A NEW METHOD FOR STAINING SYNAPTIC VESICLES AT PERIPHERAL AND CENTRAL SYNAPSES.

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Zinc iodide-osmium mixture (1, 2) was used for the first time in electronmicroscopic studies of the peripheral and central nervous system (3). The mixture consists of a saturated solution of zinc iodide containing 0.4% osmic acid; it is applied directly to tiny blocks of fresh tissue. Thus far, perfusion fixation and attempts of aldehyde prefixation have failed to provide satisfactory results. Very disturbing artifacts derived from dissection procedures may render the whole technique rather hazardous and unpredictable. Nevertheless, the data obtained from the nerve endings in the following four types of tissue were consistent and satisfactory: 1. Diaphragm (motor endplate), 2. Subfornical organ, 3. Sphincter iridis, 4. Dilator iridis.

The reaction product consists of a homogeneous electron opaque material. It is found within synaptic vesicles which belong to the category of the so-called "clear vesicles". Thus far, the "granulated" or "dark-cored" vesicles did not show any reaction. It is noteworthy, that a varying number of the clear vesicles of all four regions remained equally unstained. Of particular significance is the observation, that the reaction within nerve endings is restricted to the synaptic vesicles and spares junctional membranes as well as mitochondria. The fact that reacting substances may be found in other types of tissues (e.g. neuroglia, Golgi apparatus of neurons and other cells) remains to be further elucidated. This problem is intimately related to the question of histochemical specificity of the stain.

Of the four regions under study the following three are known to contain exclusively or predominantly cholinergic endings, i.e. the motor endplate, the subfornical organ and the sphincter iridis. The synaptic vesicles of the respective terminals react consistently if not uniformly to the zinc iodide-osmium stain (Fig. 1-3). On the other hand, the dilator iridis, known to be predominantly innervated by adrenergic sympathetic nerve fibers, has terminals with only a minimal number of positively stained synaptic vesicles. These observations suggest a correlation between the zinc iodide-osmium stain and nerve endings with cholinergic vesicles. Yet conclusive evidence is at present not available.

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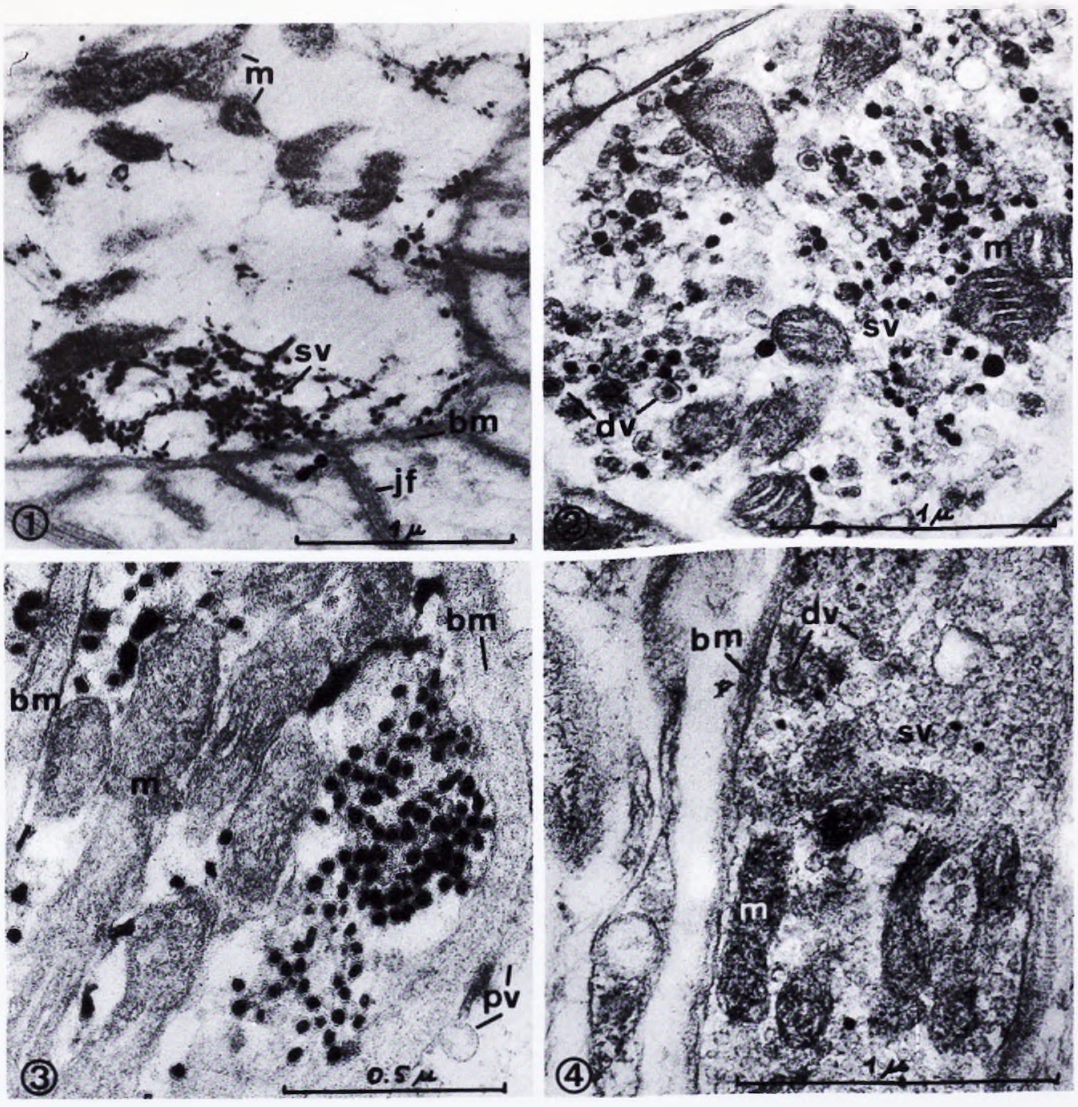

Fig. 1: Motor endplate. Mouse diaphragm. Synaptic vesicles (sv) are black. No reaction product is seen in mitochondria (m) cell membranes and basement membrane (bm) of junctional folds (jf).

Fig. 2: Nerve terminal in the cat subfornical organ, which contains a heterogeneous population of synaptic vesicles. Many but not all of the clear vesicles are stained black. The dark cored vesicles (dv) fail to show reaction. Mitochondria are equally indifferent.

Fig. 3: Nerve terminal in the sphincter iridis muscle of the cat. Many but not all synaptic vesicles are stained black. Note that the outer surface of the vesicular membrane is free of reaction product. pv = pinocytotic vesicles.

Fig. 4: Nerve ending in the dilator iridis muscle of the cat. Note the presence of clear vesicles and a few dark cored vesicles. Only few clear vesicles seem to have reacted to the stain. The dark cored vesicles remained indifferent.

AN ELECTRON-MICROSCOPIC STUDY OF ZINC IODIDE–OSMIUM IMPREGNATION OF NEURONS. I. STAINING OF SYNAPTIC VESICLES AT CHOLINERGIC JUNCTIONS*

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(Accepted October 20th, 1967)

INTRODUCTION

Maillet[10] has recently reviewed the light-microscopic and histochemical aspects of the zinc iodide–osmium (ZIO) stain; this is a useful technique to which he has substantially contributed. Its successful applicability in the realm of the autonomic nervous system has been confirmed by Jabonero[8], Taxi[16] and many others. Originally, it was thought that adrenergic structures are specifically responsive to ZIO impregnation. However, this hypothesis was soon disproved by Hillarp[7] by demonstrating that the basic histochemical reaction was unchanged after the administration of reserpine.

The present communication deals with the electron-microscopic analysis of the ZIO block stain, with special reference to its reaction in nerve endings. Specifically, the identification of impregnated structures was attempted and the problem of specificity of the histochemical reaction was approached.

METHODS

Two types of mammalian nerve tissues were examined in detail: (1) the subfornical organ; (2) the neuromuscular junction. Both were directly immersed into the ZIO mixture at room temperature and were impregnated for 16–18 h. Subsequently the tissues were rinsed in Palade buffer, dehydrated and embedded in Epon. Sections were prepared for phase contrast and electron microscopy with the aid of a Porter–Blum microtome. For electron microscopy the sections were stained with uranyl acetate[18] and lead hydroxide[9].

The subfornical organs of 8 cats were used. The tissue was carefully removed by microdissection under direct visual control. Muscle endplates were prepared from the diaphragm of 9 albino mice according to the technique described by Waser *et al.*[17].

* This material was first presented at a meeting of the Swiss Association of Anatomists at Basel on October 14, 1967.

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In addition, samples were taken from the ventral gray matter of spinal cord in cat and rat. All dissections were carried out while the animals were under nembutal anesthesia (35 mg/kg).

The ZIO mixture which gave the most satisfactory results is prepared as follows: solution A consists of 2% OsO_4 solution; solution B consists of 15 g of zinc

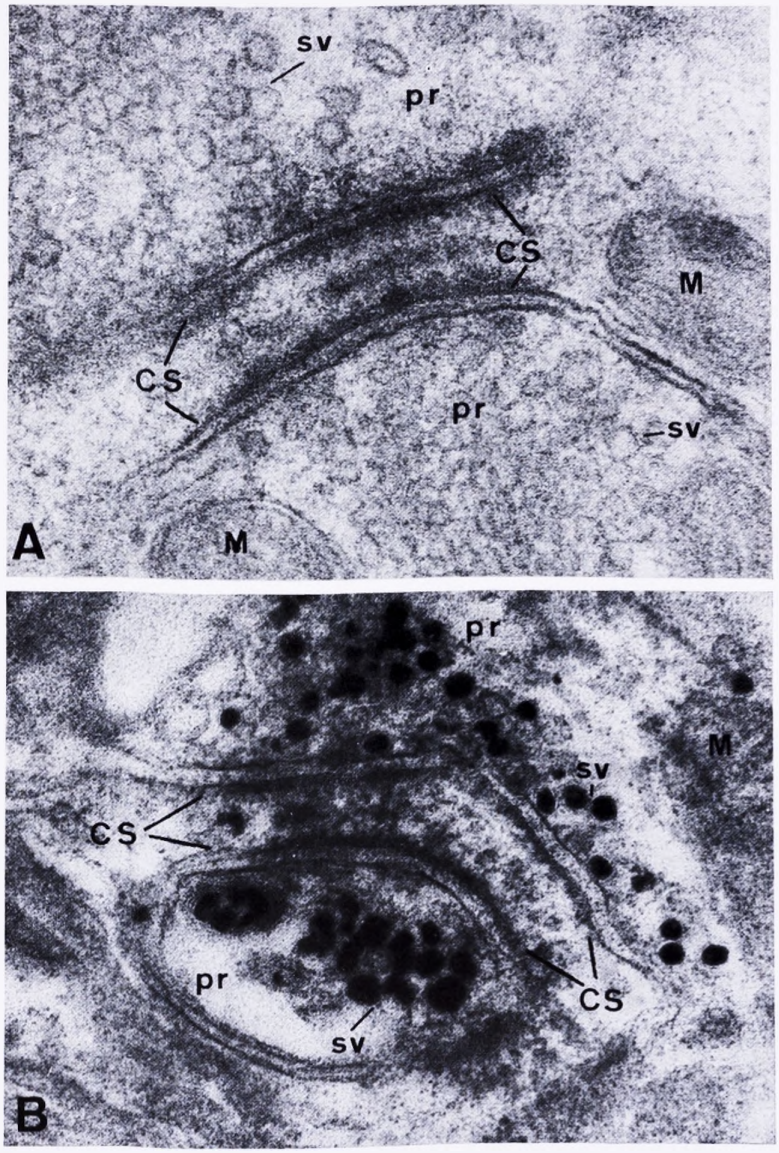

Fig. 1. Crest synapse (CS) with subsynaptic bodies in the subfornical organ (cat). A, Glutaraldehyde–OsO_4 fixation. B, Zinc iodide–osmium block staining. pr, presynaptic terminal; M, mitochondria; sv, synaptic vesicles. Primary magnification: 40,000 × for A; 20,000 × for B.

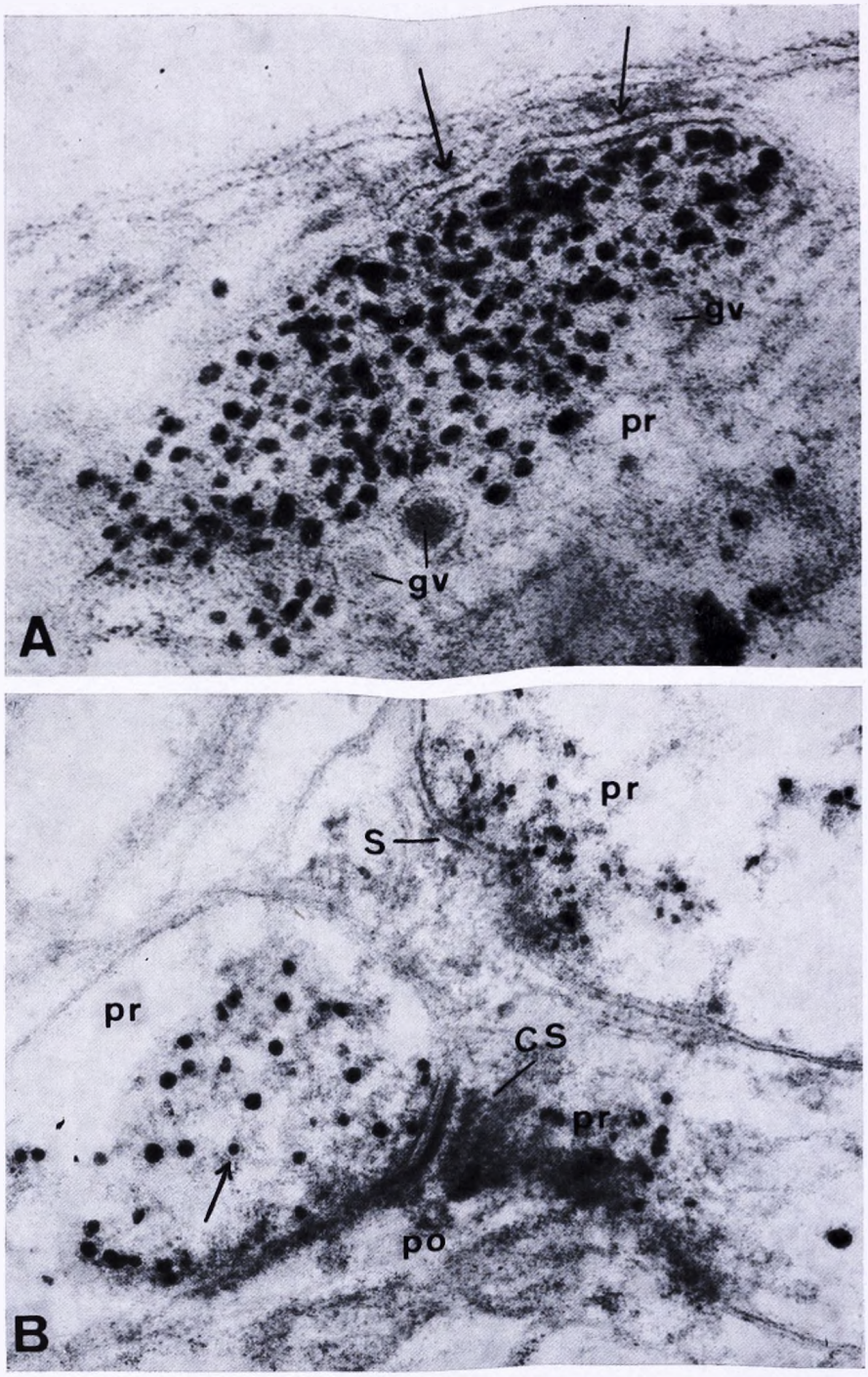

Fig. 2. nerve endings (pr) in the neuropil of the subfornical organ (cat) following zinc iodide–osmium stain. Note that the membranes of the conventional synapse (S) and the crest synapse (CS) failed to react. po = postsynaptic dendrite. A, Granulated vesicles (gv) are ZIO-negative. Arrows indicate possible synaptic sites. B, Arrow points to a stained vesicle whose outer surface of the limiting membrane is clearly free of reaction product. Primary magnification: 20,000 ×.

(powder) to which 5 g of iodine (cryst.) and 200 ml of distilled water are added; 6–8 ml of the filtered solution B is added to 2 ml of solution A shortly before use.

Control tissues were similarly obtained and fixed by immersion in glutar(di)-aldehyde (6.5%) and postfixation in OsO_4 (2%)[14]; the procedure is described in full elsewhere[3].

RESULTS

(1) Axon terminals in the neuropil of the subfornical organ

Recent electron-microscopic studies[2,3] have revealed a characteristic type of profiled junction representing about one third of all synapses in the mammalian subfornical organ (SFO). This junction—called *crest synapse with subsynaptic bodies*—is illustrated in Fig. 1A, which demonstrates a typical double synaptic plaque on a dendritic crest. The two presynaptic terminals contain clear vesicles of spheric type ranging from 300 to 800 Å in diameter. The same structure is pictured in Fig. 1B after ZIO impregnation. The junctional membranes are clearly visible, although they failed to react with the ZIO mixture. Equally indifferent is the row of subsynaptic bodies. Note that the mitochondrium in the presynaptic terminal also fails to show a positive reaction. The only structure which shows a clearcut impregnation is a conspicuous number of spheric profiles within the presynaptic terminals whose form and diameter correspond to that of synaptic vesicles.

Fig. 2 demonstrates the effect of ZIO block staining on individual SFO nerve terminals. Again, the synaptic vesicles are stained exclusively, while other synaptic organelles and the junctional membranes are reasonably well preserved but fail to show any reaction. In Fig. 2A a number of dark-core vesicles can be identified. These vesicles have a diameter of 800–1500 Å and are likely to be identical with those described in normal SFO[2] and found to be reserpine-resistant. *It is noteworthy that these granulated vesicles consistently failed to react with the ZIO mixture.*

Fig. 3 illustrates a group of less well preserved nerve endings in the SFO. Clearly, this tissue is edematous. Yet, the main interest of this picture lies in the fact that the outer dense layer of the vesicular membrane can be clearly distinguished in several instances, and that it does not in itself seem to undergo a positive reaction. The inner layer, on the other hand, coalesces with the reaction product of the vesicular content.

(2) Axon terminals at myoneural junctions

Motor endplates of mouse diaphragm were investigated with the same technique. Due to the positive reaction of nerve terminals, the endplates were found more readily than in normal control material. Fig. 4 depicts two examples. Junctional sites are clearly identified on the basis of junctional folds. In both photographs the nerve terminals contain synaptic vesicles having the black appearance shown in the SFO. It is to be noted that neither the junctional membranes nor the basement membrane of the junctional folds show any ZIO reaction.

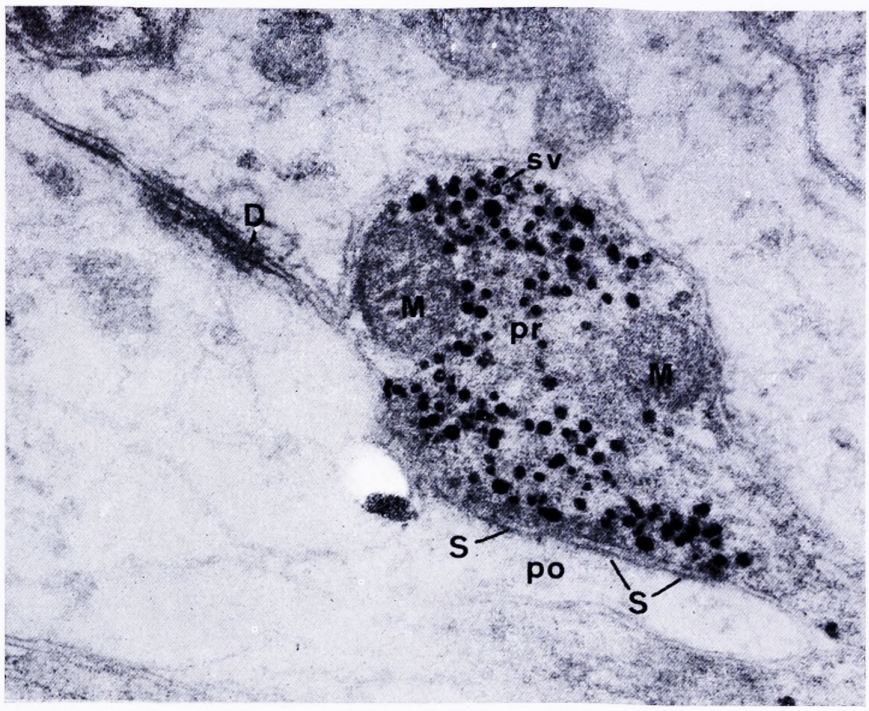

Fig. 3. Nerve ending in the neuropil of the subfornical organ (cat) following application of zinc iodide–osmium (ZIO) stain. Note the impregnation of synaptic vesicles (sv) and the sparing of membranes at S (possible synaptic sites), and D (desmosome-like junction). Mitochondria (M) remain indifferent. Also note the lack of precipitation at the outer surface of ZIO-positive vesicles. Primary magnification: 20,000 ×.

DISCUSSION

(1) The zinc iodide–osmium impregnation of nerve terminals is based on its affinity for synaptic vesicles

The main result of this study is the fact that the ZIO mixture specifically impregnates synaptic vesicles. This has been established in two different tissues: the SFO and the myoneural junction. Aside from the vesicles no other structure of the junctional region gave signs of a positive reaction. It will be necessary to verify this affinity of ZIO mixture to vesicles in many more kinds of terminal network and in different animal species in order to establish the general applicability and limitation of this method.

Fig. 5 shows a light-microscopic picture of the ZIO stain of the subfornical organ. Aside from a considerable black precipitation within the ependymal brush border it shows a fairly satisfactory impregnation of nerve terminals within the SFO neuropil. The ependymal cells are free of pericellular nerve endings, a fact which is corroborated by electron-microscopic observations. On the other hand, the stain

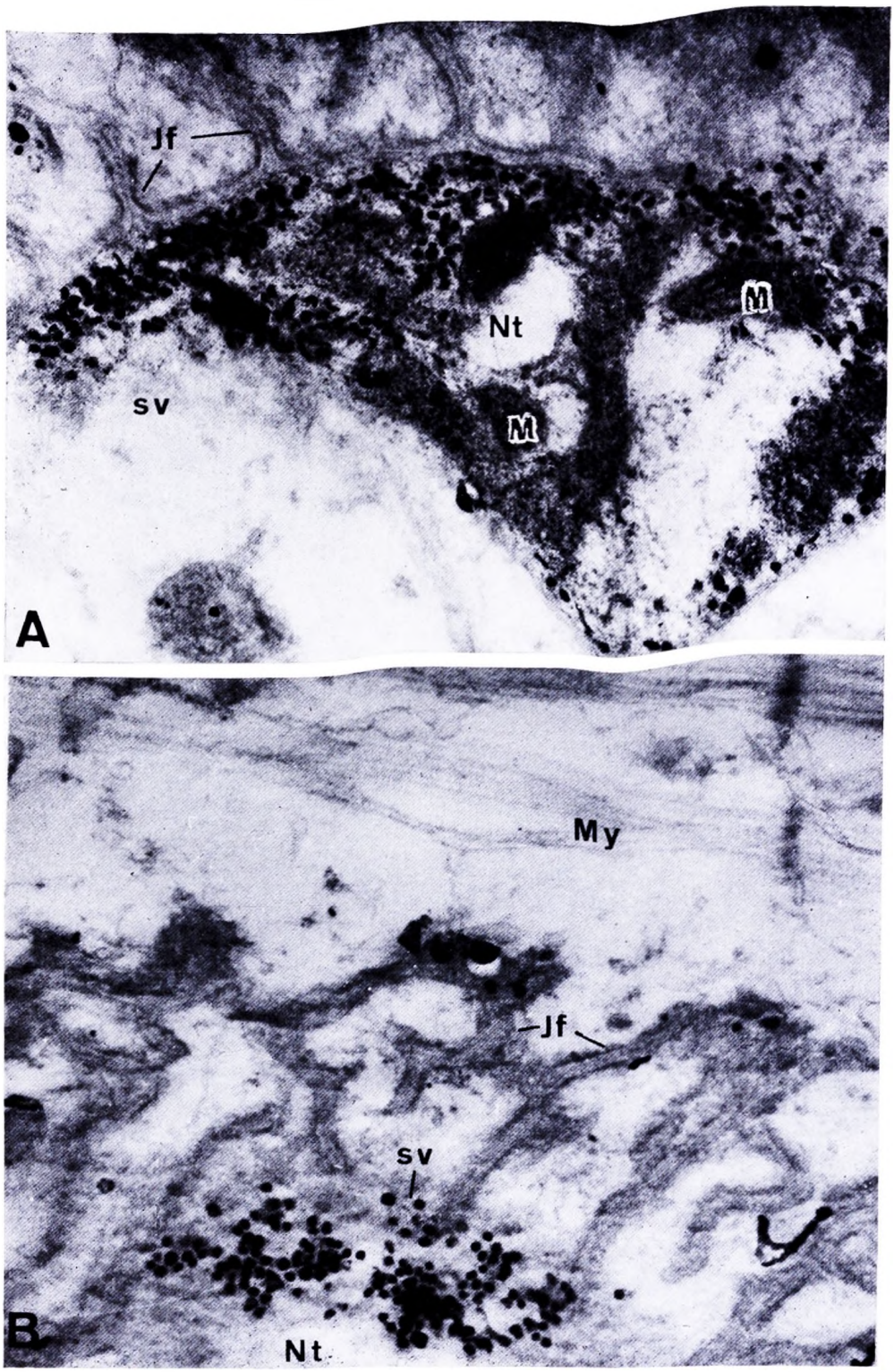

Fig. 4. Motor endplates of mouse diaphragm following application of zinc iodide–osmium stain. Jf, junctional folds; M, mitochondria; My, myofibrils; Nt, nerve terminal. Note the selective staining of synaptic vesicles (sv) in A and B. Primary magnification: 20,000 ×. Of considerable importance is the indifference of junctional membranes and cleft to the ZIO stain.

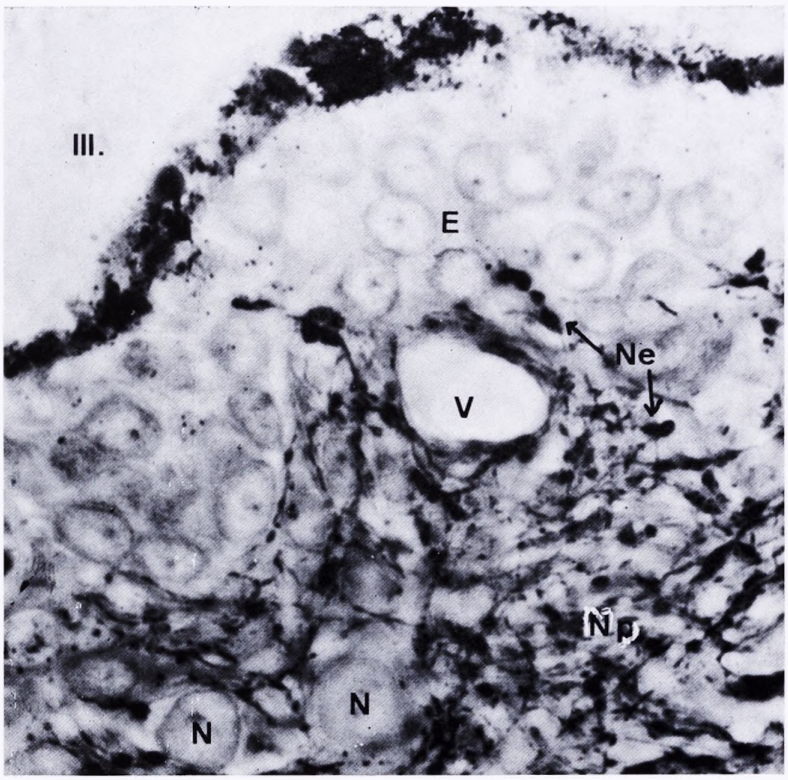

Fig. 5. Subfornical organ of cat after block staining with zinc iodide–osmium. Phase contrast (630 ×). Note the selective staining of nerve terminals. III, third ventricle; E, ependyma; N, neuron; Ne, nerve ending; Np, neuropil; V, 'vacuole'.

seems to impregnate the enlarged terminal boutons, boutons en passage (varicosities) and preterminal axons. Correlation with present electron-microscopic findings are readily established with the exception of the positive staining of preterminal axons. The electron-microscopic substrate of this latter reaction seems to be identical with extremely fine granular precipitations of electron-dense material extending along fibrillar strands in parallel with the longitudinal axis of the axon; it will be dealt with in a subsequent communication.

The light-microscopic appearance of ZIO stain reminds one of other successful stains for nerve terminals. The electron microscope has revealed that presynaptic endings generally consist of 3 main structural elements: mitochondria, neurofilaments and vesicles. It is interesting to note that the ZIO stain depicts the latter, whereas the Bielschowsky type silver impregnation methods selectionate the neurofibrils (Gray and Guillery[6]) and the well known 'synaptic stain' of Rasmussen[12] and Armstrong *et al.*[4] are likely to be mainly concerned with mitochondria. Obviously, of the 3 methods, the ZIO stain appears to be the most specific in the sense that the vesicles are more specific synaptic organelles than are neurofilaments and

mitochondria. Thus, the successful applicability of ZIO stain for the light-microscopic demonstration of normal nerve terminals has now been confirmed by the electron microscope.

(2) The problem of histochemical specificity of vesicular content

Even though the chemical composition of their content is still insufficiently known, it is generally assumed that synaptic vesicles represent the reservoir of transmitter substances[11]. In fact, there is growing evidence that granulated vesicles are associated with catecholamines while clear vesicles are said to contain acetylcholine or certain amino acids[5,13,19,21].

Does the application of ZIO stain cast new light upon these problems? First, it is necessary to examine the proof that the ZIO stain is concerned with the content of vesicles rather than with the coating of their surface. The present material seems to bear this out since the ZIO reaction product is clearly found within the vesicular profiles and since the outer layer of their limiting membrane seems to be consistently clear of electron-dense precipitations (Figs. 2B, 3). An analogous finding was recently presented by Whittaker[20] in cerebral cortical synapses; he found an electron-transparent core within otherwise agranular vesicles following treatment with gold chloride and negative staining. The relationship between this observation and the ones presented in this study remains to be clarified.

The next question is concerned with the relationship of the ZIO stain with

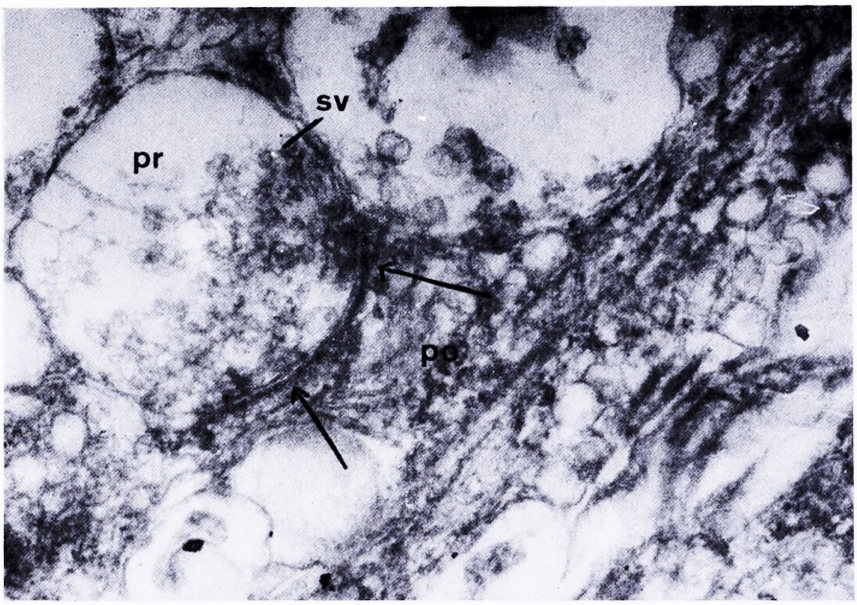

Fig. 6. Cat spinal cord, ventral gray matter following zinc iodide-osmium block staining. The tissue is not well preserved. Yet, one nerve terminal (pr) with synaptic plaque (arrows) can be readily recognized. The synaptic vesicles (sv) are of the clear spheric type; they failed to react to the stain. po, postsynaptic element. Primary magnification: 20,000 ×.

cholinergic mechanisms. The SFO, on the basis of a high acetylcholinesterase content[1,15] and of the responsiveness of some of its cells to electrophoretically applied acetylcholine (Akert, Pfenninger and Villoz, unpublished), may well contain cholinergic synapses. Much stronger and well known evidence exists in favor of the presence of acetylcholine at the myoneural junction—even though its localization within synaptic vesicles is equally hypothetical. From these considerations it becomes evident that the relationship between the ZIO vesicular stain and cholinergic transmitter mechanisms is not inconceivable, yet far from being established.

The fact that certain types of synaptic vesicles have been found to resist impregnation seems of added significance since it lends further support to the notion of specificity of the ZIO stain. One category of *ZIO-negative elements* consists of relatively large, dark-cored vesicles (Fig. 2A). Even though the chemical nature of their granules is unknown, it may be safely assumed that they differ in morphology and chemistry from the agranular ZIO-positive vesicles. A second category seems to include agranular vesicles which may not differ significantly with respect to shape from the ZIO-positive elements. These were found in samples from the ventral gray of the cat spinal cord. Fig. 6 depicts a spinal synapse with a presynaptic terminal containing vesicles of the agranular type which have failed to react with the ZIO mixture. Conceivably, this negative result may be partly due to insufficient preservation of the neuropil. However, the presence of positively staining vesicles in adjacent endbulbs would seem to weaken this argument. Large-scale studies on vesicles in non-cholinergic synaptic sites as well as experiments with cholinergic blocking agents are now under way and expected to further clarify the problem of histochemical specificity.

SUMMARY

The structural basis of the affinity of zinc iodide–osmium stain to nerve terminals was examined with the aid of the electron microscope at the mammalian subfornical organ and myoneural junction. It turned out that this affinity is based on the selective staining of synaptic vesicles, whose content forms an electron-opaque reaction product. The synaptic membranes, the basement membranes of junctional folds and the mitochondria remain indifferent. Certain types of synaptic vesicles gave equally negative results: (1) 800–1500 Å dark-cored vesicles in nerve terminals of the subfornical organ and (2) large populations of agranular spheric vesicles within spinal cord nerve terminals. These data may indicate some degree of specificity with respect to vesicular content. The possible relationship of this staining method to mechanisms of cholinergic transmission is mentioned.

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P. G. Waser, Zurich, were of considerable help to this project. Prof. G. E. Gray, London, kindly provided electron micrographs which enabled us to compare the results of his attempts at vesicular staining methods and proved useful in evaluating our own results.

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CONTRIBUTION TO THE PROBLEM OF STRUCTURAL ORGANIZATION OF THE PRESYNAPTIC AREA

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INTRODUCTION

Gray[5] first described a set of specialized presynaptic structures which he named 'dense projections'. They consist of an array of electron-dense bodies which are 'regularly spaced about 1000 Å apart along the presynaptic membrane' (ref. 7); they are more clearly visible in phosphotungstic acid (PTA)-stained material than in conventional glutaraldehyde–OsO_4 fixed material. Aghajanian and Bloom[1] have drawn attention to the possibility that these dense projections are not fully developed in junctional complexes of the immature brain and postulated a sequence of developmental stages in the morphogenesis of synapses in which changes of dense projections may furnish important criteria. Gray[7] has presented pictures suggesting that they might possibly 'guide synaptic vesicles to special localities of the presynaptic membrane'. Yet, no definitive role could be assigned to the dense projections. The present communication deals primarily with additional morphological and histochemical features of presynaptic dense projections and further emphasizes their relationship with synaptic vesicles; these results are primarily based upon impregnation effects of heavy metals (bismuth, lead and uranium) which in the proposed combination have not thus far been utilized.

METHODS

Synaptic junctions were studied in the subfornical organ[2,3] of cats, according to the following procedure: The subfornical organ is dissected with utmost care and immersed into a 6.5% glutaraldehyde solution (buffered at pH 7.4 with Palade buffer) for 2 h at room temperature. After careful washing for 6 h (4°C) in a solution containing Palade buffer the tissue block is impregnated in the following mixture: 0.5 g $BiCO_3$, 2.5 g KI, 50 ml 2 *N* formic acid, which is to be heated at approx. 50°C and filtered. It is believed that a complex consisting of bismuth and iodide is acting as staining reagent. Therefore, we will use the abbreviation B–I for this method. Impregnation of very small tissue blocks with this B–I mixture takes place at 4°C and

lasts for 12–18 h. Subsequently the tissue is dehydrated (starting in 70% ethanol) and embedded in Epon 812 (ref. 9). Ultrathin sections are stained with uranyl acetate[11] and lead hydroxide (Karnovsky[8]; Method B). The rest of the procedure is standard and has been described in detail elsewhere[3]. Control sections were prepared from glutaraldehyde–OsO_4 fixed tissue[10] as well as after phosphotungstic acid (PTA) block staining[1].

RESULTS

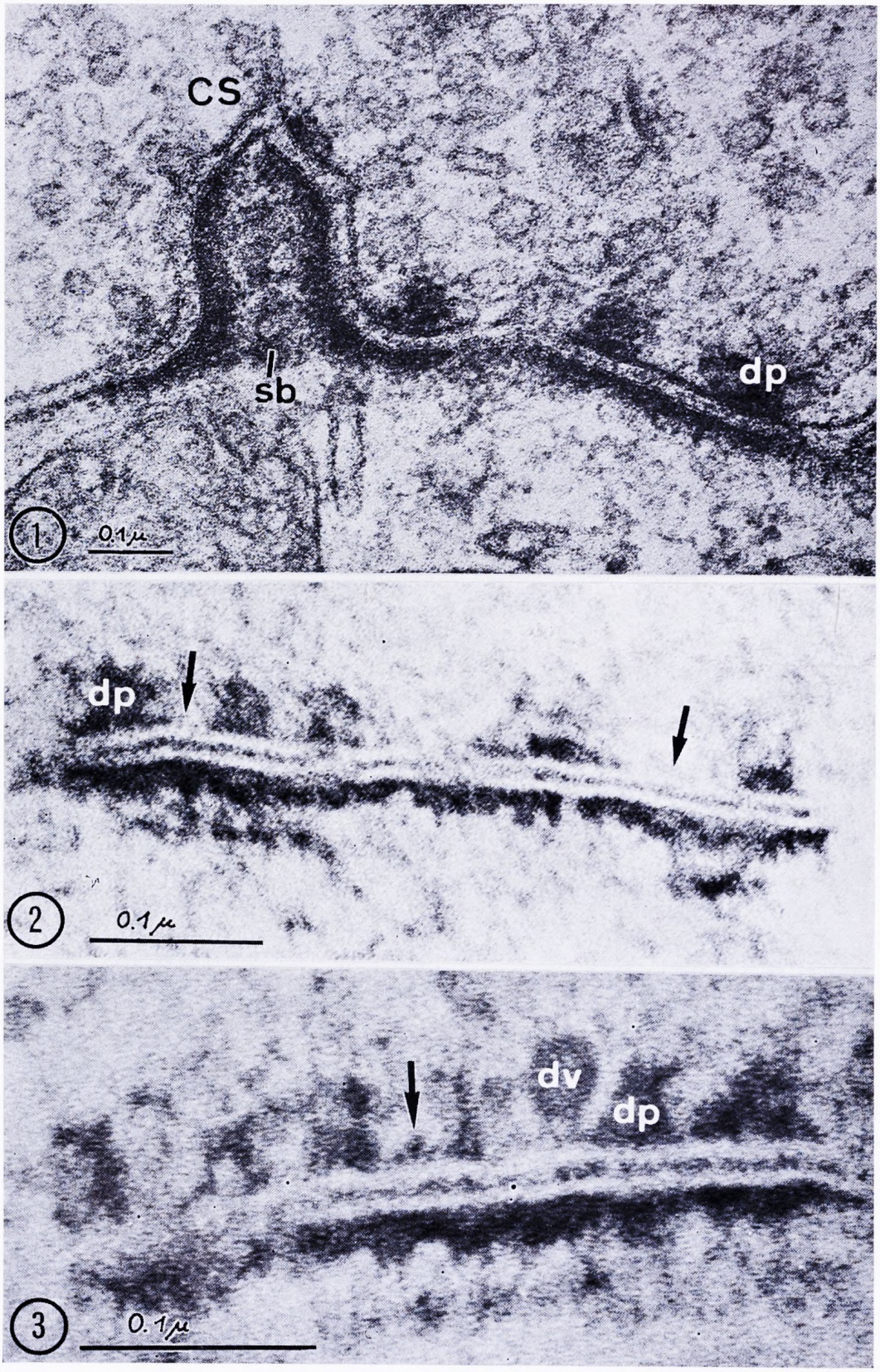
Presynaptic dense projections

Figs. 1, 2 and 3 represent synaptic junctions as visualized in control sections prepared with conventional methods. Presynaptic dense projections are seen in glutaraldehyde–OsO_4-fixed material (Fig. 1) provided that additional heavy metals (uranium and lead) are used for the contrasting of sections. A more selective impregnation is obtained with glutaraldehyde fixation and PTA block staining (Figs. 2 and 3); similar pictures have been published by Aghajanian and Bloom[1]. The synaptic membranes are spared but a medial dense layer is visible within the cleft. Note that the dense projections seem to be separated in some places (Fig. 2), and in others they appear linked by discrete filamentous extensions along the presynaptic membrane (Fig. 3).

Figs. 4, 5 and 6 show similar junctions after treatment with the new heavy metal staining technique. At first, the effect seems quite similar to that seen after PTA treatment, *e.g.* the clear differentiation between osmiophilic membranes (which are spared) and appositional densities (which are heavily impregnated). However, several improvements are worth mentioning: (1) More intense impregnation and clearer differentiation of synaptic cleft, vesicles and membrane appositions have been accomplished. (2) In contrast to Fig. 2 and in agreement with Fig. 3 it is evident that thin filaments seem to interconnect the base of dense projections in many instances; however, in other places a clear separation between peaks exists. (3) The presynaptic dense projections seem to derive their spiny and polyhedric profiles partly from the impressions of adjacent synaptic vesicles (Figs. 4 and 6).

The dimensions of presynaptic dense projections are: diameter approx. 550 Å, height approx. 600 Å. The fact that the synaptic cleft seems to contain *two separate* lines (Fig. 6) will be described and discussed in more detail in a subsequent paper[4].

Of particular interest are sections oriented in parallel with the synaptic membrane. Figs. 7 and 9 represent the profile of a nerve terminal at the level of and parallel to the presynaptic membrane; it contains a cluster of dense bodies forming the nodal points of a hexagonal network which is coextensive with the junctional area. Six outer dense projections and one in the center are interconnected by tiny filaments. The free spaces ('holes') of the network are nearly round and form a rosette (Fig. 8). The center-to-center distance between dense projections is approx. 800 Å. A similar but slightly more oblique view has been presented by Gray[6].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Synaptic vesicles

Treatment with heavy metals results in a differential reaction of clear *versus* large dark-cored vesicles in synaptic endings. The former are not clearly recognizable in the non-osmicated PTA-stained material (Fig. 3) and in B–I preparations. They seem to be spared and their negative images stand out as hollow spheric profiles within a finely granulated electron-dense matrix (Fig. 6), while the dark larger vesicles are readily identified on the basis of their impregnated core (Figs. 3, 6).

The reaction of the core is similar to that of the synaptic membrane appositions but less intense. Several dark-cored vesicles (1000–1500 Å) are pictured in Fig. 6. It seems that the osmiophilic layers of the vesicular membranes failed to be stained.

The close vicinity between synaptic vesicles and dense projections is remarkable. Fig. 3 shows a large dark-cored vesicle situated in the gap between two presynaptic dense projections and Fig. 6 illustrates a dark-cored vesicle in direct contact with the peak of a dense projection. Similar spatial relations exist with respect to clear vesicles as distinctly demonstrated by previous investigators[7,12]. They are dealt with in more detail in a subsequent paper.

DISCUSSION

The present findings on B–I staining of presynaptic dense projections corroborate the pioneering observation of Gray[5–7]. It seems that these dense projections are arranged within a coherent network or lattice. The geometrical pattern is that of a hexagon in which the dense projections occupy the nodal points and form polyhedric peaks oriented towards the cytoplasm of the nerve terminal.

One of the problems raised by Aghajanian and Bloom[1] concerns the connectivity between dense projections and their relationship to the presynaptic membrane. From Gray's[7] observations one is led to conclude that the dense projections might consist of isolated bodies 'from which fine filaments radiate and curve outward'[7], each center being individually attached to the presynaptic membrane. A somewhat different interpretation was reached by Aghajanian and Bloom[1] who prepared *non-osmicated* tissues with PTA block staining. This method enables one to distinguish more clearly between synaptic membranes and membrane appositions as pointed out before. Aghajanian and Bloom[1] demonstrated that presynaptic dense projections are often interconnected at the base by a thin dense line and

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Fig. 1. Crest synapse (CS) with subjunctional bodies (sb) in the subfornical organ of the cat. Presynaptic dense projections (dp) are clearly seen. Glutaraldehyde–OsO_4 fixation. Primary magnification: 40,000 ×.

Fig. 2. Synapse in cat subfornical organ. Note presynaptic dense projections (dp). Arrows point at holes between two peaks. PTA block staining (without the use of OsO_4). Primary magnification: 40,000 ×.

Fig. 3. Synapse in cat subfornical organ. Note dense-cored vesicle (dv) between peaks. Arrow points to filament connecting dense projections near the presynaptic membrane. PTA block staining as in Fig. 2. Primary magnification: 40,000 ×.

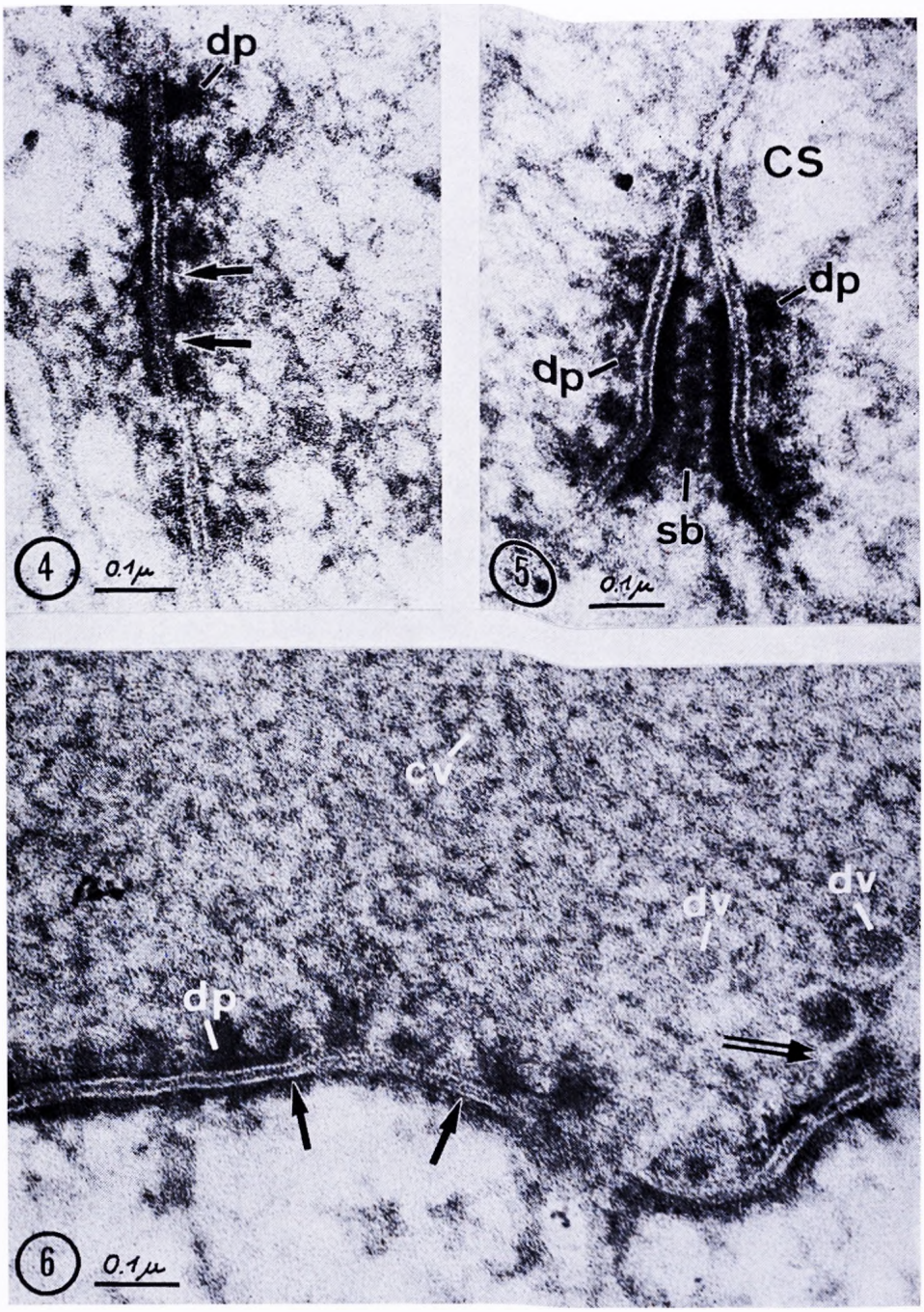

Fig. 4. Synapse in cat subfornical organ. Note presynaptic dense projections (dp). Arrows at the connecting filaments. There is a hole between second and third peak from above. Note also the double 'intracleft' line. B–I block impregnation. Primary magnification: 40,000 ×.

Fig. 5. Crest synapse (CS) with subjunctional bodies (sb) in cat subfornical organ[2,3]. Presynaptic dense projections (dp) are seen at both synaptic sites. B–I block impregnation. Primary magnification: 40,000 ×.

Fig. 6. Large synapse in cat subfornical organ. Note presynaptic dense projections (dp), double 'intracleft' line (arrows) and large dark-cored synaptic vesicles (dv). Note also the proximity between peak and dark-cored vesicles (double arrow). The clear synaptic vesicles (cv) are spared by the stain. B–I block impregnation. Primary magnification: 40,000 ×.

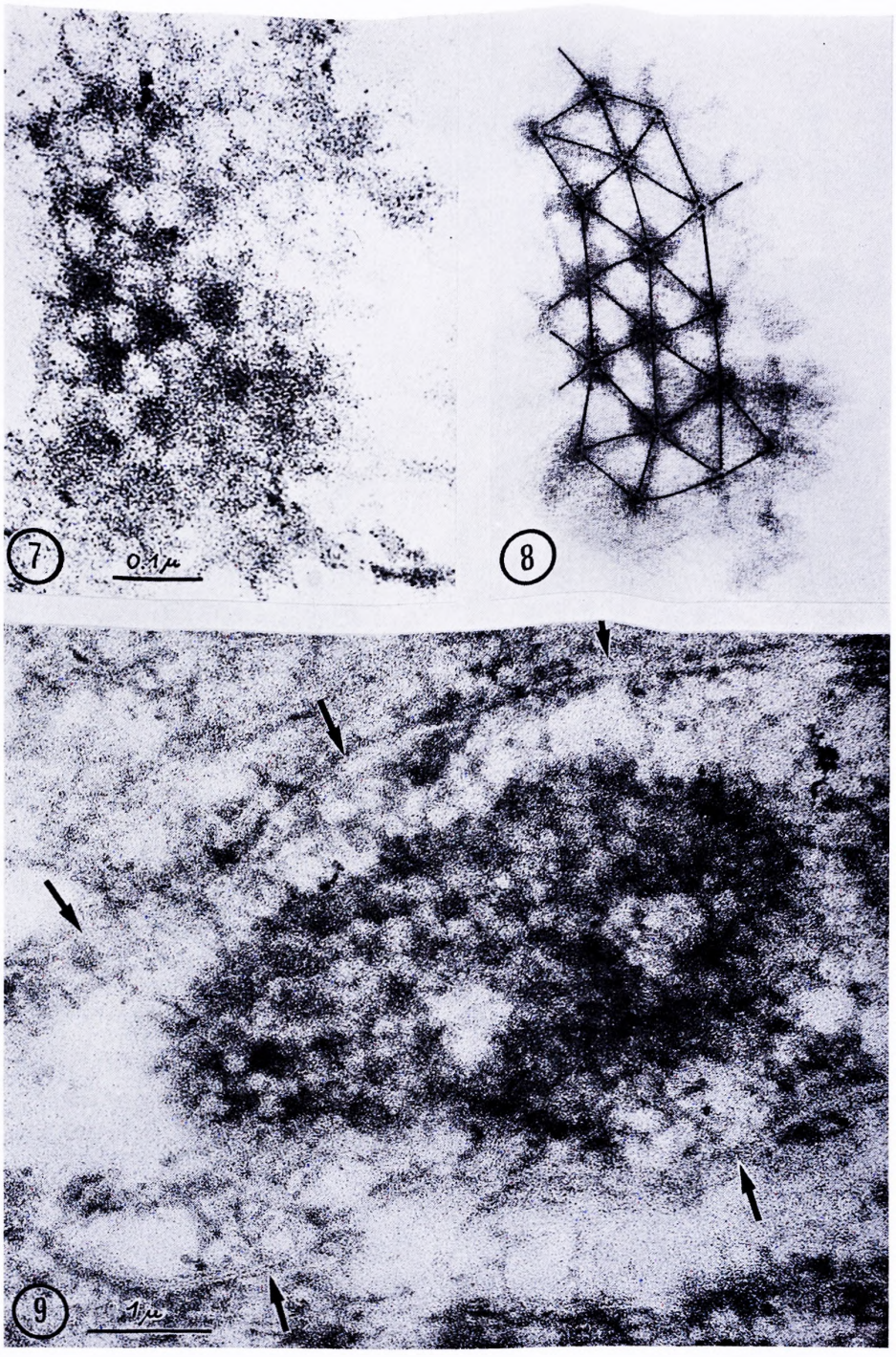

Fig. 7. Tangential section through presynaptic area. A section of the hexagonal peak-and-hole pattern of the *presynaptic grid* is clearly represented. Original photograph. B–I block impregnation. Primary magnification: 40,000 ×.

Fig. 8. Interpretative drawing of photograph in Fig. 7.

Fig. 9. Tangential (and slightly tilted) section through presynaptic site. The entire plaque and especially the *presynaptic grid* seems contained within the section. Arrows indicate approx. location of cytoplasmic membrane of nerve terminal. B–I block impregnation. Primary magnification: 40,000 ×.

suggested that the dense projections form peaks of a plate-like structure lining the presynaptic membrane. According to these authors the dense projections seem hardly distinct from the appositional density at early developmental stages and individualization of the peaks may take place with advanced maturation.

Our own findings may clarify and extend these earlier observations and interpretations. For it seems that the mature presynaptic region consists of a grilled plate with peaks and holes, the former being linked together by means of tiny filamentous extensions. Depending upon the plane of sectioning across the synaptic area one may encounter free holes between peaks or connecting dense lines. Considering the thickness of the sections it is obvious that profiles cut perpendicularly to the presynaptic membranes (Fig. 6) are ill suited to disclose the relatively fine pattern of alternating peaks, holes and filaments whose dimensions are less than 1000 Å; an additional complication is the presence of the densities within the gaps derived from synaptic vesicles which are often encountered in this region and may blurr or simulate truly existing links between dense projections. A final handicap of cross sections is that they portray the regular intervals between peaks only in those rare instances where the sections are oriented in parallel with an axis of the lattice.

The best view of the marvellous regularity and differentiation of the presynaptic area is obtained from *tangential sections* as already demonstrated by Gray[6]. Many thick and thin sections cut at varying angles were necessary to ascertain the geometrical arrangement of peaks, the presence of connecting filaments and holes as represented in the tridimensional diagram in Fig. 10.

The present interpretation does by no means contradict the developmental hypothesis of Aghajanian and Bloom[1]. *However, it suggests that the peaks of dense projections remain interconnected even at the time when maturation is reached.* It is reasonable to suppose that both peaks and filaments develop from the homogeneous appositional matrix which seems to cover the junctional membranes at an earlier stage. Nevertheless, it remains for further studies to decide whether the formation of holes represents a typical feature of synaptogenesis. Perhaps these holes — by trapping synaptic vesicles — may play an important role in the process of synaptic transmission and the dynamics of vesicles. More on this point will be presented elsewhere[4].

A final comment concerns the relations between dark cored vesicles and presynaptic dense projections which seem to have gone largely unnoticed in the past. Two findings have evolved from the present study: (1) the similar appearance of appositional densities and vesicular core material[1] after impregnation with B–I mixture as well as with PTA; (2) the proximity which occurs not infrequently between dense projections and dark-cored vesicles. In combining the two findings one is tempted to speculate *that dark-cored vesicles might be involved in the transport and deposition of dense material at the presynaptic membrane*. However, it should be pointed out that the two points mentioned are disputable to some degree. The histochemical reactions of the two structures are not consistently identical with respect to various substances: *e.g.* $KMnO_4$ fixation seems to affect them differentially by impregnating vesicular cores and sparing dense projections; even by using the conventional

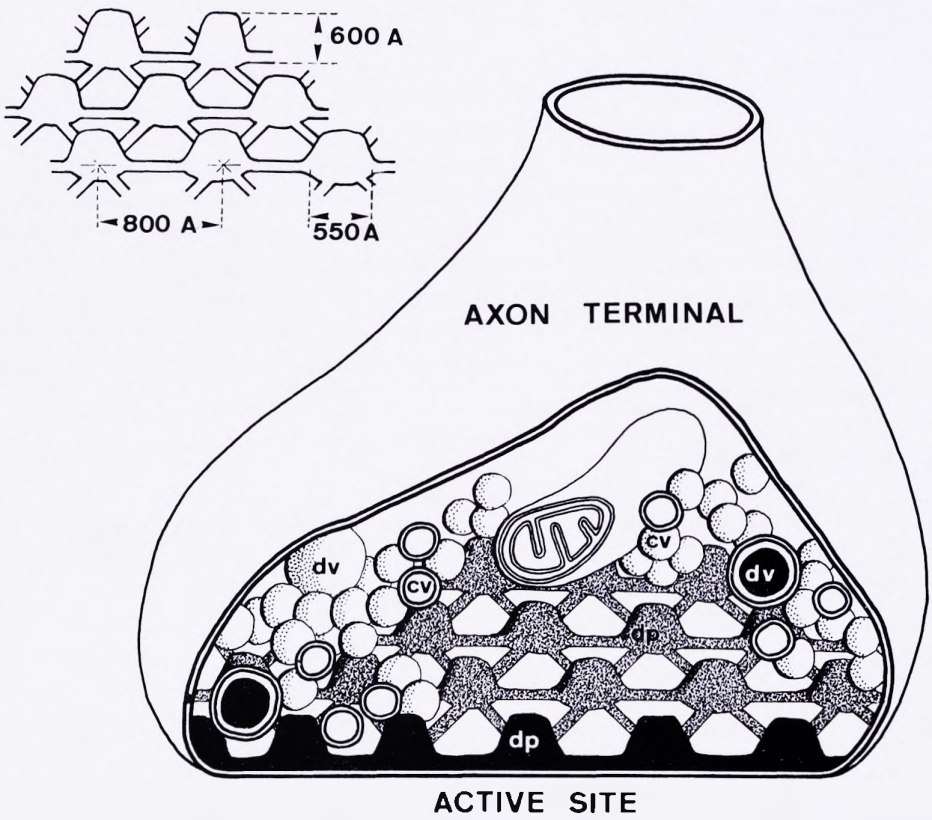

Fig. 10. Three-dimensional reconstruction of presynaptic area based upon many sections at varying angles. The hexagonal peak-and-hole pattern is demonstrated. The proportions are slightly distorted for clarification of concept. The exact dimensions are represented in the inset (upper left). Abbreviations same as in Fig. 6.

glutaraldehyde–OsO_4 fixation which impregnates both structures, one may notice a small but definite difference in opacity. On the other hand, such differential staining reactions are not sufficient to reject the hypothesis since the material which is transported by the vesicles could change its chemical constitution before reaching its final destination at the synaptic membrane. With respect to the second point it may be argued that the presence of dense projections is not consistently associated with that of dark-cored vesicles, *e.g.* myoneural junctions of smooth muscles seem to be lacking of appositional densities, yet the nerve terminals are known to contain dark-cored vesicles; the reverse situation is encountered at the postsynaptic side of regular synapses. Thus, attractive as the new 'transport' hypothesis of dark-cored vesicles may be, it seems that other possible relationships between synaptic vesicles and the presynaptic grill plate may be equally promising for future studies.

SUMMARY

A new method of synaptic staining has been developed by using bismuth–iodide block impregnation combined with uranyl acetate and lead hydroxide contrast. Synaptic junctions of the cat subfornical organ have been studied in detail with this procedure. Special emphasis was placed upon the spatial organization of presynaptic dense projections of Gray which consist of a grid with hexagonal pattern of peaks, interconnecting filaments and holes. A three-dimensional reconstruction of the presynaptic area is presented. The new impregnation spares the 'clear' vesicles but impregnates the large dark-cored vesicles (1000–1500 Å) rather well. The similar appearance of vesicular cores and presynaptic dense projections as well as their occasional proximity is discussed with respect to functional relationships between the two structures.

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