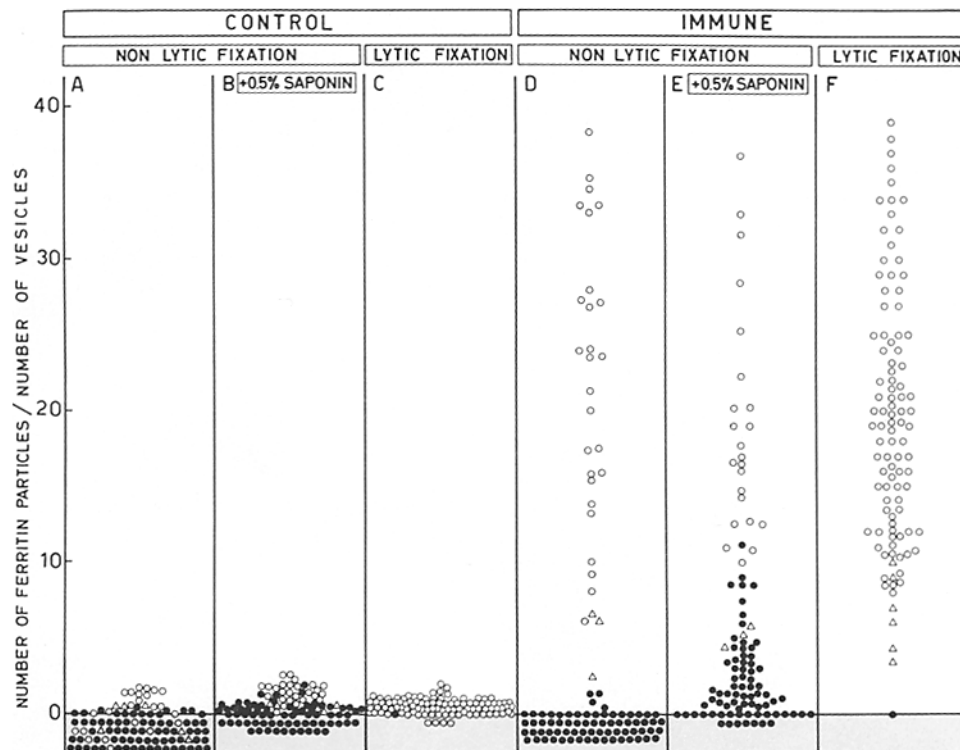


FIGURE 6 Morphometric analysis of immunoferritin labeling of nerve endings processed according to various experimental procedures. Synaptosomal preparations were fixed either in nonlytic or in lytic conditions, agar-embedded, incubated in the absence or presence of 0.5% saponin, and then immunoferritin-labeled. Control IgG (Rb) or anti-synapsin I IgG (Rb) was used at the primary antibody step of the immunolabeling. For EM analysis, randomly taken micrographs, including 100 nerve endings for each experimental condition, were used. Nerve endings were grouped into two major classes (well-preserved nerve endings or disrupted nerve endings) according to the following characteristics: well-preserved nerve endings were those with a continuous plasma membrane and a dense appearance as indicated either by the close apposition of synaptic vesicles, or by the presence of a dense cytoplasmic matrix, or by both. They are represented in the figure by solid circles. Disrupted nerve endings were those with a clear (empty) cytoplasm and dispersed vesicles. They are represented in the figure by open circles. The few nerve endings that were difficult to classify (for instance, having an interrupted plasma membrane but a rather dense cytoplasmic matrix) are represented in the figure by open triangles. Clusters of free synaptic vesicles that were not clearly part of a nerve ending remnant were not included in the count. The total number of ferritin particles and the total number of synaptic vesicles were counted for each nerve terminal. The ratios of these two numbers for individual nerve endings are presented in the figure. All values plotted in the stippled area are zeros. Data reported in columns *A*, *B*, *D*, and *E* were obtained from four agarose blocks cut out from a single synaptosome-agarose gel. The two blocks used for the data reported in columns *C* and *F* were also obtained from a single gel.

the detergent treatments indicated that a large proportion of these endings were still poorly accessible or not accessible to free ferritin, even though their membranes had been permeabilized (not shown). Lack of immunolabeling or poor immunolabeling in these endings could, therefore, be attributed even

in this case to poor penetration of marker molecules rather than to an absence of synapsin I. The lack of penetration by macromolecules, together with the dense cytoplasmic matrix visible in these endings, suggests the presence of a tight network of fixative-cross-linked cytosolic proteins. Such cross-linking

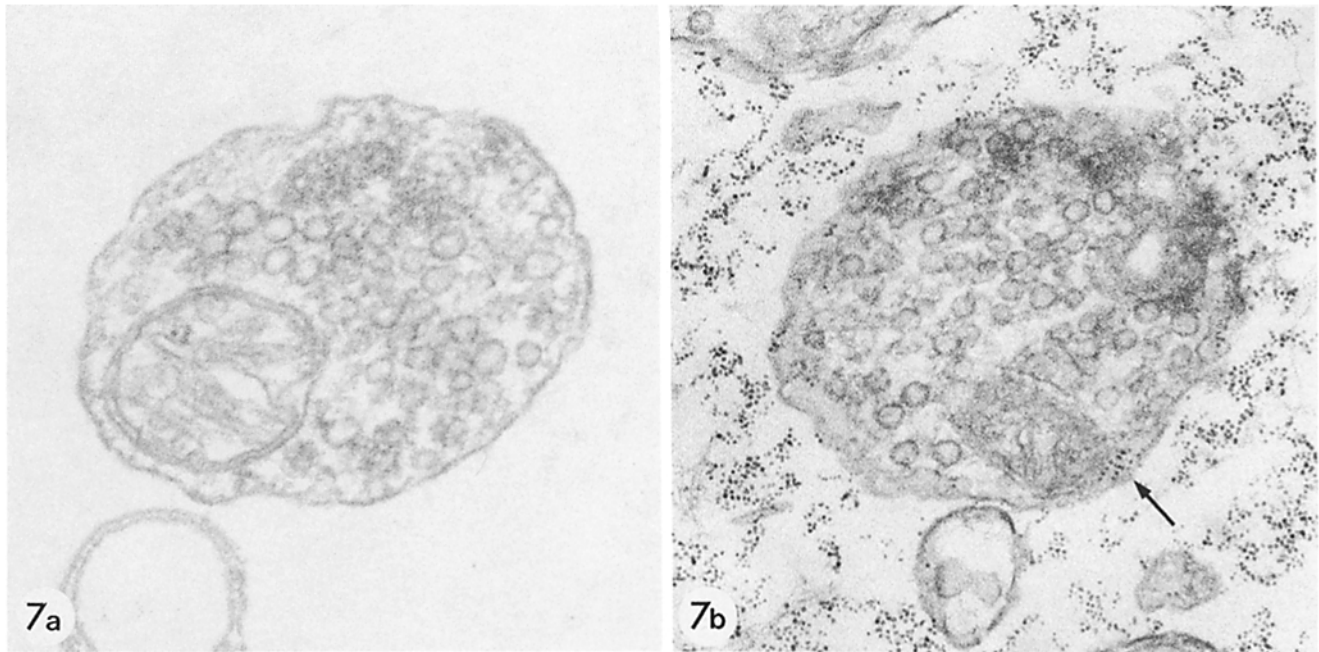


FIGURE 7 Comparison of well-preserved nerve ending not immunolabeled after the immunoferritin procedure, with nerve ending not accessible to free ferritin. (a) Nerve ending from an agarose block subjected to immunoferritin labeling for synapsin I. (b) Nerve ending from an agarose block incubated in the presence of a high concentration of free ferritin and subsequently postfixed with glutaraldehyde while still in the ferritin-containing buffer. (The few ferritin particles visible inside the profile of the nerve ending in the micrograph [arrow] are located at the very periphery of the ending, in a region where the plasmalemma is cut obliquely. The image, therefore, is compatible with the possibility that these ferritin particles are only apparently located inside the ending.) The agarose blocks used for a and b were from the same agarose gel, which contained synaptosomes fixed in nonlytic condition. a and b, $\times 100,000$.

appears to be closely associated with the preservation of good nerve ending morphology.

Synaptosomes Fixed in Lytic Conditions

MORPHOLOGY: Since optimal ferritin penetration and heavy labeling were obtained on morphologically-disrupted terminals, we attempted to find fixation conditions that would result in a disruption of all nerve endings. This was achieved by adding a large volume of hypotonic fixative solution at the final desired strength (3% formaldehyde, 0.5% glutaraldehyde, 5 mM PB) to the crude synaptosomal fraction (lytic fixation). As seen by EM, all subcellular particles, and in particular nerve endings, were highly disrupted by this treatment (Fig. 9). Synaptosomal ghosts were still recognizable by clusters of synaptic vesicles that were contiguous to, and often partially enclosed by, fragments of plasma membrane. In some cases the plasma membrane could be identified as part of a nerve ending by the presence, in the plane of the section, of an adjacent PSD. Clusters of isolated synaptic vesicles, apparently not in proximity to synaptosomal ghosts, were also visible. The occurrence of structurally well-preserved nerve endings in these preparations was extremely rare.

IMMUNOFERRITIN LABELING: When such preparations were immunolabeled for synapsin I by the indirect immunoferritin procedure, a high concentration of ferritin particles was always found at all sites where vesicles or clusters of vesicles were present (Fig. 9). The large majority of ferritin particles decorated specifically the surface of synaptic vesicles. The only unlabeled vesicles were those present in the rare, apparently undamaged, nerve terminals. A quantitative analysis of ferritin labeling in synaptosomes fixed in lytic conditions is shown in

Fig. 6, columns c and f. The data shown in this figure suggest that all nerve endings contain synapsin I.

Immunoferritin Labeling of Synaptic Vesicles after Extraction of Synapsin I from the Endings

To further test the reliability of the immunoferritin method as a specific label for synapsin I, we applied it to a synaptosomal fraction previously processed by a procedure designed to deplete synaptosomes of synapsin I. The latter can be eluted from the surface of isolated synaptic vesicles by suspending them in a large volume of 0.15 M KCl (20). Therefore, the unfixed crude synaptosomal fraction was incubated in the presence of 0.15 M KCl and 0.01% saponin. The rationale of the procedure was to open focal discontinuities in the plasma membrane by saponin (4, 31) and to extract synapsin I from its intracellular binding sites by exposing it to a large volume of 0.15 M KCl; the focal discontinuities in the plasma membranes would then allow cytoplasmic proteins, including solubilized synapsin I, to be released into the medium. After the KCl-saponin incubation, the synaptosomes were recovered by centrifugation, fixed, and embedded in agarose. Fig. 10 shows synaptosomes immunolabeled for synapsin I after such treatment. Discontinuities are visible in the plasma membranes of the endings and the cytoplasmic space of the endings appears rather "watery" (empty) as if most cytoplasmic proteins had been eluted. In spite of their apparent accessibility to marker molecules, synaptic vesicles are not labeled by ferritin. The morphology of the two synaptosomes shown in Fig. 10 is representative of the morphology of the bulk of the synaptosomes present in the preparation.

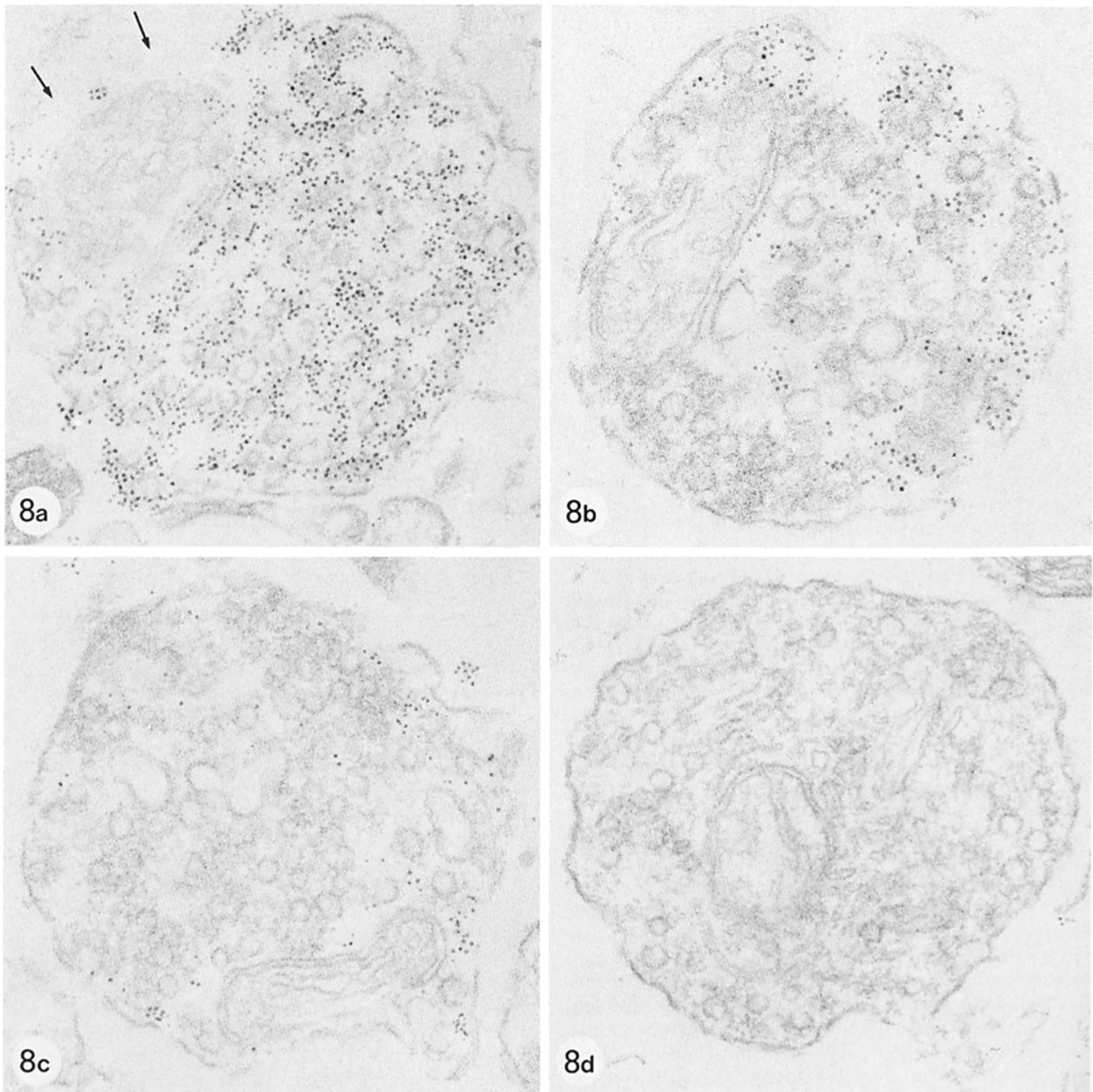
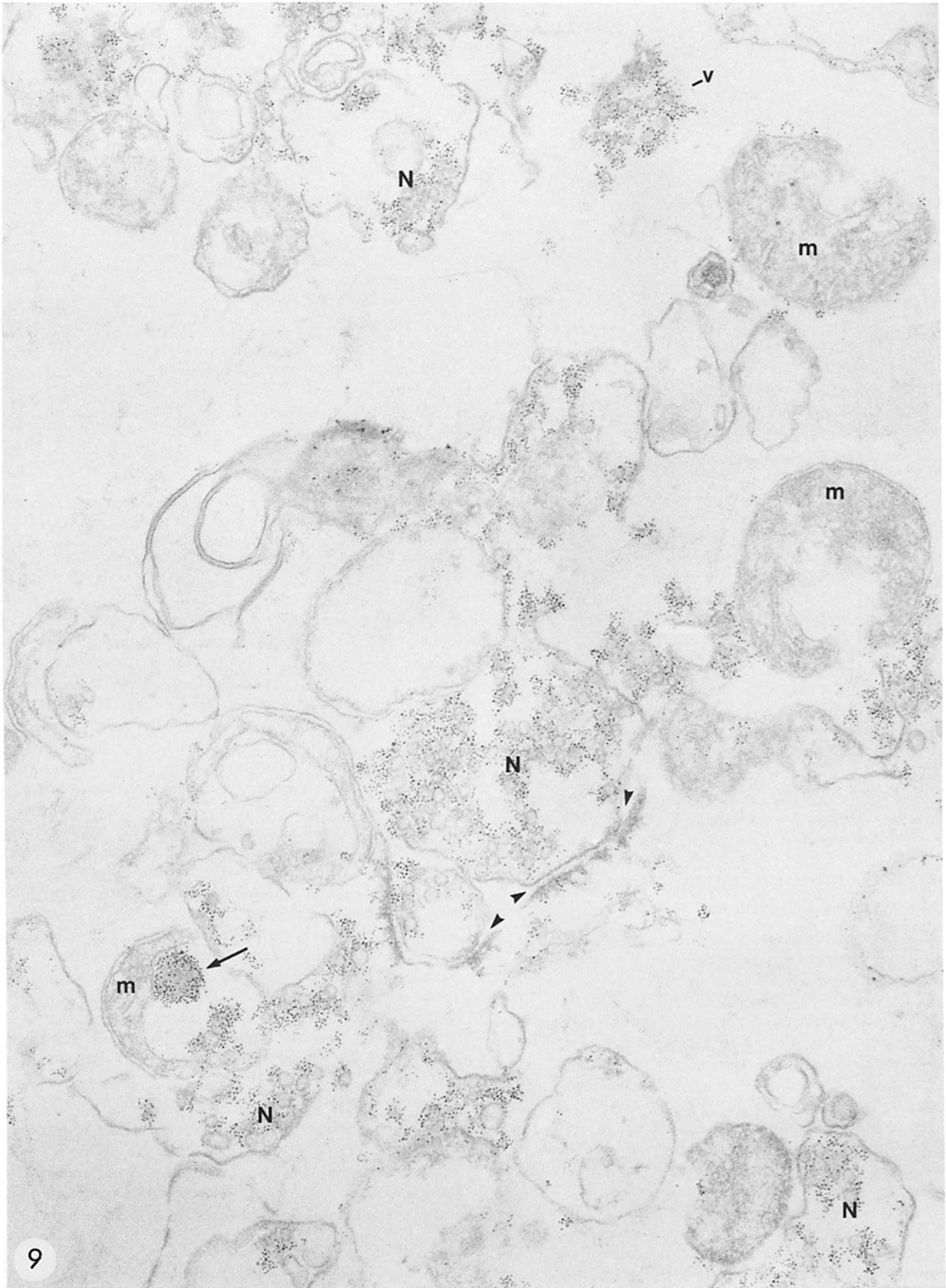


FIGURE 8 Gallery of nerve endings from a synaptosomal preparation fixed in nonlytic conditions, embedded in agarose and immunolabeled for synapsin I after saponin (0.5% [wt/vol]) treatment. The plasma membranes of the endings display the festooned profiles and the focal discontinuities known to be produced by saponin (4, 31). The nerve ending shown in *a* is a disrupted nerve ending, as can be seen from the space around vesicles which appears clear (except for the ferritin particles), and from the large interruptions of the plasma membrane (arrows). Nerve endings from *b* to *d* are characterized by an increasingly dense cytoplasmic matrix. Labeling is high in *a*; present, but scanty, in *b*; even more scanty in *c*; and absent in *d*, in reverse order to the apparent density of the cytoplasmic matrix. *a*, $\times 92,000$; *b*, $\times 107,000$; *c*, $\times 101,000$; *d*, $\times 103,000$.

FIGURE 9 Distribution of anti-synapsin I immunoferritin in a synaptosomal preparation embedded in agarose after lytic fixation. Synaptosomes and mitochondria are severely disrupted. Mitochondria (*m*) have the typical morphology observed after exposure to severe hypoosmotic conditions (12). Nerve endings (*N*) are clearly open. Clusters of vesicles (*v*) apparently not in proximity to synaptosomal ghosts are visible. Postsynaptic densities are visible at the synaptic junctions indicated by arrowheads. Note the prominent labeling associated with synaptic vesicles. The large cluster of ferritin particles indicated by an arrow does not represent specific labeling. Images like this cluster were very rare and were also seen in blocks reacted with control IgG (Rb) at the first antibody step. They may represent complexes of rabbit IgGs and ferritin-conjugated goat anti-rabbit IgGs that form as a consequence of a slow shedding of antibodies from antigenic sites. $\times 68,000$.



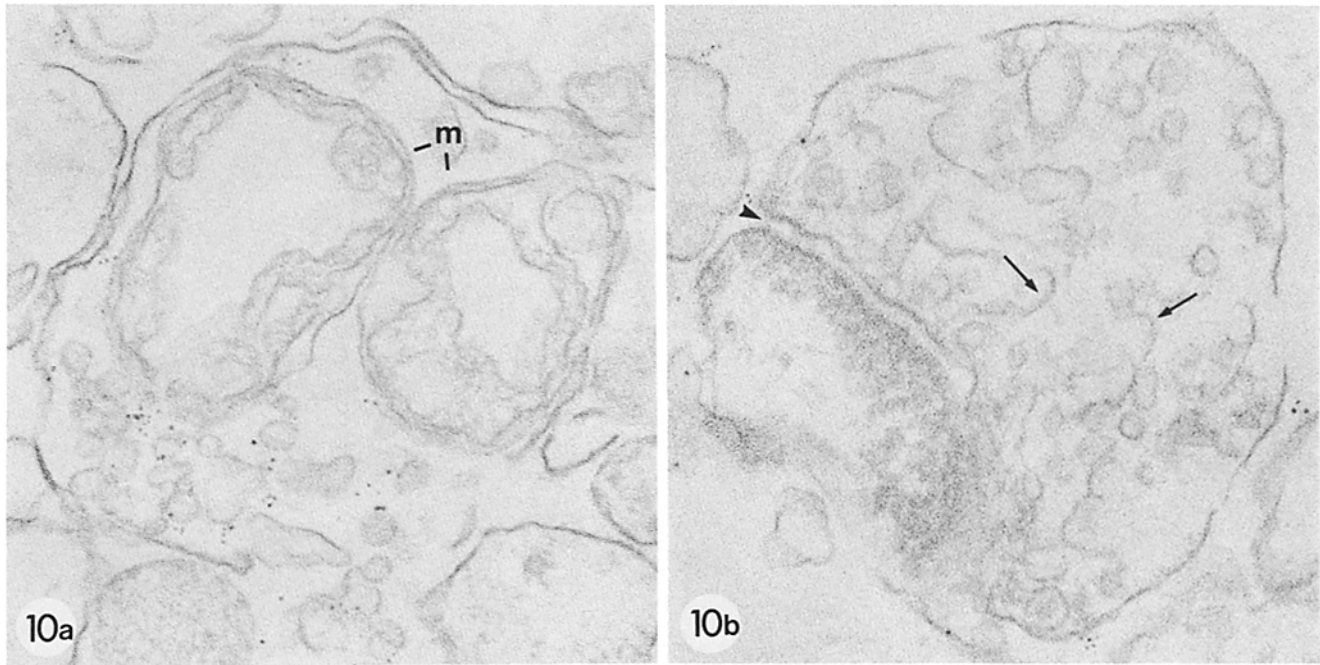


FIGURE 10 Anti-synapsin I immunoferritin labeling of nerve endings after extraction of synapsin I by the saponin-KCl treatment. In the pictures the features characteristic of saponin-treated particles (4) are visible: undulated and focally interrupted plasma membranes and ruptured synaptic vesicle membranes (arrows). Note also the clear (empty) appearance of the space among vesicles, suggesting that most cytoplasmic proteins had been released from these endings before fixation. *m*, Mitochondria. A synaptic cleft is indicated by an arrowhead. *a*, $\times 117,000$; *b*, $\times 116,000$.

Immunoferritin Labeling of PSDs

Two previous studies (2, 39) had suggested that synapsin I might be present at PSDs. Using the procedures developed in the present study, we have reexamined this question. Occasional ferritin particles were found to be associated with PSDs when they were not enclosed by a sealed plasma membrane. Such labeling, however, did not appear to be significantly different in samples reacted with control IgG (Rb) or with anti-synapsin I IgG (Rb) (Fig. 11; compare also Fig. 2*a* with Fig. 2*c*). This conclusion is supported by the quantitative data reported in Table II.

Immunoperoxidase Staining of Agarose-embedded Synaptosomes

Agarose-embedded synaptosomes fixed in either nonlytic or lytic conditions were also immunolabeled for synapsin I by an indirect (36) immunoperoxidase procedure (14).

In preliminary experiments, carried out with control IgG (Rb) at the first antibody step of the immunostaining procedure (step 3), a fine coating by peroxidase reaction product (14, 36) was often found on all surfaces of subcellular particles exposed to the incubation medium. We selected incubation times and concentrations of reagents for the peroxidase reaction that minimized this background (Fig. 12*a*). When rabbit anti-synapsin I IgG (Rb) was used at the first antibody step of the immunoperoxidase procedure (step 3), a specific, very prominent accumulation of peroxidase reaction product was observed on disrupted nerve endings (Figs. 12*b-d* and 13). Peroxidase-positive terminals constituted $<50\%$ of synaptosomal profiles observed after fixation under nonlytic conditions, while they accounted for close to 100% of the total synaptosomal population in preparations fixed under lytic conditions (Fig. 13). Reaction product was not present in well-preserved nerve

endings (Fig. 12*b* and *d*). However, when plasma membranes were made permeable by saponin or freeze-thawing, some apparently well-preserved nerve endings did show light immunoreactivity (Fig. 12*e*). Therefore, results obtained using the immunoperoxidase procedure were, in several respects, identical to those obtained by the immunoferritin procedure. However, in one respect they differed: peroxidase reaction product accumulated on all membranes directly contiguous to synaptic vesicles, irrespective of the nature of the membranes. Thus, reaction product was present on all cytoplasmic faces of membranes of disrupted nerve terminals and also on membranes of other organelles present in the crude synaptosomal fraction, which in the process of fixation and agarose embedding had become contiguous to synaptic vesicles. Peroxidase labeling of membranes other than synaptic vesicle membranes was in general concentrated on the region of the membrane nearest to and facing synaptic vesicles and was absent from portions at a distance from the vesicles. Occasionally, some small membrane fragments apparently not in proximity to synaptic vesicles were specifically labeled by the reaction product (Fig. 13). However, these membranes might have been close to synaptic vesicles out of the plane of the section.

Immunoperoxidase Labeling of Intact Tissues

In a previous immunoperoxidase study of the localization of synapsin I in intact tissues at the ultrastructural level, it was possible to demonstrate reaction product in only a small proportion of nerve endings (2). Given the information obtained in the present study of isolated synaptosomes, we have reexamined the immunolabeling of synapsin I in intact tissue by EM. For this purpose we used thick ($20\ \mu\text{m}$) frozen sections of fixed brain tissue. Attempts to immunolabel nerve endings in these sections by immunoferritin failed because of the almost complete lack of penetration of ferritin into the sections, even

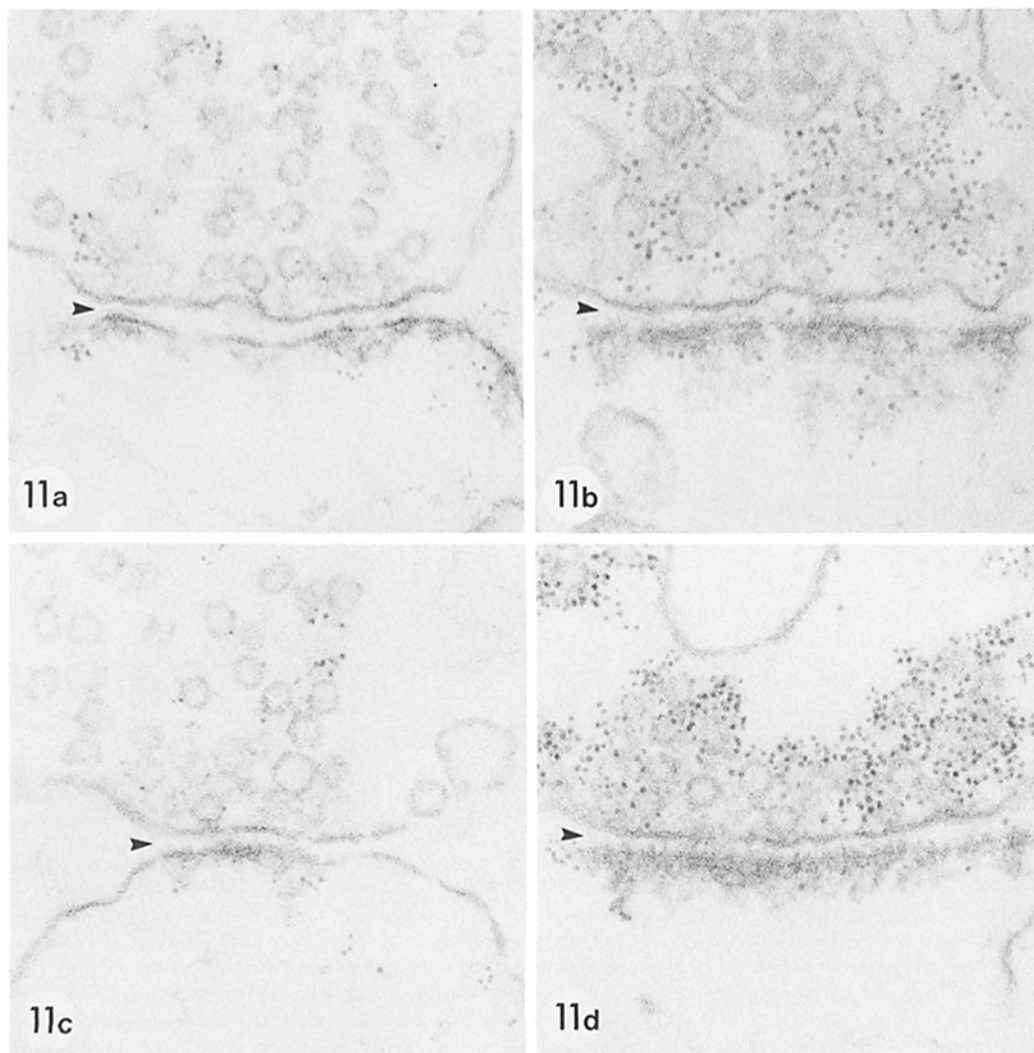


FIGURE 11 Comparison of ferritin labeling of PSDs reacted with control IgG (Rb) (*a* and *c*) and anti-synapsin I IgG (Rb) (*b* and *d*), at the primary antibody step of the immunolabeling procedure. The micrographs were obtained from synaptosomes embedded in agarose after nonlytic fixation. Scattered ferritin particles are present on all four PSDs with no apparent difference between control and immune-treated synaptosomes. In contrast, a striking difference in particle labeling is observed on vesicles in the nerve endings (disrupted nerve endings). Synaptic clefts are indicated by arrowheads. *a*, $\times 107,000$; *b*, $\times 110,000$; *c*, $\times 108,000$; *d*, $\times 106,000$.

TABLE II
Immunoferritin Labeling

	Number of PSDs counted	Total length, L μm	Total number of ferritin particles, n	n/L
Control	47	16.15	777	48.1
Immune	53	16.14	923	57.2

Quantitative comparison between immunoferritin labeling of PSDs reacted with control IgG (Rb) and anti-synapsin I IgG (Rb) at the primary antibody step of the immunolabeling procedure. The morphometric analysis was carried out as described in Materials and Methods.

after incubation with detergents. In contrast, some labeling could be obtained by the immunoperoxidase technique. Peroxidase reaction product was detected only on nerve terminals (Fig. 14) and was confined to terminals of the most superficial portions of the thick sections. Furthermore, even in the superficial layers, it was seen in only some nerve terminals. The

proportion of immunolabeled nerve terminals increased, but only to a limited extent, when the sections had been pretreated with detergent (saponin or Triton X-100) to permeabilize membranes before incubation with antibodies.

In both detergent-treated and nontreated tissue sections, those terminals that showed immunoreactivity were labeled to a variable extent by the reaction product, which in many cases was unevenly distributed in the ending (Fig. 14). Tears were often visible in those terminals or portions of terminals that were heavily stained (Fig. 14*a*).

The distribution of peroxidase reaction product in the cytoplasm of the terminal was very similar to the distribution observed in immunoperoxidase-reacted, agarose-embedded isolated synaptosomes. Thus, reaction product, rather than being confined to a specific structure of the nerve ending, occupied the whole cytoplasmic space, and labeled cytoplasmic faces of axon terminal membranes irrespective of their nature.

DISCUSSION

The aim of this study was to determine, at the ultrastructural level, the localization of synapsin I in synapses and the pro-

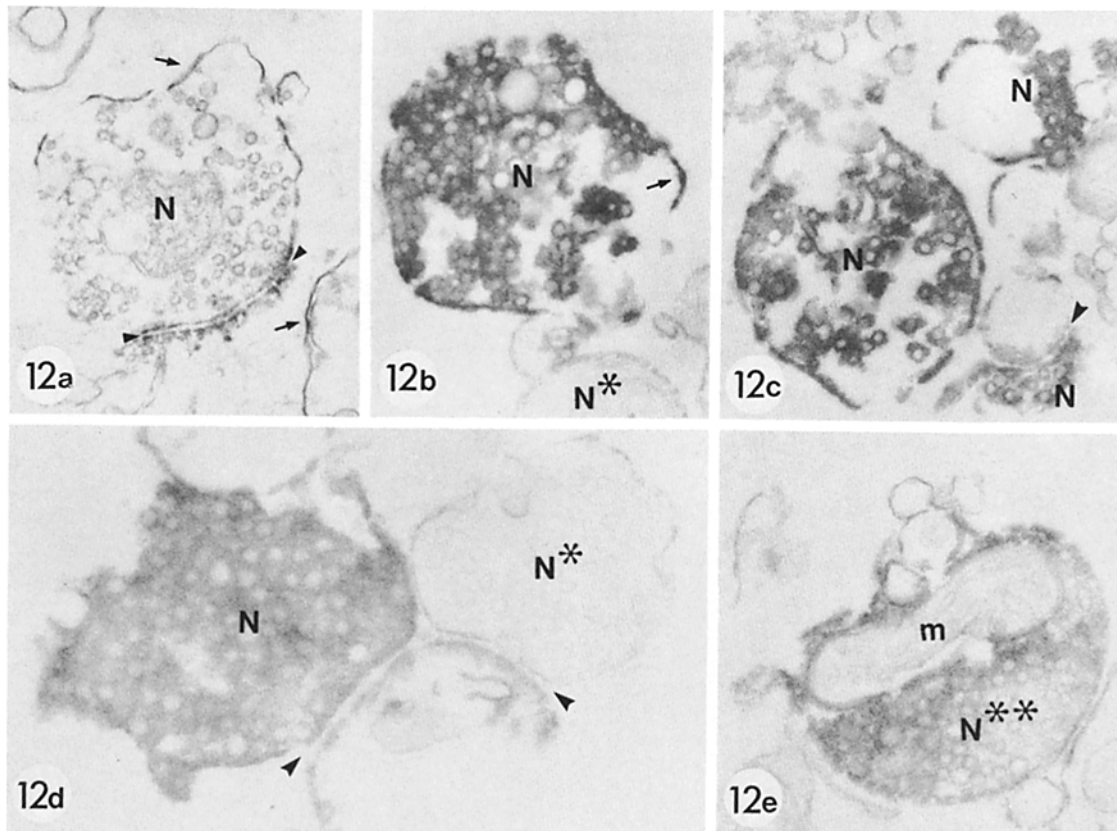


FIGURE 12 Immunoperoxidase staining for synapsin I of synaptosomes embedded in agarose after nonlytic fixation. (a) Disrupted nerve ending from an agarose block incubated with control IgG (Rb) at the primary antibody step of the immunostaining procedure. Low level background labeling by peroxidase reaction product is visible on some membranes exposed to the incubation medium, in particular plasma membranes (arrows). Some increase in density of the PSD is also visible. (b–d) Disrupted nerve endings (N) and well-preserved nerve endings (N*) from synaptosomal preparations incubated with anti-synapsin I IgG (Rb) at the primary antibody step of the immunolabeling procedure. Reaction product darkens synaptic vesicle membranes and all other membranes (e.g., presynaptic plasma membranes) exposed to the cytoplasmic space of the disrupted endings. Staining appears more prominent when vesicles are more dispersed (b and c) than when they are more densely packed (d). In the latter case, the reaction product is homogeneously distributed in the space among vesicles. Well-preserved nerve endings are unlabeled. (e) The nerve ending labeled N** is from an agarose block containing synaptosomes permeabilized by freeze-thawing before agarose-embedding. It shows a compact structure and is only partially and unevenly labeled by peroxidase. Some background labeling on membranes exposed to the incubation medium is visible in b–e. This is similar in intensity to the background labeling observed on membranes in a. Synaptic clefts are indicated by arrowheads; m, mitochondria. Micrographs shown in b–e are from thin sections not stained by uranyl acetate and lead citrate. a, $\times 47,500$; b, $\times 40,500$; c, 45,000; d, $\times 62,000$; e, $\times 53,000$.

portion of synapses at which synapsin I is present. To answer these questions we have developed an EM technique to label isolated synaptosomes embedded in agarose. Synaptosomes, which can be prepared easily from the central nervous system, contain the relevant elements of the synapse: the resealed nerve ending and the postsynaptic membrane with the attached PSD. Synaptosomes are, therefore, a suitable model system in which to study the localization of macromolecules in the architecture of the synapse. Our results indicate that synapsin I is specifically associated with synaptic vesicles and that it is present on vesicles of the great majority of (and possibly all) nerve endings.

Advantages of Agarose-embedded Synaptosomes over Tissue Sections as an Experimental Model

Immunolabeling of synaptosomes embedded in agarose offers three important advantages over traditional methods to immunostain antigens in synapses, which involve the use of fixed tissue sections: (a) prefixation manipulations and fixation can be performed under precisely defined conditions on iso-

lated synaptosomes. This possibility to manipulate the cytoplasmic matrix of the nerve endings by appropriate treatments before and during the fixation proved to be very useful in our study. (b) Synaptosomes are spatially separated from other structures and from each other by the meshwork of the agarose. All synaptosomes are, therefore, easily accessible to marker proteins (even to large probes such as ferritin-conjugated antibodies), which diffuse freely through the agarose matrix. (c) Agarose embedding of synaptosomes allows subsequent handling of the synaptosomes in the agarose blocks in a manner similar to that used for tissue specimens. This avoids synaptosomal losses or damages expected to occur upon the repeated sedimentation/resuspension steps otherwise needed to accomplish immunolabeling.

Role of the Fixation Procedure in Synapsin I Immunostaining

Satisfactory immunostaining of synapsin I in nerve endings was obtained only when the majority of the cytoplasmic proteins had been extracted. The importance of the meshwork of

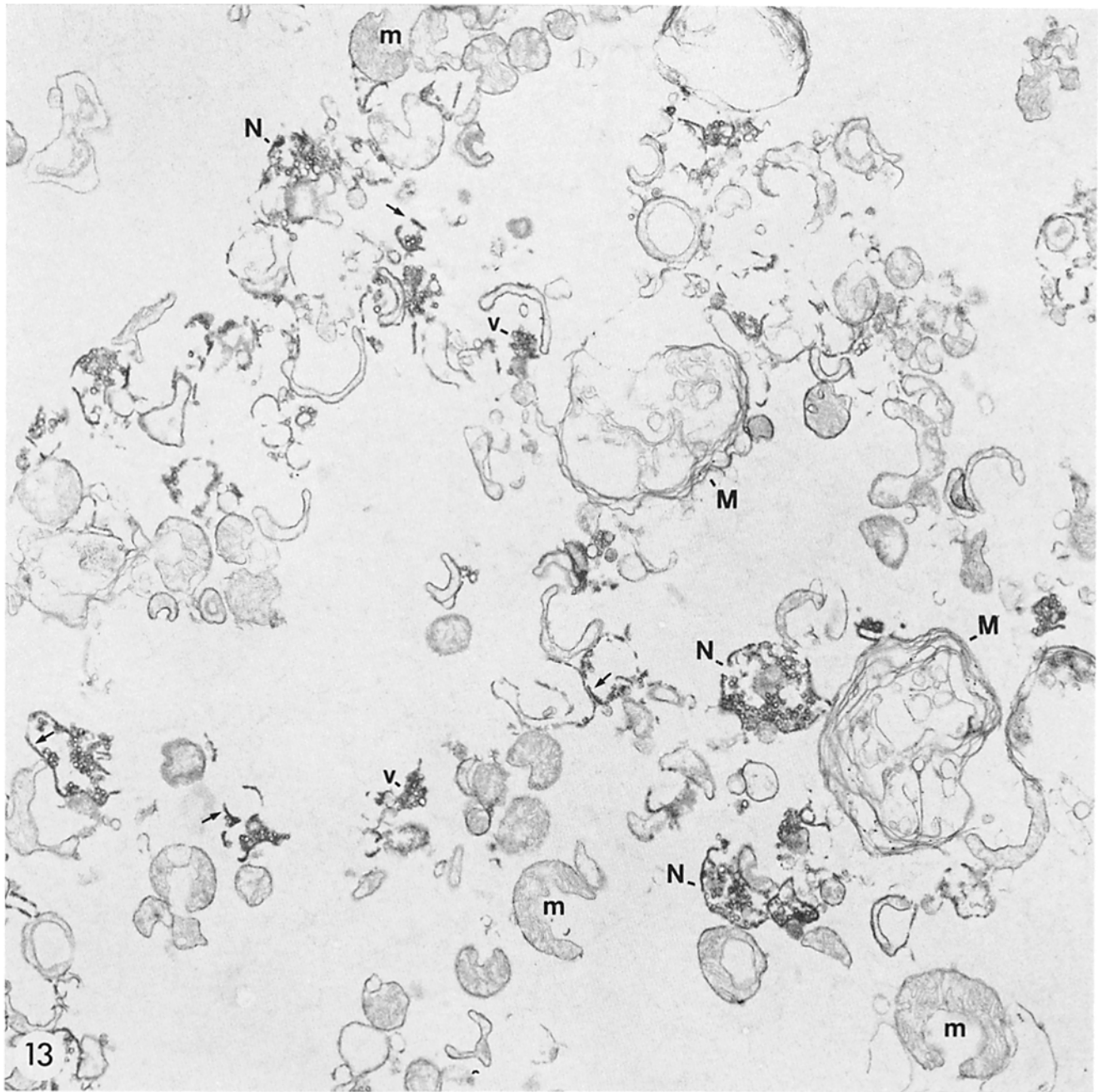


FIGURE 13 Anti-synapsin I immunoperoxidase labeling of synaptosomes agarose-embedded after fixation in lytic conditions. Subcellular particles are highly deformed and disrupted. Mitochondria (*m*) have the typical concave shape observed after severely hypotonic fixation (12). All recognizable synaptic vesicles are labeled, irrespective of their localization (within or outside nerve ending remnants [*N*]). Reaction product also decorates membrane fragments adjacent to synaptic vesicles (arrows) and occasionally membrane fragments apparently not in proximity to synaptic vesicles. *v*, Free clusters of synaptic vesicles; *M*, remnant of myelin sheaths. $\times 17,000$.

the aldehyde-cross-linked cytoplasmic proteins as a barrier to the diffusion of marker macromolecules is well known, and several methods have been developed to reduce this meshwork to a minimum (17, 31, 40). In our case this was accomplished by selectively extracting from the terminals those proteins that are soluble in low ionic strength. Under low ionic strength conditions, synapsin I is known to remain quantitatively associated with membranes (20).

When nerve endings were fixed in isotonic sucrose, heavy labeling could be observed on disrupted nerve endings but not on well-preserved nerve endings. In spite of the large interruptions often visible in the plasma membrane of disrupted nerve

endings, a large number of vesicles were still present, presumably connected by a cytoskeletal network not disrupted by the low ionic strength medium.

Prominent labeling of practically all terminals was achieved only when all nerve endings were made leaky by hypoosmotic shock before fixative action. Resuspending the synaptosomes in a large volume of $1 \times$ fixative solution, in the absence of sucrose, proved an effective way of accomplishing a generalized lysis of nerve endings. Since this occurred almost simultaneously with the fixation, the essential features of the nerve ending morphology were preserved. The fixative solution used (1 M formaldehyde, 0.05 M glutaraldehyde, 5 mM phosphate

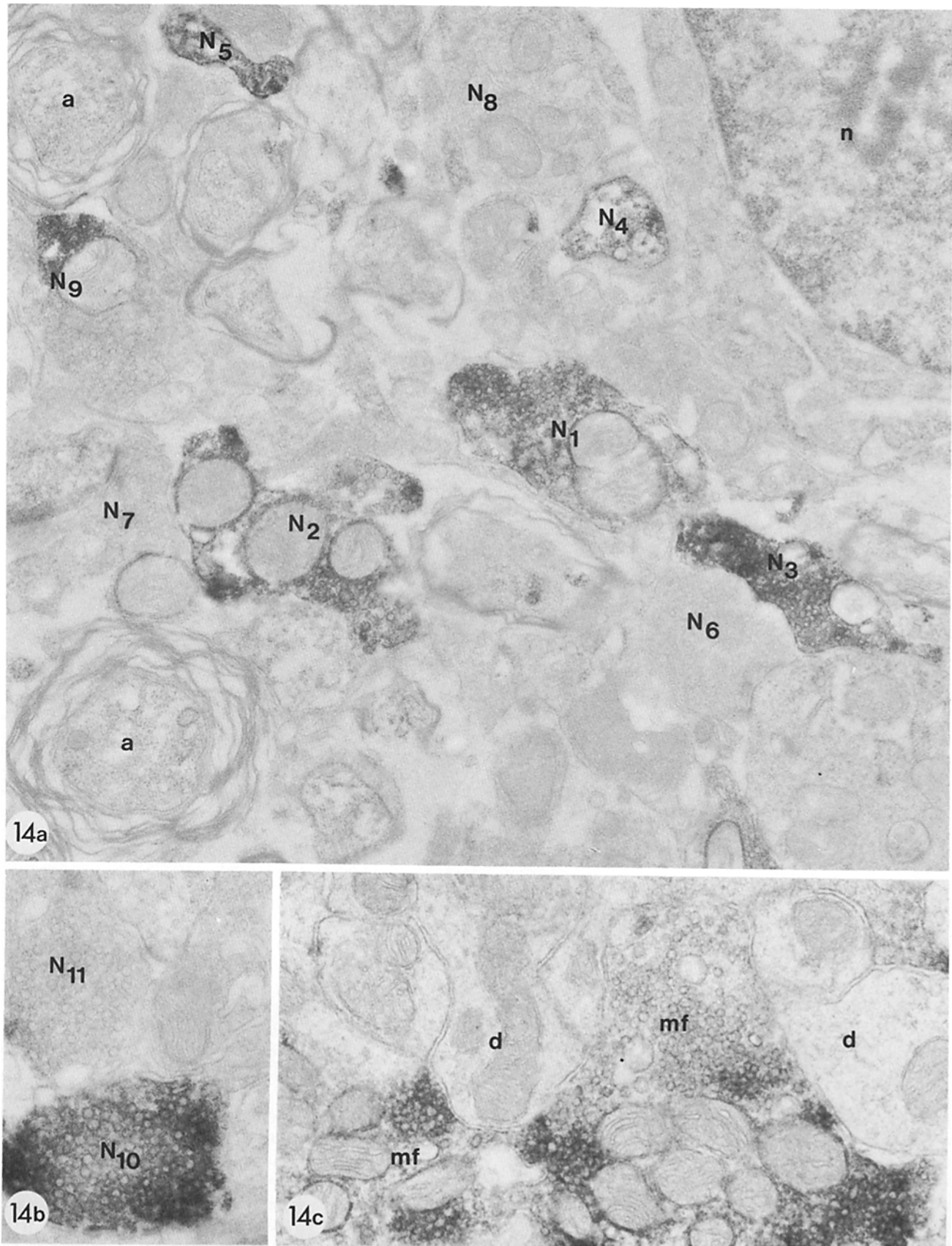


FIGURE 14 Immunoperoxidase labeling of synapsin I in frozen sections of rat brain. Frozen sections (20 μm thick) of fixed brain tissue were stained in suspension by the immunoperoxidase labeling procedure and subsequently processed for EM. (a and b) Brainstem motor nucleus. The micrographs show coexistence of labeled (for instance, N_1-N_5 in a and N_{10} in b) and unlabeled (for instance, N_6-N_9 in a and N_{11} in b) terminals in the same field. N_9 is a nerve ending labeled only in one portion. n, Nucleus; a, myelinated axons. (c) Mossy fiber terminal (mf) in the granule cell layer of the cerebellum. Peroxidase reaction product is unevenly distributed in the ending. d, Dendrites. All micrographs shown in this figure are from experiments in which detergent treatments were not included in the immunostaining protocol. a and b were taken from thin sections not stained by uranyl acetate and lead citrate. a, $\times 31,000$; b, $\times 41,000$; c, $\times 40,000$.

buffer) was strongly hypoosmotic, since formaldehyde and glutaraldehyde diffuse rapidly through biological membranes and, therefore, do not contribute to establishing an osmotic gradient (12). As formaldehyde was the predominant aldehyde component of the fixative mixture, and since the cross-linking action of formaldehyde proceeds very slowly (12), it is not surprising that a severe disruption and extraction of the terminals takes place before cross-linking occurs.

Distribution of the Ferritin Label in Synaptosomes

In immunolabeled disrupted nerve endings, an overwhelming majority of ferritin particles were associated with synaptic vesicle membranes. Very few ferritin particles were observed to be associated with other membranes. In those well-preserved nerve endings that were labeled with ferritin particles after treatments that permeabilize plasma membranes, labeling was so scanty that it did not allow any conclusion concerning the distribution of synapsin I.

The necessity to partially disrupt the molecular architecture of the cytoplasm of the terminals in order to obtain a substantial degree of labeling raises important questions concerning the physiological significance of our immunocytochemical localization. This issue of physiological significance also arises when one considers that, to prevent elution of synapsin I upon disruption of the plasma membrane, the cytoplasmic space of the nerve ending had to be exposed to solutions of low ionic strength. In the presence of 150 mM KCl (i.e., approximately the ionic strength of the cytoplasm), synapsin I was rapidly solubilized from terminals with a leaky plasma membrane (Fig. 10).

A redistribution of synapsin I in nerve endings upon exposure to low ionic strength cannot be excluded. However, two considerations suggest that the localization of synapsin I on the vesicle membrane does indeed reflect its localization *in vivo*. (a) The very specific localization of synapsin I on synaptic vesicle membranes and not on other membranes suggests a specific interaction rather than a nonspecific absorption occurring upon nerve ending disruption. (b) Light microscopic studies (2, 8, 9) and EM studies in tissue sections (2; and the present study) clearly show that synapsin I is concentrated in nerve endings. Such compartmentalization of synapsin I (a protein of the cytoplasmic space) must be interpreted in terms of a strong interaction with the equivalent of a solid substrate (membrane or cytoskeleton) present in nerve endings. Synaptic vesicles, which are highly concentrated in nerve endings, appear as an attractive candidate to be the binding site. This matter is further discussed in the third paper of this series (20).

Several technical limitations prevent using the immunoferritin results to estimate the number of synapsin I molecules on individual vesicles *in vivo*. These limitations include the indirect nature of the immunolabeling procedure, the variable accessibility of the synaptic vesicle surface, and the possible loss of antigenicity of some synapsin I molecules. However, the presence in some nerve endings of as many as 40 ferritin particles/vesicle (Fig. 6, columns *d-f*), and images like those shown in Fig. 5, suggest that more than a few synapsin I molecules are present on each vesicle. This interpretation is also supported by the very high concentration of synapsin I found in the nervous system and by the proportion (~6%) of the total synaptic vesicle protein represented by synapsin I (20).

Ferritin vs. Peroxidase

The results obtained by the immunoperoxidase procedure in

agarose-embedded synaptosomes confirmed the immunoferritin data concerning the presence of synapsin I immunoreactivity in nerve endings and the proportion of nerve endings showing positive immunoreactivity under various experimental conditions. The immunoperoxidase results, like the immunoferritin results, suggested the presence of synapsin I in all nerve endings. Peroxidase reaction product, however, did not exhibit the same specific localization. A detailed examination of the micrographs of peroxidase-stained preparations supports the idea that this less specific localization is due to the indirect nature of the labeling, which allows stain to occur at some distance from the peroxidase molecule (30). Thus, (a) membranes in close proximity to synaptic vesicles were frequently darkened, irrespective of their nature, and (b) in many instances, such darkening was limited to the portions of the membranes closest to the synaptic vesicles.

An alternative explanation for the discrepancy between the immunoferritin and the immunoperoxidase results might be that the synapsin I molecules not on synaptic vesicles are partially shielded by a web of other proteins and are accessible to the smaller peroxidase-IgG conjugate but not the larger ferritin-IgG conjugate. Arguing against this possibility, however, we found that no major web was visible on the cytoplasmic faces of certain membranes heavily stained by peroxidase but not by ferritin, such as for instance, mitochondrial outer membranes and the nonjunctional portion of the nerve-ending plasma membranes.

The distribution of the peroxidase reaction product, seen in isolated nerve endings upon immunoperoxidase labeling for synapsin I, is very similar to the one seen in fixed brain slices after the same labeling protocol. This pattern (staining of all membranes exposed to the cytoplasmic space) seems to be a common pattern for proteins located in the cytoplasmic space of the terminal (the space not included within membrane-bound organelles), irrespective of their precise subcellular localization. Thus, a similar distribution of peroxidase reaction product has been observed after staining for clathrin (6), for the 65,000-dalton protein (a synaptic vesicle membrane protein) (27), and for proteins specific to certain terminals and thought to be soluble, such as tyrosine hydroxylase (33), glutamate decarboxylase (34, 41), and cGMP-dependent protein kinase (unpublished results). The similarity of these patterns indicates that the peroxidase reaction product accumulates in the whole cytoplasmic space and is then nonspecifically adsorbed onto any nearby particulate structure, irrespective of the subcellular localization of a given protein in the cytoplasm of the terminal.

Immunocytochemistry in Tissue Sections

Immunocytochemistry in tissue sections at the ultrastructural level confirmed the specific localization of synapsin I in nerve endings. Labeling of intact tissue, however, appeared to be greatly hampered by poor penetration of marker molecules. The tissue was totally inaccessible to ferritin-conjugated proteins. After peroxidase staining, only a minor proportion of nerve endings were labeled even after treatments aimed at permeabilizing plasma membranes.

Similar results were obtained in a previous immunoperoxidase study carried out on vibrotome sections (2). Our results in isolated synapses indicate that the uneven labeling in tissue sections, observed in that study (2) and in the present study, may be artifactual. The erratic labeling is most likely dependent on an uneven diffusion of marker proteins in the neuropile, which, in turn, might be dependent on an uneven fixation.

Ruptures and tears were often visible in those endings that were most heavily stained. These ruptures might have been present before the staining (and have been at least partially responsible for the good staining) or might have been produced by the process of polymerization of the peroxidase reaction product. In either case, they seem to be the expression of an incomplete cross-linking of the cytoplasmic matrix. Nonstained terminals showed, in general, a very well preserved morphology.

Immunoperoxidase labeling of nerve terminals in thick sections of brain before Epon embedding is a widely used technique to identify and sometimes to quantitate specific types of synaptic inputs in brain regions (36). Our results indicate that caution should be used in the interpretation of data obtained by this approach. Even if the proportion of terminals accessible to macromolecules were higher with fixation conditions different from ours, it is important to consider the possibility that some of the terminals are unstained because they are inaccessible to the marker molecules.

PSDs

In an earlier study, Synapsin I immunoreactivity was observed at some PSDs facing labeled terminals (2). Similarly, in the present study, ferritin particles and peroxidase reaction products were often present on PSDs in immunolabeled samples. However, such labeling was quantitatively similar to that observed in control samples. Nonspecific labeling of PSDs is probably the result of a nonspecific trapping of marker molecules in the filamentous meshwork of the PSDs.

In tissue sections not treated with detergents, we never observed immunoperoxidase reaction product on PSDs, even when PSDs were close to the free surface of the frozen section and no intervening membranes were visible between the PSD and the surface of the frozen section. A slight darkening of PSDs, which occurred exclusively at synapses with heavily labeled terminals, was observed only in sections that had been pretreated with detergents. Labeling of PSDs, therefore, seems to be an artifact due to diffusion of reaction product from the nerve ending, the diffusion occurring when the permeability barrier represented by intact membranes is abolished.

Synapsin I and Synaptic Vesicle Function

The specific association of synapsin I with synaptic vesicles in the majority of, and possibly in all, nerve endings indicated by the present study suggests that the role of synapsin I is related to some aspect of the function of synaptic vesicles and, therefore, directly or indirectly, to the primary function of nerve endings, which is the vesicular release (via exocytosis) of neurotransmitter. The ability of synapsin I to act as a substrate for cAMP- and Ca/calmodulin-dependent protein kinases in broken cell preparations and in physiological conditions (10, 18, 19, 29, 35) makes this protein a candidate to be involved in regulation of the release process itself, since this process can be regulated both by Ca (5, 22) and by cAMP (3, 24, 28).

Given the localization of synapsin I at the cytoplasmic surface of synaptic vesicles, an attractive hypothesis is that synapsin I might mediate a specific interaction(s) of synaptic vesicles with other subcellular elements (e.g., cytoskeletal proteins or plasma membrane proteins). The observation that synapsin I is associated with the vesicle membrane via an electrostatic interaction (20) raises the possibility that this association might not be permanent but limited to some stage(s) of the vesicle cycle in the nerve ending (storage, translocation

to the plasma membrane, fusion with the plasma membrane, retrieval from the plasma membrane).

The experimental results obtained in the present study allow us to conclude only that synapsin I is associated with synaptic vesicles during their storage phase in the cytoplasm of the nerve ending. Ferritin particles were never observed unambiguously decorating the cytoplasmic face of the plasma membrane of the ending. However, the region of the nerve terminal plasmalemma at which synaptic vesicles fuse (i.e., the region of the presynaptic plasmalemma directly facing the synaptic cleft) was often lined by a web of cytoplasmic matrix and by tightly packed synaptic vesicles (see, for instance, Figs. 3, 4, and 11). Lack of labeling of this region, therefore, might be due to lack of accessibility to marker molecules rather than to absence of synapsin I.

It has been proposed that patches of synaptic vesicle membranes incorporated into the plasmalemma upon fusion are recovered as coated vesicles (16). Therefore, it would be helpful to know if coated vesicles contain synapsin I. Unfortunately, conditions required to obtain optimal labeling of synapsin I (permeabilization of the plasma membrane and exposure of the cytoplasmic space to a large volume of low ionic strength medium) lead to a disappearance of coated vesicles. Thus, as can be seen in Figs. 2-4, coated vesicles were only visible in well-preserved endings, i.e., those poorly accessible to immunolabeling. This is in agreement with our observation that clathrin, the constituent of the coat (32), is solubilized by low ionic strength buffers at neutral pH (20).

Experiments are in progress to elucidate those stages of the synaptic vesicle cycle in the nerve ending during which synapsin I is associated with the vesicle membrane. This information is expected to be very important for the identification of the function of this protein.

Clearly, much further work is needed to achieve an understanding of the precise role played by synapsin I in the function of synaptic vesicles. One important clue, however, exists: although synapsin I is present in virtually all nerve endings, it appears to be absent from nonneuronal secretory cells (8, 9). This indicates that synapsin I is involved in some aspect(s) of regulation of vesicular secretion specific to neurons rather than in those steps of vesicular secretion that are common to neuronal and nonneuronal secretory systems.

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