Synapsin I (Protein I), a Nerve Terminal-Specific Phosphoprotein. III. Its Association with Synaptic Vesicles Studied in a Highly Purified Synaptic Vesicle Preparation

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ABSTRACT Synapsin I (protein I) is a neuron-specific phosphoprotein, which is a substrate for cAMP-dependent and Ca/calmodulin-dependent protein kinases. In two accompanying studies (De Camilli, P., R. Cameron, and P. Greengard, and De Camilli, P., S. M. Harris, Jr., W. B. Huttner, and P. Greengard, 1983, *J. Cell Biol.* 96:1337–1354 and 1355–1373) we have shown, by immunocytochemical techniques at the light microscopic and electron microscopic levels, that synapsin I is present in the majority of, and possibly in all, nerve terminals, where it is primarily associated with synaptic vesicles.

In the present study we have prepared a highly purified synaptic vesicle fraction from rat brain by a procedure that involves permeation chromatography on controlled-pore glass as a final purification step. Using immunological methods, synapsin I concentrations were determined in various subcellular fractions obtained in the course of vesicle purification. Synapsin I was found to copurify with synaptic vesicles and to represent ~6% of the total protein in the highly purified synaptic vesicle fraction. The copurification of synapsin I with synaptic vesicles was dependent on the use of low ionic strength media throughout the purification. Synapsin I was released into the soluble phase by increased ionic strength at neutral pH, but not by nonionic detergents. The highly purified synaptic vesicle fraction contained a calcium-dependent protein kinase that phosphorylated endogenous synapsin I in its collagenase-sensitive tail region. The phosphorylation of this region appeared to facilitate the dissociation of synapsin I from synaptic vesicles under the experimental conditions used.

Synapsin I (previously referred to as protein I [31, 33]) is the collective name for two almost identical neuron-specific polypeptides, synapsin Ia (protein Ia) and synapsin Ib (protein Ib), which are major substrates for protein kinases present in mammalian brain (16, 17, 19, 20, 31). Synapsin I is phosphorylated at distinct sites by cAMP-dependent and two Ca/calmodulin-dependent protein kinases (16, 17, 19). It is a basic protein, with a globular "head" and an elongated "tail" (31). The state of phosphorylation of synapsin I can be altered both in vivo and in vitro by a variety of physiological and pharmacological manipulations known to affect synaptic function (10, 11, 20, 26, 27, 29).

In previous studies (2, 8) and in the two accompanying papers (6, 7), the localization of synapsin I in the nervous system has been studied by immunocytochemical techniques at both the light- and electron-microscopic levels. Synapsin I is highly concentrated in the nerve terminal region of the great majority of neurons, and possibly of all neurons, of the central and peripheral nervous systems (6). Within nerve terminals, synapsin I appears to be associated primarily with synaptic vesicle membranes, at their cytoplasmic surface (7).

To assist in studying the possible role of synapsin I in

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synaptic vesicle function, it seemed desirable to have available synaptic vesicles from mammalian brain with a high degree of purity. In the present study we describe the preparation of highly purified rat brain synaptic vesicles. The purification involves permeation chromatography on controlled-pore glass, and yields a final fraction that appears to be composed almost entirely of synaptic vesicles and to be highly enriched in synapsin I. With the use of this preparation, we have begun to characterize the nature of the association of synapsin I with the synaptic vesicle membrane, and to study the possible role of phosphorylation in this association.

MATERIALS AND METHODS

Male Sprague-Dawley rats, weighing 150–200 g, were used as brain donors. Glutaraldehyde (10% aqueous solution) and OsO_4 (4% aqueous solution) were from Electron Microscopy Sciences (Fort Washington, PA). Controlled-pore glass beads (GLY 03000B) were obtained from Electro-Nucleonics Inc., Fairfield, NJ; See product information bulletin CPG-10. *Staphylococcus aureus* V8 protease was obtained from Miles Laboratories, Inc. (Elkhart, IN), TPCK-trypsin from Worthington Biochemical Co. (Freehold, NJ), and collagenase from Advance Biofactures (Lynbrook, NY). [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (13). ^{125}I-protein A was obtained from Amersham Corp. (Arlington Heights, IL). Catalytic subunit of cAMP-dependent protein kinase, purified as described (18), was a gift of Dr. Angus C. Nairn of our laboratory. Calmodulin, purified according to Watterson et al. (35), was a gift of Dr. H. Clive Palfrey of our laboratory. Purified clathrin was a gift of Dr. E. Merisko (Yale University). All other reagents were obtained from sources previously listed (20, 31).

Purification of Synaptic Vesicles from Rat Cerebral Cortex

The procedure described below was designed using published methods for the isolation and purification of synaptic vesicles from mammalian brain (24, 37) as guidelines. The purification procedure is outlined schematically in Fig. 1.

PREPARATION OF THE HOMOGENATE: Seven rats were killed by cervical dislocation followed by decapitation. The brains were removed from the skull and placed into an ice-cold "buffered sucrose" (320 mM sucrose/4 mM HEPES-NaOH buffer, pH 7.3). From this point on, the material was kept at 4°C throughout the preparation. Each cerebral cortex was dissected free of cerebellum, brain stem, and most of the midbrain; the cerebral cortices were pooled and homogenized in 50 ml of buffered sucrose in a glass-Teflon homogenizer using 12 up-and-down strokes at 900 rpm. The whole process was repeated with another seven rats, the two homogenates were pooled, and 40 ml of buffered sucrose were added to yield a final homogenate (H) of ~150 ml.

DIFFERENTIAL AND EQUILIBRIUM CENTRIFUGATION: The subsequent steps of differential centrifugation were carried out in 50-ml polycarbonate tubes in a Sorvall SS-34 rotor (DuPont Co., Newtown, CT). The homogenate was centrifuged for 10 min at 3,000 rpm (800 g_{av}). The resulting pellet (P1) was discarded, while the supernatant (S1) was collected and centrifuged for 15 min at 10,000 rpm (9,200 g_{av}). The supernatant (S2) was removed, and the pellet (P2) was washed by resuspending in 120 ml of buffered sucrose and recentrifuging for 15 min at 10,500 rpm (10,200 g_{av}) to yield a supernatant, S2', and a pellet, P2'. (In some experiments, the supernatants S2' and S2 were pooled and the combined fractions were centrifuged at 4°C for 1 h in a Beckman 50Ti rotor (Beckman Instrument Co., Spinco Div., Palo Alto, CA) at 50,000 rpm [165,000 g_{av}] to yield a pellet fraction [P3] and a cytosol fraction [C].)

The pellet (P2') was resuspended in 13 ml of buffered sucrose. This suspension is referred to as the well-washed, crude synaptosomal fraction. This suspension was transferred to a glass-Teflon homogenizer. 117 ml of ice-cold water were added, and the whole suspension was immediately subjected to three up-and-down strokes at 3,000 rpm. The resulting P2'-lysate (L) was poured rapidly into a beaker containing 1 ml of a 1 M HEPES-NaOH buffer (pH 7.4), and the suspension was kept on ice for 30 min. It was then centrifuged for 20 min at 16,500 rpm (25,000 g_{av}) to yield a lysate pellet (LP1) and a lysate supernatant (LS1). The supernatant was collected, transferred into 12 10-ml polycarbonate tubes, and centrifuged for 2 h in a Beckman 50Ti rotor at 50,000 rpm (165,000 g_{av}). The supernatants (LS2) were removed, and the pellets (LP2) were resuspended in a total of 6 ml of 40 mM sucrose. To augment the resuspension process, the suspension was subjected to 10 up-and-down strokes in a glass-Teflon homogenizer at 1,200 rpm, followed by forcing the suspension five times back and forth through a 25-gauge needle attached to a 10-ml disposable syringe. The suspension was then layered on top of a linear continuous sucrose gradient

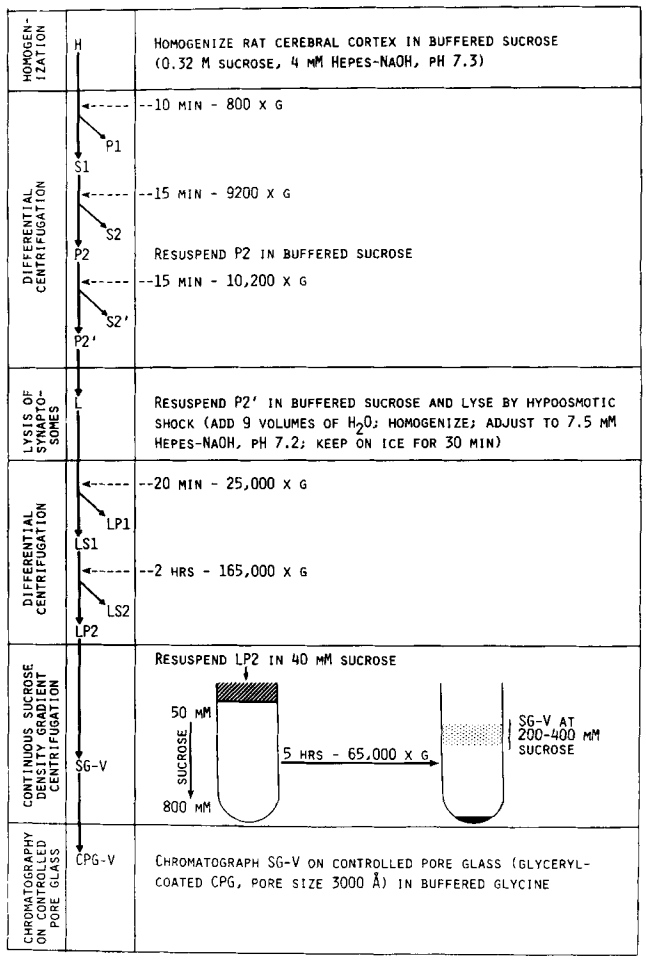


FIGURE 1 Scheme for purification of synaptic vesicles from rat brain.

generated in a 60-ml cellulose nitrate tube from 25 ml of 800 mM sucrose and 29 ml of 50 mM sucrose. Sucrose gradient centrifugation was performed for 5 h in a Beckman SW25.2 rotor at 23,000 rpm (65,000 g_{av}). At the end of the centrifugation, the gradient revealed a broad band of high turbidity in the 200–400 mM sucrose region. In initial experiments (including the experiment illustrated in Fig. 3) the gradient, except for the pellet and the densest 3 ml, was collected in 2-ml fractions through the bottom of the tube. Analysis of various fractions revealed that the material banding at the 200–400 mM sucrose region was very enriched not only in synaptic vesicles, as assessed by morphological criteria (EM analysis), but also in synapsin I. Therefore, in subsequent experiments, in which vesicles were further purified, fractions were collected from the sucrose density gradient by introducing a needle through the side of the centrifuge tube. The fractions corresponding to the 200–400 mM sucrose regions were pooled (SG-V).

CONTROLLED-PORE GLASS CHROMATOGRAPHY: For this purification step (compare references 3, 23, and 24), glyceryl-coated controlled-pore glass beads with a mean pore diameter of 3,000 Å, 74–125 μm (120/200 mesh) in size, were used. For preparative purposes, the following column was used: 420 cm^3 of dry beads were suspended in water. The suspension was degassed and poured into a column (2 cm i.d. × 150 cm) which was strongly vibrated with a vortex during packing. The column was washed with water, and was then equilibrated with "buffered glycine" (300 mM glycine/5 mM HEPES-NaOH buffer [pH 7.2]/0.02% [wt/vol] sodium azide) before use. The SG-V pool (~15 ml) was layered on top of the glass beads, overlayered with buffered glycine, and column chromatography was performed in buffered glycine at a flow rate of 40 ml/h, collecting 5-ml fractions. In some experiments, 300 mM sucrose/5 mM HEPES-NaOH (pH 7.2) was used instead of buffered glycine. The protein staining profile of synaptic vesicles purified in buffered glycine was identical to that of vesicles obtained when sucrose was used instead of glycine. Since vesicles in buffered glycine, in contrast to vesicles in buffered sucrose, could easily be pelleted, vesicles in buffered glycine were routinely used for the extraction and phosphorylation experiments described in Results. The entire preparation was routinely carried through in 1 d, with the controlled-pore glass chromatography step overnight.

Processing of Subcellular Fractions

Aliquots of fractions P1, P2′, P3, C, L, LP1, LP2, and LS2 were diluted with buffered sucrose to correspond in volume to fraction H. Fractions obtained after continuous sucrose density gradient centrifugation were used without further dilution. Fractions obtained after permeation chromatography on controlled-pore glass using buffered glycine were, unless otherwise indicated, either centrifuged in cellulose nitrate tubes at 4°C for 1 h in a Beckman SW50.1 rotor at 50,000 rpm (234,000 g_{av}) or first mixed at 4°C with TCA to a final concentration of 15% (wt/vol) and then centrifuged; the resultant pellets were processed further.

SDS PAGE: Samples were mixed with 0.5 vol of "3 ×-stop solution" (9% [wt/vol] SDS/375 mM Tris-HCl [pH 6.8]/30% [wt/vol] sucrose/10% [vol/vol] β-mercaptoethanol) or resuspended in "1 ×-stop solution" and were immediately boiled for 5 min. They were then, either directly or after freezing and reboiling, subjected to SDS PAGE in 7.5 or 10% gels according to Laemmli (21) as described previously (20, 31). After electrophoresis, gels were either fixed, stained with Coomassie Blue, destained, and dried as described (20, 31), or subjected to radioimmunolabeling for synapsin I.

RADIOIMMUNOLABELING FOR SYNAPSIN I AFTER SDS PAGE: Radioimmunolabeling for synapsin I after SDS was performed as described previously (8) by modification of the method of Adair et al. (1). After the electrophoresis, gels (7.5% unless otherwise indicated) were fixed in 46% (vol/vol) methanol/8% (vol/vol) acetic acid. All subsequent steps were performed in NaCl-Tris buffer (150 mM NaCl/20 mM Tris-HCl [pH 7.4]/0.02% [wt/vol] sodium azide) and the indicated additions. Fixed gels were washed extensively (three changes of 500 ml NaCl-Tris buffer each, 12 h total), then incubated for 12 h in a 1:300 dilution of rabbit antiserum (6, 8) raised against purified bovine synapsin I (31), washed extensively as above, then incubated for 12 h in ^{125}I–protein A (100–500 cpm/μl)/0.1% (wt/vol) gelatin, washed extensively as above, dried, and autoradiographed, using Kodak XR5 film with or without intensifying screens. Gel pieces containing radioimmunolabeled synapsin I were cut from the gel, using the autoradiogram as guide, and counted in a Beckman gamma counter.

RADIOIMMUNOASSAY OF SYNAPSIN I: Samples were mixed with 0.5 vol of 10% (wt/vol) SDS, immediately boiled for 2 min, and stored at −20°C. After thawing and reboiling, the SDS-containing samples were diluted with Nonidet P-40, and the endogenous synapsin I concentration was determined by radioimmunoassay essentially as described by Goelz et al. (14), using rabbit antiserum raised against bovine synapsin I and ^{125}I–bovine synapsin I. Purified rat synapsin I (16) was used to generate the standard curve.

ELECTRON MICROSCOPY: Subcellular fractions (with the exception of glycine-containing fractions) were fixed in suspension by the addition of glutaraldehyde in sodium cacodylate buffer (pH 7.4) to a final glutaraldehyde concentration of 2%. The final concentration of the sodium cacodylate buffer was 120 mM in the case of the P2′ fraction, and 10 mM in the case of subfractions obtained from the lysed P2′ fraction (L). After incubation at room temperature for 15 min, the fixed subcellular particles were recovered by centrifugation for 1 h at 234,000 g_{av}. Glycine-containing fractions were fixed as pellets by the addition of 2% glutaraldehyde in 10 mM sodium cacodylate buffer (1 h at room temperature). The fixed pellets were then washed with 0.1 M veronal/acetate buffer (pH 7.4) and postfixed in 1% OsO_4 at 0°C in the same buffer. Then, after a quick rinse in veronal/acetate buffer, they were dehydrated in graded ethanols and propylene oxide and embedded in Epon.

Silver sections were prepared 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.

EXTRACTION OF SYNAPSIN I: The synaptic vesicle (CPG-V) fraction was used as obtained after permeation chromatography in buffered glycine. Aliquots containing 80–120 μg of protein were incubated in polycarbonate centrifuge tubes for 30 min at 0°C in a final volume of 1.3 ml in the absence or presence of the indicated concentrations of various salts, of 1% (wt/vol) Triton X-100, or (by addition of an appropriate amount of 1 N HCl) at pH 3.0. Samples were then centrifuged at 4°C for 1 h in a Beckman 50Ti rotor at 50,000 rpm (165,000 g_{av}). Supernatants (1.2 ml) were harvested with a Pasteur pipette and transferred into 60 μl of 3 ×-stop solution; this procedure included rinsing the glass wall of the Pasteur pipette with stop solution. Pellets were resuspended directly in 300 μl of 1 ×-stop solution. Samples were then subjected to SDS PAGE followed by protein staining or by radioimmunolabeling for synapsin I.

Phosphorylation of Synapsin I on Synaptic Vesicles

Phosphorylation reactions were carried out at 0°C in cellulose nitrate tubes, which were also used for the centrifugation of the sample after termination of the phosphorylation reaction. Aliquots (500 μl) of CPG-V in buffered glycine (40–60 μg of protein) were added to centrifuge tubes containing 28 μl of concentrated reaction mixture. The addition of the synaptic vesicle suspension was performed rapidly with immediate mixing of the sample in order to avoid the exposure of membranes to high local salt concentrations. The phosphorylation reaction was started by addition to each tube of 150 μl of [γ-^{32}P]ATP (1–5 × 10^6 cpm/nmol). For experiments in which synapsin I was to be analyzed by radioimmunolabeling of gels, nonradioactive ATP was used instead of radioactive ATP. The composition of the phosphorylation reaction mixture was: 10 mM HEPES-NaOH buffer (pH 7.4)/2 mM $MgCl_2$/15 mM KCl/200 μM EGTA/5 μg/ml calmodulin and, as indicated, 500 μM $CaCl_2$/75 nM catalytic subunit of cAMP-dependent protein kinase/45 μM ^{32}P-ATP or ATP.

After addition of ATP, the samples were incubated for 30 min and then centrifuged at 4°C in a Beckman SW50.1 rotor for 60 min at 50,000 rpm (234,000 g_{av}). After centrifugation, each supernatant was quickly transferred with a fresh pasteur pipette to a glass test tube and rapidly frozen in a dry-ice/acetone bath. Minor portions of the supernatant wetting the wall of the cellulose nitrate tube were quickly removed, and 50 μl of 3 ×-stop solution were added to each pellet without contacting the wall of the tube except for its bottom part. The centrifuge tube was then rapidly frozen on dry ice.

For SDS PAGE, the supernatants were lyophilized and the residues dissolved in 150 μl of 1 ×-stop solution followed by boiling for 5 min. When necessary, an appropriate amount of LiCl was added to prevent precipitation of potassium dodecyl sulfate. The centrifuge tubes containing the pellets were cut right above the surface of the frozen 3 ×-stop solution. The pellet was suspended in 50 μl of 3 ×-stop solution by scraping it from the bottom of the tube with a solid steel needle, and the sample was transferred to a glass test tube. The cut-off bottom of the centrifuge tube was then rinsed two times, each with 50 μl of H_2O, which were afterwards added to the sample. Complete transfer of the pelleted material was checked by monitoring the radioactivity with a Geiger counter. Samples were then boiled for 5 min. After SDS PAGE, gels of ^{32}P-labeled samples were subjected to protein staining and autoradiography, and gels of nonradioactive samples (nonradioactive ATP or no ATP) were subjected to radioimmunolabeling for synapsin I. From the first type of gel, dried gel pieces containing ^{32}P-labeled synapsin I were subjected to limited proteolysis by the method of Cleveland et al. (4) as described (16, 17), followed by autoradiography.

PROTEIN DETERMINATION: Protein was assayed by the method of Lowry et al. (22).

RESULTS

Preparation of Highly Purified Synaptic Vesicles from Rat Brain

The purification procedure finally adopted for the purification of synaptic vesicles was based on established methods (24, 37) for the purification of these organelles from mammalian brain and can be divided into six major steps (Fig. 1): (*1*) homogenization of rat cerebral cortex, (*2*) differential centrifugation of the homogenate to obtain a crude synaptosomal fraction (P2′), (*3*) hypoosmotic lysis of the synaptosomes to release synaptic vesicles, (*4*) differential centrifugation of the crude synaptosomal lysate to obtain a crude synaptic vesicle fraction, (*5*) purification of the synaptic vesicles by continuous sucrose density gradient centrifugation, and (*6*) permeation chromatography of synaptic vesicles on controlled-pore glass.

Homogenization and all subsequent steps were carried out in low ionic strength media because this condition was found to be essential to recover synapsin I in particulate fractions. The aim of the primary subfractionation of the brain homogenate (step *2*) was to remove small vesicles other than the vesicles contained in nerve endings. As can be seen from Fig. 2, the vast majority of small vesicle profiles visible in the washed crude synaptosomal fraction (P2′) were synaptic vesicles in the nerve endings. Upon osmotic lysis of the P2′ fraction, these small vesicles were released into the medium. The released vesicles were separated from larger organelles and from soluble proteins of the lysate by differential centrifugation, and were then subjected to a further purification on a continuous sucrose density gradient.

When the various fractions obtained from the continuous sucrose density gradient were analyzed by electron microscopy, those fractions present in the band of high turbidity at 200–400 mM sucrose were found to have the highest content of synaptic

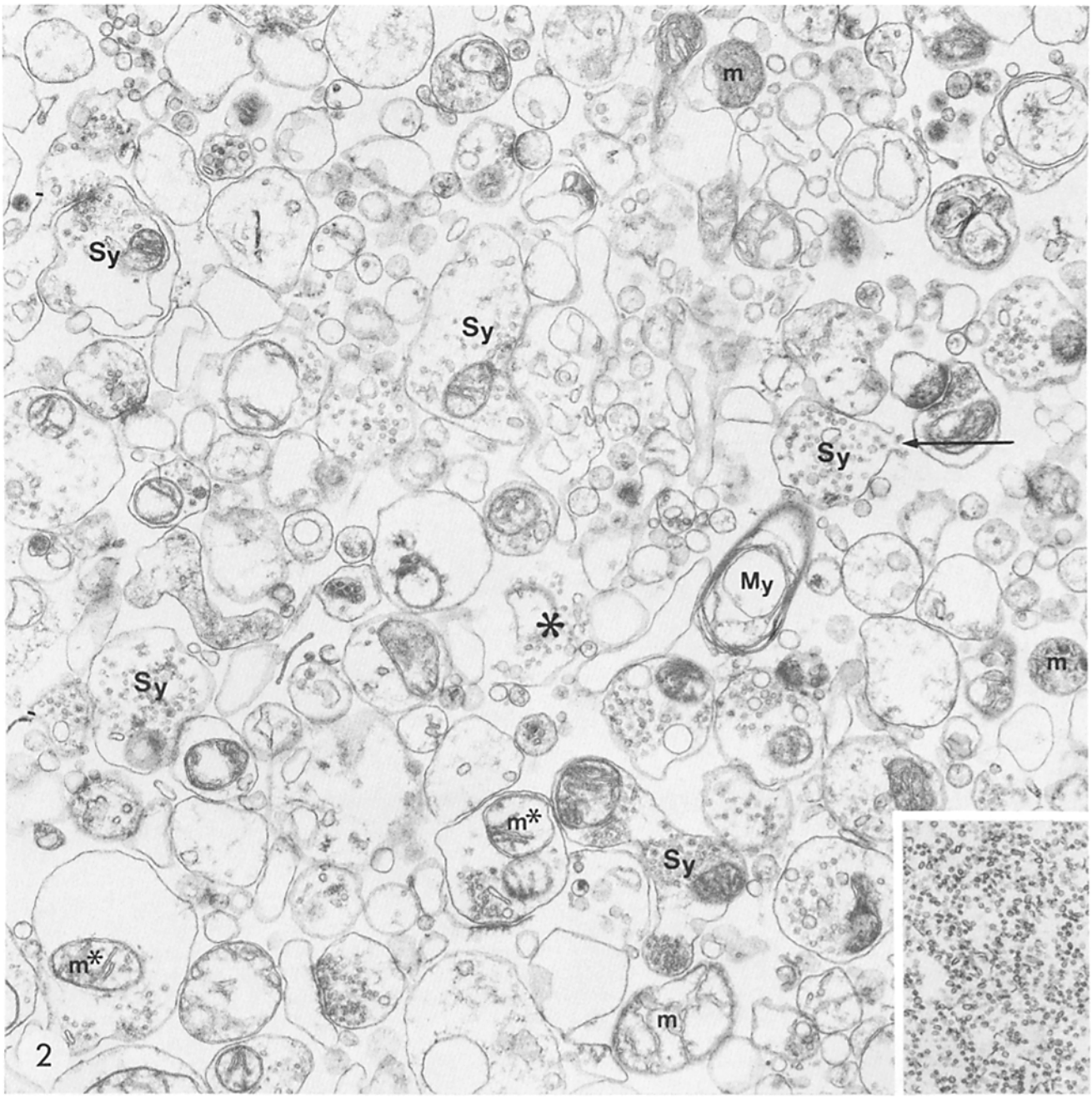


FIGURE 2 Electron micrograph illustrating the morphology of subcellular particles constituting the washed crude synaptosomal pellet (P2′ fraction). The field shown in the picture is representative of the composition of the fraction. The relative proportions of free mitochondria, myelin sheaths, synaptosomes, and other large membrane fragments were variable from the top to the bottom of the pellet, but the whole population of particles present in the fraction is represented in this field. Most of the nerve endings have a continuous plasma membrane but discontinuities are present in the plasma membrane of some of them (arrow). In spite of these discontinuities, synaptic vesicles appear to be at least partially retained inside the nerve ending profile. A group of synaptic vesicles completely stripped of the plasmalemma of the ending but still apparently connected to each other is labeled by a large asterisk. *Sy*, synaptosomes; *My*, myelin sheath; *m*, free mitochondria; *m**, mitochondria in nerve endings. *Inset*: Electron micrograph illustrating the morphology of the highly purified synaptic vesicle fraction (CPG-V). A comparison of the main picture and of the inset, which were printed at the same final magnification, shows the similarity in size between the purified vesicles and vesicles in nerve endings. × 25,000; *inset*, × 25,000.

vesicles. This sucrose concentration range represents the bouyant density reported for mammalian synaptic vesicles by others (24, 25). These same fractions were also enriched in synapsin I, as monitored both by protein staining (Fig. 3, *top*) and by radioimmunolabeling for synapsin I (Fig. 3, *bottom*). These fractions were pooled (SG-V) and subjected to the final purification step, permeation chromatography on controlled-pore glass (Fig. 4). This step yielded three protein peaks. The first peak (coinciding with the void volume of the column) and the second peak contained particulate material, while the third peak (coinciding with the low molecular weight marker, phenol red) contained a small amount of souble protein.

The particles contained in the first and second peaks obtained after permeation chromatography on controlled-pore

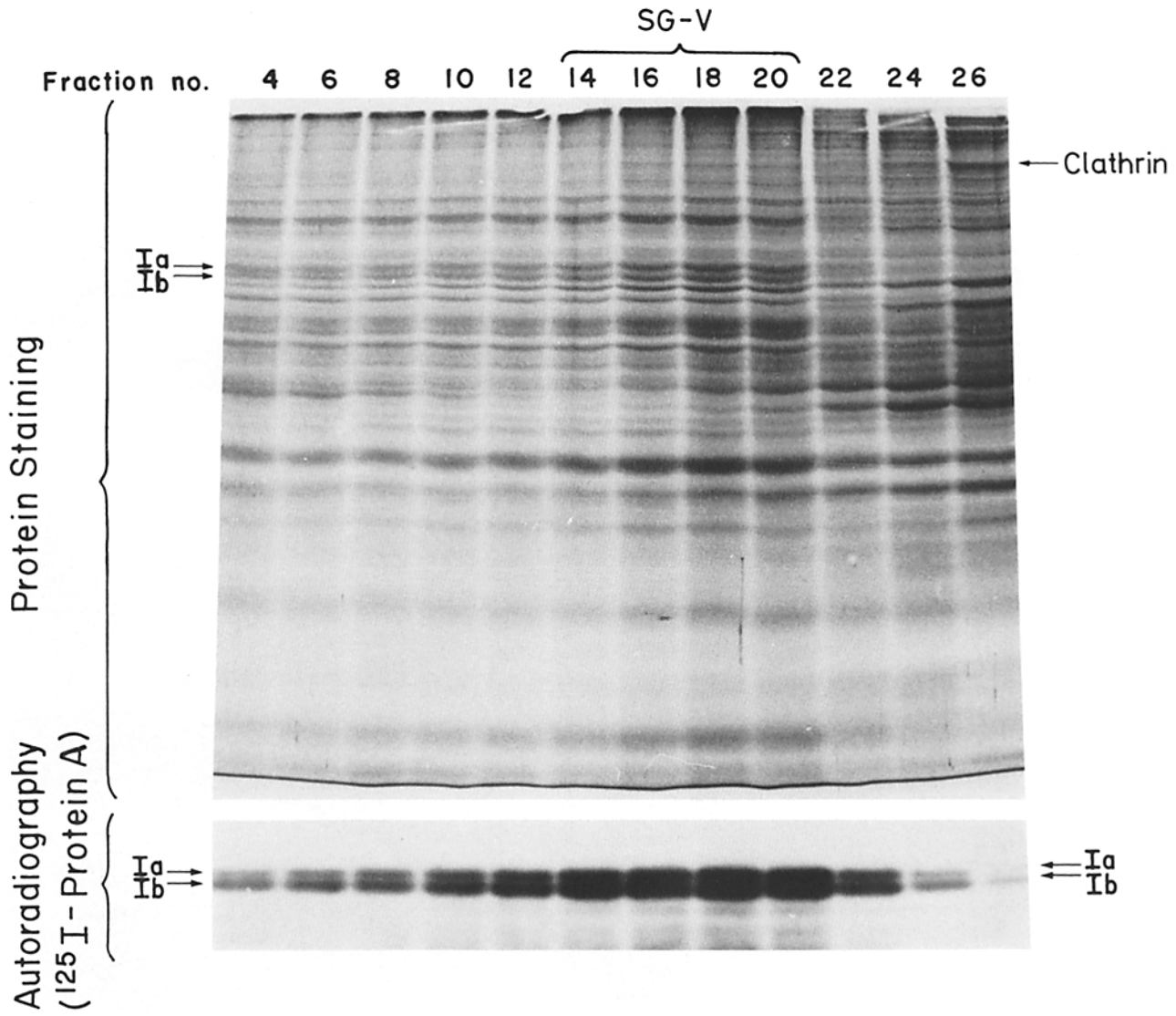


FIGURE 3 Analysis of total protein and of synapsin I in fractions obtained after continuous sucrose density gradient centrifugation of fraction LP2. Aliquots (100 μl) of alternate fractions obtained were subjected to SDS PAGE on a 10% gel, and the gel was stained with Coomassie Blue (*top*). Aliquots (33 μl) from these same fractions were also subjected to SDS PAGE on a 7.5% gel, and this gel was used for radioimmunolabeling of synapsin I with ^{125}I-protein A followed by autoradiography (*bottom*). Only the portion of the autoradiogram indicating ^{125}I-protein A binding to the gel is shown. The density of fractions decreases from left (fraction *4*, ~700 mM sucrose) to right (fraction *26*, ~40 mM sucrose). Fractions (indicated by SG-V) enriched in synaptic vesicles and synapsin I were pooled for further purification. The positions of synapsins Ia and Ib and of clathrin are indicated by arrows.

glass were analyzed by electron microscopy (Figs. 5 and 6). The void volume peak contained predominantly a heterogeneous population of vesicular structures and some small aggregates of amorphous material (Fig. 5). The vast majority of vesicular structures were much larger than synaptic vesicles, as can be seen from a comparison of Figs. 5 and 6*a*, which are at the same magnification. In contrast, the pooled fractions constituting the second peak (CPG-V) contained an almost homogeneous population of small vesicles of the same size as synaptic vesicles (Figs. 2 (*inset*) and 6). A few small membrane fragments with free edges were also present, but these always constituted a minor proportion of the particles present in the fraction. We shall refer to the pooled fractions constituting the second peak (CPG-V) as "highly purified synaptic vesicles."

Synapsin I immunoreactivity was associated only with the second peak obtained upon permeation chromatography (Fig. 4, ^{125}I-protein A autoradiogram). Analysis of the pooled fractions constituting the second peak (CPG-V), by SDS PAGE and protein staining, revealed a specific polypeptide pattern with synapsins Ia and Ib as major components (Fig. 4, *right*). Densitometric scans of this protein profile indicated that synapsins Ia and Ib constituted ~6% of all proteins present.

Synapsin I Distribution in Various Subcellular Fractions

Fig. 7 shows the results obtained when equal amounts of protein from the various subcellular fractions were analyzed by SDS PAGE followed by protein staining or radioimmunolabeling for synapsin I. It can be seen that, under the conditions of subcellular fractionation used, synapsin I was exclusively particulate. Neither the cytosol fraction (C) nor the high speed supernatant of the crude synaptosomal lysate (LS2) contained any significant amounts of synapsin I. The concentration of

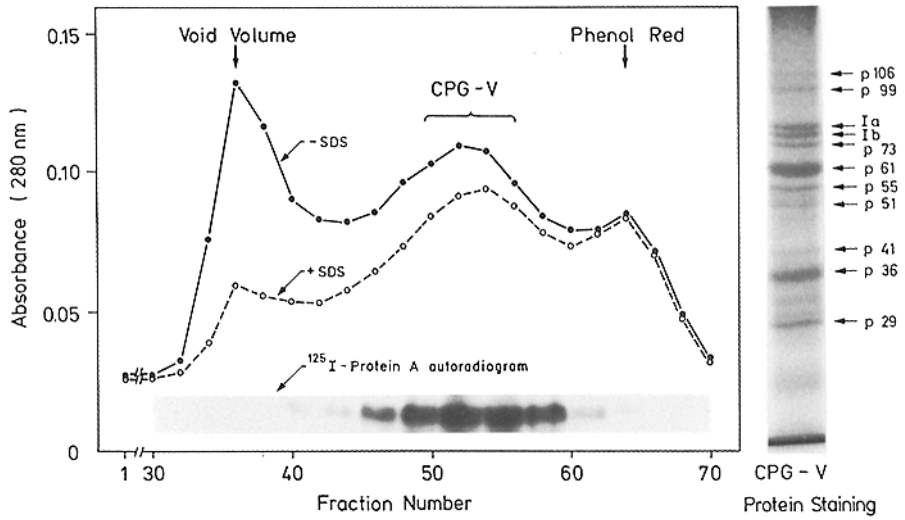


FIGURE 4 Purification of synaptic vesicles by permeation chromatography on controlled-pore glass. *Left*: fraction SG-V, obtained after continuous sucrose density gradient centrifugation, was subjected to chromatography on glyceryl-coated controlled-pore glass (pore diameter, 3,000 Å) in buffered glycine. Aliquots (1 ml) of alternate fractions were analyzed for their absorbance at 280 nm either before (– *SDS*) or after (+ *SDS*) addition of 0.2% (wt/vol) SDS. Aliquots (500 μl) of every third fraction were subjected to TCA precipitation followed by SDS PAGE on a 7.5% gel, radioimmunolabeling of synapsin I with ^{125}I-protein A, and autoradiography (*inset*). Only the portion of the autoradiogram indicating ^{125}I-protein A binding to the gel is shown. The fractions constituting the highly purified synaptic vesicle preparation (indicated CPG-V) were pooled. *Right*: An aliquot (1 ml) of the pooled (CPG-V) fractions was subjected to TCA precipitation followed by SDS PAGE on a 10% gel, and protein staining with Coomassie Blue. The positions of synapsins Ia and Ib and of other prominent polypeptide bands, designated according to their apparent molecular weights in kilodaltons, are indicated by arrows.

synapsin I clearly increased at each step of the purification of synaptic vesicles. Densitometric scans of the protein staining profiles of fractions LP2, SG-V, and CPG-V indicated that the increase in synapsin I concentration during the later stages of the purification was paralleled by a similar increase in concentration of a few other polypeptide bands that appeared to be major constituents of the purified synaptic vesicle fraction, for instance p61 and p36 (see Fig. 4).

The enrichment for synapsin I in the course of synaptic vesicle purification was quantitated using a specific and sensitive radioimmunoassay (Table I). Synapsin I represented ~0.4% of the total protein of the homogenate. In the highly purified synaptic vesicle fraction (CPG-V), synapsin I was enriched ~15-fold over the homogenate, and constituted ~6% of the total synaptic vesicle protein.

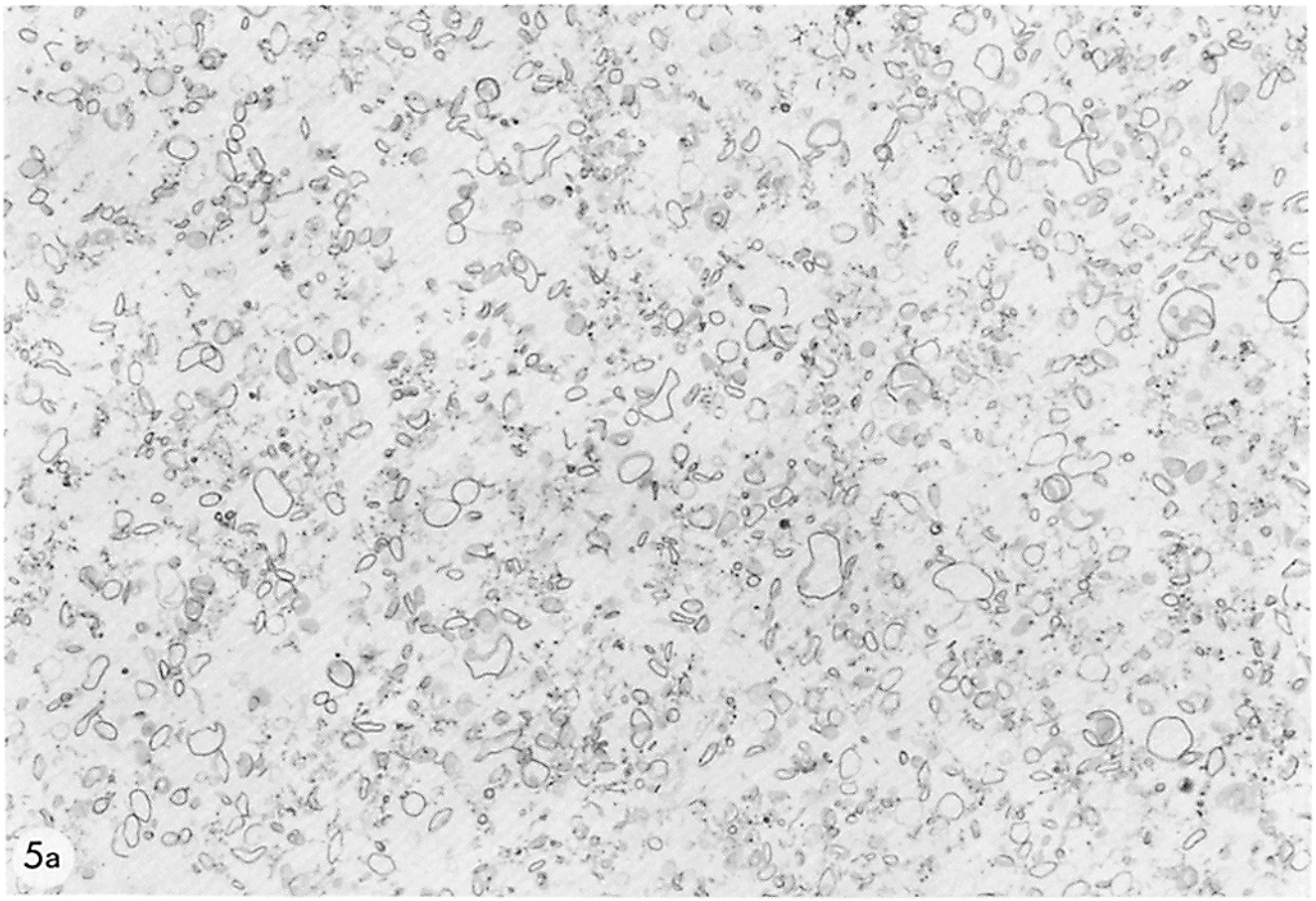
Clathrin Distribution in Various Subcellular Fractions

Since the procedure used to purify synaptic vesicles was designed to select small vesicular structures that can be released from synaptosomes upon hypoosmotic lysis, we considered the possibility that our highly purified vesicle fraction (CPG-V) might contain coated vesicles (28). However, coated vesicles were not seen by electron microscopy in this fraction (Fig. 6), and no clathrin (28) was found in SDS polyacrylamide gels of the same fraction (Figs. 4 and 7), as assessed by the absence of a polypeptide band of the same electrophoretic mobility as purified clathrin. Furthermore, analysis of Fig. 7 revealed that, following the purification procedure used to prepare CPG-V, clathrin was recovered in fractions containing soluble proteins. Thus, upon centrifugation of LS1, clathrin was recovered primarily in the supernatant fraction (LS2) rather than in the pellet constituting the crude synaptic vesicle fraction (LP2). Moreover, even the small amount of clathrin remaining in the LP2 fraction behaved as a soluble protein upon sucrose density gradient centrifugation of this fraction (Fig. 8).

The solubilization of clathrin in our purification procedure was found to be dependent upon the low ionic strength condition used. Due to this observation it is possible that "stripped coated vesicles" were present in the CPG-V fraction.

Solubilization of Synapsin I from Synaptic Vesicles

The highly purified synaptic vesicle fraction (CPG-V) has enabled us to begin an investigation of the nature of the association of synapsin I with synaptic vesicles. Fig. 9 shows the effects of various salts on the association of synapsin I with synaptic vesicles, as determined by SDS PAGE followed by protein staining or radioimmunolabeling for synapsin I of particulate and soluble material. Protein staining and radioimmunolabeling for synapsin I gave identical results concerning the proportion of soluble and particulate synapsin I after the various treatments. The Coomassie Blue–stained gels are shown for the particulate material since they also show the behavior of other vesicle proteins. The autoradiograph of the immunolabeled gel is shown for the soluble material. To avoid interference by the various salts during electrophoresis, only small portions of the supernatants were analyzed and, as a consequence, the synapsin I doublet was not easily visualized by protein staining. Under control conditions (low ionic strength, neutral pH), synapsin I was recovered in the pellet along with the highly purified synaptic vesicles. Raising the salt concentration of the medium, even to moderate levels (for instance 150 mM KCl, Figs. 9 and 10), resulted in the loss of considerable amounts of synapsin I from the synaptic vesicles. This material was recovered as intact synapsin I in the supernatant. The effect of salt appeared to be due to raising the ionic strength, as no apparent specificity of the various salts used



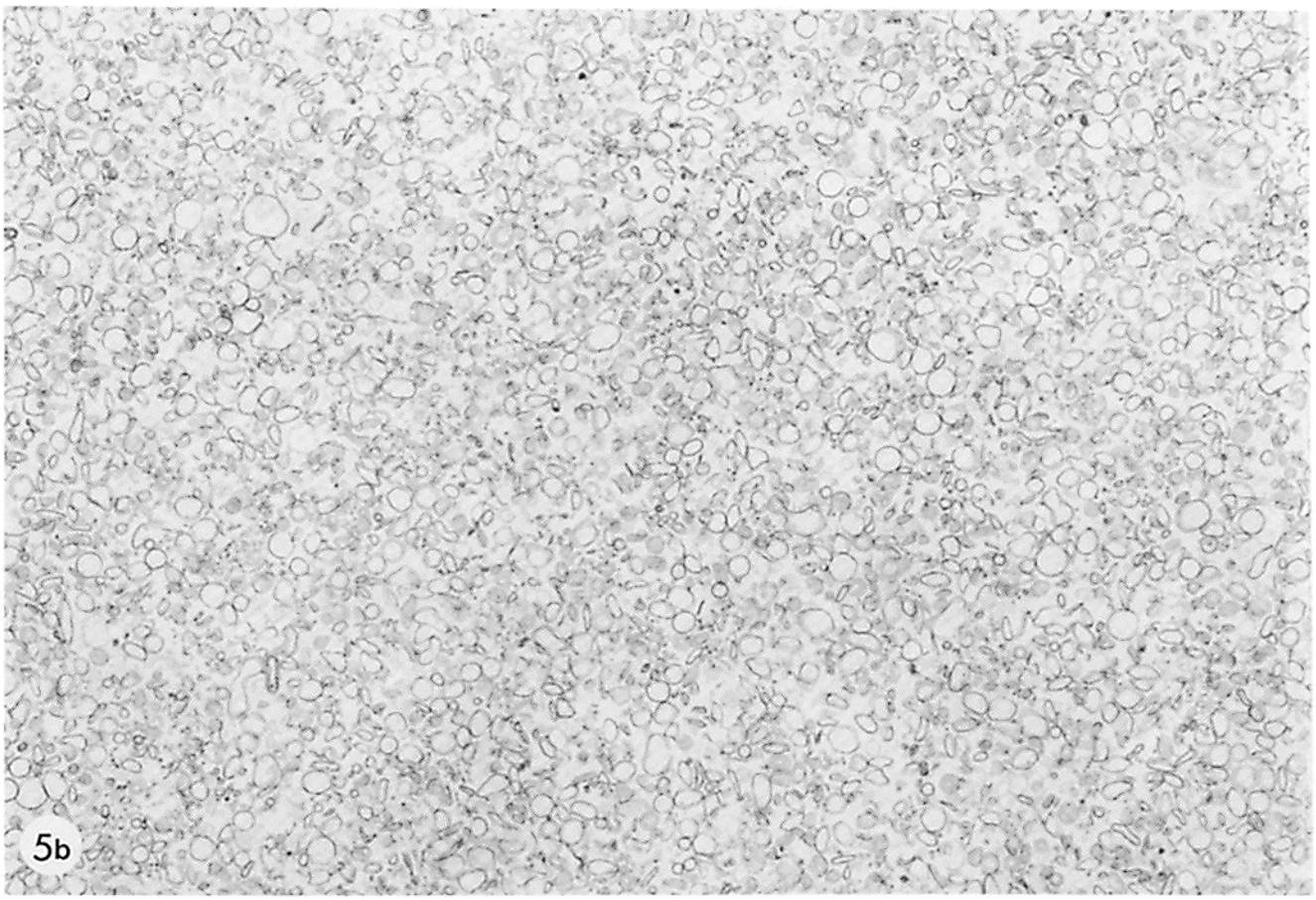


FIGURE 5 Electron micrograph illustrating the morphology of the pellet obtained by centrifugation of the fractions corresponding to the void volume of the CPG-column. (*a*) A field from the top third of the void volume pellet; (*b*) a field from the lower third of the pellet at the same magnification. The field shown in *b* is representative of the bottom two thirds of the pellet. As can be seen, the pellet is almost homogeneously composed of vesicular structures larger than synaptic vesicles (compare with Fig. 6 *a* which is printed at the same magnification). Some clusters of amorphous material can also be seen. *a* and *b*, × 18,200.

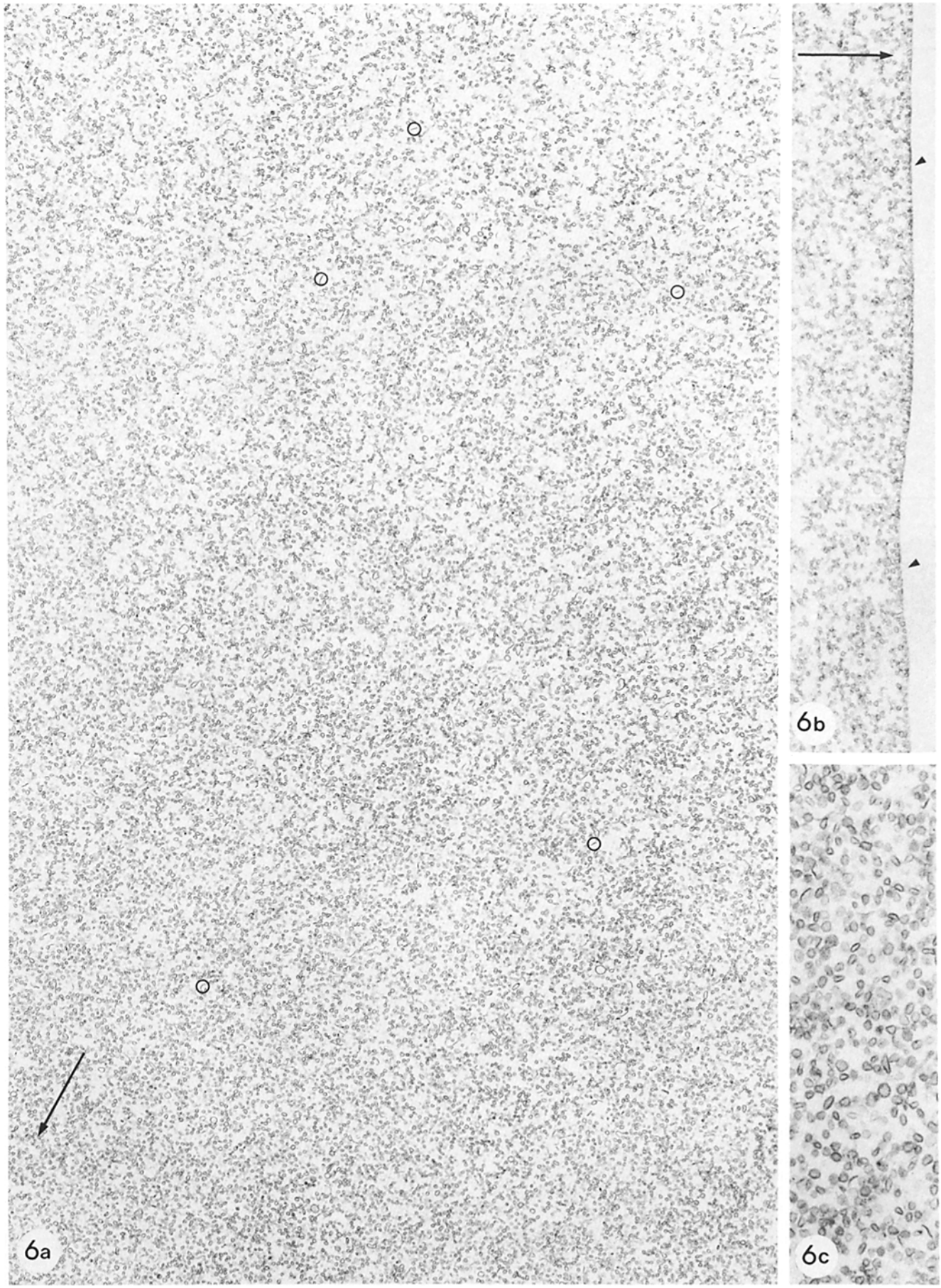


FIGURE 6 Electron micrographs illustrating the morphology of the pelleted CPG-V fraction. (*a*) Central region of the pellet; (*b*) bottom of the pellet. Arrowheads indicate the interface between the bottom of the pellet and the bottom of the centrifuge tube. Long arrows in *a* and *b* show the orientation from top to bottom of the pellet. (*c*) High magnification of a random field of the pellet. The pellet is constituted almost exclusively of small vesicles. Short, rodlike structures, probably membrane fragments, are also visible (a few are indicated by small circles). *a*, × 18,200; *b*, × 25,000; *c*, × 52,000.

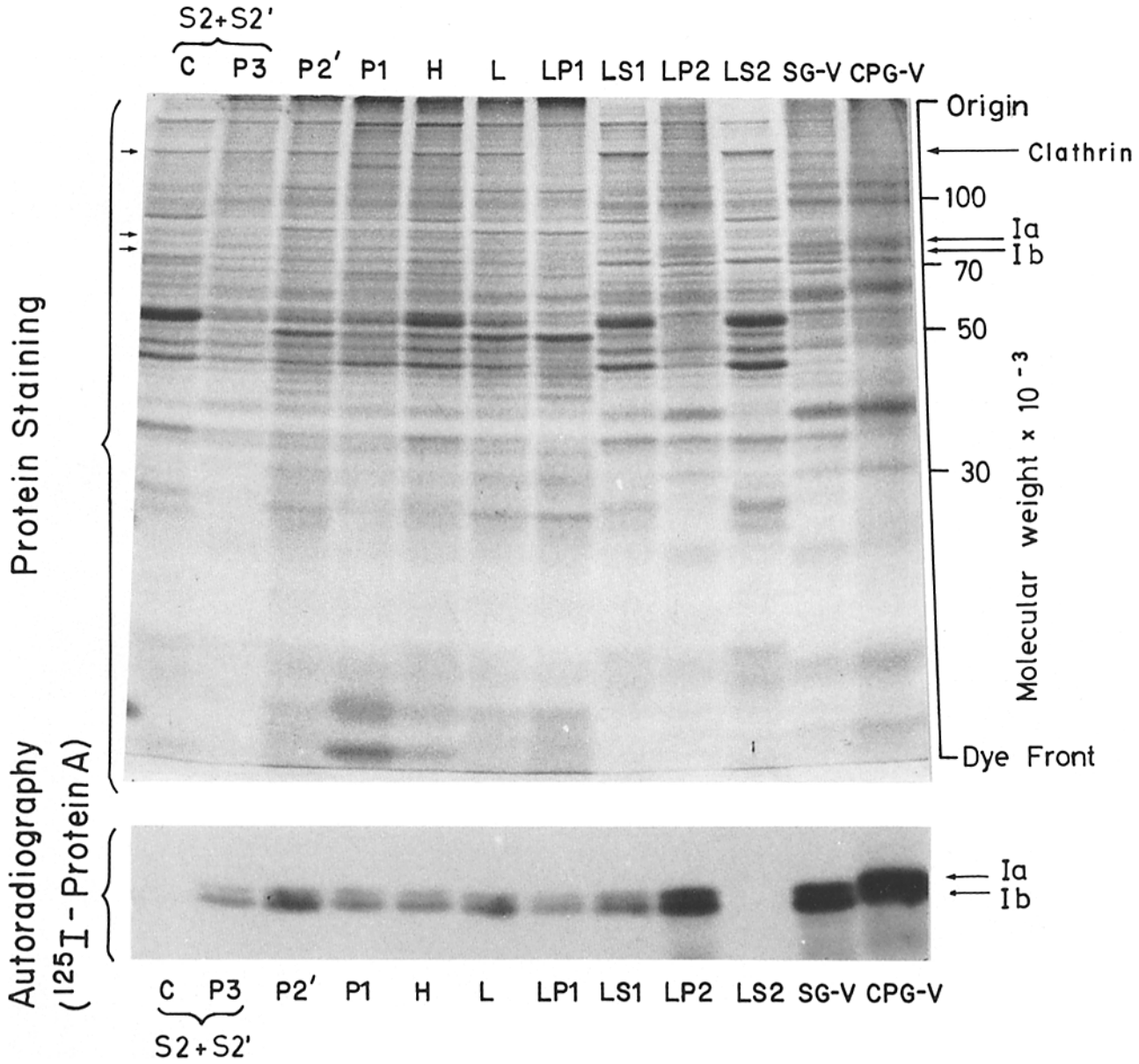


FIGURE 7 Protein staining profiles and synapsin I concentration of various subcellular fractions from rat cerebral cortex. Subcellular fractions were obtained and designated as described in Materials and Methods and in Fig. 1. Aliquots of fractions, containing equal amounts of protein, were subjected to SDS PAGE. *Top*: 10% gel used for protein staining with Coomassie Blue (25 μg of protein per lane). The positions of synapsins Ia and Ib and of clathrin are indicated by arrows. *Bottom*: 7.5% gel used for radioimmunolabeling of synapsins Ia and Ib with ^{125}I-protein A followed by autoradiography (8 μg of protein per lane). Only the portion of the autoradiogram indicating ^{125}I-protein A binding to the gel is shown. The slightly slower migration rate of synapsin I in the CPG-V fraction is attributable to the fact that the proteins in the fraction were precipitated with TCA before electrophoresis.

was observed (Fig. 9). The solubilization of synapsin I from the synaptic vesicle membrane by high concentrations of EGTA (32) or EDTA (Fig. 9) was probably also due to raising the ionic strength rather than to the chelation of divalent cations, as 1 mM EDTA or 1 mM EGTA had no effect (data not shown). Synapsins Ia and Ib were the only proteins present in the highly purified synaptic vesicle fraction that could be demonstrated to be solubilized by raising the ionic strength (Figs. 9 and 10). Exposure of synaptic vesicles to the nonionic detergent Triton X-100 in low ionic strength resulted in the solubilization of some polypeptides including the major polypeptide, p36, while several other polypeptides, including synapsin I, were still pelletable after a high-speed centrifugation (165,000 g_{av}, 60 min, 50Ti Rotor) (Fig. 10). The combination of Triton X-100 and moderate or high salt concentration produced a quantitative solubilization of synapsin I and of several other proteins.

Effect of Phosphorylation of Synapsin I on Its Association with Synaptic Vesicles

Ueda (30), using purified collagenase, reported that the interaction of synapsin I with particulate synaptic material is mediated by the collagenase-sensitive tail region of the Synapsin I molecule. We have confirmed these observations, both with synaptosomal lysates as well as with highly purified synaptic vesicles (data not shown). We have, therefore, examined the possibility that an alteration in the state of phosphorylation of the tail region of synapsin I might affect its association with synaptic vesicles. In the CPG-V fraction as

TABLE I

Radioimmunoassay of Synapsin I in Various Subcellular Fractions Obtained in the Course of Purification of Synaptic Vesicles from Rat Brain

Fraction	Synapsin I		Total protein		Synapsin I/ total protein
	Amount	Recovery	Amount	Recovery	
	μg	%	mg	%	%
H	5,460	100	1,400	100	0.39
P2′ = L	3,233	59	366	26	0.88
LP2	954	17	31	2.2	3.08
LS2	14	<1	64	4.6	0.02
SG-V	286	5.2	6.4	0.46	4.47
CPG-V	127	2.3	2.1	0.15	6.05

Subcellular fractions were obtained and are designated as described in Fig. 1 and in Materials and Methods. The amounts of synapsin I (synapsins Ia plus Ib) and of total protein are given for the whole volume of each fraction. From these numbers, the recovery of synapsin I and of total protein and the proportion of synapsin I as percent of total protein were calculated.

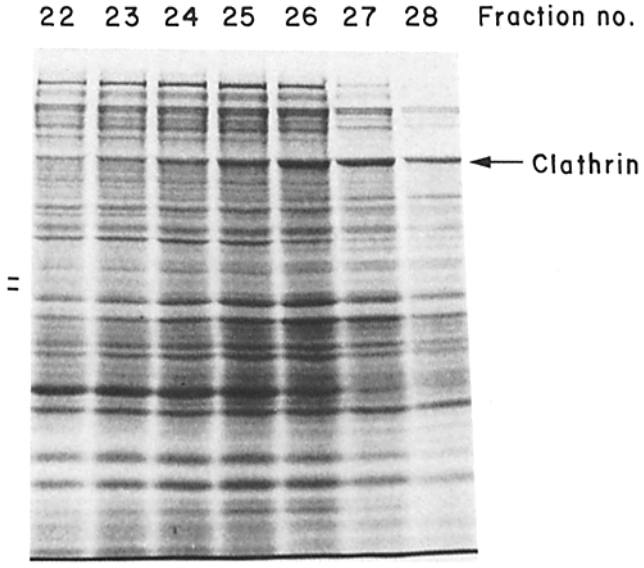


FIGURE 8 Analysis of lighter fractions of continuous sucrose density gradient illustrating presence of clathrin in fractions that contain soluble material. After continuous sucrose density gradient centrifugation of fraction LP2, 100 μl aliquots of fractions *22–28* representing the gradient from ~140 mM sucrose to the top were analyzed by SDS PAGE on a 7.5% gel followed by protein staining with Coomassie Blue. The position of clathrin, identified by comigration with purified clathrin standard (not shown), is indicated by an arrow. The peak of clathrin corresponds to the position in the gradient of the loaded fraction, which contained 40 mM sucrose, i.e., the position where soluble proteins would be expected to be found. The two marks on the left side of the gel indicate the positions where synapsins Ia and Ib migrated in fractions containing vesicles at 200–400 mM sucrose.

prepared,[1] the head region (31) of synapsin I could not be phosphorylated (15). This observation was turned to our advantage in the present investigation in that it allowed a study of the effect of phosphorylation specifically of the tail region on the association of synapsin I with the vesicle membrane.

Synapsin I from rat brain contains two sites of phosphorylation in the collagenase-sensitive tail region of the molecule (16). The phosphorylation of these two sites is stimulated by Ca ions in synaptosomal preparations (17). Moreover, the phosphorylation of the same two sites is catalyzed by a partially purified Ca/calmodulin-dependent protein kinase (19) when purified rat synapsin I is employed as substrate (16). The same two sites can also be phosphorylated by addition of supraphysiological concentrations of purified catalytic subunit (CS) of cAMP-dependent protein kinase (16). We have now demonstrated that those two sites in the tail region of synapsin I can be phosphorylated in the highly purified synaptic vesicle preparation by an endogenous Ca^{2+}-dependent protein kinase. This has enabled us to study the effect of the phosphorylation of the two sites on the association of synapsin I with synaptic vesicles. We carried out these studies using conditions of ionic strength under which synapsin I was partially membrane-associated and partially soluble. These conditions were chosen to permit measurements of shifts in either direction between membrane-associated and soluble synapsin I.

When highly purified synaptic vesicles (CPG-V) were incubated under basal phosphorylation conditions (i.e., in the absence of Ca), synapsin I was the major phosphoprotein seen in the particulate fraction and the only phosphoprotein seen in the supernatant (Fig. 11). When either Ca (in the presence of calmodulin), or supraphysiological concentrations of CS, were added to the reaction mixture, the state of phosphorylation of synapsin I was markedly stimulated. Under all conditions studied, the phosphorylation of synapsin I was found to occur exclusively in the tail region of the molecule (Fig. 11). The Ca-dependent stimulation of phosphorylation was presumably attributable to the activation of an endogenous Ca/calmodulin-dependent protein kinase (19). In support of this interpretation, we have found that trifluoperazine inhibited the Ca-stimulated phosphorylation of synapsin I (not shown).

The increased state of phosphorylation of the tail region of synapsin I seen in the presence of Ca was associated with an increase in the proportion of synapsin I recovered in the soluble form. This was observed both by protein staining and by radioimmunolabeling of synapsin I in SDS polyacrylamide gels (Fig. 12). The phosphorylation of the same portion of the tail region of synapsin I, by a supraphysiological concentration of exogenously added CS of cAMP-dependent protein kinase (see reference 16), also resulted in an increased proportion of synapsin I being recovered in the soluble form, supporting the interpretation that phosphorylation of the tail region of synapsin I decreases its affinity for synaptic vesicles (Fig. 12). The effect of CS was slightly weaker than that of Ca^{2+} on phosphorylation (Fig. 11) as well as on release of synapsin I (Fig. 12). The effect of the CS of cAMP-dependent protein kinase did not occur when heat-denatured rather than native enzyme was added or when the native enzyme was added together with protein kinase inhibitor (34) (not shown).

Further evidence that the increased solubilization of synapsin I resulted from the phosphorylation of the tail region of the molecule was provided by experiments in which ATP was omitted from the phosphorylation reaction mixture. The omission of ATP abolished the effect of Ca (Fig. 13) and of the CS of cAMP-dependent protein kinase (not shown) on the release of synapsin I from vesicles. Interestingly, even under basal conditions, the omission of ATP reduced the amount of soluble synapsin I (not shown), suggesting that the low level phosphorylation of the tail region of synapsin I seen under these conditions contributed to the observed partition of synapsin I between soluble and particulate material.

[1] The refractoriness of the head region of vesicle-associated synapsin I to phosphorylation depends on the procedure used for the purification of synaptic vesicles and is currently under investigation.

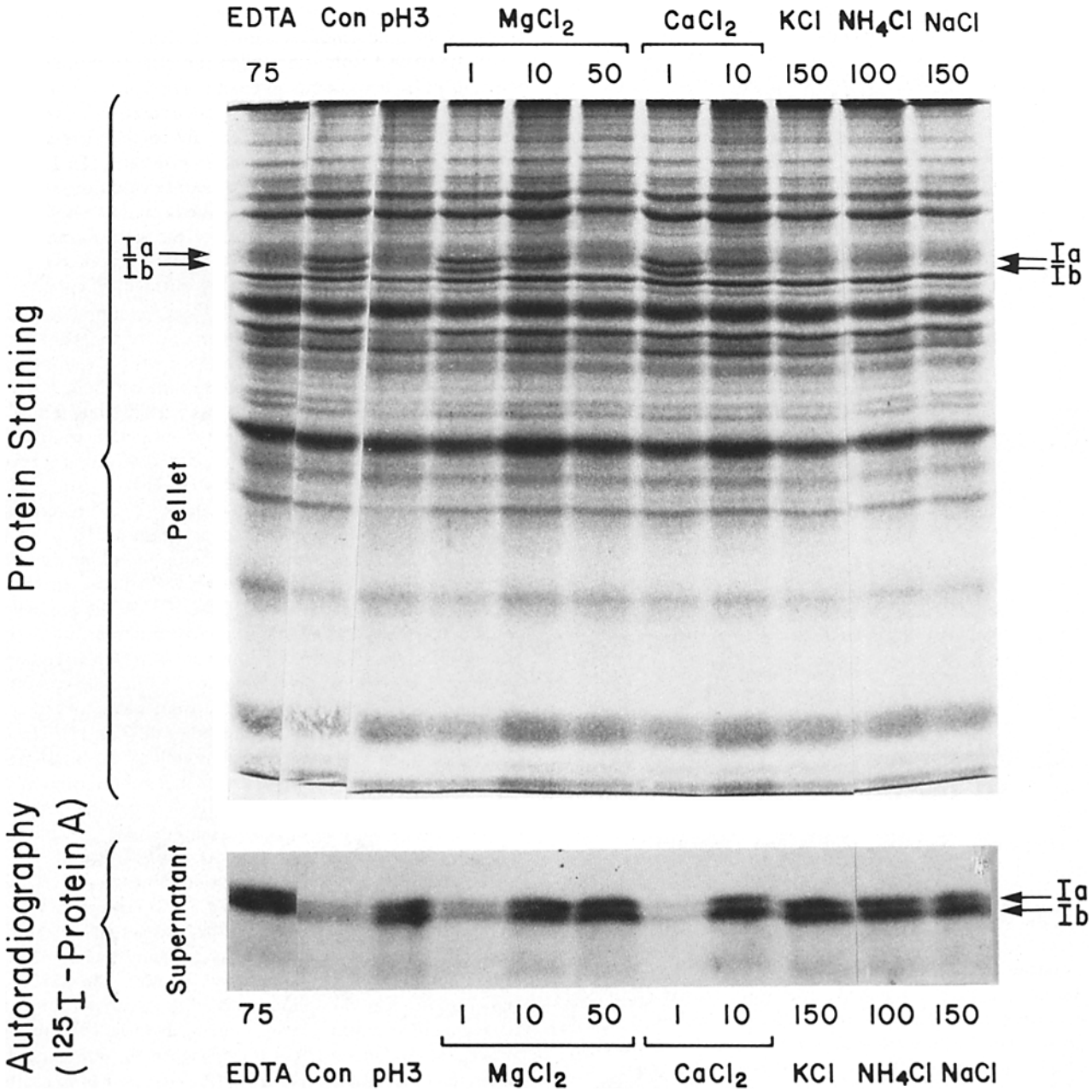


FIGURE 9 Effects of various salts on the solubilization of synapsin I from synaptic vesicles. Equal amounts (100 μg of protein) of highly purified synaptic vesicles (CPG-V) were incubated in a final volume of 1.3 ml in the absence (*Con*) or presence of the indicated final concentrations (mM) of $MgCl_2$, $CaCl_2$, KCl, NH_4Cl, NaCl, or EDTA (pH 7.0). For comparison, vesicles were incubated at pH 3, a condition known (L. J. DeGennaro and P. Greengard, manuscript in preparation) to cause nearly quantitative extraction of synapsins Ia and Ib from total particulate fractions of brain. After separation of soluble and particulate material by centrifugation, aliquots (150 μl) of supernatants and pellets were subjected to SDS PAGE. *Top*: 10% gel illustrating Coomassie Blue staining of the proteins present in the pellets. *Bottom*: 7.5% gel illustrating radioimmunolabeling of synapsins Ia and Ib, present in the supernatants, with ^{125}I-protein A, followed by autoradiography. Only the portion of the autoradiogram indicating ^{125}I-protein A binding to the gel is shown. The positions of synapsins Ia and Ib are indicated by arrows. (The small amount of synapsin I found in the control supernatant [*Con*] resulted from incomplete pelleting of vesicles.)

DISCUSSION

Co-purification of Synaptic Vesicles and of Synapsin I upon Subcellular Fractionation

We have purified synaptic vesicles from rat cerebral cortex to apparent morphological homogeneity. Considering the fact that the purification procedure was based on established procedures (24, 37), the use of morphological criteria to estimate synaptic vesicle purity appeared to be adequate for two reasons:

First, it seems unlikely that the vesicular profiles in the highly purified synaptic vesicle preparation represented organelles, other than synaptic vesicles, present in vivo. In the crude synaptosomal fraction, intrasynaptosomal vesicles were the only significant source of vesicles similar in size to those obtained in the highly purified synaptic vesicle fraction, as determined by electron microscopy of the crude synaptosomal fraction before the hypoosmotic release of intrasynaptosomal organelles (Fig. 2). The preparation of a well-washed crude synaptosomal fraction from mammalian brain by differential centrifugation before the release of synaptic vesicles represented an important selection step that was aimed at eliminating those small vesicular organelles (such as endoplasmic retic-

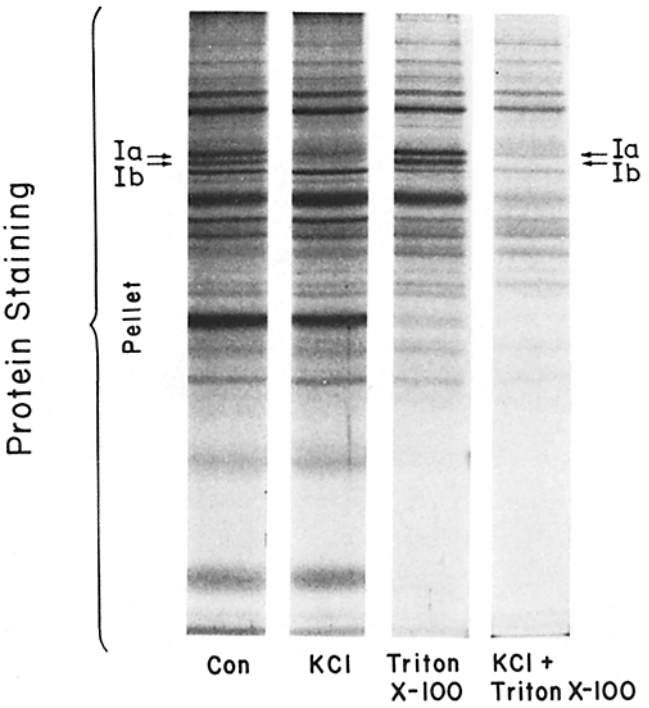


FIGURE 10 Comparison of effects of salt and nonionic detergent on the solubilization of proteins from synaptic vesicles. Equal amounts (100 μg of protein) of highly purified synaptic vesicles (*CPG-V*) were incubated in a final volume of 1.3 ml in 10 mM HEPES-NaOH (pH 7.2) in the absence (*Con*) or presence of 150 mM KCl, 1% (wt/vol) Triton X-100, or both. After separation of soluble particulate material by centrifugation, aliquots (150 μl) of pellets were subjected to SDS PAGE on a 10% gel followed by protein staining with Coomassie Blue. The positions of synapsins Ia and Ib are indicated by arrows.

ulum components or other small membrane fragments) which, by virtue of their size and density, would have behaved similarly to synaptic vesicles in further purification steps. In this respect, the approach used for the purification of vesicles from mammalian brain differed from that employed to purify synaptic vesicles from electric organs of *Torpedo* or *Narcine* (3, 24, 36). In the latter cases, synaptic vesicles were directly extracted from homogenates of whole tissue, and then purified.

Second, owing to the small size of the vesicular profiles in the highly purified synaptic vesicle preparation, it seems unlikely that they were formed by fragmentation and resealing of larger membranes.

We have used immunological methods to determine the enrichment for synapsin I at the various steps of the procedure used for the purification of synaptic vesicles. The immunological procedure seemed preferable to the enzymological approach taken previously (32) to quantitate synapsin I by phosphorylation. Using immunological methods, we found that the purification of synaptic vesicles was accompanied by an increase in the specific activity of synapsin I, resulting in an ~15-fold higher concentration of synapsin I in the highly purified synaptic vesicle preparation than in the initial homogenate (Table I). Synapsins Ia and Ib constituted ~6% of the total synaptic vesicle protein and were thus two of the major Coomassie Blue–staining bands of the synaptic vesicle preparation seen after SDS PAGE (Fig. 4; compare Fig. 1 in reference 32).

Since synapsin I appeared to be only weakly associated with the isolated synaptic vesicles, the amount of synapsin I recovered in the highly purified synaptic vesicle preparation could have represented an underestimation of the amount of synapsin I present on synaptic vesicles in vivo. However, the purification of synaptic vesicles was carried out in low ionic strength media, i.e., under conditions designed to minimize the loss of synapsin I from subcellular particles. In fact, virtually no synapsin I was detected, under our experimental conditions, in fractions containing only soluble material (Fig. 7 and Table I). Furthermore, a major "shedding" of synapsin I due to the high dilutions of the particulate material did not appear to occur, as indicated by densitometric scans of the protein staining profiles of fractions LP2, SG-V, CPG-V after SDS PAGE (Fig. 7). Thus, such scans revealed in the three fractions the

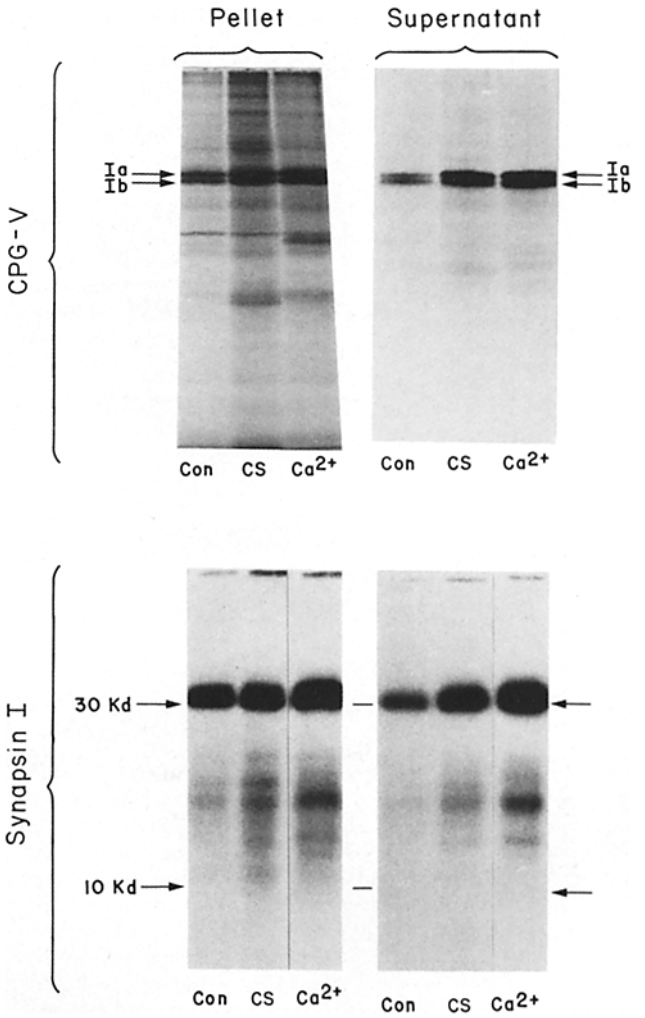


FIGURE 11 Autoradiograms showing (*top*) endogenous phosphorylation of synapsin I present in the highly purified synaptic vesicle preparation and (*bottom*) analysis of the sites of phosphorylation of synapsin I. Protein phosphorylation in the synaptic vesicle preparation (CPG-V) (50 μg of protein) was carried out in the presence of 5 μg/ml calmodulin with [γ-^{32}P]ATP, in the absence (*Con*) or presence of 75 nM purified catalytic subunit of cAMP-dependent protein kinase (*CS*) or 300 μM free calcium ions (Ca^{2+}). Upon termination of the phosphorylation reaction, soluble and particulate material was separated by centrifugation, and total supernatants and resuspended pellets (150 μl each) were subjected to SDS PAGE on 10% gels and autoradiography (upper two panels). The positions of synapsins Ia and Ib are indicated by arrows. The synapsin I (Ia plus Ib) bands were cut from the gel and subjected to limited proteolysis using *Staphylococcus aureus* V8 protease (5 μg/lane) in a 15% SDS polyacrylamide gel. The lower two panels show the autoradiograms of these proteolysis gels. As can be seen from these autoradiograms the 30-kdalton fragment, which is derived from the tail region of Synapsin I, was phosphorylated, whereas the 10-kdalton (*Kd*) region, which is derived from the head region of synapsin I, was not.

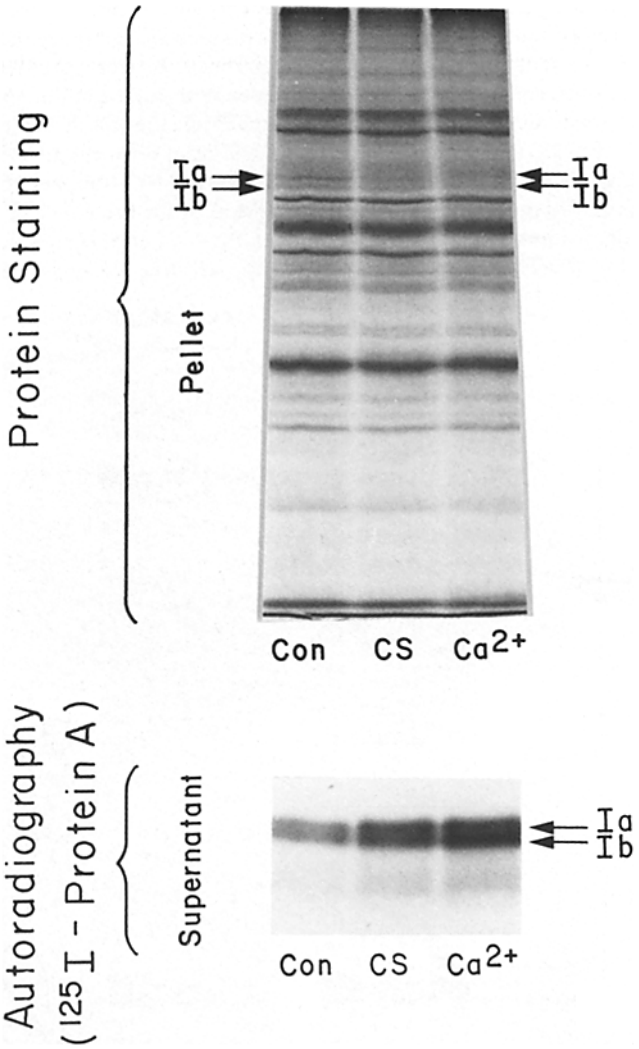


FIGURE 12 Effect of phosphorylation of synapsin I on its association with synaptic vesicles, analyzed by protein staining (*top*) and radioimmunolabeling (*bottom*). Equal amounts (50 μg of protein) of highly purified synaptic vesicles (CPG-V) were incubated in the presence of 5 μg/ml calmodulin under phosphorylating conditions, in the absence (*Con*) or presence of 75 nM purified catalytic subunit of cAMP-dependent protein kinase (*CS*) or 300 μM free calcium ions (Ca^{2+}). Samples to be used for radioimmunolabeling of synapsin I were incubated with nonradioactive ATP instead of [γ-^{32}P]ATP. Upon termination of the phosphorylation reaction, soluble and particulate material were separated by centrifugation, and total supernatants and resuspended pellets (150 μl each) were subjected to SDS PAGE. A 10% gel was used for Coomassie Blue staining of the proteins present in the pellet (*top*). (The autoradiogram of the same gel lanes is shown in Fig. 11, *top left*.) A 7.5% gel was used for radioimmunolabeling of synapsins Ia and Ib present in the supernatants with ^{125}I-protein A, followed by autoradiography (*bottom*). Only the synapsin I region of the autoradiogram is shown. The positions of synapsins Ia and Ib are indicated by arrows.

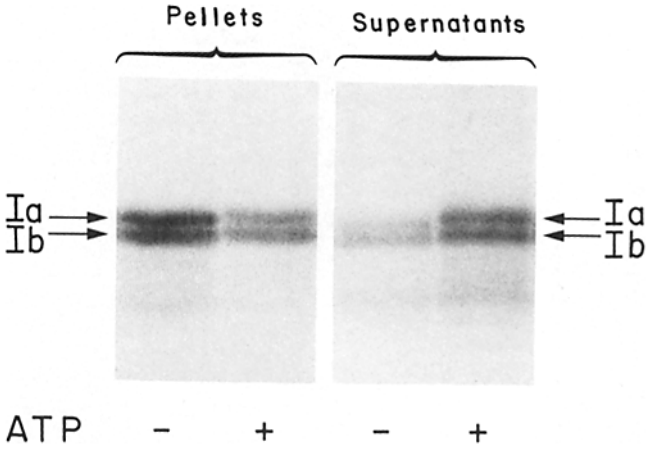


FIGURE 13 Autoradiogram illustrating that the Ca-dependent release of synapsin I from vesicles into the supernatant requires the presence of ATP. Equal amounts (50 μg of protein) of highly purified synaptic vesicles (CPG-V) were incubated in the presence of 300 μM free calcium ions and 5 μg/ml calmodulin as described for phosphorylating conditions, in the absence (−) or presence (+) of nonradioactive ATP. Upon termination of the phosphorylation reaction, soluble and particulate material were separated by centrifugation, and total supernatants and resuspended pellets (150 μl each) were subjected to SDS PAGE followed by radioimmunolabeling of synapsins Ia and Ib with ^{125}I-protein A and autoradiography. Only the synapsin I regions of the autoradiograms are shown. The positions of synapsins Ia and Ib are indicated by arrows. The slightly slower electrophoretic migration of synapsin I in the presence of ATP is characteristic of the phosphorylated form of synapsin I (unpublished observations).

existence of similar proportions of synapsin Ia, synapsin Ib, p61, and p36. The latter two proteins also appeared to be synaptic vesicle-specific. Since p61 and p36 could only be released from the synaptic vesicles by detergent treatment (Fig. 10), it is not likely that they underwent shedding during subcellular fractionation.

Nature and Specificity of the Interaction between Synapsin I and Synaptic Vesicles

The interaction of synapsin I with the cytoplasmic surface of synaptic vesicles is of an electrostatic nature and appears to involve the collagenase-sensitive domain of the molecule (30). An increase in the ionic strength of the medium promoted a solubilization of synapsin I from the synaptic vesicle membrane, irrespective of the salt used to provide the ionic strength (Fig. 9). Partial or complete solubilization of synapsin I from the synaptic vesicle membrane occurred at values of ionic strength comparable to the ionic strength range of the intracellular milieu (e.g., 150 mM KCl). (These observations may explain why synapsin I was not observed previously [9, 38] in synaptic vesicles purified in the presence of physiological or higher salt concentrations.) However, the solubilization of synapsin I from membranes at physiological ionic strength occurred, due to reasons inherent in such biochemical work, at unphysiological dilutions of subcellular organelles, i.e., at an enormously greater ratio of soluble to membranous material than that present in nerve terminals. In preliminary experiments using synapsin I at 1 mg/ml, a concentration comparable to that at which synapsin I may be present in synaptic terminals (compare Table I), 150 mM KCl did not prevent the reassociation of purified synapsin I with purified synaptic vesicles (data not shown). Nevertheless, the extensive dissociation of synapsin I from synaptic vesicles at physiological ionic strength justifies questioning the physiological significance of the association between synapsin I and synaptic vesicles that we found biochemically (this study) and immunocytochemically (7), after disruption of the nerve terminals in low ionic strength.

Several considerations (see also reference 7) suggest that the synapsin I–synaptic vesicle interaction occurs "in vivo" and is

of a specific nature: (*a*) Synapsin I is compartmentalized in the axon terminal region of nerve cells, as clearly indicated by immunocytochemical results in tissue sections at both the light microscopic and electron microscopic level (2, 6–8). This compartmentalization can only be explained by a specific, high-affinity interaction of synapsin I with particulate components of nerve endings. Synaptic vesicles, which are highly concentrated in nerve terminals, appear as attractive candidates to be the binding sites. Even when synapsin I was immunohistochemically detectable in regions of nerve cells other than the terminal (peripheral branches of sympathetic nerves, major nerve trunks at ligation sites) [6, 12], it was localized at regions where clusters of synaptic vesicles are known to be present. In addition, the immunohistochemical images were compatible with the interpretation that synapsin I is bound to the vesicles. (*b*) Radioimmunolabeling of synapsin I in SDS polyacrylamide gels of the various fractions obtained after continuous sucrose density gradient centrifugation, and after permeation chromatography on controlled-pore glass, indicated that synapsin I was present only in fractions containing synaptic vesicles (see for instance the clear co-migration of synapsin I and of synaptic vesicles in the second peak of the permeation chromatography step [Fig. 4]). This suggests that the recovery of synapsin I in synaptic vesicles was not due to an artifactual adsorption of synapsin I (a very basic protein) to all nerve terminal membranes upon hypoosmotic lysis of the terminal. The results obtained in the subcellular fractionation studies thus complement results obtained by immunoferritin in morphological studies at the ultrastructural level (7). (*c*) The association of synapsin I with the synaptic vesicle membrane seems to involve a specific domain of the synapsin I molecule located in the collagenase-sensitive tail region (31). The presence in synaptic vesicles of a Ca^{2+}-dependent protein kinase that is able to phosphorylate the domain of synapsin I involved in its interaction with synaptic vesicles further suggests that this interaction is physiologically relevant.

The apparently specific nature of the binding of synapsin I to synaptic vesicles is likely to involve a vesicle protein with a domain exposed at the cytoplasmic surface of the synaptic vesicle membrane. After nonionic detergent treatment, in low ionic strength, of the synaptic vesicle preparation, synapsin I was pelleted together with some other proteins (Fig. 10). This protein complex should contain the hypothetical synapsin I binding protein of the synaptic vesicles, since such binding is of an ionic nature and would not be expected to be affected by nonionic detergents.

The finding that synapsin I associated with synaptic vesicles could still be pelleted after nonionic detergent treatment might explain why synapsin I was previously found (32) in fractions enriched in postsynaptic densities (PSDs). These PSDs were isolated as pelleted material after Triton X-100 treatment of a synaptosomal membrane fraction. Since such synaptosomal membrane fractions still contained synaptic vesicles (see Fig. 11 of reference 5), one might expect to find, after Triton X-100 treatment, synapsin I in the pellet that contained PSDs and other Triton X-100–insoluble material.

The weak ionic association of synapsin I with synaptic vesicles is compatible with the possibility that this association may be subject to physiological regulation. Phosphorylation of the domain involved in the association of synapsin I with synaptic vesicles (the collagenase-sensitive domain) reduced the tendency of synapsin I to remain associated with the synaptic vesicle membrane in vitro (Figs. 11–13). In support of the possible physiological significance of this observation is the finding, mentioned above, that a Ca-activated protein kinase that was capable of phosphorylating synapsin I in its synaptic vesicle–binding domain was also present on the synaptic vesicles. It will be of interest to determine whether the effect of phosphorylation of synapsin I on its association with synaptic vesicles plays a role in regulation of vesicle function, and, in particular, in regulation of some aspect of the release process.

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