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Contributions of New Impregnation Methods and Freeze Etching to the Problems of Synaptic Fine Structure*

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INTRODUCTION

The present review is concerned with new information on synaptic fine structure that has been obtained recently in our laboratory by using iodide compounds of heavy metals for block staining as well as by examining synaptic areas in specimens prepared with the freeze-etching technique. The main interest revolves around synaptic vesicles, internal and external coats of nerve membranes and their specializations at the synaptic junction. It is hoped that the data might provide a basis for deeper understanding of the complex interplay of molecular mechanisms at the synapse, and that the methods involved may prove to be useful in monitoring experimental approaches to the problems of excitation, memory and individuality which remain great mysteries of living matter and especially the nervous tissue.

(1) Impregnation of synaptic vesicles

Various attempts have been made to obtain specific staining of synaptic vesicles. The most successful have been concerned with so-called granulated vesicles (see the contribution to this symposium by Tranzer *et al.*, 1969) which are known to store catecholamines and perhaps indolamines. The staining of so-called clear vesicles, especially at cholinergic sites, has recently been reported by Akert and Sandri (1968) and will be discussed here briefly.

The zinc-iodide-osmic acid (ZIO) method of Champy (1913) and Maillet (1962) was used for this purpose. It was for the first time clearly demonstrated that the black precipitation in nerve terminals and varicosities that are seen in ZIO-stained sections at the light microscopic level is based upon the specific impregnation of synaptic vesicles (Fig. 2). The problem of specificity of this method has been raised by several earlier investigators and seems far from being solved (the relevant literature was recently reviewed by Maillet, 1968).

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(a) Specificity of vesicular impregnation

Consistent results were obtained when carefully dissected tissue blocks of peripheral and central nervous systems were impregnated according to the procedure described by Akert and Sandri (1968). Within nerve terminals the reaction is limited to synaptic vesicles. However, in other components of nerve tissue one may encounter reaction products in organelles such as the Golgi zone, lysosomes and even in the glial and neuronal cytoplasm. While such observations clearly demonstrate the limitations of the method, it is noteworthy that the ZIO stain of nerve terminals can be considered the most specific of all "boutons techniques" that are presently available. The reason for this statement is that other synaptic stains (Gray and Guillery, 1966) are based on the impregnation of neurofilaments or mitochondria both of which are less specific components of nerve terminals than are synaptic vesicles.

(b) Specificity at cholinergic versus adrenergic sites

The positive correlation of vesicular impregnation at cholinergic sites and the negative correlation at certain adrenergic sites (Akert *et al.*, 1968) strongly suggest a relationship with cholinergic transmitter mechanisms. The question is naturally raised whether acetylcholine itself may provide the substrate for the reaction. At the present time there is no direct evidence available in support of this hypothesis. Some serious inconsistencies have been encountered in that vesicles at adrenergic sites (*e.g.* the pineal gland) have undergone a clear-cut reaction with the ZIO mixture. These controversial findings will be presented and discussed in detail elsewhere. At the present time, a simple explanation of observed facts is not available.

It should be stressed, however, that the most indispensable element of the staining compound seems to be the *iodide*. OsO_4 normally does not itself produce a positive reaction within synaptic vesicles at known cholinergic sites (*e.g.* motor endplate, sphincter iridis nerve terminals) with the exception of the electroplaque (Israel, 1969) whose vesicles contain a considerably higher concentration of acetylcholine. An iodide compound of acetylcholine may be formed (Stanek, 1905), which is able to reduce OsO_4. However, the positive reaction of certain non-cholinergic amine storage granules may have a different explanation. The osmium component of the ZIO mixture could react with the unsaturated bonds of catecholamines. This second

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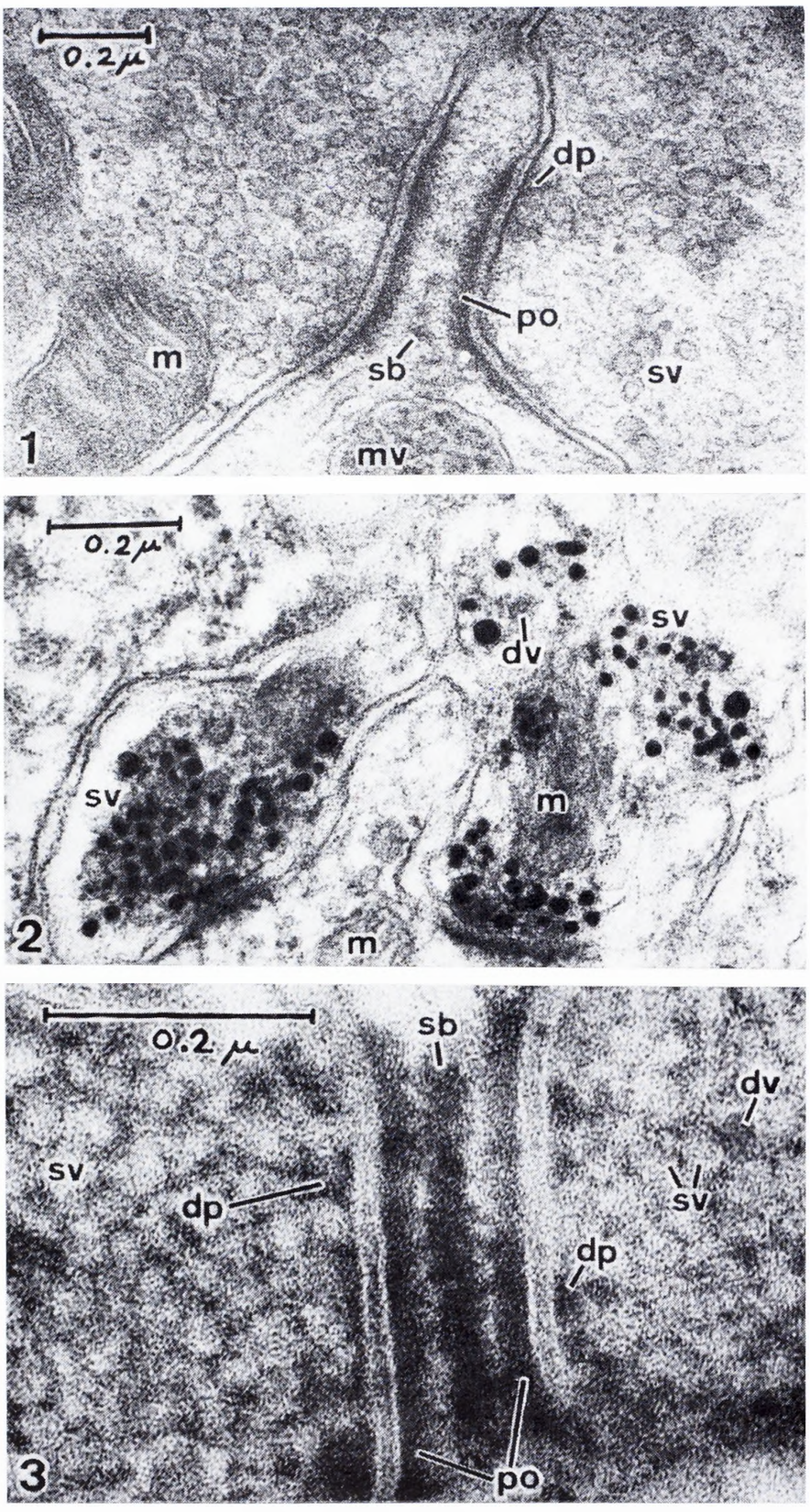
Fig. 1. Axo-dendritic synapse in the cat subfornical organ. Note the double synaptic plaque. The presynaptic dense projections (dp) are faintly visible, while the postsynaptic appositional densities (po) and the subsynaptic bodies (sb) are plainly seen. mv = multivesicular body; m = mitochondria; sv = synaptic vesicles. Glutaraldehyde/OsO_4 fixation. Primary magnification 20 000 ×.

Fig. 2. Axo-dendritic synapse in the cat subfornical organ stained with zinc iodide–osmium (ZIO) mixture (*cf.* Fig. 1). The clear synaptic vesicles (sv) are electronopaque. Dense-cored vesicles (dv), mitochondria (m) and cytoplasmic membranes are unstained. Primary magnification 20 000 ×.

Fig. 3. Axo-dendritic synapse in the cat subfornical organ (*cf.* Fig. 1) stained with bismuth iodide (BI) mixture. Appositional densities are clearly visible, especially the presynaptic dense projections of Gray (dp). Although the synaptic vesicles (sv) failed to react to this stain, they stand out in the negative because their contours are marked by a finely granular electronopaque coat. po = postsynaptic density; sb = subsynaptic bodies. Arrow = synaptic cleft with intracleft lines. Primary magnification 20 000 ×.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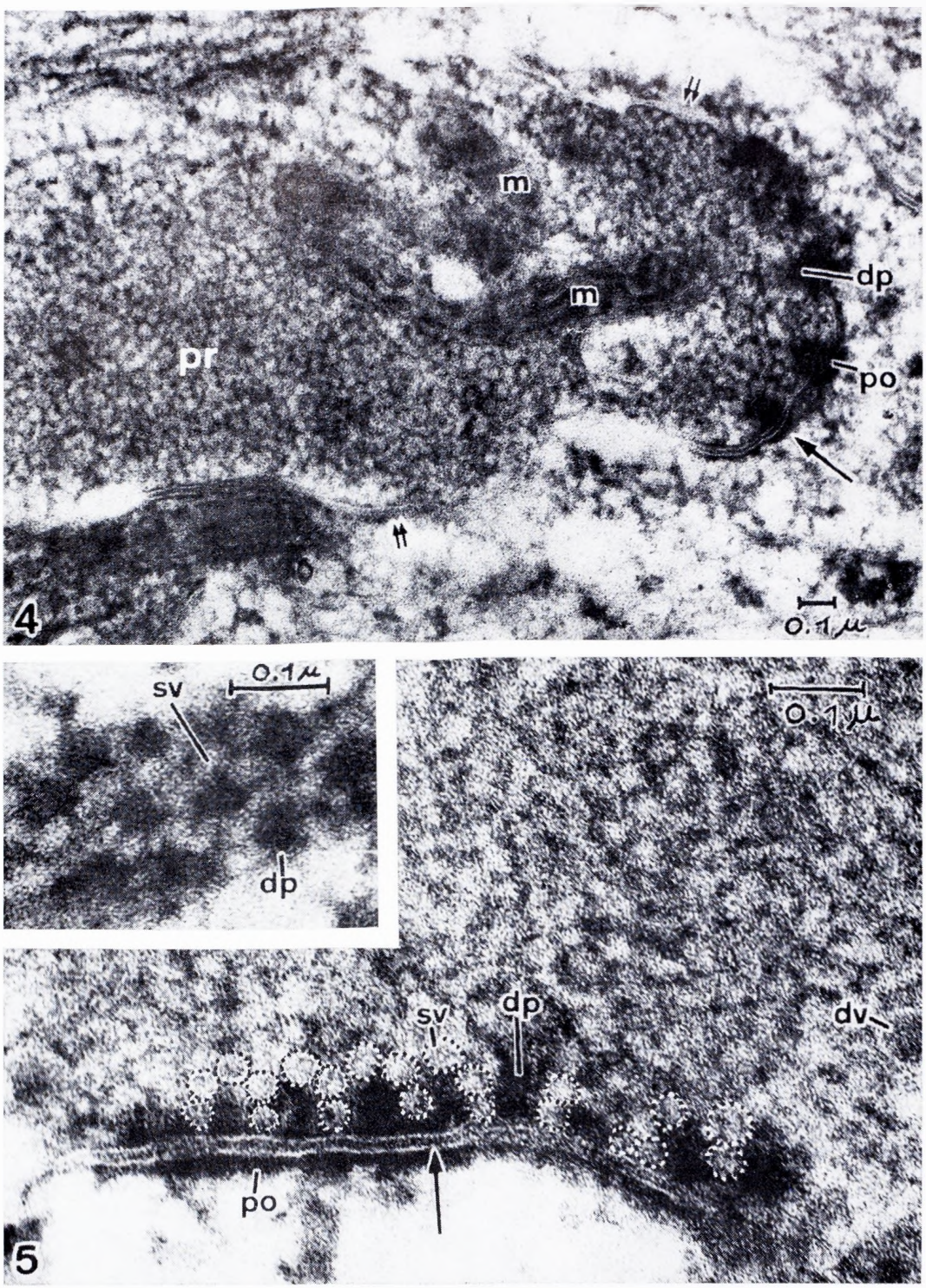

Fig. 4. The presynaptic dense projections (dp) of Gray in a cross-sectioned axon terminal (pr) stained with BI mixture. po = postsynaptic element (see also at arrow). m = mitochondria. Note the spiny contour of dense projections. Double arrows mark position of the plasmalemma. The unit membrane is spared, the inner and outer "fuzz" coats are stained. Primary magnification 20 000 ×.

Fig. 5. Presynaptic dense projections of Gray and the formation of a hexagonal *vesicular grid*. The spiny appearance of dense projections is due to "fuzz coat" surrounding the adjacent synaptic vesicles. dv = dense-cored vesicle; po = postsynaptic density; arrow = synaptic cleft with intracleft lines.

Inset: Tangential section through vesicular grid. Dark spots represent presynaptic dense projections (dp) in hexagonal arrangement with interconnecting filaments. Clear profiles between dense projections represent synaptic vesicles (sv), forming a rosette-like pattern. Note that each hole of the grid accommodates one single vesicle (*cf.* Fig. 6). Primary magnification 40 000 ×.

mechanism would seriously limit the specificity with respect to cholinergic binding capacity of the iodide component unless the catecholamines were eliminated experimentally before the reaction. Yet, so-called clear vesicles in nerve terminals of the cat dilator iris, vas deferens and spleen capsule yield positive ZIO staining reactions even after high doses of reserpine (15 mg/kg). Such vesicles, as well as their reaction product with ZIO, are morphologically not distinguishable from so-called cholinergic vesicles in the sphincter iridis terminals. They form a population within a given nerve terminal of only a few per cent, the whole of the remainder being non-reactive to ZIO (Akert *et al.*, 1968). This finding needs to be further investigated and discussed in the context of the Burn and Rand (1959) hypothesis.

(2) Impregnation of internal and external "fuzz" coats at the synaptic membranes

The term "fuzz" was recently used by F. O. Schmitt (cited by Lehninger, 1968) for the designation of an outer coat of the neuronal membrane. This coat is of low electron-density in pictures obtained with conventional ($KMnO_4$, OsO_4) fixation techniques and overlies the tramlines of the classical "unit membrane" of Robertson (1959). Application of the BI block impregnation of glutaraldehyde-fixed nerve tissue enables one to see both an external and an internal "fuzz" coat with local specializations in the region of synaptic contacts (Fig. 3). Such features can be readily detected at the postsynaptic membrane (*e.g.* the "postsynaptic web" of De Robertis, 1964), and previous studies in our laboratory (Fig. 1) have confirmed and extended the pioneering observations of Taxi (1965) and of Milhaud and Pappas (1966) with respect to postsynaptic bars and especially the subsynaptic bodies (Akert and Sandri, 1966; Akert *et al.*, 1967a, b). The following observations are primarily concerned with the "*presynaptic dense projections*" of Gray and with the *intracleft substance*.

(a) The presynaptic vesicular grid

Gray (1963, 1964) was the first to draw attention to small dense spots at the presynaptic membrane; these were particularly evident after phosphotungstic acid block staining. With the aid of the BI impregnation of non-osmicated material the densities are seen in abeyance of the membrane (Pfenninger *et al.*, 1969). In cross sections (Figs. 4, 5) one observes polyhedric bodies with spiny profiles which are spaced more or less regularly. These bodies are immediately surrounded by the contours of clear and dense-cored synaptic vesicles. Tangential sections reveal the regular hexagonal arrangement of the dense projections as well as the interconnecting filaments (Fig. 5, inset). Sections taken near the base of the grid, *i.e.* near the level of the presynaptic membrane, demonstrate that each of the free spaces between presynaptic dense projections contains one single synaptic vesicle (Fig. 5, inset). Based upon measured values of the diameters of dense projections and synaptic vesicles as well as their interdistances one may construct a three-dimensional model. It turns out that the dense projections form the nodal points of a grid and that each dense projection is surrounded by a monolayer of synaptic vesicles (Fig. 6). Thus, it seems appropriate to designate this structural complex "The presynaptic vesicular grid" (Fig. 7).

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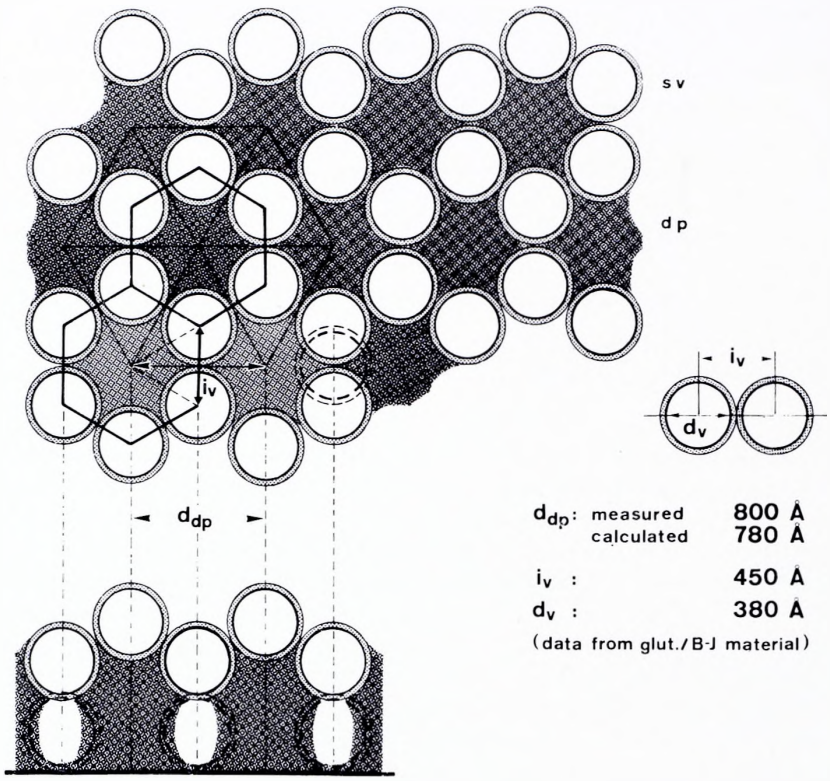

Fig. 6. The presynaptic vesicular grid. Reconstruction of geometrical relationships between presynaptic dense projections (dp) and synaptic vesicles (sv). *Upper diagram* represents tangential section of the grid, *lower diagram* represents cross-section. d = diameter; i = interval. The measured and calculated dimensions of grid and vesicles are within close range. The relationship between vesicles and dense projections as viewed in the cross-sectional reconstruction below can be detected in BI-stained cross-sections of synapses under optimal conditions (see Figs. 3 and 5).

Although not easily recognized in sample sections of synaptic areas, the vesicular grid pattern is suggestive in Figs. 3 and 5, and it was found to be present in a considerable number of electronmicrographs of our collection.

The plane of sectioning plays a decisive role for the demonstration of this unique and remarkable relationship between dense projections and vesicles. However, the basic configuration of the presynaptic grid may be truly unstable for reasons which will be discussed below, and this may provide an additional explanation for the fact that the arrangement of the vesicles as shown in the calculated reconstruction (Fig. 6) is not consistently seen in reality.

The significance of the vesicular grid at the presynaptic membrane is not yet clearly understood. The following two possibilities should be considered. (*1*) The grid arrangement "may play a role in guiding synaptic vesicles to special localities of the presynaptic membrane" (Gray, 1966). This suggestion is supported by the

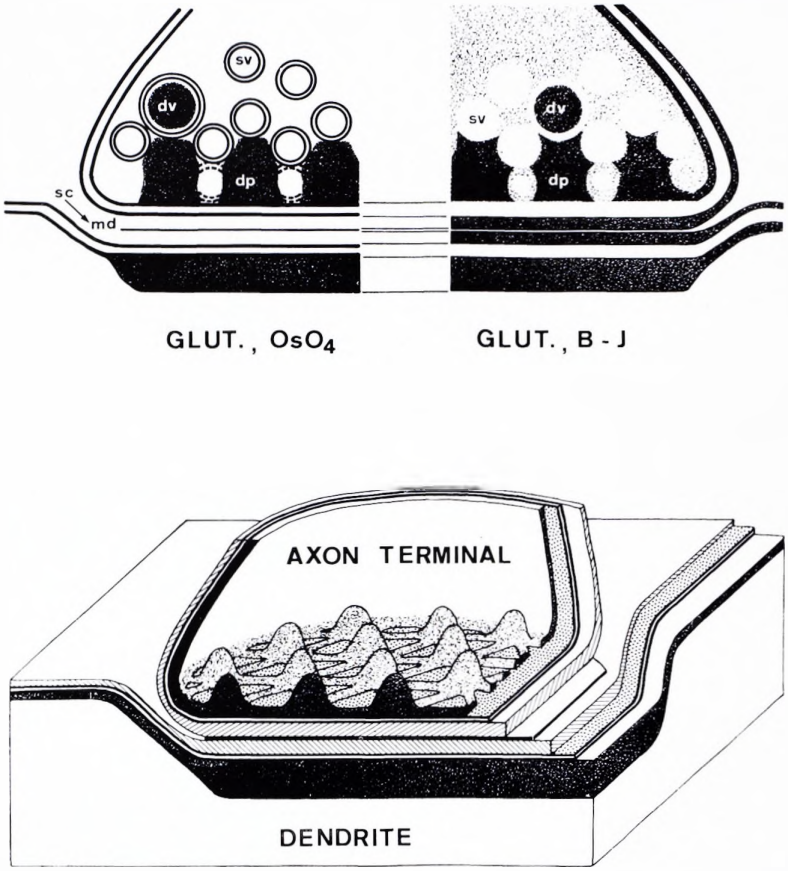

Fig. 7. Schematic reconstruction of synapse as viewed in BI-stained material. *Upper diagram*: Glutaraldehyde–OsO_4 fixed synapse (left) is compared with one prepared with glutaraldehyde fixation and BI block staining (right). Note the spiny appearance of dense projections due to the indentation by adjacent vesicles. The intracleft lines seem to form thickenings of the *outer* "fuzz" coat of the cytoplasmic neuronal membrane. The "true" synaptic cleft is minimal. *Lower diagram*: Three-dimensional reconstruction of the presynaptic grid of dense projections (without vesicles for sake of clarity) and the configuration of the synaptic cleft. Note that pre- and postsynaptic densities appear as specialized formations of the *inner* "fuzz" coat of the plasmalemma.

following morphological finding. The cytoplasmic surface of the synaptic terminal is covered by a thin "fuzz" coat (Pfenninger *et al.*, 1969) which is lacking in the holes of the presynaptic grid. Akert and Pfenninger (1969) have suggested that this situation would allow the synaptic vesicles to enter into an immediate contact with the electrically excitable membrane which is not present at other membrane sites of the terminal (Fig. 8). This constellation might favor the transmitter release from the presynaptic grid when the nerve impulse reaches the axon terminal, and may prevent it from occurring at other sites. (*2*) The dense projections may contain molecules which are of significance in the mechanisms of synthesis, release and repletion of transmitter

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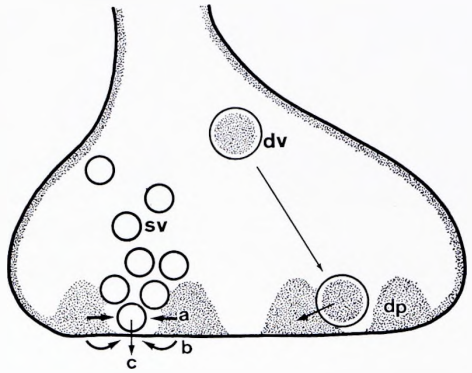

Fig. 8. Topographical relationships between vesicles and presynaptic grid. Arrows indicate functional pathways and interactions (hypothetical). *Clear vesicles* (sv) are randomly distributed in the terminal and regularly arranged within the grid. Only one single vesicle is accommodated within a grid hole and allowed to be in close contact with adjacent dense projections as well as with the excitable membrane. The internal "fuzz" coat seems to be lacking in the holes. a = postulated interaction between presynaptic dense projections (dp) and vesicles before transmitter is released. b = excitatory process involving the presynaptic membrane and triggering transmitter release (c). At least one fraction of the synaptic vesicles might be derived from the plasmalemma by micropinocytosis (see Fig. 10). *Dense-cored vesicles* (dv) may be transported by axoplasmic flow from the cell-body and deposit important molecular constituents at the dense projections.

substances. The intimate relationship between vesicles and grid seems ideally suited for processes of chemical interaction.

The presynaptic grid as a highly organized structure raises the question of its formation. Aghajanian and Bloom (1967) have recently studied the dense projections of Gray in young and adult animals and — although they have failed to recognize the geometrical finesse in the three-dimensional arrangement of dense projections in the mature state — they have reported a remarkable observation. It appears that the immature neuropil may exclusively contain junctions with *symmetrical* membrane appositions as in desmosomes or intermediate junctions. In more advanced maturational stages they encountered increasingly focalized densities at the presynaptic site which eventually attained the profiles of separated dense projections. Thus, the dense projections would not only appear as a criterion of *synaptic polarity* (Gray, 1966), but — in the view of these authors — it may well serve as an indicator of *synaptic viability*. The significance of this notion is easily appreciated. An extended version of this concept can be offered on the basis of our present findings which tend to emphasize the degree of orderliness of presynaptic organization and to correlate levels of its three-dimensional structural evolution with levels of functional differentiation. If this process parallels that of synaptic viability it seems inevitable that the formation of vesicles at the terminal might be among the dynamic factors which bring about the transformation of the solid plaque into the vesicular grid! On the other hand, it should not be overlooked that confirmational evidence of this attractive hypothesis is not available at the moment because direct observations on the transformation are difficult to obtain.

A second problem concerns the origin of appositional densities. As mentioned before, it has been demonstrated in BI-impregnated material that an internal "fuzz" coat exists throughout the axon terminal (Pfenninger *et al.*, 1969). The appositional densities at the pre- and postsynaptic membranes are continuous with, and may be considered to be specializations of, this internal "fuzz" coat. The chemical nature of the material is not known, and no information is available with respect to chemical differences between appositional densities at synapses and other forms of cell contact. Conceivably, the skeleton of these densities may be formed by similar macromolecular constituents. More specific compounds may be formed or deposited secondarily. Akert and Pfenninger (1969) suggest that dense-cored vesicles might be involved in the molecular transport from the neuronal Golgi zone to the presynaptic dense projections (Fig. 8), analogous to the transport of catecholamine-storage granules (Dahlström, 1969).

Finally, the question is raised whether the presynaptic grid is an obligatory synaptic structure or limited to certain types of junction. Our own material is at the moment limited to synapses of the cat subfornical organ. However, presynaptic dense projections have been observed by Gray (1966), Aghajanian and Bloom (1967) and others in the spinal cord and in the cerebral cortex. Thus, the vesicular grid may indeed constitute an important element of central synapses. Nevertheless, more comparative work on this problem seems highly desirable. To mention only the following possibility: Gray (1969) has recently made systematic studies on structural differences between excitatory and inhibitory synapses. Convincing evidence has been accumulated by this author with respect to the "flat vesicle story" of Uchizono (1965). Moreover, Gray's electronmicrographs seem to suggest that appositional densities differ in the two types of synapse. Those with round vesicles seem to be associated with more conspicuous appositional densities than those with flat vesicles. This observation raises the question whether the presynaptic grid formation may be typical for excitatory junctions and whether it might be absent or less developed in inhibitory synapses. The problem is now under investigation in our laboratory.

In the same context one wonders whether the presynaptic grid exists in peripheral junctions. Miledi (see Gray and Guillery, 1966) has found dense spots opposite the junctional folds at the membrane of nerve terminals of frog endplates. Recent studies in our laboratory on neuromuscular junctions in the rat diaphragm have confirmed this observation by clearly demonstrating the presence of dense projections (Fig. 9).

(b) The intracleft substance

Lehninger (1968) has recently discussed the problem of the outer "fuzz" coat of the neuronal membrane and the adjacent intercellular space. Glycoproteins and polysialogangliosides seem to play an important role. However, it was not until periodic acid–silver methenamine (Rambourg and Leblond, 1967), lanthanum (Doggenweiler and Frenk, 1965; Revel and Karnovsky, 1967) and ruthenium red (Bondareff, 1967) were allowed to penetrate the corresponding compartments that morphological evidence became available at the ultrastructural level. More recently, Pfenninger

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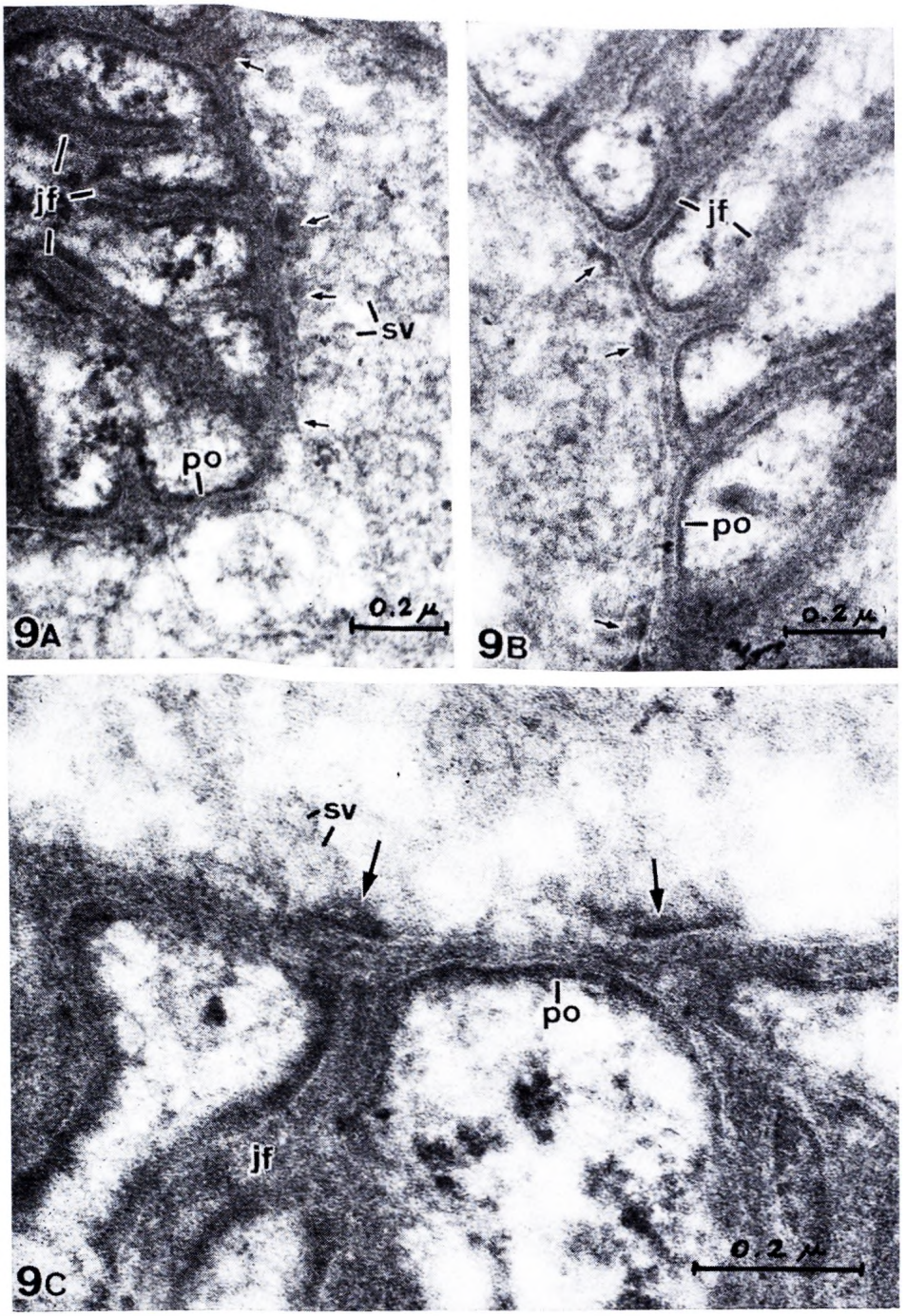

Fig. 9. Motor endplate of rat diaphragm after BI impregnation. Note that prejunctional dense spots (arrows) occur regularly opposite the junctional folds (jf). The postjunctional membrane apposition (po) is clearly seen. Synaptic vesicles (sv) are best seen in A; they seem to have a different appearance from those in presynaptic terminals (*cf.* Figs. 3, 4 and 5), and their relationship to the dense projections is less obvious. Primary magnification of A and B = 20 000 ×, of C = 40 000 ×.

et al. (1969) using the BI block stain, have obtained additional details on the outer coat of nerve membranes. *A double intracleft line* was demonstrated which can also be seen in material prepared with Westrum's combination (uranyl acetate and lead citrate) staining (Westrum, 1965a). This double line represents the outer coat of the plasmalemma which is somewhat thicker in the region of the synapse than elsewhere on the nerve terminal (Figs. 4 and 5). The findings are briefly summarized diagrammatically in Fig. 7. Further description of intracleft lines in synapses and other types of cell contact is to be found elsewhere (Akert and Pfenninger, 1969). The fact that the BI method fails to stain the basement membranes is in contrast with the specificity by which the capillary glycocalyx reacts with ruthenium red (Luft, 1966). Furthermore, the granular intracleft material demonstrated with the aid of ruthenium red (Bondareff, 1967) seems to be randomly arranged, while a clear-cut orientation of very fine electron-opaque granules in parallel with the plasmalemma is seen in BI-stained material. Thus, it seems that the outer membrane coat of neurons, especially at the synapse, may consist of a highly complex system of macromolecules (proteins, glycoproteins, glycolipids) which may be differentially affected by various histochemical reagents.

The functional significance of the intracleft substance at the synapse is at present mainly a matter for speculation. Recent results from our laboratory suggest that basic amino acids may be among the substrates of the BI reaction, and their presence in the junctional area may be critical for the "stickiness" to which the synapse owes its name.

(3) Freeze-etching of neuropil and synaptic junctions

The freeze-etching method (Moor and Mühlethaler, 1963) is known to provide high fidelity electronmicroscopic profiles of cells and tissues in the frozen state. In addition, it enables one to examine a surface view of plasmalemma and organelles. The subfornical organ (SFO) is ideally suited for such studies because it can be rapidly removed from the native brain without mechanical damage to the delicate texture of its neuropil. A further advantage is its relatively high synaptic density (Akert *et al.*, 1967b).

The investigations were based on 14 unfixed and 14 fixed cat SFOs. Fixation was performed with 3% buffered glutaraldehyde. Before the freeze-etching procedure began it was necessary to treat the fixed or unfixed specimens for 30 min in a Ringer solution containing 25–30% glycerol. Approximately 30% of the preparations turned out successfully; of these nearly 150 electronmicrographs were obtained. The majority of the pictures were derived from aldehyde-fixed material, although a sufficient number of unfixed control pictures was available for examination.

While details of this study will be reported in another communication (Moor *et al.*, 1969), we should like to present a brief summary of the principal findings with special emphasis on synaptic fine structure. Profiles of the *synaptic terminals* are readily identified on the basis of vesicles and mitochondria. *Synaptic sites* are characterized by the regularly apposed membranes, the slightly enlarged cleft and the accumulation of finely granulated material at the junctional membranes.

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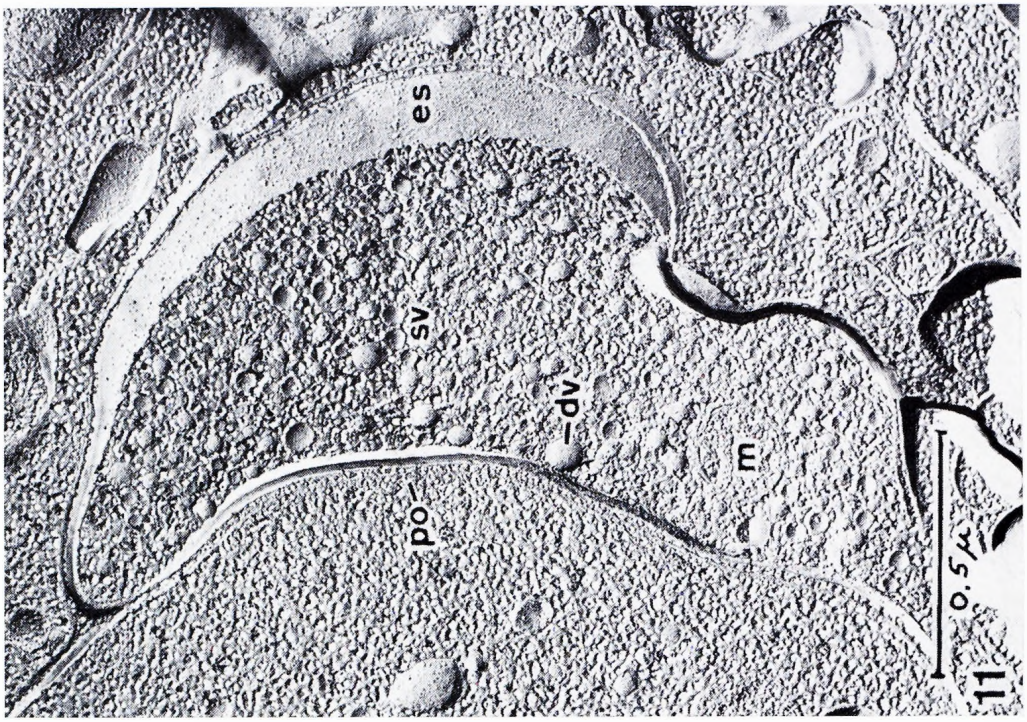
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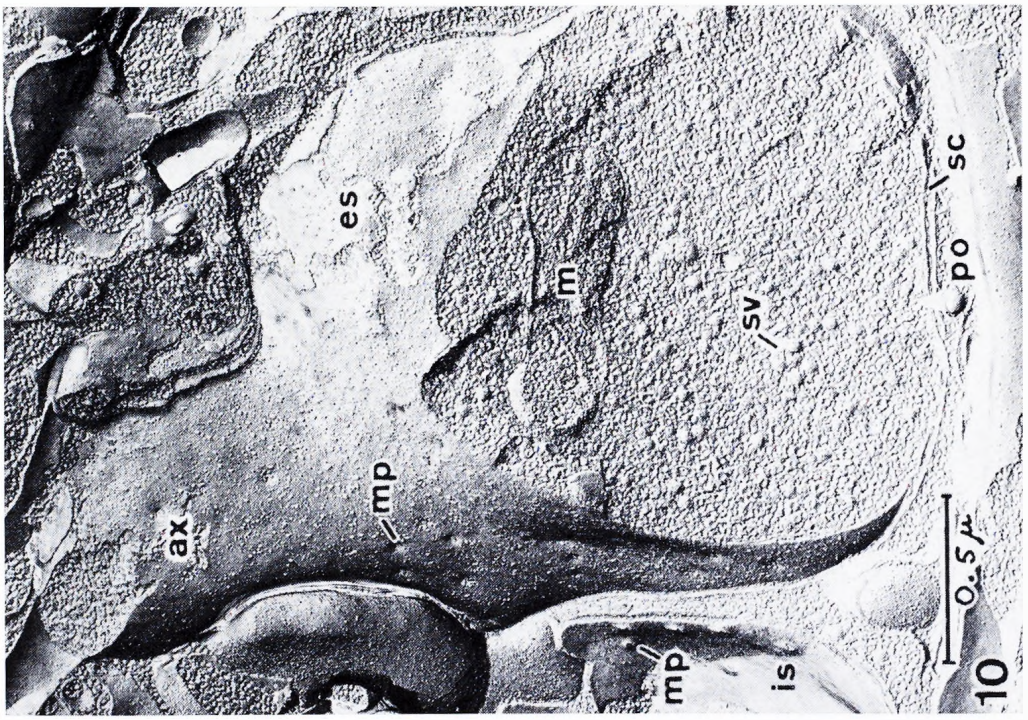
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(a) Configuration and size of synaptic vesicles

All the vesicles observed in the SFO terminals (including those from aldehyde-fixed material) have *round* profiles (Figs. 10, 11, 12 and 13). This is in accord with the results of extensive studies on conventional glutaraldehyde–OsO_4-fixed sections of the same tissue. Thus, it appears that the SFO neuropil is not suitable for examining the problem of the so-called flat vesicles and their relationship with inhibitory synapses (see Gray, this volume, p.141).

Some vesicles are seen from the inside, others from the outside. By examining a large number of vesicles one gains the impression that the outer membrane surface is somewhat smoother and contains fewer and smaller granules than the inner surface. Size and shape of granules found at the inner surface of the vesicular membrane are similar to those found at the outer side of the plasmalemma.

The size of the synaptic vesicles is of particular interest from the point of view of maximal storage capacity for the transmitter molecules (Whittaker and Sheridan, 1965). For this reason, preliminary measurements of vesicular diameters were taken, and histograms of unfixed as well as prefixed freeze-etched material could be compared with that obtained from classical aldehyde-osmium fixed sections. The range and peaks of the histograms were surprisingly similar. In unfixed freeze-etched specimens the range of synaptic vesicles (including the large dark-cored) varied between 300 and 1000 Å and the peak distribution was at 500 Å (Moor *et al.*, 1969). Thus far, dark-cored and clear vesicles can be differentiated on the basis of size only. Comparison of freeze-etched and conventional material makes it possible to define the range for diameters of dark-cored vesicles between 600 and 1100 Å. Obviously, there is an intermodal overlap between the large profiles of clear vesicles and the small profiles of the dark-cored vesicles.

(b) Plasmalemma of presynaptic terminals. Micropinocytosis

Examination of freeze-etched neuropil reveals that plasmalemmal surfaces vary with respect to basic texture and granulations. These differences can be partly attributed to different cell types, to differences between inside and outside surfaces within cell types and possibly to regional differences of the latter. The present material is sufficiently large to make some statements concerning the difference between outside and inside surfaces of the plasmalemma near and at the axon terminal.

Fig. 10. Freeze-etched preparation of presynaptic nerve terminal. Cat subfornical organ. ax = preterminal axon. Note the external surface (es) covered with scattered granules giving a *rough* appearance. Micropinocytosis (mp) at the left side. is = inner surface of neuronal (?) plasmalemma. The cut surface of the bouton contains spherical profiles of synaptic vesicles (sv) and mitochondrion (m). Synaptic cleft = sc. Postsynaptic element = po. This specimen was fixed with 3% buffered glutaraldehyde. Primary magnification 20 000 ×.

Fig. 11. Freeze-etched presynaptic nerve terminal in the cat subfornical organ. It contains numerous convex and concave profiles of synaptic vesicles (sv) and one mitochondrion (m). The larger profiles represent dense-cored vesicles (dv). At the left is presumably a synaptic contact, blurred by an uncoated (white) zone. po = postsynaptic density (note the finely granulated material). The outer surface (es) of the plasmalemma is visible at the right. This specimen was fixed with 3% buffered glutaraldehyde. Primary magnification 20 000 ×.

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mp
is
sv
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sv
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Fig. 13. Freeze-etched preparation of presynaptic nerve terminal. Cat subfornical organ. *This is an unfixed specimen*. Compare the outer rough surface of the plasmalemma (es) with that of fixed preparations in Figs. 8–10. The granules are larger and smoother. The profile of the bouton contains many large and small sized synaptic vesicles (sv). Arrow marks the site of a synaptic contact (sy). Note the *smooth* inner surface (is) of postsynaptic plasmalemma (dendrite?). Primary magnification 20 000 ×.

Fig. 12. Freeze-etched preparation of axosomatic synapse. Cat subfornical organ. The profile of the presynaptic terminal contains numerous spherical vesicles (sv). The synaptic cleft (sc) contains finely granulated material. The texture of the cytoplasm is particularly fine at the pre- and postsynaptic membranes, thus suggesting the site of appositional densities (arrows). Note the difference between the outer surface (es) of the bouton and the inner surface (is) of the plasmalemma of an adjacent element (neuron?). The latter is characterized by inward bulging pinocytotic vesicles (mp) and a small piece of adhering cytoplasm (cy). This specimen was fixed with 3% buffered glutaraldehyde. Primary magnification 20 000 ×.

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Figs. 10, 11 and 12 illustrate the *outer surface* of three different boutons terminaux. The same type of surface granulation is seen in all instances. These pictures were taken from aldehyde-fixed material. The granules appear somewhat larger and more flat-topped in the unfixed state (Fig. 13) and resemble those described by Bischoff and Moor (1967a, b) and Branton (1967) on the "rough surface" of Schwann cell and oligodendroglial plasmalemma. The second feature of the outer surface of nerve terminals are the round holes approximately 600 Å in diameter which can be identified as micropinocytotic vesicles in the stage of formation (Fig. 10). They seem to correspond in form and size to those of the capillary endothelial cells, but the latter occur in more densely and regularly arranged populations. The observation of pinocytosis on the plasmalemma of axon terminals is not new. Brightman (1967) has shown this phenomenon in conventional electronmicroscopy. Using peroxidase molecules as tracer for the transport processes, the same author has convincingly demonstrated that substances may be transported by pinocytotic activity from the interstitial space into the nerve terminal. The present study confirms these observations and adds further support to the notion that at least part of the synaptic vesicle population receives its membrane from the plasmalemma. Thus far, the pinocytotic activity of the plasmalemma has been observed in the non-synaptic sites of the terminal. On the other hand, Westrum's (1965b) investigations by means of conventional electronmicroscopy have revealed occasional vesicles at the presynaptic membrane. Present studies with the freeze-etching method are not sufficient to decide whether pinocytosis into or from the synaptic cleft is an important process.

(c) Synaptic cleft and membrane appositions

The synaptic cleft is clearly pictured in Figs. 10 and 12. The presence of a finely granular material within the cleft (Fig. 12) confirms the findings reported earlier in this communication. The presence of synaptic densities is more difficult to ascertain with freeze-etched material. Close examination of the postsynaptic area in Fig. 12 shows that the cytoplasmic texture is extremely fine within an area roughly corresponding to the zone of the postsynaptic membrane apposition. Even more difficulty exists in verifying the presence of the "vesicular grid". Evidently, more detailed studies of the freeze-etched presynaptic area are necessary before comparison and correlation with conventional EM data can be successfully accomplished.

SUMMARY

(1) A brief survey is given on the results obtained with the zinc iodide–OsO_4 impregnation of presynaptic and prejunctional nerve terminals. Synaptic vesicles were stained selectively and a positive correlation with cholinergic sites was demonstrated. Negative as well as positive results were obtained at adrenergic sites. The nature of the histochemical reaction remains to be further clarified.

(2) Impregnation of synaptic junctions with bismuth iodide and subsequent contrasting with uranyl acetate and lead hydroxide gave significant details on the presynaptic membrane and its densities (Gray). In adult animals, the presynaptic

dense projections form a *grid structure* with the dense spots hexagonally arranged and interconnected with filamentous cross-bridges. The free spaces (holes) of the grid can accommodate one single synaptic vesicle. Thus, the formation is called the "*presynaptic vesicular grid*". Plasticity and functionally significant variations of this structure may possibly occur. The same method provided important details on the outer and inner coats of the plasmalemma of the presynaptic terminal. Within the synaptic cleft a double layered subunit is described.

(3) Freeze-etching preparations of synapses confirmed the "true" existence and spheric shape of synaptic vesicles. It revealed important differences between the outer and inner surfaces of the plasmalemma of presynaptic terminals. Furthermore, the presence of micropinocytosis at the surface of boutons terminaux was demonstrated.

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