THE STRUCTURE OF POSTSYNAPTIC DENSITIES ISOLATED FROM DOG CEREBRAL CORTEX

II. Characterization and Arrangement of Some of the Major Proteins within the Structure

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ABSTRACT

An attempt was made to identify some of the proteins of the postsynaptic density (PSD) fraction isolated from dog cerebral cortex. The major protein has been tentatively labeled "neurofilament" protein, on the basis of its 51,000 mol wt correspondence to a protein found in neurofilament preparations. Other proteins are akin to some dog myofibrillar proteins, on the basis of immunological cross-reaction and equal sodium dodecyl sulfate (SDS)-gel electrophoretic mobilities. While a protein similar to dog muscle myosin is not present in the PSD fraction, a major protein present is actin, as evident from reactivity with antiactin serum, from SDS-gel mobility, and from amino acid composition. Only very little tubulin may be present in the PSD fraction, as determined by gel electrophoresis. Various treatments of the PSD fraction were attempted in order to extract some proteins, as revealed by gel electrophoresis, and to observe the structural changes of the PSD fraction residue after extraction of these proteins. The PSD is remarkably resistant to various extraction conditions, with only 4 M guanidine being found to extract most of the proteins, except the 51,000 mol wt protein. Disulfide reducing agents such as dithiothreitol (DTT), blocking agents such as *p*-chloromercuribenzoate (PCMB) (both in the presence of deoxycholate [DOC]), a Ca^{++} extractor, ethylene glycol-bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetate (EGTA), and guanidine caused an opening up of the native dense PSD structure, revealing ~10-nm filaments, presumably consisting of "neurofilament" protein. Both DTT-DOC and PCMB-DOC removed chiefly actin but also some other proteins. EGTA, in greatly opening up the structure, as observed in the electron microscope, revealed both 10-nm and 3- to 5-nm filaments; the latter could be composed of actin, since actin was still in the residue after the treatment. EGTA removed a major 18,000 mol wt component and two minor proteins of 68,000 and 73,000 mol wt. Based on the morphological and biochemical evidence, a picture is presented of the PSD as a structure partly made up of 10-nm and 3- to 5-nm filaments, held together through Ca^{++} interaction and by bonds amendable to breakage by sulfhydryl-

blocking and disulfide-reducing reagents; either removal of Ca^{++} and/or rupture of these disulfide bonds opens up the structure. On the basis of the existence of filamentous proteins and the appearance of the PSD after certain treatments as a closed or open structure, a theory is presented which envisages the PSD to function as a modulator in the conduction of the nerve impulse, by movements of its proteins relative to one another.

The preceding paper (5) described the isolation and general morphology of a postsynaptic density (PSD) fraction isolated from dog cerebral cortex. By morphological and biochemical evidence, the fraction was judged to be moderately pure. The density was described as a compact disk-shaped structure, which is made up of some filamentous proteins, and which apparently has extensions of filaments coming out of the central structure. Some of these proteins are perhaps identical to some of the proteins comprising the synaptic membrane. The molecular weights of the major proteins were determined and, on the basis of these and the filamentous appearance of the proteins, further research on this structure was deemed advisable.

In this paper, an attempt is made to characterize the major proteins of the density and to try to visualize their arrangement as part of the structure of the density. To this end, two types of experiment were performed: first, to compare, when possible, by electrophoretic, chemical, and immunological means, known proteins with some of the density proteins, and, secondly, to break up the structure of the density by various chemical treatments and to examine the resultant modified structure both morphologically by electron microscopy and chemically by gel electrophoresis. On the basis of the results, a theory of the neurological function of the PSD has been proposed.

MATERIALS AND METHODS

The methods used for the isolation of the PSD fraction, for its morphological characterization by electron microscope methods, and for a characterization of its proteins by gel electrophoresis are described in the preceding paper (5).

For the various treatments of the PSD fraction described in this paper, the following method was used. Either a PSD preparation taken directly from the gradient, or a pellet of it after centrifugation (in both cases, ~600 μg protein) was resuspended in 12 ml of the treatment solution described in the figure legends, and kept in the cold room overnight (~12 h). It was only for convenience that this overnight treatment was used, and it could be that a shorter treatment would suffice to give our results. The treated suspension was then centrifuged at 160,000 *g* for 1 h to obtain a pellet and a supernate. The pellet was cut into three parts, one for protein determination by the Lowry method, one for electron microscope visualization, and one for sodium dodecyl sulfate (SDS) treatment, all as described in the preceding paper (5). To the supernate was added cold TCA to a final concentration of 10%; the suspension was kept overnight in the cold to insure precipitation of all the proteins, then centrifuged at low speed, and the precipitated proteins were washed once with cold 5% TCA, twice with cold water, and twice with cold 90% acetone. The acetone wash was found necessary to rid the preparation of adsorbed deoxycholate (DOC) and guanidine (when used). The washed pellet was then treated with the SDS solution as described in the preceding paper (5).

Muscle fibrils were isolated from the ear muscles of a dog by the method of Perry and Zydowo (49). Myosin was prepared from this muscle by the method of Holtzer and Lowey (28) while actin was extracted and purified by the method of Rees and Young (54), carrying the procedure through the Sephadex G-200 step. Tubulin, purified from porcine brain by G. Piperno and D. J. L. Luck, The Rockefeller University[1], was a gift from Dr. G. Piperno. The purified protein preparations were characterized by SDS gel electrophoresis, and the major protein by far in each case was the one purified for (cf. Fig. 1, slot 3 for tubulin, and Fig. 3, slot 1 for actin).

Amino acid analyses on gel bands were performed by Drs. Stanford Moore and David C. Sogin, The Rockefeller University, using the Durrum 500 amino acid analyzer (Durrum Instrument Corp., Sunnyvale, Calif.). The individual bands for any one protein were excised with a razor blade, then pooled, macerated with a razor blade, and the proteins were extracted from the gel with 0.1% SDS-1% β-mercaptoethanol in 50 mM barbital buffer, pH 8.6, by stirring at room temperature overnight. About one-half of the protein in the band was recovered in the extract. The suspension was filtered on a 1.2-μm Millipore filter (Millipore Corp., Bedford, Mass.) and the filtrate was concentrated in an Amicon PM-10 membrane (Amicon Corp., Lexington, Mass.) to a volume of ~0.5 ml. Cold acetone was added to the filtrate to a 90% concentration, kept for a few hours in the cold, and the precipitated proteins were centrifuged

[1] Piperno, G., and D. J. L. Luck. 1977. Microtubular proteins of *Chlamydomonas reinhardtii*. An immunochemical study based on the use of an antibody specific for the β-tubulin subunit. *J. Biol. Chem.* **252**:383.

at low speed. The pellet was dried by evaporation, 0.5 ml of 6 N HCl in 0.2% phenol was added, and the proteins were hydrolyzed at 110°C for 22 h. The hydrolysate was taken up in either 0.1 or 0.2 ml of citrate buffer, pH 2.2, for the chromatography. It was found necessary, in order to minimize amino acid contamination, to use thoroughly washed equipment, and to wear gloves at all handlings of the gels.

Immunology

Antisera against various rat and dog PSD fractions were prepared as described in the previous paper (5). For preparation of an antiserum against muscle actin, the semipurified fraction whose SDS-polyacrylamide gel electrophoresis pattern is seen in Fig. 3 was used as a starting material. After electrophoresis, staining, and drying of the gel containing the original actin fraction (Fig. 3), the actin band was cut out and the gel strip was cut into small pieces. Protein was then eluted from this gel band as described above. The eluate volume was reduced by concentration on an Amicon PM30 filter and then by precipitation in a large volume (1:9) of ice-cold acetone. The precipitate obtained was then run on a second gel and a single band was obtained. This band was again cut out and the gel strip was homogenized in 0.9% NaCl. The homogenate was emulsified in Freund's complete adjuvant (1:2), and two rabbits received three injections intrascapularly at biweekly intervals. The protein content of one injection corresponded to 350 μg of the semipurified actin fraction and was injected in a volume of about 2 ml. 10 days after the third injection, the rabbits were bled by heart puncture. Antiactin reactivity was detected in only one of the two rabbit sera. The active serum was concentrated four times by $(NH_4)_2SO_4$ precipitation and dialysis as described in the previous paper (5).

Muscle and PSD fraction extracts were prepared as follows: washed dog myofibrils and dog PSD fractions were solubilized (to about 80%) in 0.5% DOC-1 mM *p*-chloromercuribenzoate, listed as *p*-hydroxymercuribenzoate (PCMB) in 50 mM barbital buffer (pH 8.6) by extensive homogenization in a tight-fitting Dounce homogenizer (Kontes Co., Vineland, N. J.). After centrifugation at 105,000 *g* for 1 h, the supernate was used for immunodiffusion directly or stored at −20°C. The reason for the use of DOC-PCMB as the extracting medium was that actin was found to be the major protein solubilized by this mixture from the dog PSD fraction (cf. Fig. 4) and was also solubilized from dog myofibrils (not shown). The semipurified actin was also treated similarly with DOC-PCMB, since this treatment prevented both some unspecific precipitation of actin in the immunodiffusion plates and also polymerization to actin filaments, as judged from electron microscope (EM) studies of such a treated actin fraction.

Double immunodiffusion was performed as described in the preceding paper (5), but, in the experiments described in Fig. 5, Triton X-100 was omitted from the agarose.

Chemicals

In addition to the chemicals used in the experiments of the preceding paper (5), the following were obtained: guanidine-HCl, ethylene glycol-bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetate (EGTA), and PCMB from Sigma Co. (St. Louis, Mo.); DOC from Calbiochem (San Diego, Calif.); dithiothreitol (DTT) from RSA Corp. (Ardsley, N. Y.); Sephadex G-200 from Pharmacia, Inc. (Piscataway, N. J.).

RESULTS

Tentative Identification of Some PSD Fraction Proteins

Ever since the initial finding of a colchicine-binding property in brain particulates (12), tubulin has been thought to be linked to nerve-ending membranes (1, 2, 13, 32, 37) and to be part of the synaptic junction complex (43, 59). It was of interest therefore to compare the SDS-gel electrophoretic mobilities of the polypeptides in our PSD preparation with that of tubulin purified from mammalian brain, and Fig. 1 shows this comparison. It is clear that neither the α nor the β subunit of tubulin is the major protein in the density; indeed, if tubulin is a part of the density at all (bands at 55,000 and 59,000), it is there in rather minor amounts. The absence of large amounts of tubulin is at least not due to Triton extraction, for if the synaptosomal membrane fraction (synaptosomes lysed and the membrane fraction removed from a gradient before the treatment with Triton; cf. Figs. 1 and 11 of preceding paper [5]) is electrophoresed in SDS, again only small amounts of the 55,000 and 59,000 bands are apparent (Fig. 1). Indeed, even a whole synaptosome preparation gave only little indication of the presence of tubulin (not shown). This result is in contrast to that of Blitz and Fine (2) who found large amounts of tubulin in a rat synaptosomal membrane fraction and in synaptosomes. Our finding that tubulin is not a major component of the PSD fraction is in agreement with the findings of Yen et al. (61), and with those of Therien and Mushynski (58). The claim that tubulin is the major protein of the PSD (7, 59) or of the synaptosomes (2) could be due to insufficient electrophoretic resolution between the major 51,000 protein and the tubulin subunits; to a general similarity of amino acid composition among filamentous proteins (cf. reference 47),

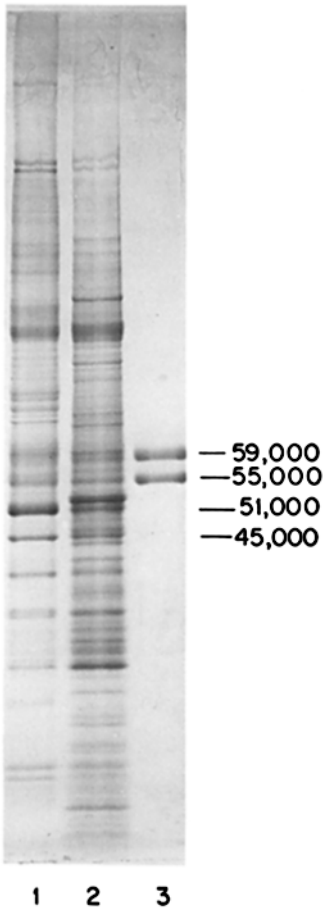

FIGURE 1 Comparison of protein gel electrophoresis patterns of the PSD and synaptosomal membrane fractions, and of purified brain tubulin. The PSD and synaptosomal membrane fractions were obtained as described in the preceding paper (5). Purified porcine brain tubulin was kindly supplied by Dr. G. Piperno. Gel electrophoresis was performed and molecular weights were determined as described in the preceding paper (5). Slot 1: PSD fraction; slot 2: synaptosomal membrane fraction; slot 3: purified tubulin.

possibly giving similar tryptic maps (2, 59); to a lack of sufficient visual contrast between the PSD structure and the oxidized diaminobenzidine deposit due to the peroxidase-linked tubulin antibody (43) in order to clearly localize the purported tubulin at the density; and finally, because we have identified actin to be one of the density proteins, to our finding[2] that in some cases the pre-immune sera corresponding to the antitubulin sera contained material reacting with purified actin in the double immunodiffusion technique. Our conclusion is that the PSD fraction, as we isolate it, does not contain tubulin among its major intrinsic proteins, but may contain minor amounts (bands at 55,000 and 59,000). However, it is possible that tubulin could be linked somewhat loosely to the basic structure and that it is mostly removed during the isolation of the synaptosomes or of the densities.

Because of the filamentous nature of some of the proteins of the density fraction, it was deemed advisable to compare the electrophoretic pattern of the density proteins with that of myofibrillar proteins, and Fig. 2 shows the result of this comparison. Mixing the SDS-solubilized proteins from myofibrils and from the density fraction and running them together on a high-resolution gel would indicate whether some degree of similarity in size existed between them. It can be seen from Fig. 2, as judged by comparing the electrophoretic patterns of the individual preparations and of the mixed preparation, that some proteins are specific to the density, some are specific to the myofibrils, particularly myosin, but that some, particularly the 45,000 one, are found in both myofibrils and the PSD fraction. Thus, some eight proteins seem to be common to both, those of 110,000, 100,000, 59,000, 45,000, 31,000, 26,00, 18,000, and 17,000, and are so indicated on the figure. However, as detailed in the previous paper (5), the 110,000, 59,000, and 17,000 bands could well be contaminants from other fractions. It is instructive to recall that of the filamentous proteins which have been characterized from mammalian muscle, α-actinin has a mol wt of 100,000 (40); tropomyosin has assigned molecular weights of 33,500 (55), 35,000 (21), and 33,000 (45); the three troponin components have molecular weights of 37,000, 24,000, and 20,000 (21), 37,000, 24,000, and 19,000 (55), 38,000, 28,000, and 20,000 (4), and 39,000, 24,000, and 18,500 (26); and actin has a mol wt of 45,000 (51). Evidence will be presented below that the 45,000 mol wt PSD fraction protein is actin. Thus, it could be that the 100,000 PSD fraction component is similar to muscle α-actinin; that the 31,000 PSD fraction component is similar to tropomyosin; and that the 18,000 component is similar to one of the subunits of troponin, the Ca^{++}-binding troponin C.

Immunological similarity was also detected be-

[2] F. Blomberg, G. Piperno, M.-C. Tzeng. Unpublished results.

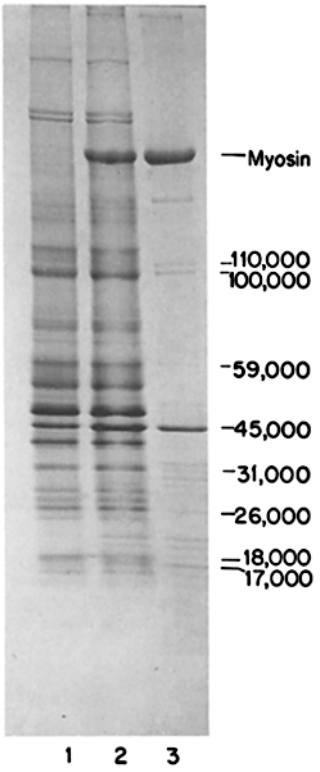

FIGURE 2 Comparison of protein gel electrophoresis patterns of dog PSD and dog muscle myofibril fractions. The PSD fraction was isolated as described in the preceding paper (5); ear muscle was dissected and myofibrils were obtained and washed as described in Materials and Methods. Gel electrophoresis was performed and molecular weights were determined as described in the preceding paper (5); the two major proteins of the myofibrils were identified as myosin by its amount and actin by its molecular weight. The depicted molecular weights on the right connote proteins thought to be common to both PSD and striated muscle (cf. text). Slot 1: PSD fraction; slot 2: mixture of 1/2 of PSD and 1/2 of myofibril fractions; slot 3: myofibril fraction.

tween some PSD fraction proteins and some myofibrillar proteins. When antisera against rat and dog PSD fractions were tested against solubilized dog myofibrillar proteins in immunodiffusion, two different cross-reacting antigens were detected (Fig. 3). Although no identification of the particular antigens can be made from this test, the results certainly suggest a high degree of similarity between some myofibrillar proteins and some PSD fraction proteins.

Evidence that one of these proteins common to both myofibrils and to the PSD fraction is actin is presented in Figs. 4 and 5 and Table I. When a semipurified actin preparation from dog muscle is electrophoresed on an SDS gel, its by-far-major component migrates exactly with the 45,000 mol wt component of the PSD fraction (Fig. 4, slots 1 and 2). When the respective bands are eluted from each of the PSD fraction or actin gels and amino acid compositions are determined on each, as given in Materials and Methods, it can be seen from Table I that the two proteins are very similar in this regard; in particular, both contain methyl histidine. Judged from the protein relatedness test of Marchalonis and Weltman (39), the muscle and density fraction actins gave sums of amino acid composition differences which fell within the value for the same protein determined in different laboratories. However, there is a report (22) that the tryptic peptide maps of chick muscle actin and chick brain actin are not identical, though very similar.

Further evidence for the presence of actin in the

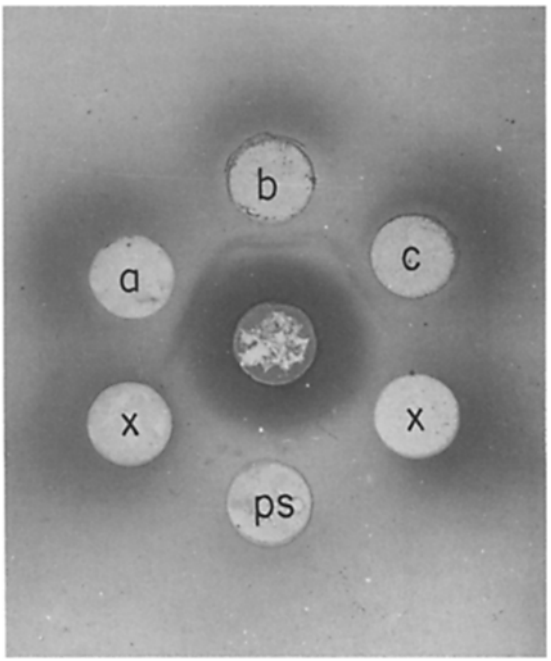

FIGURE 3 Immunodiffusion plate showing cross reaction between anti-PSD antiserum and a washed dog myofibril extract. Details are given in Materials and Methods. Center well: extract of myofibrils (~5 mg protein/ml) solubilized in 0.5% DOC-1 mM PCMB; *a*, antirat PSD; *b* and *c*, antidog PSD, in which the immunogen in *c* was treated with 0.1% SDS-1% β-mercaptoethanol before injections; ps, pre-immune serum; x, unrelated antisera. The wells contained 40 μl of the reagents.

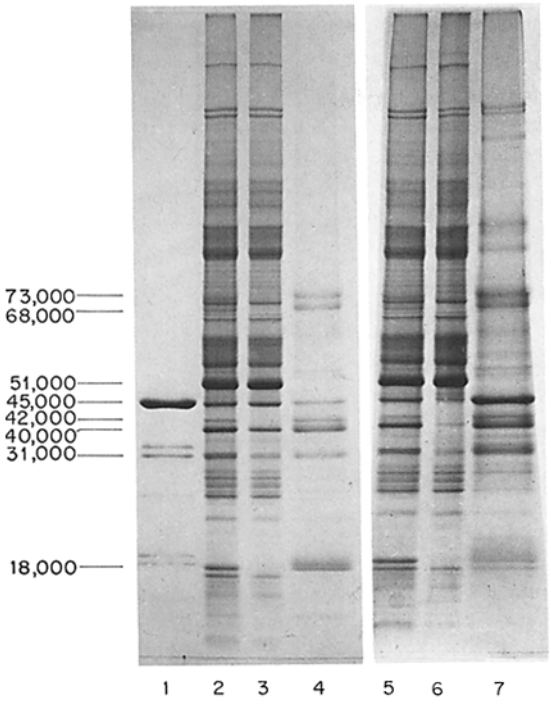

FIGURE 4 Comparison of protein gel electrophoresis patterns of PSD fraction, of purified actin, and of PSD fraction after various treatments. The PSD fraction was obtained as described in the preceding paper (5), while actin was purified as described in Materials and Methods. Gel electrophoresis and molecular weight determination was performed as in the preceding paper (5), and treatments of the PSD fractions are described in Materials and Methods. Slot 1: actin; slot 2: PSD fraction; slot 3: PSD fraction treated with 100 mM EGTA overnight in the cold and then centrifuged to give pellet; slot 4: supernate after EGTA treatment; slot 5: PSD fraction; slot 6: PSD fraction treated with 0.5% DOC-0.5 mM PCMB overnight in the cold and then centrifuged to give pellet; slot 7: supernate after DOC-PCMB treatment.

PSD fraction was obtained by using an antiserum against a highly purified muscle actin preparation. This antiserum precipitates actin from the semipurified muscle actin fraction (Fig. 5), and also precipitates a protein contained in the PSD fraction extract (Fig. 5). That the protein in the PSD fraction extract is actin is indicated by the finding that actin is the major protein present in the extract (Fig. 4, slot 7) and also by the observation of a confluence of the antisera precipitin lines against the PSD fraction extract and actin (Fig. 5).

Actin, or an actin-like protein, has been described to be present in many cell types (cf. reference 51), in nerve tissue (2, 15, 46, 52), in a synaptosomal preparation (2), and in a synaptic junction complex fraction (58). In addition, a tropomyosin-like protein has been described to be present in neurons (14) and in isolated synaptosomes (2), and there are indications that all the troponin components (53), including troponin C, the Ca^{++}-binding subunit, are also present in brain (16). While Puszkin and Berl (52) indicated that a myosin-like protein is present in whole brain, we could find no evidence for a protein like dog muscle myosin in our PSD preparation (cf. Fig. 2). Even though the estimated molecular weight of dog muscle myosin on our gel is low (~170,000), this could be in error due to an incorrect extrapolation from the 130,000 mol wt of β-galactosidase (cf. preceding paper [5]). The important point is that no band in the dog PSD preparation migrates like dog muscle myosin. Also, although it is well known that actin can be extracted from muscle tissue with low ionic strength buffer, this is not the case with the PSD fraction actin, for only higher salt concentrations or other reagents (cf. below) can remove it from the PSD fraction, indicating a binding of actin to a protein other than myosin.

Treatments of the PSD Fraction

One of the ways in which the intimate structure of the PSD can be visualized is to treat the preparation with various reagents in the hope that the reagents would change the structure, as observed in the EM, and at the same time cause a separation and differential release of some of the proteins of the PSD fraction, as observed upon gel electrophoresis. Somewhat the same procedure was employed by Cotman and Taylor (8) on synaptic junction complexes. Accordingly, treatments were instituted as given in Materials and Methods, and representative results are shown in Figs. 4–12. In the case of treatments with KCl, DTT, and PCMB, it was found necessary to add 0.5% DOC to the reagents. In the concentration used, DOC by itself or the reagents by themselves did not do much to break up the PSD; the reason for this is not known. In all the gel patterns (Figs. 4 and 6), it can be seen that the disappearance or reduction of bands in the treated PSD residues is matched by the appearance of comparable bands in the treated PSD supernates.

Fig. 6 shows the gel pattern and Fig. 7 shows a picture of the PSD fraction pellet obtained after treatment of the fraction with 0.5% DOC-75 mM KCl and subsequent centrifugation. In the low magnification inset (Fig. 7), it can be seen that

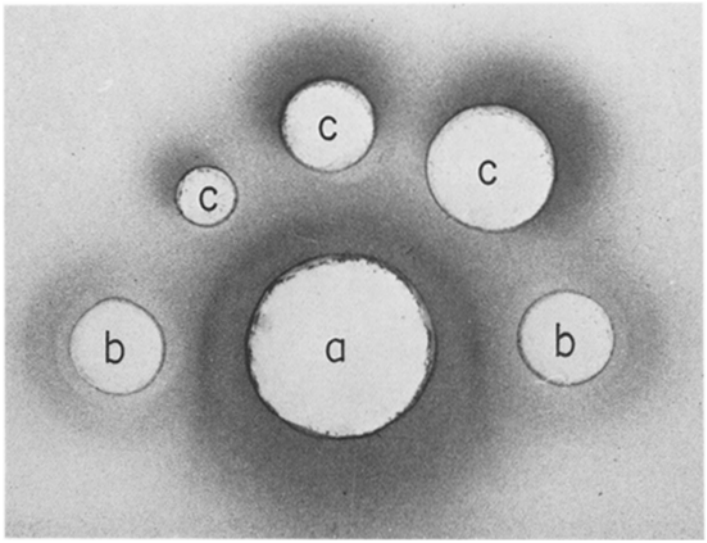

FIGURE 5 Immunodiffusion plates demonstrating the specificity of the antiactin antiserum and its cross-reactivity with an extract of dog PSD. Details are given in Materials and Methods; Coomassie Blue-stained. *a*, antiactin antiserum; *b*, semipurified dog myofibrillar actin treated with 0.5% DOC-1 mM PCMB and concentrated to ~10 mg protein/ml; *c*, extract of PSD after PSD (2 mg protein/ml) was solubilized in 0.5% DOC-1 mM PCMB. The wells from small to large contained 10, 20, 40, and 80 μl reagents. Controls using pre-immune sera were negative (not shown).

many PSD structures can still be seen, as compared to Figs. 2 and 3 of the preceding paper (5). However, they seem to be opened up somewhat and less compact, and spaces within the structure (arrows) are evident at the higher magnification. The only proteins affected by the DOC-KCl treatment are the 45,000 and 40,000 mol wt bands, with only a small part of these being lost (Fig. 6, slots 4, 5, and 6). However, the slight opening up does reveal the apparent particulate nature of the PSD, the apparent particles, of size ~13 nm in diameter, being somewhat ordered within the structure, while also visible is thin filamentous material.

When the fraction pellet was treated with DOC-DTT, the subsequent preparation was as seen in Fig. 8, with the gel picture being shown in Fig. 6, slots 1, 2, and 3. In the low magnification inset, very few recognizable densities are present; instead, the high magnification picture revealed a marked opening up and rearrangement of the structure, with less of the background filamentous material which is seen in Fig. 7 and in the untreated densities as seen in the preceding paper (Figs. 2 and 3 of reference 5). The apparent particulate nature of the interior of the density is much more apparent than that seen after DOC-KCl treatment (Fig. 7), but less thin filamentous material is seen in Fig. 8 than in Fig. 7. The PSDs appear as aggregates of ~13-nm diameter particles (Fig. 8, arrows). The gel study (Fig. 6) showed a differential loss of most of the actin, with about one-half of the 40,000, 31,000, and 26,000 mol wt bands also being removed. A tentative conclusion based on these results is that the presence of actin and the 51,000 mol wt band, and possibly also the 40,000, 31,000, and 26,000 mol wt bands, is necessary for the intactness of the structure.

However, if, instead of using a reducing agent like DTT, one uses a sulfhydryl-blocking agent like PCMB, a different appearance of the treated density fraction is obtained (Fig. 9). The low magnification picture shows still recognizable densities, enmeshed in quite a bit of background granular material (arrows, inset). It appears that some densities have opened up completely and that some are quite compact, virtually intact. Again, the PSD is observed at higher magnification to be apparently composed of dense particles, 10–20 nm in diameter (arrows), with a network of filamentous material apparent in the background. Yet, if one examines the gel pattern of the PSD fraction residue after DOC-PCMB treatment (Fig. 4, slots 5, 6, and 7), it is not too different from that obtained after treatment with DOC-DTT

TABLE I

Comparison of Amino Acid Compositions of Dog Muscle Actin and the 45,000 mol wt Band from Dog Brain PSD

	Muscle	PSD
Asp	1.00	1.00
Thr	0.65 ± 0.05	0.58 ± 0.06
Ser	0.78 ± 0.07	0.81 ± 0.02
Glu	1.28 ± 0.04	1.40 ± 0.09
Pro	0.45 ± 0.05	0.47 ± 0.03
Gly	1.20 ± 0.13	1.21 ± 0.09
Ala	0.79 ± 0.03	0.86 ± 0.06
Val	0.48 ± 0.04	0.54 ± 0.06
Met	0.25 ± 0.03	0.24 ± 0.02
Ile	0.63 ± 0.03	0.58 ± 0.02
Leu	0.82 ± 0.01	0.90 ± 0.04
Tyr	Lost	Lost
Phe	0.36 ± 0.01	0.39 ± 0.02
His	0.16 ± 0.05	0.22 ± 0.04
Lys	0.44 ± 0.07	0.51 ± 0.03
Arg	0.49 ± 0.02	0.57 ± 0.04
Me-His	0.020*	0.018*

The 45,000 mol wt gel bands from PSD and purified dog muscle actin preparations were excised, extracted, and the proteins were hydrolyzed and amino acid compositions determined as given in Materials and Methods. The values represent the mean, with standard deviations, of three determinations in each case; aspartic acid is set at 1.00. Blanks obtained from gel fractions showing no stained protein gave the same small amino acid contamination in both cases; thus the values shown below were not corrected for these blanks.

* Estimate of methyl histidine shoulder.

(Fig. 6, slots 1, 2, and 3). There is a differential loss of most of the actin as well as most of the 40,000 and 31,000 mol wt bands. In addition, the 18,000 mol wt band as well as two faint bands at 68,000 and 73,000 are mssing after DOC-PCMB treatment. It would appear, then, that not all of the thin filamentous nature of the density could be due to the presence of actin, for both DOC-DTT (Fig. 6) and DOC-PCMB (Fig. 4) remove most of the actin, and yet more thin filamentous background is seen in Fig. 9 (after DOC-PCMB) than in Fig. 8 (after DOC-DTT).

A further clue to the nature of the apparent filamentous material of the density may be had by examining the preparation after EGTA treatment. The gel pattern is shown in Fig. 4, slots 2, 3, and 4, where it can be seen that 100 mM EGTA removes rather specifically the 18,000 mol wt band (giving an apparent doublet in the supernate), the minor 73,000, 68,000, and 42,000 mol wt bands, as well as most of the 40,000 mol wt band, a small amount of the 31,000 mol wt band, and in even smaller amounts, the actin band; 50 mM EGTA worked as well as 100 mM EGTA in this regard. The EM picture (Fig. 10) shows a rather startling pattern of apparent particles 10–13 nm in diameter in ordered array, in some cases

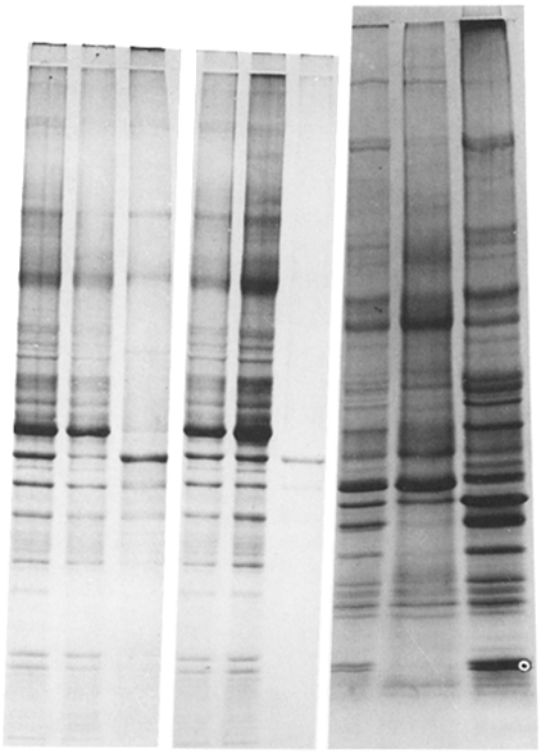

FIGURE 6 Gel electrophoresis of PSD fractions after various treatments. The PSD fraction was isolated and gel electrophoresis was performed as described in the preceding paper (5). Treatments of the PSD were done as described in Materials and Methods. Slot 1: PSD fraction; slot 2: PSD fraction treated with 0.5% DOC-50 mM DTT overnight in the cold and then centrifuged to give pellet; slot 3: supernate from DOC-DTT treatment; slot 4: PSD fraction; slot 5: PSD fraction treated with 0.5% DOC-75 mM KCl overnight in the cold and then centrifuged to give a pellet (much more of the pellet was placed on the gel as compared to the untreated PSD of slot 4); slot 6: supernate from DOC-KCl treatment; slot 7: PSD fraction; slot 8: PSD fraction treated with a solution containing 4 M guanidine HCl, 0.01 M β-mercaptoethanol, 0.05 M phosphate buffer, pH 6.4 (cf. reference 10) overnight in the cold and then centrifuged to give pellet; slot 9: supernate from guanidine treatment (more of the supernate was put on the gel in order to detect minor bands).

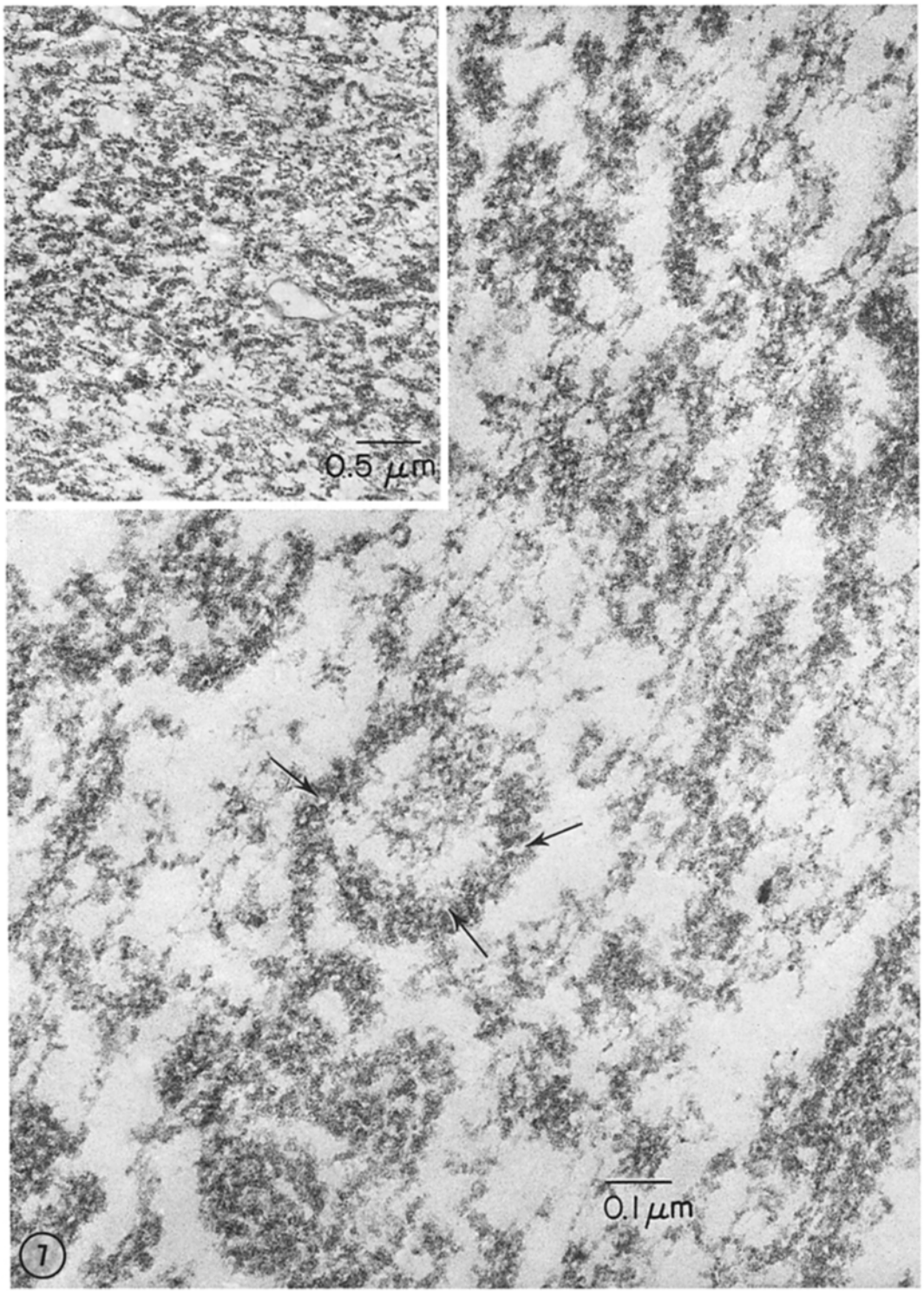

FIGURE 7 Thin-section electron micrograph of PSD pellet after treatment with 0.5% DOC-75 mM KCl. Treatment described in Materials and Methods. The PSD structure has opened up as seen by the appearance of spaces (arrows) within the structure and the lighter staining as compared to the untreated condition. × 100,000. Inset, × 20,000.

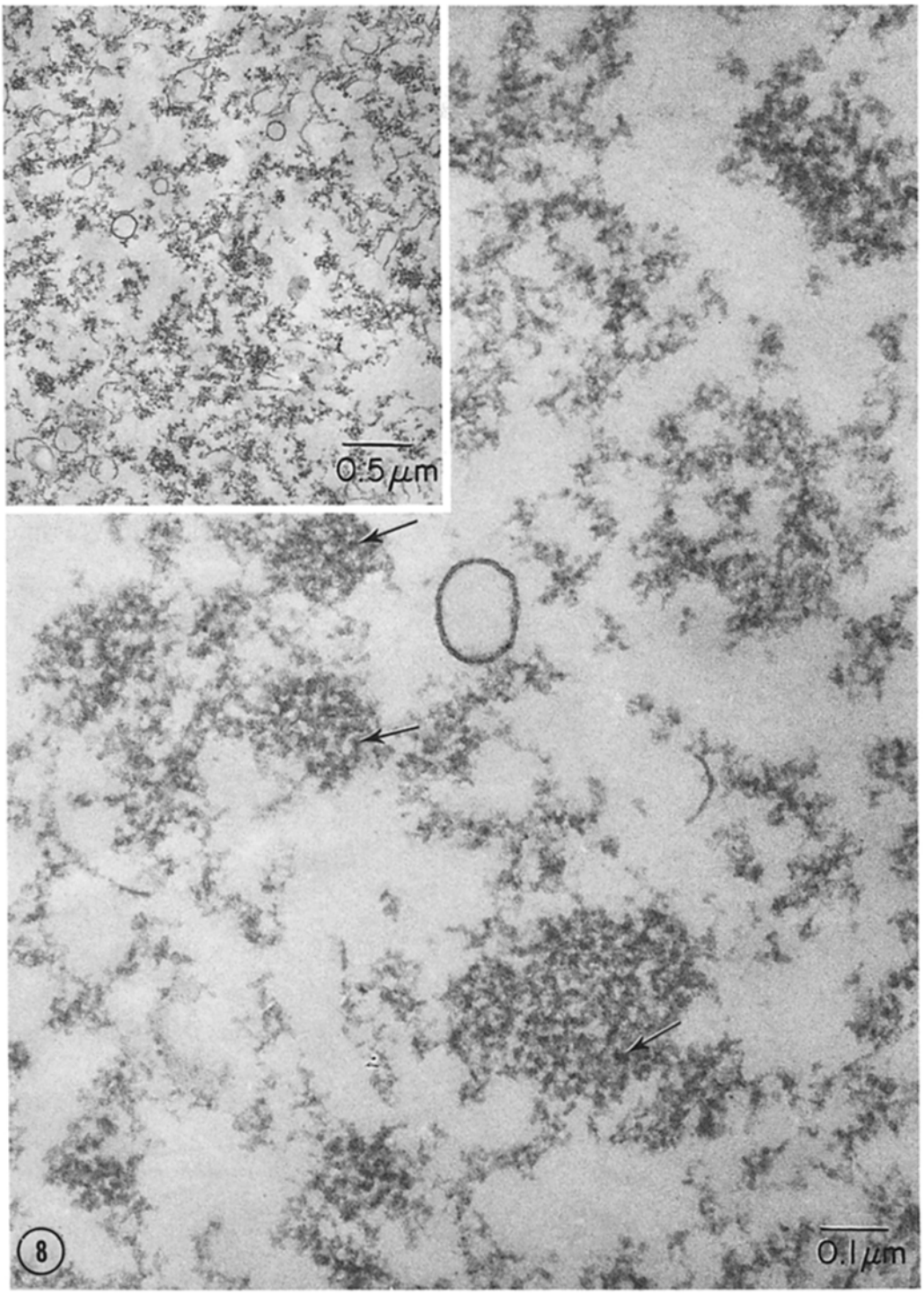

FIGURE 8 Thin-section electron micrograph of PSD pellet after treatment with 0.5% DOC-50 mM DTT. Treatment described in Materials and Methods. PSDs are seen as aggregates of ~13-nm diameter particles (arrows). Occasionally, membrane vesicles and rod-like membrane fragments are observed. × 100,000. Inset, × 20,000.

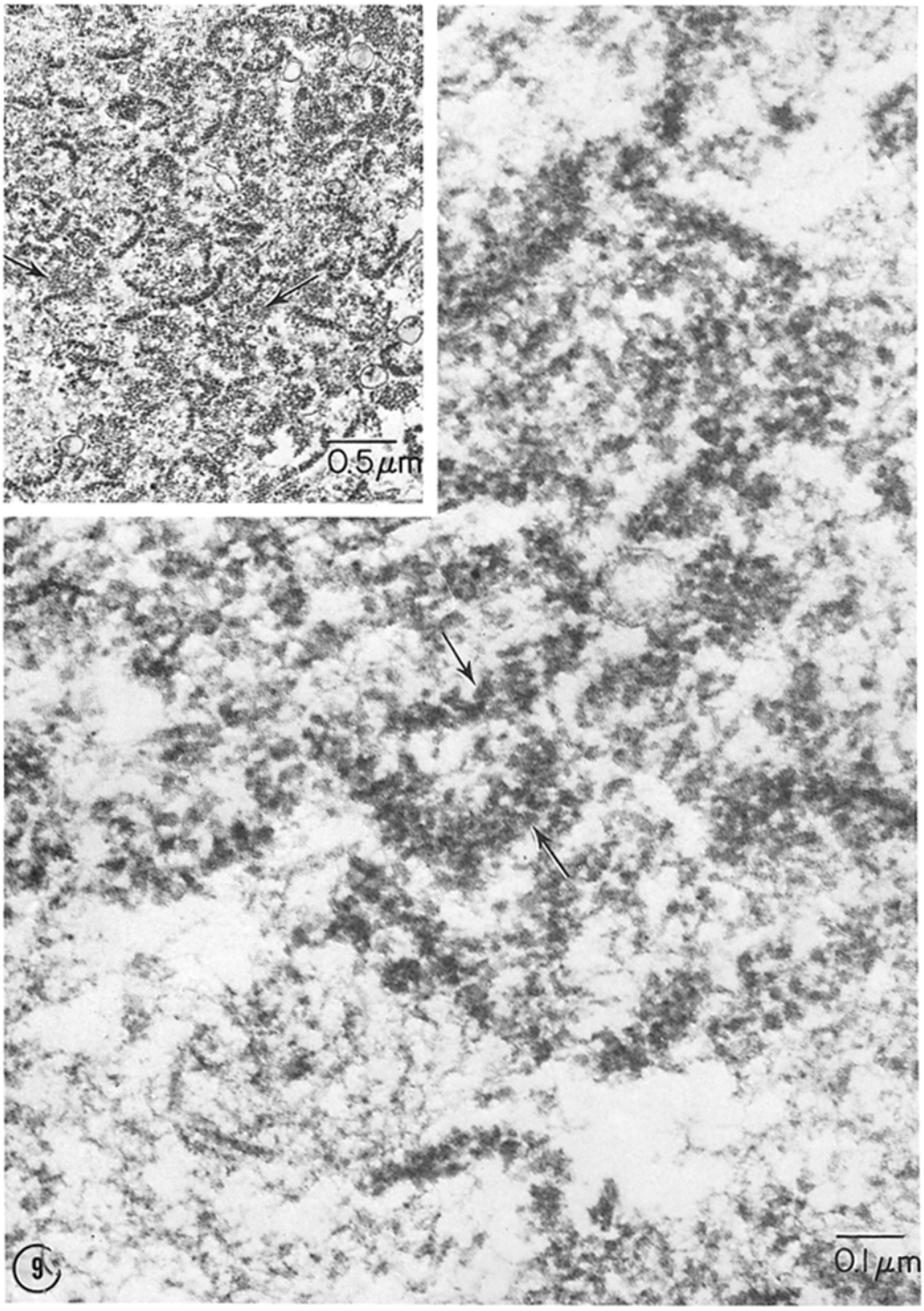

FIGURE 9 Thin-section electron micrograph of PSD pellet after treatment with 0.5% DOC-0.5 mM PCMB. Treatment described in Materials and Methods. The PSD is seen to be composed of dense apparent particles 10–20 nm in diameter (arrows). Note the background filamentous material. × 100,000. Inset: Considerable background material is seen (arrows) which may be broken-up PSDs. × 20,000.

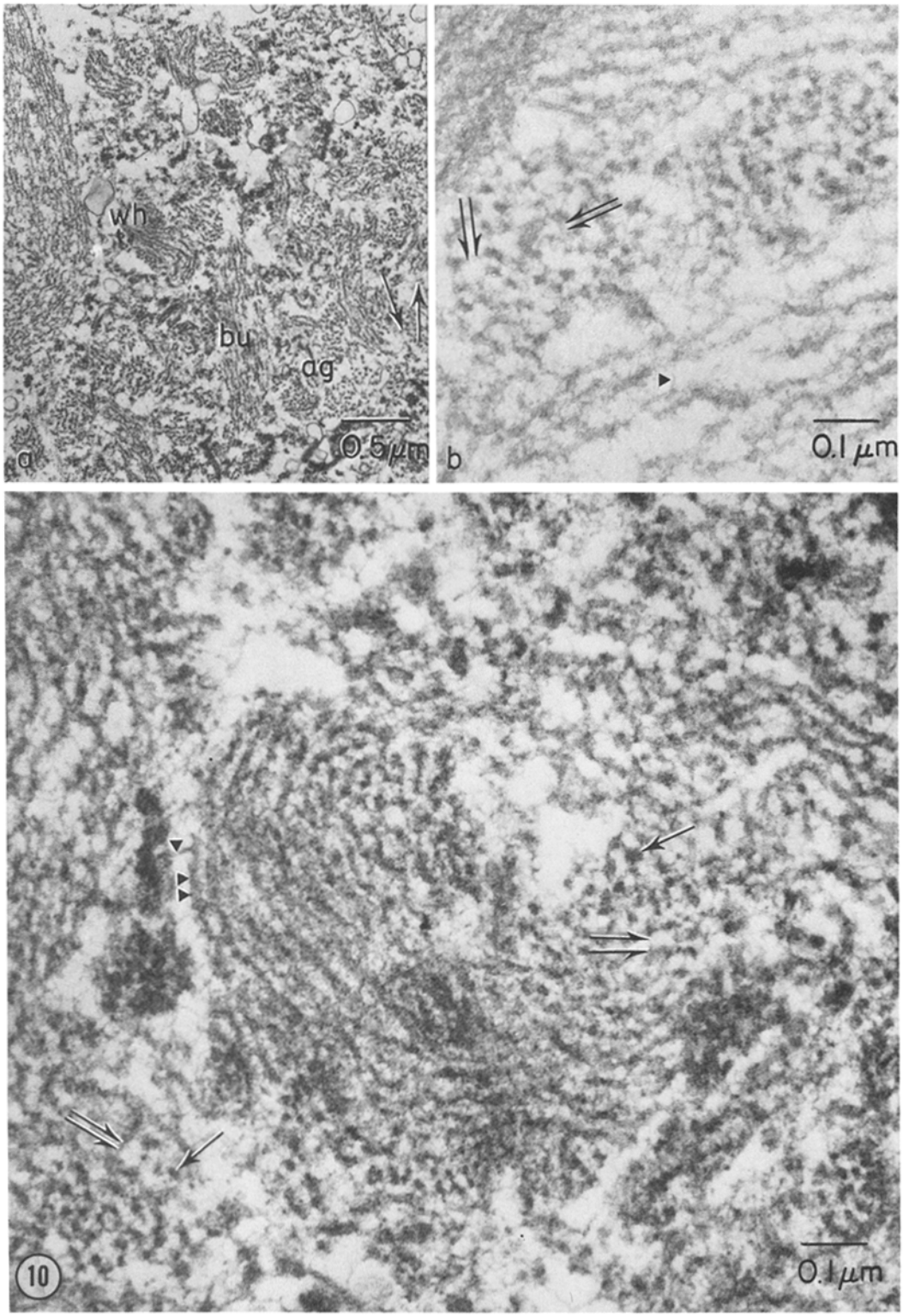

FIGURE 10 Thin-section electron micrograph of PSD pellet after treatment with 100 mM EGTA. Treatment described in Materials and Methods. In (*a*) (× 20,000) whorls (*wh*) and bundles (*bu*) of filaments are seen amongst PSDs as well as aggregates (*ag*) of particles. Extending from the 10-nm filaments (double black triangles) are thinner, shorter filaments ranging between 3 and 5 nm (single black triangles). Aggregates made up of 10–13-nm particles (single arrows) are interconnected by thinner filaments (double arrows) as can also be seen in (*b*) (× 100,000). These aggregates of apparent particles may be cross sections of the filamentous bundles, since the filament bundles seem to be continuous with the apparent particles in the center of the lower figure (× 100,000).

apparently connected by thin 3–5-nm filaments (Fig. 10*b*). Cotman et al. (7) also found that 100 mM EDTA disrupted the PSD structure. The particles can be seen as whorls, as fibrous bundles, and as aggregates. Since nearly all of the actin is still present in the preparation, we construe this to mean that the thin connecting filaments (double arrows) are composed mainly of actin, because after those treatments which release actin, DOC-DTT (Fig. 6) and DOC-PCMB (Fig. 4), the apparent particulate structure of the density is easily visible (Figs. 8 and 9) but little evidence can be seen of thin filamentous material connecting these particles. A tentative conclusion therefore is that actin, or mainly actin, comprises the filaments holding the putative particles together within the structure of the density, but that the filaments seen in Fig. 9, after DOC-PCMB treatment, are not actin, since the gel in Fig. 4 shows a virtually complete removal of the actin. A reconciliation of the filamentous and particulate aspects of the PSD (cf. preceding paper [5]) may be obtained through Fig. 10. What are seen as particles of 10–13-nm diameter (single arrows) might really be the result of a cross-section cut of filaments (double triangles) or bundles of filaments, for what is apparently a grazing cut of the material in the center of the picture reveals these apparent particles to be continuous with the long, thicker filaments.

A further clue to the identity of the thicker filaments can be obtained by examining the effects of guanidine treatment. Fig. 6, slots 7, 8, and 9 indicate that guanidine removes most of the proteins of the density, leaving most of the major 51,000 mol wt band and the 100,000 band(s) still in the modified density. The PSDs are hardly recognizable as such (Fig. 11), with only a few left intact. The remaining pellet again seems to have broken up into ~10-nm diameter particles. The structure remaining after guanidine treatment (Fig. 11) appears similar to that after DOC-PCMB treatment (Fig. 9), except for the presence of more filamentous material in Fig. 9. The gel patterns of the treated PSD preparation also appear similar (Fig. 4, slot 6 and Fig. 6, slot 8) except for the retaining of protein in the 55,000–59,000 region and in the 185,000 region in the case of DOC-PCMB-treated PSDs. It is clear that the major 51,000 mol wt band is retained after all treatments, even after guanidine.

A phosphotungstic acid (PTA)-stained image of the guanidine-treated PSD (Fig. 12) shows not only ~10-nm filamentous structures instead of particles, but also numerous rods, rings, "Y"-shaped structures, and also many longer filaments and aggregated structures. These Y-shaped structures, though larger, are seen also in replica views of the intact density (cf. Figs. 6 and 7 of the preceding paper [5]), forming a "geodesic" type of structure. Another visible feature of the filaments is the non-uniform staining along the length (Fig. 12); this is reminiscent of similar pictures obtained of 9-nm filaments from axons (10, 56) and of spread preparations (44). These filaments, wider in diameter than the microfilaments, some of which are undoubtedly composed of actin-like protein, have been reported to contain a major protein with assigned molecular weights of 60,000 (57), 57,000 (30), 54,000 (9), 51,000 (10), and 50,000 (29). Although some of these filament preparations are reported to originate from glial cells (9, 30), the morphology of our filaments (Fig. 12) most closely resembles that of the one isolated from axons (10). However, the 51,000 mol wt axonal filamentous protein was solubilized by 4 M guanidine (10) while the 51,000 mol wt band of our PSD fraction is mostly resistant to extraction by this agent. But because of its similarity to the axonal neurofilament protein, we postulate that the protein comprising all or part of the 51,000 mol wt PSD band is similar to "neurofilament" protein[3], and we suggest that it forms a

[3] We have shown recently, using the double immunodiffusion technique, that an antiserum prepared against axonal neurofilament protein, obtained by the courtesy of A. O. Jorgensen (cf. Jorgensen, A. O., L. Subrahmanyan, C. Turnbull, and V. I. Kalnins, 1976. Localization of the neurofilament protein in neuroblastoma cells by immunofluorescent staining. *Proc. Natl. Acad. Sci. U. S. A.* **73**:3192), is able to form a precipitate with an SDS-solubilized PSD fraction. This precipitin line was confluent with a precipitin line obtained when an axonal neurofilament extract prepared by us was run against the Jorgensen antiserum. Yen et al. (61) also reported that antibodies to an axonal neurofilament preparation reacted with an *N*-lauroyl sarcosinate-derived PSD preparation. Furthermore, we have prepared an antiserum against the 51,000 mol wt region from SDS-polyacrylamide gels of the PSD fraction. This antiserum gives, using the double immunodiffusion technique, a precipitate with both an SDS-solubilized PSD fraction and an extract of isolated axonal neurofilaments. These precipitin lines were again completely confluent, and were also confluent with the immunoprecipitates formed when the anti-axonal neurofilament protein antiserum reacted with the axonal neurofilament preparation or with the SDS-solubilized PSD fraction (K. Berzins, R. Cohen, and P. Siekevitz, unpublished observations).

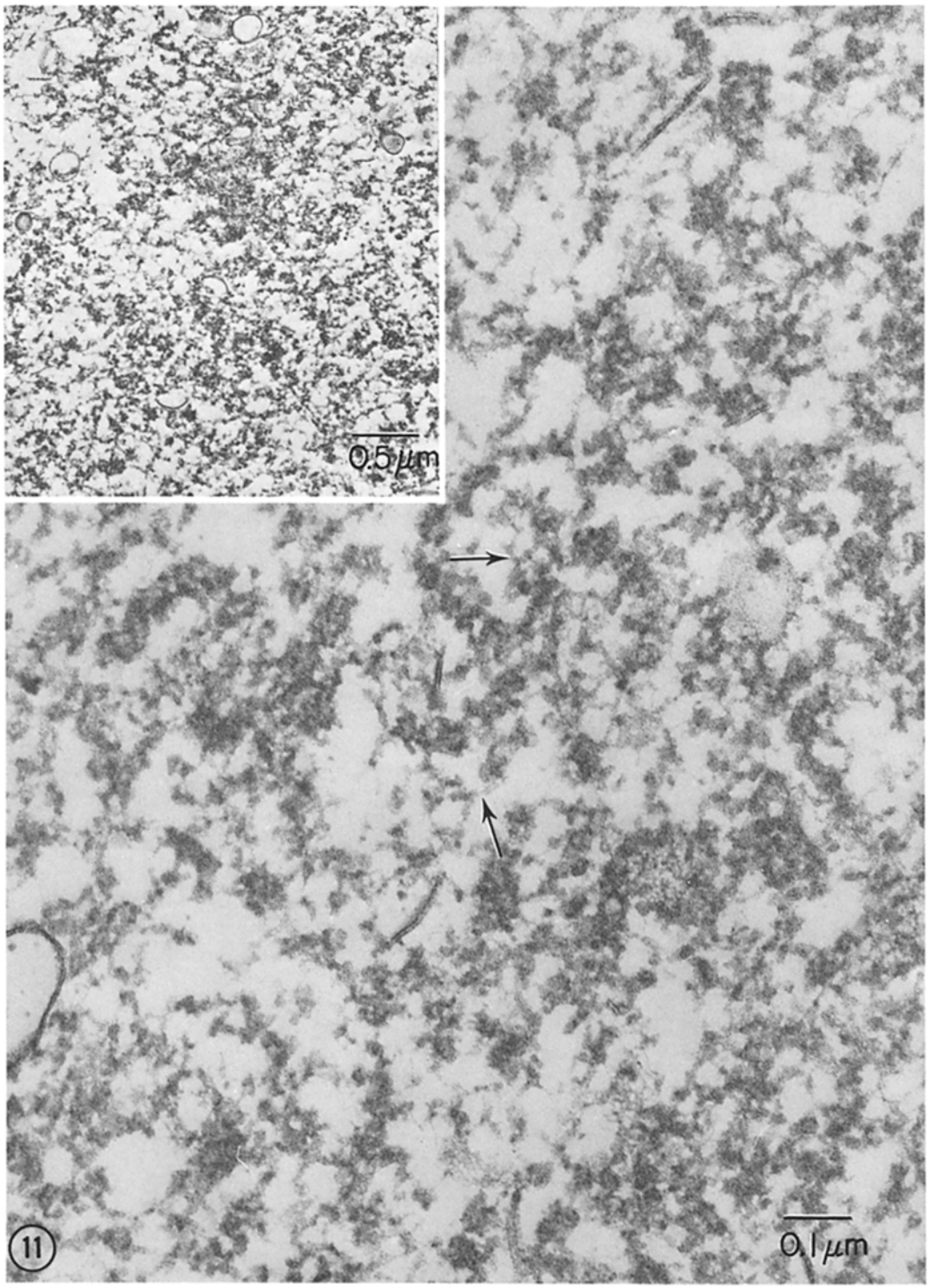

FIGURE 11 Thin-section electron micrograph of PSD pellet after treatment with the 4 M guanidine-HCl solution. Treatment described in Materials and Methods. The PSDs are almost entirely broken up. The predominant structures left are 20–30-nm particles and ~10-nm diameter filamentous structures (arrows). Occasionally membrane vesicles or rod-like membrane fragments are observed. × 100,000. Inset, × 20,000.

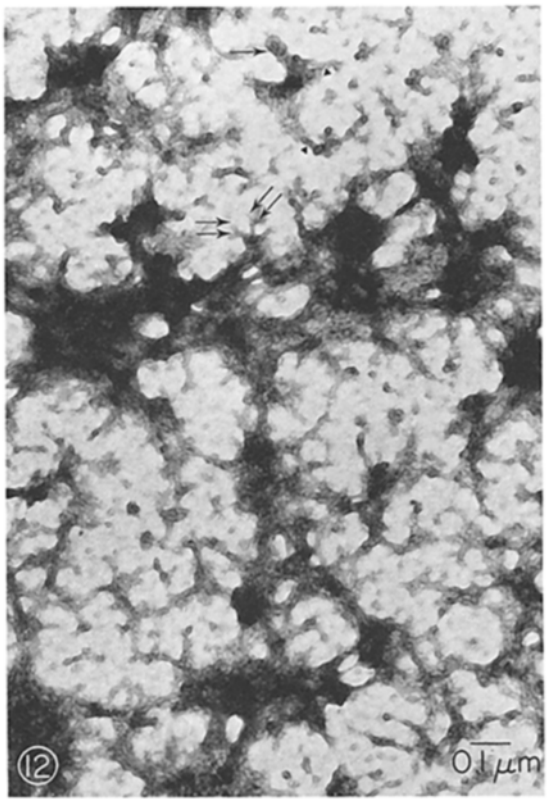

FIGURE 12 PTA-stained sonicated PSD pellet after treatment with the 4 M guanidine-HCl solution. Treatment described in Materials and Methods. In this picture, the PTA-stained matter appears dark against a light background. 10-nm filamentous structures are the major structures remaining. They are sometimes seen as rods (black triangles), as rings (single arrow), or as "Y-shaped" units (double arrows). × 100,000.

filament which occurs singly or in bundles, and which in cross section shows up as particles, much like cross sections of muscle tissue.

It is of interest that the EGTA treatment, presumably removing bound Ca^{++}, removes, among other proteins from the density, the 18,000 mol wt one and part of the 40,000 mol wt one (cf. Fig. 4, slots 2, 3, and 4). The Ca^{++}-binding component of muscle, the so-called troponin C, has reported molecular weights of 20,000 (4, 20), 19,000 (55), or 18,500 (26), and it could well correspond to our 18,000 band, particularly since a similar protein, of mol wt 18,000, has been found in whole brain (16, 60)[4]. It is also of interest that the 37,000 mol wt muscle troponin component strongly interacts with the 20,000 mol wt muscle troponin component and is separated from it by EGTA (48); it may be possible that the 40,000 PSD component removed by EGTA together with the 18,000 mol wt one is similar to the 37,000 mol wt muscle protein, since its molecular weight has been given also as 39,000 (26). Of possible significance for the function of the PSD are the 68,000 and 73,000 mol wt bands which are removed by EDTA or DOC-PCMB treatments (Fig. 4). In preliminary experiments, these proteins were found to be phosphorylated by ATP through a cAMP-dependent protein kinase[5].

DISCUSSION

From the information we have gathered together in this and the preceding paper (5), our perception

[4] An indication of the identity of this protein comes from gel electrophoresis and from Ca^{++}-binding experiments. The purified 18,000 mol wt Ca^{++}-binding protein of whole brain, obtained through the courtesy of D. M. Watterson, Duke University (cf. reference 59), co-migrates exactly with the PSD protein on SDS gels. In experiments relating to Ca^{++} binding, PSD gels were not fixed or stained, but washed repeatedly with distilled water to remove excess SDS. They were then incubated in 20 ml of 10^{-4} M $CaCl_2$ in 50 mM Tris, pH 7.0, containing 5 μCi $^{45}Ca^{++}$, for 30 min at room temperature. The gels were then repeatedly washed with the same buffer and then autoradiographed. The only darkening due to Ca^{++} binding was observed in the 18,000 mol wt region, as compared to a companion Coomassie Blue-stained gel taken through the same procedure (R. Cohen, F. Blomberg, and P. Siekevitz, unpublished results).

[5] PSD fractions were incubated at room temperature in 50 mM MES buffer, pH 6.2, containing 10 mM Mg^{++}, 1 μM ^{32}P-ATP, 1 mM isobutyl methyl xanthine (IBMX), partially purified cAMP-dependent brain protein kinase, with or without 10^{-5} M cAMP (assay of T. Ueda and P. Greengard, Yale University, manuscript in preparation). After 3 min, 100 mM EGTA was added, and the suspension was kept overnight in the cold and then centrifuged (cf. Materials and Methods). The proteins of the pellet and supernate were electrophoresed as given in Materials and Methods, and Coomassie Blue staining and autoradiography were performed. Several proteins in the EGTA supernate were phosphorylated, with the most intense by far being the 68,000 and 73,000 mol wt bands. In the presence of cAMP, the radioactivity increased in some of these proteins, with again the largest increase (approx. fivefold) occurring in the specific radioactivity of the 68,000 and 73,000 mol wt protein bands (R. Cohen, F. Blomberg, P. Siekevitz, T. Ueda, and P. Greengard, unpublished results). It is of interest in this regard that Cotman et al. (7) found a cyclic 3′-5′-phosphodiesterase activity in their *N*-lauroyl sarcosinate-derived PSD fraction.

of the structure of the PSD is as follows. Viewed from thin-sectioned material, the density is a somewhat flattened disk-like structure, 200–500 nm in diameter and 40 nm in thickness, plastered up against the postsynaptic membrane at the synaptic site, and having a network of subsynaptic web material connected to it (cf. Figs. 2, 3, and 4 of the preceding paper [5]). It can be removed free of the membrane by Triton X-100 treatment, and we assume at this time that no intrinsic PSD proteins are removed from it by this procedure. Under viewing conditions which rely on drying-down conditions of fixation, a discontinuity sometimes appears in the plane of the density, so that, instead of having a material-filled, disk-like appearance, the density has the appearance of a "doughnut" (cf. Figs. 5–7 of the preceding paper [5]). This does seem to be the situation *in situ*, as determined by serial sections of cerebral cortex (reference 50, and R. Cohen, unpublished observations). Thus, the "hole" in the doughnut would be covered by the postsynaptic membrane, and perhaps by that part of the membrane which contains the receptor for the chemical transmitters. Another result of the latter type of visualization is the appearance of filaments of various lengths and diameters, not too well seen in the thin-sectioned material (cf. Figs. 5–7 of the preceding paper [5]), except after EGTA treatment (Fig. 10) which possibly causes an alignment of the filament bundles. It is not too difficult to reconcile these two types of image, for most likely filaments would not be easily visible in the thin-sectioned material since they would tend to move in and out of the plane of the section. What are more difficult to evaluate are the great length and thickness of the filaments (cf. Figs. 5–7 of the preceding paper [5]). The seemingly inordinate length and thickness of the filaments could be due to an unraveling of filaments from a tight structure and subsequent cohesiveness during the drying-down process, or it could really reflect a natural situation. Our present belief, based on the results of the chemical treatments shown in this paper, particularly that of EGTA treatment (Fig. 10), is that the long, thick filaments appear as a result of a loosening up of the structure, of an unraveling of filamentous proteins from a tightened structure. However, it could well be that short filaments also emanate from the central "core" structure of the density, as has been remarked before (19, 25, 36, 44) and as can be seen in Figs. 5 and 6 of the preceding paper (5). Finally, it is not inconceivable that these two apparent states of the density, that of a tight structure or that of a more filamentous, open one, are static, morphological reflections of the dynamic nature of the density in the cell (cf. below).

This tight structure of the density appears to be particulate in structure. These apparent particles can be seen in untreated densities (cf. Fig. 4 of the preceding paper [5]) but much more easily in the densities which have "opened up" after various chemical treatments (Figs. 7–12). The occurrence of particles as part of an intact synapse (18), isolated synaptosomes (31), isolated junctional lattices (41, 42), or detergent-treated densities (42) has been remarked upon before. And, indeed, Cotman and Taylor (8) used various chemical treatments such as 1 M urea to dissociate isolated synaptic junctions, and found the opened-up PSDs to consist of particles enmeshed in web-like fibrous material. The apparent particle size is not uniform, varying from 10 to 20 nm; whether this range reflects their natural condition or an artifact of isolation and/or treatment is not known. As mentioned above, we think that the apparent particles are composed mostly of the 51,000 mol wt "neurofilament" protein, for the resistance to extraction of this protein by various reagents and the retaining of the apparent particulate appearance in the residues of the treated PSDs seem to go hand in hand, particularly after guanidine treatment (Figs. 6 and 11). On the basis of this putative identity between neurofilament protein and apparent particles, on the basis of Fig. 10, in which the particles seem to merge with and be continuous with filaments, as if grazing sections were made, and on the basis of Fig. 12, in which the particles seem to be largely in the form of rods, we visualize that what we observe as particles are really bundles of 10–20-nm filaments seen in cross section.

It is possible that the bundles of thick filaments are held together by thin actin filaments. Even after the loosening-up of the structure, thin filaments still appear to link the apparent particles, as witness the result of EGTA treatment which does not remove actin (Fig. 10). However, it would be pointed out that, even after removal of the actin (Fig. 6), the filament bundles, seen as particles in cross section, are still visible in an ordered array (Figs. 8 and 11), as if they are enmeshed in a supra-structure not involving actin. How this ordered arrangement is maintained is not known, but one possibility is that an even finer cytoskeleton exists which is not visible by ordinary EM

visualization and in which the filament bundles are enmeshed.

The idea of a filamentous network at the postsynaptic site of the synaptic junction or as a part of the PSD has been broached previously. Kornguth (36) writes of a filmentous network spanning the cleft into the presynaptic region; Hansson and Hydén (25) contemplate a network linked to the postsynaptic membrane, as do Metuzals and Mushynski (44); Gray (18) and also Le Beaux (38) speak of a filamentous framework in various localities of the neuron; and Guillery (23) and Gray and Guillery (19) contemplate a series of filaments running underneath the neuronal membrane and linking up with it, as do Hansson and Hydén (25) and Le Beaux (38). Indeed, Figs. 5–7 of the preceding paper (5) are reminiscent of some pictures published in the above-mentioned papers and may indicate an *in situ* condition of filaments arising out of, and being a part of, the central part of the PSD.

Much further work will have to be done to characterize biochemically and immunologically the major proteins of the density. However, we can even now tentatively identify some of these proteins and relate them to the structure of the density. The main component, that found in the 51,000 mol wt region, has been named neurofilament protein, on the basis of its size as compared to isolated neurofilament protein from axons (10), its morphological appearance in certain cases as forming filaments with a diameter ~10 nm, and our tentative immunological results (5)[3]. Thus, we tentatively identify this protein as the major constituent of the filaments/particles seen in many of the pictures, on the basis of a correlation of the retention of the filaments/particles and of the protein after various treatments, and on the basis of the premise that although the proteins in the 100,000 MW region are also retained, the molecular weight of the neurofilament protein corresponds more to a similar protein isolated from other sources. If it is the same protein as that isolated from axons, it is more tightly bound to the density than is neurofilament protein in the axon; the 4 M guanidine solution solubilizes the latter (10), but most of the protein in the density is resistant to extraction by this treatment. The only other major protein resistant to guanidine extraction is the protein(s) in the 100,000 mol wt region, and it could be that these two or more proteins form a tight complex within the density that is resistant to all methods of extraction except by SDS. We therefore look upon these proteins as forming the core of the PSD structure, with the 51,000 mol wt protein constituting the long filaments of the density, sometimes seen as particles, with perhaps the proteins in the 100,000 mol wt region somehow cross-linking the filaments and thus being responsible for the ordered, cross-sectional appearance of the apparent particles (Figs. 7–11).

With the evidence presented, on the basis of SDS-gel electrophoresis and immunological correlations, and amino acid composition, it is more certain that the 45,000 mol wt protein is actin. On the basis of a correlation of the electron micrographs with the electrophoresis patterns after DOC-DTT, DOC-PCMB, and EGTA treatments, we tentatively conclude that this protein also is attached to the neurofilament protein making up the thick filaments, but that it is attached much more loosely than the proteins in the 100,000 mol wt region. It is the protein that is most easily removed by DOC-DTT (Fig. 6), with the resultant structure shown in Fig. 8. The PSD is loosened up, the apparent particles are seen to be quite distinctly farther apart than in the intact density, and it is hard to discern thin filaments linking them up.

The above statements lead to an apparent paradox, in that we have tentatively concluded that both the actin and the 100,000 mol wt proteins link up the 51,000 mol wt neurofilament protein in a tight array, and yet EGTA treatment, which does not extract actin from the PSD preparation, loosens up the structure just as much as do other treatments such as DOC-PCMB which remove the actin. However, it can be seen that both EGTA and DOC-PCMB (Fig. 4) remove quite specifically the 40,000, 18,000, 73,000, and 68,000 mol wt proteins. The first is one of the major proteins of the density but the three other proteins are minor components, and yet we conclude that all are responsible, together with actin, in complexing with the neurofilament protein and the 100,000 mol wt proteins, resulting in the tight structure of the density. Extraction of some of the proteins, not necessarily all, leads to a loosening-up of the structure.

The various treatments used on the PSD preparation also provide some information about the binding among the various proteins. Actin was the most easily removed; 75 mM KCl could extract some of the actin, but the need for some DOC to be present along with KCl would indicate

that more than salt linkages bound actin to some other density proteins (Fig. 6). The finding that both DOC-DTT (Fig. 6) and DOC-PCMB (Fig. 4) could remove most of the actin tells us little about the state of disulfide linkages necessary for the interaction. However, Kelly and Cotman (34) did find extensive cross-linking, by disulfide bonds, of some of the proteins of the PSD preparation. But, the presumed removal of all the bound Ca^{++} by EGTA still left most of the actin in the density (Fig. 4), indicating that Ca^{++} was not necessary for its binding in the density. Again, like actin, the 40,000 mol wt protein was partially removed by DOC-DTT (Fig. 6) and almost completely by DOC-PCMB (Fig. 4); but, unlike actin, much of it was removed by EGTA treatment. This protein was more than half removed by EGTA, while the 73,000, 68,000, and 18,000 mol wt proteins were completely removed (Fig. 4), indicating the probable need for Ca^{++} to effect their complexing to other proteins of the density. Unlike DOC-DTT, DOC-PCMB was instrumental (Fig. 4) in completely removing all the above four proteins, indicating the probable use of inaccessible disulfide bonds in their interaction with other density proteins, for even sustained reduction of these bonds, as during DOC-DTT treatment (Fig. 6), did not remove these proteins. The major proteins most resistant to all these treatments were the 51,000 mol wt neurofilament protein and the proteins in the 100,000 mol wt region, even after 4 M guanidine treatment (Fig. 6).

In summary, our present tentative conclusion regarding the protein structure of the PSD, based on the results summarized in Table II is as follows: the neurofilament protein in the 51,000 mol wt region and the proteins in the 100,000 mol wt region constitute the core of the density. This protein core is held in a tight configuration through the complexing to these proteins of actin and of the 40,000 and possibly the 31,000 mol wt and 26,000 mol wt proteins. Removal either of the 40,000 mol wt protein (as after EGTA treatment) or of the actin plus possibly the 40,000, 31,000, and 26,000 mol wt proteins (as after DOC-DTT or DOC-KCl treatments) opens up the structure to reveal the apparent particles, which we think are really filaments seen in cross section, still in an ordered array relative to one another. In conjunction with these major proteins, four minor ones, the 18,000, 42,000, 68,000, and 73,000 mol wt proteins, also seem to be involved in the tightening of the structure. All these four proteins are removed by EGTA treatment, indicating a Ca^{++} binding, and are also removed by DOC-PCMB treatment, indicating the necessity of sulfhydryl groups in their attachment to other density proteins. The opening-up of the structure can occur in stages, as it is not too apparent after DOC-KCl treatment, when only the actin and the 40,000 and 31,000 bands are partially missing; but the open structure is very apparent after DOC-DTT treatment, when almost all of these three proteins are removed, and after EGTA treatment, when in addition to the 40,000 mol wt protein, the minor 18,000, 42,000, 68,000, and 73,000 mol wt proteins are removed. Extending from the core of the density are thick filaments and filament bundles which seem to be the same as those comprising the core; since the thick filaments are seen after guanidine treatment, which leaves as major components mainly the 51,000 mol wt band and those in the 100,000 mol wt region, we assume that the long, thick filaments are comprised mostly of the proteins in these regions. All of the above interpretations rely on the assumption that each and every PSD in our preparation is comprised of the same proteins. However, since it is very probable that in our PSD fraction we have a mixture of densities, some axonal, some dendritic, some a part of excitatory synapses, and some a part of inhibitory synapses,

TABLE II

Summary of Results of Treatments of PSD

Treatments	Proteins mostly solubilized	Resultant PSD structure
DOC-KCl	Actin (partially)	Somewhat opened up
DOC-DTT	Actin, and ~1/2 of 40,000, 31,000, 26,000	Mostly opened up; 10-nm filaments present
DOC-PCMB	Actin, 40,000, 31,000, 18,000, 68,000, 73,000	Partially opened up; 10-nm filaments present
EGTA	18,000, 73,000, 68,000, 42,000, 40,000 (partially)	Mostly opened up; 3–5-nm and 10-nm filaments present
Guanidine	All except 51,000 and 100,000 region	Mostly opened up; 10-nm filaments present

it is also possible that some of the proteins found in our PSD fraction, particularly the minor ones, are not present in every individual density. For example, the minor 73,000 and 68,000 mol wt proteins may only be found in some specific densities.

Finally, the possible identity of some of the PSD proteins as glycoproteins has been attempted by others. While the synaptic junction complex has proteins which bind concanavalin A, these proteins are in the molecular weight range of 100,000 and above (24, 35), with only one lower, at ~30,000 (24). The *N*-lauroyl-sarcosinate-derived PSD fraction has only one of these bands, at ~105,000 mol wt (35), and may correspond to the proteins in our 100,000 mol wt region.[6]

Does the density have any function in nerve impulse conduction? It is possible that it has only a structural role in linking together, along with synaptic cleft material and the presynaptic knobs, those membranous elements of two cells at their very important contact, the synapse, as postulated by Kornguth (36) and Cotman and Banker (6). The latter authors also visualize the density as a structure preventing the diffusion of specialized membrane transmitter-receptor proteins from the synaptic site to other areas of the postsynaptic cell membrane. The appearance of the density as disk-shaped, with an apparent hole in the center being perhaps occluded by that membrane area containing the transmitter-receptor proteins, is in keeping with this suggestion. However, on the basis of the picture of the structure of the density as we have perceived it, it behooves us to speculate upon another function for this structure. Is it not also possible that the density plays a part as a modulator of the conduction of the nerve impulse? It could do so in two ways: one by acting as an amplifier of the initial signal, and two by acting as a regulator of the biochemical impact of the signal.

Some of the proteins in the density are proteins of "movement", such as actin. Some of the proteins are filamentous in nature, such as actin, and perhaps the 51,000 mol wt protein. Some of the chemical treatments of the PSD are capable of "opening up" the structure by relaxing the adherence of some proteins to other proteins, treatments such as those with DTT-DOC, PCMB-DOC, and EGTA whose chemical results could reflect metabolic events *in situ*. The particular finding that EGTA opens up the density (Fig. 10), loosening up the complexing of some density proteins among themselves (Fig. 4), is relevant in this regard, since Ca^{++} movements are clearly implicated in sodium permeability changes (27) and transmitter release (33). Ca^{++} could also act at the postsynaptic site of the synapse, with changes in Ca^{++} concentration acting to transform the density between the open and closed states; this concept is reminiscent of the postulated role of Ca^{++} in the interaction of actin and myosin in muscle via tropomyosin and the troponin components (cf. reference 17), where actin binds to the heavy meromyosin site of myosin only in the presence of Ca^{++}. The further finding that one of the proteins that are removed by EDTA, the 18,000 mol wt one, may be similar to the Ca^{++}-binding troponin C is also relevant in this regard. Again, the preliminary evidence that the minor 73,000 and 68,000 mol wt proteins removed by EGTA are phosphorylated by a cAMP-dependent protein kinase is relevant for the role of cAMP in the central nervous system (cf. Bloom [3]) and for the resultant possibility of a structural change occurring in the density during impulse conduction. The above findings lead to a hypothesis that the coupling of a chemical transmitter to a membrane receptor protein not only can lead to a change in configuration of that protein, resulting in a change in membrane structure at that site, but also could lead to a change in configuration of some density proteins, with a resultant movement of these proteins relative to one another. The relationship of membrane proteins to density proteins is visualized as being of a kind hypothesized by Edelman (11), in which he postulates that changes in the configuration of cell membrane recognition proteins are communicated to the cell cytoplasm by a submembranous fibrillar protein assembly, consisting in part of microfilaments and microtubules. The finding (preceding paper [5]) that there is some electrophoretic and immunological similarity between some density proteins and some membrane pro-

[6] Unpublished experiments of K. Berzins, R. Cohen, and P. Siekevitz recently have confirmed the observation made by Kelly and Cotman (35) and Gurd (24) that concanavalin A-binding glycoproteins are present in the high molecular weight range of the PSD fraction. By labeling the PSD polypeptides separated in SDS-polyacrylamide gels with ^{125}I-concanavalin A, it was observed that four bands (~100,000, ~115,000, ~130,000, and ~160,000 mol wt) bound the label. The highest specific radioactivity was found in the ~115,000 mol wt band.

teins, including actin, would argue for the hypothesis that the density is part of a membrane-submembrane assembly.

Thus, it could be visualized that the reception of the chemical transmitter by its receptor initiates a chain of movements, culminating in the movements of the density proteins relative to each other. If the density is also part of a submembranous network stretching all along the cytoplasmic side of the axonal membrane, then movements of density proteins could be communicated to movements of proteins along the network. If some of the proteins of the filamentous network are connected to membrane proteins in all parts of the axonal membrane, then it is easy to visualize that movements of these proteins could cause perturbations of the axonal membrane, allowing ion channels to open and close all along the axonal membrane, and in essence amplifying in this way the Na^+ and K^+ currents which originated at the synaptic site of the axonal membrane.

Secondly, the PSD could also act as an on/off switch, deciding whether or not to respond to the signal in the manner postulated above. This regulation could depend on the strength of the signal, the amount, and the connections of the receptor protein in the postsynaptic membrane to some density proteins. It could also depend on the relative amount of certain proteins in the density, proteins which are necessary for the connection between the density and the membrane upon which it rests and of which it is, in certain aspects, a part. If one visualizes that the many densities in a single axon may have different relative amounts of these proteins, then a speculation can be proposed that in this manner certain pathways of conduction can be turned on while others are turned off, and that in time a memory circuit may be established of potentially active pathways containing these active densities.

The above hypotheses will not be easy to verify; but we believe that the structure of the density, as outlined in this and the preceding paper (5), offers an intriguing insight into various possible functions, not the least of which is the role of intracellular protein linkages, and the resultant movements of these, in the conduction of signals between various locations within the neuron. Finally, it is of historical interest that the dominant theory of nerve conduction in the nineteenth century was the neurofibril hypothesis (cf. reference 56), in which noncellular neurofibrils, as opposed to discrete cellular neurons, were the propagating elements. Perhaps we will find that we have come almost full circle, in that the neuronal theory and the filamentous theory, with the filaments now conceived to be intracellular, can be conjoined into one; it would not be the first time in science if this were so.

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