ISOLATION OF COATED VESICLES, PLAIN SYNAPTIC VESICLES, AND FLOCCULENT MATERIAL FROM A CRUDE SYNAPTOSONE FRACTION OF GUINEA PIG WHOLE BRAIN

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ABSTRACT

Two vesicular fractions and one nonvesicular fraction were prepared from crude synaptosomes by differential centrifugation and salting out with ammonium sulfate. Fraction 1 contained a mixture of coated vesicles, material thought to be derived from breakdown of the coats (shell fragments), and plain synaptic vesicles. Fraction 2 contained a mixture of plain synaptic vesicles and flocculent material. Fraction 3 contained flocculent material only. Fractions 1 and 3 were partially purified by passage through a Sephadex column. Fraction 3 contained no shell fragments but contained finer flocculent material which, it is suggested, is composed of unit particles either occurring singly or linked together into chainlike or amorphous aggregates. Each unit particle appears to have four subunits and is here referred to as a tetrasome. Tetrasomes sometimes appear to be attached to the surfaces of the plain synaptic vesicles. Also, it is possible that aggregates of tetrasomes form part of the structure of the presynaptic dense projections.

INTRODUCTION

The method of isolating synaptosomes and synaptic vesicles from brain tissue has been established with a differential centrifugation and sucrose density gradient method (Gray and Whittaker, 1960, 1962; De Robertis et al., 1962, 1963; Whittaker et al., 1964). The separation of synaptic vesicles by electrophoresis was introduced later (Kuriyama et al., 1968; Ryan et al., 1971). However, neither the sucrose density gradient nor electrophoresis methods were suitable for preparing a large quantity of synaptic vesicles for further physicochemical studies, and in addition the synaptic vesicles isolated by differential centrifugation usually contained many contaminating membrane fragments of uncertain origin. Furthermore, these methods proved unsuitable for isolation of components of the presynaptic cytoplasm other than synaptic vesicles.

Part of this difficulty was resolved (Kanaseki and Kadota, 1969) by an isolation method which gave a mixture of coated vesicles and so-called synaptic vesicles, using a combination of a differential centrifugation and Sephadex column chromatography. However, a pure fraction of each
of the two was required to clarify a possible relationship between these two types of vesicles (Bunt, 1969; Gray and Willis, 1970; Nagasawa et al., 1971). Moreover, the study of a nonvesicular fraction containing presynaptic cytoplasm would be useful in order to supplement the biochemical and functional properties of cytoplasmic and paramembranous structures in synapses (Kanaseki and Kadota, 1969; Gray and Pease, 1971; Feit et al., 1971).

The present article reports a new technique for the isolation of two synaptic vesicle fractions and one nonvesicular fraction to meet the above-mentioned requirements. The former two fractions were collected by differential centrifugation and the third was precipitated by addition of ammonium sulfate in order to separate these materials on a large scale. In order to eliminate some of their contents, the precipitated materials were passed through Sephadex columns. The main isolation medium consisted of 10 mM Triis-maleate (pH 6.5) and 10 mM KCl, to preserve the fine structures of the presynaptic materials. Sucrose in the isolation medium was kept as low as possible (below 60 mM) to avoid interference with negatively stained specimens, resulting in low contrast. The contents in the nonvesicular fraction were compared with thin sectioned material of tissue in order to obtain a better understanding of synaptic cytoplasmic constituents other than shell fragments of coated vesicles.

**MATERIALS AND METHODS**

**Primary Subcellular Fractions**

These fractions were prepared according to Gray and Whittaker (1960, 1962) and De Robertis et al. (1962). Isolated whole brains, each weighing 3.2 g on the average, were immersed in 0.32 M sucrose solution with a volume of 25 ml/brain, and ground in a Potter-Elvehjem homogenizer with a glass or Teflon pestle; the clearance was ca. 0.01 mm. The homogenization was continued for 1.5 min by moving the mortar up and down 7-8 times and rotating the pestle at approximately 1,500/min. The homogenate was centrifuged at 2,000 g for 10 min. The pellet (P1) was discarded. The supernatant (S1) was centrifuged at 10,000 g for 20 min to obtain a crude synaptosome fraction (P2) as a precipitate. The supernatant (S2 fraction) was discarded. The P2 pellets were resuspended in 0.32 M sucrose solution with the volume equivalent to that of the S2 fraction and then recentrifuged at 10,000 g for 20 min; the supernatant (S3) containing microsomes was discarded. The precipitate, fraction P3, was a washed, crude synaptosome fraction (P2), and this was the starting material for the isolation of various presynaptic structures.

**Electron Microscopy**

**Sectioned Materials**

The precipitates of fractionated materials were treated like whole tissues. The pellets were fixed by immersion in the following ways.

**Buffered aldehyde-osmium tetroxide:** The fixative, 6% glutaraldehyde with 0.1 M phosphate buffer (pH 7.3), was poured into a centrifugal tube with the adhering pellet. Several minutes later, the pellet was stripped off from the wall of the centrifugal tube with a stainless steel spatula and cut into two to four pieces. Fixation was continued for 2 h and the pieces were briefly rinsed with 0.1 M phosphate buffer (pH 7.3) and postfixed in 2% osmium tetroxide with the same buffer for 1 h (Karnovsky, 1965). Unless otherwise stated, all materials were treated with this buffered fixative method.

**Unbuffered osmium tetroxide-aldehyde:** Some of the specimens were fixed with unbuffered fixatives. The materials were immersed in 2% unbuffered osmium tetroxide for 1.5 h, briefly rinsed in distilled water, and postfixed in 12% unbuffered glutaraldehyde for 2-3 h (Kanaseki and Kadota, 1969).

For tissue sections, guinea pig brains were perfused for 30 min under sodium pentobarbital anesthesia (Palay et al., 1962) with glutaraldehyde-formaldehyde mixture (Karnovsky, 1965) in 0.1 M phosphate buffer at pH 7.3. Blocks were taken from the occipital cortex and kept in the same fixative for 2 h. Then the specimens were rinsed in 0.1 M phosphate buffer at pH 7.3 and transferred to 2% osmium tetroxide with the same buffer for 1 h. Brains of other guinea pigs were fixed by the unbuffered fixative method. The skulls were opened and specimens were dissected from the occipital cortex. The tissue blocks were then fixed by immersion with unbuffered fixative as described above.

After fixation, the pieces were washed with distilled water for 30-60 min to remove phosphate and to minimize the formation of uranyl phosphate,1 block stained with 4% uranyl acetate for 30 min (Farquhar and Palade, 1965), dehydrated in graded ethanol, and embedded in Epon 812 resin (Luft, 1961). These materials were cut with a Porter-Blum microtome (Ivan Sorvall, Inc., Newtown, Conn.), mounted on collodion-coated grids, and double stained with

1 Our observations show that this procedure causes little damage due to osmotic shock.
uranyl acetate (Watson, 1958)² and lead citrate (Reynolds, 1963).

**Negatively Stained Materials**

The negative staining method with uranyl acetate (Huxley, 1963) was applied to the various materials fractionated. The sample was dispersed, or diluted with an appropriate volume of 10 mM Tris-maleate (pH 6.5). The material was dropped on a collodion-coated grid, and then several drops of the 1-2% uranyl acetate solution were applied to the grid. The excess was drawn off with a filter paper and the grid was air dried.

**Preparation of Sephadex for Column Chromatography**

DEAE-Sephadex A-25 medium and Sephadex G-200 medium were suspended in water, using a magnetic stirrer. The suspension stood for 10 min and the supernatant was decanted. The procedure was repeated several times until the suspension became free of fine granules. The column material was then cycled through base and acid with 0.2 N sodium hydroxide and 0.4 N hydrochloric acid on a Buchner funnel and washed with water until the pH of the eluate reached that of the water. The hydrated material was equilibrated with 3 vol of 10 mM Tris-maleate buffer (pH 6.5) containing 10 mM KCl. The column material was stable for several months at 5°C.

All reagents were of the highest grade of purity commercially available and their solutions were prepared with deionized, glass-distilled water.

**RESULTS**

**Dispersion and Serial Separation of the Contents within Synaptosomes**

Electron microscopy showed that fraction P₃ consisted mainly of synaptosomes and free mitochondria (Fig. 1) (Gray and Whittaker, 1960, 1962). In addition, it contained myelin fragments and irregularly shaped membrane fragments. Fig. 2 a shows a synaptosome in P₃ at high magnification. Two types of vesicles are seen in it. The predominant type is consistent in size and shape with the so-called synaptic vesicles in tissue thin sections (De Robertis and Bennett, 1955; Palay, 1956). These vesicles shall be called “plain synaptic vesicles” (PSVs) in this article. The other type with an outer shell structure resembles the complex vesicles (Gray, 1961) and is identical with “coated vesicles” (CVs) found in a variety of tissues (Roth and Porter, 1964; Kanaseki and Kadota, 1969). Besides these vesicular structures, flocculent material (floc) is found within the synaptosome scattered irregularly throughout the axoplasm. Fig. 2 b shows a synaptosome in the same P₃ fraction but treated with the unbuffered fixative. CVs and PSVs are seen within the bag surrounded by its unit membrane. Cytoplasmic material other than vesicles forms complex networks with a rough appearance (Net).

The contents of the synaptosomes were dispersed and then separated into three fractions. The isolation steps are summarized in Fig. 3. The contents of the synaptosomes were extracted according to the method of De Robertis et al. (1963) and Whittaker et al. (1964), with modifications in the volume of water used, in pH, and in usage of KCl. The P₃ pellets were resuspended in ice-cold water 5.5 times the volume of the pellets: this volume of water was used to lower the sucrose concentration below 60 mM in the main isolation medium and it served also to minimize contaminating membrane fragments in the following fractions. The material was then dispersed with a glass homogenizer with a clearance of about 0.02 mm. The mortar was moved 3-4 times up and down and the pestle was rotated approximately at 300/min. The homogenization was completed after 20-30 s. By adding 1:40 vol of 400 mM Tris-maleate (pH 6.5), the pH of the dispersed material was rapidly adjusted to 6.5 to preserve presynaptic structures. Then, 1:100 vol of 1.0 M KCl solution was added to the material to facilitate the removal of mitochondria, partially disrupted synaptosomes, and membrane fragments as precipitate in the following centrifugation. The material was centrifuged at 20,000 g for 30 min; the tightly packed brownish pellet (P₄) was discarded and the milky white supernatant was collected as fraction S₄.

Fig. 4 shows the contents of fraction S₄. The two types of vesicle are seen. One type has an outer structure having a network-like appearance and resembles the vesicles reported by De Robertis et al. (1963). The outer diameter ranges from 700 to 1,000 Å and the shape is spheroid or ellipsoidal. This type of vesicle clearly corresponds with CVs in the sections of synaptosomes (Figs. 2 a and b). The other type of vesicle without the outer structure is the typical PSV. It has a mean diameter of 500 Å and its shape is spheroid or

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²Previous staining with uranyl acetate enhanced the second staining with lead citrate.
ellipsoid. This type of vesicle corresponds with PSVs in the sections of synaptosomes (Figs. 2a and b). Flocculent material irregular in shape and size can also be seen. Some of it appears stringlike (Fig. 4, double arrow) and some looks like meshwork (Fig. 4, asterisk). These materials are presumably derived from the flocculent material seen in the synaptic axoplasm (Fig. 2a, floe and Fig. 2b, Net). Other contents consisted of membrane fragments of irregular shape and of uncertain origin (Fig. 4, f).

Fraction S4 (Fig. 3) was centrifuged at 55,000 g for 60 min with a Beckman 30 R rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) to give the P5 precipitate, a mixture of CVs and PSVs. The resultant supernatant (S5) was centrifuged further at 80,000 g for 75 min with the same rotor to give the S5P precipitate, a mixture of vesicles equivalent to PSVs and the flocculent material, and the S5S supernatant containing the flocculent material.

Fraction P6 Containing CVs and PSVs

The P5 precipitate contained CVs and PSVs, but also many contaminating membrane fragments. A greater part of the latter were removed by DEAE-Sephadex column chromatography as follows. The P5 precipitate was resuspended with an addition of 10 mM Tris-maleate (pH 6.5) containing 10 mM KCl in a volume equivalent to 1–1.5 ml approximately per 10 g of the original wet weight of brain. The resuspension was placed on a column of DEAE-Sephadex (1.6 × 7 cm) that had been equilibrated with the same buffer containing 100 mM KCl. After washing with 14 ml of the same buffer containing 100 mM KCl, material was eluted by adding 14 ml of the same buffer containing 500 mM KCl. After one void volume (7 ml) of the same buffer passed through the column, a milky white eluate with a brownish tint ap-
peared. All this P₄ eluate was collected and its contents were spun down to give the P₅ precipitate.

The contents of the P₅ precipitate were checked with serial sections from bottom to top. Two layers were discriminated: the bottom layer, occupying four-fifths of the total volume of the P₅ precipitate, and the upper layer, occupying the remaining volume. These two layers were clearly separated from each other by an undulating line. The contents of the bottom layer were CVs, shell fragments, PSVs, and membrane fragments (Figs. 5–7). CVs and PSVs were found as a mixture at every position of the bottom layer of the P₅ precipitate. Occasionally, clumps of CVs, not contaminated by PSVs, occupied the lowest part of the bottom layer, but this was not a constant finding. There was a tendency for the larger vesicles to be located in the lowest part. The same tendency was seen for the membrane fragments 2,000–10,000 Å in size (f) (Fig. 6, the lowest part; Fig. 7, the middle part of the bottom layer). The empty shell structures of CVs (S₅) were found at every position, but the majority occurred at the top of the bottom layer. The contents of the upper layer of the P₅ precipitate were PSVs and flocculent material, the same contents as those of the S₅P precipitate to be described later in detail.

Several attempts were made to obtain separate fractions of CVs and PSVs. Modifications of centrifugal force to the P₅ eluate or fraction S₅ were not successful: CVs were always precipitated with PSVs as found in the P₅ bottom layer (Figs. 5–7). Other eluting conditions, e.g., passing the P₅ precipitate through DEAE-Sephadex at lower pH, did not yield good results: they easily caused damage to the shell structures of CVs and so it was best to keep the pH at 6.5. The more the CV shell was damaged, the more acid the medium was. Finally, the P₅ precipitate was resuspended in an appropriate volume of 10 mM Tris-maleate (pH 6.5) to give fraction P₆, a mixture of CVs and PSVs.

Fig. 5 shows the contents of the P₅ precipitate treated with the buffered fixative method. The various materials can be seen closely packed together. With application of the unbuffered fixative method, CVs, their shell fragments, and PSVs are demonstrated in high contrast and, although these appear more scattered, they do not seem to be changed in their shapes and sizes (Fig. 6). The membrane fragments of irregular shapes (Fig. 5) took on round profiles (Fig. 6). These findings suggest that the vague background seen after the buffered fixative method (Fig. 5) is due to the tangentially cut surfaces of contaminating membrane fragments.

As shown in Figs. 6 and 7, the vesicles contained within CVs have sizes ranging from 400 to 600 Å and their shells vary from 700 to 1,000 Å in size. The shell, either spheroid or ellipsoid, encloses the vesicle (which appears often very electron opaque) of the same shape, suggesting a close match of shape between the shell and the contained vesicle (CV₁ and CV₂). Other vesicles without coat structures are identified as PSVs from size, shape, and "empty" interior (Fig. 6, PSV). Some of the vesicles without coat structures look like the vesicles originally contained within CVs because of their dense appearance (Fig. 7, D).

Figs. 8–11 show the contents of fraction P₆ after negative staining. The material was prepared from the P₅ eluate and fraction P₆. CVs, their shell fragments, and PSVs are seen. Some PSVs bear fine particles ca. 90 Å in diameter (Figs. 8 and 9, arrows). In addition, strands of material are present (Fig. 8, crossed arrow). They are about 90 Å thick and several hundred angstroms long. These structures resemble the "fibrillar material" shown by Whittaker in his synaptic vesicle fraction (1966).

At high magnification the CV appears to consist of a superficial coat structure (shell) and an inner vesicular structure (contained vesicle) (Fig. 10). The skeleton of the shell is composed of a network of hexagons and pentagons whose sides have a chainlike appearance with a width of ca. 76 Å (Fig. 11). Sometimes, a part of the shell appears to have broken and the contained vesicle looks as if it was partly extruded (Fig. 9, CV₆).

It is of interest that the vesicle enclosed within the coat always takes up a central position in spite of the rigorous procedures of ultracentrifugation and column chromatography. Since the enclosed vesicle never appears eccentric, this could suggest that there are structures spanning the gap between the vesicle and its coat. So far, we have not been able to resolve these structures: this gap region always appears uniformly dark with the negative stain.

**Fraction S₅P Containing PSVs and Flocculent Material**

When the S₅P precipitate was examined in serial sections from bottom to top, it was found that PSVs are almost the exclusive occupant with very few
FIGURE 5  Contents of the lowest part of P₆ bottom layer. Buffered fixative method. A mixture of CVs, their shell fragments, and PSVs is seen vaguely in the background (CV, Sh, and PSV). Membrane fragments of irregular shape and size are scattered here and there (f). × 95,000.

FIGURE 6  Contents of the lowest part of the P₆ bottom layer. Unbuffered fixative method. CVs, their shell fragments, and PSVs are demonstrated in high contrast in an open intervesicular space (CV₁, CV₂, Sh, and PSV). CVs are spheroid (CV₁) or ellipsoid (CV₂). Membrane pieces are seen round in shape and high in contrast (f). × 95,000.

FIGURE 7  Contents of the middle part of the P₆ bottom layer. Unbuffered fixative method. CVs and their shell fragments are seen everywhere (CV, CV', and Sh). PSVs are those vesicles that have an empty interior and are without a shell structure (PSV). Occasionally, vesicles of dense appearances are seen (D). Contaminating membrane fragments (f) of round shape are fewer and smaller in this middle part than in the lowest part of the P₆ bottom layer (Fig. 6). × 95,000.

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contaminating membrane fragments (Fig. 12). CVs and shell fragments were found very rarely in the lower half of the precipitate. Fig. 14 shows the contents in the middle portion of the precipitate. Many PSVs, spheroid (PSV₁) or ellipsoid (PSV₂) in shape, are seen, and flocculent material, often complex in appearance, is scattered between the vesicles. More PSVs were located at the lower position of the precipitate, while more flocculent material was found at the higher position as shown in Fig. 13. Some of the intervesicular flocculent material appears in clumps, while other fragments lie close to neighboring PSVs as if adhering to them (Fig. 13, crossed arrow). With application of the unbuffered fixative method, these flocculent materials disappeared and material of rough appearance could be seen (Fig. 15, crossed arrow). No subunit pattern of CV shells was observed in these rough networks (Fig. 15). PSVs were seen in high contrast against the surrounding clear space.

A Millipore filtration procedure was used to attempt to remove the flocculent material from the
FIGURE 12 Contents of the middle part of the S5P precipitate. Buffered fixative method. PSVs are distributed homogeneously, and CVs or membrane pieces are scarcely found. Bar, 1 μm. X 30,000.

FIGURE 13 Contents of the upper part of the S5P precipitate, the part enriched in flocculent material of complex appearance. Buffered fixative method. Some fragments of the flocculent material appear to come into contact with PSVs (crossed arrow). X 152,000.

FIGURE 14 Contents of the middle part of the S5P precipitate. Buffered fixative method. An exclusive vesicular occupant is the PSV with an empty interior and having no shell structure. Its shape is spheroid (PSV1) or ellipsoid (PSV2). Flocculent material of complex appearance is clumped between PSVs. X 152,000.

FIGURE 15 Views of the contents of the middle part of the S5P precipitate after the unbuffered fixative method. PSVs are demonstrated in high contrast. The flocculent material is not seen, but fibrillar material of rough appearance (crossed arrow) is scattered in an open intervesicular space. X 152,000.
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Flocculent Material fraction (Figs. 13-15). The flocculent material of material shown in the thin sections of this S5P specimen corresponds to the fibrillar or granular fraction P6 containing 10 mM Tris-maleate (pH 6.5) to give fraction S5P, a mixture of PSVs and the flocculent material. Fig. 16 shows a negatively stained S5P fraction. Many PSVs are seen. Some PSVs bear fine particles that vary in number from one vesicle to another. At high magnification, this fine particle appears to be an open angular structure with sides about 90 Å long (Fig. 17). Flocculent material of irregular size and appearance is scattered in the background (Fig. 16, crossed arrow and double-headed arrow). Some flocculent material has an open angular structure about 90 Å wide (Fig. 16, double-headed arrow and inset). This appears to be the same as the fine particles attached to PSVs (Fig. 17). Other flocculent material appears as a stringlike structure about 90 Å thick and several hundred angstroms long (Fig. 16, crossed arrow); this linear structure corresponds to that found in fraction P5 (Fig. 8, crossed arrow). The other type of flocculent material was a meshwork-like structure consisting of units of irregular shape and size. Some meshwork-like structures appear to come into contact with PSVs or to project from them (Fig. 18), and others appear to have trapped PSVs within their intricately arranged networks (Fig. 19). Occasionally, the fine particles mentioned above are found in these meshworks (Figs. 18 and 19, arrows). From its relationships to PSVs, the above flocculent material in negatively stained specimens corresponds to the fibrillar or granular material shown in the thin sections of this S5P fraction (Figs. 13-15). The flocculent material of Figs. 18 and 19 was scarce in fraction P5.

Fraction S5S-P Containing Flocculent Material

The contents of the transparent S5S supernatant were precipitated by addition of an equal part of neutralized saturated ammonium sulfate solution and collected by centrifuging at 10,000 g for 20 min to give the S5S-P precipitate (see Fig. 3).

Examination of the contents of the S5S-P precipitate by serial sections showed aggregates of the flocculent material distributed throughout the layer (Fig. 20 a). No vesicles, CV shell fragments, or contaminating membrane pieces were observed. When the unbuffered fixative method was used on the S5S-P precipitate, much coarser clumps of material could be seen (Fig. 20 b), differing in appearance from the material seen with the buffered fixatives (Fig. 20 a); the former is, in appearance, a coarser aggregate of the latter. The coarse material does not contain CV shell fragments such as shown in fraction P6 (Figs. 6 and 7), but seems to correspond with the rough material demonstrated in fraction S5P (Fig. 15).

The S5S-P precipitate was passed through a Sephadex G-200 column to eliminate substances of low molecular weight (below 100,000) and to determine which substances could participate in the formation of the flocculent material shown in fraction S5S-P after negative staining. The material was prepared from S5S supernatant, fraction S5S-P, and S5S-P eluate from the Sephadex column. As shown in Fig. 21, flocculent material is seen in all parts with fragments of various size and shape. They could, however, be classified into (a) short tubelike structures (Figs. 22 a-d), and (c) meshwork-like structures (Fig. 22 e and Fig. 21, arrows). The fine particles were the smallest and are interpreted as consisting of four globules arranged in an open square with four linking rods: side length of the square, ca. 90 Å; diameter of globules, ca. 35 Å; length of linking rods, ca. 20 Å (Fig. 22 a). These fine particles appear to have the same size and appearance as those...
FIGURE 16 Contents of fraction S₄P after negative staining. PSVs are seen everywhere (PSV). Some of them bear fine particles ca. 90 Å in size (arrows). Some of the flocculent material in the background appears as a stringlike structure, ca. 90 Å wide and several hundred angstroms long (crossed arrow), and some as very small particles (doubled-headed arrow) having the same appearance and size as the fine particles borne by PSVs (arrows). Inset: a fine particle lying free in the background. X 120,000. Inset, X 800,000.

FIGURES 17a and 17b A fine particle borne by PSV at high magnification (arrow). Negatively stained. The particle has the appearance of an open quadrangle having a side length of ca. 90 Å. X 800,000.

FIGURES 18 and 19 Meshwork-like, flocculent material lying between PSVs in fraction S₄P. Negatively stained. The projections of the flocculent material appear to come into contact with neighboring PSVs (Fig. 18, crossed arrow), and appear to wrap around several of them to form a clot (Fig. 19). Minute particles equivalent to those as shown in Fig. 16 (inset) and Fig. 17 are seen built into these fragments of meshwork-like flocculent material (arrows). The bar in Fig. 19, 0.1 μm. Fig. 18, X 144,000; Fig. 19, X 260,000.
lying free or attached to PSVs in fractions P₆ and S₅₋₆ (Figs. 8 and 16, arrows; Fig. 17). These fine particles could not be derived from the CV shell structure. The fine particle is ca. 90 Å in its side length, but the chainlike units that make up the bars of the CV shell are ca. 76 Å in width (Fig. 22 a and Fig. 11). Some fine particles are adherent to PSVs, but CV shells and their fragments do not appear to be in direct contact with the vesicles (usually they encircle or lie at some distance from the vesicles) (Figs. 17–19 and 9–11). After allowing the P₆ fraction at pH 3–4 and 0°C to stand for 1 h, CV shells were destroyed completely without a relative increase in the number of fine particles. Furthermore, these fine particles appeared quite different from the small particles derived from mitochondria (Fernández-Morán, 1962; Kagawa and Racker, 1966). To check this, the various fractions were prepared equivalent to the brain fractions (Kagawa and Racker, 1966). To check this, the various fractions were prepared from guinea pig liver and kidney mitochondria. Numerous small particles were found in these fractions, and they were free or attached to membranous structures of irregular shape and size. These mitochondrial particles had a mean diameter of 110 Å and were spherical in shape and so, quite different from the present fine particles (Figs. 22 a and 17).

The short, tubelike structures (Figs. 22 b–d) appear to be built up of linear arrays as shown in the diagrams (Figs. 22 b–d). Some short tubes seem to have a spiral arrangement (Fig. 22 e). In this case the longitudinally arranged globules appear to be arrayed with some interglobular space: in other words, the tube (rectangular in cross section) appears to have openings in the side walls between the subunits. Occasionally, some short tubes are found to have a helically arranged, bifilar appearance (Fig. 22 d). More complex aggregates of these fine particles can also be seen (Fig. 21, arrows and Fig. 22 e).

The above flocculent material, seen after negative staining, no doubt corresponds with the material shown in the thin sections of the S₅₋₆ precipitate (Figs. 20 a and b) and appears to correspond with that in the S₅₋₆-P fraction (Figs. 16–19). The short tube in this S₅₋₆-P fraction looks like a shortened derivative of the stringlike structures shown in fractions P₆ and S₅₋₆ (Figs. 8 and 16, crossed arrows). Incidentally, these stringlike and short tubelike structures were found only in the brain fractions and were absent from fractions derived from kidney and liver mitochondria, as was the case for the fine particles mentioned above.

Flocculent Materials in Neocortical Nerve Endings

Fig. 23 shows a Gray's type 1 synapse in guinea pig occipital cortex perfused and fixed with the buffered fixative. In addition to CVs and PSVs,

Figure 20 Contents of S₅₋₆-P precipitate. (a) Buffered fixative method. Flocculent material of complex appearance is seen distributed. (b) Unbuffered fixative method. The flocculent material is not seen, but fibrillar material of rough appearance is scattered. × 100,000.

Figure 21 Contents of fraction S₅₋₆-P after negative staining. Flocculent materials of various sizes and appearance are scattered irregularly and abundantly. Arrows: meshwork-like flocculent material. × 180,000.

Figures 22 a–e Various structures in the flocculent material in fraction S₅₋₆-P at high magnification. Negatively stained. (a) A fine particle, the smallest of the flocculent material in this fraction. This fine particle looks like an open quadrangle consisting of four globules and having a side length of ca. 90 Å (a sketch is drawn in the right-hand column). (b) A short, tubelike structure. This appears to consist of many globules that are horizontally disposed at a distance of ca. 90 Å and are longitudinally piled up one upon the other. The diameter of each globule is ca. 35 Å, equivalent to that of the corner globule of the first particle (Fig. 22 a). A sketch of this structure is shown in the right-hand column. (c) A short, tubelike structure with a helical appearance. The globules of this structure appear to be piled up in winding array so as to give this tube a helical appearance as a whole. Globules appear to be piled up loosely and longitudinally. The sketch of this structure is shown in the right-hand column. (d) A structure looking like a doublet of the helically arranged, short, tubelike structure. (e) A smaller meshwork-like structure. This structure appears to contain many randomly arranged globules. Each globule seems to be equivalent to the corner globule of the fine particle in Fig. 22 a. An open quadrangle equivalent to the fine particle seems to be found built into this structure. The sketch of this structure is shown below. (a) × 570,000; (b), × 470,000; (c), × 570,000; (d), × 284,000; (e), × 500,000.
Flocculent material is seen scattered throughout the axoplasm. Fragments of this flocculent material are close together or apparently fused to each other. Pieces of the flocculent material sometime come close enough to neighboring PSVs so as to appear to be in direct contact with them. Presynaptic dense material (double-headed arrow) can be seen in close relation to PSVs that lie against the presynaptic membrane. From their location, these patches of dense material would seem to correspond to some part of Gray’s (1963) dense projections. Some protruding branches of the presynaptic dense material (double arrow) appear to be continuous with the distal fragments of the intervesicular flocculent material. The above flocculent material appears to correspond to that of synaptic vesicles lying against the presynaptic membrane (Fig. 24 a, double-headed arrow and Fig. 24 b). The above fibrillar material resembles that of fractions (Figs. 13 and 20 a).

Figs. 24 a and b show a synapse in guinea pig occipital cortex excised and treated with the unbuffered fixative method. Small clumps of coarser fibrillar material are found here and there between CVs and PSVs demonstrated in high contrast. This material of rough appearance would seem to be scattered also at the region where PSVs lie crowded against the presynaptic membrane (Fig. 24 a, double-headed arrow and Fig. 24 b). The above fibrillar material resembles that of fractions (Figs. 15 and 20 b).

**DISCUSSION**

In the present work we have attempted to isolate, by ultracentrifugation, salting out, and column chromatography, what we believe to be several distinct entities from the presynaptic bag. These we have studied by (a) thin sectioning using buffered and unbuffered fixatives, and (b) negative staining. One of our endeavors was to isolate a pure fraction of CVs and also of PSVs. We obtained a pure fraction of PSVs (S6P) but we could not separate a pure fraction of CVs. We always obtained a mixture of CVs, PSVs, and shell fragments (P6). Observations on our negatively stained material suggest that some of the vesicles contained within the CVs emerge from the shell. Therefore, it is thought possible that the P6 fraction initially contained mostly CVs, and that these vesicles had emerged artificially from the shell during the subsequent technique and had apparently become indistinguishable from the PSVs. This interpretation appears to be consistent with the hypothesis that PSVs are formed from those contained within the CVs (Bunt, 1969; Gray and Willis, 1970; Gray and Pease, 1971; Nagasawa et al., 1971).

However, we believe that it is possible that the PSVs may differ from those contained within the CVs, since we invariably obtained a separate and distinct fraction of PSVs (S5P) almost free of CVs and completely free of material suggesting CV origin, i.e., the shell fragments. Furthermore, we have evidence that the PSVs differ biochemically from the CVs and so may have a function distinct from that of the others. For example, the PSV fraction (S5P) had a higher acetylcholine content than the CV-rich fraction (P6), whereas choline was associated only with the latter (Kadota et al., 1970; Kamiya et al., 1972).

The flocculent material in the fractions S6P and S5S-P must be derived from synaptosomes as are CVs and PSVs, since the same material was obtained from the purified starting fraction P6 (prepared from either neocortex or whole brain by the preparation method of Gray and Whittaker [1962]) that appeared under the electron microscope to be essentially free of any material other than synaptosomes (with small numbers of free mitochondria and microsomes). Since the flocculent material did not consist of polygons (hexagons and pentagons) either in sections or after negative staining, we make the assumption that this material differs from the shell (coat) fragments of the CVs.

From our studies we can clearly demonstrate the

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**Figure 23** A Gray's type 1 synapse in occipital cortex of guinea pig. Perfused. Buffered fixative method. Both CVs and PSVs are seen. Intervesicular flocculent material material (crossed arrow) is clumped here and there. Double-headed arrow: presynaptic dense material. Double arrow: contacting point of the intervesicular flocculent material and presynaptic dense material. × 160,000.

**Figures 24 a and b** A synapse in occipital cortex of guinea pig. Immersed. Unbuffered fixative method. (a) Fragments of fibrillar material of rough appearance are scattered irregularly in the presynaptic axoplasm and appear to be found between synaptic vesicles lying against the presynaptic membrane (double-headed arrow). (b) A high magnification of the area shown by the double-headed arrow in Fig. 24 a. (a), × 52,000; (b), × 260,000.
substructure of the flocculent material only in the negatively stained specimens. The same material in sections after fixation by either buffered or unbuffered fixative has a fibrillar or coarser granular appearance (Figs. 20 a and b). The substructure is best seen in fraction S5-P after negative staining. It consists of units about 90 A wide. These lie either free or attached to the surface of synaptic vesicles. High resolution studies suggest that the individual unit consists of four subunits arranged in a quadrangle—possibly held together by bars (see Fig. 22 a). We here term these unit structures “tetrasomes” and are investigating further their fine structure, with high resolution goniometry.

In the same fraction as the unit tetrasomes, we have also seen chains and aggregates of the material. High resolution microscopy suggests that these are linear or amorphous aggregates of the unit tetrasomes (Figs. 22 b–e). The linear aggregates correspond to those seen scattered freely in the vesicular fractions (Figs. 8 and 16, crossed arrows), and the amorphous aggregates agree with those lying wrapped round some of the vesicles in the S5-P fraction.

Our observations suggest that the presynaptic axoplasm and the dense projections of Gray (1963) are at least in part composed of the flocculent material, which in sections appears different depending on whether buffered or unbuffered fixatives are used. This is the material that can be seen, when negatively stained, to be composed of the tetrasome units. From our other observations, coat fragments (hexagons and pentagons) also sometimes appeared to constitute part of the dense projections (see Gray and Willis, 1970). It is possible that the dense projections of Gray (1963) contain two sorts of material, i.e., the above-mentioned flocculent material and shell fragments of the CVs.

At present, we have no direct evidence which would enable us to relate the tetrasomes to some defined structures in whole tissue. However, the fact that the tetrasomes can exist in linear arrays reminiscent of neurofilaments or microtubules indicates that they might be derived from either of the latter two structures or from both (see Wuerker, 1970; Hunecus and Davison, 1970; Feit et al., 1971).

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