FINE STRUCTURE OF THE SYNAPTIC DISCS

SEPARATED FROM THE GOLDFISH MEDULLA OBLONGATA

GUIDO ZAMPIGHI AND J. DAVID ROBERTSON

From the Department of Anatomy, Duke University Medical Center, Durham, North Carolina 27710

ABSTRACT

Synaptic discs are structures localized in the club ending synapses on the Mauthner cell lateral dendrite of the goldfish medulla oblongata. The synaptic discs present a hexagonal array of particles ~8.5 nm center-to-center when observed in *en face* view. This lattice covers the entire surface Divalent cations are important in the stabilization of this particular hexagonal array of particles When a synaptic disc-rich fraction is treated with chelating agents (EDTA or EGTA), definite changes occur in the hexagonal lattice. First, the synaptic membranes show zones without particles interspersed with zones covered with the hexagonal array of particles Second, the synaptic discs break down and a new structure characterized by two parallel dense bands (7 nm each), separated by a 4 nm gap, is observed. The negative stain fills the gap region showing striations spaced ~10 nm center-to-center crossing the gap, but it does not penetrate the dense bands This "double band" structure is interpreted as an edge on view of a fragment of the synaptic membrane complex. Further treatment of this fraction with a chelating agent plus 0.3% deoxycholate produces an increase in the number of double band structures. However, EDTA plus Triton X-100 (a treatment known to produce solubilization of membrane proteins) *never* shows such double band structure An ordered material was observed associated with the cytoplasmic leaflets of the double bands This material consists of rows of beads ~4 nm in diameter and spaced at intervals of ~7 nm. Each of these beads is joined to the band by a thin stalk.

INTRODUCTION

In 1963 a new type of synaptic membrane-complex specialization was discovered in the club endings on the Mauthner cell lateral dendrites in goldfish medulla oblongata (28, 30). These complexes were called "synaptic discs." The synaptic discs were characterized by a close apposition of the external leaflets of the pre- and post-synaptic membranes, in disclike regions 0 3–0 5 μm in diameter. The overall thickness of the synaptic complex was ~15 nm and in *en face* views a hexagonal array of particles spaced ~9 nm center-to-center was present. Later, Brightman and Reese (6) using a variety of fixation techniques, demonstrated the presence of a gap (~2 nm wide) between the pre- and post-synaptic membranes of the synaptic discs. They showed that the gap could be filled with lanthanum by the technique described by Revel and Karnovsky (26).

This particular type of membrane complex is widespread in a variety of tissues (13–17) in different animal species In mammals it occurs in liver (1, 2, 14), heart (22, 34), and smooth muscle (9) Crayfish giant synapses (25, 27) and synapses on pacemaker neurons in certain fish brains (3–5) present a similar close apposition of unit membranes The term "gap junction" is commonly applied to this kind of membrane complex.

The close contact between pre- and postsynaptic membranes observed in synaptic discs was immediately thought of in terms of electrical transmission

(28). This interpretation was supported by the electrophysiological work of Furakawa and Furshpan (10) and Furshpan (11) These authors demonstrated that club ending synapses on the Mauthner cell lateral dendrite transmit electrically The previous work of Furshpan and Potter (12) and the studies by Bennett et al (3–5) on a variety of tissues gave additional support to this interpretation

The array of particles observed in *en face* views of both thin sections and by negative staining (1, 2) was interpreted as a pattern localized on the outside layer of the unit membrane However, new evidence obtained particularly from freeze-fracture techniques has challenged this intrepretation (8, 21a).

We now present new information about the organization of isolated synaptic discs This information was obtained by treatment of a synaptic disc-rich fraction with chelating agents and several combinations of chelating agents plus ionic or nonionic detergents.

MATERIALS AND METHODS

Common goldfish (*Carassius auratus*) 4 inches long were obtained from local suppliers 10–15 animals were anesthetized successively by immersion in an ice bath The brain was fully exposed by careful removal of the superficial tissues and was freed by cutting the nerve roots and the spinal cord. The emergence of the fifth and seventh nerve root was located as a cranial point of reference using a dissecting microscope. The emergence of the eighth nerve pair was used as a caudal reference. Transverse sections through these points permitted a separation of the medulla oblongata from the rest of the brain The attached vagal lobes and cerebellum were then removed This preparation will be called "dissected medulla oblongata" (DMO).

Separation of a Synaptic Disc-Rich Fraction

Fresh pooled DMO's were minced and homogenized with 10 vol of 0 3 M sucrose in a smooth-walled Perspex and Teflon homogenizer pestle (20 mm diameter) A clearance of 0.3 mm and a speed of rotation of 500 rpm were used. The procedure described by Whittaker (36) was used for the separation of the synaptic discs Modifications were introduced in order to produce a better yield of synaptic membranes The mitochondrial pellet was suspended in 6 ml of 0 001 M bicarbonate buffer pH 7.5 and the sample was layered on top of a 9 ml, three step, discontinuous sucrose gradient (d 1.14, d 1.16, d 1 18, 1:1:1) and centrifuged at 100,000 g for 2 h in a SW-41 Ti rotor of the Beckman ultracentrifuge (model L2-65B, Beckman Instru-

ments, Inc, Spinco Div, Palo Alto, Calif.) The preparation separated into three bands (one at each interface of the gradient) and a pellet

The material at the interfaces and the pellet were successively resuspended in bicarbonate buffer and centrifuged three times at 50,000 g for 30 min in order to wash away the sucrose These fractions were prepared for electron microscope study.

Fixation and Embedding

Fixation was carried out in 3% glutaraldehyde in 0 2 M cacodylate buffer Postfixation was done in a 1:1 mixture of 4% OsO_4 in water and 0 1 collidine buffer (pH 7 35) The pellets were dehydrated with ethanol and passed through propylene oxide and embedded in Epon 812 according to Luft (20).

Negative Staining

Negative staining was carried out with 1 or 2% neutralized phosphotungstic acid (PTA) at room temperature on 400-mesh grids coated with carbon films. A Phillips EM 300 microscope was used with a 50 μm objective aperture.

Treatments

The synaptic disc-rich pellet was suspended in EDTA (ethylene-diaminetetraacetic acid disodium dihydrate, Mann Research Labs, Inc, New York) at a concentration of 5–20 mM at pH 6, or EGTA (ethyleneglycol-bis[β-aminoethyl ether] N,N'-tetraacetic acid, Sigma Chemical Co, St Louis, Mo) at a concentration of 1–10 mM at pH 6 The suspension was spun down at 50,000 g for 30 min The pellets were examined by negative staining.

Detergents

The synaptic disc-rich pellet was suspended in Triton X-100 (Sigma Chemical Co) or sodium deoxycholate (DOC, Sigma Chemical Co) diluted to a final concentration of 0.1 or 0.3%. The suspension was dialyzed overnight against bicarbonate buffer, spun down at 50,000 g for 30 min, and observed by negative staining The EDTA-treated fraction was suspended in both ionic (DOC) and nonionic (Triton X-100) detergents at the same concentrations as before

Freeze Fracture

Several DMO's were fixed for 2 h at room temperature in 3% glutaraldehyde with 0.1 M collidine buffer at pH 7 35 After a brief rinse in collidine buffer, the specimens were trimmed into 1 mm blocks and immersed in 10–20% glycerol for 2–10 h. The blocks were then placed in liquid nitrogen-chilled Freon 22

(E. I. du Pont de Nemours and Co., Inc., Wilmington, Del.) and brought to liquid nitrogen temperature. A Balzers high vacuum freeze-etch unit (BA 360M, Balzers High Vacuum Corp., Santa Ana, Calif) was used according to the technique described by Moor (23). The frozen tissues were cleaved at −100°C and replicated without etching or after etching (30 s to 2 min). The platinum-carbon replicas were carefully cleaned and mounted on uncoated grids.

RESULTS

Electron microscope observations showed that the largest amount of synaptic disc membrane was found in the pellet recovered from the bottom of the discontinuous sucrose gradient Thus, all our subsequent observations were limited to this fraction.

Thin sections of the crude sucrose-free pellet showed a fair number of transverse sections of synaptic discs (Fig. 1) They were observed as a hepta-lamellar structure 18 nm in cross-section which was resolved as two closely apposed unit membranes (each 7 nm thick) separated by a 3–4 nm gap. While the original micrographs of synaptic discs in goldfish medulla fixed in $KMnO_4$ did not show the gap, subsequent work using aldehyde fixation showed this feature as well as the hexagonal arrays of subunits The synaptic disc has essentially the same structure as the gap junctions described by Revel and Karnovsky (26).

The negative stained preparations of the crude sucrose-free pellet showed *en face* views of the junctional membranes Fragments of synaptic discs (Fig 2) with irregular outlines were usually found. In these fragments, a characteristic hexagonal array of particles ∼6 nm in diameter and spaced ∼8 5 nm center-to-center was observed This hexagonal lattice makes the synaptic discs easily distinguishable from other sorts of membranes present in the fraction The complete synaptic discs are folded membranes 1 5–2 μm in diameter.

The hexagonal array composing the synaptic disc is not a perfect lattice. There are only limited regions of perfection and these yield two orders of hexagonal diffraction in an optical diffractometer (Fig. 3) The spots are stronger in one axis (meridional) This effect is probably due to imperfections present in the hexagonal lattice or tilting of the membrane from which the optical transform was obtained. Spots corresponding to the third order are usually seen, but they are not exactly located in the hexagonal net.

No substructure has been resolved either by viewing the prints obliquely or by optical diffraction. At the level of resolution so far reached, each

FIGURE 1   Thin section of a synaptic disc of the fraction isolated from the goldfish brain. Glutaraldehyde-OsO_4 fixation. × 192,000.
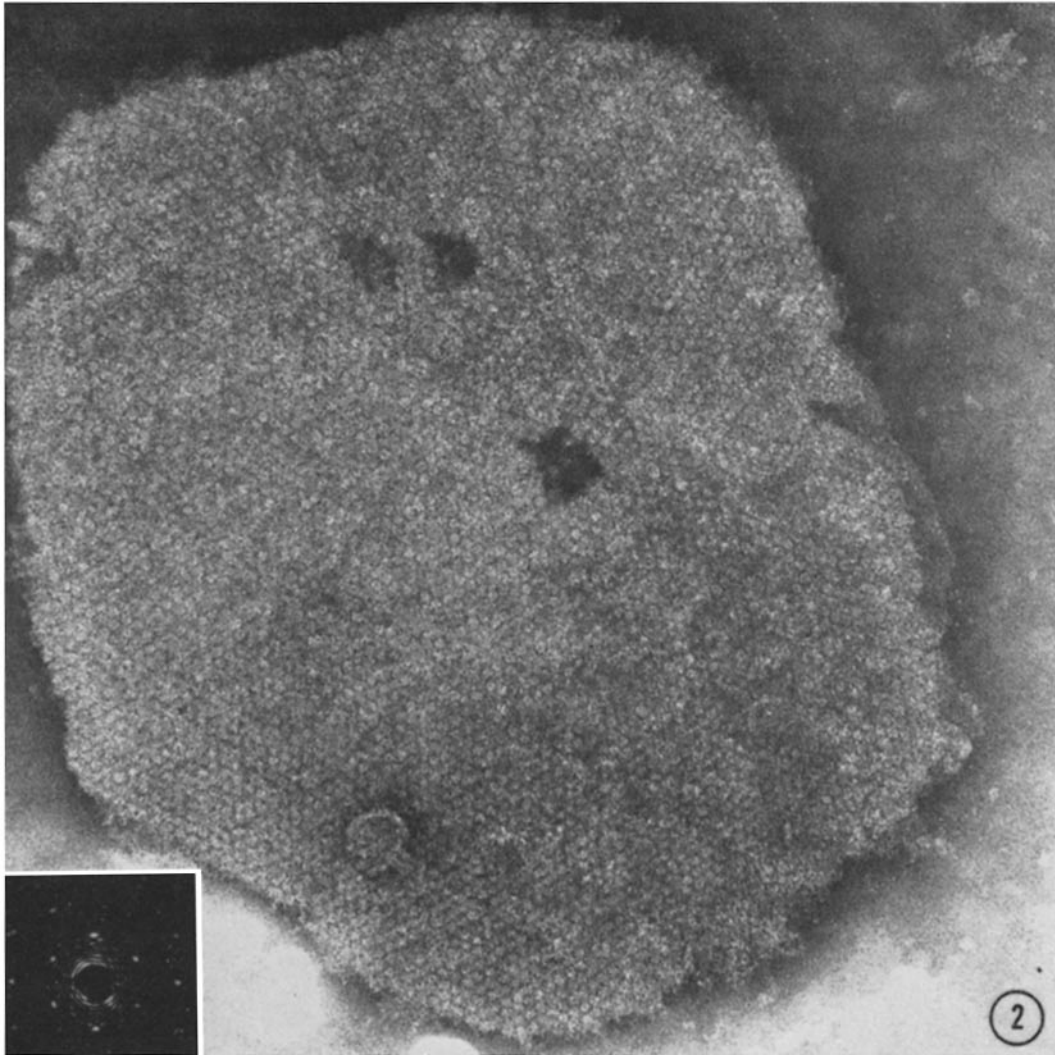
FIGURE 2 PTA negative stain preparation of the fresh, untreated synaptic disc fraction. Fragments of membranes displaying the hexagonal array of particles are observed covering the membrane surface completely. × 205,000.

6 nm particle has not been found to be made up of smaller subunits like those described in the hexagonal array of urinary bladder membranes (35). No pentagonal packing has been observed in our preparations as described in mouse liver gap junctions by Bennedetti and Emmelot (1) and in thin sections of synaptic discs (28)

Freeze-fracture replicas of the medulla oblongata showed regions having a hexagonal array of particles, ~9 nm center-to-center. The particles were coplanar with the fracture face A of the mem-

branes (Fig 4) and they have a complementary junctional B face.

Chelating Agents

The withdrawal of Ca^{++} by EDTA or EGTA caused an interruption of intercellular communication as shown by Lowenstein (19) and Oliveira-Castro and Loewenstein (24) in several electrically coupled systems such as leech cells, *Chironomus* salivary gland cells, and mouse liver cells It thus

seemed appropriate to determine whether or not these agents would produce alterations in our synaptic disc preparations. The synaptic disc-rich fraction was accordingly washed in EDTA (5–20 mM at pH 6) or EGTA (1–10 mM at pH 6) and then studied by negative staining After these treatments definite changes in the hexagonal array of the synaptic discs were found. The synaptic discs
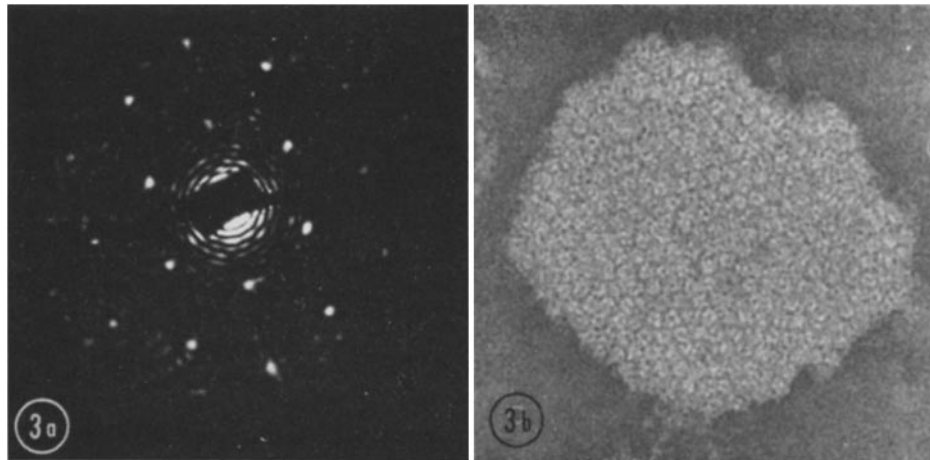

FIGURE 3 Optical diffraction of the synaptic disc. (a) A piece of synaptic membrane from which the optical transform was obtained. × 250,000. (b) The optical transform consists of two diffraction orders of spots with hexagonal symmetry.
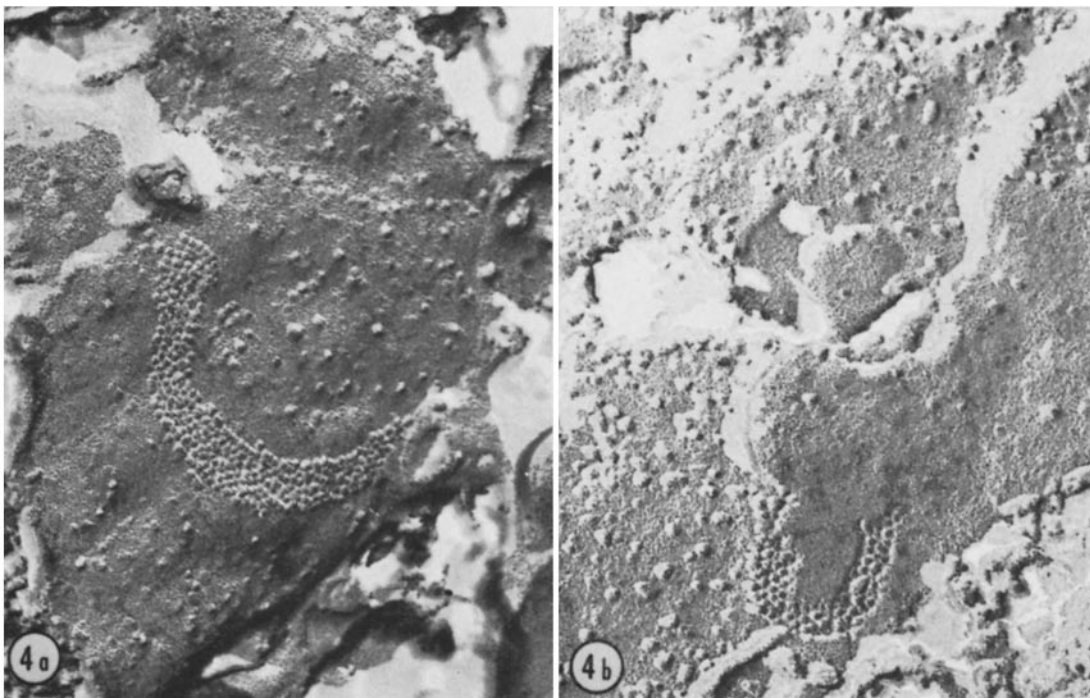
FIGURE 4 Freeze-fracture replica obtained from the goldfish medulla oblongata. (a) A belt of particles, hexagonally arrayed, are coplanar with a fracture face in which particles are scattered on a smooth surface. × 132,000. (b) The same array of particles is observed in the fracture face. × 128,000.

showed zones without particles interspersed in zones covered with the hexagonal array (Fig 5) The center-to-center spacing of the particles was found to be increased from 8 5 to 10 nm The diameter of the 6 nm particles was unchanged but the space between the particles was greater. The central dot of the particle present in sectioned material and confirmed by freeze-fracture technique (21) is poorly preserved. In Fig 6, an irregular-shaped fragment of a synaptic disc has a structure of ~18.5 nm in overall thickness associated with it This structure is characterized by two dense parallel bands 7 nm thick, separated by a ~4–4 5 nm gap Fine striations ~6 nm wide cross the gap at regular intervals of 10 nm.

An intensification of the bridges between the dense bands was obtained in preliminary tilting experiments, performed with the goniometer stage of the Philips EM 300 with the tilted axis roughly parallel to the double band. Occasionally, rows of beads ~4 nm in diameter spaced at regular intervals of 6 nm were observed associated with the external face of the dense bands Each of these beads was joined to the bands by thin stalks

Detergents

The action of detergents, both ionic (DOC) or nonionic (Triton X-100) were studied in the fresh fraction or in fractions previously treated with che-
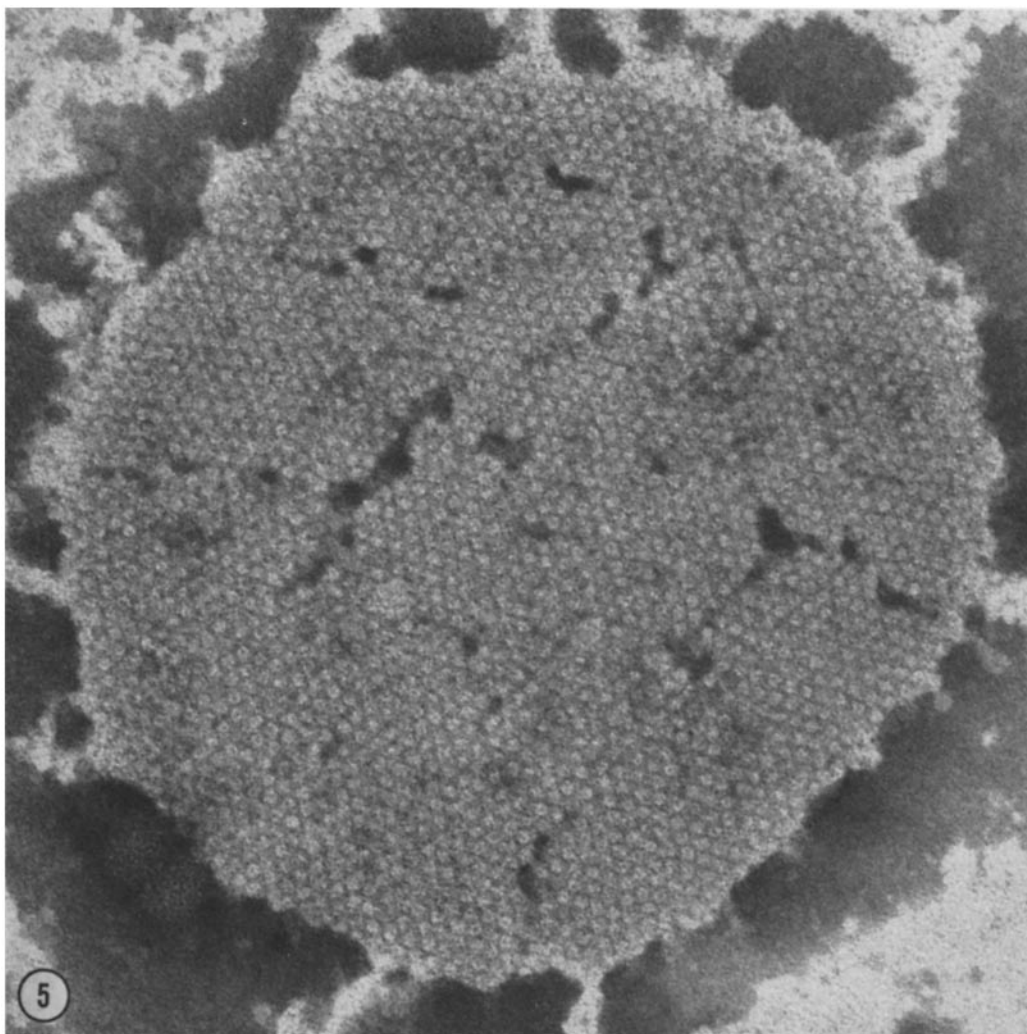

FIGURE 5   High magnification of a synaptic disc membrane after treatment with EGTA. Note the imperfections of the lattice produced by the chelating agent. × 210,000.
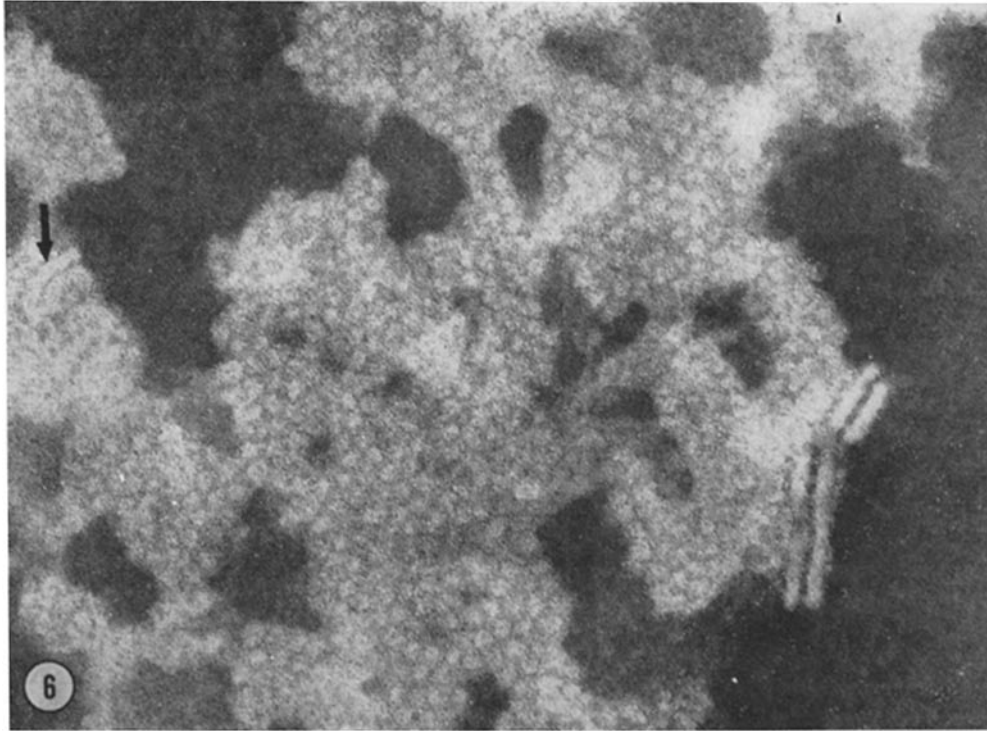
FIGURE 6 High magnification view of a synaptic disc treated with EGTA. A double band structure associated with the hexagonal arrays of particles is observed although very rarely. The arrow shows a second inconspicuous structure present in the same membrane. × 370,000.

lating agents. DOC at concentrations of 0.1–0 3% selectively solubilized mitochondrial and synaptosome membranes. Synaptic discs were less affected, but an increased degree of disorganization was observed in the hexagonal lattice.

In the fraction treated with chelating agents, DOC produced a sequence of changes in the organization of the membranes with the particulate arrays. The synaptic disc was broken into small, independent, irregular-shaped fragments ~0 3 μm in diameter, each one of which conserved the hexagonal array of particles (Fig. 7). Upon increasing the concentration of the detergent (Fig. 8), the synaptic discs were progressively disassembled into poorly preserved networks of hexagonal particles associated with large numbers of the double band structures previously observed (Fig. 6) without detergent treatment.

The bridges crossing the 4 nm gap were usually seen as fine lines of low negative contrast (Fig. 6). However, in certain micrographs an increased negative contrast of the bridges was observed (Fig

9). This effect was probably due to superposition of the bridge material in a direction parallel to the electron beam

A material organized periodically is associated with the cytoplasmic leaflets of the double band structure (Fig 10), obtained by the treatment with EDTA plus DOC. This material consists of beads ~4 nm in diameter separated ~4 nm from the surface of the dense band and spaced ~8 nm center-to-center.

Triton X-100, like DOC, produced an apparent enrichment of synaptic disc membranes presumably by destruction of less resistant membranes. When Triton X-100 was used associated with a chelating agent, its action was highly accentuated and the preparations were more enriched in synaptic discs. However, these preparations showed consistent fragmentation of the synaptic disc structure into small circular patches 0.3–0.5 μm in diameter (Fig. 11) No double band structures were resolved after Triton X-100, used alone or in combination with chelating agents.
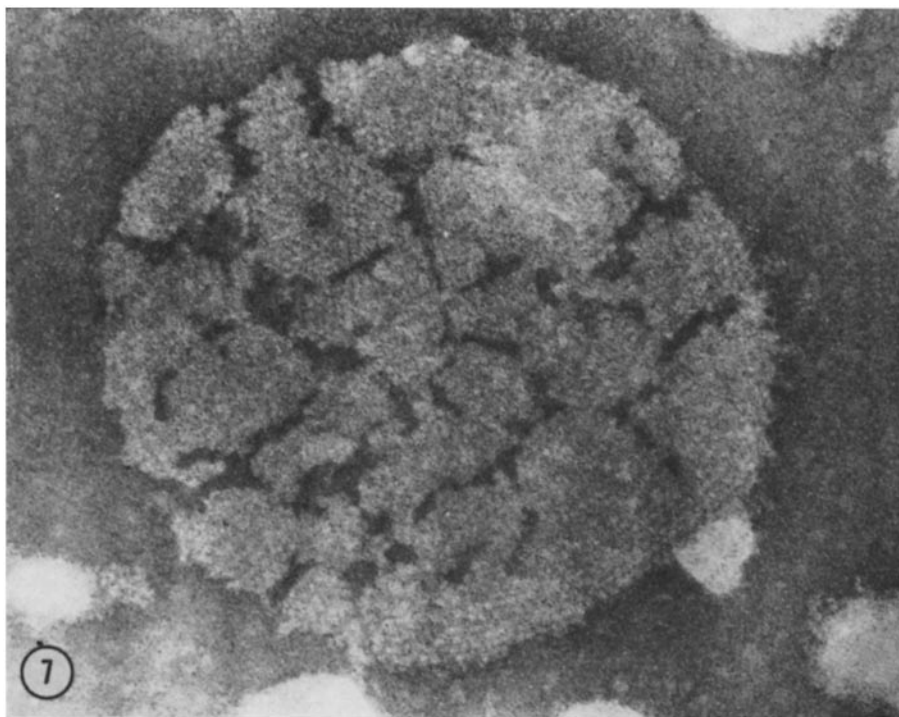
FIGURE 7 Synaptic disc fraction treated with a combination of EDTA and DOC. A fragmentation of the synaptic disc into pieces of irregular shape and size is observed. × 61,600.

DISCUSSION

General Morphology and the Effect of Chelating Agents

The synaptic discs display a hexagonal array of particles 8 5 nm center-to-center when they are observed in negative stained preparations. A similar array of particles is present in freeze-fracture replicas obtained from goldfish medulla oblongata Therefore, the synaptic discs seem to share the same kind of organization described for the liver gap junctions by thin sectioning (14) and negative staining (1, 2)

It is concluded from the present results that the synaptic discs are maintained in a stable configuration at least in part by means of divalent cations This effect could be due to crosslinking between the surface subunits by divalent cations Thus, the withdrawal of divalent cations with chelating agents (EDTA or EGTA) results in definitive changes in the hexagonal lattice These changes consist of: (a) a uniform increase of the space surrounding the subunits; (b) a nonuniform and much larger increase in the spacing between some rows of subunits occurs giving the effect of irregular interconnecting cracks in the otherwise regular lattice These cracks are shown up in good contrast by the negative stain Sometimes the edges of the cracks do not match well, suggesting a detachment of the particles from the synaptic discs. The cracks are random in distribution. (c) The appearance of a new structure referred to as double band

The double bands are only found associated with the hexagonal lattice (see Fig. 6) and their number increases in proportion to the disarray of the lattice. Thus, it is reasonable to assume that the double band structure originates from the synaptic discs and in fact represents small fragments standing on edge Furthermore, it is reasonable to suppose that when chelating agents (EDTA or EGTA) break up the whole synaptic disc structure, rows of subunits separate laterally along with the underlying membrane components. Those rows are often three or four subunits wide and may be, occasionally, seen edge on at 90° and appear as double bands in negative stained preparations. Therefore,
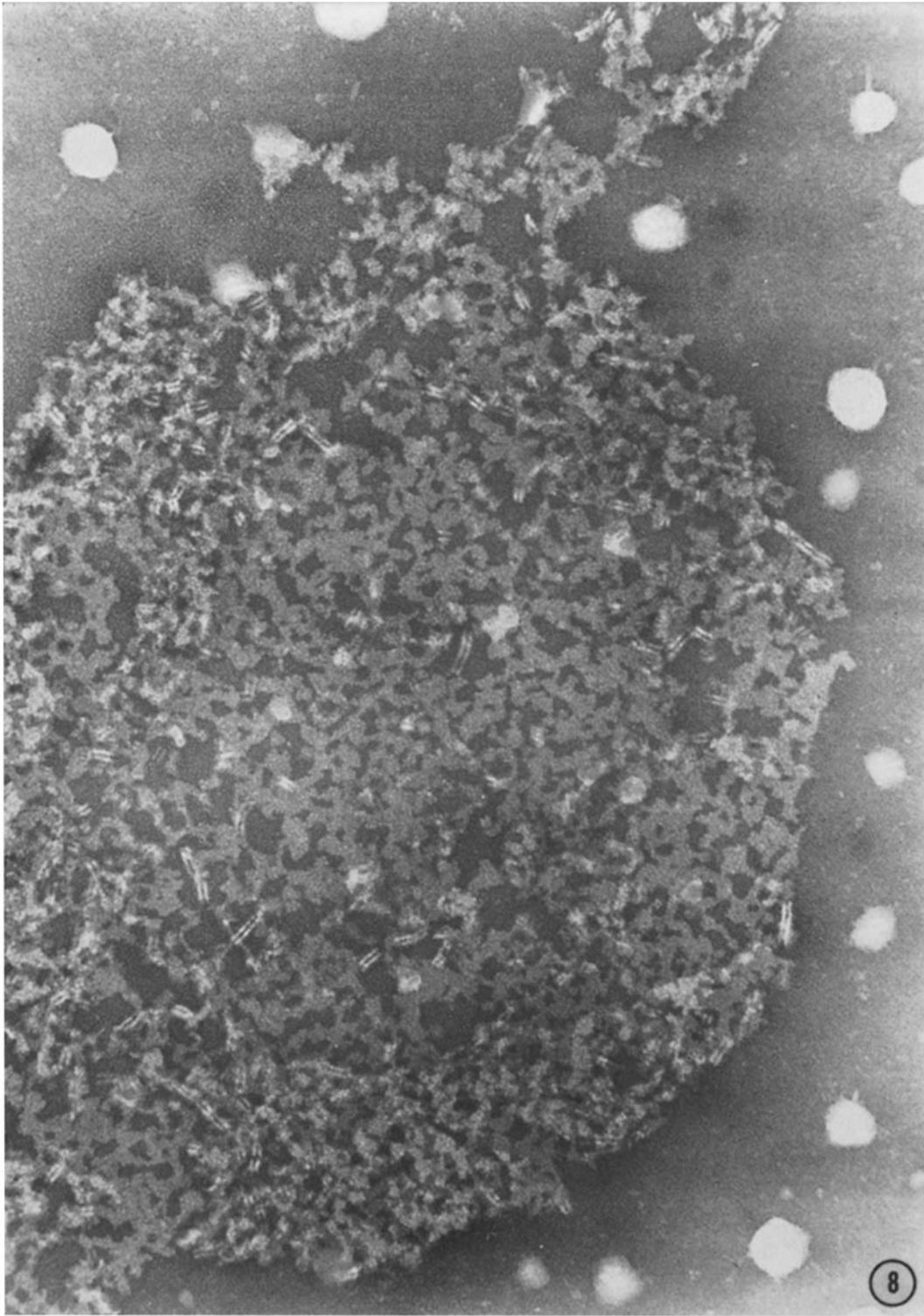
FIGURE 8 In some membranes the effect of the treatment with EDTA and DOC is more conspicuous. The hexagonal lattice is almost completely disrupted giving a number of double band structures. × 87,800.
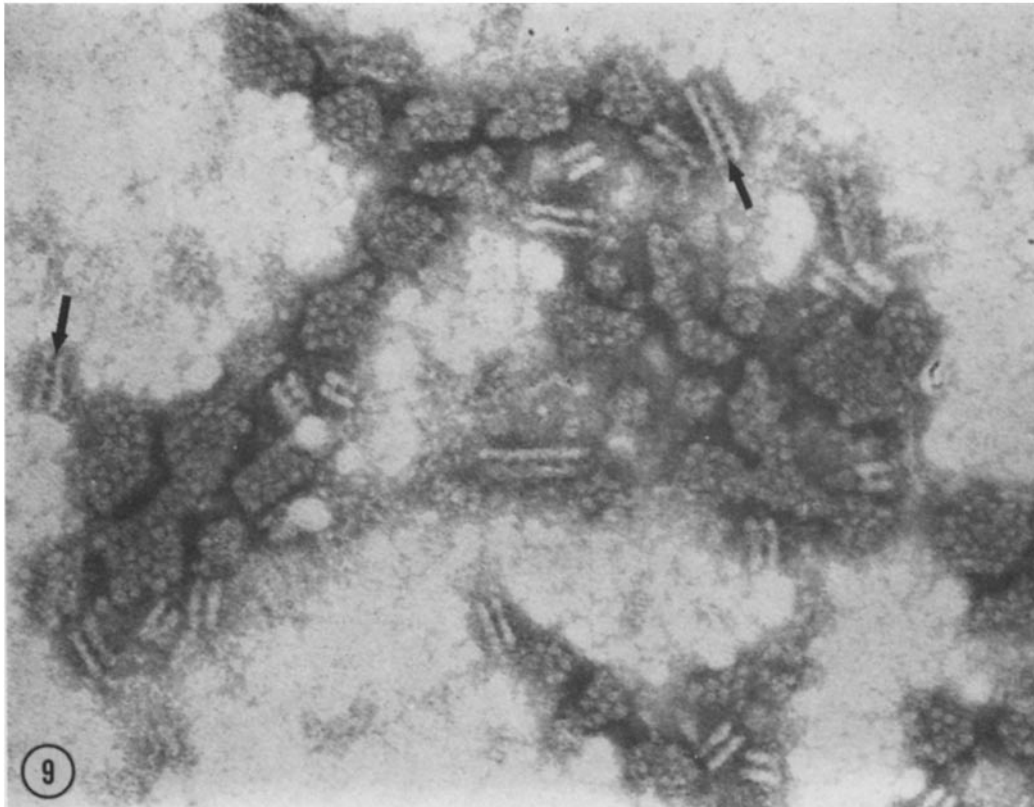
FIGURE 9 High magnification view of EDTA and DOC treatment. Note an intensification of the bridges (arrows) crossing the dense bands of the structure. × 204,000.
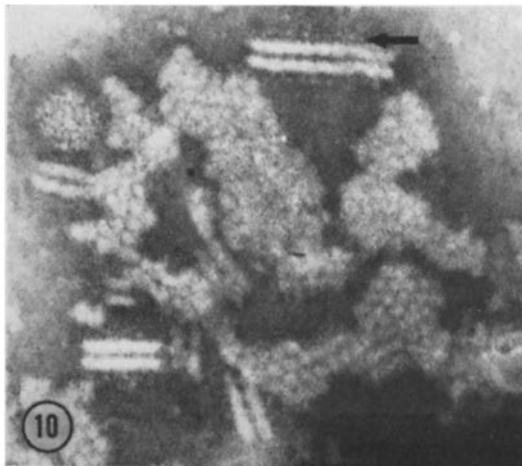

FIGURE 10 Appearance after EDTA and DOC treatment. Note the periodicity of the material (arrow) associated with the cytoplasmic leaflets of the double band. × 200,000.

we regard the double band as representative of an intermediate step in the disassembly of the synaptic discs

According to this interpretation of the nature of the double band structure, each dense band ∼7 nm wide represents a unit membrane and the ∼4 nm gap corresponds to the intermembrane gap observed in sectioned material The overall thickness of the double band is 18 5 nm This is close to the cross-sectional dimension of the synaptic discs observed in sectioned material The hexagonally arranged particles observed in negative stain and in sectioned preparations could be represented in the double band by the less dense striations observed crossing the ∼4 nm gap These striations are the only repeating material observed in the double band displaying a periodicity similar to the hexagonally arranged particles

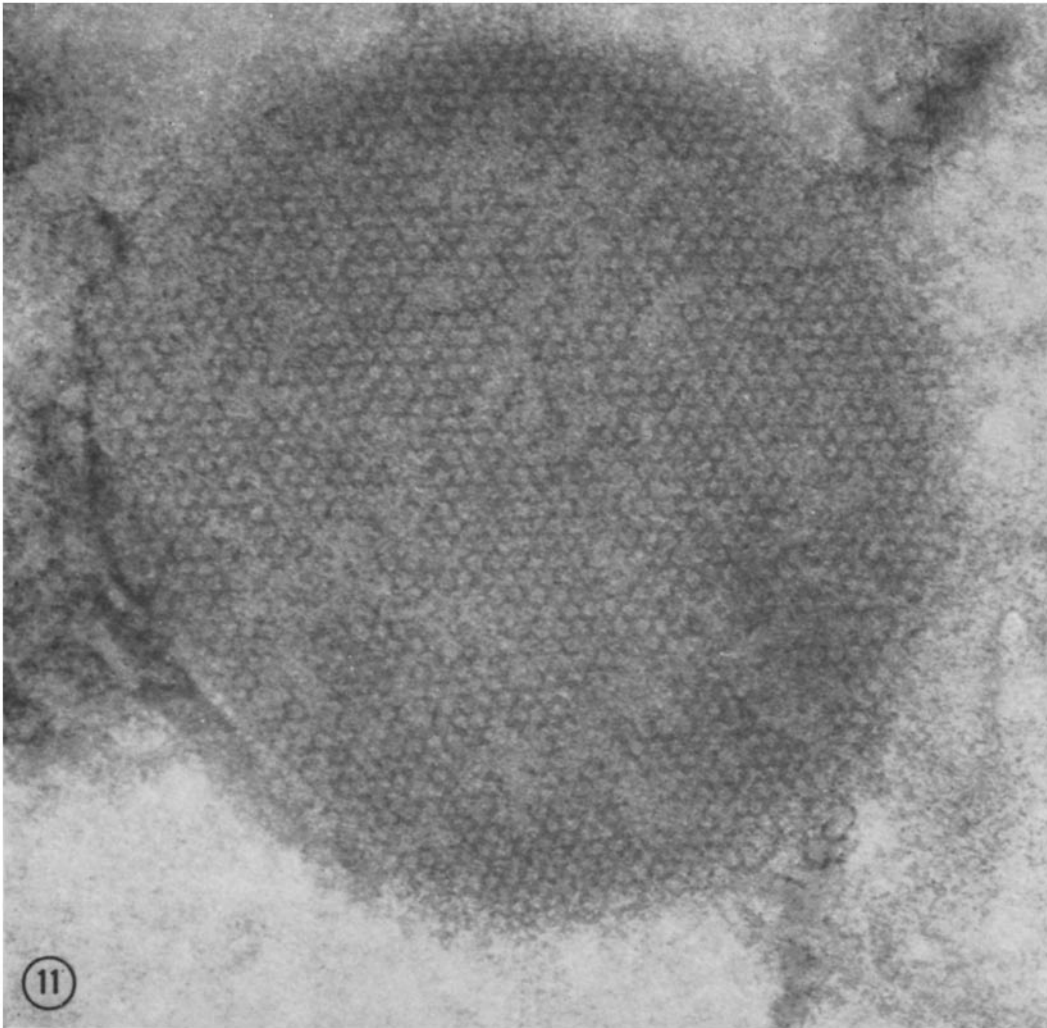This observation is interesting because it shows that the negative stain penetrates between the pre-

FIGURE 11 Treatment of the synaptic disc fraction with EDTA and Triton X-100. Empty areas resulting from the detachment of the ~6 μm particles from the surface of the membrane are observed. X 362,000.

and postsynaptic membranes of the synaptic discs Thus, the gap should be considered in part a space filled with hydrophilic material. On the other hand, the dense bands ~7 nm wide that we have taken as a cross view of the unit membrane are not penetrated by the negative stain However, in some micrographs (Fig. 10) a scalloping of the "dense band" is sometimes observed.

The material associated with the cytoplasmic surface of the double band (Fig. 10) is difficult to account for. It appears as a well-organized pattern

of beads that is better observed by tilting the specimens Its meaning is still unclear to us.

Modifications of the pattern related to the temperature of the negative stain solution (PTA) have been reported by Bennedetti and Emmelot (2) in liver gap junctions. In our experiments the PTA was always used at room temperature. In these conditions changes in the pattern of the control preparations, as those described as temperature-dependent, were never observed. Thus, the changes described in the hexagonal lattice of the

synaptic discs are better related with the action of the chelating agents.

The Effect of Detergents

The gap junctions are structures resistant to the detergent action Bennedetti and Emmelot (2) showed that liver gap junctions were not significantly damaged after treatment with 1% DOC. Goodenough and Stoeckenius (16) have reported that Sarkosyl does not produce morphological changes in liver gap junctions, while selectively destroying other membranes. These authors succeeded in isolating a pure fraction of gap junction membranes using 0.5% Sarkosyl. Similarly, we have observed that Triton X-100 does not destroy the synaptic discs (37) but produces solubilization of other membranes of the fraction

The property of high resistance of the synaptic discs to detergent action is greatly reduced when the fraction is previously treated with chelating agents Combined treatment of EDTA and DOC produces an almost complete disassembly of the structure very largely into the double band fragments It is known that DOC extracts phospholipids from bacterial membranes (31). Since DOC aids in the breakup of synaptic discs induced by chelating agents alone it may be that the initial separation of the surface particles resulting from removal of divalent cations makes some of the underlying membrane phospholipids more accessible to DOC Perhaps these are a less specific component of the membrane lipids not specifically bound to the surface particles.

On the other hand, a combination of EDTA and Triton X-100 also fragments the synaptic discs although in a very different way. Here the effect seems to be one of complete disruption of the structure progressively at its edges We came to this conclusion for two reasons. First, the discs are smaller and more round and second, the double bands do not appear. Triton X-100 produces solubilization of membrane proteins, as has been observed in nerve ending fractions obtained from rat brain (18) and in bacterial plasma membranes (33) Used in combination with EDTA on $E\ coli$ by Schneitman (32) it produces a four times increase in protein solubilization We thus believe that the primary effect of Triton X-100 on the synaptic disc may be protein dissolution with lipid dispersion occurring secondarily. In this case, dissolution of the protein components leads to complete disruption even of the double bands. It seems reasonable to suppose that if the protein molecules in the surface lattice are soluble in Triton X-100 the ones at the edges would be the first to go into solution because they present a larger fraction of their areas to the solvent. The underlying, newly exposed lipid molecules would then be expected to go into solution.

The particles we have seen in the negative stain preparations lie side-by-side in a regular lattice in the center of the double band structures. The negative stain has not revealed a deep penetration of the
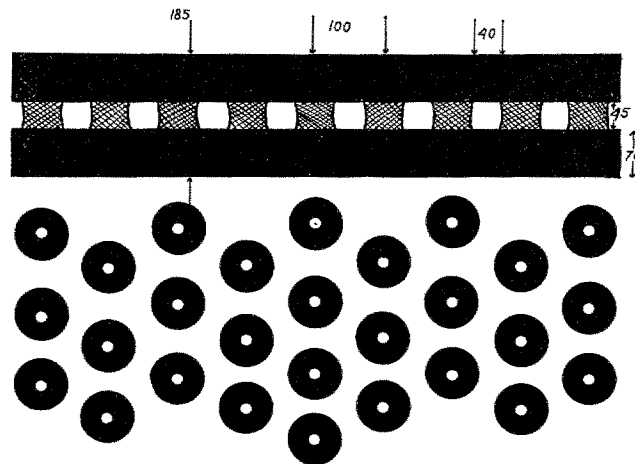

FIGURE 12 Diagrammatic representation of the hexagonal array and the double band structure. The main point in our interpretation is that the double band is visualized by an edge on view of fragments of the synaptic membrane complex.

particles into the cores of the adjacent membranes This is difficult to correlate with our limited freeze-fracture evidence. However, more freeze-fracture evidence is now being accumulated and a future paper will deal with this in detail

The speculative interpretations of our data (Fig 12) can best be tested by obtaining larger quantities of a very pure fraction and our current efforts are directed to this immediate goal.

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