are similar to, if not identical with, those of the native vesicles. These results show that the acetylcholine receptor retains all of the cholinergic characteristics after osmotic shock. The shock procedure is therefore a mild one.

The present studies bear resemblance to studies on red-cell ghost membranes after hemolysis. Red cells are usually hemolyzed with 20–40 volumes of ice-cold buffer of low osmolarity, and resealing of the lysed ghost membranes only occurs upon incubation above 15–20 °C and with the ionic strength reconstituted to >15 mM NaCl (Bjerrum, 1979; Johnson, 1975; Steck, 1974). We have observed a rapid resealing (within 15 s) of acetylcholine receptor rich membranes. In our experiments, osmotic shock was carried out at 20–25 °C, and ionic strengths of the solution never decreased below 100 mM NaCl. One would therefore expect a rapid resealing of the membranes after shocking. Indeed, instantaneous resealing was also observed with red cells by Bodemann & Passow (1972) when hemolysis was carried out at 37 °C.

The results of the present study suggest the possibility of loading molecular probes into the interior of these vesicles. This could open up new opportunities to study the cytoplasmic surfaces of electroplax membranes if used in conjunction with the right side out vesicles described by Hartig & Raftery (1979). We have shown that $^{22}Na^+$ and larger molecules can be readily loaded into the aqueous interior of these vesicles by a simple procedure: exposure of vesicles to hypotonic solutions containing the tracer molecules. This mild procedure does not alter vesicle sideness or acetylcholine-receptor function. Therefore, it could provide a powerful tool not only for studying inner surfaces of these vesicles but also for manipulating transmembrane ion distributions for studies of membrane functions.

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New Cleavable Photoreactive Heterobifunctional Cross-Linking Reagents for Studying Membrane Organization†

Charles L. Jaffe, Halina Lis, and Nathan Sharon*

ABSTRACT: The synthesis is described of four new cleavable, photosensitive, heterobifunctional cross-linking reagents for use in examining membrane organization: 4′-azidoazobenzene-4-oxysuccinimide ester (**1**), *N*-[4-(*p*-azidophenylazo)benzoyl]-3-aminopropyl-*N*′-oxysuccinimide ester (**2**), *N*-[4-(*p*-azidophenylazo)benzoyl]-6-aminohexyl-*N*′-oxysuccinimide ester (**3**), and *N*-[4-(*p*-azidophenylazo)benzoyl]-11-aminoundecyl-*N*′-oxysuccinimide ester (**4**). Two photoaffinity-directed cross-linking agents were prepared by attaching reagents **1** and **2** via their activated ester groups to soybean agglutinin (subunit M_r 30000). Irradiation of the lectin derivatives resulted in a decrease in their absorption spectra at 360 nm due to photolysis of the bound reagents. Cross-linking of soybean agglutinin subunits following irradiation of the soybean agglutinin derivative to which reagent **2** had been coupled was observed by the appearance of new Coomassie blue staining material (60000, 90000, and 120000 daltons) on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. On polyacrylamide gel electrophoresis in the absence of sodium dodecyl sulfate, soybean agglutinin oligomers were observed. Cleavage of the cross-linked soybean agglutinin with 0.1 M sodium dithionite for 25 min at room temperature resulted in the disappearance of the high molecular weight bands and an increase in the amount of uncross-linked material. The use of the photoaffinity-directed agents for probing membrane organization is discussed.

Cross-linking agents are becoming increasingly important tools for understanding membrane structure and organization [reviewed by Peters & Richards (1977), Friedman (1979), and Ji (1979)]. Homobifunctional cross-linking agents, while sufficient for examining the conformation and structure of protein molecules, when applied to cell membranes give, on polyacrylamide gel electrophoresis, complex patterns which

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are difficult to analyze. The introduction of cleavable homobifunctional cross-linking reagents in conjunction with two-dimensional gel electrophoresis has greatly simplified the analysis of the cross-linked membrane products and has stimulated the wide use of these reagents. The available reagents suffer, however, from several serious drawbacks; among these are long reaction times, random collisional cross-linking, and inability to control the cross-linking, all of which result in nonselective cross-linking of membrane components. A great step toward solving the problems inherent in conventional cross-linking reagents is the development of heterobifunctional reagents in which one of the reactive functional groups is photolabile and the other is chemically reactive. Cross-linking with such reagents is easily controlled since the first step, the attachment of the agent via the chemically reactive functional group, can be carried out in the dark independent of the second, photosensitive cross-linking step. Cross-linking is then accomplished when desired by a rapid photolysis, fixing the membrane components.

Photoaffinity cross-linking reagents can be prepared by attaching the heterobifunctional reagents via the chemically reactive group to macromolecular ligands which bind to specific receptors on the cell surface. Unlike the conventional cross-linking reagents which react indiscriminately with many membrane constituents, photoaffinity cross-linking reagents are directed by the ligand moiety to specific sites on the cell surface. Thus, the latter reagents can be used effectively to probe membrane receptors and their environment in intact cells.

We have synthesized a series of new cleavable, photosensitive heterobifunctional cross-linking reagents which are derivatives of 4′-aminoazobenzene-4-carboxylic acid. This compound was chosen because it can be converted to a photolabile azide which is readily cleaved by dithionite under mild conditions. Moreover, chemical derivatization of the carboxyl group permits the preparation of photoreactive homologues of increasing chain length which, in addition, are easily activated to react with proteins in the dark.

In this publication we describe the synthesis and properties of the following four new reagents: 4′-azidoazobenzene-4-oxysuccinimide ester (**1**),[1] *N*-[4-(*p*-azidophenylazo)-benzoyl]-3-aminopropyl-*N*′-oxysuccinimide ester (**2**), *N*-[4-(*p*-azidophenylazo)benzoyl]-6-aminohexyl-*N*′-oxysuccinimide ester (**3**), and *N*-[4-(*p*-azidophenylazo)benzoyl]-11-aminoundecyl-*N*′-oxysuccinimide ester (**4**) (Figure 1). We also describe the preparation of two photoaffinity cross-linking reagents obtained by coupling compounds **1** and **2** to soybean agglutinin (SBA), a lectin which binds specificially to cell surface glycoproteins and glycolipids containing terminal galactose or *N*-acetyl-D-galactosamine residues (Lis & Sharon, 1977; Gordon et al., 1977; Tunis et al., 1979).

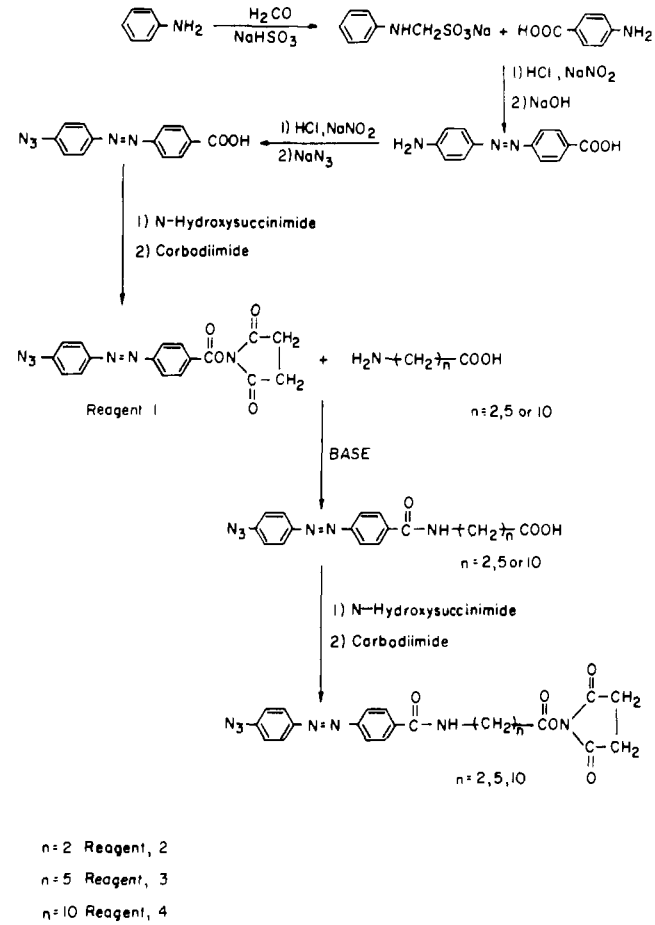

FIGURE 1: Synthesis scheme for the cleavable, photoreactive, heterobifunctional cross-linking reagents.

Materials and Methods

All ^1H NMR spectra were recorded in $CDCl_3$ at 80 MHz on a Varian FT-80A NMR spectrometer system using tetramethylsilane as an internal standard. Infrared spectra were taken in KBr by using a Perkin-Elmer Model 237 grating infrared spectrometer calibrated with polystyrene. Ultraviolet spectra were recorded in $CHCl_3$ on a Cary 18 ultraviolet spectrophotometer. Melting points were determined on a Fisher-Johns melting point apparatus. Purification was carried out by column chromatography on silica gel 60 (70–230 mesh, ASTM) and by thin-layer chromatography on silica gel 60 F_{254} plates (E. Merck A.G., Darmstadt).

Soybean agglutinin was purified by affinity chromatography as described by Gordon et al. (1972), except that HCl-treated Sepharose (Ersson et al., 1973) was used for specific binding of the lectin. The concentration of the lectin was determined either by absorbance with the value $\epsilon_{280nm}^{1\%} = 12.8$ cm^{-1} (Lotan et al., 1974) or by a modified Lowry procedure (Markwell et al., 1978) using purified SBA as a standard. The amount of reagent covalently bound to SBA was calculated on the basis of the molar absorption coefficient at 360 nm of the free reagents and the molecular weight of SBA (120000). Immunodiffusion was performed by the Ouchterlony method (1948) in 1% agar noble in 0.01 M sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl. The hemagglutinating activity was tested with rabbit erythrocytes by using a titer plate assay. All work involving the photoreactive azides and the lectin derivatives was carried out in a darkroom under red light.

Synthesis of the Cross-Linking Reagents. (*a*) *4′-Azidoazobenzene-4-oxysuccinimide Ester* (**1**). An ice-cold aqueous solution of sodium nitrite (50 mg in 0.1 mL) was added dropwise to 160 mg of finely ground 4′-aminoazobenzene-4-carboxylic acid (Theil & Peter, 1913) suspended in 1 mL of 10 M HCl at 4 °C. After the mixture was stirred for 15–20 min, the reaction reached completion as demonstrated by a positive test with starch–iodine paper, whereupon an ice-cold

[1] Abbreviations used: reagent **1**, 4′-azidoazobenzene-4-oxysuccinimide ester; reagent **2**, *N*-[4-(*p*-azidophenylazo)benzoyl]-3-aminopropyl-*N*′-oxysuccinimide ester; reagent **3**, *N*-[4-(*p*-azidophenylazo)benzoyl]-6-aminohexyl-*N*′-oxysuccinimide ester; reagent **4**, *N*-[4-(*p*-azidophenylazo)benzoyl]-11-aminoundecyl-*N*′-oxysuccinimide ester; SBA, soybean agglutinin; PBS, 5 mM sodium phosphate and 0.15 M NaCl, pH 7.2; M^1-SBA, reagent **1** derivative of soybean agglutinin; M^2-SBA, reagent **2** derivative of soybean agglutinin; $NaDodSO_4$, sodium dodecyl sulfate.

aqueous solution of sodium azide (45 mg in 0.1 mL) was added slowly to the diazotized azo dye. After standing for 1 h in the cold, the brick red 4′-azidoazobenzene-4-carboxylic acid was collected by filtration, washed extensively with ice-cold water, and dried. The product was used without further purification.

Carbodiimide (130 mg) was added to a solution containing 4′-azidoazobenzene-4-carboxylic acid (116 mg) and *N*-hydroxysuccinimide (70 mg) in dry dimethylformamide (4.0 mL). The reaction mixture was cooled to 4 °C and left overnight. The urea formed was removed by filtration, the filtrate was lyophilized, and the resulting orange product was purified by column chromatography in chloroform–ethyl acetate (1:1) to give 60 mg of the title compound (yield was 25% based on 4′-aminoazobenzene-4-carboxylic acid). After recrystallization from chloroform–ether, compound **1** had the following properties: R_f in chloroform–ethyl acetate (1:1), 0.48, and in chloroform–ethyl acetate (4:1), 0.53; mp 190–191 °C dec; IR 2110 (N_3) and 1770 and 1740 cm^{-1} (*N*-oxysuccinimide ester); UV λ_{max} 365 nm (ϵ 33 000); NMR δ 8.00 (aromatic 6 H, m), 7.21 (aromatic 2 H, d), 2.93 (aliphatic 4 H, s). Anal. Calcd for $C_{17}H_{12}N_6O_4$ (M_r 364.33): C, 56.04; H, 3.22; N, 23.07. Found: C, 56.25; H, 3.31; N, 23.15.

(*b*) *N-[4-(p-Azidophenylazo)benzoyl]-3-aminopropyl-N′-oxysuccinimide Ester* (**2**). A solution of **1** (100 mg in 20 mL of dry dioxane) was added with stirring to 0.28 mmol (25 mg) of 3-aminopropionic acid in 5 mL of 1% sodium bicarbonate. The solution was stirred overnight at room temperature, evaporated to 3 mL, and cooled in an ice bath. After the pH was adjusted to 1 with concentrated HCl, the solid product, *N*-[4-(*p*-azidophenylazo)benzoyl]-3-aminopropionic acid, was filtered off, washed extensively with ice-cold water, and used without further purification. Carbodiimide (38 mg) was added with stirring to a solution containing the 3-aminopropionic acid derivative (62 mg) and *N*-hydroxysuccinimide (21 mg) in dry dimethylformamide (7 mL). The solution was cooled and allowed to stand overnight at 4 °C. After filtration and lyophilization, the product **2** was purified by column chromatography in chloroform–ethyl acetate (1:1) and recrystallized from chloroform–ether to give 40 mg of the title compound (yield was 33% based on compound **1**). Compound **2** had the following properties: R_f in chloroform–ethyl acetate (1:1), 0.25, and in chloroform–ethyl acetate (4:1), 0.14; mp 158–159 °C; IR 2110 (N_3) and 1790 and 1755 cm^{-1} (*N*-oxysuccinimide ester); UV λ_{max} 360 nm (ϵ 17 000); NMR δ 7.94 (aromatic 6 H, t), 7.17 (aromatic 2 H, d), 3.89 (CH_2 2 H, m), 2.97 (CH_2 2 H, t), 2.87 (aliphatic 4 H, s). Anal. Calcd for $C_{20}H_{17}N_7O_5$ (M_r 435.41): C, 55.17; H, 3.93; N, 22.52. Found: C, 55.30; H, 4.01; N, 22.53.

(*c*) *N-[4-(p-Azidophenylazo)benzoyl]-6-aminohexyl-N′-oxysuccinimide Ester* (**3**). The title compound was prepared from **1** by the procedure described for **2** but using 6-aminohexanoic acid instead of 3-aminopropionic acid. Following purification by column chromatography in chloroform–ethyl acetate (1:1), **3** was crystallized from chloroform–ether to give 30 mg of the title compound (yield was 30% based on compound **1**). It had the following properties: R_f in chloroform–ethyl acetate (1:1), 0.31, and in chloroform–ethyl acetate (4:1), 0.17; mp 156–157 °C; IR 2110 (N_3) and 1785 and 1745 cm^{-1} (*N*-oxysuccinimide ester); UV λ_{max} 357 nm (ϵ 28 000); NMR δ 7.97 (aromatic 6 H, d), 7.17 (aromatic 2 H, d), 6.47 (NH 1 H, s), 3.49 (CH_2 2 H, m), 2.82 (aliphatic 4 H, s), 2.65 (CH_2 2 H, t), 1.64 (CH_2 6 H, m). Anal. Calcd for $C_{23}H_{23}N_7O_5$ (M_r 477.49): C, 57.85; H, 4.85; N, 20.53. Found: C, 57.96; H, 4.97; N, 20.37.

(*d*) *N-[4-(p-Azidophenylazo)benzoyl]-11-aminoundecyl-N′-oxysuccinimide Ester* (**4**). 11-Aminoundecanoic acid (38 mg) was dissolved in a minimum amount of dry dimethyl sulfoxide by heating to 80 °C. To the hot solution, triethylamine (83 μL) and compound **1** (68 mg dissolved in a minimum amount of dry dimethyl sulfoxide) was added and the solution allowed to cool to room temperature. After 12 h the reaction mixture was worked up by adding 25 mL of chloroform to the reaction mixture and extracting with three 50-mL portions of an aqueous solution of 0.15 M NaCl. The combined water extracts were washed once with chloroform and the chloroform extracts were combined. After being dried over $CaSO_4$, the chloroform was removed by evaporation and the product, *N*-[4-(*p*-azidophenylazo)benzoyl]-11-aminoundecanoic acid, was processed without further purification. *N*-Hydroxysuccinimide (11 mg) and the aminoundecanoic acid derivative (46 mg) were dissolved in dry dimethylformamide (5 mL), and carbodiimide (20 mg) was added with stirring. After standing overnight at 4 °C, the solution was filtered and lyophilized. The product **4** was purified by column chromatography in chloroform–ethyl acetate (4:1) and recrystallized from chloroform–ether to give 32 mg of the title compound (yield was 31% based on compound **1**). It had the following properties: R_f in chloroform–ethyl acetate (1:1), 0.48, and in chloroform–ethyl acetate (4:1), 0.40; mp 157.5–158.5 °C; IR 2110 (N_3) and 1780 and 1735 cm^{-1} (*N*-oxysuccinimide ester); UV λ_{max} 358 nm (ϵ 35 000); NMR δ 7.97 (aromatic 6 H, d), 7.17 (aromatic 2 H, d), 6.0 (NH 1 H, s), 3.51 (CH_2 2 H, m), 2.82 (aliphatic 4 H, s), 2.54 (CH_2 2 H, t), 1.36 (CH_2 10 H, m). Anal. Calcd for $C_{28}H_{33}N_7O_5$ (M_r 547.62): C, 61.41; H, 6.07; N, 17.90. Found: C, 61.52; H, 6.20; N, 17.87.

Soybean Agglutinin Derivatives. Soybean agglutinin (8 mg/mL in 0.1 M $NaHCO_3$, 0.15 M NaCl, and 0.2 M D-galactose) was reacted in the dark with a 30-fold molar excess of **1** added as one aliquot in 100 μL of dioxane. After 6 h at room temperature with shaking, the solution was centrifuged and applied to a Sephadex G-25 column, and the column was eluted with phosphate-buffered saline (PBS: 5 mM sodium phosphate and 0.15 M NaCl, pH 7.2). Fractions absorbing at both 280 and 360 nm were pooled, and the product (M^1-SBA) was further purified by affinity chromatography on HCl-treated Sepharose.

Reagent **2** was attached to SBA in the dark by using a procedure essentially as described for peanut agglutinin (Jaffe et al., 1979a). The reagent **2** (0.1 M in dimethylformamide) was added in five aliquots (5 μL each), one aliquot every 15 min, to 12 mg of SBA dissolved in 1.5 mL of 0.1 M $NaHCO_3$, 0.15 M NaCl, and 0.2 M in D-galactose. After 1.5 h at 4 °C, the solution was centrifuged, applied to a Sephadex G-25 column, and eluted with PBS. Fractions absorbing at both 280 and 360 nm were pooled, and the modified SBA (M^2-SBA) was further purified by affinity chromatography on HCl-treated Sepharose.

Irradiation. (*a*) *Reagent* **1**. A solution (1 mg/mL of compound **1** in dry chloroform) was divided into two 1-mL aliquots and one aliquot was set aside as a dark control. Irradiation was carried out by using the 365–366-nm line of a super-pressure Hg lamp (200 W, Osram). After predetermined periods of time, aliquots (5 μL) were removed from the irradiated sample and added to 1 mL of dry $CHCl_3$, and the ultraviolet spectra of the chloroform solutions were taken. Infrared spectra and thin-layer chromatograms of samples before and after irradiation for 30 min were also compared.

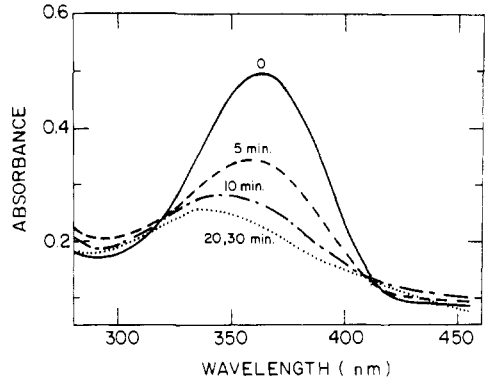

FIGURE 2: Irradiation of 4′-azidoazobenzene-4-oxysuccinimide ester (**1**). Reagent **1**, 1 mg/mL in dry chloroform, was irradiated by using the 365–366-nm line of a super-pressure mercury lamp (200 W, Osram). Following the indicated time periods, five 1-μL aliquots were removed and dissolved in 1 mL of dry chloroform, and the ultraviolet spectra of the diluted solutions were recorded.

(*b*) *M^1- and M^2-SBA.* Irradiation at 365 nm of solutions of M^1-SBA and M^2-SBA (0.59 mg/mL and 0.71 mg/mL, respectively) in 0.1 M $NaHCO_3$, pH 8.0, was carried out in a glass cuvette at 25 °C using the lamp described above. After irradiation for increasing periods of time, up to 15 min, the spectra of the modified lectins were measured. Experiments carried out at pH 7.2 gave identical results.

In order to examine whether cross-linking of the lectin derivatives occurs following irradiation, a solution of M^2-SBA, 4 mg/mL in 0.15 M NaCl and 0.1 M in $NaHCO_3$, was divided into four equal aliquots. Two aliquots were irradiated for 3 min and the remaining two aliquots were set aside as dark controls. Prior to dialysis against 2 × 1000 mL of doubly distilled water, one of the dark controls and one of the irradiated samples were treated with 0.1 M sodium dithionite (see below). After dialysis the samples were lyophilized and examined by gel electrophoresis both with and without Na-$DodSO_4$.

Cleavage. Cleavage of irradiated M^1-SBA and M^2-SBA was carried out by incubating the SBA derivatives at room temperature in 1 mL of 0.1 M sodium dithionite in 0.15 M NaCl, buffered at pH 8.0 with 0.1 M $NaHCO_3$ for 25 min.

Polyacrylamide Gel Electrophoresis. Gel electrophoresis of the native and modified lectins, 60 μg each, was carried out under both nondissociating conditions at pH 4.5 (Reisfeld et al., 1962) and dissociating conditions (Fairbanks et al., 1971).

Results

Synthesis and Irradiation of the Cross-Linking Reagents. The synthesis scheme for the cross-linking reagents is shown in Figure 1. The azo dye, 4′-aminoazobenzene-4-carboxylic acid, was prepared by coupling diazotized *p*-aminobenzoic acid to aniline-ω-methylsulfonate as described by Thiel & Peter (1913). After removal of the methylsulfonate group by alkaline hydrolysis, the azide was synthesized by diazotization of the azo dye and the addition of sodium azide. The shortest heterobifunctional cross-linking reagent, **1**, was obtained, without further purification of the intermediate, by preparing the *N*-hydroxysuccinimide activated ester of 4′-azidoazobenzene-4-carboxylic acid. Additional cross-linking reagents were synthesized by first coupling compound **1** with an appropriate amino acid spacer and then preparing the activated ester, without purification of the intermediates, to give compounds **2**–**4**. These reagents all contain a chemically reactive oxysuccinimide ester and a photolabile azide and are cleavable by sodium dithionite under mild conditions.

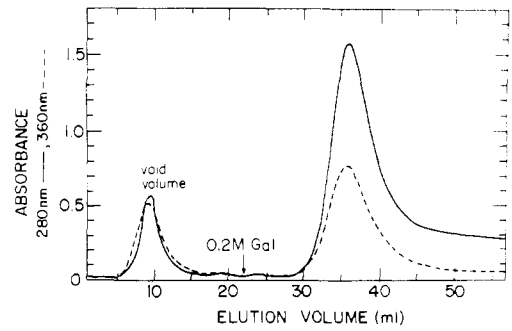

FIGURE 3: Purification of M^2-SBA by affinity chromatography. Soybean agglutinin (12 mg) to which reagent **2** was covalently attached was applied to a column (1.5 × 6 cm) of HCl-treated Sepharose, the column was washed with 0.15 M NaCl, and the adsorbed M^2-SBA was eluted with 0.2 M galactose in 0.15 M NaCl. Absorbance at 280 nm (—); absorbance at 360 nm (- - -).

Irradiation of reagent **1** resulted in a decrease and a shift in λ_{max} of the ultraviolet spectrum of the compound (Figure 2), both of which reached a maximum after 20 min. These changes were accompanied by the disappearance of the azide stretching band at 2110 cm^{-1} in the infrared spectrum and the appearance of at least five products moving slower than the starting material on thin-layer chromatography.

Preparation and Irradiation of the SBA Photoaffinity Derivatives. Two SBA derivatives, M^1-SBA and M^2-SBA, were prepared by coupling reagents **1** and **2**, respectively, to the lectin in the dark. Each reaction was terminated by centrifuging the solutions to remove insoluble material and applying the supernatant to a Sephadex G-25 column. The first peak, absorbing at both 280 and 360 nm and containing the derivatized lectins, was pooled and purified by affinity chromatography (Figure 3). Approximately 76% of the SBA applied to the column was recovered in the active fraction eluted from the affinity column by D-galactose.

The spectra of M^1-SBA and M^2-SBA are given in Figure 4. The amount of reagent bound to the lectin was calculated based on the ϵ_{max} of the respective reagent and the molecular weight of SBA (120 000). Reaction conditions were chosen to give between 3 and 4 mol of reagent bound/mol of protein (3.3 for M^1-SBA and 3.5 for M^2-SBA).

In order to obtain this molar ratio with compound **1**, it was necessary to increase the time and temperature of the reaction, since this reagent is less reactive than the reagents which contain the aliphatic spacers. Both M^1-SBA and M^2-SBA gave the same hemagglutinating activity (results not given) as the native unmodified lectin when tested on rabbit erythrocytes.

Irradiation of M^1-SBA and M^2-SBA for 15 s caused greater than 80 and 50% photolysis, respectively, of the lectin derivatives (Figure 4B). Complete photolysis was accomplished by irradiating M^1-SBA for 30 s and M^2-SBA for 2.5 min. No further change in the spectrum of M^2-SBA was observed upon longer irradiation. In the case of M^1-SBA, longer irradiation resulted in a small increase in the absorption due to increasing turbidity of the solution. However, if the photolyzed samples were incubated with 0.1 M sodium dithionite, the remaining absorption at 360 nm disappeared, indicating that the azo bond, which remained intact after irradiation, was cleaved.

Cross-Linking of M^2-SBA. When a concentrated solution of M^2-SBA (4 mg/mL) was irradiated for 3 min and run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, cross-linking of the lectin subunits was observed as evidenced by the appearance of new Coomassie blue staining bands (Figure 5A) migrating with molecular weights corresponding

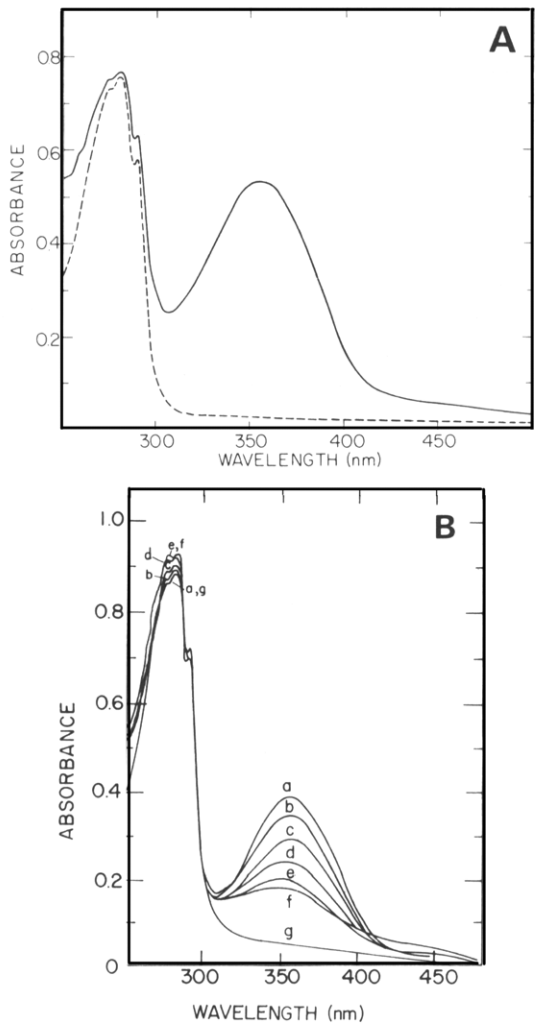

FIGURE 4: (A) Absorbance spectra of SBA (---) and M^1-SBA (—). (B) Absorbance spectra of M^2-SBA before and after irradiation. (a) No irradiation; (b) irradiation for 5 s; (c) irradiation for 15 s; (d) irradiation for 30 s; (e) irradiation for 1.0 min; (f) irradiation for 2.5, 5, and 15 min; (g) irradiation for 15 min, followed by cleavage by incubation with 0.1 M $Na_2S_2O_4$ buffered at pH 8.0 for 25 min at room temperature.

to subunit dimers, trimers, and tetramers (60 000, 90 000, and 120 000). Gels of the dark control revealed only one major band (30 000 daltons) corresponding to the SBA subunit and a minor slow-moving band (60 000 daltons) which was also present in samples of the native lectin and may represent traces of incompletely dissociated subunits. Treatment of the cross-linked lectin with sodium dithionite cleaved the subunit–subunit azo dye cross-links to regenerate the subunit monomer. No cross-linked subunit oligomers were observed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis of M^2-SBA irradiated at a low protein concentration (100 μg/mL), suggesting that the cross-linking at higher concentrations of the modified lectin is intermolecular. This was demonstrated when identical M^2-SBA samples were irradiated and examined by gel electrophoresis in the absence of sodium dodecyl sulfate. Irradiation caused the appearance of high molecular weight bands, greater than 120 000 (Figure 5B), corresponding to lectin oligomers resulting from intermolecular cross-linking. This photo-cross-linked material migrated identically with the high molecular weight aggregates formed upon the storage of native SBA in the lyophilized state (Lotan et al., 1975). The cross-links could be cleaved as in the previous experiment by treatment of the irradiated samples with sodium dithionite.

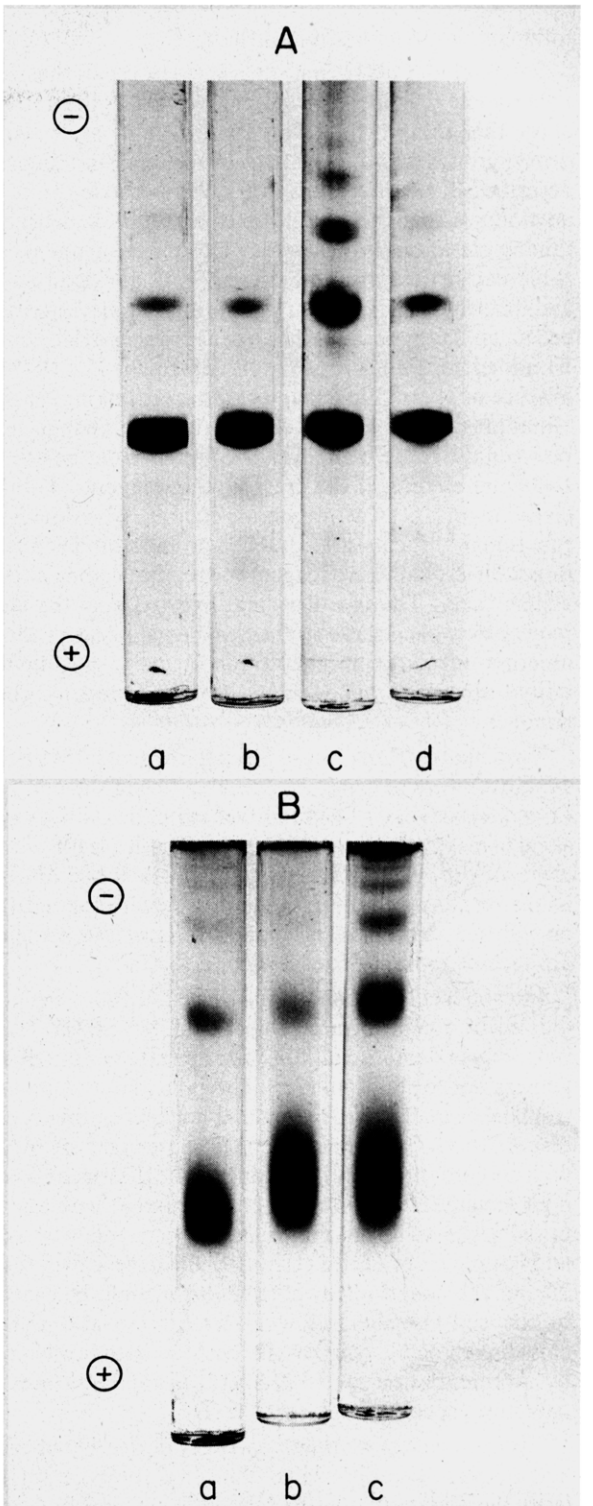

FIGURE 5: Polyacrylamide gel electrophoresis of M^2-SBA before and after irradiation. (A) Sodium dodecyl sulfate gel electrophoresis at pH 7.4 according to Fairbanks et al. (1971). (a) Native SBA; (b) M^2-SBA, no irradiation; (c) M^2-SBA, irradiation for 3 min; (d) M^2-SBA, irradiation 3 min and then cleaved by incubating in 0.1 M $Na_2S_2O_4$ (buffered at pH 8.0) for 25 min at room temperature. (B) Polyacrylamide gel electrophoresis at pH 4.5 according to Reisfeld et al. (1962) under nondenaturing conditions. (a) Native SBA; (b) M^2-SBA, no irradiation; (c) M^2-SBA, irradiation for 3 min.

Discussion

In this paper we describe a new class of cross-linking agents, with spacers of varying length. These reagents are heterobifunctional, containing a photoreactive aryl azide and a chemically reactive *N*-oxysuccinimide ester. In addition, the cross-linking reagent can be cleaved at the azo linkage under

mild conditions by sodium dithionite.

Fasold et al. (1971) first suggested the use of the azo group in cleavable reagents for studying protein conformation. We chose this group rather than the disulfide or the vicinal hydroxyl groups found in other cleavable cross-linking reagents described in the literature (Ji, 1979) because of the unique advantages that the azo group offers. Unlike with SS-containing cleavable reagents, disulfide reducing agents such as β-mercaptoethanol can be present both during cross-linking and following irradiation during the analysis of the cross-linked products. Dithiothreitol, because it reduces azides, may only be added following irradiation (Staros et al., 1978). The absence of a disulfide linkage in the cross-linking reagent also eliminates the possibility of disulfide interchange reactions between the reagent and SH or SS groups on the cell surface following binding of the cross-linking reagent. This is especially important in affinity cross-linking, when the heterobifunctional reagents are attached to macromolecules which direct the cross-linking reagent to specific binding sites on the cell surface. The use of vicinal hydroxyls as the cleavable moiety may also cause problems when analysis of the sugars on cross-linked membrane glycoproteins or glycolipids is required, since cleavage with periodate also destroys the sugar residues.

Two photoaffinity cross-linking reagents (M^1-SBA and M^2-SBA) were prepared by coupling two of the new reagents (**1** and **2**) to SBA. The modified lectin derivatives have the same hemagglutinating activity as the native lectin with rabbit erythrocytes, precipitate with anti-SBA antiserum (unpublished results), and migrate identically with the native lectin on polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate.

Intermolecular cross-linking occurred following the irradiation of a concentrated solution of M^2-SBA. The cross-linking was demonstrated by the appearance of subunit oligomers and of high molecular weight material on gel electrophoresis under dissociating and nondissociating conditions, respectively. Cross-linking could be reversed by incubation with sodium dithionite, resulting in the disappearance of the high molecular weight products concurrent with an increase in the un-cross-linked material. Experiments with M^2-SBA and lymphocytes (Jaffe et al., 1979b) have shown that up to 26% of the bound photoreactive lectin could be cross-linked to the cell surface following irradiation and that lectin cross-linking to the lymphocytes could be specifically prevented by preincubation with *N*-acetyl-D-galactosamine or D-galactose, specific inhibitors of SBA.

Experiments using a photoaffinity derivative of reagent **2** and peanut agglutinin have identified the receptor for this lectin in sialidase-treated ^{3}H-labeled human erythrocyte ghosts (Jaffe et al., 1979a). Irradiation of M^2-peanut agglutinin bound to the erythrocytes generated lectin–glycoprotein cross-linked complexes which could be isolated from membranes solubilized in 0.5% sodium deoxycholate by immunoprecipitation with antipeanut agglutinin rabbit antiserum. The complexed glycoprotein was tentatively identified as asialoglycophorin, following cleavage with sodium dithionite.

The new cross-linking agents described in this publication may be coupled via the reactive *N*-oxysuccinimide ester functional group to a variety of other effectors including hormones, toxins, and antibodies which bind to cell surface receptors. These macromolecular photoaffinity cross-linking reagents can be used to examine cell surface receptors and their environment by adjusting the length of the photoreactive probe.

Unlike other methods for receptor isolation or identification, photoaffinity cross-linking does not require the initial solubilization of the membrane components as a first step toward identifying the cell surface receptors. Cross-linking is carried out while the membrane or cell is still intact, preserving both the receptor and the membrane structure and organization. Once covalent cross-linking is accomplished, a specific inhibitor may be added to prevent reequilibration of effector binding with new "receptors" released following membrane solubilization. Therefore, "cryptic receptors" which may be unmasked following solubilization of the membrane and isolated by other methods would not be identified as "receptors" by affinity cross-linking.

In addition, since photoaffinity cross-linking does not require purification of the plasma membrane prior to identification of the cell surface receptors, the tedious work of preparing pure plasma membrane fractions is not necessary and the danger of cross contamination of the plasma membrane fraction by other subcellular fractions is obviated.

A final advantage of photoaffinity cross-linking is that activation of the reagents can be readily controlled and that the ensuing reaction is very rapid. Photoaffinity reagents prepared with a series of reactive probes of differing lengths can thus be used not only in examining the static environment around restricted sites on the cell surfaces but also, perhaps more importantly, for examining the changing environment around receptors during dynamic membrane processes such as malignant transformation, lymphocyte stimulation, cell development, and intercellular adhesion.

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Structural Reorganizations in Lipid Bilayer Systems: Effect of Hydration and Sterol Addition on Raman Spectra of Dipalmitoylphosphatidylcholine Multilayers†

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ABSTRACT: Vibrational Raman spectroscopy was used to investigate the conformational behavior of dipalmitoylphosphatidylcholine (DPPC) bilayers perturbed by cholesterol and water, two membrane components whose lipid interactions involve different regions of the bilayer matrix. Upon the addition of cholesterol, an intrinsic membrane constituent, to an anhydrous bilayer in concentrations varying from 7 to 30 mol %, modifications in lateral chain interactions were observed by monitoring spectral changes in the methylene C–H stretching and the CH_2 deformation regions. The perturbation in the 1460-cm^{-1} region was not spectroscopically observed until after the addition of 7 mol % of the sterol. Although chain–chain interactions are altered, no additional trans/gauche isomerization is developed along the hydrocarbon chains. Water, a peripheral bilayer component, was added to the multilayer assembly in the hydration range of 0.3 to ~4 molecules of water per lipid molecule. Vibrational spectra characteristic of motions in the head-group, interfacial, and acyl chain regions of the lipid bilayer were observed. These data indicate that hydration confers a mobility to the head-group, glycerol, and carbonyl moieties. Shifts in the CN symmetric and PO_2^- antisymmetric stretching modes, occurring on the addition of approximately four molecules of water, indicate a conformational rearrangement within the polar head group. After approximately four molecules of water are added to the DPPC system, the spectral features of the gel system [70% (w/w) water] indicate that no further head-group changes nor increases in either acyl chain trans/gauche or lattice disorder arise on further hydration.

Since the conformational and dynamical behavior of biological membranes reflects the concerted interactions of both intrinsic and extrinsic bilayer components, the origins of structural rearrangements within the lipid matrix are often difficult to resolve in liposomal systems. In particular, effects from either lipid head-group or acyl-chain perturbations may be propagated throughout the molecule, leading to structural reorganization of the entire phospholipid system. Of the wide variety of physical techniques available for investigating bilayer interactions and lipid rearrangements, vibrational Raman spectroscopy offers a sensitive analytical means both for monitoring inter- and intramolecular membrane disturbances and for identifying the region of the lipid molecule responding to the perturbation [see, for example, Wallach et al. (1979) and references cited therein]. The Raman scattering effect provides spectral features whose frequencies correspond to molecular vibrations which are assigned to motions within structurally distinct areas of the phospholipid molecule: for example, the lipid polar head-group region (symmetric C–N and PO_4^- stretching modes), the interface region (the inequivalent C=O stretching modes and the glycerol backbone C–C stretching mode), and the hydrophobic hydrocarbon chain region (CH_2 stretching, twisting, and deformation modes and the skeletal C–C stretching modes). By following changes in the spectral frequencies and intensities of these vibrational transitions as a function of either temperature or addition of a membrane component, one is able to assess the conformational modifications and lattice packing variations assumed by the lipid assembly under given bilayer conditions.

Of particular interest in the present study is the comparison between two membrane components, cholesterol and water, whose bilayer interactions involve different molecuar sites within the phospholipid assembly. By monitoring the Raman spectrum of an anhydrous dipalmitoylphosphatidylcholine (DPPC) lattice perturbed by quantitatively adding cholesterol and water, either singly or together, we are able to survey simultaneously the various bilayer events occurring within the three structurally distinct regions of the lipid molecule. For the DPPC–water system we follow specifically the lipid inter- and intramolecular changes induced by adding 0.3 to ~4 molecules of water per lipid molecule. Although we emphasize in this study the changes in relatively rigid lipid lattices defined by low water contents, we stress the important point that the head-group conformation, structural arrangements about the fatty acid carbonyl groups, and the trans/gauche chain disorder (at a given temperature) are developed by approximately the first four water molecules of hydration and do not change appreciably under conditions of excess water [70% (w/w)].

A relatively detailed view regarding the structural and motional requirements of cholesterol in lipid bilayers has been generated from a wide variety of biochemical and physical studies emphasizing, for example, the modification of mem-

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