COATED-VESICLE SHELLS, PARTICLE/CHAIN MATERIAL, AND TUBULIN IN BRAIN SYNAPTOSOMES

An Electron Microscope and Biochemical Study

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ABSTRACT

Coated vesicles (CVs), plain synaptic vesicles (PSVs), and nonvesicular flocculent material were isolated from synaptosomes and examined with goniometry and high-resolution electron microscopy after either negative staining or various biochemical procedures. The flocculent material (i.e. the presynaptic matrix material except CV shells) is largely composed of particulate or elongated (chainlike) structures; some of this material (here referred to as particle/chain material) is attached to PSVs. The results obtained were: (a) the proteinaceous properties of the CV coat (also referred to as CV shell) and the particle/chain material were demonstrated with chymotrypsin; (b) the CV shell, studied with various negative-staining techniques, differs from the particle/chain material since it has no 3–4-nm globular subunits and reacts differently to alkaline pH; (c) the particle/chain material consists of aggregates of 3–4-nm globular subunits, four of which yield 8–10-nm fine particles; and these particles can be further aggregated into chains 8–10 nm wide and up to 30–60 nm long showing a "hollow" core; (d) vinblastine sulfate induced ringlike or helical crystalloid precipitates closely resembling the vinblastine-induced microtubule crystals reported in the literature, but vinblastine had no effect on either the CV shell material or the particle/chain material.

The coated vesicles (CVs) in the presynaptic bags are thought by some workers to play a role in the formation of the plain synaptic vesicles (PSVs) and in the membrane recycling phenomena related to transmitter release (Kanaseki and Kadota, 1969; Douglas et al., 1970; Heuser and Reese, 1973; Holtzman et al., 1973; Turner and Harris, 1974). Besides the CVs and PSVs, nonmembranous material (including dense projections) having the appearance neither of neurofilaments nor of microtubules is also present in the presynaptic bags (Gray and Willis, 1970; Jones and Brearley, 1972; Kadota and Kadota, 1973 a). Some of this material has an appearance similar to that of the coats (shells) of the coated vesicles and therefore has been interpreted as being the fragments of coats that were discarded when the PSVs were formed from the CVs (Kanaseki and Kadota, 1969). This hypothesis, however, is considered to be not entirely satisfactory (Gray, 1972, 1973; Ceccarelli et al., 1973). The picture becomes even more complicated when one considers the developing or degen-

erating presynaptic bags in which the presence of neurofilaments, microtubules, and (actin-like?) microfilaments in addition to the CVs and PSVs has often been reported (Gray and Guillery, 1966; Jones and Rockel, 1973; Gentschev and Sotelo, 1973; Potter and Hafner, 1974; Cuénod et al., 1972; Yamada et al., 1971; Chan and Goldman, 1973; Wessells, 1973).

Recent biochemical work has shown the presence of the microtubular protein, tubulin, in the (nonmembranous) matrix fractions derived from brain synaptosomes (Feit et al., 1971; Lagnado et al., 1971; Blitz and Fine, 1974). Other researchers have reported the isolation of the CVs and their empty shell fragments, and have described their fine structure and biochemical properties (Kadota and Kanaseki, 1969; Kanaseki and Kadota, 1969; Kadota and Kadota, 1973 a-c). Kadota and Kadota (1973 a, b) were able to distinguish the CV shell material from other presynaptic matrix materials, conveniently referred to as "flocculent material." This material was seen to contain particulate and elongated profiles which were either free in the matrix, attached to the PSVs (the ordinary synaptic vesicles), or formed into amorphous aggregates. In the present effort, these profiles were studied with high-resolution electron microscopy

and goniometry when 3-4-nm globular subunits could be seen composing this material. The latter is called particle/chain material, because the particles (8-10 nm across) usually consist of four subunits and the elongated profiles (up to 30-60 nm) are chain-like aggregates of these particles. We compared this particle/chain material with the coats of the CVs. In addition, vinblastine-induced microtubular crystals deriving from the matrix material were studied and shown to be distinct from both the coat (CV shell) material and the particle/chain material. Various negative staining techniques and biochemical treatments were employed in this study.

MATERIALS AND METHODS
Fractionation of Material within Presynaptic Bags

Fractions were prepared from synaptosomes of guinea pig whole brain as previously described (Kadota and Kadota, 1973 a; see Fig. 1). Vesicles and nonvesicular material were extracted from the presynaptic bags by suspending the precipitate of the crude washed synaptosomes (P_2) in cold water at 5.5 times the volume of the pellet. After the addition of 400 mM Tris-maleate (pH 6.5) and 1.0 M KCl at a final concentration of 10 mM,
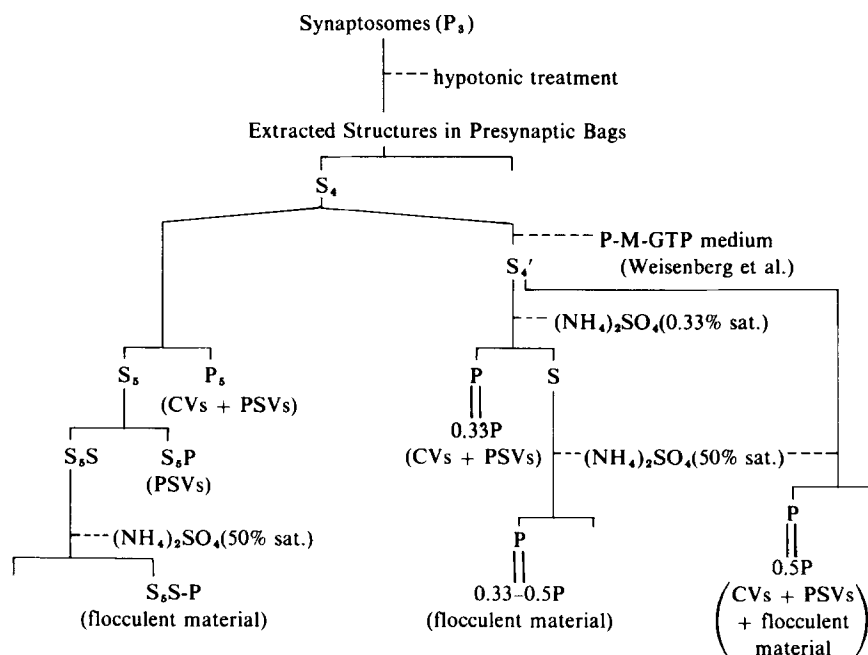

FIGURE 1   Scheme summarizing the steps in the fraction separation of CVs, PSVs, and flocculent material. See text for details.

the homogenized mixture was centrifuged at 20,000 g for 20 min with a 50 × 8 rotor in an MSE 18 centrifuge to remove the synaptic debris containing mitochondria, synaptosomal ghosts, and membrane fragments of uncertain origin. The resultant supernate (S_4) containing coated vesicles (CVs), plain synaptic vesicles (PSVs), and nonvesicular flocculent material was centrifuged at 55,000 g for 60 min with a 10 × 10 rotor in an MSE 65 centrifuge to precipitate a crude CV fraction (P_5). The supernate (S_5), containing PSVs and flocculent material, was centrifuged at 100,000 g for 60 min. The precipitate obtained was a PSV fraction (S_5P) consisting of PSVs and a small amount of flocculent material. The resultant supernate (S_5S), containing only the flocculent material, was brought to 50% saturation with ammonium sulfate by the addition of an equal part of a neutralized saturated solution of this salt, and then the mixture was centrifuged at 10,000 g for 20 min to give a precipitate of the flocculent material (S_5S-P). Unless otherwise stated, the pellets prepared as described above were suspended in an appropriate volume of 10 mM tris-maleate at pH 6.5.

For the experiments on microtubular protein(s), the vesicular and flocculent materials were delineated from the synaptosomes by modifying the method of Weisenberg et al. (1968) for isolating colchicine-binding brain protein. After the S_4 fraction had been obtained, the following substances were added: 10 mM sodium phosphate buffer (pH 6.5); 10 mM $MgCl_2$; 0.1 mM guanosine 5'-triphosphate (GTP); and 0.22 M sucrose. The mixture was centrifuged at 16,000 g for 30 min. The precipitate containing membrane fragments was discarded. The supernate (S_4' fraction) was brought to 33% saturation with ammonium sulfate by the addition of a half-part of a neutralized saturated solution of this salt, and then centrifuged at 10,000 g for 20 min to give a precipitate containing CVs and PSVs but little flocculent material (0.33 P fraction). The supernate was then brought to 50% saturation with ammonium sulfate by the addition of one-third of a part of the ammonium sulfate solution, and the precipitate was collected at 10,000 g for 20 min to give a precipitate containing the flocculent material (0.33–0.5 P fraction). In some cases, the S_4' fraction was brought to 50% saturation with ammonium sulfate and then centrifuged for 20 min to give a precipitate containing the CVs and PSVs together with the flocculent material (0.5 P fraction). Unless otherwise stated, these fractions were suspended in an appropriate volume of 10 mM sodium phosphate buffer (pH 6.5) to be treated with vinblastine sulfate. The protein in the fractions was determined according to the method of Lowry et al. (1951), using bovine serum albumin as standard.

Electron Microscopy

NEGATIVELY STAINED MATERIAL: Suspensions of the various fractions were dripped onto grids covered with a collodion film, and then several drops of 1% aqueous uranyl acetate (UA) at pH 4 were applied to the grids. The excess was drawn off with filter paper and the grids were air dried.

The other stains tested were an aqueous solution of 1% phosphotungstic acid (PTA) at pH 4, or brought to pH 7 with 1 N NaOH, and an aqueous solution of 2% ammonium molybdate (Am-Mo) adjusted to pH 7 with 1 N NaOH. Except where stated, the material used in this study was stained with UA.

SECTIONED MATERIAL: The vesicular fractions were spun down at 100,000 g for 60 min. The precipitates obtained were treated with buffered (aldehyde-osmium tetroxide in 0.1 M phosphate buffer, pH 7.3) or unbuffered (osmium tetroxide-aldehyde) fixatives as previously described (Kadota and Kadota, 1973 a). The pieces were block stained with 4% aqueous UA, dehydrated in an ethanol series, and embedded in Araldite. The serial sections from the bottom to the top of the pellets were double stained with UA (Watson, 1958) and lead citrate (Reynolds, 1963). The specimens were examined with a Philips 300 electron microscope. Most of the material was photographed with through-focus series from 100 nm underfocus, via near focus to 100 nm overfocus. Control observations for the negatively stained material were carried out in the same way, except that the collodion film was examined without a specimen on it. For the goniometric study, the material was examined with tilt angles varying from −45° to +45°. Magnifications were calibrated with a crossed diffraction grating replica.

Biochemical Treatments of the Presynaptic Material

PROTEASE AND ALKALINE PH TREATMENTS: The medium for the proteolysis of the CV shells and the particle/chain material contained the following: A P_5-fraction (1–2 mg protein/ml), chymotrypsin (10 mg/ml), 500 mM KCl, and 10 mM tris-maleate (pH 6.5) in a final volume of 2 ml. Incubation was continued at 30°C for 60 min, after which time the reaction mixture was cooled at 0°C. The control run was performed without adding the protease. Other proteases tested under the same conditions were trypsin, pronase, and Nagase.

The effects of alkaline pH on CV shells and the particle/chain material were studied by incubating the P_5-fraction (1–2 mg protein/ml) in 0.25 M bicarbonate buffer (pH 9.6) at 0°C for several hours. The control run was carried out as described above, except that here the medium was a 10 mM tris-maleate (pH 6.5). For further examination of the pH effects on the material, some specimens were incubated under the same conditions but in a 50 mM tris-maleate adjusted to various pHs, i.e. 9.5, 9.0, 8.5, 8.0, 7.5, 7.0, and 6.5.

VINBLASTINE SULFATE TREATMENT: By a modification of the procedure of Marantz and Shelanski (1970) or that of Feit et al. (1971), the material containing CVs, PSVs, or flocculent material was incubated in the following medium: a 0.33 P, 0.33–0.5 P, or 0.5 P fraction (1–2 mg protein/ml), 0.5 mM vinblastine sulfate,

a 10 mM sodium phosphate buffer (pH 6.5), 50 mM MgCl$_2$, and 0.1 mM GTP in a final volume of 1 ml. The reaction mixture was allowed to stand at 0°C for 10–60 min (preincubation). Incubation was continued at 37°C for 60 min. The control run was carried out without adding *Vinca* alkaloid.

For testing microtubule reassembly, the 0.33 P, 0.33–0.5 P, or 0.5 P fraction (1–2 mg protein/ml) was suspended in a buffer solution of 2-(N-morphino) ethanesulfonic acid (MES) at pH 6.5 and incubated in either the medium of Weisenberg (1972) or that of Shelanski et al. (1973).

Reagents

All reagents were of the highest grade of purity commercially available from the British Drug House or the Sigma Chemical Co., St. Louis, Mo., and the solutions were prepared with glass-redistilled water. The vinblastine sulfate was the product of Eli Lily & Co., Indianapolis, Ind.

RESULTS

CV-Shells and Particle-Chain Material Attached to PSVs Revealed with Various Negative Stains

Various negative stains at pH 4 or 7 were used on the P$_s$ fraction containing CVs and PSVs (Figs. 2–5). CVs, CV shells, or the particle/chain material attached to PSVs could be observed in all the methods tested. Acidic UA and PTA gave better contrast than that obtained with neutral pH. PTA, and Am-Mo. Of the two acidic stains, UA gave a crisper picture than PTA. After delineation with Am-Mo, the overall staining is good but lacks the contrast obtained with UA. The stain with Am-Mo was most suitable for fairly large organelles, e.g., mitochondria, whereas UA was more satisfactory for delineating the fine particles.

High Resolution and Goniometric Studies on the Particle/Chain Material Attached to PSVs

The particle chain material is seen more frequently attached to PSVs than lying freely in the background (approximately a 6:1 ratio). Sometimes, this material is seen attached to two or three PSVs bridging gaps between the vesicles. Occasionally, PSVs are seen apparently trapped within amorphous aggregates of the flocculent material containing the particle/chain material (Figs. 6–11).

As seen in the photographs mentioned above, the particle/chain material is found to lie in close relationship with the vesicle surface. A goniometric study showed it to lie within a distance of not more than 5 nm from the vesicle surface (Figs. 9, 10). In contrast to this, the CV shell was found to surround the vesicle with an intervening gap of 10–20 nm (Figs. 2 and 12).

Through-focus studies on the fine particles attached to PSVs clearly showed 3–4 nm globular subunits which are distinct from the granularity seen at under- or overfocus of 10 nm. These globular subunits are seen arrayed in a space of 8–10 nm thickness consisting of the fine particle (Fig. 9). In contrast, the framework of the CV shell is seen to be made up of linear units of 6–7 nm thickness (Figs. 2 and 12). The chains consist of globular subunits which are identical in size to those of the fine particles. Some chains are only 10–15 nm long, and so could equally well be described as fine particles. Many of the fine particles have "empty" centers, but the chains do not always show an internal "cavity." However, tilting the chain in the beam often reveals an apparently empty core, indicating the tubular nature of the particle/chain material (Figs. 6–10).

Effects of Chymotrypsin or Alkaline pH on the CV-Shell and the Particle-Chain Material

The inability to see any CVs, CV shells, or particle/chain material after chymotrypsin treatment indicates their proteinaceous property (Figs. 12, 13). The same observation was obtained in the sectioned material, except that the particle/chain material is not clearly seen in sections (Figs. 15, 16). Essentially similar findings were obtained when other proteases, trypsin, Pronase, or Nagase, were used.

After the alkaline pH (9.6) treatment, neither CVs nor CV shells were found, whereas the particle/chain material could be seen preserved on the PSVs or scattered in the background (Fig. 14). The disappearance of the CV and CV shell in the alkaline pH medium could also be seen in the sectioned material (Figs. 15 and 17). From other experiments with various pHs, ranging from 6.5 to 9.5, it was found that the CV and CV shell were preserved up to pH 7.5, were broken between 7.5 and 8.5, and had completely disappeared above pH 9.
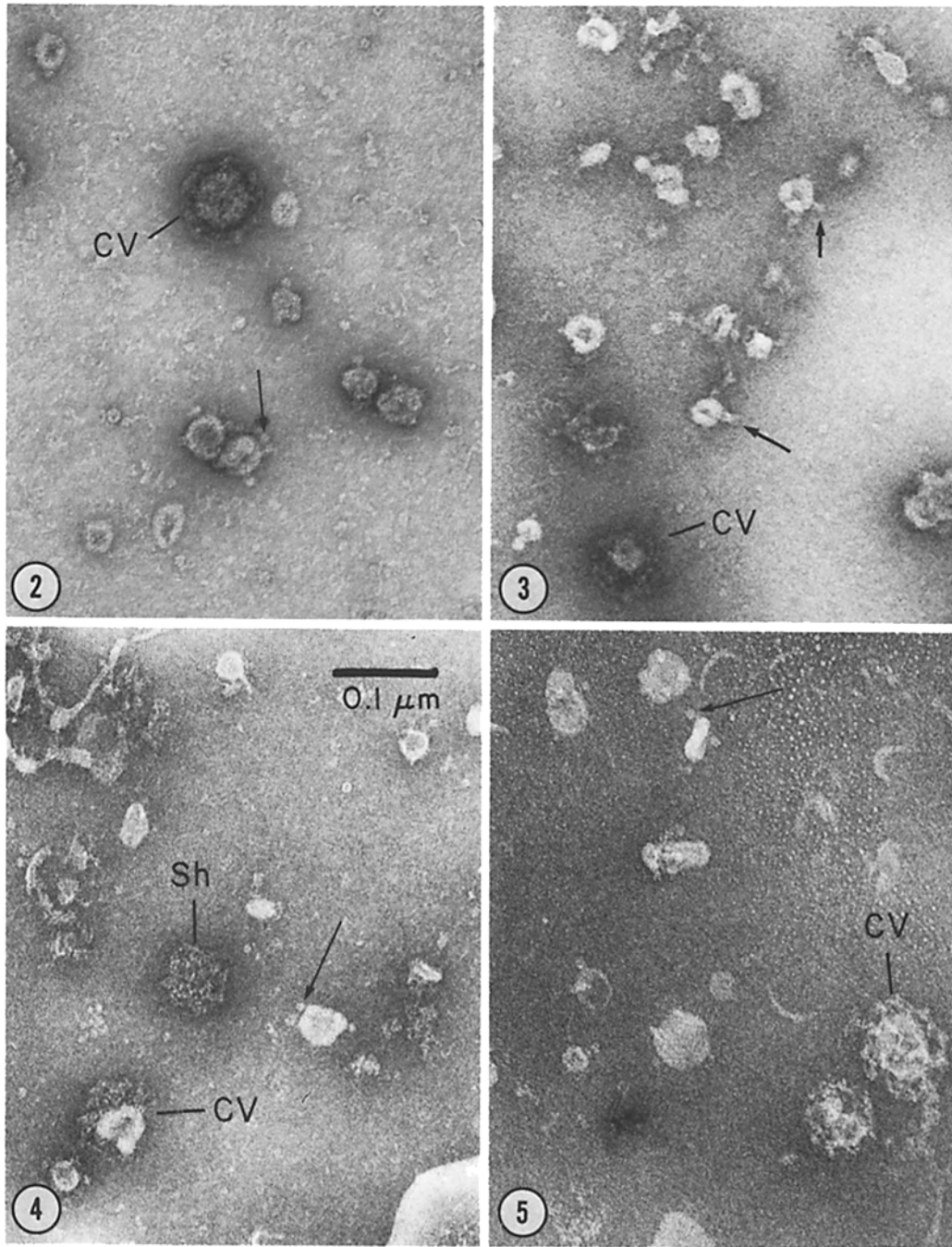
FIGURES 2, 3, 4, and 5 CVs, CV shells, and particle/chain material attached to PSVs revealed with various negative stains at pH 4 or 7. CV fraction (P_5). Fig. 2, UA at pH 4. Fig. 3, PTA at pH 4. Fig. 4, PTA at pH 7. Fig. 5, Am-Mo at pH 7. *Sh* = CV-shell. Arrows, particle/chain material attached to PSVs. Figs. 2–5, × 150,000.
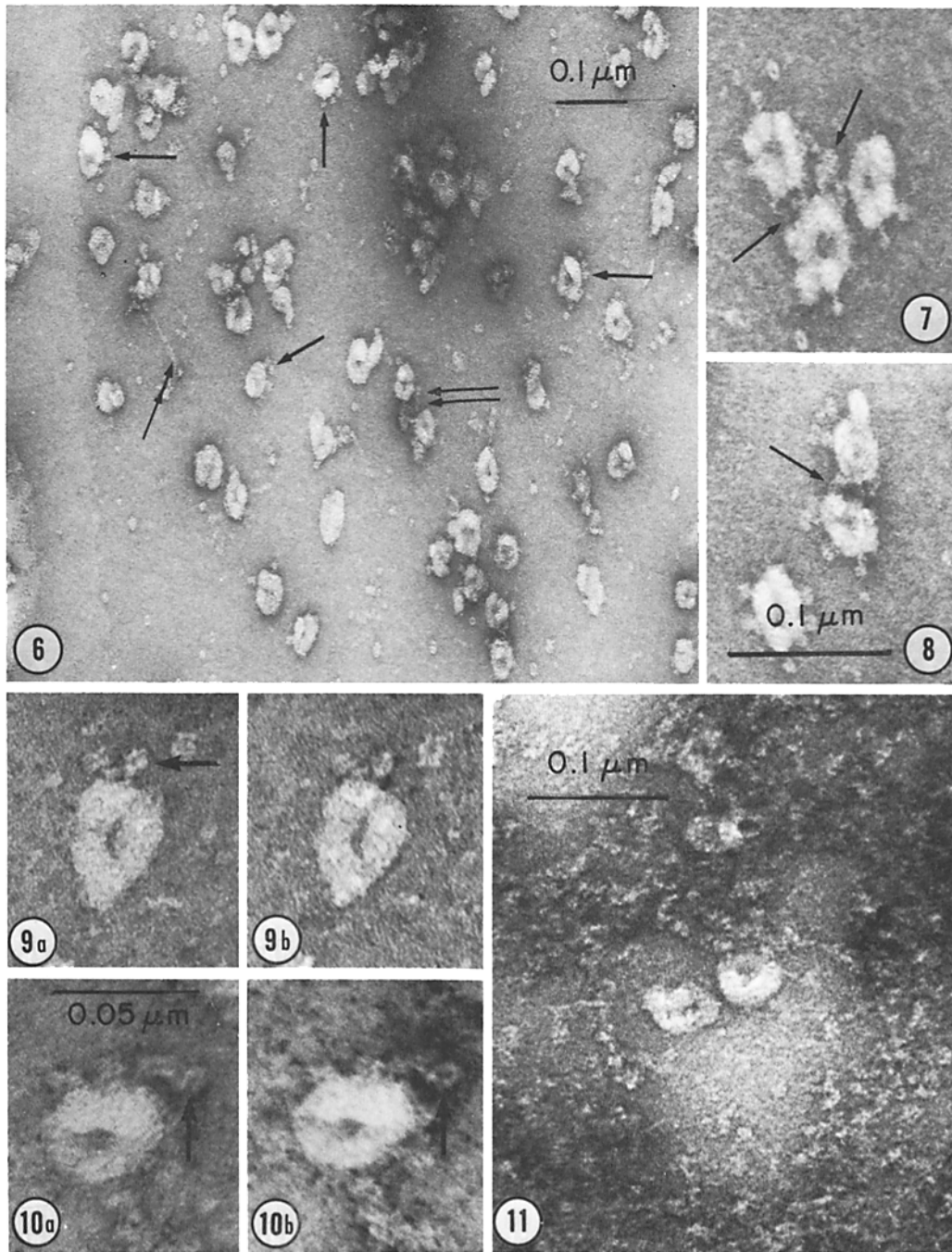
FIGURE 6 Particle/chain material attached to PSVs. Negatively stained. PSV fraction (S₆P). Arrows, fine particles attached to PSVs. Double arrow, a chain attached to PSVs. Double-headed arrow, a chain lying freely in the background. × 100,000.

FIGURES 7 and 8 Particle/chain material (arrows) attached to two or three PSVs. Negatively stained. PSV fraction. (S₈P). Figs. 7 and 8, × 230,000.

FIGURE 9 Goniometric examination of the fine particle attached to a PSV. Negatively stained. PSV fraction (S₈P). Tilt angle: a, 0°; b, −45°. Four globular subunits, each 3–4 nm thick, are seen composing a fine particle 8–10 nm in size. Arrow, one of the globular subunits. × 420,000.

FIGURE 10 Goniometric examination of the chain attached to a PSV. Negatively stained. PSV fraction (S₈P). Tilt angle: a, 0°; b, +15°. Globular subunits 3–4 nm in thickness are seen making up the chain. Arrow, an empty core of the chain. × 420,000.

FIGURE 11 PSVs trapped in amorphous aggregates of the flocculent material. Negatively stained. PSV fraction (S₈P). × 200,000.
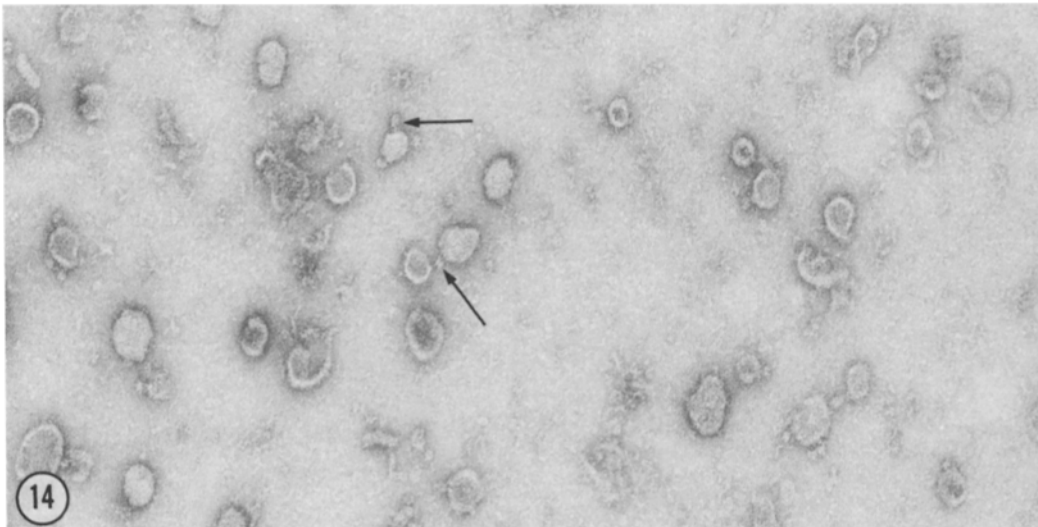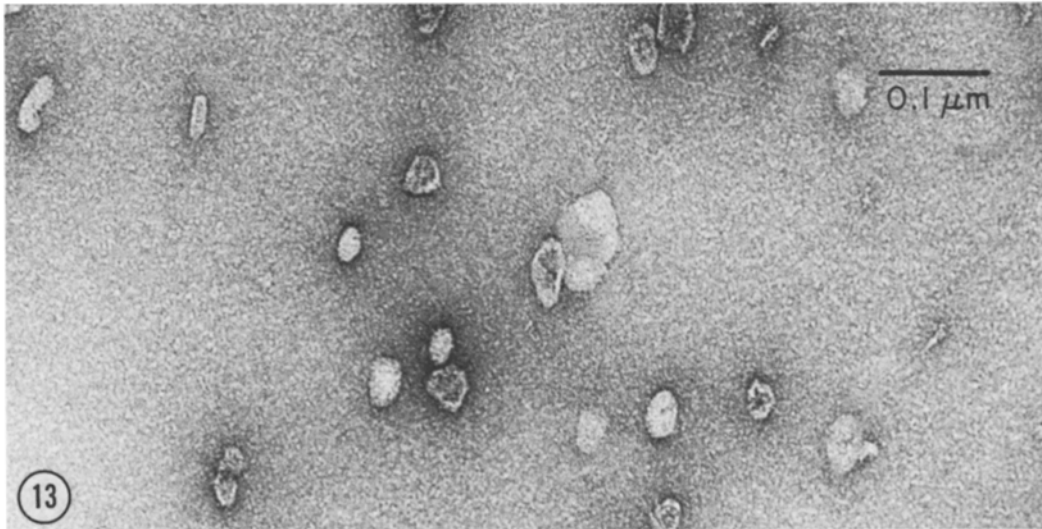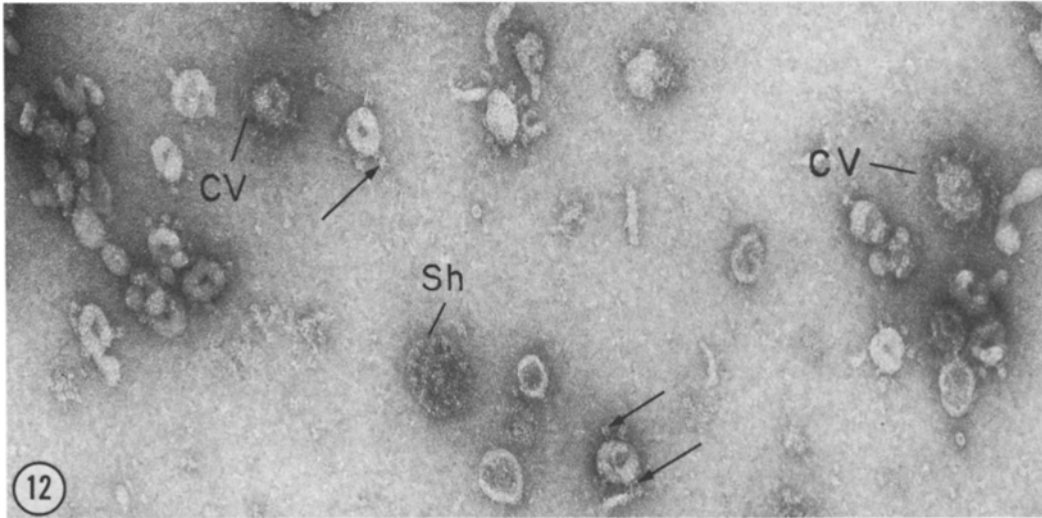
Vinblastine-Induced Crystalloid Structures in the Flocculent Material

After treatment with vinblastine sulfate, many ringlike crystalloid structures were seen scattered freely throughout the background in the 0.5 P fraction containing CVs and PSVs together with the flocculent material. The vesicular structures in the mixture were seen to be well preserved. The CV, CV shell, and particle/chain material attached to the PSVs remained intact after treatment. The vinblastine sulfate induced the crystalloid structures in the presynaptic matrix material fraction containing the flocculent material (0.33-0.5 P) but not in the vesicular fraction containing CVs and PSVs with little flocculent material (0.33 P). In addition to these ringlike crystalloids, helically arranged structures of variable length were sometimes found induced in the matrix material fraction (0.33-0.5 P) (Figs. 18-21).

The ringlike crystalloid has an outer diameter of 28-34 nm and consists of 23-26 globular subunits each about 4-4.5 nm in size. The helix has a width of 33-37 nm, and appears to consist of a pair of twisted strands. The distance between two turns of the helix is 22-37 nm (Figs. 21, 22). Some of the ringlike crystalloids are not completely circular but spiral in appearance (Fig. 18, S, and Fig. 22, S). When the material was examined in other areas there were some short helices which had spirally arranged rings at the ends. These ringlike or helical structures closely resemble the vinblastine-induced microtubular crystals shown in whole tissue materials or in isolated tubulin preparations (see Bensch and Malawista, 1969; Marantz and Shelanski, 1970; Nagayama, 1972; Shelanski, 1973; Dales et al., 1973; and Warfield and Bouck, 1974).

In earlier experiments, the induction of crystalloids by vinblastine was attempted in a medium containing 10 mM phosphate buffer (pH 6.5), 10

mM $MgCl_2$, and 0.5 mM vinblastine sulfate by the method of Feit et al. (1971) (P-M medium). Under these conditions, however, just a few crystalloid profiles were found to be induced in the nonvesicular flocculent material fraction (S_8S-P). Subsequently, the experimental procedure was modified into the present one which always contained GTP both in the isolation medium and in the incubation mixture. In addition, the present incubation medium (P-M-GTP medium) was slightly different from that of Marantz and Shelanski (1970) in that it contained $MgCl_2$ at a higher concentration (50 mM) than theirs did (10 mM); a magnesium content below 50 mM often gave unsatisfactory results. Another factor affecting crystalloid formation was the preincubation time at $0^{\circ}C$ and the incubation time at $37^{\circ}C$; long preincubation at $0^{\circ}C$ (30-60 min) led to predominant induction of the rings immediately after starting the incubation at $37^{\circ}C$, whereas short preincubation (1-5 min) followed by long incubation (30-60 min) led to the formation of helices of various lengths with few ringlike crystalloids.

No microtubules were found throughout the present vesicular and matrix fractions, except on rare occasions when short fragments with microtubular profiles, 18-20 nm in width, were detected as shown in Fig. 23. Microtubule assembly was attempted by incubating the presynaptic material in the medium of Weisenberg (1972) or that of Shelanski et al. (1973) but, under the present experimental conditions, no microtubules could be assembled although lamellated profiles were found induced in the flocculent material (Fig. 24). In contrast, the microtubule assembly was successful in the same medium of Weisenberg (1972) with a brain subcellular supernate prepared by high-speed centrifugation (1 h at 100,000 g) from a mitochondrial supernate i.e. the S_2 fraction described in our previous work (Kadota and Kadota, 1973 a) (Fig. 25).

FIGURE 12 Control material for chymotrypsin or alkaline pH treatment. Negatively stained. CV fraction (P_5). *Sh*, CV shell. Arrows, particle/chain material attached to PSVs. × 150,000.

FIGURE 13 The same material after chymotrypsin treatment. Negatively stained. CV fraction (P_5). CVs, CV shells, or particle/chain material are not seen. The background granules seen in this photograph taken at near focus are due to the added chymotrypsin molecules. × 150,000.

FIGURE 14 The same specimen after alkaline pH (9.6) treatment. Negatively stained. CV fraction (P_5). Neither the CV nor the CV shell is seen, whereas the particle/chain material is left intact. Arrows, the particle/chain material attached to PSVs. × 150,000.
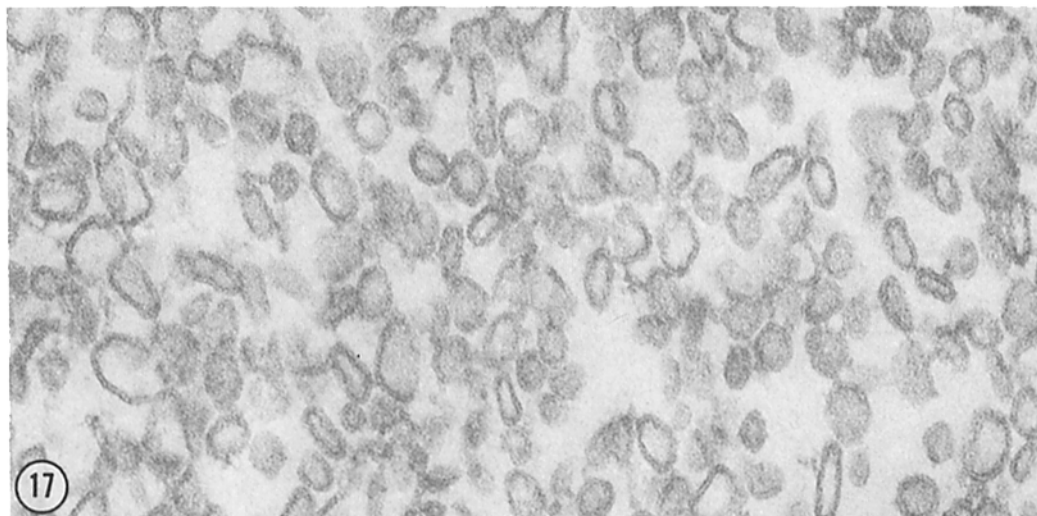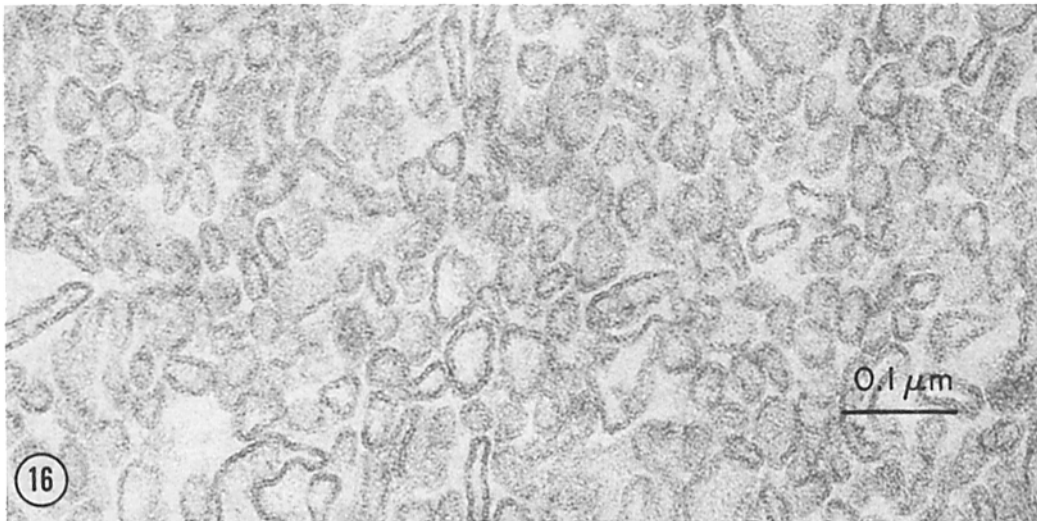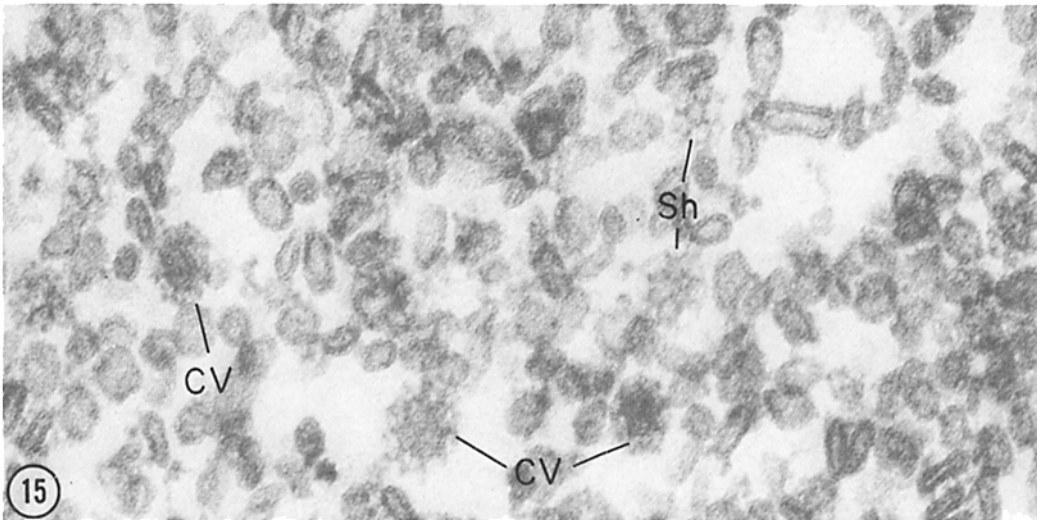
FIGURE 15 Control specimen for treatment with chymotrypsin or alkaline pH medium. Sectioned. CV fraction (P_6). CVs and CV shells are found, but the particle/chain material is not clearly seen. *Sh*, CV-shell. × 150,000.

FIGURE 16 The same material digested with chymotrypsin. Sectioned. CV fraction (P_3). Neither CV nor CV shell is seen. × 150,000.

FIGURE 17 The same specimen treated with alkaline pH (9.6) medium. Sectioned. CV fraction (P_5). Neither the CV nor the CV shell is found. × 150,000.
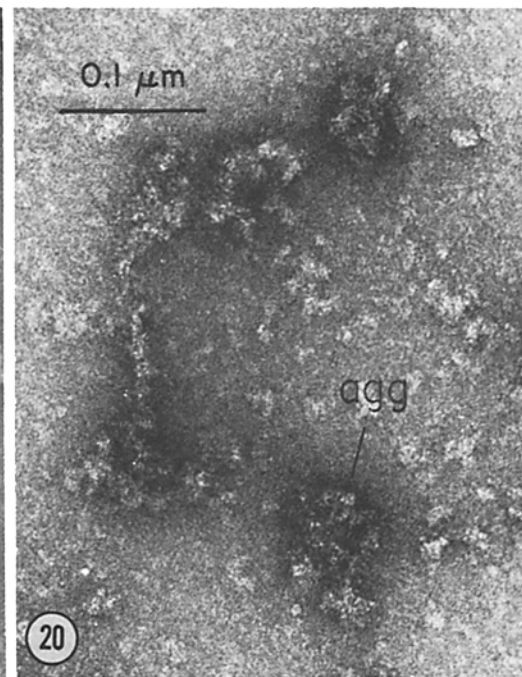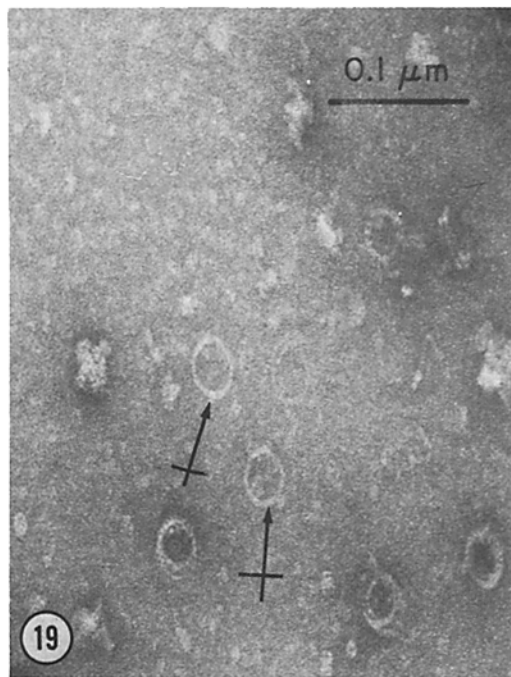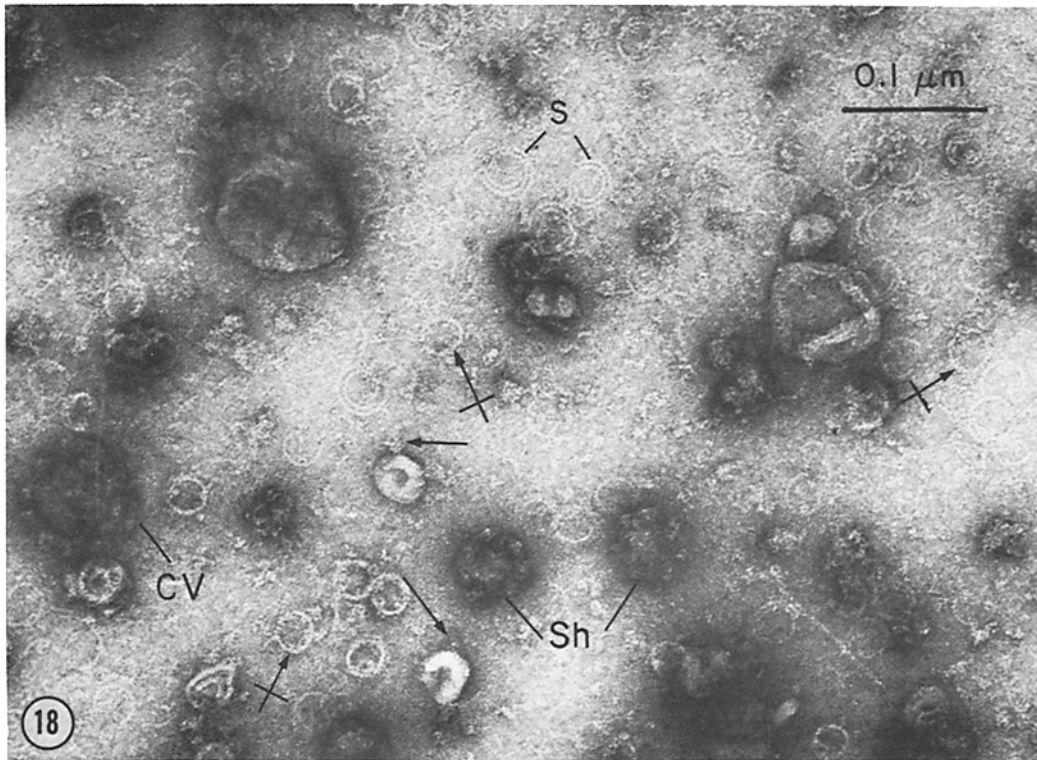
FIGURE 18   Ringlike crystalloid structures induced by vinblastine sulfate. Negatively stained. 0.5 P fraction. *Sh*, CV shell. Arrows, particle/chain material attached to PSVs. Cross-arrows, ringlike crystalloid structures. *S*, ringlike crystalloid structures, spirally arranged. CVs and CV shells are densely stained due to phosphate buffer present in the incubation medium. Contaminating membrane pieces, 150–200 nm in size, are seen in upper half corner. × 190,000.

FIGURE 19   Induction of the ringlike crystalloid structure by vinblastine sulfate in the presynaptic matrix material containing the flocculent material. Negatively stained. 0.33–0.5 P fraction. Cross-arrows, ringlike crystalloids. × 190,000.

FIGURE 20   The presynaptic matrix material before the vinblastine treatment. Negatively stained. 0.33–0.5 P fraction. The flocculent material of various sizes and shapes is seen scattered. Several amorphous aggregates of this material are found densely stained (*agg*) (cf. Fig. 11). × 190,000.

0.05 μm

0.05 μm

S

22

0.1 μm

23

0.05 μm

24

0.05 μm

25

618
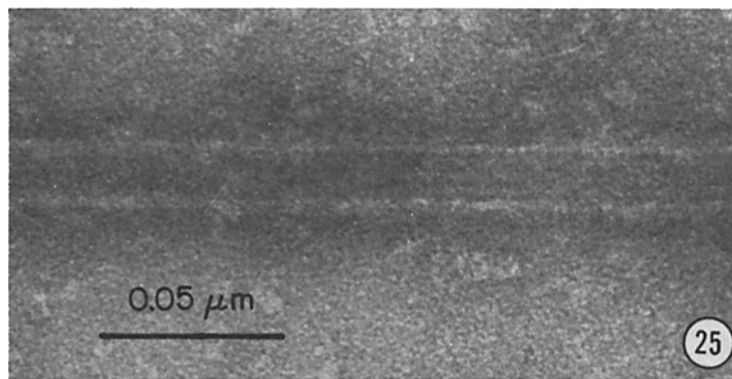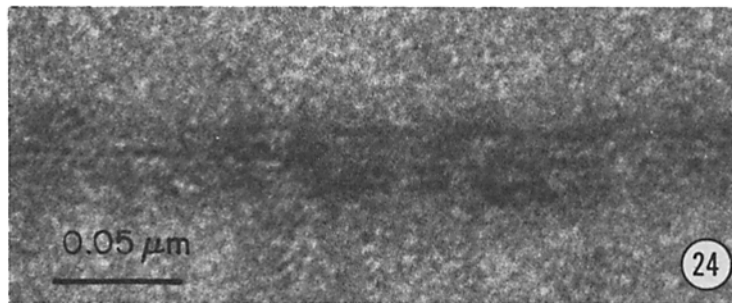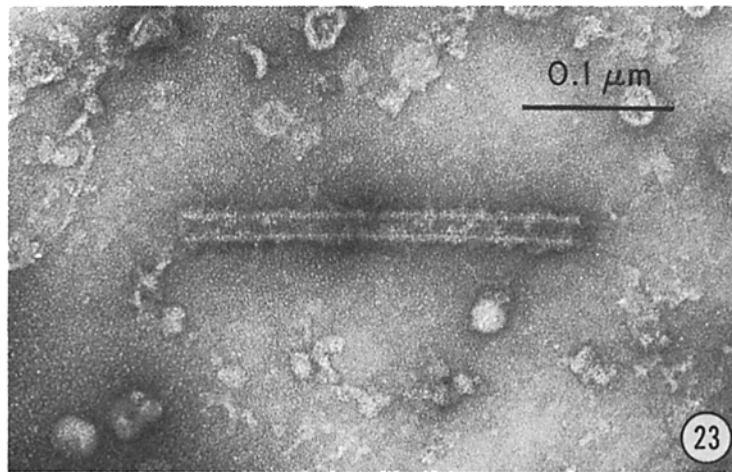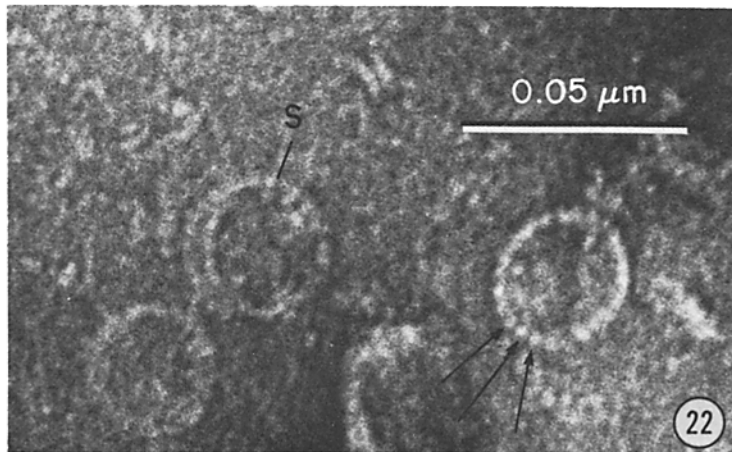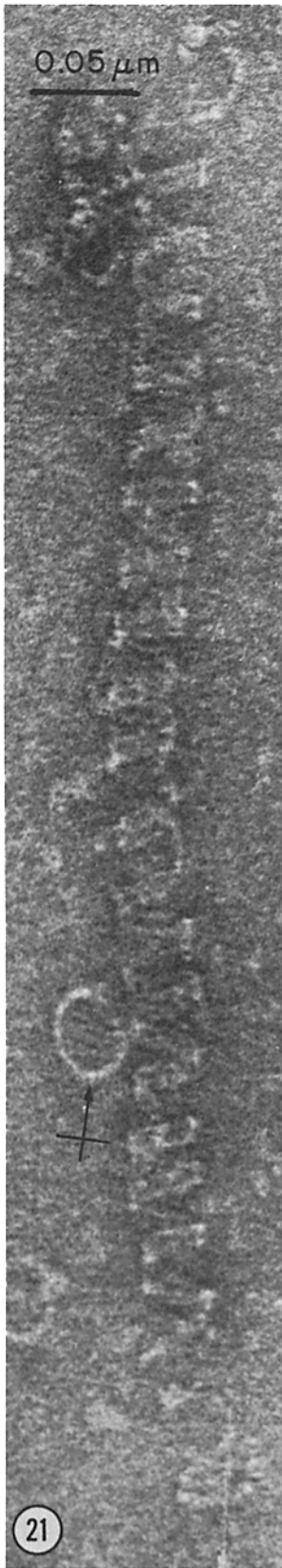
DISCUSSION

In synaptosome-derived material, we endeavoured to distinguish the coated-vesicle shell material from the particle/chain component of the flocculent material with the electron microscope, using negative staining and biochemical techniques, and we attempted to determine whether or not the shell or the particle/chain material was related to tubulin. All our results from the use of negative stains at neutral or acidic pH indicated that the proteinaceous material of the shell (coat) of the coated vesicle differed from the particle/chain material (also proteinaceous) in both dimensions and appearance. The particle/chain material (8–10 nm across) was seen to be composed of 3–4-nm globular subunits, whereas the shell material was found to be composed not of globular subuntis but of linear units of 6–7-nm thickness. After treatment with an alkaline pH medium, the shell material was completely destroyed whereas the particle/chain material was not; the latter could still be seen attached to plain synaptic vesicles or lying free in the surrounding medium. In addition, the particle/chain material seemed to coagulate the plain synaptic vesicles by bridging the gaps between them, whereas the coated-vesicle shells were not usually seen to form clots.

Attention was then concentrated on the arrangements of the globular subunits of the particle/chain component of the flocculent material, in particular, the material attached to the plain synaptic vesicles since this showed a better contrast than that in the amorphous aggregates. Through-focus high-resolution studies showed that the globular subunits appeared as particles consisting of clusters of four subunits or as chains which gave them a fibrillar appearance. Goniometry and through-focusing of the fibrillar arrangements confirmed our previous observations that the chains are tubule-like structures with a thickness of 8–10 nm (see Kadota and Kadota, 1973 a,b).

In our present synaptosome nonvesicular fraction, we were unable to identify positively microtubular subunits or microtubular fragments in the negatively stained or sectioned material, although the results of biochemical experiments have suggested the presence of tubulin in brain synaptosomes (Feit et al., 1971; Lagnado et al., 1971; Blitz and Fine, 1974); nor were we able to achieve a complete microtubule assembly from our subfraction by the method of Weisenberg (1972). Possibly, this failure was due to denaturation during our isolation and fractionation procedures or to a failure of the pre-existing nucleus to initiate the formation of regularly arrayed tubules (see Stephens, 1968; Borisy and Olmsted, 1972; Burns and Starling, 1974). However, vinblastine sulfate induced ringlike and helical crystalloid precipitates in the flocculent material but did not affect the coated-vesicle shell or the particle/chain material. These precipitates were morphologically indistinguishable from the sectioned and negatively stained vinblastine-induced paracrystals which exhibited high binding affinities for colchicine and the antibodies against tubulin (Bensch and Malawista, 1969; Marantz and Shelanski, 1970; Krishan and Hsu, 1971; Behnke and Forer, 1972; Nagayama, 1972; Tyson and Bulger, 1973; Dales et al., 1973; Warfield and Bouck, 1974). Marantz and Shelanski (1970) showed that the walls of the* ringlike crystalloids appeared to consist of special bodies, 3.7–4.3 nm across, which they suggested were identical to the microtubular subunits revealed by negative staining (Pease, 1963; Grim-

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FIGURE 21 A double helix induced by vinblastine sulfate in the presynaptic matrix material. Negatively stained. 0.33–0.5 P fraction containing the flocculent material. Cross-arrow, ringlike crystalloid. × 300,000.

FIGURE 22 Higher magnification of ringlike crystalloid structures induced by vinblastine sulfate in the presynaptic matrix material. Negatively stained. 0.33–0.5 P fraction. Arrows, globular subunits of the ringlike crystalloid. S, a ringlike crystalloid, spirally arranged. × 600,000.

FIGURE 23 A short fragment with microtubular profiles, found in the presynaptic material. Negatively stained. An intermediate fraction containing vesicles and matrix material (S₄). × 200,000.

FIGURE 24 A lamellated structure seen after incubating the presynaptic matrix material in the microtubule reassembly medium of Weisenberg (1972). Negatively stained. 0.33–0.5 P fraction. × 350,000.

FIGURE 25 Tubular profile found induced after incubating a brain subcellular supernatant fraction in the microtubule reassembly medium of Weisenberg (1972). Negatively stained. The fraction used here was obtained after centrifuging a mitochondrial supernate at 100,000 g for 1 h. × 500,000.

stone and Klug, 1966; Gall, 1966). In addition, a few workers have been successful in revealing microtubules in presynaptic bags in whole tissue (Smith et al.,1970; Gray, 1975). As judged from the works of the above authors and from our own results, it seems that tubulin may be present in our presynaptic matrix fraction (the flocculent material fraction) as a proteinaceous entity distinct from the coated-vesicle shell as well as from the particle/chain material. However, further characterization of the vinblastine-induced crystalloids is essential in order to determine whether they are specifically related to the protein tubulin. Moreover, the possibility has to be considered that a tubulin-like polypeptide present in the postsynaptic densities might contaminate the present fraction during the isolation procedures (see Wilson et al., 1970; Fine and Bray, 1971; Bunt, 1973; Banker et al., 1974).

Thus, from the present work with negative staining, we have been able to distinguish two varieties of proteinaceous material which are unaffected by vinblastine sulfate: (a) the shell material that forms the coat of the coated vesicle (or the shell fragments not associated with vesicles); and (b) the particle/chain material, consisting of globular subunits, which is seen either attached to the plain synaptic vesicle or lying free in the surrounding matrix. To determine whether this material is a polymorphic variety of tubulin or neurofilament subunits will require further investigation (see Wisniewski et al., 1968; Schochet et al., 1968; Wisniewski et al., 1971; Shelanski, 1973; Kadota and Kadota, 1973 a, b).

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