THE "VESICLE IN A BASKET"

A Morphological Study of the Coated Vesicle Isolated from the Nerve Endings of the Guinea Pig Brain, with Special Reference to the Mechanism of Membrane Movements

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ABSTRACT

Five points are discussed regarding the vesicular structure isolated by fractionation techniques from the brain and liver of the guinea pig. 1. One type of vesicle, fixed by OsO_4 and shown in thin sections, is identified with the coated vesicle that has been observed in many varieties of tissues. 2. The vesicle contained in a spherical polygonal "basketwork" shown by the negative-staining techniques is identical with the coated vesicle shown in sections. 3. Despite minute observation of this basketwork we could not confirm the existence of "hairlike projections" extending from the convex cytoplasmic surface of the vesicle. We are inclined to believe, therefore, that the hairlike projections are actually the superimposed visual images of the regular hexagons and pentagons of the network composing the basketwork. 4. We repeat the hypothesis originally advanced by Roth and Porter (1) that the "coating" of the coated vesicle plays a role in the mechanism of the infolding and fission of the membrane; we suggest that these events are caused by the transformation of the regular hexagons (of the coating) into regular pentagons. 5. Finally, we make a suggestion as to the nature of those vesicles which have on their surface subparticles which look like "elementary particles (2)."

INTRODUCTION

This article deals with the advanced hypothesis originally put forth by Roth and Porter (1) on the role of the "bristle coating" of "coated vesicles." They suggested that when a coated vesicle appears to be formed by a pinocytosis of the apical plasmalemma, the "bristles (spines)" extending radially from the convex cytoplasmic side of the vesicle may be associated with the mechanism of infolding and pinching off. They stated that "such a mechanism using specific molecular spacing of the spines, can be envisioned to account for a gradual repulsion of the unattached ends of the spines and the consequent infolding of the membrane producing the pit, and the cytoplasmic vesicle."

Although pinocytotic vesicles seem to occur arbitrarily anywhere on cell surfaces, the cell does nonetheless acquire its essential nutrition. Hence, many cytologists have concentrated their attention upon the outer surfaces of the membranes to look for some special adsorbing apparatus that would

function as the binding sites of the food from outside. The glycocalyx (3) is supposed to have such functional aspects, and many studies have speculated on the structure and function of the surface coat (4–6) in accord with this supposition. Roth and Porter (7) also suggested that it is more fruitful to consider the adsorbing nature of the bristle border with respect to specific materials than to speculate on the general role of bristles in the mechanism of membrane vesiculation. Bowers (8) suggested further that the coatings may act as the binding sites for protein.

It seems, however, that the binding of protein occurs first on the outer surface of the membrane, even though the bristles are located on the cytoplasmic side of the membrane. Since the coated vesicles are distinguished by the possession of hair-like material projecting from both sides of the membrane, a consideration of the intercalated unit membrane existing between the fuzzy substance and the bristle material may lead to the conclusion that the functional aspects of these two coatings differ. It is true that the coated vesicles have a specific capacity to incorporate protein in their lumina, but lipid, too, was recognized in the apical pits by Palay and Karlin (9). Nor could Cardell, Badenhausen, and Porter (10) entirely deny the existence of lipid contained in the pits on the apical cell surfaces. Considering these cases, one might argue that the existence of triglyceride in the pits results only from an overdose of lipid. But the fact that engulfing of lipid by apical pinocytosis did occur is important.

In this article, we suggest that the coatings of the coated vesicles are exclusively an apparatus to control the infolding and fissioning mechanism of the membrane. Marshall (11) and Brandt (12) observed that a stimulus such as positively charged protein given to amoeba sets off an active process of pinocytosis by itself. Also, Friend and Farquhar (13) reported that the formation of coated vesicles from Golgi membranes increases when the cell absorbs peroxidase-protein in the epithelium of the vas deferens. Considering these observations together with the fact that even triglyceride-lipid is sometimes contained in the apical pits, and recognizing that the surface coating of the membrane has strong affinity for adsorption of protein, one may conclude that the "bristle coats" associate exclusively with a mechanism to form apical pit-vesicles.

According to Beams and Kessel's (14) discussion of the Golgi apparatus, J. D. Jamieson (Ph.D. thesis) suggests that the bristle coat functions as a contractile device in the buckling mechanism of the membrane. Booij stated (15), however, that "one might suppose that a myosin-like contractile protein plays a role (in the mechanism of membrane movements), but the difficulty is that such a protein must be anchored to other proteins in a very *definite structure*." But, the bristles which appear in the negatively stained material that we are about to deal with *are* shown "in a very definite structure," so we agree with Jamieson in considering the myosin-like material as a "contractile device" in the case of the formation of coated vesicles.

Readers of this article may suspect that the fraction we isolated from nerve endings of the brain contained too many coated vesicles. But Novikoff (16) says that the GERL-complex in the neurons has many coated vesicles. So the coated vesicles in our fraction may at least be partially contaminated by vesicles from the perikaryon. We do not deal with this problem, but merely suggest that the "annular vesicles" or "ring vesicles" isolated by De Robertis et al. (17) from the nerve endings of the brain may be coated vesicles. Nor do we discuss the differences in appearance between the "rough surfaced vesicle" shown by negative-staining techniques by Mollenhauer et al. (18) and by Cunningham et al. (19) and the negatively stained coated vesicles in our material.

Finally, there is the question of vesicles which have "elementary particle"-like subparticles. We think that these vesicles may not really be contaminated mitochondrial cristae (20), but rather that these vesicles are synaptic vesicles as they appear in certain of their functional stages.

MATERIALS AND METHODS

Isolation of the Materials

The brains (mean weight 3.2 g) of adult guinea pigs were first homogenized with a glass homogenizer in 25 ml of 0.32 M sucrose for 1 min and then centrifuged at 2,000 *g* for 10 min. After centrifuging the supernatant obtained from this process at 10,000 *g* for 20 min, a precipitate consisting of crude nerve endings was obtained (P2 fraction). This P2 fraction was resuspended in 0.32 M sucrose, a volume equivalent to that of its supernatant, and centrifuged at 10,000 *g* for 20 min, which generated the P3 fraction in the sediment. The P3 fraction was hypotonically treated for 20–30 sec with glass-redistilled water in a glass homogenizer, so as to extract the synaptic vesicles

(17, 21). The pH of the homogenate was adjusted to 6.5 by using 400 mM Tris-maleate and 1.0 M KCl in volumes equal to 1/20 and 1/100, respectively, of the homogenate itself. After the pH-adjusted homogenate was further centrifuged at 20,000 *g* for 30 min, the precipitate (P4 fraction) was resuspended in the above-mentioned buffer. The supernatant was again sedimented under a gravity of 55,000 *g* for 60 min. The precipitates obtained (ca. 3 g, the same as the weight of the original wet tissue) were resuspended in 0.7 ml of 20 mM Tris-maleate buffer with pH 6.5 (P5 fraction). The aliquots of 1.6 ml of the P5 fraction were applied to a column of DEAE-Sephadex (Pharmacia, Uppsala, Sweden) measuring 1.6 × 3.5 cm, which had been equilibrated with the same buffer. After the aliquots entered the column, 7.0 ml of the above-mentioned buffer containing different concentrations of KCl ranging from 10 mM to 2.0 M was added stepwise to the column to elute the sample. The P5 fraction eluted by 500 mM KCl was centrifuged at 100,000 *g* for 60 min. The sediment obtained by this final process was named the P6 fraction.

A piece of guinea pig liver (9.6 g of wet tissue) was homogenized in 75 ml of 0.32 M sucrose. The subsequent processes used to fractionate this homogenate were identical with the processes by which the P6 fraction was obtained. This final fraction obtained from the liver will be called the "liver-fraction," from now on.

Electron Microscopy

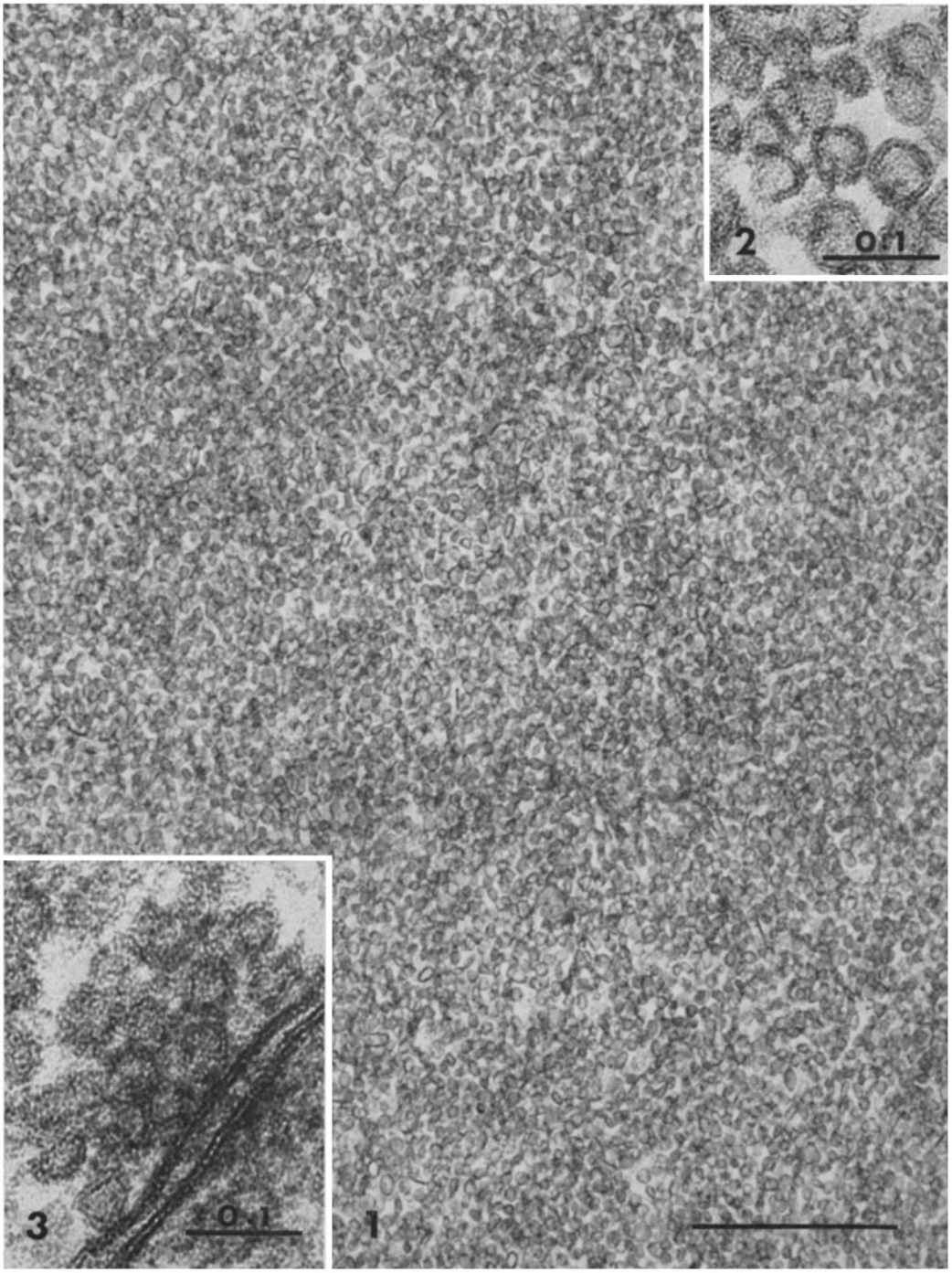
The pelleted P6 fraction and the liver-fraction were fixed with cold, unbuffered 4% OsO_4 for 2 hr. After brief rinsing with distilled water, these materials were each postfixed with 12% unbuffered glutaraldehyde (22) for another 2 hr (23; T. Kanaseki, Y. Uehara, M. Imaizumi, and K. Hama. In preparation.) and then treated with 2% uranyl acetate in a water solution (24, 25) for 2 hr before dehydration. The small pieces of the cerebrum of the guinea pig were fixed with cold 2% OsO_4 in 0.1 M phosphate buffer (26) and in 0.1 M *s*-collidine buffer (27), pH 7.3, for 2 hr. These same processes were also used on small pieces of the gastric mucosa of a mouse. After brief rinsing with distilled water, all these tissues were postfixed with 12% unbuffered glutaraldehyde for 2 hr and then treated with 2% uranyl acetate for 30 min before dehydration. (The efficiency of the "OsO_4-glutaraldehyde fixation" method for the preservation of tissues will be discussed in another paper (T. Kanaseki et al. In preparation.).) The pellets and tissues were embedded in Epon 812 (28) and sectioned with a glass knife on a Porter-Blum MT-I ultratome. These sections were stained with Millonig's lead stain (29). The P6 fraction and liver-fraction were negatively stained with 2% uranyl acetate (30), after the pellets of these fractions were resuspended in 10 mM KCl. The sections and negatively stained specimens were examined in a Hitachi 11-A electron microscope operating with 75 kv and 100 kv, and with a 30-μ objective aperture.

OBSERVATIONS

Examining the OsO_4-fixed P6 fraction from surface to bottom, one finds in sections that it is composed of three layers: A layer, B layer, and C layer.

The A layer (Fig. 1), occupying the surface of this fraction, appears to be the thickest. Fig. 2 is a high-power electron micrograph of the vesicles shown in Fig. 1. These empty vesicles with diameter of ca. 500 A are surrounded by a limiting membrane and resemble the synaptic vesicles (400–500 A) taken from the cerebrum of a guinea pig (Fig. 3).

The C layer, the layer occupying the bottom of the P6 fraction, is shown in Figs. 5 and 13. In addition to showing vacuoles with a diameter of ca. 3000 A and vesicles (1000 A or more) containing an electron-opaque granule in their lumina (Fig. 5; *gv*), Fig. 5 shows many vesicles of the same type (500–1000 A) surrounded by "coronets" consisting of radially arranged, sticklike material extending from the vesicle membranes. As judged from Fig. 7, which shows this kind of vesicle in a high-power electron micrograph, the vesicle with a diameter of ca. 500 A appears to have several projections, like the spokes of a wheel, sticking out of its inner leaflet. At the distal ending of each projection, a bar, shown as a dotted line connecting two such endings, can be seen. Therefore, observed as a whole, such a vesicle looks like a "wheel." This wheel has an outer diameter of ca. 1000 A, and its "spokes" show, in some places, a trilaminate structure with a thickness of about 70 A. The parts where this trilaminate structure is most conspicuous, outside the limiting membrane, are the parts of the spokes connecting to the "rim" of the wheel. However, this trilaminate pattern is lost wherever a structure looking like a small ring and having a diameter of less than 80 A is shown instead (Fig. 7, arrow). Aside from these membranous structures, there are in the C layer many networks showing, in some places, trilaminate structures having a thickness of ca. 70 A, which probably are not biological membranes (Fig. 5, short arrows; Fig. 13). This network of unknown nature, in many cases, overlaps the vesicle, forming the radial sticks of a coronet or the spokes of the wheel. Therefore, it can be surmised that the simple network without membranous elements is



FIGURES 1–3 Fig. 1, The A layer of the P6 fraction fixed by OsO_4-glutaraldehyde, thin-sectioned material. The vesicles have a diameter of ca. 500 A. × 30,000. Fig. 2, A high-power electron micrograph of the vesicles shown in Fig. 1. × 180,000. Fig. 3, A synapse taken from the cerebrum of a guinea pig. OsO_4 glutaraldehyde-fixed, thin-sectioned material. × 180,000.

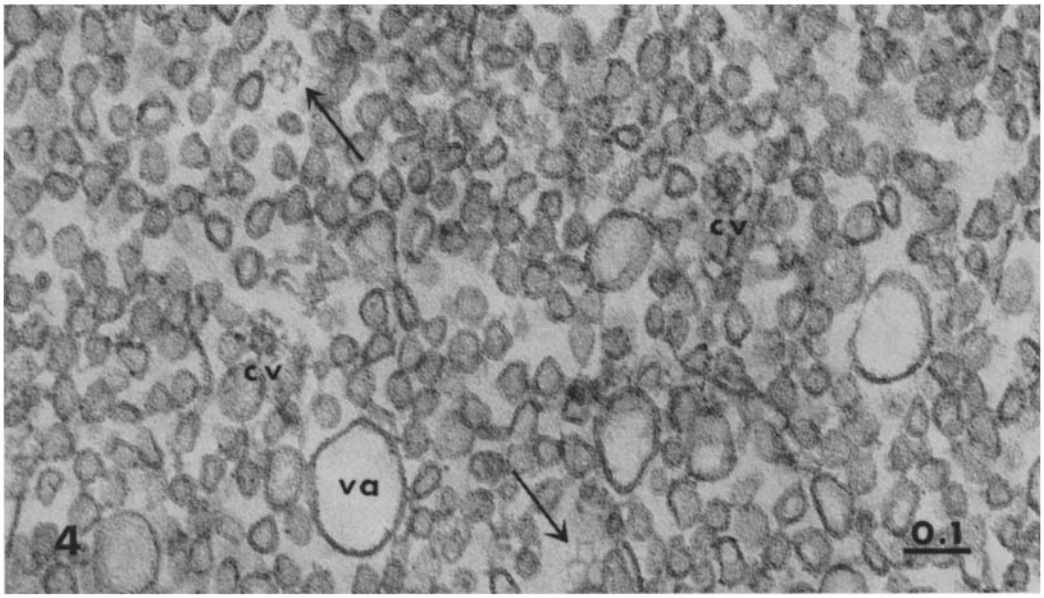


FIGURE 4 The B layer of the P6 fraction fixed by OsO_4-glutaraldehyde, thin-sectioned material. Vesicles having a diameter of ca. 500 A (as shown in Fig. 1), vacuoles having a diameter of 3.000 A or more (*va*), vesicles surrounded by "coronets" of radially arranged sticks extending from vesicle membranes (*cv*), and networks made up of unknown material (arrow) are observable. × 90,000.

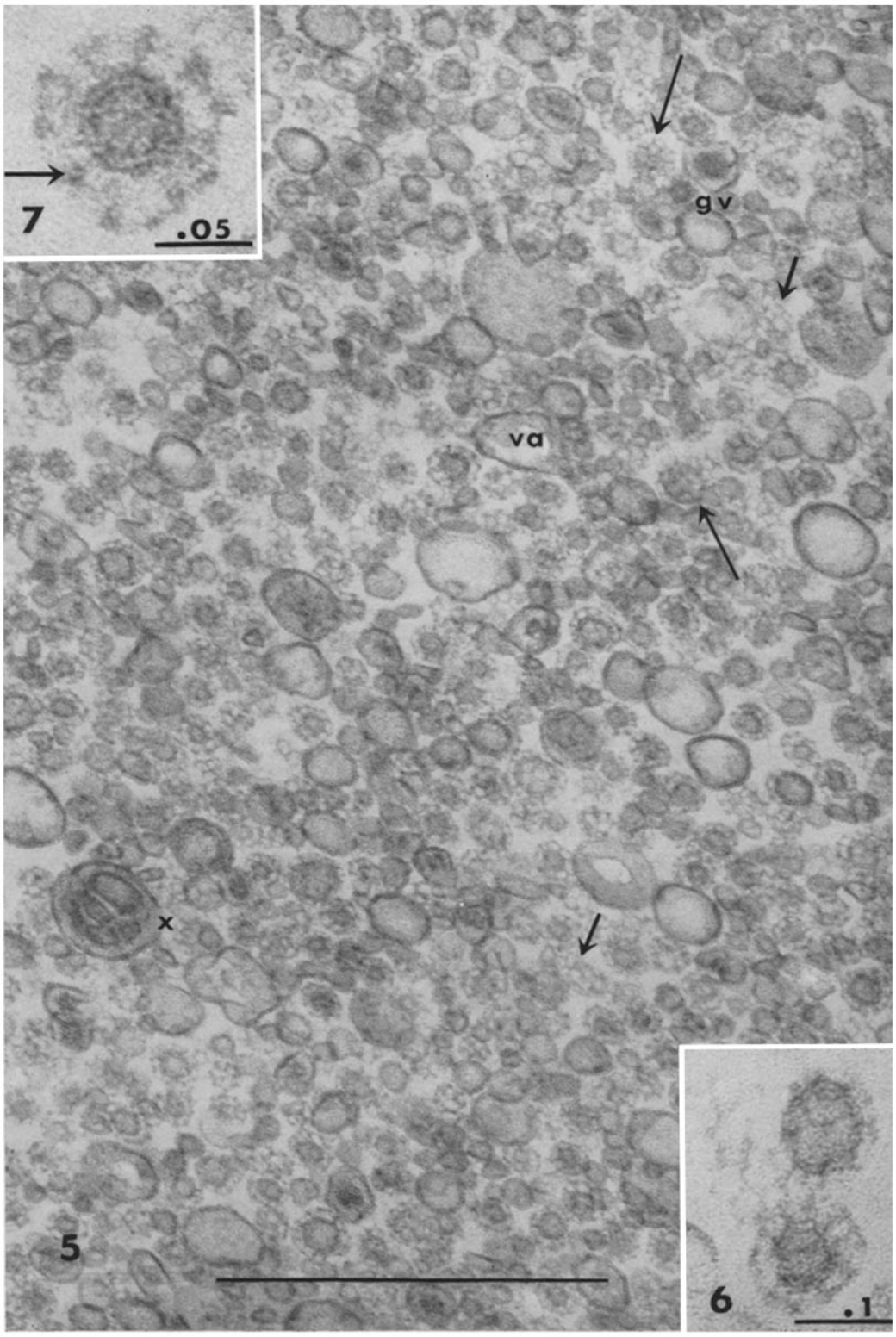
actually part of the coronet whose vesicle is outside the present section. Some of these vesicles that are surrounded by their coronets are shown in Figs. 5, 7, 13, and 14. Their appearance is similar to that of "annular vesicles" or "ring vesicles" in isolated materials (17) and especially to that of "coated vesicles" (7) or "alveolate vesicles "(31) in tissues fixed by chemical reagents.

The micrograph in Fig. 4 shows the middle, or B layer; and the structures composing the B layer are mixtures of the structures contained in the A and C layers, that is: vacuoles, small empty vesicles, vesicles with coronets, and networks.

Thus, the P6 fraction appears to be composed of the structures contained in these three layers. It was impossible to separate these layers into three completely pure fractions.

After the pelleted material of the P6 fraction in

FIGURES 5–7 The C layer of the P6 fraction fixed by OsO_4-glutaraldehyde, thin-sectioned material. Fig. 5 shows, aside from the vacuoles (*va*) ca. 3,000 A in diameter and the vesicles containing a dense granule in their lumina (*gv*), many vesicles of the same type (500–1,000 A) surrounded by coronets of radially arranged sticks extending from the vesicle membranes. Another peculiar configuration shown in this figure is the polygonal networks (short arrows). These networks seem to overlap some vesicles as if trying to make radial components of the coronets (long arrows). The nature of the structure labeled *x* is not known, but as judged from the thickness of the membrane, it is probably not a mitochondrion. ×60,000. Fig. 6, A high-power electron micrograph of superimposed images of a network and a vesicle. ×160,000. Fig. 7, A high-power micrograph of a vesicle surrounded by a coronet on its surface. The coronet is composed of many projections sticking out from the surface of the membrane of the vesicle. Each projection has laterally arranged bars at its distal end. A bar connects any two projections at their vertexes, so that as a whole the vesicle together with its coronet looks like a "wheel." Each "spoke" of the wheel has a diameter of ca. 70 A, and in some places shows a trilaminate pattern. At the part of each spoke that connects to the "rim" of each wheel, a "small ring" having a diameter of less than 80 A is shown, though not very clearly (arrow). ×300,000.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.05
gv
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6
.1

10 mM KCl was homogenized, the structures of the P6 fraction were negatively stained with uranyl acetate and are shown in Fig. 8. Four types of structures are observed:

1. The first type is a vesicle having a diameter of 500–1000 A and a smooth outline. By careful observation, one may find on some of these vecicles small particles attached to their surfaces[1] (Figs. 8, 10–12) that look like the "elementary particles" found on mitochondrial membranes (2). These "subparticles" are attached to vesicles having a diameter of ca. 500 and of 1000 A. There are from one to eight of them per vesicle. It is difficult to describe the appearance of these subparticles with certainty; they are probably hollow cylinders or polygonal pipes 100 A in diameter, and 100 A in height. Also, it is difficult to find out how they are attached to the surface of the vesicles (Fig. 10–12); Sometimes each subparticle has a stemlike structure which links it with a vesicle (Fig. 11).

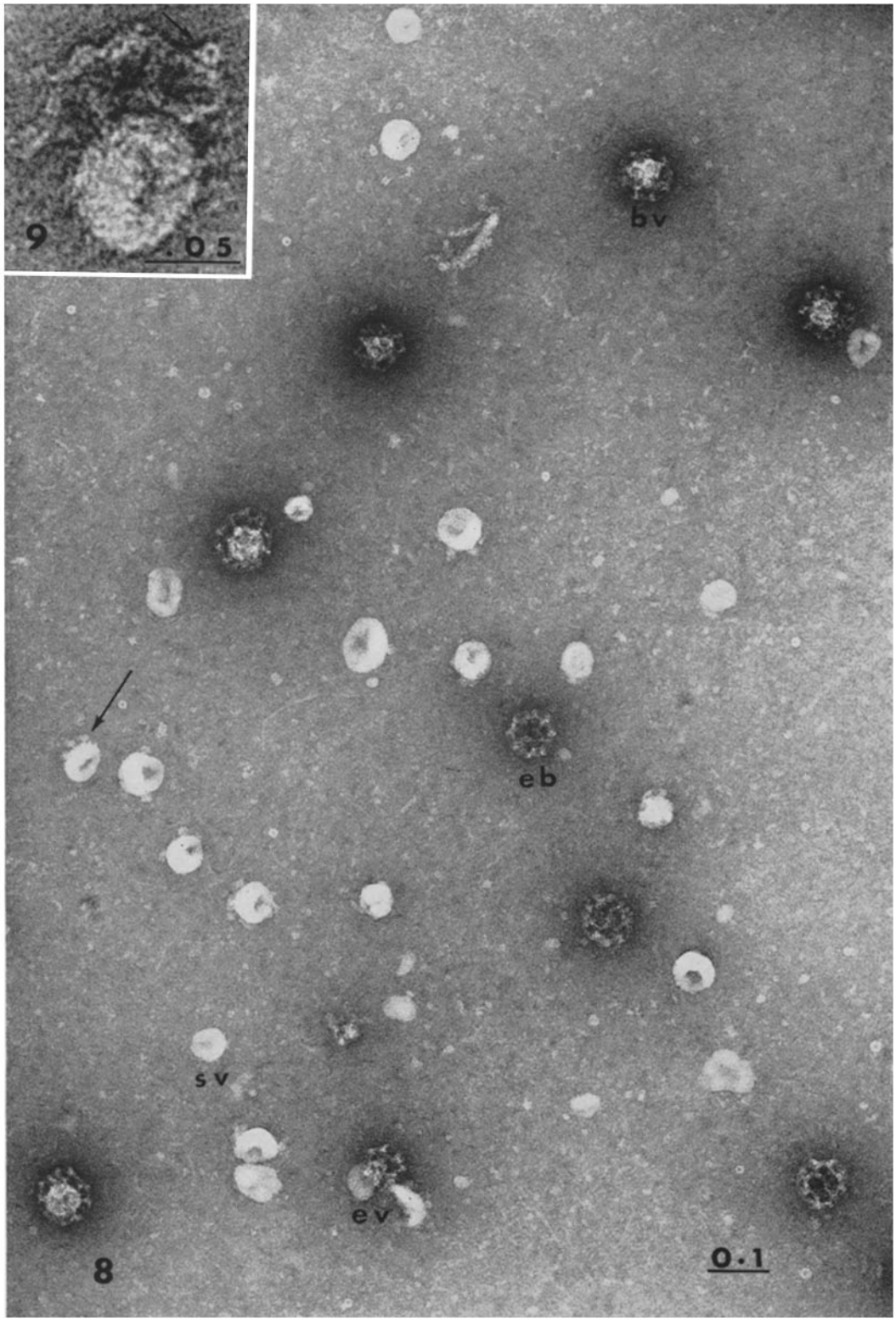
2. The second type of structure observed is a vesicle having a diameter of ca. 500 A and which is contained in a structure which looks like a spherical "basket," or network of polygons (Fig. 8, *bv*). There is an especially dense condensation of electron-opaque material around these vesicles. As judged from the high-power electron micrograph of this "vesicle-in-a-basket" (Figs. 15–17), the basketwork itself has an outer diameter of ca. 1000 A and is composed of a network of regular pentagons and hexagons whose sides are of equal length, that is, about 240 A. The material of this network looks like a series of small chains having a diameter of ca. 70 A. These "small chains" are especially noticeable in the corners of the polygons, where they sometimes form small rings having a diameter of ca. 80 A. It is important that no supporting structures can be seen between the surface of the vesicle itself and the polygonal network around the vesicle (Figs. 8, 15, and 16). We could not find any structure that would make up the walls of the "honeycomb" which Palay (31) thought to observe on alveolate vesicles viewed in intact cytoplasm (see also pp. 210–218 of the Discussion section below). Nor could we find any sign of columns holding the network above the surface of the vesicle. The vesicle appears simply to be ensconced, to float as it were, in the center of the spherical basketwork. However, (it should be noted that) the vesicle which is inside such a basketwork suffers some distortion, as if it were being compressed (tightly) by the network covering it and by some unidentified material lying in between (Fig. 8, *bv*), even though this material cannot be observed.

3. The third type of structure is a vesicle which appears as if it were trying to move out from a partially broken basketwork (Fig. 8, *ev*). A high-power micrograph of such a structure is shown in Fig. 9, though in this micrograph it is not easy to trace the remaining polygons of the network and is not possible to discover any intermediate material. The micrograph does not tell us either what kind of material holds the vesicle in position inside a whole basketwork, as in Fig. 8 (*ev*), or what prevents the vesicle from escaping completely once the basketwork is broken, as in Fig. 9.

4. The fourth type of structure consists of a basketwork which does not contain any vesicle. As in Fig. 8 (*eb*), these basketworks may show partial damage in some areas, but they are still spheres composed of polygonal networks. Fig. 26

[1] These vesicles not having small subparticles are rather rare.

FIGURES 8–9 The contents of the P6 fraction negatively stained with 2% uranyl acetate after the pelleted P6 fraction is resuspended in 10 mM KCl. *sv*, The vesicle having a diameter of 500–1,000 A. The arrow indicates small particles attaching to the surface of these vesicles and looking like elementary particles of mitochondria. *bv*, Vesicle within a spherical "basketwork" composed of polygonal networks. Note the heavy accumulation of electron-opaque material around these networks and the irregular outline of the vesicle inside compared to the outline of *sv*. Many small rings having a diameter of ca. 80 A can be seen at the corners of polygonal networks of the spherical "basketwork." *eb*, Basketwork without a vesicle, an "empty basket." *ev*, This vesicle appears as if trying to move out from a partially broken basketwork. Fig. 8, ×100,000. Fig. 9, A high-power electron micrograph of *ev*; the network of a broken basket is composed of a series of small chains having a diameter of ca. 70 A. A small ring ca. 80 A in diameter is indicated by the short arrow. ×330,000.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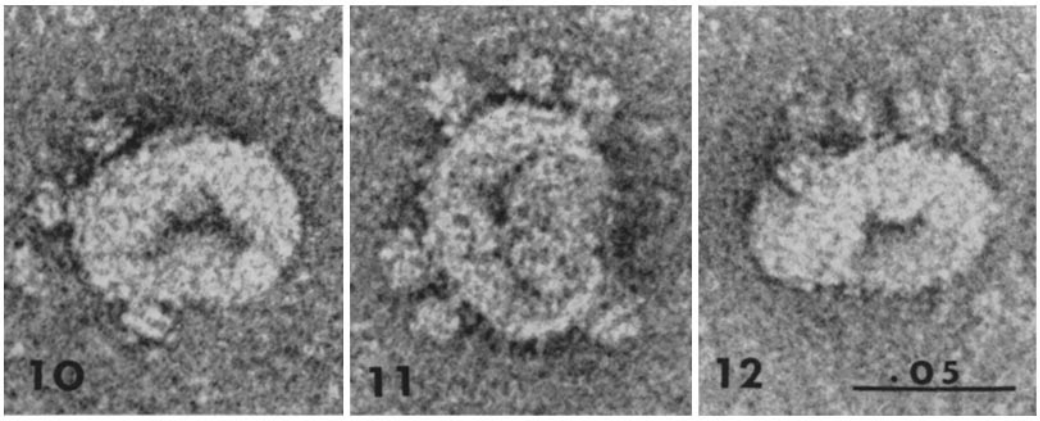


FIGURES 10–12 High-power electron micrographs of *sv* shown in Fig. 8. Negatively stained materials. Cylinders or polygonal pipe-shaped substructures ca. 100 A in diameter and ca. 100 A in height are attached to the surface of the vesicles; in Fig. 10 they are lying along the surface of the vesicles, whereas in Fig. 12 they are standing on the vesicle, and in Fig. 11 they are linked with the vesicle by a stemlike projection. Figs. 10–12, ×420,000.

is a high-power electron micrograph of such an "empty" spherical basket. These empty baskets are very convenient for investigating the structure of the network of the basket (see the explanations of Figs. 18, 19, and 26).

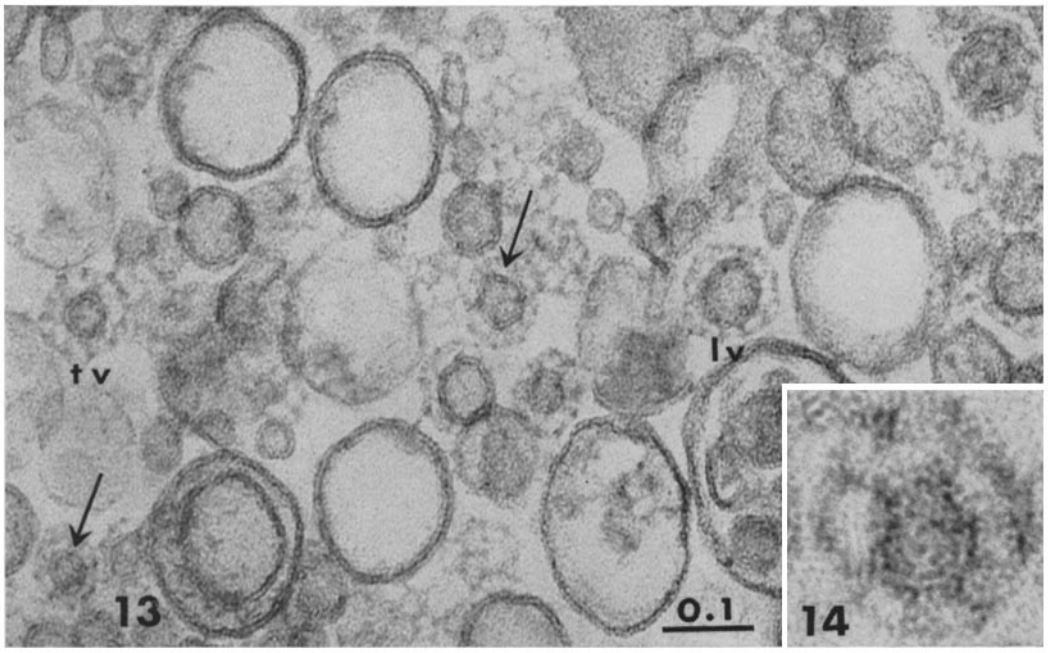
We may compare the appearance in sections of the OsO_4-fixed structures with that of negatively stained materials. For instance, the structure shown in Fig. 18 (and Fig. 19) is a negatively stained polygonal network composed of a series of small chains each having a diameter of ca. 70 A. In Fig. 20, this network appears exactly to be a positive picture of what is shown in Fig. 18 (and Fig. 19). In Fig. 20, it can be observed that the polygonal network is made up of a fine cordlike material with subunits that has a diameter of ca. 70 A. Continuing this comparison of the negative and positive images, we find the following: 1. The small rings, each having diameter of less than 80 A observed at the end of the "spokes" where they connect to the "rim" of the "wheel," are easily found in the corners of the polygons which form the spherical basketwork shown in negatively stained material (Figs. 8, 9, 15, and 16). 2. The superimposed images of the vesicle and the overlapping network in sections (Fig. 5, long arrow; and Fig. 6) show up more clearly in Figs. 16 and 17 made with negative-staining techniques. 3. The distortion of the "basketed vesicle" shown in Fig. 8, presumably caused by compression from some material lying over the vesicle, is observable in sections (Fig. 13, arrow; and Fig. 14). The profile of the vesicle-membrane seems to suffer, in places, some pressure either from the coronet itself or from some unidentified material lying between the coronet and the vesicle.

The vesicles in basketwork have also been separated from the liver of guinea pig by the same method by which the P6 fraction is acquired (Fig. 21). The polygonal network shown in Fig. 21 appears essentially to have the same pattern as that of the basketed vesicles separated from the brain.

DISCUSSION

When De Robertis et al. (17) isolated empty (synaptic) and filled (cored) vesicles in the negatively stained materials of the brain nerve endings, they also isolated certain more complex types of vesicles which they named "annular vesicles" or "ring vesicles" because a fine particulated or microvesicular material was observed surrounding each central vesicle. The "annulus" has an outer diameter of ca. 900 A, but the diameter of the inner vesicle measures 400–500 A. The perivesicular material itself "might correspond to parts of the matrix of the ending plastered around some vesicles."

Two kinds of vesicles are usually found in the nerve endings of vertebrates in thinly sectioned specimens of tissue which are fixed by chemical reagents. One kind is empty and is called the "synaptic vesicle" (Fig. 3), having a diameter of



FIGURES 13–14 The C layer of the P6 fraction fixed by OsO_4-glutaraldehyde, thin-sectioned material. Fig. 13, The irregular profiles of *cv*-vesicles are shown by arrows. The distortion seems due to compression by a compact structure surrounding the vesicle. Many polygonal networks without membranous elements are seen. Note that the *lv*-vesicle is larger than the *tv*-vesicle, but what are called "hairlike projections" of the *lv*-vesicle are shorter than those of the *tv*-vesicle. ×120,000. Fig. 14, A high-power electron micrograph of a distorted vesicle. ×270,000.

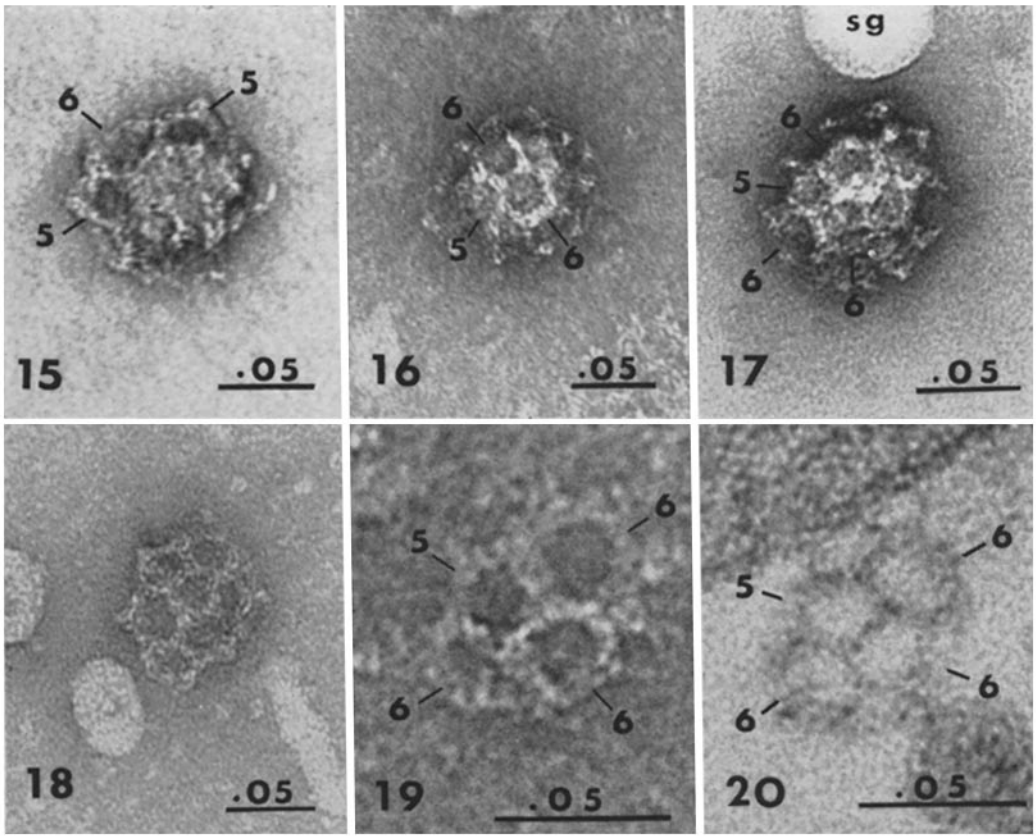
ca. 450 A; the other is called the "cored vesicle" (Fig. 5, *gv*), each one containing an electron-opaque granule in its lumen. It is reported, however, that a third type of vesicle also occurs in considerable amount and this vesicle is a coated one (32, 33).

The coated vesicles (7) of nervous tissue have been observed along the plasmalemma of the perikaryon and the dendrites, and around the cytoplasmic Golgi area and the region occupied by the agranular endoplasmic reticulum. These vesicles are called "complex" by Gray (33), "dense-rimmed" by Brightman and Palay (34, 35), and "alveolate vesicle" by Palay (31).

In sections, these coated vesicles can be recognized because they are wrapped in an amorphous coating ca. 20 mμ thick on the cytoplasmic surface of the vesicle (36). This coating consists of many bristles sticking out from the external leaflet of the vesicle-membrane (1); Friend and Farquhar (13) have counted eight to 13 bristles around vesicles having a diameter of 750 A. Sometimes these hairlike projections appear to protrude through the membrane of the vesicle into its lumen (37).

According to Palay, who described these vesicles as "alveolate vesicles" after minute observation of them in thin sections (31), these vesicles are distinguished "by a set of radially arranged striae extending 15 mμ from the outer surface of the limiting membrane." He suggested that the radially arranged striae which show the trilaminate structure of the unit membrane represent the vertical walls of a honeycomb structure making up the limiting membrane of the vesicle. Bowers (8) emphasized the honeycomb-like structure of the coated vesicle observed in the pericardial cells of aphids. She suggested that in surface view the structure of the coating consists of polygons, probably the combination of many pentagons or irregular hexagons (see Fig. 7 of her paper), and she counted 40 alveoli around one vesicle.

As judged from the appearance of "the bristle-projections extending from the outer surface of the vesicle" as described by Roth et al., and from the



FIGURES 15–17 High-power electron micrographs of *bv* ("basketed vesicles") shown in Fig. 8. Negatively stained materials. The networks of baskets are here superimposed on vesicles. The networks are composed of regular pentagons and hexagons with sides of equal length (ca. 240 A). *5*, regular pentagon. *6*, regular hexagon. *sg*, Sephadex granule. Fig. 15, ×260,000; Fig. 16, ×220,000; and Fig. 17, ×250,000.

FIGURES 18–20 Figs. 18 and 19, Negatively stained materials of the polygonal networks. Fig. 18, ×230,000; Fig. 19, ×470,000. Fig. 20, An OsO_4-glutaraldehyde–fixed and thin-sectioned material of the polygonal network. ×450,000. The networks are shown by negative-staining techniques as a series of small chains having a diameter of ca. 70 A. In section, the rings of the chains appear as a cordlike material having dense subunits. The images made by these two different methods appear exactly in reverse relationship, like positive and negative images in a photograph.

existence of the alveolate structure seen in oblique sections as reported by Palay and by Bowers, the vesicles with a coronet in the C layer of our P6 fraction are undoubtedly these same coated vesicles. (What is described by Palay as "the trilaminate structure of the striae on the alveolate vesicle" however, seems to us to be a series of small chains. Compare Fig. 19 and Fig. 20.) We have already pointed out that there are strong structural resemblances between the coated vesicles in sections and the *vesicles contained in a spherical basketwork* of a polygonal network consisting of regular pentagons and hexagons which are shown in our negatively stained materials. And we surmise that "annular or ring vesicles," the complex types of vesicles isolated by De Robertis et al., are identical with our vesicle in a basket. We believe, therefore, that the basket-like structure of the vesicle which is shown in clear outline by our negative-staining techniques is the true structure of the alveolate or coated vesicle. (The coated vesicles taken from the liver showed a structure identical with that of the coated vesicles isolated from the brain. See Fig. 21.)

There are, in the negatively stained specimen shown in Fig. 8, vesicles which appear as if they

were trying to move out from their partially broken basketworks (Fig. 8, *ev*; and Fig. 9). It may be reasonable to imagine from the appearance of these *escaping vesicles* that each of the baskets now empty formerly contained one vesicle. That is, these empty baskets may be simply the "coatings empty of vesicles" after some violent fractionating procedures have caused the removal of the contents. Figs. 18, 19, and 26 are high-power electron micrographs of such empty baskets. The networks of these baskets that look like spheres shown in Fig. 8 are composed of a combination of regular hexagons and pentagons (the sides of these polygons having the same length). Comparing Figs. 24, 25, and 26, one finds in their polygonal pattern some interesting resemblances between the vesicle basket and a kind of soccer football sold on the market whose spherical surface is also made up of hexagons and pentagons.

It is a well known fact both from direct observation (7, 38, 39) and from experimental work (36, 37, 40–42, 43, 49) that when the coated vesicles function as apical pits and perform apical pinocytosis they carry extracellular protein into the cytoplasm by the fission of the apical cell membrane. It is also clear that the coated vesicles transport the contents of the Golgi cisternae to some other parts of the cytoplasm (44–47) and to the cell surfaces (13) by pinching off from the hairy portion of the membranes of the Golgi complex. According to the schematic drawing by Roth and Porter (7) of the protein uptake of the mosquito oocyte, the first sign of membrane vesiculation leading to formation of the coated vesicles is recognized on the apical cell membrane where bristles occur on its inner leaflet. The second sign is the process of invagination of the cell membrane in the shape of a neck elongating into the cytoplasm; and the last sign is the formation of a closed vesicle with a bristle coating caused by the pinching off of the neck of the invaginated plasmalemma. This same process takes place in the membrane vesiculation leading to the formation of the coated vesicles from Golgi lamella (48). We have already observed (as shown in Fig. 8) that the form of the basketed vesicles suffers distortion compared to that of the free vesicles (Fig. 8, *sv*). This distortion has also been found in the OsO_4-fixed material (Figs. 13 and 14), and it is thought to occur by the compression of the vesicle membrane caused by the tight network covering the vesicle and some unknown material lying between this net and the vesicle itself and maintaining a distance of 150–200 A.

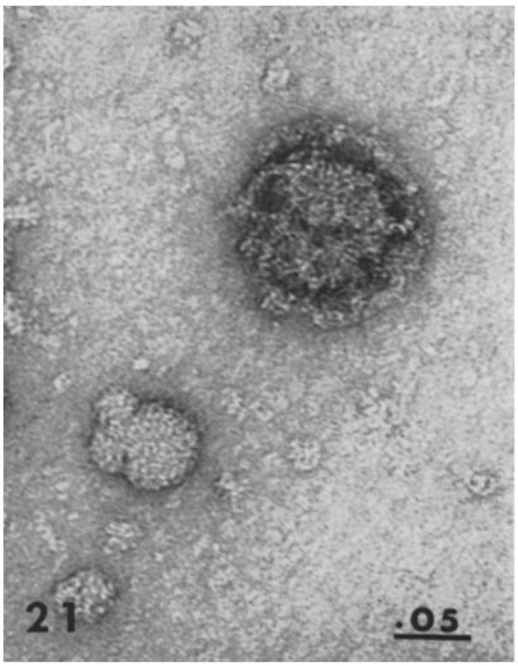


FIGURE 21 Negatively stained material isolated from a guinea pig liver by the same method by which the P6 fraction was produced. The pattern of the polygonal network is considered to be identical with that of Figs. 15–17. × 200,000.

When the apical cell membrane or the Golgi membrane transforms itself into the coated vesicle, according to the pattern shown in Fig. 22, the first stage of this transformation probably takes place at the hairy part of the membrane; and from the surface view, one may infer that this part is composed merely of a network of regular hexagons of the same size[2] (Fig. 23. See also Fig. 10 of Slautterback, D. B. 1967. *J. Cell Sci.* **2:** 571.). In the second stage, a hemisphere is formed at the bottom of the invagination by the transformation of the hexagons, which are in certain fixed positions (like the black hexagons in Fig. 22, *1*), into regular pentagons. Finally the formation of a beautiful polygonal sphere containing a vesicle is completed by

[2] The resultant polygonal sphere is actually composed of regular hexagons and pentagons having sides all of the same length. When the coating covers a plane there can be no pentagons, because it is geometrically impossible for a plane surface to be divided into a mixture of hexagons and pentagons.

the transformation of certain fixed hexagons into pentagons at the neck of the invagination. That is to say, a membrane spreading over the plane surface is invaginated and made to vesiculate passively by the transformation of the polygonal network of the coating. (Remember that the membranes of the coated vesicles in Figs. 8, 13, and 14 were compressed by their coatings.) A completed polygonal sphere of a coated vesicle has 32 surfaces (Bowers counted 40 alveoli around a vesicle) and, since the length of a side of a polygon is about one-fourth the diameter of the sphere, if there is a network of hexagons having a side length of 250 A, the diameter of the coating containing a vesicle will be about 1000 A. Since the coated vesicles are generally thought to have bristles with a length of 150–200 A, the diameter of the inside vesicle, that is, the coated vesicle, should be about 600–700 A. This hypothesis about the formation of the coated vesicles is, in fact, very applicable to the formation of small vesicles[3] (500–1000 A), for the length of a side in the polygonal network has been determined, by our observations, to be 240–270 A.

The simple hexagonal network spreading over a plane surface directly under the inner leaflet on the cytoplasmic side of a membrane is shown, though not very clearly, in a few hexagonal patterns having a side length of about 250 A (Fig. 23). One of the pictures given by Slautterback (58) shows the pattern of this kind of network better, though he does not interpret it as such.

Our hypothesis about the formation of the coated vesicle is supported, though indirectly, by three points.

1. According to Maunsbach (36), who studied ferritin absorption in the renal proximal tubule cells of *Necturus*, though specific plasma membrane invaginations had coatings, when ferritin contents of these invagination were found in the small apical vacuoles in the cytoplasm (49) the membranes of these apical vacuoles were no longer covered by the amorphous cytoplasmic coatings. Roth and Porter also have suggested that those cytoplasmic vesicles which contain yolk appeared not to be surrounded by bristle coats (7). Two electron micrographs of the coated vesicles are shown in a recent paper by Palade and Bruns (50). According to these micrographs, when the coated vesicle is about to fuse with the surface membrane it is free of its fibrillar coating in the area close to the plasmalemma. However, when the invaginating vesicle moves away from the plasmalemma it acquires an elongating neck which is entirely surrounded by fibrillar coatings. These observations

[3] In some cases of coated vesicles, the diameter of a vesicle tends to be in reciprocal proportion to the length of the projections (Fig. 13, *lv*, *tv*). However, in the case of vesicles more than 1500 A in size the combination of the polygons seems to be different from that of our model.

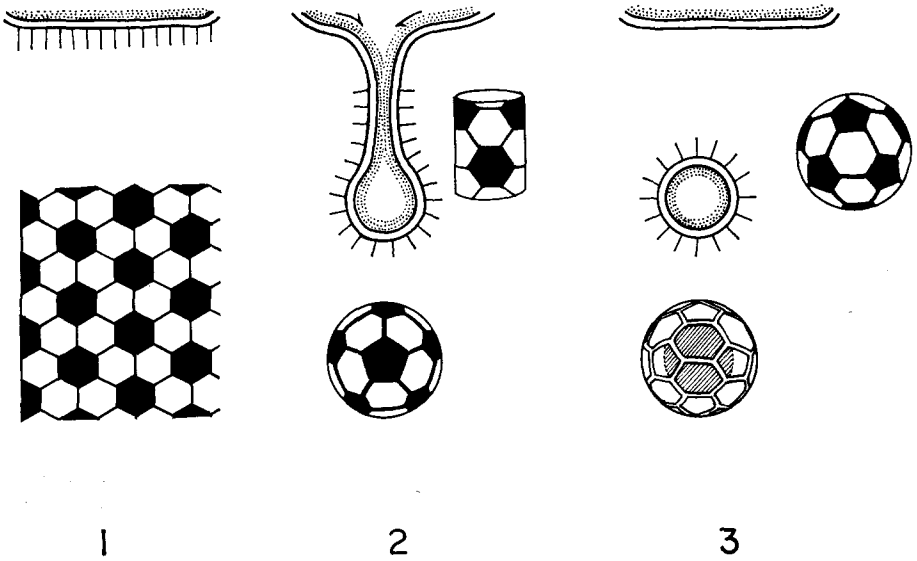


FIGURE 22 A schematic drawing illustrating the special role played by "coating" in the formation of the coated vesicle. See the interpretation on pp. 213 of the Discussion.

seem to suggest that the coatings perform an active, important part in the formation of vesicular structures by the membranes.

2. The configuration of a cytoplasmic vacuolar system is always governed by the amounts of its contents. For example, a granular endoplasmic reticulum can take a flattened shape only if the amount of amorphous materials in its cistern is small enough to permit such a low-volume form. McIntosh and Porter (51) have suggested that the structural elongation of the nucleus of spermatids is produced by the spirally arranged microtubules composing the caudal sheath. It seems likely that the elements which will determine the form of a membranous structure are not in the leaflets which are 20-A electron-opaque laminae, but are on the outer or the inner surfaces of these dense laminae.

3. The coated vesicles in Fig. 8 seem to us to be tightly compressed on their surfaces both by their polygonal-sphere baskets and by some unknown material surrounding the vesicles themselves, and lying within the baskets.

It is impossible, however, to know, at this stage of our hypothesizing, exactly how those regular hexagons transform into regular pentagons.

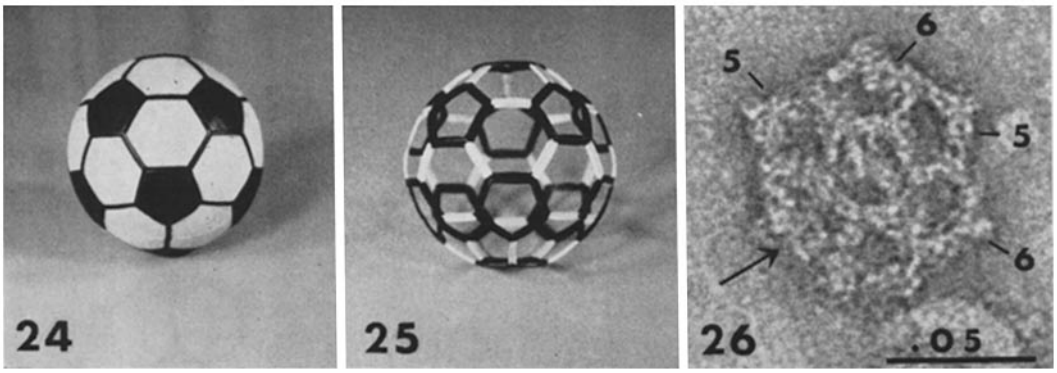
What substances can there be to maintain the interval of 20 mμ from the surface of a vesicle to the polygonal network of its basket? Palay suggests that the hairlike projections which seem to appear in views of sections represent the vertical walls of a honeycomb structure, but this does not seem to agree with the results shown by our negative-staining techniques. Furthermore, it is likely that, as supporting material extending from the surface of a vesicle, such bristles (if they exist) would terminate their distal endings in the corners of the polygons of a basket. According to the "football" model, there should be 60 such corners on this polygonal sphere; but the negatively stained materials have never confirmed the existence of that many bristles on any one vesicle. (If there were that many projections on a vesicle, a much more complicated pattern would be shown by the negative-staining techniques. See Figs. 15–17.)

Rather, we think that it is simpler to consider that the appearance of eight to 13 bristles on a small (750 A) coated vesicle (13) may be due to superimposed images of parts of the polygonal network of the basket, caused by the thickness of a section[4] (Figs. 27–30. Compare carefully Fig. 28 and Fig. 30).

A coated vesicle is always located in the center of a spherical basketwork, at a distance of 150–200 A from the inner surfaces of the polygonal

FIGURE 23 Would it be correct to suppose that there is a certain polygonal coating attached to the cytoplasmic side of the inner leaflet of the membrane? The longer arrow indicates what could be the profile of such a coating. Where two tilted membranes are buried in the present section, it appears that several hexagonal patterns (short arrow) with a side of ca. 250 A cover the membranes from inside just as the "scales" of the flagella (61) cover the membrane from outside. Epithelial cells of the gastric mucosa of the mouse. OsO_4 glutaraldehyde–fixed and thin-sectioned material. × 80,000.

[4] We have hypothesized that the "projections" which appear in sectional views are not true bristles, but rather are merely superimposed images of parts of the polygonal basketwork. In terms of Fig. 28 and Fig. 30, this hypothesis seems highly plausible. However, Friend and Farquhar counted 16–25 such "bristles" around large (more than 1000 A) coated vesicles. Our hypothesis will not account for such a number. Therefore, in the case of these larger vesicles it is possible that true bristle-like projections actually do exist. Or, it is possible that the combination of the polygons in the basketwork of such large (more than 1000 A) vesicles is much more complex than that which we have shown in our illustrations.



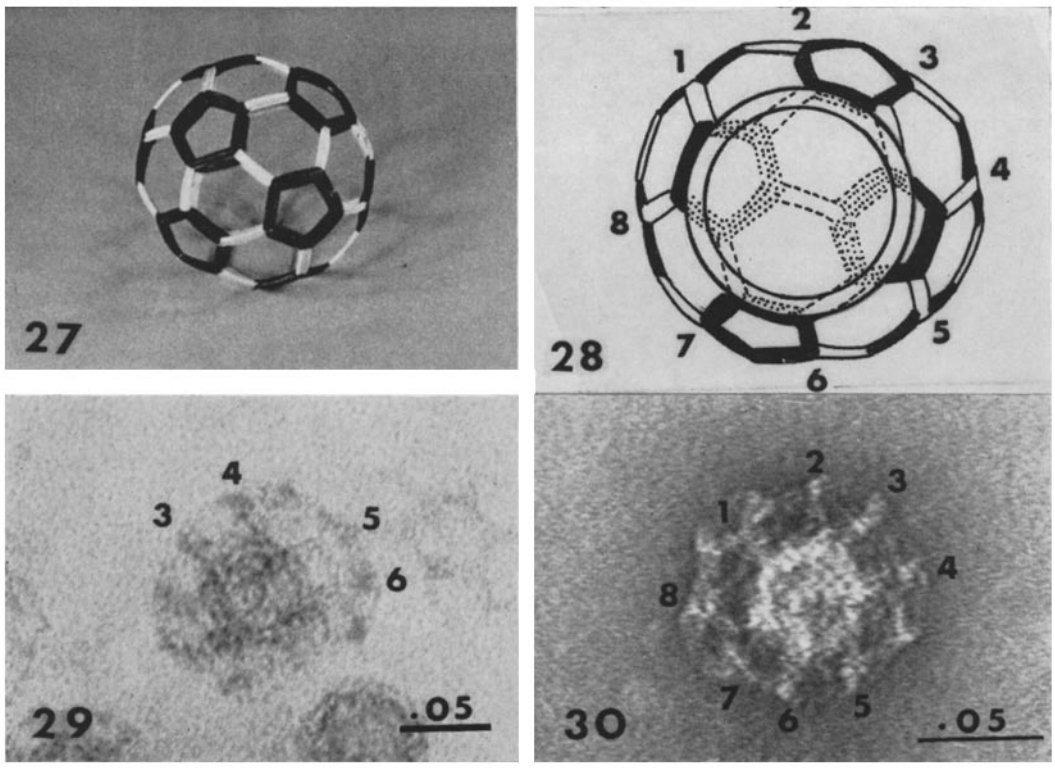
FIGURES 24–26 Fig. 24, An ordinary commercial football whose spherical surface is made up of pentagons and hexagons. Fig. 25, A hand-made model of an "empty basket." Fig. 26, A high-power electron micrograph of an empty basket. Fig. 26, ×400,000. Fig. 26, The arrow indicates the partial damage to the basket. *5*, regular pentagon. *6*, regular hexagon. The surface of the football shown in Fig. 24 has 32 planes: 12 regular pentagons and 20 regular hexagons. There are 120 sides and 60 corners.

planes of the basket. It is surprising that the coated vesicles of our fractionated materials have withstood the severe stress of 10^5 *g* (Fig. 7) and still appear in their natural condition, as they were in the cytoplasm, that is, properly ensconced in the centers of their baskets. It is therefore difficult not to suppose the existence of some substance between the basket and the vesicle. In this space there is some mysterious denseness due to an electron-opaque material. However, this supposition does not entirely refute the view that this material is either the honeycomb-like structure or the bristles that are filling up this space.

According to Marchesi and Palade (52), the localization of ATPase activity on the red cell ghost is in the filamentous material attaching to the inner leaflet of a ghost membrane. In another histochemical demonstration of ATPase activity on the cytoplasmic leaflet of a membrane, Oda and Ski (53) showed an electron micrograph of the cytochemical deposition of lead on intestinal microvilli. As an illustration of ATPase itself attaching to a biological membrane, Kagawa and Racker (54) singled out mitochondrial elementary particles. Fleischer et al. (55), too, suggested that the mitochondrial elementary particles are likely to be ATPase. The histochemical demonstration of ATPase activity on the cytoplasmic leaflet of the coated vesicle has not yet been made. However, from various cytological observations, it is very clear that membranous structures with "hairs" on their cytoplasmic side are particularly active in many cells (1, 5, 8, 9, 13, 31, 32, 42, 45, 56–58, 59). In Fig. 8 there are some vesicles without a basketwork (*sv*). Many of these vesicles have on their surfaces three or four subparticles that resemble elementary particles (Figs. 10–12). It is very probable that these vesicles with subparticles are not coated vesicles but belong to another system of vesicles, perhaps synaptic vesicles. However, as is suggested in Fig. 31, these subparticles may be either the remnants of partially broken baskets or the columns which hold the basket networks on their vesicles (hairlike projections . . . ?) and which remain on the surfaces of the vesicles. Besides, the configurations of the subparticles shown in Figs. 10–12 are very similar to those shown by Kagawa and Racker (54). We think that the polygonal networks typically seen around the coated vesicles have an important role in the vesiculation of the membrane. It is also of interest that ATPase activity has been reported (59, 60) in the synaptic vesicle fraction isolated by sucrose density gradients. According to the suggestion of Kadota et al. (60), the alteration of the ATPase activity that depends upon the ionic strength of Na^+ or K^+ in this fraction is very similar to that of the actomyosin ATPase.

On the other hand, it is highly probable morphologically that vesicles having subparticles correspond to the synaptic vesicles in sections, for the following reasons:

1. An electron microscopical observation failed to reveal 450–1000 A vesicles with elementary particle-like subparticles in the liver material isolated by the same method as the P6 fraction. This



FIGURES 27–30 As shown in Fig. 29 (sectioned material, ×250,000) and Fig. 30 (negatively stained material, ×340,000), the hairlike projections do not always extend from the surface of the vesicle in a correct radial pattern. Fig. 27 shows a model of the empty basket photographed from an angle to show the simplest pattern of its network. Fig. 28 shows a model of a vesicle contained in the model of Fig. 27, making superimposed images of a vesicle and its network. Note the way in which the projections stand up on the vesicle; compare the pairs of numbered projections shown in Fig. 30 and those in Fig. 28: *1* and *2*, standing in almost parallel lines. *2* and *3*, making an acute angle. *3* and *4*, standing in almost parallel lines. *4* and *5*, making an obtuse angle. As shown in Fig. 28 and Fig. 30, in this simplest condition of the superimposed images, the number of projections is eight.

appears to be strong evidence that the vesicles shown in Figs. 10–12 have not originated from mitochondrial cristae.

2. We could not find a mitochondrion in the C layer in section, though this layer contains many 3000 A vacuoles.

3. Among vesicles having a diameter of 450–1000 A in negatively stained materials of the P6 fraction, nearly all the vesicles had subparticles. (Vesicles without subparticles were rather few.) On the other hand, in the contents of the sectioned material of this fraction the largest volume was occupied by the synaptic vesicles.

4. As evidence counter to what is shown in Fig. 31, the well preserved coated vesicles in negatively stained material did not show any structures corresponding to "elementary particles" having a diameter of about 100 A or more.

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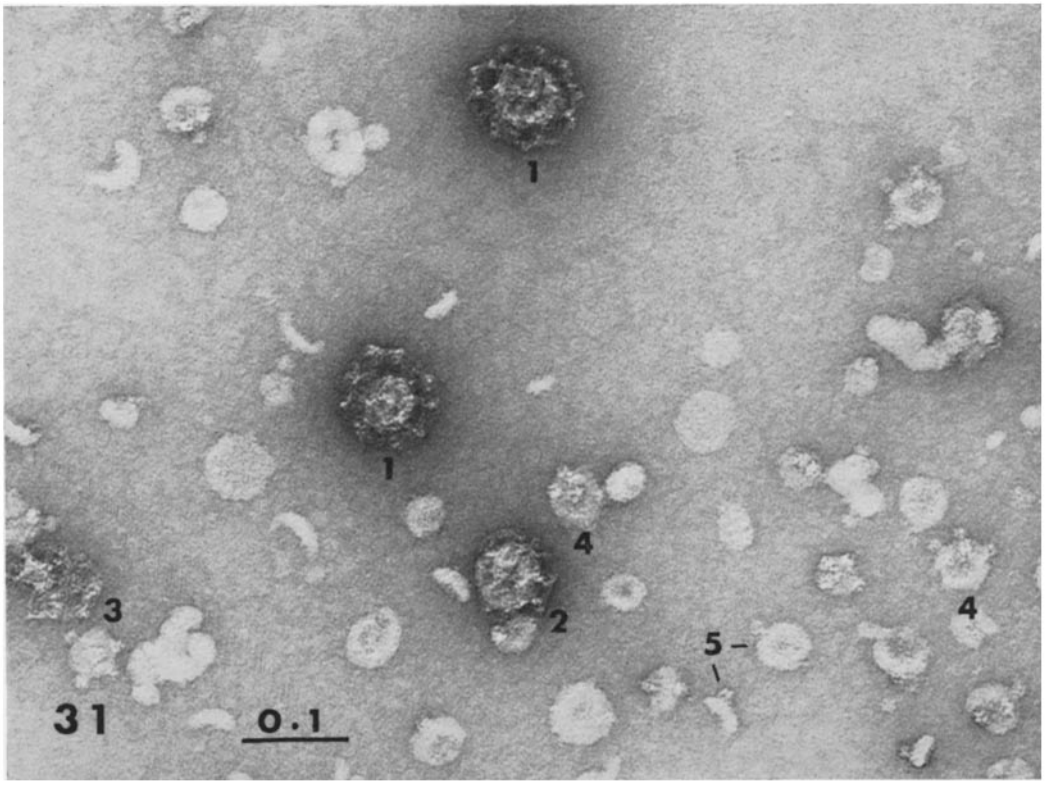

FIGURE 31 Negatively stained material from the P6 fraction. ×140,000. *1*, "coated vesicles" well preserved. *2*, coated vesicles suffering from partial damage. *5*, a vesicle having one elementary particle-like substructure. May the morphological transformations of *1* → *2* → *3* → *4* → *5* be simply the result of some violent fractionating procedures?

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