Diffusion and Patching of Macromolecules on Planar Lipid Bilayer Membranes†

David E. Wolf,‡ Joseph Schlessinger,‡ Elliot L. Elson,§ Watt W. Webb,*,‡ Robert Blumenthal,# and Pierre Henkart¶

ABSTRACT: We have developed a model system for biological membranes in which amphipathic macromolecular antigens are bound to planar lipid bilayers. The effect of antibodies on the diffusion and distribution of these antigens has been studied. The antigens used are derivatives of dextran (mol wt 82 000) to which controlled amounts of fatty acid, rhodamine, and the antigenic hapten, 2,4,6-trinitrophenyl (TNP), have been covalently bound. These fluorescent amphipathic antigens bind to artificial planar lipid bilayer membranes from the aqueous medium. The diffusion coefficients of these macromolecules were measured by fluorescence photobleaching recovery. Membrane-bound antigens at $\sim 10^2$ molecules/μm^2 were distributed homogeneously and had a lateral diffusion coefficient $D = (2.1 \pm 0.9) \times 10^{-8}$ cm^2/s on oxidized cholesterol and $(4.5 \pm 2.2) \times 10^{-8}$ cm^2/s on egg phosphatidylcholine. On the addition of anti-TNP, small visible clumps formed similar to the "patches" observed on cell membranes. At a higher level of membrane bound antigen, $\sim 10^4$ molecules/μm^2, the distribution was again homogeneous but lateral diffusion was slower with $D = (3.1 \pm 1.0) \times 10^{-9}$ cm^2/s on oxidized cholesterol and $(6.8 \pm 2.4) \times 10^{-9}$ cm^2/s on egg phosphatidylcholine. At this concentration, the addition of anti-TNP reduced the diffusion coefficient and immobilized a fraction of the antigens, but did not cause patching. However, further cross-linking by anti-Ig resulted in a complete cessation of lateral diffusion and at higher concentrations in some cases the formation of patches. Similar phenomena were observed using concanavalin A as the cross-linking ligand. The diffusion of a fluorescent lipid probe was unaffected by either the addition of macromolecules or their subsequent cross-linking. Cross-linking one population of dextran derivative retarded the diffusion of another non-cross-reacting population. Sodium azide, colchicine, and cytochalasin B did not affect the motion of these molecules on oxidized cholesterol bilayers.

Molecules on the surface membrane of a variety of cells have been shown to undergo spatial rearrangements (Taylor et al., 1971; Bretscher and Raff, 1975; Edelman, 1976; Nicolson, 1976) and changes in mobility (Schlessinger et al., 1976a-c) when cross-linked by external macromolecules. Many surface receptors appear by fluorescence initially to be randomly dispersed in the membrane, but the addition of multivalent antibody or lectin molecules, which bind these receptors under physiological conditions, often results in a rearrangement into small clusters or patches. In some cases, such as lymphocyte surface immunoglobulin, a more dramatic rearrangement occurs in which the bound receptors form a tight "cap" in one area of the cell surface. Cap formation is generally believed to require metabolic energy from the cell (Taylor et al., 1971; Nicolson, 1976). While their biological significance remains a mystery, the phenomena of patching and capping have been interpreted as evidence for the fluid character of the membrane, in which membrane molecules diffuse freely within the plane of the lipid bilayer. Recent evidence, however, has shown that not all membrane constituents are mobile (Schlessinger et al., 1976a-c; Axelrod et al., 1976b), and that the binding of one receptor population can modulate the behavior of another (Yahara and Edelman, 1972; Edelman, 1976). These observations suggest strongly that cytoplasmic elements may interact with some surface receptors (Edelman, 1976; Nicolson, 1976; Schlessinger et al., 1976a-c).

In considering the molecular mechanisms for capping and patching, we decided that it would be advantageous to devise a simple model system in which cell surface phenomena might be reproduced and studied in a more controlled and quantitative manner than is possible in cell culture and in which phenomena involving cell metabolism or the cytoskeleton would play no role. In this paper we describe the model system which we have developed and preliminary observations on it.

We have bound fluorescence labeled synthetic amphipathic macromolecules to artificial bilayer lipid membranes, BLMs.[1] Modification of these molecules by attachment of haptens provided the means for cross-linking with antibodies. Their lateral motion in the presence and absence of cross-linking ligands was studied by the technique of fluorescence photobleaching recovery, described previously (Axelrod et al., 1976a; Koppel et al., 1976). This method has recently been used in our laboratory and elsewhere to study the mobility of cell surface components (Zagyansky and Edidin, 1976; Jacobson et al., 1976; Schlessinger et al., 1976a-c; Axelrod et al., 1976b).

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‡ School of Applied and Engineering Physics, Cornell University, Ithaca, New York 14853.

§ Department of Chemistry, Cornell University, Ithaca, New York 14853.

Laboratory of Theoretical Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014.

¶ Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014.

[1] Abbreviations used: FPR, fluorescence photobleaching recovery; FCS, fluorescence correlation spectroscopy; BLM, bilayer lipid membrane; Con A, concanavalin A; diI, 3,3′-dioctadecylindocarbocyanine iodide; TNP, 2,4,6-trinitrophenyl; Rho Con A, tetramethylrhodamine isothiocyanate labeled Con A; SD, stearoyl-dextran; TSD, TNP-stearoyl-dextran; TRSD, TNP-rhodamine stearoyl-dextran; AcRSD, acetylrhodamine stearoyl-dextran; TRD, TNP-rhodamine dextran; AcRD, acetylrhodamine dextran; AD, amino-dextran; TNBS, 2,4,6-trinitrobenzenesulfonate; TFSD, TNP-fluorescein stearoyl-dextran.

Materials and Methods

Lipid Bilayer Membranes. The membranes used in this study were formed on 100 or 200 mesh copper or gold electron microscope grids by an extension of the technique of Mueller et al. (1962). The grids were held horizontally by a stainless steel rod in a 2.2-mL stainless steel or Plexiglass cup. The cup was equipped with a glass window underneath. All membranes were formed at room temperature in Dulbecco's phosphate-buffered saline without calcium and magnesium, obtained from Grand Island Biological Co. Because of their high stability under subsequent treatment, the majority of the BLMs were made of oxidized cholesterol dispersed in *n*-octane at 30 mg/mL by the method of Tien (1974) from Sigma cholesterol. In other experiments, membranes were made using egg phosphatidylcholine obtained from Applied Science in *n*-hexadecane or *n*-octane, 10 mg/mL. The hydrocarbon solvents were all of the highest purity obtainable from Aldrich. Membrane thinning was observed by transmitted light, the cup being placed on a microscope stage and illuminated from below by an incandescent microscope source.

Materials were added to the aqueous medium surrounding the preformed BLM using a micropipet. Mixing was effected by repeatedly pipetting a significant volume of the solution. The effectiveness of this mixing procedure was demonstrated by the speed with which solutions with colored additives reach homogeneity. Dilution of solutions was achieved by addition of buffer and allowing the mixture to overflow the chamber containing the membrane and solution.

Antibodies and Con A. Affinity pure IgG antibodies to TNP were obtained from the serum of a sheep hyperimmunized to TNP-hemocyanin. The antibodies were purified by ammonium sulfate precipitation, affinity purification on DNP-lysine-Sepharose, and gel filtration on Sephadex G-200. The association constant of these antibodies with DNP-lysine determined by measuring the quenching of intrinsic antibody fluorescence upon hapten binding (Velick et al., 1960) was between 10^7 and 10^8 M^{-1}. The IgG fractions of rabbit anti-sheep IgG and goat anti-Con A serum, as well as Con A, were obtained from Miles Yeda and used without further purification. Purified anti-TNP antibodies and Con A were labeled by the procedure of Goldman (1968) with tetramethylrhodamine isothiocyanate obtained from Curtin Matheson. With the Con A it was necessary to label in 10% sucrose solution to prevent aggregation. In general it was found best to centrifuge Con A and the dextran derivatives before using to remove aggregates.

Lipid Probe. 3,3′-Dioctadecylindocarbocyanine iodide, diI, was used as a probe of lipid diffusion. It was the generous gift of A. S. Waggoner. This probe has been used as a lipid probe in various cell membranes (Sims et al., 1974; Schlessinger et al., 1976c) and recently in BLMs (Fahey et al., 1976).

Modified Dextran Preparations. All dextran derivatives used in the present study were made from a dextran fraction (from *Leuconostoc mesenteroides* B512) with a weight average molecular weight of 82 000 (Sigma). The stearic ester, stearoyl-dextran (SD), was prepared by mixing 5 cm^3 of 10 mg/mL dextran in dry Me_2SO, 1 cm^3 of dry pyridine, and 50 μL of stearoyl chloride (Sigma) by a modification of the technique of Hammerling and Westphal (1967). The reaction was carried out in a sealed glass tube at room temperature for 2 days. The product was dialyzed for 2 days against phosphate buffered saline, precipitated from 80% ethanol, and redissolved in phosphate-buffered saline. Insoluble material was removed by centrifugation at 12 000*g* followed by filtration through a 0.22-μm Millipore filter. Concentrations of dextrans and their derivatives were determined by the phenol–sulfuric acid assay for hexose (Dubois et al., 1956).

TABLE I: Composition of Dextran Derivatives.

Molecule	No. of groups per dextran molecule			
	Fatty acid[a]	TNP	Rhodamine	Acetyl
SD	1.4	0	0	0
AcRSD	1.3	0	3	85
TSD	1.3	87	0	0
AcRD	0	0	3	86
TRD	0	86	3	0
TRSD	1.3	85	3	0

[a] Roughly 50% stearic acid and 50% palmitic acid. These are mean values for the dextran derivative preparations. It is likely that the membrane will select for molecules with higher numbers of fatty acids.

Dextran derivatives containing TNP and rhodamine groups were synthesized via the *N*-(2-aminoethyl)carbamoylmethyl derivative which we refer to as amino-dextran, AD (Inman, 1975). The carboxymethylation reaction was carried out as described, at 37 °C for 4.5 h. The final product AD was found to contain 177 mmol of amino groups per mol of hexose using the method of Fields (1972) for estimating amino groups. This corresponds to roughly 1 amino group per 6 glucose units, or 89 amino groups per dextran molecule.

Stearoylation of AD was carried out as follows: The lyophilized powder was dissolved in water at 10 mg/mL and the pH adjusted to 10.0 with 1 N NaOH. The polysaccharide was precipitated by addition of ethanol to a final concentration of 80%, and the resulting precipitate was washed once with absolute ethanol. This precipitate was dissolved in dry Me_2SO by brief heating at 56 °C and the final concentration adjusted to 5–10 mg/mL. This solution was dried over molecular sieves (Type 4A, Fisher Chemical Co.) overnight and the stearoylation reaction carried out as previously described for dextran. For analysis of the fatty acid content of SD and stearoyl-AD, aliquots were hydrolyzed in 4 N HCl, 100 °C for 5 h. The free fatty acids were extracted with chloroform, converted to methyl esters, and determined by gas chromatography. As seen in Table I, 1 to 2 mol of fatty acid per mol of dextran was found in the preparations used in this study.

Rhodamine B derivatives of AD or stearoyl-AD were prepared by reaction of rhodamine B isothiocyanate (Sigma) dissolved in absolute ethanol at 10 mg/mL, with the dextran derivative dissolved in 0.04 M borate, pH 9.0, at 10 mg/mL. A volume ratio of 1:50 of these solutions was used and the reaction carried out at 37 °C for 1–2 h. This was followed directly by addition of either: (a) 0.25 mL of 100 mM TNBS (pH 6.5) per mL of dextran solution (roughly 200-fold molar excess over dextran), or (b) 4 μL of acetic anhydride per mL of dextran solution (roughly 350-fold molar excess over dextran). After an additional 2 h of incubation at 37 °C, the dextran derivative was dialyzed vs. phosphate-buffered saline, and AG 1-X2 beads (Bio-Rad Laboratories). These reaction conditions resulted in 2–3 rhodamine groups per dextran and complete substitution of the amino residues by TNP or acetyl groups. For the synthesis of stearoyl-dextran with TNP but without rhodamine, reaction with rhodamine B isothiocyanate was omitted. There are thus about 85 TNP or acetyl groups on these modified dextrans.

To facilitate understanding we have adopted a simple no-

menclature for these dextran derivatives. Each of the possible constituents is given a simple abbreviation: Ac for acetyl groups, D for dextran, R for rhodamine groups, T for TNP groups, and S for stearoyl groups, and the composite macromolecule is referred to by grouping the abbreviations of the constituents together. For instance, TRSD refers to dextran to which TNP, rhodamine, and stearoyl groups have been attached. All of the dextran derivatives employed in this study are listed in Table I along with the assayed quantities of each of the constituents.

Instrumentation. Our apparatus and the techniques of FCS and FPR have been extensively described elsewhere (Elson and Webb, 1975; Axelrod et al., 1976a; Koppel et al., 1976). Rhodamine fluorescence was excited by an attenuated 10^{-5}-W, 520.8- or 568.2-nm laser line from a Coherent Radiation krypton laser. Fluorescein fluorescence was also excited by the krypton laser using the 476.5-nm line. The optical filtering used to separate fluorescein and rhodamine fluorescence have been described elsewhere (Schlessinger et al., 1976b). The beam was focused through the vertical illuminator and 40× water immersion objective (numerical aperture 0.75) of a Zeiss Universal fluorescence microscope. The illuminated spot was of Gaussian intensity profile $I(r) = I_0 \exp(-2r^2/w^2)$, where w is the $I/I_0 = \exp(-2)$ radius. The values of w used in this series of experiments were in the range of 1 to 3 μm. The excited fluorescence was collected through the microscope optics, filtered, and detected by a photomultiplier tube. The small illuminated spot was exposed for 0.01 to 0.5 s at a laser power of 0.1 to 3.0 mW. The course of the fluorescence recovery is monitored with the laser intensity attenuated 1000-fold until it reaches a stable plateau. The time which it takes the fluorescence to recover half way to its final value is called the recovery halftime, $\tau_{1/2}$. In the case of recovery by diffusion the diffusion coefficient, D, is related to $\tau_{1/2}$ by the simple relation $D = \gamma(w^2/4\tau_{1/2})$, where γ is the parameter which accounts for the degree of bleaching. For these experiments $\gamma \simeq 1.3$ (Axelrod et al., 1976a). Failure of the fluorescence to recover fully to its initial value is interpreted as indicating the existence of a fraction of fluorophore immobile on the experimental time scale. We interpret the fraction fluorescence recovery as a measure of the fraction of mobile fluorophore.

Heat absorbed by the membrane during the bleaching pulse is rapidly conducted away by the surrounding aqueous medium. An equilibrium temperature is reached in a few microseconds, a time much shorter than the bleaching pulse. For conditions used in these experiments, the localized heating on the membrane is in the worst possible case less than 0.5 °C (Axelrod, 1977).

In FCS the stochastic temporal fluctuations of the fluorescence intensity are measured and analyzed with the help of a PDP-11 computer to obtain their normalized autocorrelation function $G(t)$. For diffusion in two dimensions $G(t) = [N(1 + t/\tau)]^{-1}$, where $\tau = w^2/4D$, and where N is the number of fluorescent particles in the beam. The values of τ and N are easily obtained from the straight line plot of G^{-1} vs. t.

The concentration of a fluorophore on the surface of a membrane was estimated by comparing the fluorescence of the illuminated spot to that of a solution of known concentration of the fluorophore. The solution was added to a microslide of 50-μm optical path length obtained from Vitro Dynamics. The microscope was brought to a focus at the center of the microslide. The level of solution fluorescence was recorded using exactly the same optics, photomultiplier, and recording gain as was used to measure the membrane fluorescence. The fluorescence excited in an infinitesimally thin layer of solution perpendicular to the laser beam is proportional to the concentration times the total laser power, and is thus independent of beam size and shape. Dividing the solution fluorescence by the pathlength and the concentration gives a calibration of the fluorescence per mol/cm^2. Dividing measured membrane fluorescence by this calibration factor gives mol/cm^2 of membrane. Since the rhodamine groups are attached to the hydrophilic dextran, we are assuming that the quantum efficiency of fluorescence of the rhodamine dextran conjugates is the same when in solution during the standardization as when adsorbed to the bilayer.

Data Analysis. Values of lateral diffusion coefficients measured by FPR were obtained from the average $\tau_{1/2}$ of FPR curves taken from three separate membranes, generally three or four measurements at the same spot on each membrane. Since there may be some variations in beam size and since it was impractical to measure the beam size before each set of experiments, we instead adopted a normalizing standard. At the beginning of each set of experiments, we measured the diffusion of TRSD at high concentration (see below) on oxidized cholesterol BLMs, so that all subsequent experiments could be normalized to that standard and compared with other sets of experiments. In this way we assured the relative accuracy of diffusion constants. The errors quoted in the text are derived by propagating the standard deviation of the measured $\tau_{1/2}$'s and reflect this relative accuracy. The absolute accuracy of our diffusion constants is further dependent on the accuracy of our beam radii determinations. The values for the beam radii quoted above are accurate to 20% so there is an additional 40% uncertainty in the absolute diffusion constants.

All dil diffusion measurements were made by FCS using a similar sample set. To maximize precision in the measurement of diffusion coefficients, all of the raw data from each sample were merged to obtain a single correlation function from which the best value of D was then found. The uncertainty was estimated by calculating the variation of the values of D for individual experiments about the D of the merged function.

Results

This series of experiments was planned to aid in the understanding of lateral diffusion experiments made in our laboratory on cell membranes (Schlessinger et al., 1976a–c,; Axelrod et al., 1976b) which led us to the following questions: How do the dextran derivatives bind and distribute themselves in BLMs? What effect do dextran concentration and lipid composition have on their mobility on the BLM? How is their distribution and mobility altered by the addition of cross-linking ligands? How does the addition of these macromolecules to a BLM and their subsequent cross-linking affect the mobility of the lipids or that of a population of macromolecules with a different specificity? Do drugs alter membrane fluidity and thus the mobility of these macromolecules?

The Adsorption of Dextran Derivatives to Lipid Bilayers. When exposed to aqueous solutions of TRSD or AcRSD for 30 min and then largely washed free of unbound material, the BLMs acquired a rhodamine fluorescence. This fluorescence was uniformly distributed (similar in appearance to Figure 2, left panel) and visible only in the sharply defined plane of the bilayer. While the Gibbs–Plateau border, which surrounds the membrane, was also stained brightly, it did not interfere with measurements on the membrane, because of the collection geometry of the optics.

To prove that binding of TRSD and AcRSD was dependent on the presence of stearoyl groups, we prepared the unstear-

TABLE II: Diffusion in Planar Lipid Bilayer Membranes.

Fluorescent probe	Type of membrane	Treatment of membrane	Diffusion constant ± SD (cm^2/s)	% recovery
TRSD	OC[c]	0.14 μg/mL washed yields $\simeq 10^2$ molecules/μ^2	$(2.1 \pm 0.9) \times 10^{-8}$	100
TRSD	EPC	0.14 μg/mL washed yields $\simeq 10^2$ molecules/μ^2	$(4.5 \pm 2.2) \times 10^{-8}$	100
TRSD	OC	40 μg/mL washed yields $\simeq 10^4$ molecules/μ^2	$(3.3 \pm 1.0) \times 10^{-9}$	100
TRSD	EPC	40 μg/mL washed yields $\simeq 10^4$ molecules/μ^2	$(6.8 \pm 2.4) \times 10^{-9}$	100
TRSD	EPC	40 μg/mL washed + 25 μg/mL anti-TNP	$(5.8 \pm 1.7) \times 10^{-10}$	35 ± 5
TRSD	EPC	40 μg/mL washed + 25 μg/mL anti-TNP + 100 μg/mL anti-sheep IgG	$\leq 10^{-11}$	0
AcRSD	OC	40 μg/mL washed	$(4.8 \pm 3.1) \times 10^{-9}$	100
Con A (rhodamine labeled)	OC	SD at 20 μg/mL washed + 100 μg/mL rhodamine labeled Con A washed	$(1.1 \pm 0.4) \times 10^{-9}$	50-100
Con A (rhodamine labeled)	OC	SD at 20 μg/mL washed + 100 μg/mL rhodamine labeled Con A washed + 100-200 μg/mL anti-Con A	$\leq 10^{-11}$	0
dil	EPC	1:10^3 in membrane forming solution	$(4.4 \pm 0.8) \times 10^{-7}$	*a, b*
dil	OC	1:10^3 in membrane forming solution	$(8.2 \pm 1.3) \times 10^{-8}$	*a*
dil	OC	dil as above + 20 μg/mL	$(6.8 \pm 0.9) \times 10^{-8}$	*a*
dil	OC	dil + 20 μg/mL + 100 μg/mL Con A	$(5.5 \pm 1.1) \times 10^{-8}$	*a*

[a] Information not available since measurement was made by fluorescence correlation spectroscopy. [b] From Fahey et al. (1976). [c] OC, oxidized cholesterol; EPC, egg phosphatidylcholine.

oylated derivatives AcRD and TRD. In the case of AcRD, negligible nonspecific staining was observed (<1% that of AcRSD). TRD did bind to BLMs, but unlike the uniform homogeneous binding of TRSD, TRD bound only unhomogeneously in large clumps. We presume that, in the case of TRD, the hydrophobic TNP groups may interact with the membrane.

The amount of membrane fluorescence and consequently surface concentration depended on the original concentration of TRSD in solution. In the present series of experiments, we have studied bilayers exposed to two different TRSD solution concentrations, 0.14 and 40.0 μg/mL (see Table II). After a subsequent approximately ninefold dilution, these resulted in TRSD surface concentrations of approximately 10^2 and 10^4 molecules/μm^2, respectively, which we hereafter refer to as low and high densities. These should be compared with characteristic lipid surface densities of 10^6 molecules/μm^2. We estimate on the basis of reasonable molecular dimensions that the high density case corresponds to the BLM being essentially completely covered with sugar.

Diffusion of TRSD and AcRSD Adsorbed to Lipid Bilayers. The two-dimensional diffusion coefficients of the dextran derivatives in oxidized cholesterol BLMs measured by FPR are as follows: TRSD at low density $(2.1 \pm 0.9) \times 10^{-8}$ cm^2/s, TRSD at high density $(3.3 \pm 1.0) \times 10^{-9}$ cm^2/s, AcRSD at high density $(4.8 \pm 3.1) \times 10^{-9}$ cm^2/s. For egg phosphatidylcholine BLMs formed from a dispersion in octane, we measured $D = (4.5 \pm 2.2) \times 10^{-8}$ cm^2/s at low density and $(6.8 \pm 2.4) \times 10^{-9}$ cm^2/s at high density. In all cases, 100% recovery was observed. A typical recovery curve of this type is shown in Figure 1 and also in Figure 3a.

It was important to show that the behavior observed was due to diffusion. As a first test, we compared the $\tau_{1/2}$ at $w = 1.2$ μm with that at $w = 2.2$ μm and found the characteristic w^2 dependence of $\tau_{1/2}$ for the diffusion. As a further test, we fitted experimental data to the theoretical recovery curves for diffusion as shown in Figure 1. Where flow is present, curves take on a sigmoidal shape distinguished by a zero slope at early times and a rapid approach to a 100% recovery asymptote.

Cross-Linking Membrane-Bound TRSD with Anti-TNP. Exposure of oxidized cholesterol BLMs labeled with the low

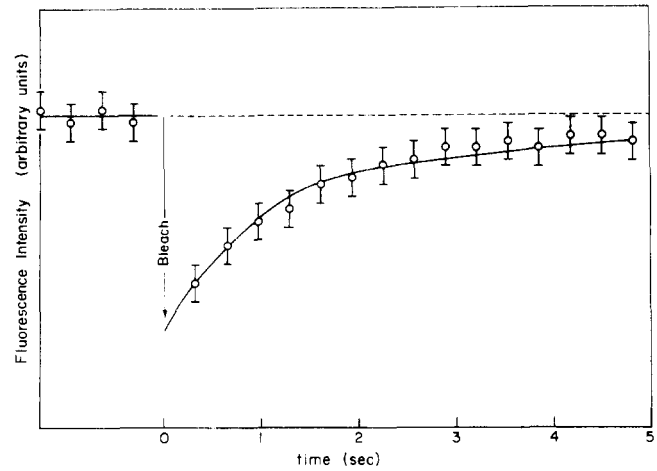

FIGURE 1: Fluorescence photobleaching recovery curve of TRSD adsorbed at high concentration to a phosphatidylcholine membrane. Open circles are experimental points taken by averaging data from eight experiments. Brackets are the standard deviations of the measurements. Solid line is the best theoretical fit of the data to recovery by diffusion; see Axelrod et al. (1976a). $\tau_{1/2} = 0.90$ s, $w = 1.2$ μm, $D = 5.2 \times 10^{-9}$ cm^2/s.

density of TRSD to anti-TNP in solution at a concentration of 2 to 3 μg/mL led to the formation of small patches ≤ 1 μm in diameter over the course of about 10 min. Raising the solution antibody concentration to 10 to 30 μg/mL (0.067 μM to 0.2 μM) caused the patches to enlarge and bunch together. The appearance of the patches is illustrated in Figure 2 (right panel). The further addition of antibodies to a concentration of 300 μg/mL had no further effect on the appearance of these patches. When the antibody was initially introduced at a concentration of 30 μg/mL, the same transition of homogeneous fluorescence progressing to small patches and then to long chains or bunches of patches was observed to occur during about 1 min. Similar patching was observed on egg phosphatidylcholine membranes.

To demonstrate that the patching was due to cross-linking by bivalent antibodies, we exposed BLMs labeled with the low density of TRSD to the monovalent Fab fragment of the sheep anti-TNP antibody at concentrations from 0.08 to 0.8 μM. No

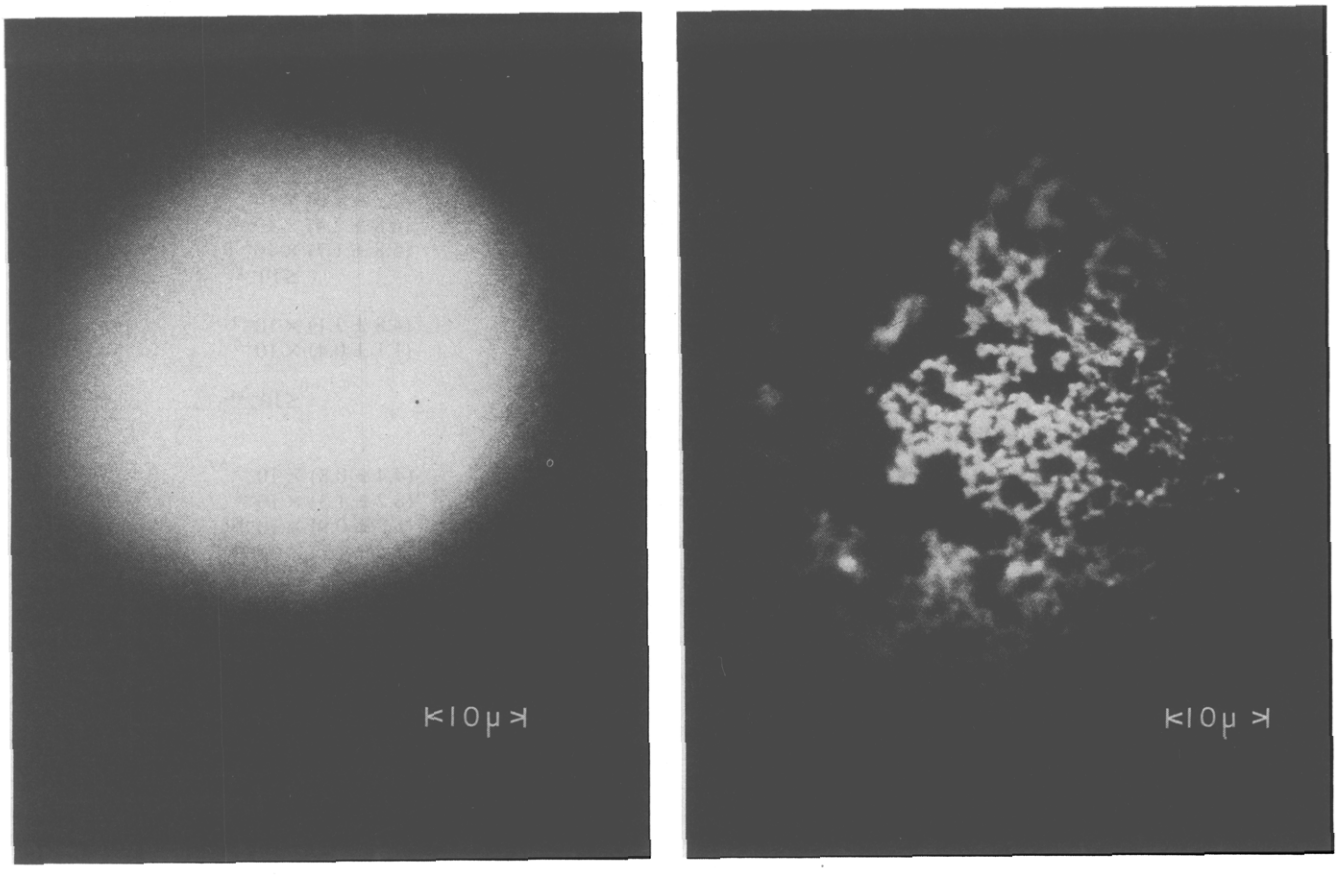

FIGURE 2: Fluorescence photomicrographs showing patching of membrane-bound SD by Con A. (Left) Oxidized cholesterol membrane was exposed to SD in solution at 20 μg/mL for 30 min and then partially washed free. Membrane was then exposed to Rho Con A at 100 μg/mL for 20 min and then partially washed free. Photograph shows a portion of the membrane under an expanded laser beam. Rho Con A fluorescence is seen to be homogeneously distributed. (Right) The membrane was then exposed to anti-Con A at a concentration of 200 μg/mL. Fluorescence became patchy.

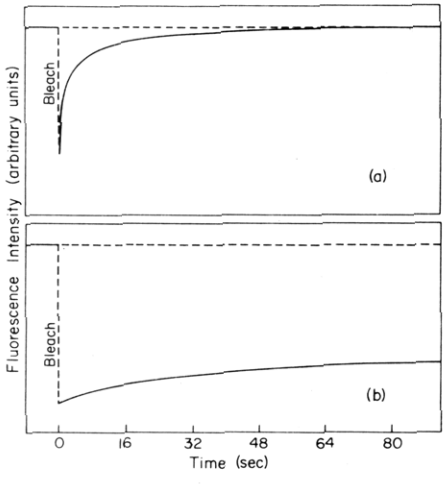

FIGURE 3: Fluorescence photobleaching recovery curves. (a) TRSD adsorbed at high concentration to a egg phosphatidylcholine membrane, $\tau_{1/2}$ = 1.5 s, w = 1.8 μm, γ = 1.3, $D = 7 \times 10^{-9}$ cm^2/s. (b) The same membrane after the addition of 25 μg/mL anti-TNP IgG, $\tau_{1/2}$ = 17.6 s, w = 1.8 μm, γ = 1.5, $D = 7 \times 10^{-10}$ cm^2/s fractional recovery = 30%.

patching was observed. However, subsequent addition of rabbit anti-sheep IgG resulted in extensive patching, demonstrating that the Fab was bound to the TRSD and that cross-linking is necessary for patching.

Bilayer membranes labeled with the high density of TRSD were also exposed to anti-TNP in solution. Antibody concentrations of 0 to 200 μg/mL caused no change in the uniform distribution of the fluorescent label on the membrane in contrast to the observations on low densities of TRSD. The lateral diffusion constant of TRSD was also measured as a function of added antibody concentration. A typical experiment showing the effects of antibody cross-linking on high density TRSD is shown in Figure 3. Two significant effects are observed on both oxidized cholesterol and egg phosphatidylcholine membranes: (1) the lateral diffusion constant systematically decreased with the addition of antibodies until a maximum of tenfold decrease was reached at about 10 μg/mL added antibodies; (2) an immobile fraction developed reaching ~65% at about 10 μg/mL added antibodies (see Figure 3). (In the case of oxidized cholesterol, these measurements were sometimes complicated by the occurrence of slow flow in the membrane. These experiments were rejected. This problem did not arise with egg phosphatidylcholine.)

The effect of an additional layer of cross-linking was investigated by the further addition of rabbit anti-sheep IgG to BLMs previously treated with the high density of the TRSD and then a saturating concentration of sheep anti-TNP, which remained in the solution during the measurement. This treatment should cross-link the anti-TNP antibodies. When rabbit anti-sheep IgG was added at a concentration of 100 μg/mL and incubated for 15 min, FPR showed essentially no recovery of the bleached spot, implying a "freezing" of the TRSD molecules into an immobile network. The appearance of the fluorescence was still uniform. Only when the rabbit anti-sheep IgG concentration was raised to 200 μg/mL was the uniform surface fluorescence seen in some cases to "break up" forming dark round holes of diameter $\leq$10 μm. This

mottled appearance was similar to, but not as extensive as, the rearrangement of fluorescence induced by Con A and anti-Con A (see Figure 2).

Cross-Linking of Membrane-Bound SD by Con A. Oxidized cholesterol BLMs were labeled with SD by exposing them to a solution of SD at a concentration of 20 μg/mL for 30 min and then diluting it threefold. Rhodamine labeled Con A, Rho-Con A, was then added at a concentration of 100 μg/mL and incubated with BLMs for 15 to 20 min. The solution was then diluted threefold. The BLMs were seen to be labeled uniformly with a Rho-Con A surface concentration of about 2×10^4 molecules/μm^2 that exhibited a diffusion coefficient $D = (1.1 \pm 0.4) \times 10^{-9}$ cm^2/s (see Figure 2, left). Percent recoveries in this case were 60 to 100%. Identical treatment with Rho-Con A without pretreating with SD produced only negligible nonspecific staining.

On the addition of goat anti-Con A to a concentration of 100 to 200 μg/mL, the membrane fluorescence pattern became mottled after 5 to 10 min due to the formation of dark fluorescence free areas. With further time, a definitely patchy quality of the fluorescence stain was observed. This is shown in Figure 2 (right). An upper limit for the lateral diffusion coefficient of the bound Rho-Con A patches is 10^{-11} cm^2/s. The patches observed here are identical in appearance with those formed at low TRSD density by anti-TNP antibodies.

The Effect of Cross-Linking of Macromolecules on Lipid Probe Diffusion. Recently in our laboratory Fahey et al. (1976) measured by FCS the diffusion of several lipid probes in BLMs of various compositions. The extremely rapid motion of dil in BLMs required that FCS rather than FPR be used to measure its motion. Their measurements of the diffusion coefficients for the probe dil in egg lecithin formed by the Mueller–Rudin technique from lecithin in *n*-octane gave $D = (4.4 \pm 0.8) \times 10^{-7}$ cm^2/s. We similarly prepared membranes from a dispersion in *n*-octane with a dil:cholesterol ratio of $1:10^3$. It was clear from visual observation that the majority of dil partitioned out into the torus. Comparison of the fluorescence per particle obtained in oxidized cholesterol with similar data of Fahey (private communication) in egg lecithin led us to conclude that in oxidized cholesterol dil is probably present as small aggregates of about ten molecules. We measured the lateral diffusion coefficients of those dil aggregates in the membrane to be $(8.2 \pm 1.3) \times 10^{-8}$ cm^2/s. Although the diffusion coefficients of these aggregates may differ considerably from the coefficient of self diffusion of the oxidized cholesterol in the BLM, dil aggregates should still be a sensitive probe of changes in fluidity.

After exposure of the membrane to a high density of SD (see Table II), we measured a diffusion constant for dil of $D = (6.8 \pm 0.9) \times 10^{-8}$ cm^2/s; after the SD was cross-linked with 100 μg/mL of Con A, the lateral diffusion constant for dil was reduced slightly to $D = (5.5 \pm 1.1) \times 10^{-8}$ cm^2/s.

The Retardation of the Diffusion of One Membrane Macromolecular Species by the Cross-Linking of Another. A dramatic effect on the motion of one species of membrane macromolecule was observed when a second coexisting species was cross-linked. For this experiment we prepared a molecule, called TFSD, analogous to TRSD, but which is labeled with fluorescein isothiocyanate rather than rhodamine. Egg phosphatidylcholine membranes were incubated for 30 min in a solution of 20 μg/mL TFSD and 20 μg/mL AcRSD and after incubation the solution was diluted 27-fold. The diffusion of each species was measured separately by exciting fluorescein at 476 nm and rhodamine at 568 nm. In a typical experiment, we found D(TFSD) $= (5.1 \pm 1.6) \times 10^{-9}$ cm^2/s and D(AcRSD) $= (6.2 \pm 1.9) \times 10^{-9}$ cm^2/s, with both showing complete recovery. These values should be compared with the $D = (6.8 \pm 2.4) \times 10^{-9}$ cm^2/s for TRSD at high density on similar membranes. The TFSD was then cross-linked by the addition of anti-TNP at 25 μg/mL. The diffusion constant of the TFSD was found to be reduced to $D = (8 \pm 3) \times 10^{-10}$ cm^2/s with only $10 \pm 10\%$ of it remaining mobile. Independent measurements of the AcRSD motion showed that it too was diffusing slower with $D = (11 \pm 5) \times 10^{-10}$ cm^2/s and $18 \pm 8\%$ of it remaining mobile. These values should also be compared with diffusion of TRSD under the identical conditions, where $D = (5.8 \pm 1.7) \times 10^{-10}$ cm^2/s and $35 \pm 5\%$ is mobile. In a control experiment, we found that the diffusion of AcRSD alone in a membrane was unaffected by anti-TNP antibodies, thus only the TFSD is capable of binding anti-TNP antibodies. These experiments show that the cross-linking of one species of membrane macromolecule that slows its diffusion can also impede the diffusion of another coexisting but non-cross-linked molecule.

The Effect of Drugs on the Motion of TRSD. In studies of cell surface phenomena, various drugs known to affect cytoskeletal components and metabolism have been used to explore the relationship between these structures and processes and cell surface mobility. It has been suggested that some of the effects observed might be due to intercalation of the drug into the lipid bilayer in a way which alters the membrane's fluidity. To explore this possibility, we have measured the effects of drugs which have been used in studies of patch and cap formation on cells on the intrinsic diffusion of TRSD at high surface concentration. None of the drugs tested, sodium azide at 10^{-2} M, colchicine at 10^{-4} M, and cytochalasin B at 10 μg/mL in 1% Me_2SO, had any measurable effect on the diffusion of TRSD in oxidized cholesterol membranes. These results are consistent with experiments done in our laboratory on cell membranes.

Discussion

Our studies of dispersed and cross-linked dextran derivatives in BLMs invite comparison with studies made in our laboratory and elsewhere into the behavior of proteins in animal cell membranes. Our model facilitates the isolation of an individual membrane process for study without interference from competing processes that may introduce ambiguities and obscure the interpretation of mechanisms. The surface density, molecular weight, and antigenic valence of the incorporated macromolecules, and the lipid composition of the BLMs are systematically variable parameters. This inherent control and flexibility of parameters cannot be achieved using available membrane proteins and natural membranes.

As is the case with all model systems, our system of dextran derivatives in BLMs in some respects resembles and in others differs from authentic cell membranes. While the structure of a "typical" membrane protein is as yet unclear, we may be confident of some similarities to the dextran derivatives. The molecular weight of our model molecules is in the range of known membrane proteins (Marchesi et al., 1976). However, most membrane glycoproteins are largely protein while our molecules have no amino acids and are largely polysaccharide. Like membrane proteins the dextran derivatives are amphipathic, possessing a hydrophobic portion buried within the lipid membrane and a hydrophilic sugar portion which extends into the aqueous phase. Since antibody and polysaccharide Con A binding sites interact directly with soluble ligands, one would expect them to be attached to the hydrophilic portion of the molecule. The fact that AcRSD binds extensively to mem-

branes while AcRD does not shows that the stearoyl groups are cause for membrane binding. Hence we infer that these fatty acids intercalate into the lipid bilayer. The binding of Con A and antibodies indicates that some of the sugar moieties and haptens are accessible to the aqueous phase. The slowing of diffusion and the formation of patches on the cross-linking of the dextran derivatives in BLMs is similar to phenomena observed for cell membrane proteins. Furthermore, Henkart and Neels (private communication) have shown that, when these dextran derivatives are adsorbed to cell membranes, cross-linking ligands induce both patching and capping. Thus in some regards the behavior of these macromolecules in membranes is very similar to that of proteins. Thus this simple system of an amphipathic macromolecule interacting with a BLM appears to provide a reasonable model for several interesting aspects of cell membrane behavior.

We have shown that patching can occur as a purely passive phenomenon. Furthermore, we have demonstrated patching under two different conditions: one at low receptor concentration where one layer of cross-linking ligand is sufficient, and the other at high receptor concentration where an additional cross-linking ligand is required. The former condition appears to be the more analogous to the patching observed on mammalian cells (Taylor et al., 1971) and hypothesized (Taylor et al., 1971; dePetris and Raff, 1973) to be a purely passive phenomenon resulting from the formation of an equilibrium distribution of ligand receptor aggregates comparable to the three-dimensional molecular aggregates of multivalent antigen and antibodies observed in classical precipitin reactions. In common with precipitin reactions we did not find "soluble complexes" in the zone of antibody excess. We are presently testing the possibility that TRSD molecules with fewer TNP groups would form patches which dissolve in excess antibody, similar to the case in some hemagglutination systems (Zmijewski and Fletcher, 1972).

At higher receptor densities, cross-linking agents induce a more complex set of changes. The patching that we have observed on the introduction of a secondary cross-linking ligand is probably caused by mechanical tearing. We suspect that, as additional cross-links are added, the network, which may be thought of as a taut fabric on a molecular scale, becomes more highly stressed. Small tears in the adsorbed dextran layer caused by disruption of a group of neighboring bonds are extended by the stresses to become larger holes. We visualize the mottled stage of this patching as an accumulation of a large number of these holes, and the final coalescence into large patches as the culmination of the mechanical failure as the binding sites are saturated. This phenomenon does not appear to involve the Gibb's plateau border of the planar BLM because a fissure is seen under the fluorescent microscope to separate the torus from the bilayer region under conditions of high cross-linking.

The observation that patching is induced by one ligand at low but not high receptor site density is suggestive of the finding of Stackpole et al. (1974) of an inverse relationship between capping and site density in cells, and it is tempting to draw parallels between the requirement of two cross-linking layers for patching at high receptor density and a similar requirement for optimal induction of caps in cells with some antigens and receptors. However, since the basic process of cap formation in cells is not understood, we feel that it is premature to draw any conclusions.

We have found a large concentration dependence of the diffusion coefficients of the dextran derivatives at such high concentrations that membrane coverage is almost complete. In this limit these molecules are not free to move independently. We are presently exploring whether this effect is due to specific sugar interactions or to general excluded volume interactions.

Cross-linking the dextran derivatives dramatically slows their diffusion rate and can cause a fraction of them to become immobile. This is probably due to the formation of large scale ligand–receptor networks, and is similar to the behavior of cell membrane proteins seen by Schlessinger et al. (1976a,b).

The immobilization of the receptor by cross-linking is a most provocative result. It shows that a cytoskeleton is not necessary to explain the existence of an immobile fraction of a membrane component. Note, however, that Schlessinger et al. (1976a–c) have observed immobile fractions in several cell systems where the proteins have not been extrinsically cross-linked; for example, proteins on L-6 myoblasts labeled with fluorescein isothiocyanate or with TNBS and then the Fab portion of anti-TNP IgG antibodies (Schlessinger et al., 1976c), and the Fc receptors on mast cells (Schlessinger et al., 1976b). Furthermore, they have observed a pronounced slowing of the protein diffusion in the presence of cytochalasin B (Schlessinger et al., 1976a–c). Axelrod et al. (1976b) have observed an immobile fraction of acetylcholine receptor labeled with fluorescent α-bungarotoxin in developing myotubes. These results would seem to implicate other cellular structures, possibly the cytoskeleton, acting as an anchor.

We have found that none of the drugs, sodium azide, colchicine, or cytochalasin B, has an effect on the fluidity of oxidized cholesterol membranes as indicated by the diffusion coefficients of labeled dextran derivatives. This result implies that the effects of these drugs on the cell membrane are really due to their effects on internal components as is generally supposed and not due to a change of viscosity of the lipid matrix and is consistent with the observation of Schlessinger et al. (1976c) of the diffusion of dil in myoblast membranes.

Despite extensive cross-linking of the dextran derivatives at high concentration, we have observed only a negligible effect on the diffusion of a lipid probe. On the other hand, we observed considerable immobilization and reduction of the diffusion of a second non-cross-reacting membrane-bound lipopolysaccharide. This result implies that the second dextran derivative may be trapped in the molecular network formed by cross-linking the first.

This system of synthetic macromolecules in BLMs offers a useful model for studying the behavior of membrane components under conditions approximating certain aspects of cell membranes. We intend to explore the dependence of mobility and cross-linking of these dextran derivatives on valence and molecular weight. We believe that these molecules may be useful as probes in cell membranes where they can be added as independent antigens, which may not be tied to internal structures.

Conclusions

The major conclusions of our analysis of the dextran derivatives in BLMs model system are that:

(1) Patching can occur as a purely passive phenomenon induced by cross-linking ligands. Depending on the surface concentration and valences one or two layers of cross-linking ligand are sufficient to induce patching. At low receptor concentration, one layer of ligand is sufficient, but at high receptor concentration two are required.

(2) Receptor diffusion is retarded by cross-linking.

(3) Cross-linking can be sufficient for receptor immobili-

zation; therefore, involvement of a cytoskeleton is not necessary.

(4) Lipid motion is unaltered by cross-linking the dextran derivatives in the membrane.

(5) Cross-linking one species of dextran derivative receptor impedes the motion of a second coexisting derivative, that is not itself cross-linked.

(6) Patching is not necessary for retardation of diffusion by cross-linking.

(7) The diffusion constant for the dextran derivatives is concentration dependent.

(8) The drugs sodium azide, colchicine, and cytochalasin B have no measurable effect on the fluidity of oxidized cholesterol membranes.

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