Effect of Bisimidate Cross-Linking Reagents on Synaptosomal Plasma Membrane†

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ABSTRACT: The bifunctional amino reagents dimethyl malonimidate, dimethyl adipimidate, and dimethyl suberimidate were tested for their ability to link covalently components of purified synaptosomal plasma membrane. Dimethyl malonimidate, the shortest of the three molecules, had no observable effect on the membrane at concentrations up to 50 mM. Dimethyl adipimidate, at concentrations of 10–50 mM, resulted in the disappearance from $NaDodSO_4$ gels of three polypeptides with molecular weights of approximately 25 000, 65 000, and 75 000, and in the appearance of three new species with molecular weights of about 45 000, 125 000, and 200 000. Dimethyl suberimidate, at concentrations of 10–50 mM, resulted in the partial or complete disappearance of nearly all the polypeptide species; most of the new species formed had molecular weights greater than 10^6. The effects of dimethyl suberimidate were maximal at pHs greater than 9, showed a slight time dependence over a period of several hours at 25 °C, and were relatively unaffected by temperature in the range 0–45 °C. The results of two types of control experiments suggested that the occurrence of cross-linking between membranes was insignificant except under extreme conditions. Treatment of the membrane with 50 mM dimethyl suberimidate had no effect on the activities of any enzyme tested, except (Na^+,K^+)-ATPase, which was drastically inhibited by either dimethyl suberimidate or by the monofunctional reagent ethyl acetimidate. These observations suggest that many of the polypeptides of synaptosomal plasma membrane exist in the native membrane as monomers, and that individual molecules of most species come into close proximity with several neighbors. The large size of the complexes formed during extensive cross-linking is consistent with either stabilization of previously existing ordered arrays, or with random collisions of protein molecules following lateral diffusion in the lipid bilayer. Our results suggest that both mechanisms are involved to some extent.

Bifunctional reagents, which are capable of reacting with and covalently linking two other molecules, have proved to be useful probes of membrane structure (Steck, 1972; Capaldi, 1973; Ji & Nicolson, 1974; Mikkelsen & Wallach, 1976; Tinberg & Packer, 1976). Membrane components that become linked by these reagents can be detected by their disappearance from characteristic positions in standard resolving systems, such as $NaDodSO_4$–polyacrylamide gels (Steck, 1972), and the composition of such linked complexes can be determined by analysis of the new, high molecular weight species that result (Ji, 1974). By treating membranes with cross-linking reagents of different length, it is thus possible to infer the distances separating various species from one another. These reagents have also been used to supply evidence for the importance of motion of individual enzyme subunits during catalytic activity (Tinberg & Packer, 1976).

Synaptosomal plasma membrane (SPM[1]) encloses the nerve terminals in brain and plays a key role in the transmission of impulses between neurons. It has a complex composition of polypeptides and is rich in a variety of functional activities (Gurd et al., 1974; Smith & Loh, 1977a). In this paper, we report some of the effects on this membrane of several bisimidate reagents. These molecules react at both ends with free amino groups; in this study, we have used dimethyl malonimidate (DMM), in which the reactive carbons are separated by a single additional carbon atom, dimethyl adipimidate (DMA), possessing a four-carbon bridge, and dimethyl suberimidate, with a six-carbon bridge.

Materials and Methods

Materials. Malononitrile, adiponitrile, and suberonitrile were from Aldrich. Ethyl acetimidate (EAI) was from Sigma, triethanolamine (TEA) was from Mallinkrodt, and Sepharose 2B was from Pharmacia. Sources of other materials have been given previously (Smith & Loh, 1977a).

Isolation of SPM. SPM was prepared from mouse brain homogenates as previously described (Smith & Loh, 1977a).

Preparation of Hemoglobin. Hemoglobin was prepared by lysis of washed bovine erythrocytes (a gift of Ms. Carol Glasgow) in 5 mM sodium phosphate, pH 8.0, and was not further purified.

Synthesis of Bisimidate Reagents. DMM was synthesized by the procedure of McElvain & Schroeder (1949). DMS was synthesized by the procedure of Davies & Stark (1970). DMA was synthesized, from the appropriate reagents, by the same method used for DMS. Each compound was identified by melting point determination and by chemical analysis. They were stored in solid form, and stock solutions (200 mM in 1 M TEA, pH 9) were made up immediately before each experiment.

Treatment of SPM with Bisimidate Reagents. Under standard conditions, used in most of the experiments reported below, SPM (1.0 mg of protein/mL) was incubated 1 h at 25 °C in 0.25 M TEA, pH 8.5, in the presence of various concentrations of bisimidate reagents; modifications of these conditions are described in the Results section. Following incubation, samples were centrifuged at 50 000*g* for 10 min in a Sorvall RC2-B centrifuge; the pellet, resuspended in a small volume of water, was used for further analysis.

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

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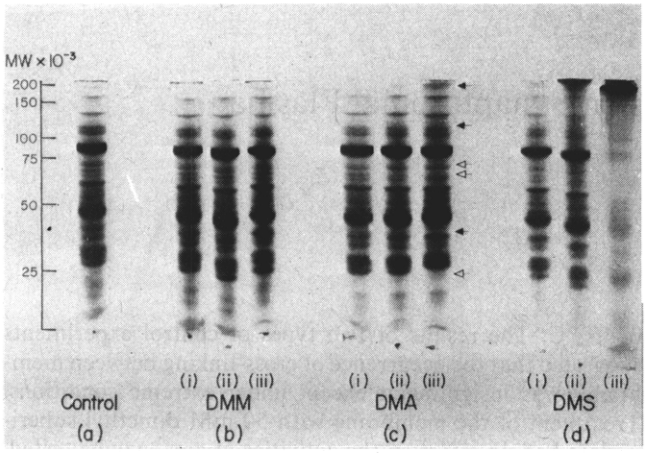

FIGURE 1: Effect of DMM, DMA, and DMS on SPM polypeptides. SPM (1.0 mg/mL) was incubated with various concentrations of bisimidate for 1 h at pH 8.5 and 25 °C. Following incubation, samples were centrifuged at 50 000*g* for 10 min, resuspended in a small volume of water, and analyzed by electrophoresis on 5.7% acrylamide gels in $NaDodSO_4$, as described by Smith & Loh (1977a). (a) Incubation control; (b) DMM treated; (c) DMA treated; (d) DMS treated. In b–d: (i) 2 mM reagent; (ii) 10 mM reagent; (iii) 50 mM reagent. Load on each gel was about 100 μg of protein, and gels were stained with Coomassie Blue. The black mark near the bottom of each gel in this and in subsequent figures is in the position of the tracking dye. The hollow arrows adjacent to Figure 1c (iii) mark the position of bands absent from this gel but present in the control gel (Figure 1a); the solid arrows mark the position of bands present in Figure 1c (iii) but not in Figure 1a.

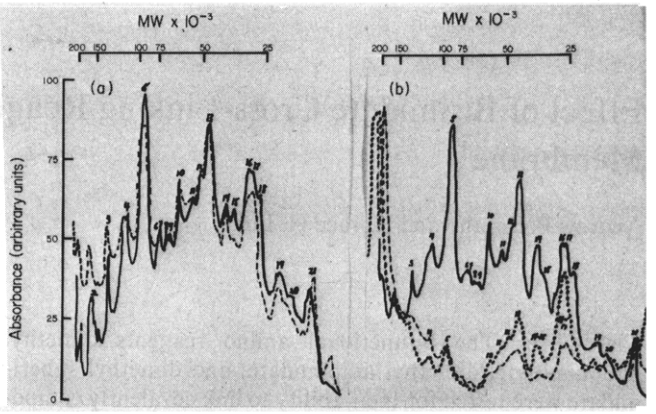

FIGURE 2: Densitometric scans of gels of cross-linked material. Some of the gels shown in Figures 1 and 2a were scanned at 600 nm, as described in Smith & Loh (1977b). (a) Incubation control (—); 50 mM DMA (- - -); (b) 2 mM DMS (—); 50 mM DMS (- - -); 10 mM DMS, pH 9.0 (• • •). In b, the 2 mM DMS sample serves as a control since virtually no cross-linking occurs under these incubation conditions.

Treatment of Hemoglobin with Bisimidate Reagents. Solutions of hemoglobin, 1.0 mg/mL, were incubated with bisimidates under the same conditions used for SPM. The reaction was terminated by the addition of $NaDodSO_4$ to 1%, β-mercaptoethanol to 1%, and boiling.

Sepharose 2B Filtration. Samples of untreated or cross-linked SPM, about 5 mg of protein, were dissolved in 2% $NaDodSO_4$, 1% β-mercaptoethanol, and boiled for 2 min. The samples were then loaded onto a 24 × 1.5 cm column of Sepharose 2B which had previously been equilibrated with 0.1% $NaDodSO_4$–20 mM TEA, pH 8.5, and eluted at 8–12 mL/h with the same buffer. Fractions of 1.0 mL were collected and their absorbance read at 280 nm. V_0 and V_t of the column were determined by eluting a mixture of blue dextran and bromophenol blue.

Other Procedures. The procedures for assay of protein and various enzymes have been given before (Smith & Loh, 1977a). Polyacrylamide gel electrophoresis in $NaDodSO_4$ was also carried out essentially as described previously (Smith & Loh, 1977a,b), including staining of gels and densitometric scanning of stained gels; however, the acrylamide concentration was 5.7%, unless noted otherwise, and the bis(acrylamide) concentration was reduced proportionately.

Results

Effect of Various Concentrations of Bisimidates on SPM Polypeptides. SPM was incubated 1 h at 25 °C with 2, 10, or 50 mM of DMM, DMA, or DMS, at pH 8.5 (Figure 1). DMM, the shortest molecule, had no detectable effect on any of the polypeptide species in the membrane, even at the highest concentration (Figure 1b). DMA had virtually no effect at concentrations as high as 10 mM, but at 50 mM resulted in the disappearance of species of molecular weights approximately 25 000, 65 000, and 75 000, and in the appearance of new species of molecular weights 45 000, 125 000, and 200 000 (Figure 1c (iii)). DMS at concentrations of 2–10 mM had effects somewhat similar to those of 50 mM DMA (Figure 1d (i and ii)). DMS at 50 mM resulted in the partial or complete disappearance of most of the visible species in the gel; most of the new material formed was excluded from the gel (Figure 1d (iii)).

Densitometric scans of gels of cross-linked SPM are shown in Figure 2. The gel of SPM treated with 50 mM DMA displayed an absorbance profile essentially identical with that of the control gel, except for the disappearance or appearance of the minor species noted above, and thus demonstrates the reproducibility of the method (Figure 2a). In SPM cross-linked with 50 mM DMS, the bands numbered 4–18, covering the molecular weight range 100 000–25 000 were all markedly decreased in absorbance relative to controls, while the absorbances of bands 19–21 were affected little or not at all (Figure 2b). A similar pattern was seen with SPM cross-linked with 10 mM DMS at pH 9 (see also below); however, in this sample the absorbances of bands 16 and 17 were also decreased only slightly, and the absorbances of bands 11–15 showed somewhat less of a decrease, relative to controls, than did those of bands 5–10. These patterns must be interpreted with some caution, since the extent of dye binding is not necessarily proportional to protein concentration, but they suggest a certain degree of selectivity with respect to the polypeptides joined into the large complexes.

Effect of pH, Time of Incubation, Membrane Concentration, and Temperature on the Cross-Linking Process. The effect of independently varying several parameters on the cross-linking process was examined, using 10 mM DMS (Figures 3 and 4). When 1.0 mg/mL membrane was incubated at 25 °C for 1 h, the effect of DMS appeared to be virtually the same at pH 7.5, 8.0, or 8.5 (Figure 3a (i–iii)); at pH 9.0, however, there was a dramatic increase in its effectiveness, resulting in the partial disappearance of all species, and in the appearance of a heavy band of material at the top of the gel (Figure 3a (iv)).

When the time of incubation was varied, at pH 8.5 and 25 °C, the amount of cross-linked material formed appeared to increase slightly over a 3-h period, and still further over a 24-h period at 0 °C (Figure 3b). There was no significant effect of varying the concentration of SPM from 0.1 to 3.0 mg/mL, in a 1-h incubation at pH 8.5 (Figure 4a). When the temperature of a 30-min incubation was varied, only a slight increase in the extent of cross-linking was observed over the interval 0–45 °C (Figure 4b).

Effect of Cross-Linking on SPM Enzyme Activities. SPM

TABLE I: Effect of Bisimidate Cross-Linking Reagents on Enzymatic Activities of SPM.[a]

	ATPase	5′-Nuc.	Alk. Phos.	AChE
Control	100 ± 4 (3)	100 ± 8 (3)	100 ± 12 (3)	100 ± 3 (3)
DMM, 2 mM	ND	ND	81 ± 30 (2)	100 ± 8 (2)
10 mM	86 (1)	96 ± 6 (2)	90 ± 14 (2)	83 (1)
50 mM	50 (1)	87 ± 9 (3)	100 ± 14 (2)	100 (1)
DMA, 2 mM	ND	ND	86 ± 22 (2)	90 ± 11 (2)
10 mM	100 (1)	95 ± 4 (2)	97 ± 13 (2)	113 (1)
50 mM	72 ± 12 (3)	89 ± 15 (3)	100 ± 4 (2)	104 (1)
DMS, 2 mM	ND	ND	93 (1)	98 ± 7 (2)
10 mM	93 ± 10 (2)	84 ± 6 (2)	98 ± 15 (2)	111 (1)
50 mM	29 ± 19 (4)	82 ± 18 (4)	127 ± 6 (2)	96 (1)
EAI, 2 mM	ND	ND	ND	ND
10 mM	100 ± 0 (2)	ND	ND	ND
50 mM	19 ± 2 (2)	ND	ND	ND

[a] SPM was incubated 1 h at 25 °C with various concentrations of DMM, DMA, or DMS, at pH 8.5, as described in Materials and Methods. At the end of incubation, the samples were centrifuged; the pellets were resuspended in a small volume of water, and assayed for various enzymes, as described. Each value is listed as the mean ± standard deviation, with number of experiments in parentheses, and is expressed as the % of the value found for incubation controls. The absolute values of the latter, in μmol of substrate utilized min^{-1} (mg of protein)$^{-1}$ were: (Na^+,K^+)-ATPase, 0.75; 5′-nucleotidase, 0.032; acetylcholinesterase (AChE), 0.16; alkaline phosphatase, 0.16. ND, not determined.

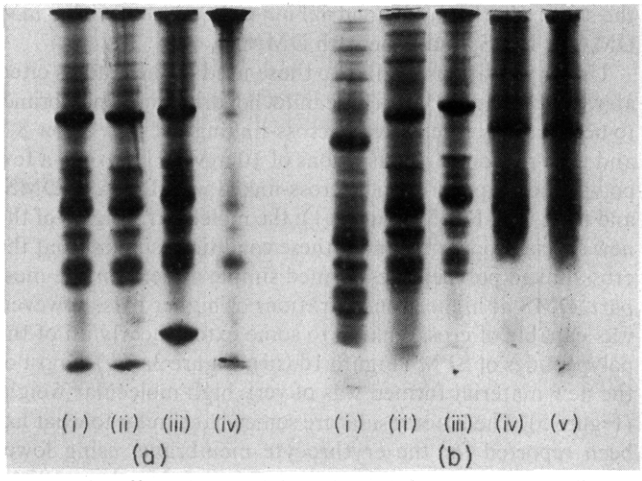

FIGURE 3: Effect of pH and time of incubation on SPM cross-linking. SPM (1.0 mg/mL) was incubated with 10 mM DMS at 25 °C under various conditions described below. Following incubation, all samples were further processed exactly as in the legend to Figure 1. (a) Effect of pH. Incubation was for 1 h at a pH of: (i) 7.5; (ii) 8.0; (iii) 8.5; (iv) 9.0. The pH was adjusted with 0.1 M sodium phosphate in i and ii and with 0.25 M TEA in iii and iv. (b) Effect of time of incubation. Incubation was at pH 8.5 for (i) 10 min; (ii) 30 min; (iii) 1 h; (iv) 3 h; (v) 24 h (at 0 °C).

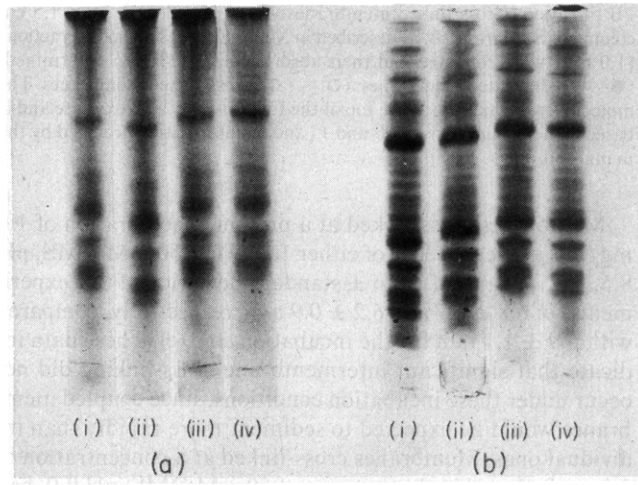

FIGURE 4: Effect of membrane concentration and temperature on SPM cross-linking. SPM was incubated with 10 mM DMS at pH 8.5 under various conditions described below. Following incubation, all samples were further processed exactly as in the legend to Figure 1. (a) Effect of membrane concentration. Incubation was for 1 h at 25 °C, with an SPM concentration of (i) 0.1 mg/mL; (ii) 0.3 mg/mL; (iii) 1.0 mg/mL; (iv) 3.0 mg/mL. (b) Effect of temperature. Incubation was for 30 min with a membrane concentration of 1.0 mg/mL, at (i) 0 °C; (ii) 25 °C; (iii) 37 °C; (iv) 45 °C.

incubated 1 h at 25 °C, pH 8.5, with several concentrations of DMM, DMA, or DMS was assayed for the activities of several membrane-bound enzymes (Table I). 5′-Nucleotidase (EC 3.1.3.5), alkaline phosphatase (EC 3.1.3.1), and acetylcholinesterase (EC 3.1.1.7) were essentially unaffected by even 50 mM DMS. (Na^+,K^+)-ATPase was greatly inhibited by 50 mM DMS, but was also inhibited by this concentration of DMM, DMA, or the monofunctional reagent ethyl acetimidate (EAI).

The Extent of Cross-Linking between Membranes. When testing the effects of cross-linking reagents on membranes, it is important to discriminate between reaction between different components of the same membrane, and that occurring between components situated on different membranes. The data in Figure 4a suggest that the latter process contributed insignificantly to the extent of cross-linking observed overall, because cross-linking between membranes would be expected to increase with increasing membrane concentration. The very large molecular weight of the complexes formed by extensive cross-linking (Figure 1d (iii); Figure 5, below) also would appear to be inconsistent with the importance of intermembrane bridges, which would produce complexes larger than dimers only with small probability.

As a further test of this possibility, however, membranes treated with DMS under several different conditions were subjected to sedimentation analysis. Following incubation with DMS for 1 h at 25 °C, the membranes were pelleted by centrifugation at 50 000*g* for 10 min, and resuspended in 5.0 mL of 0.1 M sodium phosphate, pH 7.0. The suspensions were then centrifuged at 700*g* for various periods of time, and, from protein assays of the resulting pellets and supernatants, the time required for half the material to sediment (T_s) was determined for each sample.

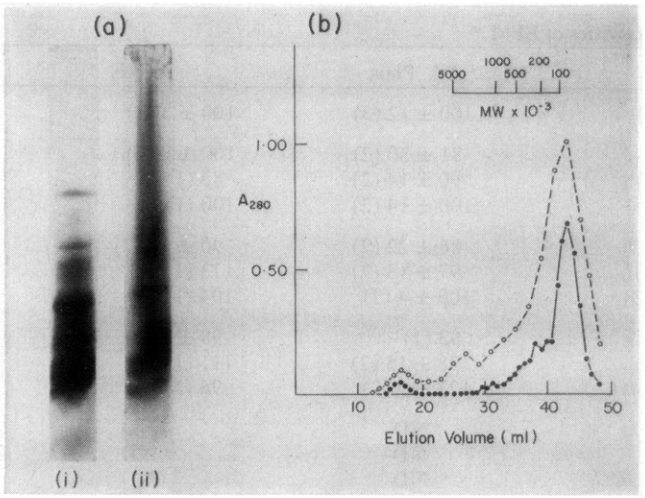

FIGURE 5: Molecular weight analysis of cross-linked SPM. SPM (1.0 mg/mL) was incubated with 50 mM DMS at pH 8.5 for 1 h at 25 °C. Following incubation and centrifugation, the cross-linked membranes were analyzed as described below. (a) $NaDodSO_4$ gel electrophoresis. Samples were analyzed exactly as given in the legend to Figure 1, except that the acrylamide concentration was 3.0%. (i) Incubation control; (ii) cross-linked SPM. In ii, a compact, intensely staining "button" of material appeared at the top of the gel, but was not recorded by the camera. (b) Sepharose 2B filtration. Cross-linked membranes were filtered on a 24 × 1.5 cm column of Sepharose 2B, as described in Materials and Methods. Fractions (1.0 mL) were collected and their absorbances at 280 nm determined. (●—●) Untreated membranes; (○ - - - ○) cross-linked membranes. The molecular weight scale at the top of the figure is only approximate and is based on determinations of V_0 and V_t and on information provided by the manufacturer.

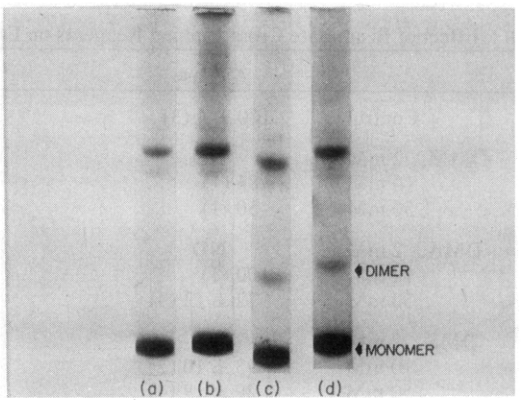

FIGURE 6: Cross-linking of hemoglobin. Hemoglobin, prepared as described in Materials and Methods, was incubated with bisimidate reagents under the same general conditions given in the legend to Figure 1. Following incubation, samples were immediately processed for electrophoresis. (a) Incubation control; (b) 10 mM DMM; (c) 50 mM DMM; (d) 10 mM DMS. The arrows indicate the position of hemoglobin monomer and dimer. The three light bands appearing about half way down in each of the gels are impurities present in the hemoglobin preparation.

Membranes cross-linked at a protein concentration of 1.0 mg/mL in the presence of either 10 mM or 50 mM DMS, pH 8.5, had T_s values (mean ± standard deviation of two experiments) of 6.8 ± 0.7 and 6.2 ± 0.9 min, respectively, compared with 6.9 ± 1.1 min for the incubation control. These data indicate that significant intermembrane cross-linking did not occur under these incubation conditions, since coupled membranes would be expected to sediment more rapidly than individual ones. Membranes cross-linked at a concentration of 3.0 mg/mL and in the presence of 50 mM DMS, pH 9.0, had a T_s value of 1.7 ± 0.6; thus significant intermembrane cross-linking may have occurred under these conditions, although the increase in sedimentation rate might also have resulted from an increase in density of individual membranes, brought about by extensive intramembrane cross-linking.

Analysis of Cross-Linked Material. Membranes cross-linked in the presence of 50 mM DMS, pH 8.5, were analyzed by $NaDodSO_4$ gel electrophoresis and by Sepharose 2B filtration (Figure 5). When analyzed on 3% polyacrylamide gels, most of the cross-linked material was observed to be excluded from the gel (Figure 5a); globular species of less than approximately one million molecular weight will enter this system. Sepharose 2B analysis was complicated by the fact that DMS absorbs at 280 nm, so that cross-linked membrane displayed an increase in total absorbance relative to untreated membrane (Figure 5b). However, the former material clearly showed a high molecular weight peak, corresponding to about 3–4 × 10^6 molecular weight for a globular protein, that was not present in the latter.

Discussion

The bisimidate reagents used in this study have been applied with different degrees of success to other membranes. Ji (1974) and Ji & Nicolson (1974) were able to cross-link to some extent most of the major species of the erythrocyte membrane with 1 mM DMM or DMA at pH 7.5. Tinberg & Packer (1976), in contrast, were able to cross-link only a small proportion of the species in the mitochondrial inner membrane with 8 mM DMA or DMS, and none with DMM.

Under conditions similar to those used in the studies cited above, we found SPM, like the mitochondrial inner membrane, to be relatively insensitive to cross-linking. At pHs below 8.5 and with reagent concentrations of 10 mM or less, only a few polypeptides appeared to be cross-linked with DMA or DMS, and none with DMM (Figure 1); the molecular weights of the new species appearing under these conditions suggest that the cross-linked polypeptides formed simple dimers for the most part. DMS at higher concentrations or higher pHs, however, was capable of cross-linking to some extent nearly all of the polypeptides of SPM (Figure 1d (iii); Figure 3a (iv)); most of the new material formed was of very high molecular weight (Figure 5). The latter results are somewhat similar to what has been reported for the erythrocyte membrane, using lower concentrations of bisimidate (Ji, 1974; Ji & Nicolson, 1974), suggesting similarities between the two systems in the lateral relationships of their proteins.

Surprisingly, such massive cross-linking had no significant inhibitory effect on any enzyme tested, except (Na^+,K^+)-ATPase, in which instance inhibition was due largely or entirely simply to amino blocking (Table I). Because the unaffected enzymes—5′-nucleotidase, alkaline phosphatase, and acetylcholinesterase—are probably all present in the membrane as minor species, it is not possible on the basis of our gel data to conclude that they were cross-linked into higher molecular weight complexes. This seems likely, however, and assuming it is so, the data in Table I suggest that some restriction of motion of these enzymes is not incompatible with their normal functioning.

Both DMM and DMA are theoretically long enough to join subunits within a single protein molecule, and have been demonstrated to do so in the case of soluble proteins (Davies & Stark, 1970; Figure 6). Their failure to cross-link all but a few minor polypeptides in SPM is thus surprising, for this membrane presumably contains many multisubunit proteins. Even supposing DMM and DMA to be ineffective for some reason other than their length, one would expect such multisubunit complexes to become cross-linked by DMS more readily than would separated species, forming new species of relatively low molecular weight; very few such species are

observed, however. A possible explanation is that these complex proteins, which in many cases are probably functional species such as enzymes and glycoproteins (Singer, 1974), are present as minor bands not easily observed on gels; their detection would in fact not be possible if they comigrated with other, more prominent species, as many of the glycoproteins appear to do (Wang & Mahler, 1976; Smith & Loh, 1977c).

The large complexes formed by treatment of SPM with high concentrations of DMS appeared to have molecular weights of several million (Figure 5). This value is only approximate, but it seems reasonable to conclude that these complexes contained a dozen or more individual polypeptides, most of which have molecular weights of less than 100 000 (Figure 1). The joining of such a large number of species into a single complex could reflect the existence of ordered arrays of closely spaced protein molecules in the membrane, or could result from random collisions of laterally-diffusing species (Frye & Edidin, 1970; Singer & Nicolson, 1972). Our results do not rigorously distinguish between these two possibilities, but suggest that both make a contribution to the cross-linking observed. The large, fairly uniform size of the complexes formed with DMS (Figure 5b), the failure of DMM and DMA to promote extensive cross-linking (Figure 1b,c), and the virtual lack of effect of temperature on the cross-linking process (Figure 4b) are all more consistent with the first alternative; the last observation is perhaps the most compelling one, since low temperatures appear to reduce lateral diffusion greatly in this and in other membranes (Edidin & Fambrough, 1973; Kelly et al., 1976).

On the other hand, the fact that cross-linking continues to occur over a period of time much greater than that required for chemical reaction (Figure 4c; Peters & Richards, 1977) suggests that diffusion is involved in some of the cross-linking. The densitometric data in Figure 2, furthermore, give some support to both alternatives. On the one hand, a large number of species are complexed to a nearly uniform extent, which fact favors a diffusional mechanism; on the other hand, a few species are cross-linked much less readily than the others, which is not consistent with their freely diffusing in the membrane. Finally, the results of a lectin-binding study by Kelly et al. (1976) also support both alternatives. These researchers found that an ordered array of immobile lectin receptors was present in a restricted portion of SPM—its attached postjunctional membrane—while randomly oriented, freely diffusing receptors were present in the remainder of the membrane.

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