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SYNAPTIC FINE STRUCTURE AND NEURAL DYNAMICS*

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Even though present knowledge on the fine structure of synapses is still in a relatively crude stage, it seems that electron microscopic research has in recent years succeeded in establishing certain correlates of bioelectrical and biochemical phenomena associated with synaptic events. A good example is the finding of synaptic vesicles [7, 13] which seem to furnish a suitable structural basis for chemical transmission processes. On the other hand, there are electron microscopic observations whose functional counterparts are painfully missing, the most conspicuous being the appositional densities at pre- and postsynaptic membranes [11, 15].

Our own interest in synaptic fine structure was aroused by the discovery of peculiar synapses in the mammalian subfornical organ [2, 3] and the successful application of zinc-iodide-osmium stain to its afferent innervation [4]. It soon became apparent that iodide compounds either alone or in combination with heavy metals may provide useful contrast to certain synaptic structures. A short account on the work done in our laboratory during the last three years may demonstrate that improved morphological techniques may help in the detection of details near the threshold of "just noticeable structural differences" that are likely to accompany the various categories of synaptic events. Special attention will be given to the organization of the presynaptic area and to the problem of membrane subunits vesting the synaptic cleft.

METHODS

Staining Procedures

Synaptic and nonsynaptic junctions were studied in the subfornical organ of cats according to the following procedures:

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 hours at room temperature. After careful washing for 6

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hours (4°C) in Palade buffer the tissue block is impregnated in the following mixture: 0.5 gm of $BiCO_3$, 2.5 gm of KI, 50 ml of formic acid (2 *N*). This mixture is heated at approximately 50°C and filtered before use. It is believed that a complex consisting of bismuth and iodide $[BiI_4]^-$ acts as staining reagent. The abbreviation B-I is used to designate this method. Impregnation of very small tissue blocks with B-I mixture is carried out at 4°C for 12–18 hours. Subsequently the tissue is dehydrated (starting in 70% ethanol) and embedded in Epon 812. Ultrathin sections are stained with uranyl acetate and lead hydroxide. The rest of the procedure is standard and has been described in detail elsewhere [2].

In a second series of experiments, "synaptosomes" were obtained by fractionation and compartmentalization by density gradient according to Whittaker *et al.* [17] with slight modifications. Homogenized rat cortex was used. The synaptosome fraction was subjected to the impregnation methods described above and prepared for electron microscopic examination in an analogous manner.

Control sections of SFO tissue blocks and rat forebrain synaptosome fractions were prepared from glutaraldehyde–OsO_4-fixed material as well as after phosphotungstic acid block staining according to Aghajanian and Bloom [1].

Additional controls were made by subjecting the B-I impregnated material to a 2% OsO_4 solution in 0.1 Veronal acetate buffer and 6.8% sucrose (pH 7.4) for 90 minutes at 4°C. Prior to osmication the tissue was washed in 0.1 *M* acetate buffer (pH 3.5–4.0) for 90 minutes at 4°C and subsequently placed into 0.1 *M* Veronal acetate buffer with 6.8% sucrose (pH 7.4) for 30 minutes at 4°C. This combined procedure is designated B-I/OsO_4.

Measuring Procedures

The material consisted of photographic plates taken at 40,000 and 80,000 ×. These plates were examined in a stereomicroscope at 20 ×, and distances were measured with the aid of a micrometer ocular. The following measurements were taken: width across the two intracleft "iodophilic" lines; inner and outer diameter across the junction in osmicated sections; and distance between outer bounds of "iodophilic" coat lining the cytoplasmic surface of cell membranes.

Since the two impregnation methods exert a differential influence upon the tissue, the respective measurements are not directly comparable. For this reason a correction procedure was applied to the values obtained with B-I method.

A theoretical "shrinkage-factor" (sf_T) was calculated from the

quotient of total synaptic widths (W) measured on the basis of the two sets of staining material:

$$sf_T = \frac{W_{OsO_4}}{W_{B\text{-}I}} \qquad (1)$$

W was calculated from the arithmetic means of distances between inner and outer bounds of respective lines across the entire synapse.

The actual "shrinkage-factor" (sf_A) was derived from the quotient of inner synaptic diameters (d) of osmicated versus combined B-I/OsO_4-treated material. These diameters were taken between the outer bounds of osmiophilic tramlines.

$$sf_A = \frac{d_{OsO_4}}{d_{B\text{-}I/OsO_4}} \qquad (2)$$

THE PRESYNAPTIC GRID

Gray [9] was the first to draw attention to an array of presynaptic spots, which he named "dense projections." This structure has recently been studied in more detail in our laboratory, and it turned out that the B-I method gave additional detail [14]. These observations will be briefly reviewed and expanded in this section.

Staining with B-I complex differentiates between presynaptic dense projections and presynaptic unit membrane, the former being intensely impregnated and the latter remaining invisible. The two-dimensional aggregate of dense projections is most remarkable because of its hexagonal cristalloid array of dense spots and interconnecting filaments giving the impression of a grid (Fig. 1).

Typical profiles of synaptic junctions as visualized in Figs. 2 and 3 closely resemble the ones presented earlier by Gray [9, 10] and by Aghajanian and Bloom [1]. The synaptic dense projections consist of polyhedric bodies, approximately 550 Å in diameter and 600 Å in height. They are spaced at intervals of approximately 800 Å (center-to-center) and are surrounded by clusters of synaptic vesicles. Dark-cored vesicles of 1000–1500 Å diameter are not infrequently seen in close vicinity or even touching the peak of dense projections. Patches of clear vesicles as well as solitary dark-cored vesicles are accumulated in the holes of the grid.

The presynaptic grid offers two important aspects: The first is concerned with its positive image, the *peaks* and *filaments*. The second aspect relates to the *holes* and deserves more detailed consideration. Even though the dense projections in Fig. 4 are sectioned in parallel and at the level of the cytoplasmic membrane of a nerve terminal, it appears

that free spaces occur between the dense projections. These free spaces seem to form almost round holes and are separated from each other by tiny filaments that interconnect the dense projections. Thus, it seems as though the presynaptic area communicates directly with the synaptic cleft through the holes of the grid. This is of course a false impression due to the fact that the osmiophilic leaflets of the presynaptic membrane

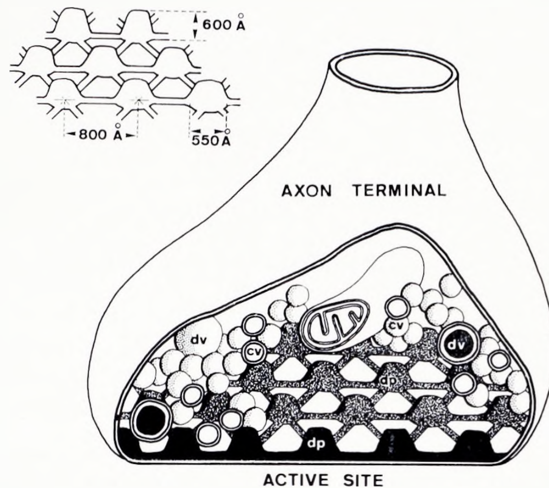


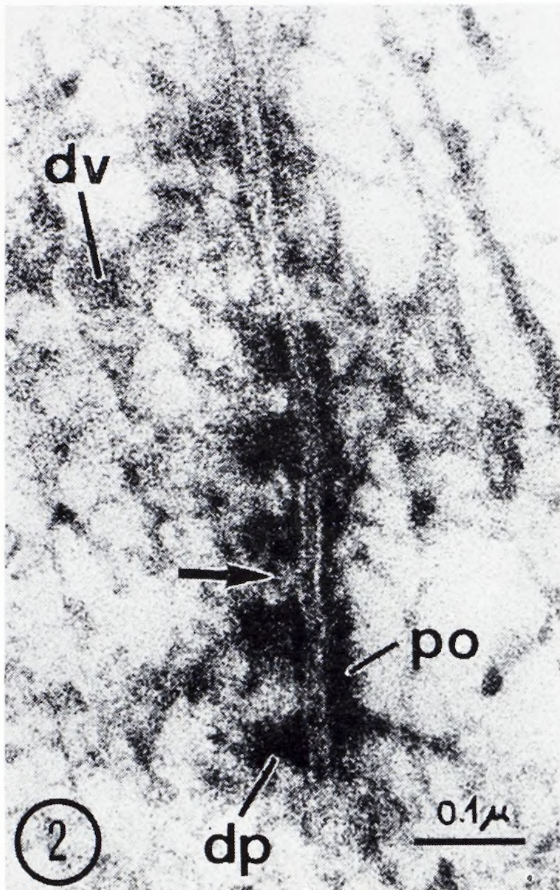
FIG. 1. Three-dimensional reconstruction of the presynaptic grid as demonstrated in B-I stained material. *cv*, Clear vesicles; *dp*, dense projections; *dv*, dense-cored vesicles. From Pfenninger *et al.* [14].

FIG. 2. Synapse stained with B-I method. Note the heavy contrast of pre- and postsynaptic densities. The presynaptic dense projections are spaced at regular intervals. Iodophilic cytoplasmic layer seems to be missing at arrow. *dp*, dense projection; *dv*, dense cored vesicle; *po*, postsynaptic membrane apposition. Primary magnification: ×40,000.

FIG. 3. Synapse stained with B-I method. Note the trapping of dense-cored vesicles (*dv*) in the holes of the presynaptic grid. D indicates a symmetrical thickening of synaptic membranes at lower end of the plaque. Primary magnification: ×40,000.

FIG. 4. Tangential section at the level of presynaptic membrane. The peak-and-hole pattern of the presynaptic grid is hexagonally arranged. *dp*, Dense projections; *f*, filamentous extensions. B-I method. Primary magnification: ×40,000.

FIG. 5. Presynaptic axon terminal (*ax*) filled with clear synaptic vesicles whose contours are marked by B-I stained material. Note that cytoplasmic membrane surfaces are coated by a narrow B-I-positive layer (arrows). A very discrete reaction is barely visible in the cleft. This is *not* a synaptic region, but regular cell contact. Primary magnification: ×40,000.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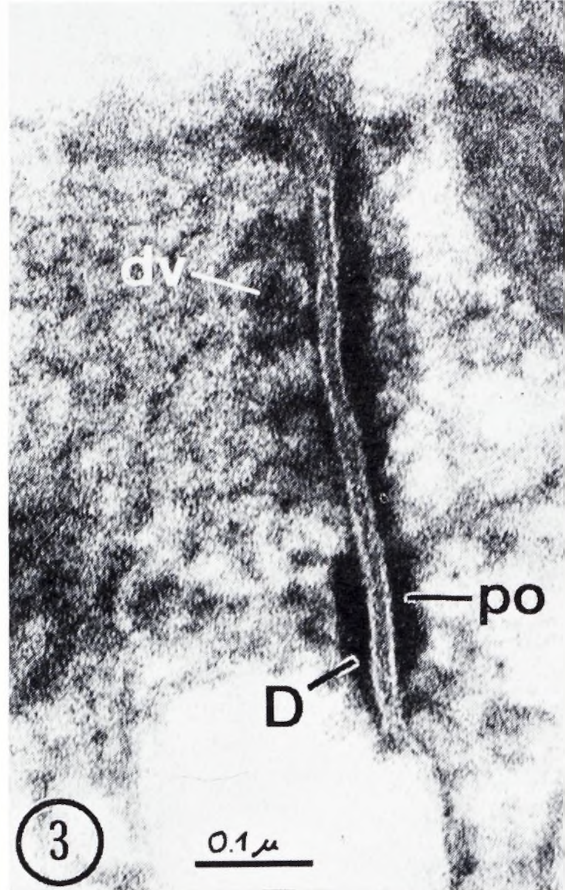
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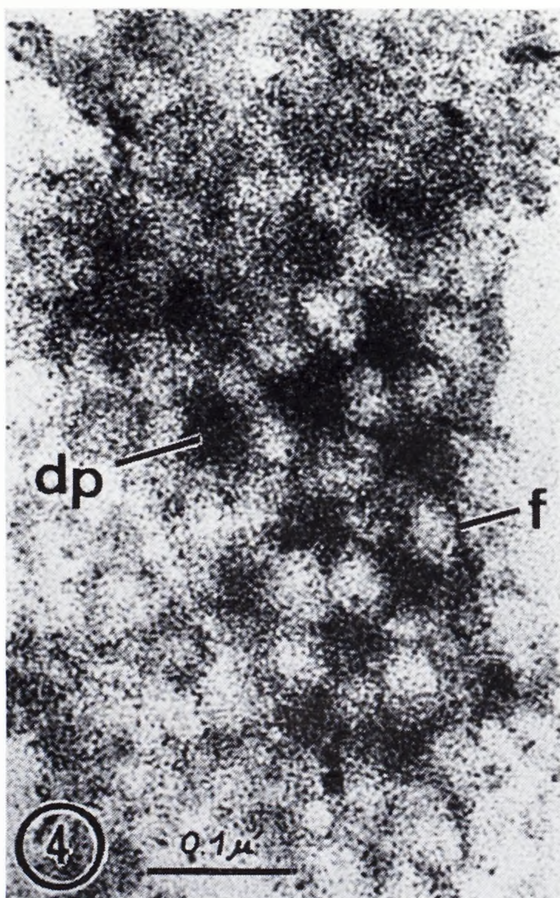
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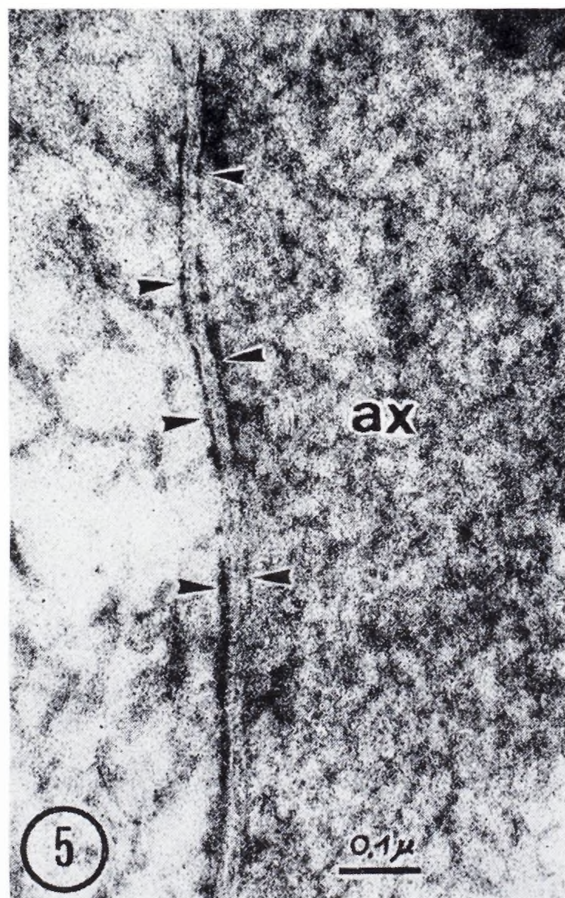
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remained unstained and the "iodophilic" layer is missing between dense projections and their filamentous extension. However, this situation is clearly unusual since, within the holes of the presynaptic grid, *the synaptic vesicles may touch directly upon the uncoated presynaptic membrane.* Figure 5 is a reminder that in all other sections of the presynaptic terminal there is a "iodophilic" layer on the inner surface of the cytoplasmic membrane which separates it from synaptic vesicles. This situation is schematically represented in Fig. 6.

Comments

The present findings on B-I staining of presynaptic dense projections have greatly profited from Gray's pioneering investigations [9, 10]. The admirable regularity of its structural arrangement raises the problem of functional interpretation. Only conjectures can be offered at the present. In the first place the consistent relationship between dense projections and synaptic vesicles must be mentioned; it has already been recognized and commented upon by Gray [11]. Wherever presynaptic membrane appositions occur in association with synaptic vesicles, a grid with hexagonal peak-and-hole pattern seems to develop. This is, of course, true in all chemical synapses with the exception of autonomic myoneural junctions where appositional densities are unknown. The presence of presynaptic dense projections in the motor end plate has been suggested by Miledi [cited in Gray, 11] and firmly established by means of the B-I method in our laboratory. In contrast, desmosomes and intermediate junctions which are equipped with appositional dense material fail to be associated with vesicles and never develop any holes within the membrane appositions. It would be of interest to know which of the two elements appears first during development of a synapse. Unfortunately, both *in vivo* and *in vitro* studies of synaptogenesis have thus far not paid sufficient attention to presynaptic densities. Bunge *et al.* [6] have concluded from their work and from that of others that densities and vesicles are closely related during maturation and that membrane appositions may precede the appearance of the first synaptic vesicles. Aghajanian and Bloom [1] have recently drawn attention to the possibility that the dense projections are not fully developed in junctional complexes of the immature brain and postulate a sequence of developmental stages in the morphogenesis of synapses in which changes of dense projections from solid plate to peak-and-hole patterns may furnish important criteria. When confronting the statement made by the Bunge's and the hypothesis put forward by Aghajanian and Bloom one is tempted to combine these notions into a coherent story whereby presynaptic densities may preexist in the form of a solid membrane thickening and gradually

develop into a grid from the moment where they become closely associated with synaptic vesicles.

What is the significance of this relationship? Three possibilities merit consideration: (1) The presynaptic grid may serve as a receptacle or trap of synaptic vesicles. *The vesicles which are stuck in the holes of the grid might undergo important changes under the influence of substances* (e.g., enzymes) *contained within the dense projections.* (2) The grid may be instrumental in regulating the transmitter release mechanism. In this context the lack of the "iodophilic" coat of the presynaptic mem-

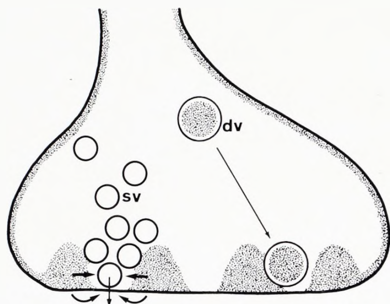


FIG. 6. Hypothetical relations between presynaptic grid, synaptic vesicles, and iodophilic coat at cytoplasmic membrane. A pathway of dense-cored vesicles (*dv*) from perikaryon to presynaptic grid is indicated. Clear synaptic vesicles (*sv*) may interact in a different way with the grid. They may be influenced by chemical compounds stored within the dense projections (arrows). Note also the absence of "iodophilic" layer within the holes; here, the synaptic vesicles make direct contact with the cell membrane. Possibly, there is an interaction between vesicles and cell membrane at the arrival of a nerve impulse triggering the transmitter release (arrows). Stippled areas represent B-I-positive material.

brane within the region of the holes is perhaps of significance. *The absence of this layer raises the question whether the direct contact of synaptic vesicles with the osmiophilic components of the cytoplasmic membrane may be an important prerequisite of transmitter release.* It would explain the fact that transmitter may be released from synaptic vesicles exclusively within the critical area of the synaptic cleft. (3) A somewhat different relationship may exist between dense-cored vesicles and synaptic dense projections. Pfenninger *et al.* [14] have put forward a new hypothesis, namely the possibility that *these vesicles may transport the chemical constituents (enzymes etc.) of dense projections from the perikaryon to the nerve endings.* The suggestion was made on the basis of the following four observations: (a) Vesicles of similar appear-

ance are often seen in the vicinity of Golgi apparatus. (b) Dense-cored vesicles and dense projections seem to react similarly to B-I stain. (c) Dense-cored vesicles are often seen in close contact with synaptic dense projections. (d) Dense-cored vesicles are by no means restricted to adrenergic endings but occur in almost all presynaptic terminals both in vertebrate and invertebrate nervous system. Unfortunately, the argument is weakened by the fact that dense-cored vesicles occur in regions where no presynaptic dense projections are known to exist, e.g., the autonomic neuromyal junctions. Further studies are necessary to test the hypothesis of presynaptic grid functions as summarized in Fig. 6.

SUBUNITS WITHIN THE SYNAPTIC CLEFT

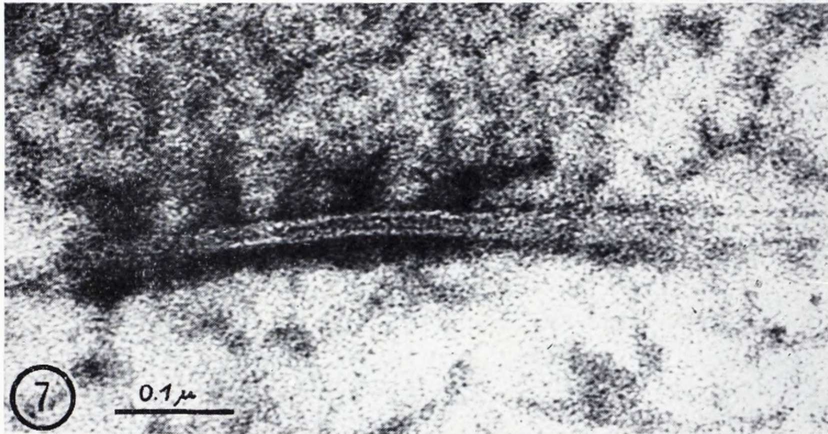
Everyone admits that the synaptic cleft is not an empty space. Strong anonymous forces prevent the rupturing of the synapse during ultracentrifugation and osmotic stress, and electron microscopic investigations with conventional techniques have clearly shown that the gap is filled with material of unknown identity and arrangement. The best-known models of synaptic cleft seem to prefer structural arrangements oriented vertically to the plane of synaptic membranes, e.g., the cross striations of De Robertis [8] and the spirals of Gray [11]. Neurophysiologists seem satisfied with the fact that the synaptic cleft has a width of 200 Å and have used this dimension for the calculations of electric currents and fields in connection with ionic transport phenomena. All these findings, of course, are based upon $KMnO_4$- and OsO_4-fixed material, and they remind us that present models are far from being satisfactory.

It is our impression, based on a large collection of conventionally prepared vertebrate and invertebrate synapses, that an electron dense finely granulated material exists in the synaptic cleft and is often arranged in parallel with the membrane surfaces in the form of a middle dense layer. In cross sections an interrupted "intracleft line" appears. This observation has been made by numerous previous authors.

By means of the B-I method we recently obtained additional contrast and details with respect to the morphology of the synaptic cleft. It seems to contain two separate layers, which are shown in Figs. 7 and 8. The double layer can be demonstrated not only with the B-I method, but with uranyl acetate and lead citrate staining as well [16]. Perhaps the most convincing evidence of the double-layered structure is provided by an electron micrograph taken from a synaptosome fraction which has been treated by the B-I method (Fig. 8). It can be readily recognized that the two layers bifurcate near the lateral edge of the synaptic plaque

and tend to follow the cytoplasmic membranes of pre- and postsynaptic elements, respectively.

The double-layered intracleft material can be equally well identified in *nonsynaptic* cell junctions, treated with the B-I mixture. Figure 12 gives an example of junctional areas between ependymal cells of the subfornical organ. A transition of intermediate and tight junction is seen. The former is characterized by symmetrical appositional densities on the cytoplasmic side of the membranes and a double-layered intracleft line, while the latter has a thin but conspicuous coat of "iodophilic" sub-



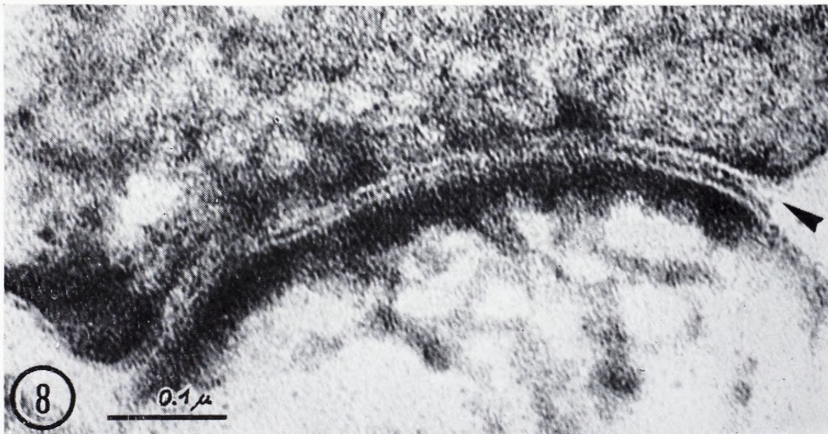


FIG. 7. Synapse, treated with B-I stain. Double-layered intracleft line is clearly visible. Primary magnification: ×40,000.

FIG. 8. Synaptosome obtained by density-gradient fractionation. Double-layered intracleft line is clearly visible and bifurcates at both ends of the synapse, particularly well seen at the right (arrow). Primary magnification: ×40,000.

stance on the cytoplasmic surface and a single-layered intracleft line. Thus, it seems that "iodophilic" substance is present even in the cleft of so-called tight junctions. However, the two coats seem to be fused into one (Fig. 10). Figure 9 may offer an explanation to this surprising fact: A tight junction between a nerve cell and a cell process (dendrite?) is demonstrated after conventional glutaraldehyde–OsO_4 treatment. Clearly, the outer osmiophilic leaflets of the membranes are not fused, but a small gap exists between them. This gap may be occupied by the iodophilic material, as seen in Fig. 10. If both staining procedures are combined (Fig. 11), the intracleft line appears heavy and the conclusion seems reasonable that both osmiophilic and "iodophilic" layers are impregnated and appear fused into one.

Finally, examples of high-resolution photographs of synaptic junctions are presented. Again, the tissue was treated with glutaraldehyde–OsO_4 (Fig. 13), with B-I mixture (Fig. 14), and with the combination of the two (Fig. 15). Similar photographs have been prepared in larger numbers and formed the basis of measurements and calculations summarized in Fig. 17. The results are briefly as follows (see also Fig. 16):

1. It seems that the osmiophilic and "iodophilic" lines are either completely separated or overlap only to a minimal degree. This is seen when the average values obtained with the two respective stains are compared, and particularly when the values of the B-I material are corrected for shrinkage. The same is true when the data derived from the combined OsO_4 B-I procedure are considered. Again, the two sets of lines appear side-by-side with only small overlap. The latter could be real; however, it lies within the error range of measurements.

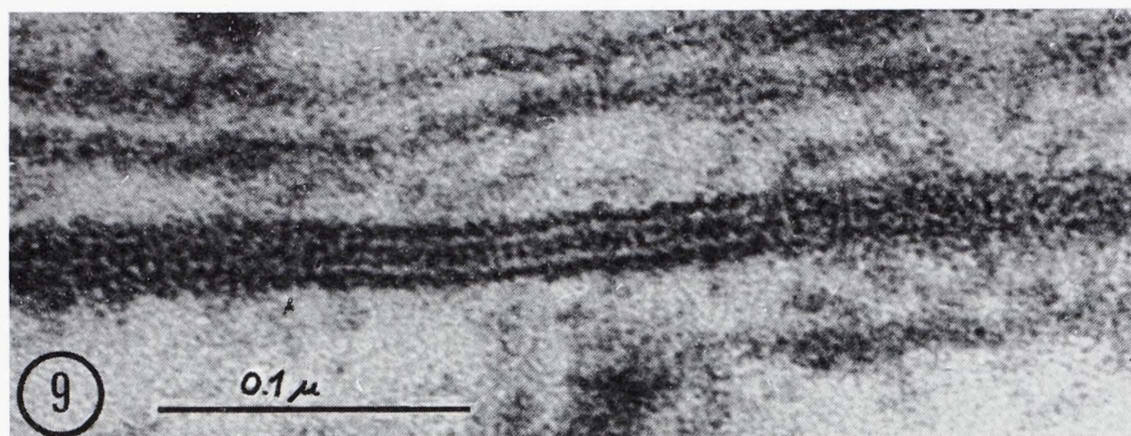
2. The results indicate that the "iodophilic" intracleft lines have a diameter of 60 Å and occupy the space considered as the "synaptic cleft"

FIG. 9. Tight junction between two cell processes (ependymal cell and glial cell?), glutaraldehyde–OsO_4 fixation. Note that the outer surfaces of unit membranes are not fused. A narrow gap is barely visible. Primary magnification: ×80,000.

FIG. 10. Tight junction between two ependymal cells; B-I impregnation. Note the thin single line in the cleft. Primary magnification: ×80,000.

FIG. 11. Tight junction between glial processes treated with combined B-I OsO_4 method. The middle line is clearly heavier than in Fig. 13. Primary magnification: ×80,000.

FIG. 12. Transition between intermediate and tight junctions of ependymal cells, treated with B-I stain. A double-layered intracleft line is visible at the level of the intermediate junction (*ij*); it appears to be fused into one single line at the tight junction (*tj*). Primary magnification: ×40,000.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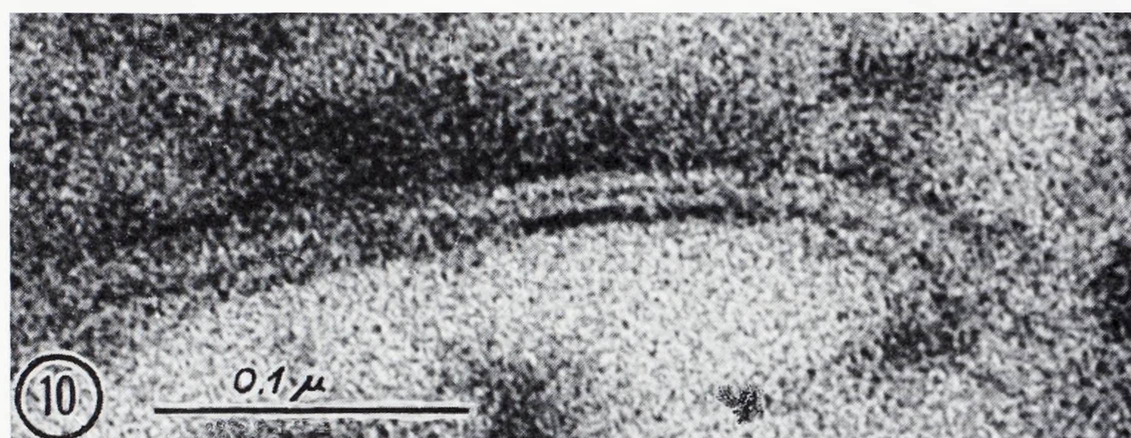
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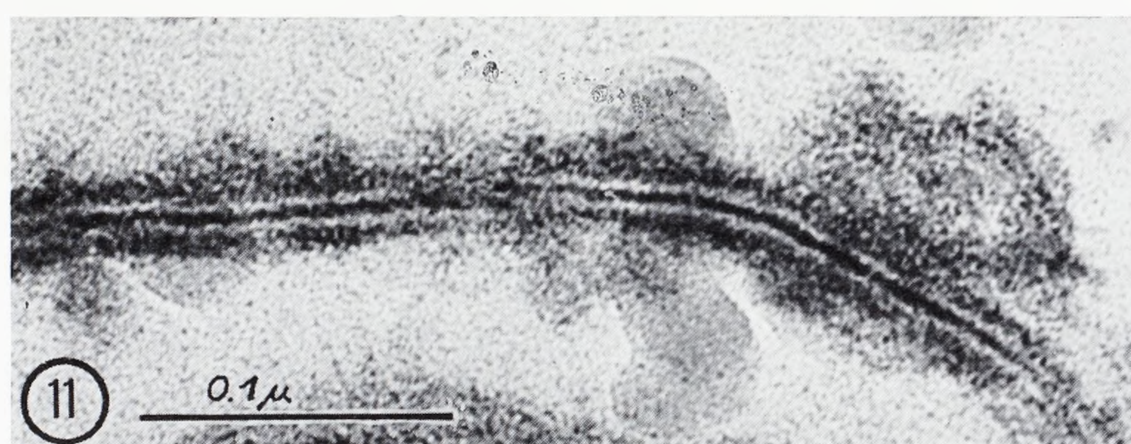
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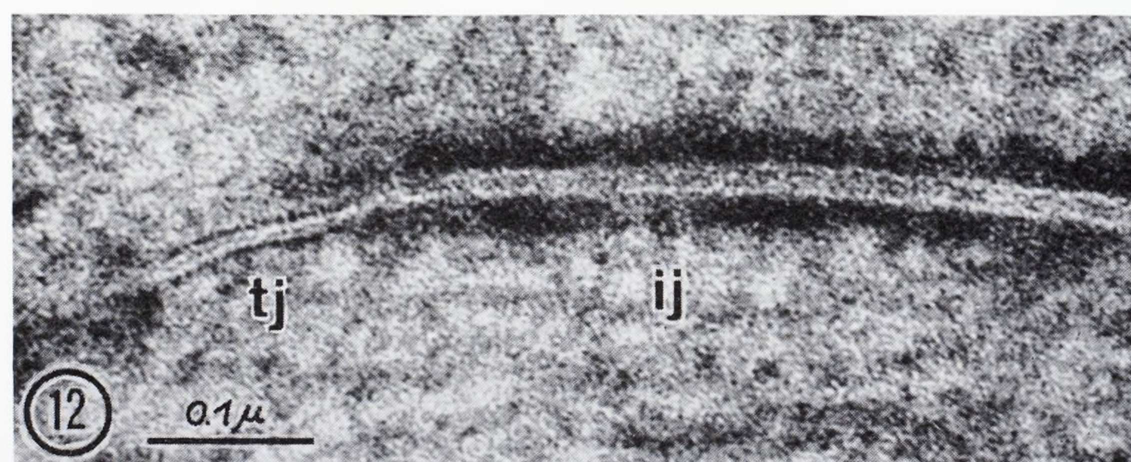
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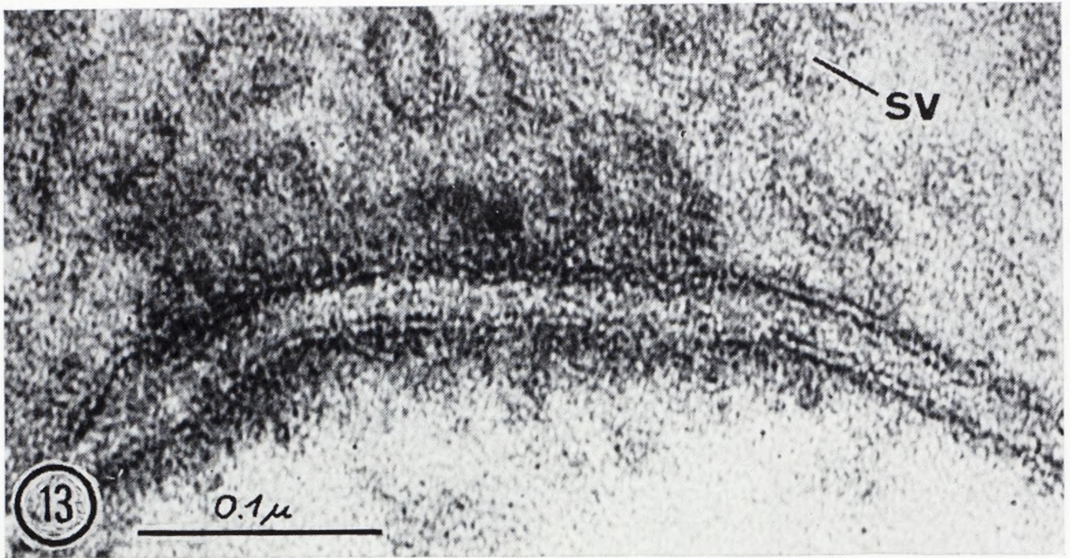
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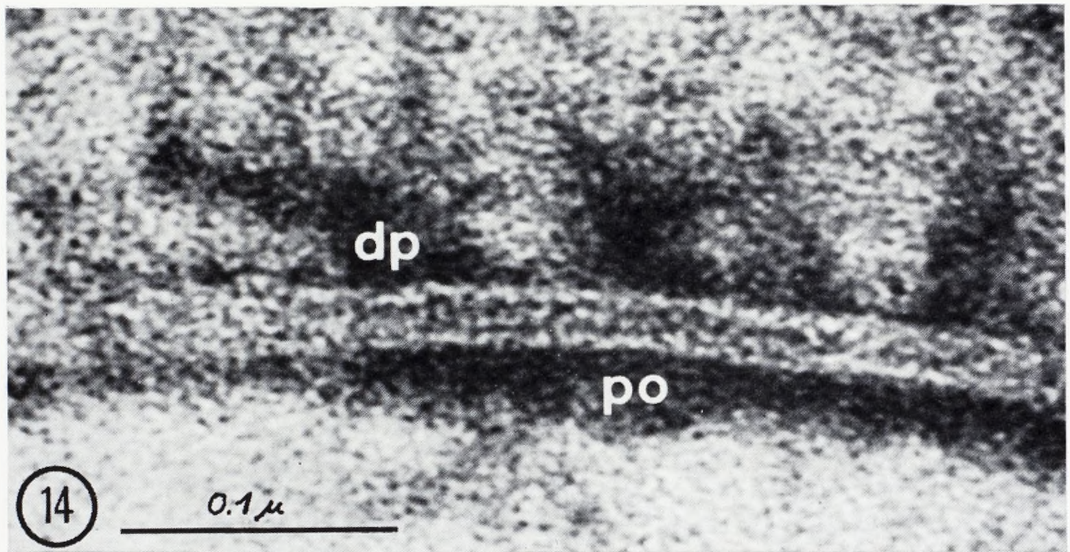
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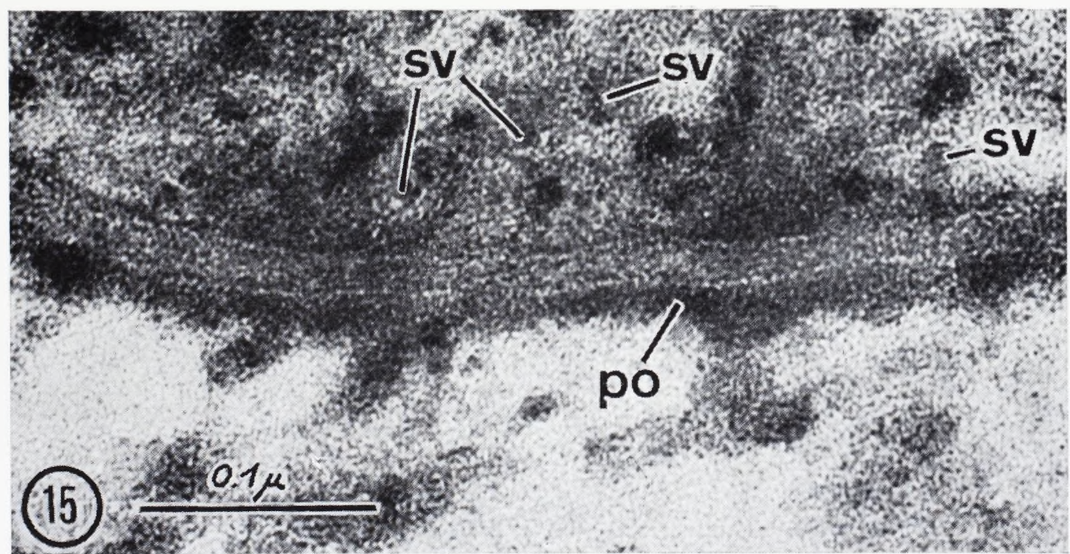
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in conventionally fixed material. Conversely, the osmiophilic lines are centered within the free gap between the "iodophilic" lines, although they seem to occupy a slightly wider space. This overlap of calculated 7 and 10 Å, respectively, lies within the error range of the method.

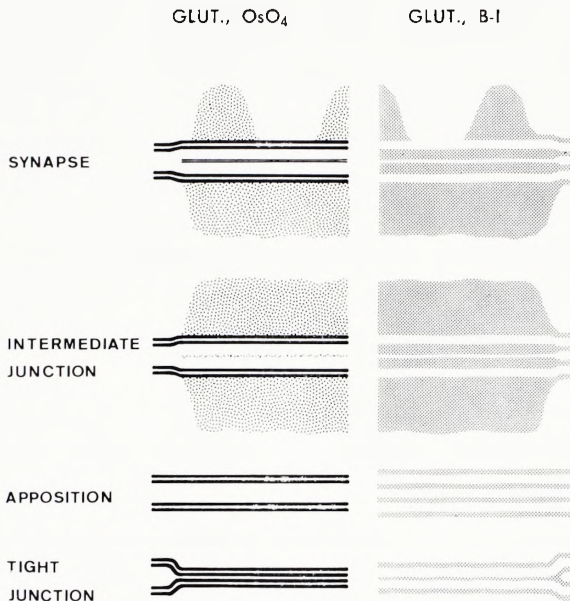

FIG. 16. Schematic representation of findings in previous electron micrographs.

3. The "iodophilic" lines are separated by a narrow gap, which might be considered as the "real" synaptic cleft. Its width could not be measured with satisfactory accuracy; it is estimated at about 20–30 Å. However, this area may be identical with that stained faintly in osmicated sections (cf. Fig. 13). More sensitive methods are needed to examine this problem.

FIG. 13. Synapse, glutaraldehyde–OsO_4 fixation. Osmiophilic tramlines and synaptic cleft are clearly visible. Primary magnification: ×40,000.

FIG. 14. Synapse, treated with B-I method. The space occupied by osmiophilic lines remains unstained. Instead the more medially situated iodophilic lines become visible. *dp*, Dense projection; *po*, postsynaptic density. Primary magnification: ×40,000.

FIG. 15. Synapse, combined B-I/OsO_4 treatment. Osmiophilic and iodophilic lines are situated side-by-side. The synaptic cleft is now virtually filled with subunits. *sv*, synaptic vesicles (positive iodide/OsO_4 reaction is faintly visible). Primary magnification: ×80,000.

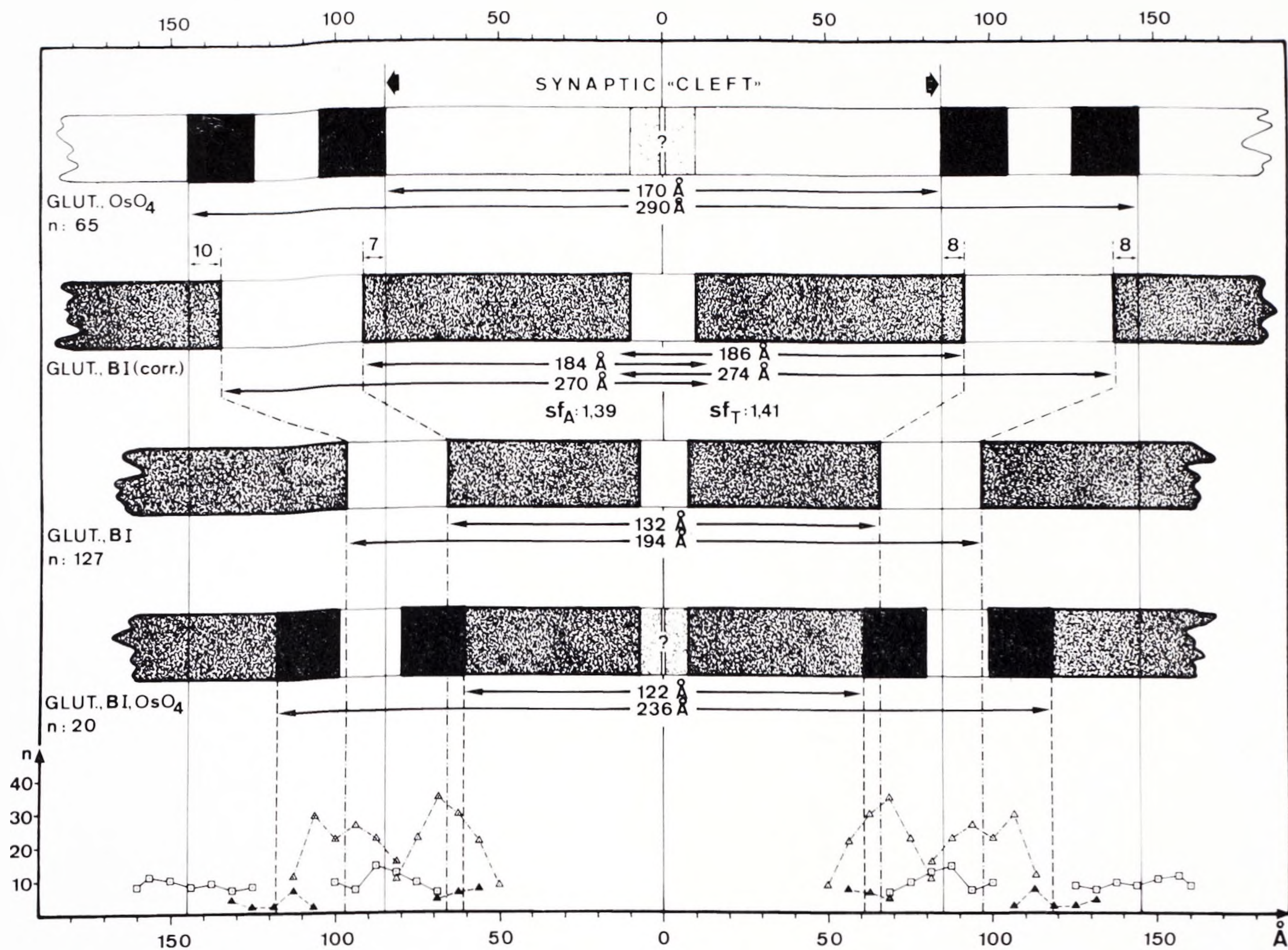

FIG. 17. Comparison of osmiophilic and "iodophilic" lines at synaptic sites. The upper part represents the mean dimensions of width and periodicity of units derived from the histograms below (see vertical lines); *n* = number of measured junctions. The lower part represents a histogram of unit and subunit dimensions. ⊡——⊡, Glutaraldehyde-OsO_4 material; △·–·–△, B-I material; ▲——▲, combined B-I/OsO_4 method.

Comments

The presence of "intracleft lines" poses the usual problems of artifactual distortion. Foremost is the question whether the "iodophilic" lines are basically identical with or separated from the osmiophilic tramlines of synaptic membranes. It seems that the measurements represented in Fig. 17 have provided an acceptable answer: while the spaces occupied by the two sets of layers seem not to be mutually exclusive, they are not ideally complementary either. The conclusion is that they represent two structural systems, which are separable at least to a major extent. This conclusion is further borne out by the fact that *the "iodophilic" layer is missing within the region of the holes* in the presynaptic grid and that it is therefore not a substitute form of osmiophilic leaflets. The next question is that of subunits. Is the "iodophilic" system to be considered as a membrane subunit or as an appositional coat like the glycocalyx in the junctional folds of motor end plates? It seems that the present information is not sufficient to provide an answer to this question. Too little is known about the chemical constituents of the "iodophilic" layers and their relations to the molecular components of the classical unit membrane. Nevertheless, two facts are noteworthy in this context. (a) The "iodophilic" layer is seen not only in the synaptic cleft region, but appears as a thin coat along the entire cell surface and is encountered within nonsynaptic cell junctions. (b) Recent observations on synaptic junctions treated with the freeze-etching method of Moor and Mühlethaler [12] have confirmed the presence of a thin coat lining the outer cell surface within the synaptic cleft region. These results will be reported in more detail elsewhere [5].

It would be equally premature to make any statements about the functional significance of the "iodophilic" layer in the synaptic cleft. If further corroborated, it would seem that this observation deserves consideration in all matters concerning transport activities within the synaptic region. And since the "iodophilic" intracleft lines as a paired structure seem to represent the most advanced frontier of macromolecular constituents of synapsing nerve cells, it can be expected that future research would profit from the study of their specificity.

SUMMARY

Electron microscopic investigations of synaptic structures were undertaken on material prepared with conventional as well as with a new bismuth-iodide staining method. Special attention was given to the structural organization of the *presynaptic grid* and the problem of its

functional interaction with synaptic vesicles, clear and dark cored. The latter may be involved in transport functions from the perikaryon to the nerve endings and provide the dense projections of the presynaptic grid with important constituents.

A so-called iodophilic layer was found on both sides of the synaptic membrane. It seems to form *a paired subunit (60 Å periodicity) in the cleft.* Its absence from the region of the holes of the presynaptic grid and its accentuation within the synaptic cleft raise problems of functional interpretations which are briefly discussed.

Note Added in Proof

After this manuscript had gone to press the authors became aware of a paper by M. W. Brightman and T. S. Reese, Junctions between intimately apposed cell membranes in the vertebrate brain [*J. Cell. Biol.* **40**, 648 (1969)]. Based on this evidence we may conclude that the close apposition of cell membranes forming a 7-layered structure (Fig. 9) should be referred to as "gap junction" rather than as "tight junction."

Acknowledgment

The technical assistance of Miss Clara Sandri in the preparation of electron microscopic material, and the untiring efforts of Miss Regula C. Hug in providing density-gradient fractionations and illustrations, as well as the many helpful interventions of Mr. A. Fäh are gratefully acknowledged.

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