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Chemical and biochemical crosslinking of membrane components

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I. Introduction

It is the purpose of this review to survey the variety of ideas in applications of crosslinking reagents to membranes. A number of earlier reviews on crosslinking reagents have appeared. Wold [1] and Fasold et al. [2] reviewed the subject of protein crosslinking in general. Peters and Richards [3] and Wold [4] have reviewed the chemistry of crosslinking. Applications of crosslinking in biology, with emphasis on identifying receptors for ligands, have been discussed by Das and Fox [5]. Applications to cell membranes are reviewed by Ji [6] and crosslinking of erythrocyte proteins by Shaw and Marinetti [7]. The synthesis (Radhakrishnan et al. [8]) and applications (Bayley and Knowles [9], Robson et al. [10]) of photosensitive reagents are the subjects of three reviews.

This review will be divided into sections on biophysics, chemistry, photochemistry, and biochemistry of crosslinking, followed by a section on selected applications. Applications of small molecule affinity reagents to membrane proteins are not covered here except when they provide supporting data for other crosslinking studies. Hydrophobic labeling [11,12], for instance by adamantyldiazirine, is covered only briefly because it is, and has been, subject matter for a separate review in itself. The 'Applications' section emphasizes recent, representative studies, but does not attempt to be comprehensive. Again, the aim is to present the variety of ideas, not every application of them. For example, recent syntheses have provided reagents which are fluorescent [13] or spin-labeled [14], that become fluorescent after photolysis [15] and that transfer radioactivity from a macromolecular ligand to its receptor [16–19]. The location of photoreactive lipids in a membrane and their reactivity with hydrophobic peptides is being determined at the amino acid analysis level [8,20–24]. Transglutaminase [25] has been employed as a reagent to examine penetration of complement proteins through membranes [26] and several disulfide-linked acetylcholine receptor dimers have been combined with electron microscopy data to yield information on the arrangement of subunits [27,28]. Use of crosslinking reagents to identify cell-surface receptors for macromolecular ligands has become an established technique, with antibodies directed against the crosslinker [29] as a new addition.

II. Biophysics of crosslinking in membranes

While synthesis of new crosslinking reagents and empirical approaches to applying them have received much attention in the last decade, a number of questions have arisen that require quantitative answers. For instance, in the use of hydrophobic reagents, what assumptions can be made about the location of the reagent in a membrane? The need for attention to this problem is apparent when the results of applying several hydrophobic reagents to a single membrane protein are compared. Hydrophobic labeling of selected complement components differs when hydrophobic re-

agents of varied structure are used (see subsection VIB-1a). Similarly, the selectivity of photoreactive reagents for subunits of the acetylcholine receptor is different for three hydrophobic reagents (see subsection VIB-2b). For reagents that partition between membrane and water, crosslinking may be favored relative to monofunctional attachment of the reagent by using high concentrations of membrane so that the membrane-associated location of the reagents is favored. When crosslinking reagents are used to examine the oligomeric state of a membrane protein, the special geometry of the membrane suggests that planar aggregates of proteins should provide the best theoretical models. And finally, when information is available on the kinetics of reaction of the functional groups on a bifunctional molecule, calculations can be made to suggest the optimum reagent concentration that favors crosslinking. This section on biophysics of crosslinking discusses experiments in which quantitative information on reagent location in a membrane, oligomeric state of a membrane protein and kinetics of reaction of bifunctional reagents have been provided. The subject of location of reagents in membranes has also been addressed chemically for photoreactive phospholipids (see subsection IVA).

IIA. Location and orientation of reagents

Several spin-labeled bifunctional and crosslinking molecules were synthesized so that the orientation of the reagents in a lipid bilayer could be examined by electron paramagnetic resonance (EPR) spectroscopy [14,30]. It was anticipated that the EPR spectra could be used to distinguish three modes of interaction of the reagents with membranes: (1) tumbling in the hydrophobic interior; (2) a surface orientation with both reactive groups on one side of the membrane; and (3) a transmembrane orientation with reactive groups on opposite sides of the membrane. Background for the latter possibility was provided by the observation that long, spin-labeled dicarboxylic acids take up an orientation extending across a bilayer in phospholipid bilayers.

Figs. 1 and 2 show examples of EPR data on orientation of bifunctional reagents. The diacid (I) assumes two arrangements – with the long-chain

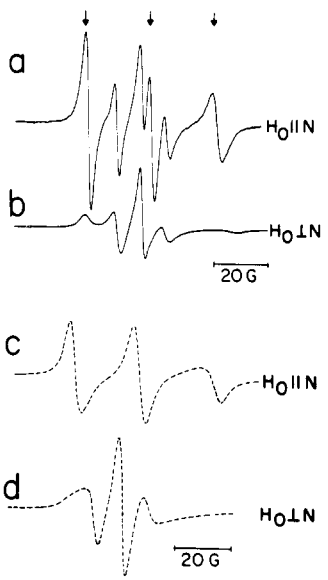
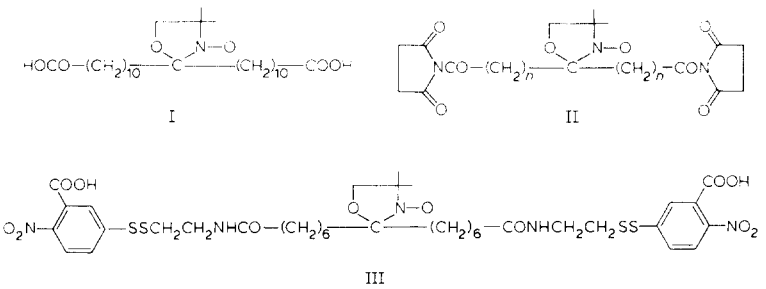


Fig. 1. Experimental paramagnetic resonance spectra resulting from oriented bilayers of dilauroylphosphatidylcholine containing one diacid spin label, I, for 100 lipids are shown in (a) and (b). The magnetic field is applied parallel to the membrane normal in (a) and perpendicular to it in (b) (H designates magnetic field; N designates the membrane normal). The arrows in (a) are at the positions of the spectral features which arise from the fraction of molecules that are in the transmembrane orientation. Spectrum (b) also contains contributions from molecules in two orientations in the same macroscopically aligned sample from which spectrum (a) was obtained. Although spectrum (b) accidentally resembles a spectrum from an isotropic distribution of membranes, it should not be confused with one. The relative contributions of molecules in the two orientations is about 4 to 1 in favor of the transmembrane orientation, based on the peak heights and line widths shown in (a). Computer-simulated spectra are shown in (c) and (d) for the spectra which would arise from the transmembrane conformation with the magnetic field applied parallel (c) and perpendicular (d) to the membrane normal. A wider distribution of label orientations is assumed in the simulated spectra (c and d) than is apparently observed in the experimental spectra (a and b). From Ref. 14, Fig. 1.

axis both perpendicular and parallel to the membrane surface. None of the di-*N*-hydroxysuccinimide esters of chain lengths 15 to 29 atoms (II) takes up the orientation extending across the bilayer, nor does a disulfide-exchange reagent (III).

Further definition of the location of these labels was required to determine whether they were located entirely on the surface of the membrane or extended partially into the hydrocarbon interior.



I II III

To do this, partition coefficients between dimyristoyl phosphatidylcholine membranes and water were measured above and below the phase-transition temperature of the lipid. The result was that, although a molecule such as (II) has a partition coefficient of over 500 in favor of membranes, this coefficient is completely insensitive to the phase transition of the lipids. In contrast, spin labels known to tumble in the lipid interior have partition coefficients very sensitive to the phase transition of synthetic lipids. This, together with EPR data, suggests that reagents (II) and (III) prefer a location at the interface between membrane and water. Obviously it is not practical to include the spin-label group in the synthetic steps for all crosslinking reagents, but the experiments cited above suggest ways that the location of other reagents in membranes can be determined. First, the partition coefficient between membrane and water indicates whether or not the reagent binds significantly to membranes. Second, if a label which binds to membranes has a partition coefficient that is insensitive to the phase transition in model membranes, the reagent probably lies at the membrane/water interface. It is possible, however, that insensitivity of the partition coefficient to the phase transition could also arise if a reagent were equally soluble in the hydrocarbon regions of 'solid' and 'liquid' phases of the membrane [31].

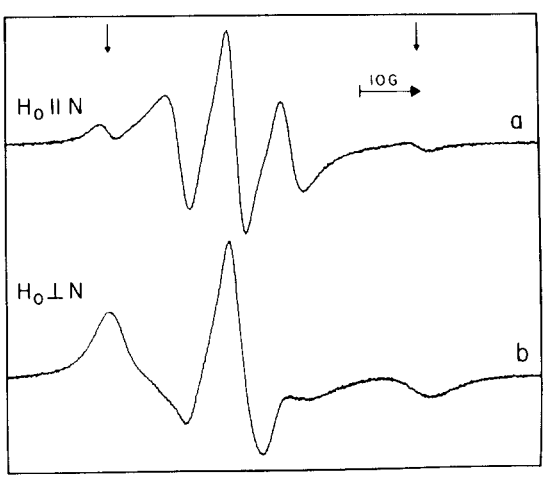


Fig. 2. EPR spectra are shown for the bis(*N*-hydroxysuccinimide ester), II, in oriented bilayers of dilauroylphosphatidylcholine. In (a) the magnetic field is parallel to the bilayer normal, and in (b) it is perpendicular. The arrows in (a) indicate the positions of spectral components from molecules that may have the transmembrane orientation. From Ref. 14, Fig. 2.

Other physical measurements have also been used to examine the orientation of reagents in membranes. The alignment of fluorescent probes in oriented membranes has been determined [32]. The recently synthesized fluorescent crosslinker [15] might be used in a similar manner to determine the location with respect to a lipid bilayer of the products of reaction of this reagent. Also, optical and Raman measurements are useful for determining the orientation of polyene molecules in membranes [33].

IIB. Crosslinking of oligomeric vs. monomeric membrane proteins

Models for the function of rhodopsin raise the question of whether the protein is monomeric or oligomeric in the dark-adapted state. The problem has been addressed by comparing experimental results of crosslinking with theoretical predictions [34].

Theoretical predictions for random crosslinking of monomers are shown in Fig. 3. The parameter μ is related to the all-over degree of crosslinking by

$$\mu = \sum_n (n-1) \cdot (\text{fraction of } n\text{-mer})$$

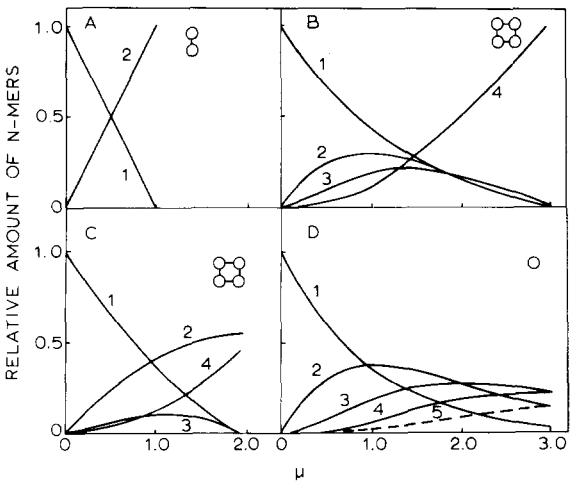


Fig. 3. The patterns of oligomeric distribution predicted for planar arrays of monomeric vs. oligomeric proteins are shown. The curves show the depletion of monomer and formation of n-mers as the degree of crosslinking (μ) increases. Calculations were done for native proteins in dimeric (A), tetrameric with identical binding domains (B), tetrameric with D2 symmetry and probability ratio for crosslinking between two binding domains of 1:4 (C), and monomeric (D) proteins. From Ref. 34, Fig. 3.

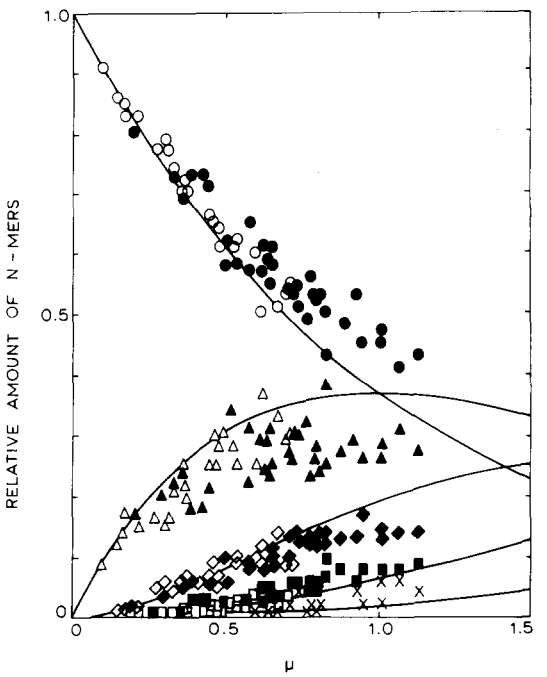


Fig. 4. The oligomeric distributions resulting from glutaraldehyde crosslinking of intact retinas (open symbols) and isolated rod outer segment membranes (closed symbols) are shown. The mass fractions of opsin and its oligomers were determined from SDS-polyacrylamide gel electrophoresis. The degree of crosslinking is μ. Data are plotted for monomers (○, ○); dimers (△, △); trimers (◆, ◇); tetramers (■, □) and pentamers (X for outer segment membranes only). The solid lines show the distribution predicted for random crosslinking of monomers. From Ref. 34, Fig. 2.

where $(n-1)$ is the number of crosslinks formed initially between two species. Using the Poisson approximation to the binomial distribution, the dependence of the fraction of any n-mer on the degree of crosslinking is given by

$$\text{fraction of } n\text{-mer} = \frac{e^{-\mu} \cdot \mu^r}{r!}$$
$$(\text{with } r = n-1)$$

Experimental results justified the use of the Poisson approximation: the observed extent of crosslinking was only 1.2 after several hours (approx. 10^4 s) of crosslinking, while the collision frequency of freely-diffusing rhodopsin is approx. 10^5 s^{-1}. Thus, the probability of forming a crosslink must be on the order of 10^{-9} per collision.

The experimental results of crosslinking rhodopsin with glutaraldehyde are shown in Fig. 4. Apparently, the reactive sites on the proteins are fully occupied by reagent by the time that the extent of crosslinking has reached about 1. The important feature of the results is that there is a generally decreasing fraction of n-mer with increasing n. These results are inconsistent with a dimer model (Fig. 3A), compatible with the monomeric random collision model (Fig. 3D), but not unambiguously different from the tetrameric model (Fig. 3B) for a limit of $\mu = 1$. Crosslinking was also performed with Cu^{2+}-*o*-phenanthroline and with diisocyanatohexane. Again, the results favored the random collisional model. Measurements of the transient decay of dichroism of rhodopsin [35] showed that the diffusing species increased in size at the earliest stages of crosslinking – again consistent with a monomeric state for rhodopsin in the dark-adapted form.

IIC. Theory of ligand-induced crosslinking of receptors

Crosslinking of multivalent antibodies by bivalent ligands is a subject with a large literature. It is

of importance to membrane studies involving triggering mechanisms that depend on induced receptor clustering. For instance, histamine release by basophiles and mast cells is induced by adding multivalent IgE to the cells or by crosslinking previously bound monomeric IgE with bivalent ligands. The aid of theoretical predictions for this situation is to simulate the bell-shaped experimental plot of histamine released vs. concentration of bivalent ligand added. In an extended sense, these theories apply to the subject matter of this review, because they cover the concentration dependence of monovalent and bivalent reactions with available sites on membrane proteins. Determining the optimum concentration of crosslinking reagent to favor formation of crosslinked product is an integral part of studies of the type discussed in the previous subsection.

The theoretical treatment of receptor clustering has been done for bivalent ligands with like and with different functional groups [36,37]. An example of the theoretical result is shown in Fig. 5. Here the concentration of crosslinks (M) is plotted as a function of concentration (C) of ligand in solution for various times during the course of reaction. The calculation was done for $2 \cdot 10^4$ receptor sites/cell and relevant kinetic and diffusion constants. The ligand was taken to be heterobifunctional with one rapidly and one slowly dissociating functional group. As the time of crosslinking increases, the plot shifts to the left until it reaches a limiting value at equilibrium. Experimentally determined histamine release curves, in one case, were identical for times of 2.5, 5 and 15 min. Thus, it seems safe to conclude that equilibrium had been achieved in each experiment.

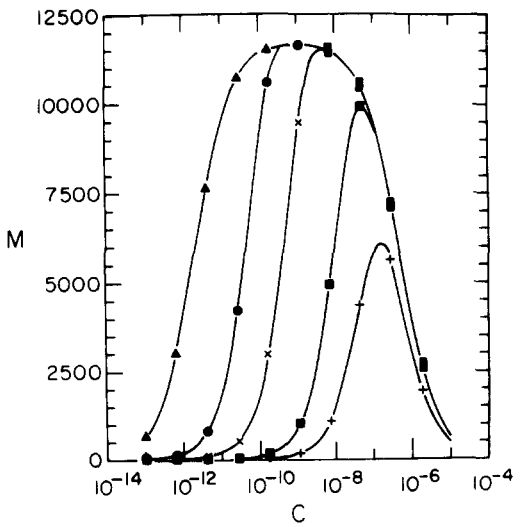


Fig. 5. The extent of receptor crosslinking when a hapten has one rapidly and one slowly dissociating functional group is shown. M is the concentration of double bound ligand and C is the external ligand concentration. Different curves are for times of 1 s (+); 5 s (■); 10^2 s (×); 10^3 s (●) and equilibrium (▲). The receptor sites were taken as $2 \cdot 10^4$/cell. Kinetic and diffusion constants are given in Ref. 37. A solid rectangle indicates points where (+) and (■) overlap. Figure is redrawn from Ref. 37, Fig. 5.

III. Chemical crosslinking

Functional groups used most often in crosslinking reagents for membrane studies are directed to amino groups on proteins. The imidoester functionality, in particular, is capable of undergoing a diverse set of reactions which depend on pH and which can lead to crosslinking even by the monofunctional imidoesters. These reactions are tabulated in the review by Peters and Richards [3]. In general, the reactivity of chemical functional groups is similar for membrane proteins and water-soluble proteins. A book on protein-modification reagents is available [38].

The development of successful crosslinking reagents for membranes has included attention to (1) rendering the molecules cleavable, (2) spacer arms, (3) polarity of reagents, (4) use of spin label, optical and fluorescent chromophoric groups and (5) introduction of radioactivity. At this point, a reagent for a specific purpose often can be designed by a 'mix and match' approach using synthetic steps for pieces of previously prepared molecules. Since much of this chemistry has been reviewed before, structures that apply to each of the categories above are collected in Tables I–V (subsection III.A). Only a few examples of the versatile reagents that have had recent applications will be discussed in detail (subsection III.B). These examples provide a summary of many of the concepts relevant to crosslinking in general.

IIIA. Synthesis of reagents

IIIA-1. Cleavable groups

When multicomponent membranes (e.g., cell membranes) are labeled, positive identification of the components in a crosslinked oligomer is facilitated when the crosslinking reagent can be cleaved. The typical analysis procedure employs a two-dimensional acrylamide gel: the sample with crosslinks intact is run in the first dimension (a tube gel or slice of slab gel), and this gel is bonded to a slab gel by an agarose layer that contains the cleaving reagent [39]. The proteins that have not been crosslinked appear in a diagonal row on the slab, while those crosslinked fall to the higher-mobility side of the diagonal and lie directly under the position of the oligomer from which they were derived in the first dimension. A two-dimensional gel for a control treated in the same manner as the sample, except for omission of crosslinking reagent, serves as a check for native oligomers which are subject to cleavage in the second dimension. Occasionally, a protein will appear on the low-mobility side of the diagonal when the electrophoretic mobility of that species is an anomalous function of molecular weight [39].

The chemical reactions that have been used in cleavable crosslinking reagents are summarized in Table I. The cleavage conditions, in general must be compatible with protein structure and an aqueous environment.

IIIA-2. Spacer arms

Spacer arms can be added to extend the length of a crosslinking reagent, to change its solubility and to introduce radioactivity. The general ideas under this heading are the same as those used in developing affinity chromatography [44]. Examples listed in the table below (Table II) include only those that are representative of applications in research on crosslinking membrane proteins. Reactions which promote dimerization of cysteine

TABLE I

CLEAVABLE GROUPS

Crosslinking product	Cleaving conditions	Cleavage products	Side reaction	Ref.
R–SS–R′	reducing agent	R–SH + R′–SH	disulfide interchange	39,40
R–S(=O)–S(=O)–R′				
↓protein SH R–SS–Prot	reducing agent	R–SH + prot–SH	disulfide interchange	39
R–N=N–R′	dithionite ($Na_2S_2O_6$)	$R–NH_2$ + $R'–NH_2$		18,19,41
R–C(–OOH)–C(–OOH)–R′	periodate ($NaIO_4$)	RCHO + R′CHO	cleavage of sugars; Schiff base formation; reaction may be difficult	42
R–C(=NH)–NHR′	1 M NH_4OAC 5 M NH_4OH	$R'NH_2$ + RC(=NH)NH_2	proteolysis; results variable	16
$R–NHCH_2OCO–CH_2–R'$	0.1 M NaOH, 15 min, RT	$R–NHCH_2OH$ + $R'CH_2COOH$	mild proteolysis	43

TABLE II

SPACER ARMS ON CROSSLINKING REAGENTS

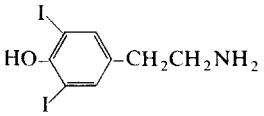
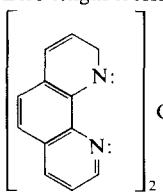
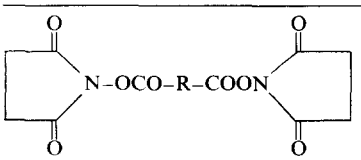
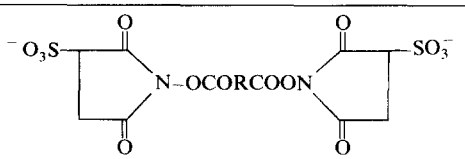
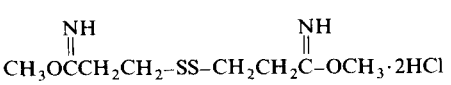
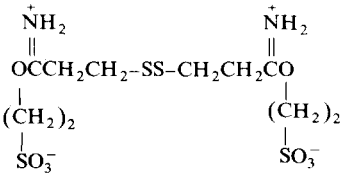
Reagent	Comment	Ref.
Chain extenders		
$NH_2-(CH_2)_n-COOH$	available in n = 1 to ≈ 10	e.g. 14
and aromatic amino acids	use: reaction with amino directed, symmetrical crosslinker gives new crosslink precursor longer by $2n+4$ atoms	
HOCO–CH(OH)–CH(OH)–COOH (tartaric acid)	cleavable	42
 (diiodotyramine)	available with ^{125}I	45,46
$HO-(CH_2)_n-OH$		47
$HOCH_2CH_2-SO_2-CH_2OH$		47
Zero-length crosslinking		
 Copper-*o*-phenanthroline	promotes disulfide formation	3,48
$(CH_3)_2NCON{=}N-CON(CH_3)_2$ (diamide)	promotes disulfide formation	49
$NO_2-C(NO_2)_2-NO_2$ (tetranitromethane)	forms tyrosine dimers	50

TABLE III

GROUPS TO VARY POLARITY OF CROSSLINKING REAGENT

Less polar reagent	More polar reagent	Ref.
		51
		52
$RO-(CH_2)_5-OR$	$ROCH_2CH_2SO_2CH_2CH_2OR$	47

or tyrosine side-chains are included in this table under the heading of 'zero-length' crosslinking [3,48–50].

IIIA-3. Variation in polarity

N-Hydroxysuccinimide esters (NHS esters) are less subject to hydrolysis than are imidoesters so

TABLE IV

CHROMOPHORIC GROUPS

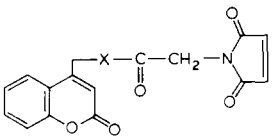
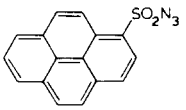
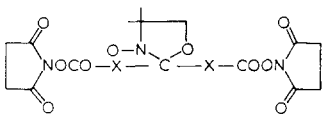
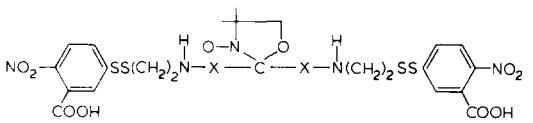
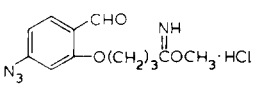
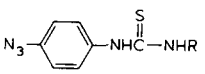
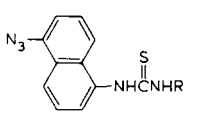
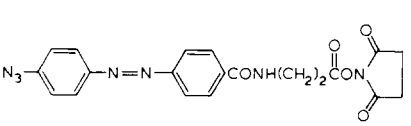
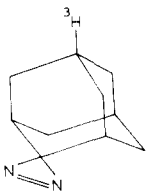
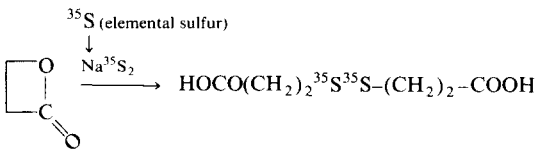
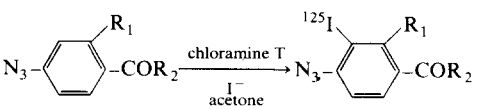
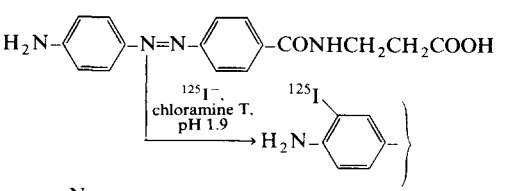
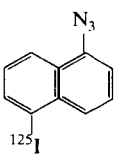
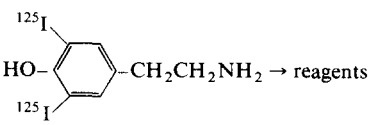
Reagent	Spectroscopy used	Ref.
	fluorescence after photolysis (emission maxima (460–550 nm) depending on X	15
	fluorescence sensitive to conformation	13
	electron paramagnetic resonance (orientation, motion, and environmental information)	14,30
		14,30
	UV/vis ($\varepsilon_{325} = 10500\ M^{-1}\cdot cm^{-1}$) reduction of aldehyde, or Schiff's base, gives loss of 325 nm absorption	16
 (isothiocyanate group already reacted with RNH_2)	UV/vis λ_{max} at 256 and 283 nm, $\varepsilon_{283} = 16000\ M^{-1}\cdot cm^{-1}$ after photolysis λ_{max} 256 nm is diminished	53
 (isothiocyanate group already reacted with RNH_2)	UV/vis λ_{max} 308 nm ($\varepsilon = 13200\ M^{-1}\cdot cm^{-1}$); diminished by photolysis	53
	UV/vis λ_{max} 358 (ε = 35000 $M^{-1}\cdot cm^{-1}$); photolysis gives diminished λ_{max} and ε	18

TABLE V

INTRODUCTION OF RADIOACTIVITY INTO CROSSLINKING REAGENTS

Isotope	Reagent or reaction	Ref.
^{3}H	Schiff's base: $R_1-CH=N-R_2 \xrightarrow{NaB^3H_4}$	16
		54
	[^{3}H]ethanolamine precursor for biosynthetic lipid	107
^{14}C	[1-^{14}C]glucosamine → glycolipid reagent	55
	[^{14}C]palmitic acid in reactive lipids	8
	$R-CH_2I \xrightarrow{^{14}C\equiv N} RCH_2{}^{14}CN \rightarrow RCH_2{}^{14}COOH$	8
^{35}S	$H_2N-CH_2CH_2^{35}SH$ (cystamine) as a spacer	14
		40
	$H_2NCH_2CH_2{}^{35}SO_3$ (taurine)	56
^{125}I		137
		19
		57
		45

they are attractive as homobifunctional reagents to minimize monofunctional attachment of the label followed by hydrolysis. However, NHS esters are considerably less water-soluble than are imidoesters. Addition of a sulfonate group to the structure of a crosslinking reagent renders it more soluble and also impermeant to membranes [51].

IIIA-4. Chromophoric groups

When crosslinking reagents include chromophores, these groups can be used to quantitate the number of probes attached to protein, to follow the course of reaction [15,16,18,53], to determine the affinity and orientation of the probe for the membrane [14,30] and to examine conformational changes of the labeled protein [13].

IIIA-5. Introduction of radioactivity

Many crosslinking studies are carried out with radiolabeled proteins to facilitate analysis. However, to demonstrate that the crosslinked product is actually the result of the crosslinking reagent, it is useful to employ radioactive reagents, or to use radioactivity in a way that is specific for the crosslinking reaction (i.e., borohydride reduction of Schiff's bases).

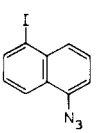
IIIB. Application: Heteromultifunctional, cleavable crosslinkers

The motivation for preparing the crosslinking reagents that contain several different chemically reactive groups (heteromultifunctional) and are cleavable is the desire to transfer a label from a macromolecular ligand to its receptor – thereby identifying the receptor. This approach has been taken to label the cell surface receptor for Sindbis virus, as will be discussed later. Two reagents (IV) [16,17] and (V) [18,19] have been designed for identifying receptors according to this plan. The chemistry involved in applying these reagents to receptor labeling encompasses many of the considerations used with simpler reagents, so this chemistry will be outlined here as a summary of many of the concepts relevant to crosslinking in general. Scheme I gives reactions of IV with proteins and Scheme II presents reactions of V.

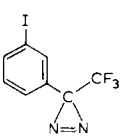
Both reagents, IV and V, have been tested for receptor labeling in well-defined and understood systems. As a test of IV, ^{125}I-labeled IgG was labeled in both chains and was then covalently attached to receptors on JY cells by reductive amination with borohydride [17]. The only band on gels which was specific for the presence of the cells during crosslinking was one of 66 kDa, identified as IgG heavy chain (52 kDa) crosslinked to β2-microglobulin (12 kDa). Label V was tested using the complex formed by protein A and IgG [19]. Protein A-Sepharose beads were labeled in 34% yield (based on incorporation of ^{125}I into the beads) via the *N*-hydroxysuccinimide ester when the reagent was limiting. After the beads had been thoroughly washed, the photochemical reaction with either human serum or purified rabbit IgG was performed and the resulting material attached to beads was treated with dithionite. This resulted in release of ^{125}I into the supernatant to account for 1.1% of the total radioactivity attached originally to protein A as IgG heavy chain and approx. 0.3% of the iodine label in light chain. Predominant labeling of the heavy chain is consistent with the known binding site of protein A. No other labeled proteins were released into the supernatant after dithionite reduction.

IV. Photochemical crosslinking

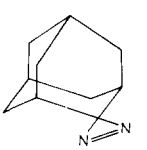
A number of monovalent reagents with specificity for the membrane-embedded domains of proteins have been developed. Examples are shown below. Reagent (VI) forms a nitrene on photolysis [57] and reagents (VII) and (VIII) give carbenes [58,12].



VI

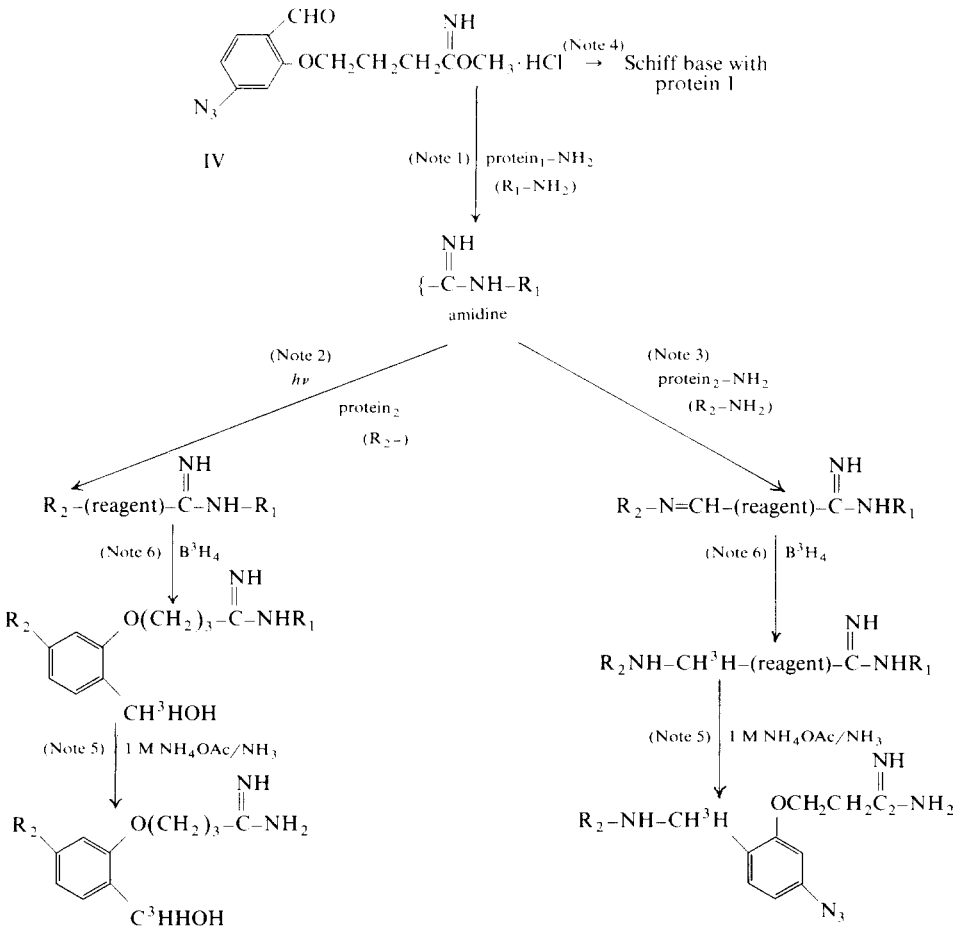


VII



VIII

Nitrenes and carbenes are attractive for crosslinking of proteins or lipids to proteins because they are not highly specific for particular amino acid functional groups and thus are potentially better reporters of simple proximity than are the non-photoreactive protein modification reagents. Khorana and co-workers [8,10] have reviewed the chemistry of photosensitive reagents. Therefore, the discussion below will be devoted to summariz-

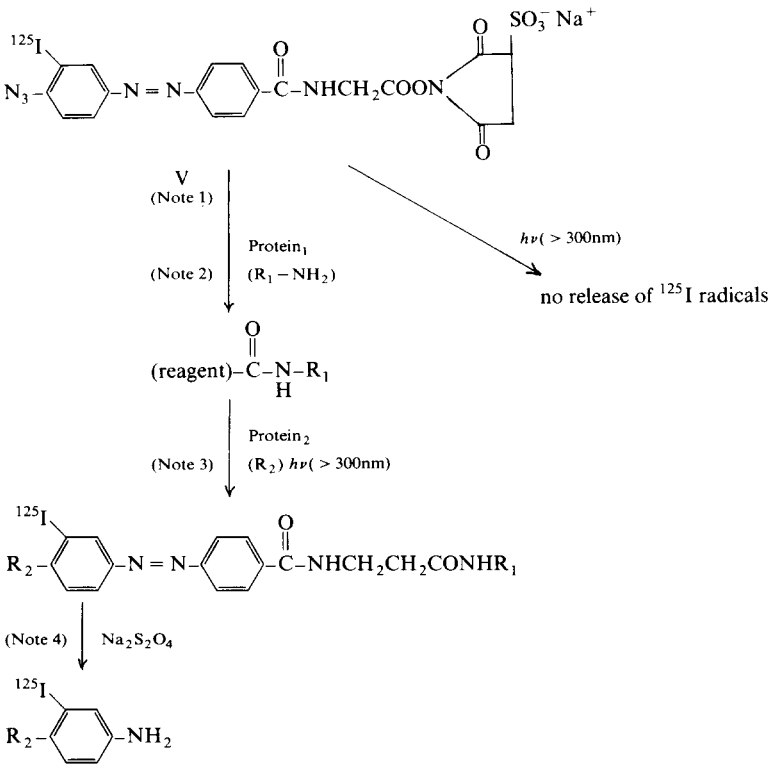


Scheme I. (Reagent IV [16,17].) Notes. (1) Absorbance of the 6-formyl-4-azidophenoxy chromophore at 325 nm can be used to quantitate the number of reagent groups incorporated into protein R_1. The reagent has $\varepsilon_{325} = 10500\ M^{-1} \cdot cm^{-1}$. Borohydride reduction abolishes the absorption at 325 nm. (2) Photochemical crosslinking was found much less efficient than chemical crosslinking by reductive amination. (3) This reaction is suppressed by 60 mM Tris or ammonium ions. Lower concentrations of these ions favor specific crosslinks between proteins. (4) The side-reaction of the aromatic aldehyde with protein amino groups during the first step of using reagent (IV) can be reversed by conditions which cleave Schiff bases but not amidines, for instance by exhaustive dialysis against 1 M acetic acid. (5) Some proteins are not stable to these harsh conditions. (6) A limitation in use of this reagent is that monofunctionally-derivatized proteins which are not part of a crosslinked complex are labeled with NaB^3H_4, necessitating two-dimensional SDS-polyacrylamide gel electrophoresis analysis to reveal crosslinked components.

ing experimental evidence for the location of these reagents in membranes and the sites of their reactions with membrane proteins.

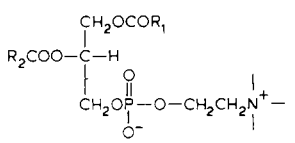
IVA. Determining the location of photoactivatable reagents in membranes: photochemical crosslinking lipids to lipids

Photoreactive lipids make use of the defined structure of a lipid bilayer to direct the reactive groups to either the head-group region or to the hydrocarbon interior. However, in view of the known high mobility and flexibility of lipids, the exact location of the reagents cannot be assumed without additional proof. The reactions of photolabile phospholipids (IXa) and (IXb) with selectively deuterated fatty acid chains in model membranes have been used to define the location of the reagents in membranes [21,22,59]. Subsequently, the sites of reaction with transmembrane regions



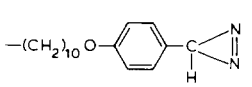
Scheme II. (Reagent V [19].) Notes. (1) Carrier-free ^{125}I was incorporated into the label two steps before the final one in the synthesis. The iodinated product, purified three times by thin-layer chromatography, contained 10% of the ^{125}I used in the reaction. Subsequent steps were diazotization and formation of the *N*-hydroxysulfosuccinimide ester. The high specific activity of the label allows a ligand protein to be labeled at a single site and gives high sensitivity for detection of receptor proteins. (2) This reaction is inhibited by 0.1 M β-alanine. Dithiothreitol is incompatible with the reaction because the azide group is reduced; 2-mercaptoethanol and glutathione may be used because they react more slowly than dithiothreitol. (3) The possibility that introduction of iodine into the arylazide leads to triplet, rather than singlet, nitrene intermediates has been considered, but not examined in detail. (4) Successive additions of dithionite to 0.2 M are necessary for quantitative cleavage, presumably due to side-reactions of dithionite.

of membrane proteins have been determined [22–24].

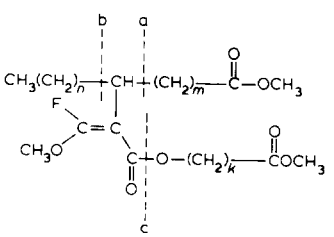


R_1 = palmitoyl or myristoyl

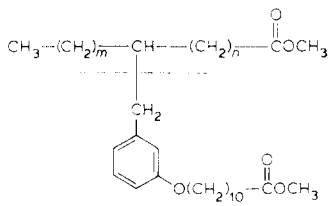
(IXa) R_2 = $-(CH_2)_{11}-OC-C(=N_2)-CF_3$

(IXb) or 

Reaction products of photolysis of lipid bilayers composed solely of (IXa) or (IXb) were determined by (1) in some cases gel filtration to separate monomer and dimer phospholipids, (2) transesterification and (3) mass spectrometric analysis of the resulting diesters [20,21,59]. The structures of two of the diesters analysed by mass spectrometry are shown below.



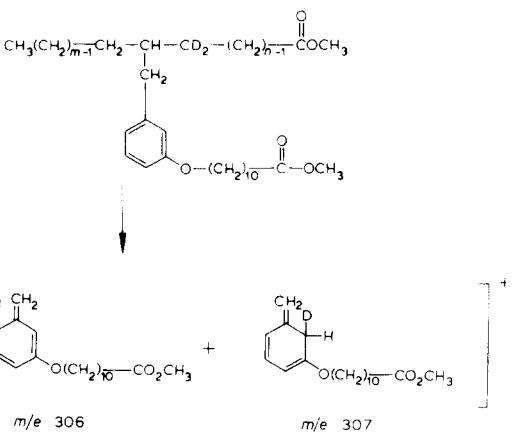
(X)



(XI)

The major sites of fragmentation of (X) and (XI) are indicated by dashed lines. Mass spectra of diesters showed that when the 2-acyl chain of the phospholipid carried the 2-diazo-3,3,3-trifluoropropionoxy group at the $(k + 2)$ carbon atom, the 1-acyl chains were labeled from carbon atoms 6 to the methyl end of the chain. The maximum in the distribution of reaction sites in the 1-acyl chain was at the position two carbons closer to the terminal methyl than the position occupied by the photoreactive group in the 2-acyl chain. An exception to this generality occurred when the position of the reactive group on the 2-acyl chain extended past the terminal methyl of the 1-acyl chain. These results are consistent with other evidence for a conformation of phospholipids in bilayers that has the 1-acyl chain extending more deeply into the bilayer than the 2-acyl chain by 2–4 carbon atoms [20]. In addition, they establish that the sites of reaction of these reagents in a bilayer are predictable from the structure.

The mass spectrum analysis of products formed between 2-acyl chains containing the photosensitive ω-[*m*-(3*H*-diazirino)phenoxyl group and fatty acid chains deuterated at specific carbon atoms makes use of a rearrangement which results in specific transfer of hydrogen (or deuterium) from one chain to the other. The mass spectral fragmentation of the crosslinked product gave cleavage at the benzylic site followed by γ-hydrogen abstraction from the carbon at the crosslink site. As a result, the benzylic ion in the mass spectrum was at *m/e* 307 for those crosslinked products in which deuterium was adjacent to the crosslink site and at *m/e* 306 when attachment was at another site [21], as illustrated below.



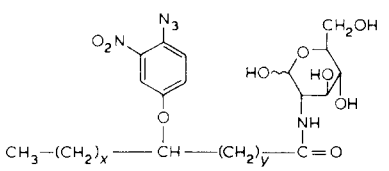
m/e 306 *m/e* 307

The results of the analysis showed that reagent (X) reacted at atoms C_7-C_{12} with the degree of labeling increasing to C_{12}. 1-Acyl chains with deuterium at C_{13} to $C_{terminal}$ were not studied.

IVB. Photochemical crosslinking of lipids to membrane proteins

Analysis of the products of crosslinking photoreactive lipids to proteins is facilitated by the fact that the products generally are stable to the conditions of protein sequencing. However, nonspecific C-H insertion is not the only mode of reaction of carbenes. Nucleophilic amino acids are preferred sites of reaction and base labile crosslinks are formed by reactions at carboxyl and peptide carbonyls [60].

Several methods are available for introducing photoreactive phospholipids into membranes. Biosynthetic incorporation of photoreactive fatty acids into a fatty acid auxotroph of *Escherichia coli* to the extent of 21–43% of the fatty acid content has been demonstrated [61,62]. Membranes from cells grown on the photoreactive fatty acids and [^{32}P]phosphate were photolysed. Two-dimensional isoelectric focusing gels of the photolysis products showed that a number of proteins were labeled. Other approaches used to introduce the photoreactive lipids are reconstitution and exchange with the phospholipid exchange protein [23,24]. Another way of introducing a photoreactive lipid is available when the lipid is fairly water soluble, as in the case of the nitrene-generating glycolipid (XII) [55,63]. Labels of this general structure



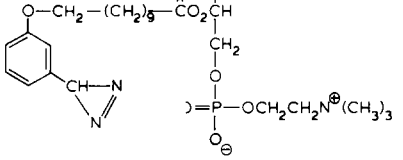
(XII)

distribute between both sides of the lipid bilayer in red blood cells [138], in spite of the polarity of the glucosamine group.

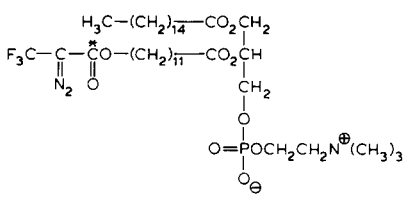
IVB-1. Cytochrome b_5

Reconstitution of membranes containing cytochrome b_5 leads to different conformations of the protein, depending on the details of the reconstitution procedure. Loosely and tightly bound forms of cytochrome b_5 can be prepared. The carboxy terminus is accessible to carboxy peptidase in the former, but not the latter, preparation [64]. In both, the amino-terminal, catalytic, portion of the protein is located in the external aqueous phase. Conceptual models for conformations of the protein in the two forms include varying degrees of penetration of the protein into the bilayer. These models have been tested by crosslinking with lipids bearing photoactivatable precursors of carbenes [23,24].

The following radioactive and photoactive phospholipids were incorporated on both [23], or only one [24], of the sides of reconstituted cytochrome b_5 membranes. The asymmetric distributions were achieved by using phosphatidyl transfer, or exchange, protein.



(IXb*)



(IXa*)

(* indicates position of [^{3}H] or [^{14}C] label)

When lipids IXa* and IXb* were randomly distributed on both sides of membranes which also contained dipalmitoylphosphatidylcholine and cytochrome b_5 in the loosely bound form, photolysis resulted in incorporation of 0.23 mol of IXb* per mol of protein or an only slightly higher amount of IXa*. Since IXa photolyses more slowly than IXb, the longer irradiation times led to some protein photopolymerization which could be minimized by using aqueous tryptophan as a photoprotectant. The distribution of labeling sites was tested first by determining the amount of radioactivity in proteolysis fragments and, second, by subjecting appropriate fragments to Edman sequencing. Trypsin digestion showed that at least 85% of the protein bound radioactivity was located in the hydrophobic segment from residues Leu-91 to the carboxy-terminus Asp-133 of cytochrome b_5. The sequence analysis showed a broad distribution of label from Ser-104 to Met-130, a highly hydrophobic region. Similar results were obtained when label (IXb*) was used to label the tightly bound form of cytochrome b_5 [24].

To obtain evidence for the transmembrane orientation for the tightly bound form, membranes were labeled asymmetrically with ^{14}C (outside) and ^{3}H (inside) derivatives of X. The ratio of $^{14}C/^3H$ in proteolysis fragments was thus a measure of the location of that segment in the membrane. The ratio decreased as the location of the proteolysis fragment approached the carboxy terminus, consistent with a transmembrane orientation for this form [24].

One further experiment was done [24] with tightly bound cytochrome b_5 in vesicles containing unlabeled phosphatidic acid (PA). In this case, the extent of labeling was 32% that of experiments with phosphatidylcholine vesicles and the label was excluded from segment 113–133. Since the cytochrome b_5 hydrophobic segments ($\approx$ 97–126) end with arginine only on the carboxy-terminal site, this result suggests that an arginine-PA complex could partially shield the hydrophobic domain from reaction with the carbene lipid.

IVB-2. Glycophorin A

The photoreactive and radioactive phospholipids IXa* and IXb* were allowed to react with erythrocyte glycophorin in reconstituted and native membranes. The primary site of reaction was Glu-70, which is at the beginning of the 23-

amino-acid hydrophobic segment previously determined to extend across the membrane [22].

IVB-3. Phospholipid exchange protein

As a side-line in studies of membrane proteins, photoreactive phosphatidylcholine (IXb) was photolysed while bound to phospholipid exchange protein [65]. In this affinity labeling experiment, the hexapeptide from Val-171 to Phe-176 was labeled specifically.

IVB-4. Alamethicin

The radioactive and photoreactive phospholipid (X*) has been used to label the hydrophobic domain of the linear eicosapeptide alamethicin [66]. The photoproduct forms membrane channels which are similar to those for the unlabeled peptide in most respects. Chromatography of hydrolysis products from the photoproduct showed that the radioactivity was located exclusively in the portion of the peptide from amino acids 2- to 13-, consistent with a selected portion of alamethicin being embedded in the bilayer.

IVB-5. M13 coat protein

A photoreactive glycolipid (XII) reacted with the hydrophobic domain of coliphage M13 coat protein in dimyristoylphosphatidylcholine vesicles in preference to lipids [55]. This result suggested that the reactive species may be an electrophilic intermediate, for instance an azacycloheptatetraene. In addition, it was shown that virtually no reagent reacted with the N-terminal, hydrophilic octapeptide, thus establishing the specificity of the probe for the hydrophobic domain of transmembrane proteins.

V. Biochemical crosslinking

VA. Transglutaminase

Calcium-dependent transglutaminases (endo-γ-glutamine: ε-lysine transferases) have been found in a number of cells [25]. In erythrocytes, the protein can be activated by adding millimolar calcium, together with calcium ionophore, to the outside of the cells [67]. Substantial crosslinking of cytoskeleton and membrane proteins are observed under these conditions. Since no procedure is available for cleaving the crosslinks, a method other than two-dimensional gels is necessary to determine the individual components of the crosslinked product. This is provided by the fact that histamine, and other monofunctional amines can prevent the crosslinking by substituting for lysine side-chains [72]. At lower levels of ^{14}C histamine, some crosslinking also occurs, but radioactivity is incorporated into monomers of band 3 and spectrin. Given the preponderance of these proteins in erythrocyte membranes, it is not clear whether they are more heavily labeled on a molar basis than are some of the minor bands, but they clearly are substrates for transglutaminase.

Factor XIII in platelet cytosol is also a transglutaminase and has been used as well for calcium-induced crosslinking of platelet proteins [73]. In addition, isolated transglutaminase has been used as a reagent to study the assembly of complement on membranes [26].

A possible solution to the problem of determining components of the transglutaminase-crosslinked complex would be to use cleavable diamines as, for example, the following:

$H_2NCH_2CH_2SSCH_2CH_2NH_2$

VB. γ-Diketone neuropathy

Exposure of humans to the solvent, hexane, results in axonal swellings similar to those seen after exposure to acrylamide and carbon disulfide. It has been proposed that the effect of hexane results from the metabolic product, 2,5-hexanedione. This diketone can react with a lysine amino group and then cyclize to a pyrrole. Subsequently, pyrrole oxidation leads to crosslinking of proteins. The diketone, 3,4-dimethyl-2,5-hexanedione, is an even more potent neurotoxicant and protein crosslinker [74].

VI. Applications

VIA. Nearest-neighbor analysis in cell membranes

One type of experiment designed to examine the role of proteins in cell membranes is 'nearest-neighbor analysis' [39]. Here, crosslinking reagents are used to determine which proteins are in close

proximity to each other. A large fraction of the new crosslinking reagents prepared in the last decade have been designed to improve the membrane nearest-neighbor analysis and red blood cell membranes have been the testing ground for many of these crosslinkers. The subject has been reviewed [3,5,6] and thus will be presented only briefly here.

Two of the major problems in analyzing the neighbors in cell membranes are (1) the multitude of proteins and (2) the need to distinguish random interactions due to diffusion from real macromolecular complexes [76]. The first problem is best resolved by using cleavable reagents and running two-dimensional acrylamide gels with the cleaving agent present only in the second dimension [39]. An example of a two-dimensional gel of red blood cell membranes is shown in Fig. 6. In this case, membranes were crosslinked with the spin-label, disulfide exchange reagent III [30]. Proteins on the diagonal were monomers in each dimension. Proteins having faster relative mobility in the second dimension than the first were oligomers during electrophoresis in the first dimension. On this gel a prominent off-diagonal, band-3 component is apparent at a location corresponding to a dimer of band-3. (In the nomenclature of Steck, band 3 is a major erythrocyte transmembrane protein and is an anion channel.) The other apparent crosslinked products result from a very high-molecular-weight complex in which bands 1 and 2 (spectrin) and numerous lower-molecular-weight species are found. In the experiment shown, the presence of crosslinking reagent in the band 3 dimer was confirmed using an ^{35}S-labeled crosslink [30].

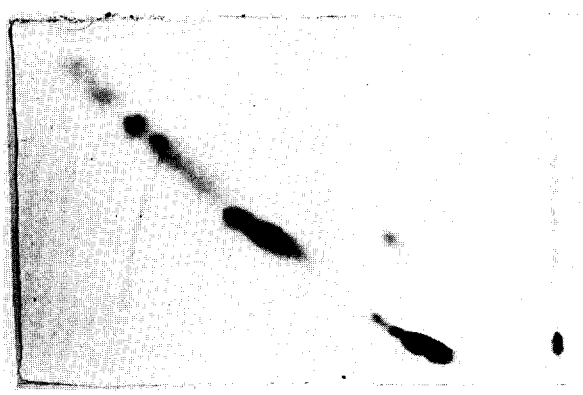


Fig. 6. A two-dimensional SDS-polyacrylamide gel is shown for a sample of red blood cells crosslinked with reagent III. Electrophoresis was performed with 3.2% acrylamide gels in the first dimension. A slab gel with a 2 cm agarose layer containing 10% β-mercaptoethanol on top of a resolving gel of 5.6% acrylamide formed the second dimension. (Willingham, G.L., Thesis, Johns Hopkins University, 1982).

Secondly, because of the very high effective concentration of proteins in membranes, the possibility of crosslinks during random collisions is a serious one. For soluble proteins, the approach would be to study crosslinking as a function of protein concentration [75]. For proteins constrained to a native membrane, the available variables are ones that increase the rate of crosslinking relative to that of diffusion. Photochemical crosslinkers give significant yields of products with red cells after photolysis for less than 1 ms at room temperature and dimers of band 3 and other proteins are seen under these conditions [76].

There is a third problem, the importance of which is becoming apparent in crosslinking studies with intact cells. Transglutaminase is stimulated by elevation of Ca^{2+} to crosslink cytoskeletal and membrane proteins in a number of cells as noted in the previous section [67–72]. In human red cells, transglutaminase activation gives a polymer apparently composed largely of band 3 and spectrin – similar to results in many experiments with chemical crosslinking. In platelets, complexes involving glycoprotein complexes are observed by both chemical crosslinking and induction of transglutaminase activity by calcium [74,77]. Clearly it is important to employ controls for enzymatic crosslinking when using chemical crosslinking reagents. The formation of transglutaminase crosslinks can be inhibited by iodoacetamide [26] or by the presence of monofunctional amines such as histamine [73]. Of course, use of histamine is only a good control when the chemical crosslinking reagents employed are not specific for amino groups.

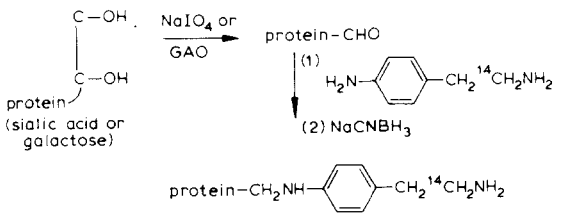
Photoinduced crosslinking is also to be considered when procedures involving photolysis are being used. Erythrocyte ghosts labeled with fluorescein were found to undergo extensive crosslinking in the presence of oxygen [78]. Cysteamine and reduced glutathione were found to inhibit this effect that is probably a result of formation of singlet oxygen. Photosensitized crosslinking has

also been observed in unlabeled red-cell ghosts [79].

VIA-1. Red blood cells

The questions that have been a major focus of red-cell crosslinking involve formation of band 3 dimers and possible association of glycophorin with other proteins. In general, as shown in the SDS-polyacrylamide gel electrophoresis gel of Fig. 6, band 3 dimers are accompanied by high-molecular-weight bands which include spectrin. In these cases, the reagent distributes across the membrane and reacts at both sides. To rule out involvement of the cytoskeleton in promoting band 3 crosslinks, reagents to which membranes are impermeant have been developed [47,51,52,80], and band 3 crosslinking is still observed.

In a different approach, the crosslinking potential of red cell glycoproteins is increased by adding spacer arms with amino groups to the carbohydrate residues [81]. The treatment should give elevated levels of band 3 or glycophorin crosslinking if these proteins are associated in the membrane. The chemistry of amino-group supplementation is outlined below.



Glycophorins and band 3 were amino-supplemented separately via aldehydes generated by oxidation of carbohydrates with periodate and galactose oxidase, respectively. (In control samples, aniline was coupled to oxidized carbohydrates). Crosslinking was performed with di-*N*-hydroxysuccinimide esters. Although amino group supplementation enhanced crosslinking of glycoproteins in isolated membranes, intact cells gave less than 2% crosslinking, even with amino group supplementation. This experiment leaves open the possibility that band 3 molecules are not associated as dimers on the outside of intact, metabolically functional red cells. The possible association of glycophorin A with band 3 was also not observed in crosslinking after amino group supplementation [81]. Other evidence for this association has been obtained in several ways including, recently, measurements of protein mobility in red-cell ghosts [82]. The rotational mobility of band 3 was slowed when glycophorin A was selectively crosslinked with antibodies, but anti-glycophorin A-Fab binding left band 3 mobility unaltered.

VIA-2. Platelet membranes

When platelet membranes are crosslinked [75] with reagents which can pass through the membrane (diamide [49]; dithiobissuccinimidyl propionate (DTSP) [40]), two-dimensional gel analysis shows homopolymers of cytoskeletal proteins and high-molecular-weight proteins of mixed composition. Complexes of glycoproteins IIb and III were also found with DTSP. Thrombin activation of platelets gives increased amounts of IIb, III and myosin in the high-molecular-weight material. Membrane-impermeable crosslinkers have been used to examine platelet adhesion to collagen [83]. Here, glycoproteins IIb and IIIa were crosslinked to collagen. Calcium-dependent crosslinking of platelets has also been studied. Platelet factor XIII is a transglutaminase in the cytosol. Since calcium-dependent crosslinking is prevented by histamine, transglutaminase action is implicated in this example of crosslinking [74]. Using photoactivatable glycolipids, (XII), three new platelet membrane proteins, probably intrinsic, were found (60, 50 and 45 kDa) [139].

VIA-3. Other cell membranes

Applications of crosslinking to proteins in the membranes of other whole cells have begun to appear. Some of these are covered in earlier reviews [5,6]. Recent reports include crosslinking of bacterial outer membrane porins [84], of outer membrane proteins in *Neisseria gonorrhoeae* [85] and of proteins in isolated cilia of *Tetrahymena* and *Aequipecten* [86].

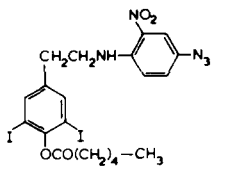
VIB. Complexes and oligomers of isolated membrane proteins

VIB-1. Complement

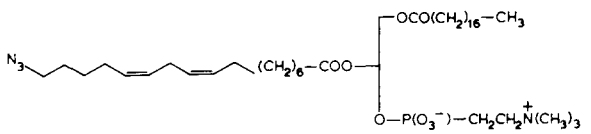
Membrane lysis by complement [87,88] involves complement components C5b, C6, C7, C8 and C9.

Assembly of a tight complex of these components is initiated by the enzymatic cleavage of C5 to C5b. Subsequent steps leading to membrane lysis are non-enzymatic. If components C5b to C9 are assembled in the presence of a biological or model membrane, a complex (C5b-9) that can lyse membranes is formed. The complex can be assembled stepwise starting with C5b6 (a stable complex of C5b and C6). The complete complex can be extracted from membranes with deoxycholate or Triton X-100 and the extracted complex has a stoichiometry [C5b, 6, 7, 8, 9_n]. The geometry of components within this complex and the mechanism by which membranes are lysed are subjects that have been addressed in experiments using crosslinking reagents as well as other approaches.

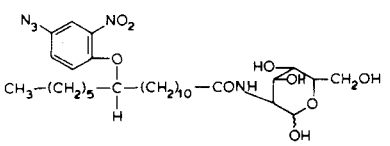
VIB-1a. Which complement components insert into lipids? Three different photoactivatable molecules XIII–XV have been employed in seeking answers to this question [89–92].



XIII



XIV



XV

The results with the three labels are in agreement that C9 labeling predominates when the complete C5b-9 membrane-bound complex is subjected to photolysis in the presence of the probes. Labeling by the photoactive glycolipid, XV, could be achieved in 2 min of irradiation by the 366 nm emission of mercury [91,92]. It was therefore possible to detect a change in labeling patterns of the C5b-C9 membrane-bound complex over a period of 30 min. 30 min after adding C9 to C5b-C8 complex, virtually only C9 was labeled by the probe. The site of labeling was further localized to the larger C9b thrombin cleavage fragment of C9. C9a was unlabeled.

Labeling of other components of these complexes varied in absolute and relative amounts for the three labels. With the C5b-8 complex, label XIII was found predominantly on C8 α-γ and β fragments [89] and label XV was found predominantly on C5b with substantial amounts labeling C6, C7 and C8 also [90–92]. Relative amount of label on C5b was the most variable result in comparing reactions of reagents XIII–XV with the C5b-8 complex.

There are several possible reasons for the different results with these three labels that have an affinity for the hydrophobic regions of membranes. Probably the most significant difference is that labels XIV and XV were used with complement components assembled on phospholipid model membranes while XIII was applied to complexes formed during lysis of erythrocytes. It is possible that some of the erythrocyte proteins, or lipids, protected C5b from labeling by XIII. In addition, the locations and reactivities of the three labels may differ somewhat. Nevertheless, the combined results support the concept that C8 and C9 are the predominant proteins inserted in the membrane during complement-mediated lysis.

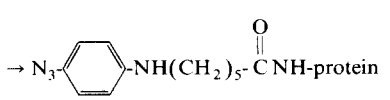
VIB-1b. Do complement components penetrate to the cytoplasmic side of the membrane? The answer to this question is required to distinguish models for the mechanism of membrane lysis by (C5b-9). Enzymatic crosslinking by transglutaminase trapped inside antibody-sensitized erythrocyte ghosts carrying complement components C1-7 (GAC1-7) was employed to detect penetration by C8 and/or C9 when these components were added to GAC1-7 [26]. Iodinated derivatives of purified C8 and C9 were used and the extent of crosslinking was taken as the amount of ^{125}I-labeled component not entering the gel. C8 and C9 were inserted into ghosts containing trapped transglutaminase by 30 min incubation in Hepes/

EDTA/ATP buffer of GAC1-7 with C8 and by 10 min incubation of GAC1-8 with C9. Crosslinking was initiated by addition of calcium. Crosslinking reached a maximum within 15 min, an effect which was shown to result from the rapid inactivation of the majority of the transglutaminase with a half-time of 3.9 min. A dose-response curve showed that there is a direct relation between the extent of C9 crosslinking and the amount of trapped transglutaminase for enzyme levels of $(5–32) \cdot 10^4$ units/10^9 ghosts. At the higher amount of enzyme, 11% of C9 was crosslinked within the time that the enzyme was stable. With addition of C8 to GAC1-7, a statistically significant, but low level (1%) of C8 crosslinking was observed with $28.8 \cdot 10^4$ units/10^9 ghosts.

Important controls in these experiments established that crosslinking was enzyme-catalyzed (it is inhibited by iodoacetamide) and that it did not result from transglutaminase that had leaked to the outside of the membrane. The latter point was made by examining crosslinking from without of C8 or C9 in membrane complexes with dimethylcasein added to the external medium. The presence of dimethylcasein completely inhibited external crosslinking of C8 or C9. Since C9 and C8 crosslinking by internal transglutaminase is also observed when dimethylcasein is present externally, evidence is provided that certainly C9, and probably C8, penetrates to the cytoplasmic side of the ghost membranes.

VIB-1c. Nearest-neighbor analysis of complement (C5b-9) components. The association of complement proteins C8 and C9 with the membrane-bound trimeric complex C5b-7 was examined by attaching a photoreactive group to C8 or C9 [93]. The reagent used was *N*-succinimidyl-6-(4′-azido-2′-nitrophenylamino)hexanoate which reacts chemically with protein amino groups to give the derivative shown below.

N-hydroxysuccinimide ester + H_2N-protein



The derivatized protein is thus rendered photoreactive. Separate derivatives of C8 and C9 were prepared and these contained 6–8 and 3–4 mol of photoreactive groups/protein, respectively. Further, because C8 can be separated into α-γ and β subunits, derivatives bearing the photoreactive group on either subunit could be prepared also. The photoreactive derivatives of C8 and monomeric C9 retained essentially all of the ability of their unlabeled counterparts to bind to the erythrocyte bound complexes of the preceding proteins, although derivatized C9 retained only 10% of its hemolytic activity. The results of photolysis of C5b-9 complexes in which one component was photoreactive were analysed on SDS-polyacrylamide gel electrophoresis. On photolysis of complexes in which C8 was labeled, approx. 30% of C8 was crosslinked to macromolecules, while 45% of C9 formed high-molecular-weight complexes when the complexes contained derivatized C9. However, using a combination of ^{125}I and photoreactive derivatives, it was shown that no crosslinks between C8 and C9 were formed, regardless of which subunit of C8 was photoreactive or whether the photoreactive label was on C8 or C9. In addition, using C8 label separately on the (α-γ) or (β) subunit, no inter-subunit crosslinks were found. These results suggest that C8 and C9 are not closely associated in the assembled membrane bound C5b-9 complex, although a transient interaction of C8 and C9 during formation of (C5b-9) is still possible.

VIB-2. Acetylcholine receptor (AChR)

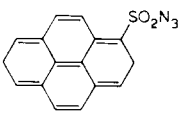
The nicotinic acetylcholine receptor [94] is a pentamer with subunit composition $\alpha_2\beta\gamma\delta$. The receptor from two sources, *Torpedo californica* and *Torpedo marmorata* electric organs, is used for biochemical and biophysical studies. There are extensive regions of homology in the amino acid sequences of the subunits [95] and each subunit is thought to traverse a lipid membrane four to six times and to contribute to the structure of the central ion channel [96]. In electron micrographs, receptors appear as rosettes with a central pit. Crosslinking has been employed to examine the arrangement of subunits around the central channel [27,28,94] and hydrophobic labeling has been applied to identify regions of the receptor in the membrane bilayer [13,97–99]. The numerous examples of specific affinity labeling of AChR are outside the scope of this review except to mention

that all four subunits of *T. marmorata* are reactive in time-resolved photolabeling with chlorpromazine during a time (less than 1 s) when the channel remains open [100]. Selective labeling by affinity reagents of some subunits relative to others has been employed often to reveal conformational differences between the resting, sensitized and desensitized states of the receptor.

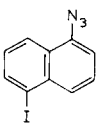
VIB-2a. What is the arrangement of subunits in AChR? Karlin and co-workers [27,28] have examined the geometry of subunits in dimers of the AChR pentamer. The native receptor is a dimer with a single disulfide bond between δ-chains. This dimer can be reduced to monomers and a different dimer is formed when the monomers are oxidized by diamide. The artificial dimer is β-β linked. The α-subunits can be visualized in electron micrographs of the receptor by applying succinylated avidin to AChR to which is bound biotinylated toxin (specific for α-chains). The angle between two α-subunits on the same monomer is found to be $114 \pm 29°$ in β-β dimers and $109 \pm 28°$ in δ-δ dimers. These results, as well as the location of minima in electron density [94], suggest that the α-subunits are not contiguous. The positions of α-subunits relative to the crosslinked subunits in the dimers was used to determine the location of the other subunits. In both of the dimers, α-subunits are found both close to the contact between monomers and opposite the contact, suggesting that neither β nor δ subunit lies between the two α's. The γ-subunit is thus favored as the one that lies between the α-subunits, although chemical crosslinking with disuccinimidyl tartrate (Fig. 6 in Ref. 94) shows that regions of the γ- and δ-subunits are within 6 Å of each other.

VIB2-b. What regions of AChR insert into lipids? The homology of AChR subunits includes the hydrophobic regions which are presumed to span the membrane. Thus, it is possible that each subunit has similar exposure to lipid. Indeed, the carbene generated photochemically from adamantanediazirine VIII does label all four subunits [99]. The α-subunits were labeled three times more heavily than the β-, γ- and δ-subunits. It was further shown that adamantanediazirine labels AChR nonspecifically by carrying out photolysis first with non-radioactive label at high concentration and second with ^3H label at lower concentration. Covalent incorporation of ^3H label was unchanged.

Several other reagents, which yield nitrenes on photolysis, have also been applied to AChR. The reactions were more subunit-selective than the reaction of adamantyldiazirine. Pyrenesulfonylazide (XVI) reacted with the β- and γ-subunits predominantly when receptor-enriched membranes were labeled, while the α- and β-subunits were the primary sites of reaction when AChR was solubilized with Triton X-100 [97]. Iodonaphthyl-1-azide (XVII) photolysis yields primarily labeled α-subunit [98]. A 13 kDa fragment from receptor labeled via iodonaphthylazide has been isolated and it bears the iodonaphthyl group [98].



XVI

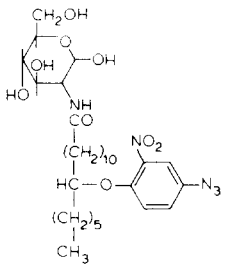


XVII

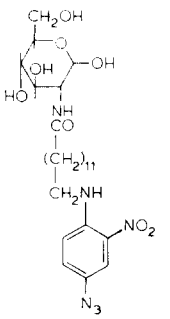
VIB-3. Cholera, diphtheria and ricin toxins

Several protein toxins have peptides (A subunits) that interact enzymatically to kill cells, but to do so, they must penetrate the cell membrane. The toxins also have cell-surface recognition components (B subunits). The mechanisms by which the toxins insert an A peptide into the membrane have been investigated by several groups using photoreactive lipids of several structures [101–105]: The subunit compositions of the toxins studied are: cholera toxin ($\alpha\beta_5\gamma$ or A_1-A_2B_5) diphtheria toxin (AB) and ricin toxin (AB).

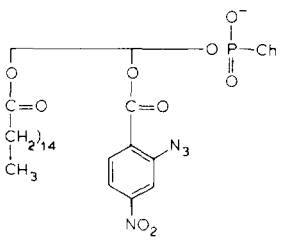
The receptor for the cholera toxin B-subunit is the ganglioside, G_{M1}. Intact toxin bound to artificial membranes (phosphatidylcholine-cholesterol-G_{M1}) is labeled in the β- and γ- (or B and A_2) subunits by the lipid with a photoreactive moiety near the head group (XX) [104]. The intact toxin, in the artificial membrane system, is not labeled by the lipids with photoreactive groups in the hydrophobic region of membranes (XIX and XXI). This result contrasts with studies of cholera toxin bound to membranes of Newcastle disease virus (NDV) [101,102]. In this case, photoreactive glycolipid (XVIII) labeled the A subunits in a time-dependent manner. Reduction of the disulfide link between A_1- and A_2- (or α- and γ-) subunits is



XVIII (101–103)

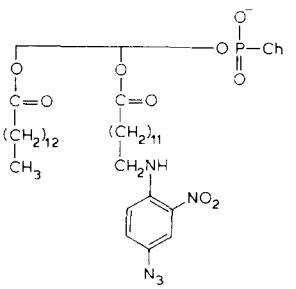


XIX (104)



Ch = choline

XX (104)



XXI (104)

apparently necessary for penetration of the A subunit into lipid because, under reducing conditions, the hydrophobic photolabels (XIX and XXI) do react with the A (α) subunit [104]. These labels, and (XVIII), are unreactive with the B (β) subunits under either reducing or non-reducing conditions.

Using an intense light source, cholera toxin bound to NDV could be labeled in 15 s, allowing a study of the time-course of insertion when the toxin is newly added to the virus [102]. The reason for using the lipid enveloped virus as a target membrane is that the quantitative aspects of photolabeling can be expressed as the ratio of label in toxin subunits to viral proteins. The ratio of labeling of toxin A subunit to labeling of viral proteins increased with up to 1 min of irradiation and then decreased to about 50% of the maximum ratio of labeling. This result was interpreted to mean that the A_1 subunit enters the membrane within 1 min and some rearrangement follows.

Quasielastic light-scattering (QELS) measurements of native cholera toxin and dimethyl-3,3′-dithiobis(propionimidate)-crosslinked toxin complexed to lipid vesicles indicate that a major subunit rearrangement is not necessary for A_1 to penetrate the lipid bilayers [106]. This conclusion was based on the observation that the amount by which the radius of the vesicles increased when toxin covered much of the surface was the same, regardless of whether the subunits were crosslinked or not before addition to vesicles. Rearrangements of the portion embedded in the membrane, as suggested by experiments with the photoreactive glycolipid, would probably not be detected by the QELS measurement.

Hydrophobic photolabeling of diphtheria toxin was performed to test a model (Kagan et al. 1981) which proposes that the B fragment forms a membrane channel through which the active A fragment passes on entering a cell [105]. The results of photolabeling showed that both subunits were accessible to a glycolipid probe in the membrane, thus demonstrating that, although a pore is formed, the pore does not have a structure in which the A subunit is protected from lipid by the B subunit.

Experiments similar to those in which the photoreactive glycolipid was used to label diphtheria and cholera toxins were carried out with ricin toxin [103]. Both A and B chains were labeled in whole toxin or separated chains. The time-dependence of appearance of labeled chains was much slower than that of cholera toxin – labeling of ricin subunits increased more than 2-fold between 15 and 30 min of interaction with membranes. Labeling of whole toxin or B-subunit was receptor-dependent as demonstrated by inhibition of the process by galactose in the medium.

VIB-4. Lipid-enveloped viruses

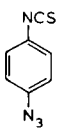
A direct method of determining which protein components of a lipid-enveloped virus are either on or in the lipid membrane is to use amino-specific crosslinkers to crosslink the lipid phosphatidylethanolamine to accessible proteins [107]. The viral phosphatidylethanolamine can be rendered radioactive by growing the virus in cells which have been allowed to incorporate [^{14}C]ethanolamine into cellular lipids. Location of viral proteins which have been crosslinked to lipid is made by autoradiography of gels. Membrane-associated proteins identified in this way include VSV-G and M (but not N and L) and proteins P19 and P15 of avian and murine leukemia viruses, respectively

[107]. Similar results were obtained in crosslinking [^{3}H]palmitate-labeled lipids to VSV-G and M proteins [108].

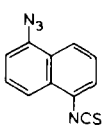
Crosslinking of protein to protein is also a means of determining which viral proteins are proximal to the membrane if one species has previously been identified as a membrane protein. In VSV, 5-[^{125}I]iodonaphthyl-1-azide labels the G protein, identifying it as the membrane protein. Cleavable, amino-specific crosslinking and two-dimensional SDS gels then identify M as a protein neighbor of G [109]. Mutants in the M protein have altered interaction with the membrane in these reactions [110].

VIB-5. Bacteriorhodopsin

The amino groups of lysines embedded in a lipid bilayer are probably unprotonated unless they are shielded from lipids. Thus, a hydrophobic, amino-directed reagent should label these groups with high specificity. Two reagents, (XXII) and (XXIII) fitting this description have been applied to bacteriorhodopsin [111,112].



XXII



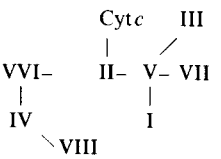
XXIII

The phenyl-derivative undergoes reaction of the isothiocyanate moiety with amino groups more quickly than does the naphthyl derivative. In contrast, photolysis of (XXIII) proceeds more rapidly than of (XXII). These reagents are specific for lysine-41 in bacteriorhodopsin [113]. Photolysis yielded dimers, trimers and high-molecular-weight complexes of the protein, depending on the concentration of crosslinker used [53].

VIB-6. Mitochondrial membrane protein complexes

Mitochondrial ubiquinone cytochrome c reductase complex (complex III). Complex III of inner mitochondrial membrane is involved in electron transport. Two disulfide crosslinking reagents have been used to examine nearest-neighbor interactions in the complex [114]. Complex III components are: core protein I (50 kDa), core protein II (46), cytochrome *b* (31.5), cytochrome *c* (29), non-heme iron protein (25), protein VI (14), protein VII (12.5) and antimycin binding protein (9). The crosslinking reagents used were dithiobissuccinimidylpropionate and dimethyl-3′-dithiobispropionimidate. The longer spans about 11 Å. Although both reagents reacted similarly, it is notable that the former produced a high yield of 310 kDa product representing the entire complex. This demonstrates that crosslinks are intracomplex. At lower levels of crosslinking, dimers were produced of I + II, II + VI, I + non-heme iron protein and VI + VII. Trimers of I and II with non-heme iron protein or protein VI were also seen.

Cytochrome c oxidase. The multisubunit protein, cytochrome *c* oxidase [115], has been crosslinked with several cleavable reagents and derivatized cytochrome *c* has been photocrosslinked to its apparent binding site on the complex [116,117]. A scheme which depicts the nearest neighbors determined by crosslinking, and consistent with physical measurements, is shown below [117].

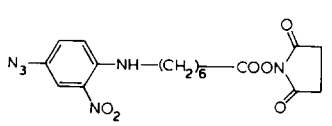


Photolabeling of cytochrome oxidase subunits in contact with lipids has also been examined [118,119].

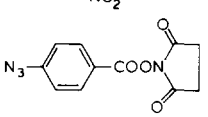
VIC. Identification of cell-surface receptors

VIC-1. Antibodies directed against the crosslinking reagents

When receptor concentration is low in a membrane, immunoprecipitation is usually employed to isolate receptor-ligand, crosslinked complexes. A method [29] to generalize this approach employs antibodies directed against the aromatic groups in the crosslinking reagents (XXIV) and (XXV). This approach avoids the necessity of making antibodies to each separate macromolecular ligand.



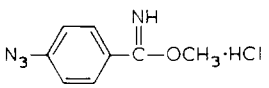
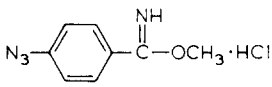
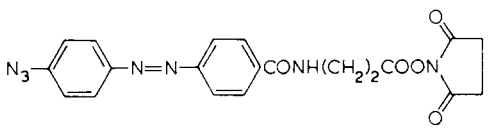
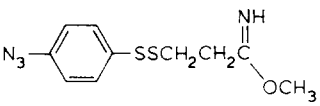
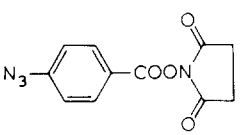
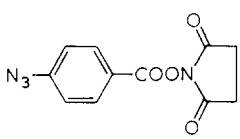
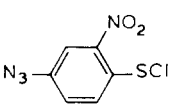
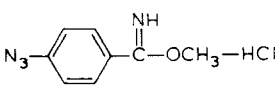
(XXIV)



(XXV)

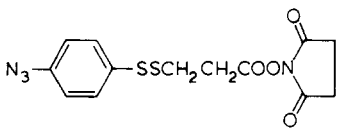
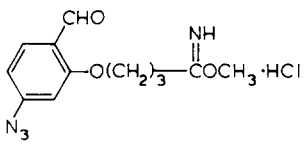
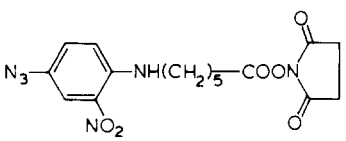
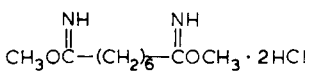
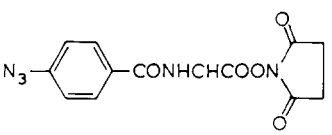
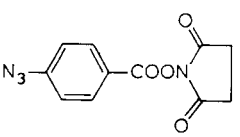
TABLE VI

LIGAND RECEPTOR CROSSLINKS

Ligand	Crosslinking reagent	Molecular mass (receptor) (RDa)	Ref.
Concanavalin A		—	121,29
Calmodulin		~150 (Ca-Mg ATPase)	124
Peanut agglutinin		46.58	125
Epidermal growth factor		184	126
Insulin		130	127
Prolactin		36	128
Glucagon		—	129
Glucagon		—	136

(continued)

TABLE VI (continued)

Ligand	Crosslinking reagent	Molecular moss (receptor)(RDa)	Ref.
Sendai virus		17	130
Sindbis virus		90	17
N—Formyl peptide		50 + 60	131
Cholecystokinin		80	132
Human choriogonadotropin		multiple bands	133
Nerve growth factor		87	134,135

The reagents were coupled to human γ-globulin to raise antibodies. The approach was tested on concanavalin A-erythrocyte receptor complexes and proteins corresponding to the major erythrocyte glycoproteins were found in the immunoprecipitate. The authors suggest that the cleavable crosslinker (V) [18] could be employed as an improvement of this procedure.

VIC-2. Sindbis virus

The heteromultifunctional reagent (IV) [16] was used in two different ways to identify the cell surface receptor for Sindbis virus [17] (see Scheme I, p. 300). In one approach, the imidoester end of the reagent was coupled to ^{125}I-labeled Sindbis virus. Subsequently, the modified virus was allowed to attach to receptors on JY and Daudi cells at pH 7.2 for 90 min and to form the Schiff's base between the reagent and receptor. Crosslinking was induced by addition of sodium cyanoborohydride. Virus in the absence of cells served as a control in these steps. Only when cells were present was a 140 kDa band observed on gels. The likely origin of this band is a dimer of virus-50

kDa and cell-90 kDa proteins. In the second approach, nonradioactive virus was modified by the imidate and then crosslinked to cells using NaB^3H_4. The results were analysed using two-dimensional SDS-acrylamide gels. The amidate crosslinks were reversed by ammonolysis for the second dimension of the gel and a tritiated protein of 90 kDa was revealed off the diagonal, confirming the result obtained with ^{125}I-labeled virus. Possible involvement of HLA antigens [120] as Sindbis receptors was ruled out because the 90 kDa receptor was found on Daudi cells which are HLA and HLB negative [17].

One limitation of the second approach, above, is that monomeric proteins (presumably of the virus) are heavily labeled by NaB^3H_4 as well as the crosslinked ones – thus labeled receptor is a minor fraction of labeled proteins present. Reagents with much higher specific activity such as the label (V) that transfers ^{125}I are designed to mitigate this type of problem: they can be used at the level of one reagent molecule per protein [19].

VIC-3. Other receptors

There are a number of studies in which a cell surface receptor is identified by an approach similar to the one discussed above for ^{125}I-labeled Sindbis virus. That is, the iodinated ligand is coupled to a receptor in a two-step procedure with a heterobifunctional reagent. This employs the ligand as a photoaffinity probe. As long as the receptor-ligand complex has a molecular weight that allows it to enter an SDS-polyacrylamide gel, the results can be analyzed without actual transfer of radioactivity from ligand to receptor [5,121]. Tests for functional activity of derivatized ligand are, of course, necessary. It is not necessary to use cleavable reagents for the analysis, although they are useful for demonstrating that crosslinked products are indeed due to the reagent and that the presumed ligand-receptor complex is a dimer, rather than a higher oligomer. Table VI lists data on ligands which have been coupled to membrane bound receptors using crosslinking reagents. Many of the cleavable reagents listed could be modified so that they could transfer radioactivity to the receptor – for instance, the aryl azide rings could be iodinated, as was done for reagent (V) [19], or ^{35}S could be used in the aryl half of the disulfide-cleavable reagents [40]. However, use of aromatic disulfides is complex because of their ability to catalyze, and take part in, disulfide exchange. There are also immunological, biochemical pharmacological approaches that, for example, have been used to identify receptors for lactate dehydrogenase [122], and rabies viruses [123] – without intervention of crosslinking reagents. The advantage of using a crosslinking reagent is that the effects of nonspecific adsorption during the experimental procedures can be more readily assessed. The ultimate use of the reagents will be to map regions of the receptor involved in contact with ligand.

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