TOPOGRAPHY OF THE SYNAPTOSOMAL MEMBRANE

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ABSTRACT

The composition and disposition of the constituent polypeptides of rat cerebral cortical synaptosomal membranes were analyzed on SDS acrylamide gels. Of 20 bands readily detected, 11 account for >93% of the total protein analyzed. These are: (molecular weight x 10^{-3} in parentheses) band 1 (>250); 2 (225); 3 (175); 4 (doublet, 137); 5 (doublet, 97); 6 (68); 7 (61); 8 (54); 9 (44); 10 (37); and 11 (33). Bands 5 and 8-10 are the most prominent and account for >60% of the protein mass or 0.67 of its molecular fraction.

By lactoperoxidase iodination, the bulk of the proteins in bands 3, 5, 6, and 8 and a portion of band 11 appear to be located on the external (junctional) face of the membrane of intact synaptosomes; proteins in bands 1, 2, 7, 9, and 10 appear to be localized on the internal (synaptoplasmic) face and become labeled only when synaptosomes are lysed. Further confirmation of the topographical distribution is provided by evidence that bands 3-6, 8, and 11 contain glycoproteins susceptible to labeling in intact synaptosomes by oxidation with galactose oxidase or periodate followed by reduction with NaB₃H₄. Evidence is provided for significant contributions by tubulin- and actin-like molecules to bands 8 and 9, respectively, suggesting that a substantial fraction of the tubulin in the synaptosomal membrane is disposed externally (accessible to iodination) whereas most, if not all, of the actin appears to exhibit the opposite topography. Similar though weaker inferences can also be drawn with regard to the location of tropomyosin and troponin.

Preliminary evidence is provided that postsynaptic densities exhibit a protein and iodination profile distinct from that of the synaptosomal membrane.

Recent studies on plasma membranes, initially of red cells, but since extended to other eukaryotic cells, have served to define the disposition or topography of their constituent proteins (for reviews see references 14, 16, 78, 79). When brain tissue of vertebrates is subjected to mild homogenization and subsequent fractionation, nerve terminals are first pinched off and then resealed. Subsequently, they can be isolated relatively free of contaminating organelles, in a state of high structural and functional integrity, with conservation of many of the most characteristic attributes of the living terminal (5, 19, 27, 40, 95). These "nerve ending particles" or "synaptosomes," some of which still retain part of the postsynaptic apparatus (20, 54, 65), are confined by a continuous plasma membrane which is responsible for maintaining the osmotic and transport properties of this artificial organelle (see, e.g., references 5, 10, 12, 76, 91, 92, 93, 95, 96). The particles can be opened and their contents released by mechanical or osmotic means and their synaptosomal plasma membranes
(SPM)\textsuperscript{1} isolated. Several recent studies (21, 45, 50, 68, 69) have indicated that such preparations can now be obtained reproducibly and with a purity sufficient to make feasible investigations into the topography of the membrane proteins in the intact and lysed particle. This communication reports on the results of such an investigation. Some of the results have already been communicated in preliminary form (60, 61).

MATERIALS AND METHODS

Subcellular Fractions

Synaptosomes and Membranes—Method A

This constitutes a slight modification of the method of Gurd et al. (43). The sucrose solutions and Ficoll-sucrose solutions used here are buffered with 1 mM phosphate and 0.1 mM EDTA at pH 7.4. Rats (30–40 days old) were decapitated and the cerebral cortices were removed and homogenized in 10 vol 0.32 M sucrose. The homogenate was centrifuged at 1,000 g for 7.5 min and the supernate saved, while the pellet was washed once with 0.32 M sucrose and again centrifuged. The two supernates were combined and the P\textsubscript{2} (crude synaptosome) fraction was obtained by centrifugation at 9,000 g for 20 min. The P\textsubscript{3} fraction was washed three times with 0.32 M sucrose and then subjected to a flotation gradient with 3 ml 0.32 M sucrose on top and 15 ml 7.5% Ficoll-0.32 M sucrose layered just above the P\textsubscript{2} suspension (20 ml, 14% Ficoll-0.32 M sucrose), and centrifuged at 97,000 g for 2 h. Synaptosomes were obtained from the interface of 7.5% and 14% Ficoll. They were diluted with 3 vol 0.32 M sucrose and the synaptosomal pellet was obtained after centrifugation at 97,000 g for 30 min. The particles were then washed once with incubation buffer (121 mM NaCl, 4.8 mM KCl, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 2.5 mM MgCl\textsubscript{2}, 16.5 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 7.4). When required, these washed synaptosomes were iodinated by lactoperoxidase (see below) and sedimented by centrifugation at 1,000 g for 10 min. The iodinated or control (uniodinated) synaptosomes were washed once with 0.32 M sucrose and then resuspended in 0.32 M sucrose (1 ml/g brain). The particles were lysed by adding 10 vol cold 1 mM phosphate-0.1 mM EDTA, pH 7.4, kept at 4°C for 20 min and were sheared five times by forcing the suspension through a 14 G hypodermic. A crude SPM fraction was then isolated by centrifugation at 75,000 g for 30 min. For the “lysed synaptosomes” control, iodination (see below) was carried out at this stage. The membrane pellets were frozen overnight, thawed, and applied to a discontinuous sucrose gradient (0.6 M–0.8 M–1.0 M–1.3 M) next morning. The gradient was centrifuged at 43,000 g for 90 min, and the interface between 0.6 M and 0.8 M was removed and sedimented after dilution with cold distilled water. The pellet so obtained is believed to be enriched in synaptic plasma membrane (SPM) on the basis of its buoyant density (43), its appearance in electron micrographs (11, 43), and various positive and negative enzyme markers (43).

Synaptosomes and Membranes—Method B

This constitutes a modification of the method of Jones and Matys (49). Rats (30–40 days old) were decapitated, and their cerebral cortices were removed and homogenized in 10 vol 10% (wt/wt) sucrose solution. The homogenate was centrifuged at 800 g for 20 min, and the resulting supernate was subjected to 9,000 g for 20 min. The pellet was washed once with 10% sucrose and twice with incubation buffer. This crude synaptosomal fraction was then subjected to iodination before or after lysis by 5 mM Tris, pH 8.1 (5 ml Tris per gram brain). The lysate was applied to a flotation gradient with 4 ml 10% sucrose on the top, 15 ml 28.5% sucrose in the middle, and 34% sucrose solution containing the lysate at the bottom. The SPM was obtained from the interface between 28.5% and 34% sucrose after centrifugation at 60,000 g for 110 min, pelleted at 97,000 g for 30 min, and stored in the freezer.

In this procedure the synaptosomes are subjected to iodination or other surface labeling procedures (see below) at a stage when their morphology and integrity, including that of the junctional apparatus, are well preserved (Fig. 1a,b). Subsequent steps using these fractions as starting material remove the mitochondrial particles and permit the isolation of SPMs virtually indistinguishable from those obtained by more laborious procedures (Fig. 1c,d, and data below; reference 66).

Rough and Smooth Endoplasmic Reticulum

Rats (30–40 days old) were decapitated, and their cerebral cortices were removed and homogenized in 4 vol 0.88 M sucrose. The homogenate was centrifuged at 25,000 g for 20 min and the supernate mixed with an equal volume of 1.76 M sucrose. 10 ml of this suspension were then overlaid with 23 ml of 1.23 M sucrose, and 5 ml of 0.15 M sucrose were placed on top. The discontinuous gradient was centrifuged at 105,000 g for 16 h and the three fractions were obtained which have been designated smooth endoplasmic reticulum (SER), light rough endoplasmic reticulum (LRER), and heavy rough endoplasmic reticulum (HRER) by Tamai et al. (82). These three fractions were diluted with distilled water to a final concentration of 0.32 M sucrose and sedimented at 10,000 g for 2 h. The fractions were washed once with

\textsuperscript{1} Abbreviations used in this paper: ER, endoplasmic reticulum; PSD, post synaptic density; SDS, sodium dodecyl sulfate; SJC, synaptic junctional complex; SPM, synaptosomal plasma membranes.
incubation buffer and then iodination was carried out before and after lysis by exposure to 10 vol cold distilled water.

Preparation of Synaptic Junctional Complexes and Postsynaptic Densities

These procedures are modifications of the preparation devised by Walters and Matus (87, 88). Synaptosomes were iodinated before lysis in 5 mM Tris, pH 8.1, containing 50 μM CaCl₂, and the labeled SPM was prepared according to Method B, but with all sucrose solutions buffered with 5 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.4, containing 50 μM CaCl₂. The isolated SPM was then resuspended in distilled water (6 mg/ml SPM) and mixed with 2 vol of a solution containing 150 mg/ml sodium deoxycholate, 50 μM CaCl₂, and 0.2 mM phenylmethylsulfonylfluoride. The mixture was incubated at 37°C for 2 h and the postsynaptic densities (PSD) were obtained after dilution with two-thirds of 1 vol distilled water and centrifugation at 95,000 g for 1 h.

For the preparation of iodine-labeled synaptic junctional complexes (SJC), labeled SPM was incubated in 10 mM Tris, pH 7.4, containing 12 mg/ml of sodium deoxycholate at an SPM concentration of 1 mg/ml, at 4°C overnight, and the SJC were obtained by centrifugation at 95,000 g for 1 h.

Enzymatic Iodination of Subcellular Fractions

Lactoperoxidase was obtained from Sigma Chemical Co., St. Louis, Mo. Carrier-free Na[I] was purchased from New England Nuclear, Boston, Mass. (5 mCi/0.25 ml 0.1 N NaOH). Usually, the crude synaptosomes from 5 g brain tissue were resuspended in 1 ml incubation buffer if particles were prepared by Method A (about 12 mg/ml protein) and from 1 g/ml brain tissue for those obtained by Method B (about 7 mg/ml). The final concentration of lactoperoxidase was about 5 U/ml. 300 μCi of Na[I] were added and iodination was initiated by adding 5 μl of 0.01% H₂O₂ every 30 s to a total of 25 μl. The reaction was terminated by addition of 20–25 vol of 6 mM NaI in incubation buffer, and the mixture was incubated 37°C for 4 min. The synaptosomes were then spun down and the pellet was washed once with incubation buffer and once either with 0.32 M buffered sucrose (Method A) or with 10% sucrose (Method B).

For the iodination of ER, the lysed or unlysed microsomal fractions derived from 10 rats were resuspended in 0.5 ml incubation buffer (about 20 mg/ml protein for rough ER, 60 mg/ml for light and heavy smooth ER) and then treated with lactoperoxidase (5 U/ml). 205 μCi Na[I] were added and the iodination was carried out as described for synaptosomes. The iodination was stopped by adding 15 vol 6 mM NaI in incubation buffer, and a membrane pellet was obtained by centrifugation.

Labeling of Glycoproteins

Sialic Acid Labeling: SPM prepared by Method B was washed twice with isotonic phosphate buffer (pH 7.4) and then frozen for storage. After thawing, the SPM (~350 μg/ml) was agitated in isotonic phosphate buffer containing 0.43 mg/ml of solid NaIO₄ for 12 min at room temperature. The SPM was then sedimented and washed twice with isotonic phosphate buffer. The oxidized membrane was resuspended in isotonic phosphate buffer, and NaBH₄ (208 mCi/mmol) was added to a final concentration of 1.5 μmol/ml and kept at room temperature for 20 min. The tritiated SPM was then spun down, washed twice with 50 mM phosphate (pH 8.5), and stored as a frozen pellet.

Galactose Labeling: Isolated SPM was washed and resuspended in 5 ml of 50 mM phosphate, 150 mM NaCl, pH 8.0, to a protein concentration of 350 μg/ml. An equal volume of 50 mM PO₄ (pH 8.2) containing galactose oxidase (5 U/ml) was added and the incubation carried out at 37°C for 1 h. NaBH₄ was then added to a final concentration of 1.5 μmol/ml and the mixture maintained for 20 min at 37°C. The labeled SPM was sedimented and washed twice with 50 mM PO₄ (pH 8.5) and stored frozen.

Gel Electrophoresis

Procedures

The gel system described by Fairbanks et al. (30) was used in this study. The sample was mixed with an equal volume of dissociation buffer (20% glycerol, 10% β-mercaptoethanol, 6% SDS, 10 mM Tris, pH 8.0) and incubated in a boiling water bath for 5 min before applying to the gel. Usually, about 50 μg of protein (based on the assay by Lowry et al., reference 59) were used. Bromphenol blue was used as the tracking dye, gels were cylindrical (10 cm, ID 6 ram), 5.6% in acrylamide, and 0.0022% Coomassie blue-10% acetic acid overnight, followed by 10% acrylamide and development was carried out for about 4 h at a constant current of 6 mA per tube. After electrophoresis was completed, gels were prepared either for visualization by staining or for counting of radioactivity as follows. Gels were stained and fixed in 0.04% Coomassie brilliant blue-25% isopropyl alcohol-10% acetic acid overnight. They were then shifted to 0.004% Coomassie brilliant blue-10% isopropyl alcohol-10% acetic acid for 8 h and destained with 0.0022% Coomassie blue-10% acetic acid overnight, followed by 10% acetic acid for more than 24 h with several changes of this solvent. Gels were scanned with a Gilford spectrophotometer equipped with gel scanner and linear transport accessory (Gilford Instrument Laboratories, Inc., Overlin, Ohio), sliced into 2-mm slices, and counted in a Beckman Biogamma Counter (Beckman Instruments, Inc., Fullerton, Calif.) with an efficiency of 75%.

Molecular Weight Standards

The following proteins with their source as indicated were used as molecular weight standards (the molecular weights are indicated in parentheses):

- Myosin (200,000)
- Bovine serum albumin (67,000)
- Ovalbumin (45,000)
- Lysozyme (14,300)
- Transferrin (7500)
- Apoferritin (440,000)

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weights ($\times 10^{-3}$ in brackets) are those cited in the references: phosphorylase A from rabbit muscle [94] (85), bovine serum albumin [68] (83), ovalbumin [45] (17) (all from Sigma Chemical Co.). DNA polymerase I from E. coli [105] (51) and RNA polymerase from E. coli [165, 155, 39] (48) were kindly provided by Prof. J. Richardson of this department. Myosin [200] (73), actin [45] (73), tropomyosin [35] (18), two components (23), and troponins T, I, and C [37, 24, 18] (41), all from rabbit muscle, were kindly provided by Prof. A. Szent-Györgyi of Brandeis University. Spectrin of human erythrocytes [240, 215] (62, 79) was a gift of Prof. T. Steck of the University of Chicago, and rat brain tubulin, purified by two cycles of thermally induced assembly and disassembly [56, 53] from pig brain by use of a different gel system (33). Tubulin from rat brain, giving a single band at $-55$ in the system used here (11), was kindly provided by Mr. Garrett Crawford of this laboratory.

**Electron Microscopy**

Pellets formed by centrifugation of particles suspended in 10% sucrose, 5 $\mu$M CaCl$_2$, HEPES buffer at pH 7.4 were resuspended in 6% glutaraldehyde, 0.1 M sodium cacodylate buffer, pH 7.4, in a Potter-Elvehjem homogenizer. This suspension was centrifuged for 30 min at 10,000 $g$ in narrow-tipped BEEM capsules to form solid pellets; the capsule tips with the pellets were cut off to aid penetration and left in the fixative for 1 h. Pellets were then gently pushed out of the cylindrical capsule tips with sharpened wooden sticks and washed overnight in 0.1 M sodium cacodylate buffer. Subsequent steps included 75 min in 1% OsO$_4$ in 0.1 M sodium cacodylate buffer, two 10-min washes in 0.05 M sodium maleate buffer, 2 h in the dark in 2% uranyl acetate in 0.05 M sodium maleate buffer (52), three 10-min washes in 0.05 M sodium maleate buffer, dehydration in ethanol and propylene oxide, and embedding in Araldite. Thin sections were cut, placed on 200-mesh grids, then stained with uranyl acetate and lead citrate, and observed with a Philips 201 electron microscope.

**Materials**

Lactoperoxidase (~$45$ U/mg) was purchased from Sigma Chemical Co.; galactose oxidase (~$78$ U/mg) was obtained from Worthington Biochemical Corp., Freehold, N. J.; carrier-free Na$^{131}$I (20 $\mu$Ci/ml) was obtained from New England Nuclear. Na$^{3}$H$_4$ (208 mCi/mmol) was purchased from International Chemical Nuclear Corp., Des Plaines, Ill.

**RESULTS AND DISCUSSION**

A *Catalogue of Membrane Proteins*

When SPM isolated by the method of Gurd et al. (43) are denatured, separated, and displayed by electrophoresis on acrylamide gels in the presence of SDS, their proteins exhibit the pattern shown in Fig. 2 B. By using staining intensity with Coomassie brilliant blue, 4 groups of major ($5a,b$, 8, 9, and 10) and 12 groups of minor bands (1,
FIGURE 2 Electropherogram of standard SPM preparations in acrylamide in the presence of SDS. Preparations of SPM and application to and electrophoresis on acrylamide were performed as described in Methods and Materials. Gel B (bottom), preparation A, the modified method of Gurd et al. (43); Gel A (top), Preparation B, a modification of the method of Jones and Matus (49). A scan of this gel is shown as well to indicate the various modes of representation and the numbering system used.

2a,b, 3, 4a,b, 5.1, 5.2, 5.3, 6, 7, 11, 12, and 13) can be distinguished by their mobility and, hence, their molecular weight (30, 71, 77, 94; but see references 2 and 28). In addition, there are faint components seen on gels and scans but not explicitly designated on the figure, which we call 1.1, 1.2, 2.1, 2.2, 2.3, 2.4, 3.1, 4.1, as well as band 14, with one more band (8.1) present in some, but not all, preparations. For many experiments we used the more rapid and convenient method of Jones and Matus (49) which affords a somewhat less pure SPM (Fig. 1), but in better yield; the membrane proteins obtained in this fashion are also displayed in Fig. 2 A. Except for certain minor variations, the qualitative patterns are similar and in agreement with those reported by others (3, 11, 69, 87, 92). It should be noted that all the polypeptides have entered the gel column, facilitating the analysis of the low mobility (high molecular weight) region near the origin. Also, among the various subfractions of P2, from which synaptosomes and SPM are derived, these particular electrophoretic patterns are quite characteristic of and specific to the SPM. Specifically, myelin and mitochondria (both free and intrasynaptosomal) generate patterns that differ from that of the SPM in their quantitative and qualitative aspects.

We have assigned molecular weights to the various components on the basis of the usual linear correlation of their mobilities with the logarithm of the molecular weight of standards, run both in parallel (Fig. 3) under identical conditions, and concurrently in the same gel (see, e.g., Fig. 8, below). The standards employed include a number of protein subunits frequently used for this purpose, as well as the dissociated forms of the rabbit muscle proteins, myosin, actin, tropomyosin, and troponins T, C, and I, tubulin (with its associated high molecular weight components), as well as spectrin of human red cells. The molecular weights of these standards are discussed in Methods and Materials. In spite of the good correlations obtained, as well as the use of internal standard, the molecular weights assigned could not necessarily be taken as absolute values. Rather, they serve as useful parameters for defining and distinguishing the various bands observed.

Dividing the relative integrated staining intensity (proportional to its mass fraction) by the molecular mass of a constituent provides a measure of its relative molecular abundance in the total membrane protein. The results of such calculations for the most abundant membrane proteins in the two standard SPM preparations are shown in Table I. It is clear that the species described—which, it must be emphasized, may well represent molecular weight classes rather than single species—account for about 95% of the total protein mass and virtually all of its total molecular fraction in the membrane. In turn, bands nos. 5a,b, and 8-10 account for 63% of the total mass or 75% of the total molecules among the 11 protein classes described in Table I.

2 We have made no attempt to obtain accurate molecular weights for polypeptides in the regions of bands 1 and 2. To do so would require standards of known masses between 2 and 4 x 10^6, and band mobilities and separations greater than can be achieved with the system used here. Such studies are, however, currently in progress. The gel system used here is also not optimal for the separation and quantitation of rapidly migrating species (mobilities > band 11).
**Surface Proteins Identified by Enzymatic Iodination**

One of the standard methods for the identification of membrane proteins situated on the outer aspect of surface membranes consists of their exposure to radioiodine in the presence of lactoperoxidase (16). This method, first developed by Phillips and Morrison (72), takes advantage of the impermeability of intact membranes to the relatively large enzyme molecule, resulting in the iodination of tyrosine and histidine residues exclusively on proteins exposed on the same side as the bathing solution containing the enzyme. Application of this method in the present instance involves (a) exposure of intact purified synaptosomes to brief enzymatic iodination, followed by lysis of the particles and isolation of the iodinated SPM, and its comparison with (b) a second aliquot of the same synaptosome preparation, treated similarly except for its iodination, which is performed after lysis of the synaptosome, from which the SPM is then obtained. Provided adequate controls can be devised, proteins iodinated in (a) would be expected to be located on the exterior aspect of the membrane, facing the synapse in the case of the junctional part of the SPM; proteins iodinated in (b) but not in (a) are probably localized on the interior (synaptosomal) surface. Any proteins not iodinated under either condition may (provided they can be iodinated at all) be occluded within the hydrophobic interior of the membrane. The results of one set of six such experiments are shown in Fig. 4 A, B. The results
Table 1

| Band no. | Mass fraction × 100" | Molecular fraction × 100 |
|----------|----------------------|-------------------------|
|          | a   | b   | a   | b   |
| 1        | 1.0 ± 0.6 | 2.4 ± 1.0 | 0.3 | 0.5 |
| 2        | 1.0 ± 0.4 | 1.8 ± 0.3 | 0.3 | 0.5 |
| 3        | 5.3 ± 1.0 | 2.4 ± 0.3 | 1.9 | 0.8 |
| 4        | 9.5 ± 0.5 | 6.0 ± 1.0 | 4.4 | 2.5 |
| 5        | 14 ± 2.2 | 14 ± 3.5 | 8.4 | 7.5 |
| 6        | 4.6 ± 0.4 | 4.8 ± 0.6 | 4.4 | 4.1 |
| 7        | 5.5 ± 0.1 | 7.6 ± 0.2 | 5.8 | 7.3 |
| 8        | 24 ± 2.2 | 27 ± 1.0 | 28 | 29 |
| 9        | 13 ± 2.0 | 11 ± 0.9 | 19 | 15 |
| 10       | 12 ± 0.7 | 9.8 ± 0.6 | 22 | 16 |
| 11       | 3.0 ± 0.1 | 10 ± 0.4 | 5.9 | 17 |
|          | 92.9 | 96.7 | 100.3 | 100.2 |

* Results based on integrated staining intensity with Coomassie blue.

a, SPM prepared by Method A: numbers represent means and standard deviations from three experiments.
b, SPM prepared by Method B: numbers represent means and standard deviations from six experiments.

for the two types of preparations are qualitatively similar, although differences between uncleaved and cleaved synaptosomes are somewhat accentuated when the preparation is subjected to less manipulation, i.e. when it is isolated by the more rapid Method B (Fig. 4 A). Iodination as described under Materials and Methods was usually performed at an enzyme concentration of 5 U/ml. No significant amount of label is incorporated if the enzyme is omitted. Qualitatively similar labeling patterns are obtained when the enzyme concentration is varied between 1.5 and 15 U/ml with the total amount of label as well as the amount per band varying monotonically and proportionally with enzyme concentration. The bands uniodinated at the lowest enzyme concentration still remain free of label at the highest, indicating that their inability to become iodinated is not due to an intrinsically slower rate of reaction.

This group of experiments has led to the following conclusions. (a) Proteins in bands 3–5, 5.1, 6, 8, and 11 can be labeled in intact synaptosomes and thus face the exterior (junctional) side. The extent of labeling of other bands (1, 9, and 14) is much less and of doubtful significance. (b) At least one of the proteins in bands 1, 9, 13, and 14 becomes labeled only or predominantly after lysis, and is therefore probably localized largely on the synaptosomal side of the membrane. (c) The labeling of proteins in bands 3, 5.1, 6, 8, 11, and perhaps 5, is intensified by prior lysis; thus similar components are disposed on either membrane surface, or polypeptides in these bands may span the membrane. (d) There is no unambiguous evidence that any of the major protein bands are inaccessible to iodination, although proteins in bands 2a, b and 10 appear relatively resistant to this treatment and may be at least partially protected by the hydrophobic core. Conversely, then, proteins in all bands visualized by Coomassie blue can also be iodinated enzymatically, albeit to different extents. The last finding is not unexpected, considering the variable efficiencies of iodination reported even for soluble proteins (39). The susceptibility of all bands to iodination is particularly evident when the SPM has first been exposed to a mildly denaturing detergent such as 0.1% SDS (results not shown).

Internal Controls — Synaptosomal Proteins are not Iodinated

Valid interpretation of surface labeling experiments such as those described above is, of course, predicated on the integrity and impermeability of the membrane subjected to the enzyme treatment. Although the differential susceptibility to iodination of different protein classes before and after lysis provides an implicit demonstration of membrane integrity, a more direct proof appears essential. Such a proof is provided by a demonstration that the soluble (and particulate) cytoplasmic contents of the intact synaptosome (the synaptoplasm and its vesicles and mitochondria) are resistant to iodination, but become accessible to this reaction when penetration is facilitated by lysis or treatment with mild detergents (56, 57). One experiment documenting this phenomenon for a lysate obtained by osmotic shock is shown in Fig. 5. The majority of the proteins in such a lysate are associated with intrasynaptic particles (mitochondrial and vesicles) and are unlabeled; among the collection of soluble proteins (>15 bands can be seen in gels [not shown]), only a few become labeled to the small extent shown in the figure. Unlike the closed saccs and cisternae in liver microsomes studied by Kreibich and collaborators (56, 57), synaptosomes exposed to 0.05% deoxycholate do not expose their internal content to iodination (not shown). The results just described would tend to rule out "leakiness" of synaptosomes as an explanation for the bulk of the labeling obtained with untreated particles. Still, penetration of the reagents into a minority of such damaged particles can clearly not be excluded. However, in view of the differential labeling pattern observed, this supposition also requires the additional assumption...
that this minority population is unusual, not only in having lost its synaptoplasm but also in either the content or accessibility of its proteins ordinarily resistant to surface labeling. Although there is no doubt that the population of synaptosomes used here is grossly heterogeneous with respect to transmitter type, there is no evidence whatever that this is reflected in a heterogeneity of the major polypeptides of their SPM.

The conclusions concerning composition and especially disposition of SPM proteins are probably not affected greatly by contamination of the latter by any residual adhering synaptic vesicles (Fig. 1D). On the basis of published data (e.g., references 95, 96) and preliminary experiments, total synaptic vesicles account for <20% of the amount of protein found in synaptic membranes (49, 60). Furthermore, in labeling experiments these iso-
Enzymatic iodination of synaptoplasm. (i) Synaptoplasm was obtained from radioiodine-labeled intact synaptosomes prepared according to Method B after osmotic shock and centrifugation at 35,000 g overnight. The supernate was removed and dialyzed against 0.1 M (NH₄)₂CO₃ for 3 days with three changes. The material was then vacuum dried and resuspended in 0.32 M sucrose-1 mM EDTA. About 30 μg of synaptoplasm was applied to the gel (O-B-B-B). (ii) Synaptoplasm was also obtained from radioiodine-labeled intact synaptosomes prepared after lysis and centrifugation by a modification of Method B in which all sucrose solutions contained 5 mM HEPES, pH 7.4, and 50 mM CaCl₂ at 90,000 g for 7 h. About 30 μg of synaptoplasm were applied to the gel (O-B-B-B). (iii) Synaptoplasm was obtained as above (ii), and then iodinated in the presence of lactoperoxidase and ¹²⁵I. About 30 μg synaptoplasm plus lactoperoxidase were applied to the gel. The principal peak in the labeled material (x-x-x-x) is contributed by lactoperoxidase. This region of the gel is relatively free of label in membrane preparation.

External Controls—Microsomal Membranes Exhibit Different Iodination Patterns

Granted that most of the synaptosomes are intact during their exposure to iodination, the relative unavailability of some of their membrane proteins to labeling under these conditions may still not be due to a difference in their topographical disposition on the membrane surface. Instead, all these proteins may be localized on the external face but may be inaccessible to iodination because of their inter- or intramolecular geometry, which renders them accessible only after a structural rearrangement brought on by lysis and/or the subsequent purification procedure. The latter treatment is innocuous, as witnessed by an identical iodination pattern of the membrane component of freshly lysed synaptosomes and of the isolated, purified SPM (not shown). However, lysis alone may be sufficient to bring about this rearrangement and expose novel species of external proteins. To rule out this model, it would be useful to study a structurally related membrane which has never been subjected to potential membrane modification by hypo-osmotic lysis, and then to examine its proteins after an analogous iodination regime. Brain microsomes may furnish such a model membrane. First, microsomal membranes from rat cerebral cortex have been isolated, subfractionated, and studied in detail by Tamai et al. (82). Studies in our (44, 50) and other laboratories (92) have demonstrated that the major proteins of these membranes, particularly those of the
smooth ER, exhibit a pattern very similar to that of the SPM. Kreibich et al. (56) have demonstrated that integral membrane proteins of rat liver microsomes appear susceptible to relatively “in-discriminate” iodination, in the sense that they become labeled—although less extensively—even in the absence of lysis or detergents. In addition to the explanations offered by Kreibich et al., namely that these proteins may either span the membrane or that the same species may be localized on either membrane face, one must also entertain the possibility, particularly in the case of brain microsomes, that proteins of such membranes are accessible from either side because the membranes themselves have become linearized or inverted in the course of isolation.

In any event, if it can be shown that cerebral cortex microsomes resemble liver microsomes in the indiscriminate nature of their labeling pattern, they might provide a means for testing the hypothesis that intrinsic accessibility of membrane proteins, rather than a secondary structural change brought about by hypotonic conditions, is the principal parameter governing their iodination. This expectation is borne out by the experiment in Fig. 6. Of particular relevance are the data of panel A dealing with the iodination of light smooth ER membranes (82). This is a fraction we know to be less contaminated by small synaptosomes and synaptosomal membranes than is the heavy fraction, and at the same time to be enriched in membranes containing the nicotinic acetylcholine receptor and thus, presumably, to be of postsynaptic (dendritic spines?) origin (75). We therefore feel justified in the inference that the differential accessibility of the two classes of proteins in the SPM does reflect their intrinsic and predominant, though not necessarily exclusive, localization on the two membrane faces.

The results just described also render unlikely the possibility that most of the labeling obtained with intact synaptosomes is actually due to “microsomal” contamination, including postsynaptic elements, whether containing or lacking attached postsynaptic densities. Contamination of synaptosomes by simple postsynaptic membranes is considered unlikely for two reasons: the lack of discrimination in their labeling pattern, and the rate and density sedimentation properties expected for such entities. Contamination by more complex entities is not likely because of analogous considerations as well as the absence of such structures on electron micrographs (Fig. 1 a,b).

Surface Labeling and Membrane Glycoproteins

Glycoproteins are found in all surface membranes, where they fulfill many essential functions, among them the response of the cell to a changing external environment, whether constituted by other cells or by solutes in the surrounding medium (reviews in references 46 and 58). This is probably the reason that most if not all glycoproteins appear to be situated on the outer surface of such membranes (14, 78, 86). Glycoproteins have been implicated as antennae for recognition signals in the nervous system also (e.g., references 45, 47, 67, and 84). Thus, identification of a synaptosomal protein as a glycoprotein provides an additional means for its topographical localization (5). Previous studies, in Baronde’s (4, 5, 29, 98), Morgan’s (69, 70, 97), Cotman’s (3, 20, 21, 22), Margolis’s (63), and our own laboratories (43, 44) demonstrated that SPMs contain glycoproteins, identifiable either by characteristic staining reactions (30) or by incorporation of appropriate carbohydrate precursors, such as fucose, in vivo. Results of some of these studies also indicated an apparent coincidence in electrophoretic mobility of these proteins with at least some of the proteins identified above as being localized on the outer surface. In order to provide further substantiation of these suggestive identities, we labeled two classes of SPM glycoproteins with 3H, viz. those containing terminal sialic acid and galactose by oxidation with periodate and galactose oxidase, respectively, followed by their reduction with NaB3H4 (16, 37, 86). The gel patterns obtained are summarized in Fig. 7. The possibility that the labeling is due to glycolipids is rendered unlikely by the observation that the radioactivity of the membrane pellet remained unaffected by prior extraction with chloroform-methanol (either 2:1 or 1:2). Combining these results with those of our earlier studies, we conclude that bands 4 (a doublet centered at 137,000 daltons), 5 (a doublet in the range 95,000–100,000 daltons), 6 (68,000 daltons), 8 (the most abundant component, centered at about 54,000 daltons), and 10 (33,000 daltons) and, possibly, band 3 all contain components that qualify as glycoproteins on the basis of all the criteria applied (Table II). It will be remembered that proteins in these same bands are also accessible to surface iodination in intact synaptosomes. Taken in conjunction, the inference appears warranted that proteins in these bands are...
Fm~6 Iodination patterns of smooth and rough endoplasmic reticulum. Light, heavy-smooth, and rough ER were prepared as described in Methods. The ER preparations were iodinated both before and after lysis with distilled water and analyzed on gels by the standard procedure. A (top), 55 µg of rough ER were applied: iodination before lysis O-O-O; iodination after lysis O-O-O. B (center), 60 µg heavy-smooth ER were used: iodination before lysis O-O-O; iodination after lysis O-O-O. C (bottom), 75 µg of light smooth ER were applied: iodination before lysis O-O-O; iodination after lysis O-O-O.

truly localized on the external aspect of the SPM. Conversely, proteins resistant to surface iodination on synaptosomes are probably internal rather than surface proteins protected against labeling by their localization, although such protected (glyco)proteins may, of course, exist, e.g., in the synaptic cleft.

Disposition of Fibrous Proteins

The presence of fibrous proteins (in either their monomeric or their polymeric state) such as tubulin and actin in close association with plasma membranes now appears well established on the basis of both biochemical and morphological studies.
lqrORB 7 Labeling patterns of glycoproteins in the SPM. SPM was prepared according to Method B and oxidized by either periodate or galactose oxidase, and then reduced by NaB₄H₄ and subjected to electrophoresis as described under Methods. -O-O-: 170 μg of SPM treated with galactose oxidase; -O-O-: 110 μg of SPM treated with IO₄⁻; -x-x-x-x-x-: incubation with B₄H₄ without prior oxidation.

TABLE II

| Band no. | Galactose | Sialic acid | Fucose | PAS | ³⁵S (external) |
|----------|-----------|-------------|--------|-----|---------------|
| 1        | ?         | ?           | +(??)  | +(?)|               |
| 3        | ?         | +           | +      |    | ++            |
| 4*       | +         | ++          | ++     | ++  | ++            |
| 5*       | ++        | ++          | ++     | ++  | ++            |
| 6        | ++        | +++         | +++    | +++ | +++           |
| 8        | ++        | ++++        | ++++   | +++ | +++           |
| 9        |           |             | +(??)  | +(?)|               |
| 11       | +++       | +++         | +      |    | ++            |

* Doublet.

Galactose by oxidase, followed by NaB₄H₄; sialic acid by IO₄⁻ plus NaB₄H₄; fucose by labeling in vivo (44); PAS = periodic acid-Schiff reaction.

(for reviews, see references 14, 73, and 78). In addition, there is also evidence suggestive of a similar localization for myosin (above, and [13]) or myosin-like molecules, such as spectrin in erythrocytes (79) and filamin (90) in smooth muscle and other tissues, including brain. In most of these instances, these proteins appear localized on, and relatively easily detached from the cytoplasmic face of the membrane. In the case of nerve cells, tubulin (9, 11, 55, 66, 88), myosin (11, 74), actin (6, 7, 8, 11, 74), tropomyosin (6, 7, 11, 34, 35, 74), and the subunits of troponin (6, 7, 11, 36).

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94) all have been claimed to form part of the SPM.

Our own studies have demonstrated that the proteins of bands 8 and 9 (Figs. 2 and 3) comigrate with authentic brain tubulin and muscle actin (Fig. 8), the former as the characteristic α,β doublet in appropriate solvent systems (60). If band 9 is indeed composed of, or at least contains actin, these molecules appear to be predominantly localized on the interior (synaptosomal) surface of the membrane. This localization would be in accord with the suggested function of these molecules in synaptic vesicle positioning, and/or depolarization-induced transmitter release (6–8, 74).

The situation with respect to the protein tentatively identified as myosin on the basis of its electrophoretic mobility (11) appears more complex. We find no evidence of any SPM protein comigrating with authentic myosin of rabbit muscle ($M_R = 200,000$) (Figs. 3 and 8). Instead, the five or so polypeptides that we can identify in this gel region all exhibit mobilities considerably below that marker. This apparent discrepancy may be due to (a) anomalous mobility or molecular weight of brain myosin, (b) its exclusive association with synaptic vesicles, as suggested by Berl and his collaborators (6, 7, 8), or (c) a loss or modification of the molecule under the particular preparation procedures used (no protection of sulfhydryl groups, no Mg$^{2+}$, no ATP). These possibilities are currently under investigation, as are the labeling patterns and the nature of the high molecular weight components. Some of these (five out of six), at least, appear to be associated with both cytosolic (Fig. 8 B) and membrane-associated tubulin, and may therefore be identical with the dynein-like and other entities identified in brain microtubules by Burns and Pollard (15), Gaskin et al. (38), and Keates and Hall (53).

An internal localization can probably also be assigned to bands comigrating with two of the three components of troponin (troponin T and I at 37 [band 10] and 24,000 daltons [band 13], respectively). If band 10 at 37,000 daltons either consists of or contains troponin T, this protein is particularly resistant to iodination, while (internal) tropomyosin in band 11 at 33,000 daltons may well be the component in this band that is responsible for the enhanced iodination after lysis. The possibility also exists that one of the components of band 2 is identical to the actin-binding protein of rabbit macrophages described by Stossel and Hartwig (81). If so, this protein is completely resistant to labeling from outside.

In contrast, the proteins in band 8 are readily iodinated in the intact synaptosome, and if tubulin does indeed—as seems highly likely—make a significant contribution to this band, and if it is one of the labeled components, some of it at least must be on the junctional face. This inference is strengthened by the recent demonstration by Feit and Shelanski (32, see also reference 64) that at least part of brain tubulin bears a carbohydrate prosthetic group, and by the glycoprotein nature of some of the component(s) of band 8 (Table II). However, some caution is in order since the Na$^+$-K$^+$ ATPase of the SPM—presumably the entity responsible for one of the proteins in band 54—in addition contains a glycoprotein with a mobility that may place it in band 8 (24).

An entity closely related to tubulin has now been shown to be the predominant protein (31, 66, 88) of a prominent structural component in any attached postsynaptic material, variously called the postsynaptic density (PSD) (1, 20) or the postsynaptic junctional lattice (89). Thus, the possibility must be entertained that some of the tubulin identified in the SPM, and in particular of that fraction localized on the external surface, is of postsynaptic origin. However, the quantitative contribution by such structures cannot be large. A significant portion of the isolated SPMs are free of postsynaptic material (Fig. 1 c, d), and these PSD structures probably do not account for more than 5% (and its tubulin no more than 3%) of the SPM protein (1, 19 and Fig. 9, below). On the other hand, the proteins in band 8 account for about 25% of the membrane proteins (Table I), and we estimate from iodination data before and after lysis (Fig. 4) that $\geq 80\%$ of this band, corresponding to about 20% of the total SPM protein is localized on the external surface. Now, if we assume (a) that tubulin accounts for $\geq 50\%$ of the total in this band, and (b) that all the postsynaptic tubulin of intact synaptosomes is susceptible to labeling, at least $0.5 \times 17/20$ or $>43\%$ of band 8
FIGURE 8 (a, Left) SPM was mixed with various fibrous protein, an equal volume of dissociation buffer was added, and the mixture was subjected to electrophoresis after boiling at 100°C for 5 min. Gel no. 1 (7 μg SMP + 8 μg myosin), gel no. 2 (14 μg SMP), gel no. 3 (7 μg SMP + 10 μg tubulin), gel no. 4 (4 μg actin + 7 μg SMP), gel no. 5 (8 μg tropomyosin + 14 μg SMP), gel no. 6 (1-2 troponin + 14 μg SMP). The SPM was prepared by Method B. The preparation and the source of the various proteins are described in Methods and Materials. (b, Right) The top portions (regions of bands 1-3) of gels nos. 1-3 and of a gel containing 10 μg tubulin have been enlarged photographically to show the various components.

must be of presynaptic origin. The actual value is probably considerably higher than this, on the basis of the observation (see below) that the tubulin of PSD still attached to intact synaptosomes is, in fact, resistant to iodination.

More precise identification and quantitation of the contributions made by these various fibrous proteins to the structure of the SPM and additional details concerning their topography can be obtained by means of their extraction, purification, and characterization by peptide maps. Such studies are now in progress.

Surface Proteins in Subsynaptosomal Structures

Considerable additional information is being obtained from studies on simpler components or derivatives of the SPM fraction that represent principally the junctional portion of the membrane system. Such preparations are of interest not only because of their intrinsically greater simplicity but also because, under appropriately controlled conditions, they provide an enrichment in components of the junctional apparatus, relative to extrajunctional proteins of the membrane or adventitious contaminants in that fraction. Such derivative fractions are usually obtained by selective solubilization of extrajunctional material by means of detergents such as Triton X-100 or deoxycholate in low concentrations to produce a subfraction that has been referred to as the synaptic junctional complex (SJC) (19, 25, 26). More complete extraction of membranous material by means of deoxycholate at high concentration (87, 89) or with sodium laurylsarcosinate (20) leaves behind a structure identified as the postsynaptic density (PSD), an integral and characteristic part of the synaptic junctional lattice (65).

We applied the methods just described for the isolation of SJC's and PSD's to synaptic plasma.
membrane preparations, which, while still in place on the intact synaptosome, had previously been subjected to surface labeling by the lactoperoxidase technique. In this manner, we hoped to examine both the protein composition of these more highly refined structures and their disposition and accessibility to iodination. The results of such an experiment are shown in Figs. 9 and 10. The protein patterns (Fig. 9) resemble those published previously by others with the PSD showing characteristic enrichment of band 8. In this instance the protein responsible is known to be closely homologous to tubulin (35, 66, 88). Coincidentally, all polypeptides of smaller size are removed, including those in band 9 as well as in band 6. The pattern in the region of band 5 is altered and there are other minor quantitative changes in the molecular weight region between 1 and $2 \times 10^5$. At least some of the high molecular weight components (band 2—band 1 is too close to the origin in this

Figure 9 Electropherograms of postsynaptic densities (PSD) and synaptic junctional complexes (SJC) on acrylamide gels in the presence of SDS. Synaptosomes and SPM were isolated by a modification of Method B in which all sucrose solutions contained 5 mM HEPES buffer, pH 7.4, and 50 mM CaCl$_2$, and the lysis buffer contained 50 mM CaCl$_2$. SPM, PSD, the extract in the preparation of PSD (SPM-PSD), and SJC all were prepared as described under Methods (see also Fig. 10), and applied to gels at approximately equal protein concentration. The extract contains >95% of the total protein of the SPM.
The synaptosomes were iodinated, and the SPM (Method B) was purified as described in Materials and Methods except that all sucrose solutions contained 5 mM HEPES, pH 7.4, and 50 μM CaCl₂, and the lysis buffer also contained 50 μM CaCl₂. The iodinated SPM so obtained was then treated with 10% deoxycholate-50 μM CaCl₂-0.1 mM phenylmethylsulfonylfluoride at 37°C for 2 h. The extract (SPM-PSD) and the residue (PSD) were separated by centrifugation at 100,000 g for 1 h and analyzed on gels. PSD (22 μg) O-O-O, SPM-PSD (21 μg) O-O-O. Note (Fig. 9) that the actual ratio of proteins in PSD:SPM-PDS is less than 5:95.

gels) associated with tubulin in microtubules also remain associated with the PSD. SJC, as also reported earlier, appear intermediate between the SPM and the PSD with a greater similarity to the latter. If, as has been suggested, this similarity of protein profiles in these subfractions is indicative of common structural features within junctional regions in general, irrespective of transmitter types (87, 88), the proteins of which this junctional lattice is composed appear to include not only tubulin-like components but also some of the high molecular weight entities usually accompanying brain tubulin in other structural contexts (15, 38, 53). In contrast, all of the muscle protein-like entities found in the SPM appear to be excluded from this lattice.

The iodination profile of the PSD fraction (Fig. 10) is quite remarkable. Iodination is restricted to just two (band 4, and part of band 6) of the five externally disposed bands of the SPM (Fig. 4), retained in the PSD (Fig. 9). That this is not an artifact is demonstrated by the fact that, as a result of conversion of SPM to PSD, the “missing” iodinated bands (bands 3 and 5 and especially the labeled portion of the tubulin band 8) appear in the detergent-treated supernate. Thus, in spite of its ostensibly easy accessibility on the exposed postsynaptic side, this protein appears resistant to enzymatic iodination. An intriguing possibility, currently under investigation, is that this resistance is a reflection of the participation of tubulin (perhaps in conjunction with other proteins) in a peculiar condensed form to provide the structural framework undergirding the synaptic junction.

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