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Mobile and Immobile Proteins of Synaptosomal Plasma Membrane†

Andrew P. Smith* and Horace H. Loh

ABSTRACT: The effects of several cross-linking reagents on mouse brain synaptosomal plasma membrane and synaptic junction fractions were examined and compared. The rate of cross-linking by dimethyl suberimidate of many synaptosomal plasma membrane polypeptides was inversely correlated with their size, and these species were found together in cross-linked complexes fractionated on Sepharose 2B; several low molecular weight species, however, believed to include tubulin and actin, were cross-linked relatively slowly and were preferentially found in fractionated complexes of highest molecular weight. In contrast to those of synaptosomal plasma membrane, most of the polypeptides of synaptic junctions were cross-linked with the shorter molecule dimethyl adipimidate, and the rate of this process did not bear any marked relationship with molecular weight. Many of these species were found to be cross-linked in situ with disulfide bonds, as previously reported by others; the molecular weight of these in situ complexes was greater than 40×10^6. Most of the in situ cross-links were cleaved by treating intact synaptic junctions with B-mercaptoethanol, but species of such reduced preparations were still cross-linked with adipimidate or *o*-phenanthroline-Cu^{2+}. These results suggest that many polypeptides in synaptosomal plasma membrane are freely mobile and probably randomly distributed in the lipid bilayer, while several structural proteins may exist in ordered arrays. All of the species in the synaptic junction, in contrast, appear to be immobilized; some of these polypeptides are linked by disulfide bonds, while others are joined by noncovalent bonds and may form bridges between disulfide-linked species. The synaptic junction complexes may form one continuous network in this specialized membrane.

The lateral disposition of proteins in biological membranes is information essential to understanding the latter's functions, but is difficult to obtain (Peters & Richards, 1977). Examination of freeze-fractured membranes by electron microscopy reveals numerous electron-dense particles generally believed to be proteins (Branton, 1969), but the identification of these particles with specific species is generally not possible except in the simplest systems. Examination of cells labeled with such reagents as fluorescent antibodies or radioactive lectins has demonstrated that many species are freely mobile in the lipid bilayer (Frye & Edidin, 1970; Taylor et al., 1971; Kelly et al., 1976), while others are restricted to certain areas (Kelly et al., 1976); however, such methods necessarily can give information about only a few species.

In an earlier study (Smith & Loh, 1978), we demonstrated that most of the proteins of synaptosomal plasma membrane (SPM),[1] a complex system of crucial importance to nervous transmission, can be cross-linked with dimethyl suberimidate (DMS). Our observations led us to conclude that some, but not necessarily all, of these species were freely mobile in the lipid bilayer. In the present study, we have examined this question more thoroughly, by compositional analysis of the cross-linked material, and by comparing the cross-linking characteristics of SPM proteins with those of the synaptic junction (SJ); the latter is a small specialized area isolated from SPM which contains many polypeptides known to be immobilized in situ (Kelly & Cotman, 1976).

Materials and Methods

Preparation of SPM and SJ's. SPM was prepared as pre-

† From the Department of Pharmacology, University of California, San Francisco, California 94122. *Received May 10, 1978.* This work was supported by National Institute of Drug Abuse Research Fellowship Award DA05029-01. H.H.L. is the recipient of National Institutes of Health Research Scientist Career Development Award K2-DA-70554.

[1] Abbreviations used: SPM, synaptosomal plasma membrane; SJ, synaptic junction; DMM, dimethyl malonimidate; DMA, dimethyl adipimidate; DMS, dimethyl suberimidate; $NaDodSO_4$, sodium dodecyl sulfate; TEA, triethanolamine.

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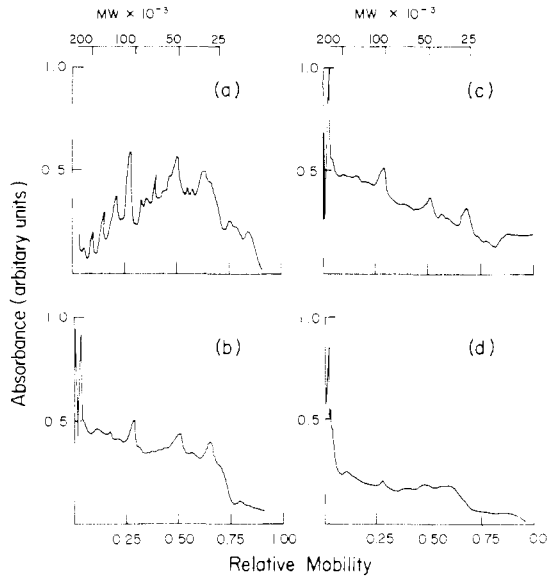

FIGURE 1: Cross-linking of SPM species as a function of time. SPM (1 mg/mL) was incubated at 25 °C in 0.20 M TEA, pH 8.5, with 10 mM DMS for various lengths of time. Following incubation, samples were centrifuged, resuspended in a small volume of water, and analyzed by $NaDodSO_4$ gel electrophoresis as described previously (Smith & Loh, 1977b). Scans of stained gels are shown. (a) Incubation control; (b) 30 min; (c) 1 h; (d) 3 h. Gel loads were about 100 μg in each case.

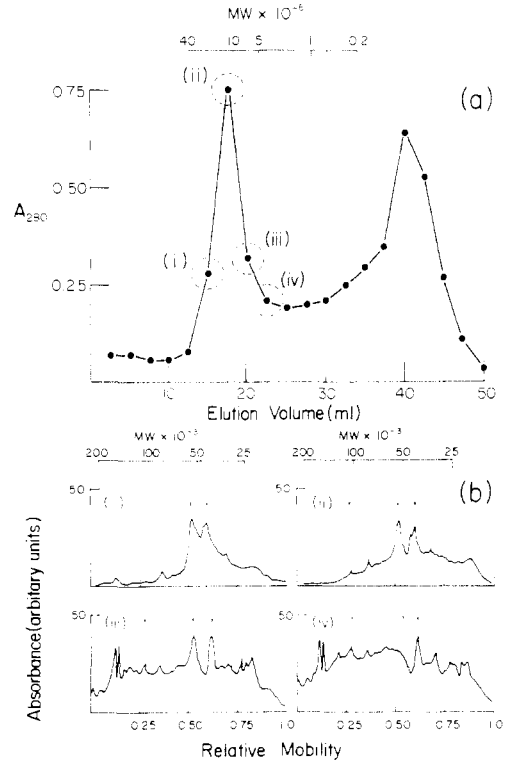

FIGURE 2: Analysis of cross-linked SPM species. (a) SPM was incubated for 30 min with 50 mM DMS, as described in the legend to Figure 1, and then centrifuged. The pellet was resuspended in a small volume of water, dissolved in 2% $NaDodSO_4$-1% β-mercaptoethanol, and analyzed by Sepharose 2B filtration in 0.1% $NaDodSO_4$ (Smith & Loh, 1978). (b) Fractions of the DMS-treated sample of a were incubated in NH_4OH as described in Materials and Methods and then analyzed by $NaDodSO_4$ gel electrophoresis. i–iv are the gel scans of fractions so labeled in a. Gel loads ranged from 50 to 100 μg. Arrows indicate positions of major species.

viously described (Smith & Loh, 1977a). SJ's were prepared from SPM by the method of Kelly & Cotman (1976). Our SJ fraction was not characterized morphologically, but it had a $NaDodSO_4$ gel profile comparable to that reported by Kelly & Cotman (1976; see Results). More importantly, since this profile is not too different from that of SPM, much of the protein of our SJ preparation failed to enter a $NaDodSO_4$ gel in the absence of reduction with β-mercaptoethanol (see Results). This feature clearly distinguishes it from SPM and most other possible contaminants.

Cross-Linking. Cross-linking was carried out with the bisimidate reagents dimethyl malonimidate (DMM), dimethyl adipimidate (DMA), or DMS, essentially as previously described (Smith & Loh, 1978). Cross-linking was also carried out with *o*-phenanthroline-Cu^{2+}, which promotes disulfide bond formation (Steck, 1972). All cross-linking experiments were carried out in 0.20 M triethanolamine (TEA), pH 8.5, at 25 °C, unless otherwise noted; following incubation for various lengths of time, samples were centrifuged at 15 000*g* for 3 min in a Brinkmann Zentrifuge, and the pellets were resuspended in a small volume of water for further analysis. Other details are given in Results.

Reversal of Cross-Linking. $NaDodSO_4$ solutions containing cross-linked proteins were reduced in volume by heating; they were then made 25% in NH_4OH and 1% in β-mercaptoethanol (final concentrations) and incubated for 2h at 25 °C to cleave bisimidate cross-links (Ji, 1974). Following incubation, NH_3 was removed by overnight evaporation.

Other Procedures. All other procedures were carried out as previously described (Smith & Loh, 1977a,b, 1978). In some experiments, however, noted in Results, β-mercaptoethanol was omitted from samples prepared for $NaDodSO_4$ gel electrophoresis or Sepharose 2B filtration; this was done in order to preserve native disulfide bonds.

Materials. Sources of most materials have been given previously (Smith & Loh, 1977a, 1978). Copper sulfate and *o*-phenanthroline were from Sigma; NH_4OH was from Baker.

Results

Cross-Linking of SPM Species as a Function of Size. SPM was incubated with 10 mM DMS for various lengths of time, then analyzed by $NaDodSO_4$ gel electrophoresis; the gels were stained and scanned at 600 nm (Figure 1). As we observed previously (Smith & Loh, 1978), cross-linking (manifested by the disappearance of polypeptides from their usual positions, and by the formation of new, high molecular weight material) increased steadily over a 3-h period; furthermore, the rate at which individual species disappeared from gels was in many cases inversely related to their size. This was especially clear for the species in the molecular weight range 100 000–55 000; in gels of control samples (Figure 1a), staining intensity increases continually as one moves through this region, but, in gels of cross-linked samples (Figure 1b–d), staining intensity generally decreased, indicating preferential removal of low molecular weight material. On the other hand, several species of lower molecular weight appeared to be relatively resistant to cross-linking, as we reported previously (Smith & Loh, 1978); these included prominent bands of about 55 000, 45 000, and 30 000 molecular weight. Observation of species of molecular weight greater than 100 000 was obscured by the presence of new, high molecular weight material in this region of the gel.

Compositional Analysis of Cross-Linked SPM Species. SPM cross-linked with 50 mM DMS for 30 min was analyzed by Sepharose 2B filtration in $NaDodSO_4$ (Figure 2a); a broad peak of material of about 10×10^6 molecular weight was observed in such treated samples, but not in controls. The frac-

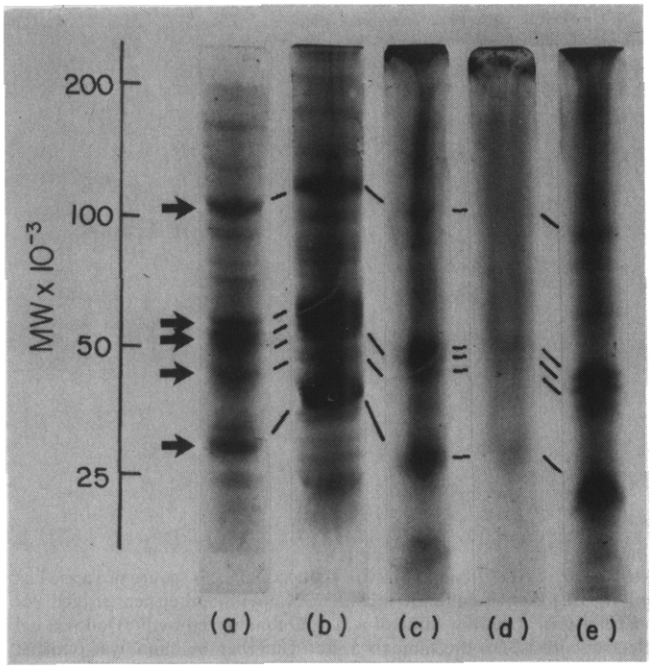

FIGURE 3: Cross-linking of SJ species. SJ's (1 mg/mL) were incubated at 25 °C and pH 8.5 with 10 mM DMM, DMA, or DMS and then processed as in the legend to Figure 1. (a) Incubation control; (b) DMM; (c) DMA; (d) DMS; e was an incubation control from which β-mercaptoethanol was omitted during preparation for electrophoresis. Gel loads were about 100 μg in each case. Arrows indicate positions of major species; lines connect equivalent bands.

tions comprising this peak were incubated with NH_4OH to cleave cross-linking, as described in Materials and Methods, then analyzed by $NaDodSO_4$ gel electrophoresis. As shown in Figure 2b, fractions comprising the highest molecular weight range of the cross-linked complexes contained primarily two major species of molecular weights 55 000 and 45 000, and some lower molecular weight species (Figure 2b, i and ii); other fractions contained these species but most or all other SPM polypeptides as well (Figure 2b, iii and iv).

Cross-Linking of SJ Species. SJ's were incubated with 10 mM DMM, DMA, or DMS for 30 min at 25 °C, pH 8.5, and then analyzed on $NaDodSO_4$ gels (Figure 3). DMM had a very slight effect, while DMA under these conditions cross-linked many of the polypeptides, and DMS cross-linked virtually all of them (Figure 3b–d). Many of these species also failed to enter the gel if untreated SJ's were prepared for electrophoresis without reduction with β-mercaptoethanol (Figure 3e); in fact, the resulting profile was highly similar to that of the sample treated with 10 mM DMA. This result suggests that many SJ species are cross-linked in situ by disulfide bonds, an observation first recorded by Kelly & Cotman (1976).

Effect of Time, Temperature, and Reagent Concentration on SJ Cross-Linking. SJ's were incubated for 30 min with several concentrations of DMA (Figure 4a). Many of the high molecular weight species were observed to be cross-linked at low DMA concentrations, while other species, particularly three prominent ones of 45 000–55 000 molecular weight and two of about 30 000 molecular weight, were relatively resistant. The latter were effectively cross-linked by 50 mM DMA, however (Figure 4a, iv).

The same pattern was observed if SJ's were incubated with 2 mM DMA for various periods of time (Figure 4b,c). Within 30 min, most of a prominent band of molecular weight approximately 110 000 had been cross-linked, along with several other high molecular weight bands. The prominent low molecular weight species, however, were relatively unaffected except during long incubation periods. The rate of cross-linking of all species was reduced by lowering the temperature to 0 °C (Figure 4b).

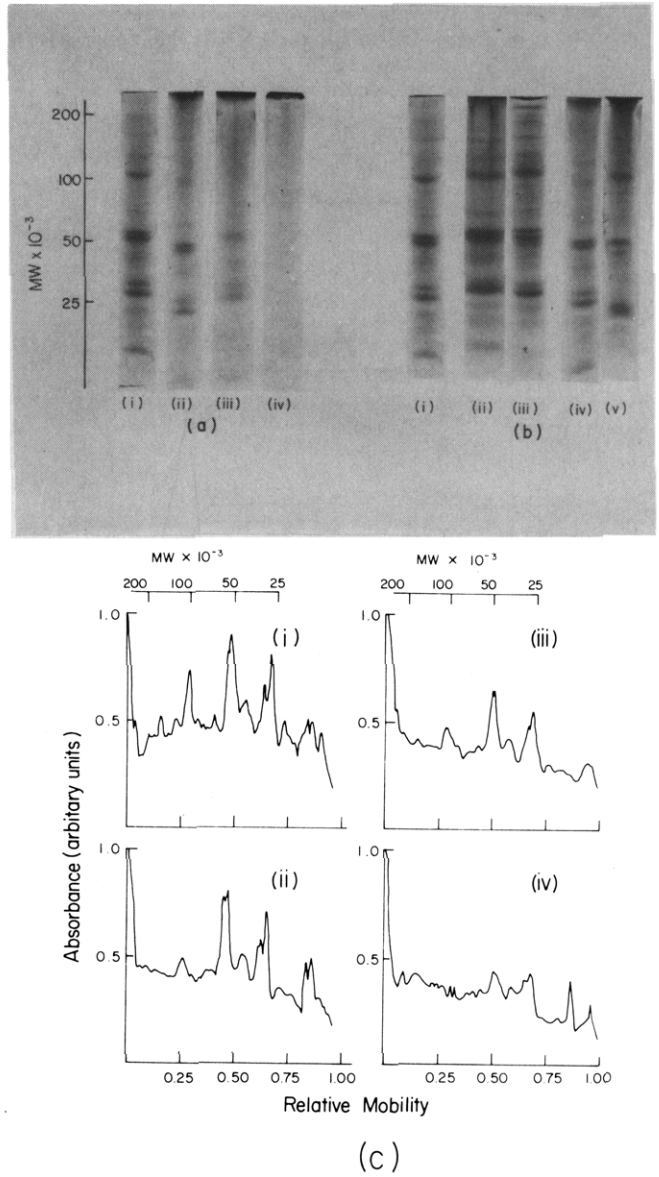

FIGURE 4: Cross-linking of SJ species as a function of reagent concentration, time, and temperature. (a) Reagent concentration. SJ's were incubated for 30 min at 25 °C, pH 8.5, with various concentrations of DMA and then analyzed as in the legend to Figure 1. (i) Incubation control; (ii) 2 mM DMA; (iii) 10 mM DMA; (iv) 50 mM DMA. Gel loads were about 60 μg in each case. (b) Time and temperature. SJ's were incubated with 2 mM DMA at pH 8.5 and then analyzed as given above. (i) Incubation control; (ii) 25 °C, 30 min; (iii) 0 °C, 30 min; (iv) 25 °C, 3 h; (v) 0 °C, 3 h. Gel loads were about 80 μg in each case. (c) Time. SJ's were incubated at 25 °C as in b and processed as given above. Gel scans are shown. (i) Incubation control; (ii) 30 min; (iii) 1 h; (iv) 3 h. Gel loads were about 80 μg in each case.

Analysis of Cross-Linked SJ Complexes. SJ's, dissolved in 1% $NaDodSO_4$ and 1% β-mercaptoethanol, and then analyzed on Sepharose 2B in $NaDodSO_4$, were found to consist mostly of material of less than 200 000 molecular weight (Figure 5a). If they were dissolved in $NaDodSO_4$ without β-mercaptoethanol treatment, however, much of the material was excluded from the column, indicating a molecular weight of greater than 40×10^6 (Figure 5b). SJ's treated with 10 mM DMA prior to solubilization in $NaDodSO_4$ and β-mercapto-

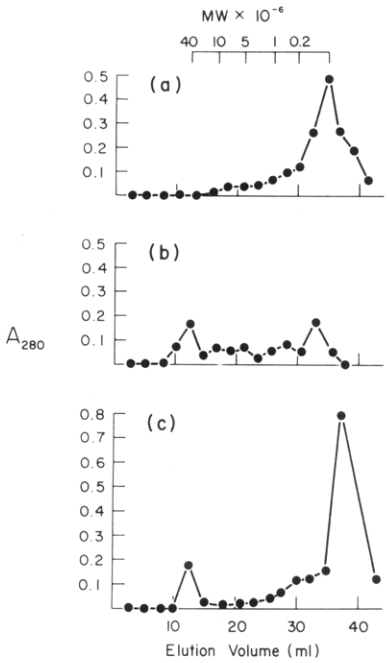

FIGURE 5: Analysis of cross-linked SJ complexes. SJ's were dissolved in $NaDodSO_4$ with or without β-mercaptoethanol and then eluted from Sepharose 2B as previously described (Smith & Loh, 1978). (a) Incubation control; (b) the same as a, except β-mercaptoethanol was omitted dur- preparation for gel filtration. (c) SJ's incubated for 30 min at 25 °C, pH 8.5, with 2 mM DMA. Loads were about 1 mg in each case. The A_{280} values of low molecular weight fractions in c are very high and shifted to the right because of the presence of free and univalently attached DMA.

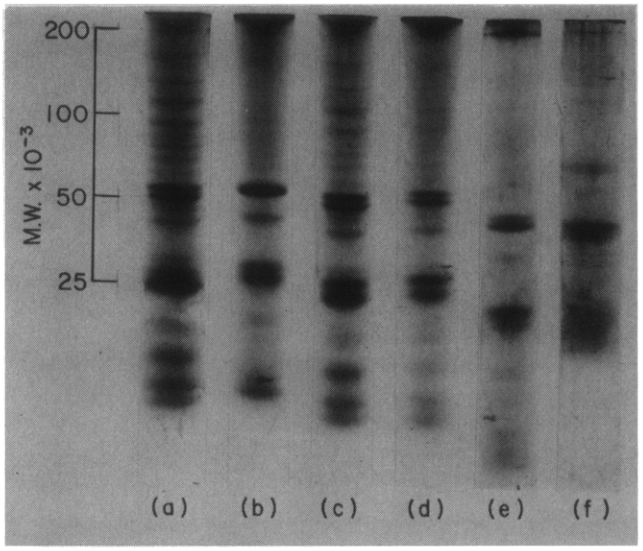

FIGURE 6: Cross-linking of in situ reduced SJ's. SJ's were processed as follows. (a) Incubated for 30 min at 37 °C, pH 8.5, then centrifuged, resuspended in a small volume of water, and analyzed by $NaDodSO_4$ gel electrophoresis; (b) the same as a, but β-mercaptoethanol was omitted during preparation for electrophoresis; (c–f) incubated as in a, but in the presence of 1% β-mercaptoethanol; they were then centrifuged, washed once by resuspending in TEA and recentrifuging, and then further processed as follows. (c) Analyzed by gel electrophoresis; (d) resuspended in TEA, incubated 30 min, centrifuged, and analyzed by gel electrophoresis; (e) the same as d, except that after 30-min incubation, DMA was added to 10 mM final concentration, and incubation was continued for 30 min; sample was then processed for electrophoresis; (f) the same as e, but, during final incubation, sample contained 100 μM o-phenanthroline–20 μM $CuSO_4$. Gel loads were about 80 μg in each case.

ethanol also showed material excluded from the column (Figure 5c).

Cross-Linking of in Situ Reduced SJ Species. Intact SJ's were incubated at 37 °C for 30 min with 1% β-mercaptoethanol, washed by centrifugation, then dissolved in $NaDodSO_4$ without β-mercaptoethanol, and analyzed by gel electrophoresis. Under these conditions, most of the polypeptides were observed to enter the gel (Figure 6c), although many minor high molecular weight bands appeared to be somewhat reduced in amount relative to those of controls (Figure 6a); this result demonstrates that most of the disulfide cross-links were cleaved in situ. Incubation of such reduced SJ preparations in buffer for 30 min at room temperature did not result in any observable re-formation of these cross-links (Figure 6d), but extensive cross-linking occurred upon incubation with 10 mM DMA or with 100 μM o-phenanthroline–20 μM $CuSO_4$ (Figure 6e,f).

Discussion

A major purpose of this study was to determine the manner in which the polypeptides of synaptosomal plasma membrane are cross-linked. It is extremely difficult to distinguish between a mechanism involving random collisions of mobile species and one involving stabilization of an ordered array (Kiehm & Ji, 1977). Previously we suggested that observation of a significant time and temperature dependence of cross-linking would support the existence of the first model (Smith & Loh, 1978), for the diffusion rate of some membrane proteins has been reported to decrease by a factor of 100 when the temperature is reduced from 25 to 0 °C (Edidin & Fambrough, 1973). It is not established, however, that such a marked decrease is a general phenomenon, and, since cross-linking of a large, immobile array would involve many, separate diffusion-dependent collisions, such cross-linking would also demonstrate to some extent time- and temperature-dependent effects. In fact, we have observed such dependence in the cross-linking of synaptic junction species (Figure 4), many of which are known to be immobilized (Kelly & Cotman, 1976; Figure 3e), as well as with hemoglobin subunits (A. P. Smith, unpublished data). Such dependence has also been reported for species in the erythrocyte membrane (Steck, 1972), many of which are believed to be cross-linked with neighboring subunits (Kiehm & Ji, 1977).

For this reason, we looked at other parameters of the cross-linking process in SPM, and when possible compared them with those observed in SJ's. Three independent observations appear to support the existence of random collisions as the primary mechanism by which many SPM species are cross-linked. First, these species showed an inverse correlation between their molecular weight and the rate at which they were cross-linked (Figure 1); since these species appear to exist as monomers in the membrane (Smith & Loh, 1978), this result suggests that their cross-linking is diffusion dependent. SJ polypeptides, in contrast, showed no such clear-cut relationship (Figure 4), which is consistent with the fact that many of them are immobilized in situ. Secondly, fractionation of cross-linked SPM complexes suggests that many of them had a random composition of polypeptides, representative of the population present in native membrane (Figure 2b, iii and iv); this result at the very least makes the existence of well-separated linear arrays of homogeneous composition extremely unlikely for most of these species. And finally, as we observed in an earlier study, cross-linking reagents shorter than DMS were completely ineffective in forming large complexes in SPM under usual conditions (Smith & Loh, 1978). In light of our present studies with SJ's, we feel this last observation to be the most compelling one; since DMA was able to cross-link all SJ species (Figure 4), its virtual lack of effect in SPM strongly suggests

that few of the latter's polypeptides [which in nearly all cases appear to be identical with SJ species (Kelly & Cotman, 1977)] are in intimate, permanent contact with one another. Without such contact, it is difficult to understand how ordered arrays could be maintained in the lipid bilayer.

Several SPM species, however, of molecular weights 55 000, 45 000, and 30 000, appeared to behave differently toward DMS. These species were cross-linked unusually slowly, relative to their size (Figure 1), and were enriched in the cross-linked complexes of highest molecular weight (Figure 2). These species are believed to be tubulin, actin, and tropomyosin, respectively (Blitz & Fine, 1974; Wang & Mahler, 1976; Kelly & Cotman, 1977), and, as the latter proteins are known to be components of larger complexes in other systems (Edelman et al., 1973; Wessells et al., 1973; Bretscher & Raff, 1975), their exceptional behavior in SPM may be explained by their being present there in large arrays. If this is so, however, their insensitivity to DMA is puzzling, especially since these same species exist in SJ's (Kelly & Cotman, 1977), and there can be cross-linked with this reagent, although relatively slowly (Figure 4).

Our results also shed light on the interaction of SJ components. As first observed by Kelly & Cotman (1976), many of these components are cross-linked in situ with disulfide bonds; the molecular weight of the cross-linked complexes is greater than 40×10^6 (Figure 5b), and thus they probably contain at least several hundred species. Most of these disulfide bonds can be reduced in situ by treating intact SJ's with β-mercaptoethanol (Figure 6c), but many of the species of such reduced SJ's can still be cross-linked with either DMA or with *o*-phenanthroline-Cu^{2+} (Figure 6e,f). This observation suggests that bonds other than disulfide ones, such as electrostatic interactions, normally hold these polypeptides together in the membrane; in support of this conclusion, we have found that incubation of β-mercaptoethanol-reduced SJ's in 1 N NaCl markedly decreases the ability of species to be cross-linked by subsequent DMA treatment (A. P. Smith, unpublished data). Furthermore, since several species in SJ's are not cross-linked in situ by disulfide bonds, but can be cross-linked by DMA and are thus probably in intimate contact with neighbors, it is tempting to speculate that these latter species form bridges between disulfide-bonded polypeptides.

We conclude that (1) many of the polypeptides of SPM, which consists primarily of extrajunctional membrane, are freely mobile and probably randomly distributed in the lipid bilayer, while several low molecular weight structural proteins are probably immobilized in some manner; and (2) all of the SJ species are immobilized by disulfide and/or noncovalent bonds, probably into one continuous network. Both of these conclusions are consistent with the results of an electron microscopic study by Kelly et al. (1976), who found that lectin receptors are randomly distributed in extrajunctional membrane and immobilized at the junction. It remains to be seen how these differences in organization are brought about and maintained in two continuous areas of membrane which contain many identical species of polypeptides.

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