Structural and Dynamical Aspects of Membrane Immunochemistry Using Model Membranes†

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ABSTRACT: Three different phospholipid haptens have been synthesized, in which the haptenic group is the paramagnetic nitroxide (spin-label) group. These lipid haptens differ from one another in the length and composition of the molecular chain linking the 2,2,6,6-tetramethylpiperidinyl-*N*-oxy moiety to the phosphodiester group of the lipid. These lipid haptens have been incorporated at low molar concentrations (0.01 to 0.5 mol %) in liposomes containing various proportions of cholesterol and dipalmitoylphosphatidylcholine (DPPC). A study has been made of specific antinitroxide IgG (and Fab) binding to these liposomes, and the fixation of complement. From these studies we conclude: (a) For lipid haptens whose possible extension above the bilayer plane is limited (e.g., ~10–20 Å), antibody binding and complement fixation depend strongly on the hapten structure and host lipid composition, because of steric limitations on the accessibility of lipid haptens to the binding sites in the protein. (b) Complement fixation by specific IgG antibodies directed against the nitroxide group as part of a lipid hapten depends strongly on the lateral mobility of the lipid hapten when its molar concentration in the plane of the membrane is of the order of 0.1 mol % or less. It is likely that this conclusion applies to many lipid haptens, and possibly other membrane components. (c) The inclusion of cholesterol in lipid membranes has at least two distinct effects on complement fixation involving lipid haptens. Through a steric effect on bilayer structure (probably involving lateral molecular ordering) cholesterol in phosphatidylcholine bilayers can enhance hapten exposure to antibody binding sites, enhance antibody binding, and thereby enhance complement fixation. It is likely that cholesterol also affects complement fixation at low hapten concentrations through a modification of membrane fluidity.

The immunochemistry of cell surface membranes is an important aspect of the general problem of cell surface recognition. In at least some instances, the immune recognition of a cell surface membrane as "nonself" and the immunochemical response involve three distinct factors. First, the cell surface membrane must contain a foreign molecule, antigen or hapten. Second, the foreign molecule must be sufficiently exposed above the surface of the membrane as to be able to interact with antibodies and/or other components of the immune system. Finally, it can be safely assumed that the lateral mobility, distribution, and concentration of cell surface membrane components can affect immune recognition and response.

In recent work we have undertaken a study of antibody binding and complement fixation by liposomal membranes having variable lipid compositions and variable hapten concentrations in an attempt to define quantitatively some of the membrane structural and kinetic factors that are significant for these immunochemical reactions (Humphries and McConnell, 1975; Brûlet et al., 1977; Brûlet and McConnell, 1976a). In most of our work we have selected spin-labeled lipids as the haptens of choice, since specific antibodies directed against nitroxide moieties (e.g., spin labels) can be prepared (Humphries and McConnell, 1976) and magnetic resonance, chemical, as well as freeze-fracture electron microscopic methods can be used to provide quantitative information on the motion, distribution, and exposure of these haptens.

In a recent report we presented evidence that the lateral mobility of a spin-label lipid hapten (II, see below) was important for complement fixation by liposomal membranes when the mole percent [c] of lipid hapten in the host lipids was low, i.e., below a critical value [c*] $\lesssim$ 0.1 mol % (Brûlet and McConnell, 1976a). In addition to the role of hapten lateral mobility, the present paper is concerned with two additional factors that affect antibody binding and complement fixation, namely (i) the importance of the physical extension of the haptenic group above the surface of the membrane, and (ii) the effect of the inclusion of cholesterol in a bilayer membrane. Although effects i and ii have been noted previously (Alving et al., 1974a; Humphries and McConnell, 1975), the present work indicates an interesting relation between the two and, furthermore, attempts to provide interpretations of these effects in terms of antibody and membrane structure.

The present work takes advantage of early observations that complement is fixed by cholesterol-rich lipid carriers containing specific lipid antigens (Kolmer, 1928; Rapport, 1970), and also takes advantage of the observations of Kinsky and colleagues that hapten-sensitized liposomes can be damaged by specific antibodies and complement (Kinsky, 1972). Although our studies to date have mostly involved measures of complement depletion, and Kinsky and colleagues have measured complement-mediated marker release from liposomes, a number of our observations appear to be closely related to those obtained by Kinsky (1972), and Alving et al. (1974a), as discussed later. The early studies of Kinsky and colleagues were aimed at obtaining an understanding of the molecular events involved in membrane damage (Kinsky, 1972). Our central aim has been to relate lipid composition, hapten distribution and mobility to antibody binding and C1 activation, the latter by assumption being related at least qualitatively to the complement depletion measured in our experiments. (Haxby et al. (1969) have shown an absolute requirement for C2 and C8 in glucose release from liposomes prepared from sheep erythrocyte lipids, using human complement components and rabbit anti-sheep erythocyte serum.)

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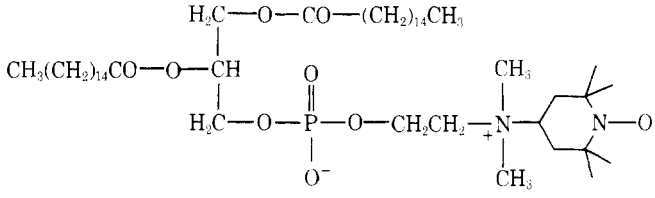
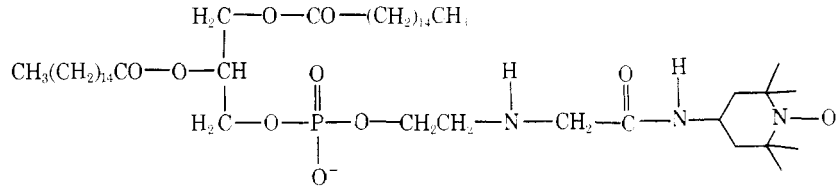
Materials and Methods

Spin Label I. The spin label phospholipid I was prepared by the method of Kornberg and McConnell (1971).

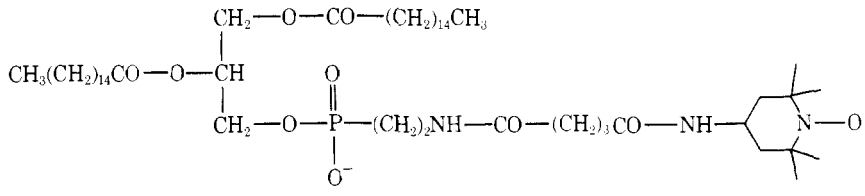


I

Spin Label II. The spin label phospholipid II was prepared as described by Brûlet and McConnell (1976a). Phospholipid II was the reaction product of an iodoacetamide spin label, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide and L-α-dipalmitoylphosphatidylethanolamine. A second reaction product II′, a biradical, was also isolated and purified. The biradical II′ arises from the attack of *two* iodoacetamide spin labels so as to replace both of the amino hydrogen atoms of phosphatidylethanolamine. See Brûlet and McConnell (1976a).



II



III

Spin Label III. The spin label phospholipid III was prepared as follows. Methyl 4-chloroformylbutyrate (11.7×10^{-4} mol) (Aldrich) in 3 mL of dried and distilled benzene was added to an equivalent amount of 4-amino-2,2,6,6-tetramethylpiperidinyl-*N*-oxy (Aldrich) in 3 mL of benzene containing 300 mg of distilled triethylamine. After 4 h at room temperature, the mixture was dried and dissolved in chloroform and purified on 30 g of silicic acid (Bio-Sil A, 200–325 mesh, Bio-Rad). The product appeared as a single spot on silica thin-layer chromatogram (Analtech) which was developed in chloroform–methanol–water 65:25:4 and stained with sulfuric acid.

The compound, dried on a rotoevaporator and taken into 2 mL of freshly distilled methanol, was added to a mixture of 50 mL of H_2O–10 mL of MeOH. One molar NaOH solution (6 mL) was added and the reaction left overnight at room temperature. The pH was adjusted to 3.0 by adding 1 N HCl and the compound immediately extracted with $CHCl_3$ (3 × 300 mL). The solution was rotoevaporated and dried successively from ethanol, toluene, and chloroform. The compound crystallized from chloroform (mp = 134 °C) was stored in 50 mL of chloroform. An oxalic acid impregnated silica thin-layer chromatogram of this solution was developed in chloroform–methanol–HCl (12 M), 87:13:0.5, v/v. It showed a single spot. The paramagnetic resonance intensity of the solution gave a total of 1.16×10^{-3} mol of nitroxide. The infrared spectrum showed the characteristic bands for the amide and acid functions.

This solution (10 mL containing 2.32×10^{-4} mol of spin label) was dried on a vacuum line and dissolved in 2 mL of freshly distilled pyridine. A tenfold excess of dried *N′,N′*-dicyclohexylcarbodiimide (Schwarz/Mann) was added prior to 150 mg of dipalmitoyl-L-α-phosphatidylethanolamine (Calbiochem) dissolved in pyridine. The mixture was stirred for 4 h. Water was added and stirred for 30 min; then the mixture was shaken with 150 mL of $CHCl_3$ and 40 mL of 0.5 N H_2SO_4 and centrifuged. The lower phase was washed successively with 40 mL of 0.5 N H_2SO_4, 40 mL of H_2O, 200 mL of CH_3OH–$NaHCO_3$ (2%) (1:2, v/v), 200 mL of CH_3OH–H_2O (1:2), evaporated under reduced pressure, dissolved in $CHCl_3$–MeOH (1:1), and applied to a column (2 × 35 cm) containing silicic acid (Bio-Sil A, 200–325 mesh, Bio-Rad). The column was washed with chloroform and the compound eluted with a gradient of chloroform–methanol. The column purification was reated twice. The compound, stored in ethanol, gave a single spot on a silica thin-layer chromatogram developed in $CHCl_3$–MeOH–H_2O (65:25:4) and stained with H_2SO_4. The phosphate content of the stock solution was determined by the method of McClare (1971) and gave 3.5 μmol/mL (total of 125 μmol). The electron paramagnetic resonance intensity gave 3.65 μmol/mL. The compound was further identified by its resonance spectra in a phospholipid matrix.

Tempo[1] was a gift of Dr. P. Rey, and Tempo-choline chloride was prepared by the method of Kornberg and McConnell (1971).

Preparation of Antibodies Directed against Nitroxide Spin Labels. New Zealand white rabbits were immunized, an IgG fraction was separated, and a specific affinity purified IgG and Fab fraction was prepared, all as described before (Humphries and McConnell, 1976; Brûlet et al., 1977; Brûlet and McConnell, 1976a). In the present work both the whole IgG fraction and the nitroxide specific IgG fraction have been used. Sera from different rabbits were not mixed. Prior to immunization each rabbit was bled to give control sera. For clarity in

[1] Abbreviations used are: DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; IgG*, an affinity purified fraction of IgG antibodies directed against the spin-label nitroxide group; Dnp-PE, dinitrophenylated phosphatidylethanolamine; Dnp-cap-PE, dinitrophenylated aminocaproylphosphatidylethanolamine; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; Tempo-choline, *N,N*-dimethyl-*N*-(2′,2′,6′,6′-tetramethyl-4′-piperidinyl-1-oxy)-2-hydroxyethylammonium chloride; PBS, 10 mM phosphate-buffered saline, pH 7.3, 150 mM NaCl; VBS, Veronal-buffered saline, prepared according to Kabat and Mayer (1971).

the following text, the affinity purified IgG fraction is referred to as IgG*.

Antibody–Hapten Binding Assays. We have used paramagnetic resonance to assay antibody–hapten binding. The binding of a nitroxide hapten by the active site of an antibody molecule strongly affects the motion of the former and thereby changes the resonance line width. The spectral consequences of the binding of low-molecular-weight water soluble nitroxide haptens by antibodies have been discussed in detail elsewhere (Humphries and McConnell, 1976; Rey and McConnell, 1976). Our assay for the binding of antibodies to hapten-sensitized liposomal membranes is described below.

The binding of IgG to a small nitroxide hapten, such as Tempo, or Tempo-choline chloride, was determined using paramagnetic resonance for a stock solution of IgG (e.g., 1.94 mg of IgG*/mL) in PBS. (Binding constants of small water soluble nitroxide haptens are of the order of 10^6 L/mol; Rey and McConnell, 1976.) For this purpose, x μL of IgG solution (x = 0–90) was added to 10 μL of a 5×10^{-5} M solution of the nitroxide hapten. A solution of IgG* reduces the signal intensity of a 5×10^{-5} M Tempo solution by 80%. The volume was made up to 100 μL and the intensity of the low-field hyperfine signal recorded. The plot of spectral intensity vs. the amount of antibody provided a standard for titration of unbound antibodies.

The assay for the binding of antibodies to hapten-sensitized liposomes consisted of mixing x μL of antibodies with y μL of lipids, increasing the volume to 90 μL and incubating, usually at 32 °C, for 30 min. The membranes were centrifuged at 300g for 10 min and the supernatant was carefully removed. Ten microliters of 5×10^{-5} M Tempo solution was added to each supernatant and unbound antibody was assayed as above. Controls involved membrane without antigen, and membrane with antigen and nonspecific IgG. With 20 μL of stock suspension of lipid (40 mM), 10–20% of the IgG was found adsorbed nonspecifically. Due to the volume of the pellet and the various manipulations, the signal of free Tempo after centrifugation, without antibody, was higher than the signal of the same quantity of Tempo in exactly 100 μL. Therefore, the intensities were appropriately scaled. One titration experiment using 0.5% II in DPPC–cholesterol (50:50 mol %) and IgG* showed a break, indicating ~10% of the haptens were exposed on the outer surface of the liposome.

Additional, different binding assays were also performed primarily to confirm the results obtained with the binding assay described above. As shown previously (Humphries and McConnell, 1976; Brûlet et al., 1977), the binding of specific antibodies to spin-labeled lipids can be detected directly by their effects on the resonance spectra of the lipid haptens. In this analysis involving the resonance spectra of the antigen spin label itself, x μL of lipid and y μL of antibody were mixed as above. The solution was drawn into a 50-μL capillary tube that was subsequently sealed. The spectra were usually recorded at 32 °C. No experiments of this type were carried out with IgG*; some experiments were carried out with whole IgG and DPPC liposomes containing 50 mol % cholesterol together with 0.5 mol % II; the loss of signal amplitude within 5 min was consistent with there being about 10–15% of the haptens exposed on the outer surface. This second binding assay method suffers from the potential difficulty that nonspecific antibody binding *might* perturb the spectra of externally exposed spin-labeled haptens. The second binding assay did establish unequivocally that there was no significant penetration of the antibodies through the lipid bilayers during the assay period (e.g., 5 min).

Some experiments were also carried out with a third binding assay, involving a direct competition of antibodies for membrane bound haptens and Tempo-choline chloride. The latter hapten is charged and does not penetrate the bilayers at a significant rate. In these experiments x μL of membranes, y μL of antibodies, and 10 μL of 5.0×10^{-5} M Tempo-choline chloride and PBS were mixed to a final volume of 100 μL. The high-field hyperfine peak intensity was used. The interpretation of this assay is rendered difficult by the overlap of the spectral lines from the two nitroxide groups (a difficulty that could be obviated through the use of ^{15}N in Tempo-choline chloride). Results of a few assays using this method are consistent with results obtained with other assays, particularly with respect to the protection of internal lipid haptens.

Complement Fixation. Numerous complement fixation assays have been described previously (Kabat and Mayer, 1971). Nonetheless we give a brief description of the assay used here since many of the results we have observed are highly sensitive to experimental conditions, so these conditions are described in as much detail as seems necessary.

The standard cell preparation contained 1 mL of washed sheep red blood cells (10^9 cells/mL), 1 mL of calibrated, suitably diluted hemolysin (described later), and 118 mL of VBS, with 0.1% bovine serum albumin. The source of complement (sometimes fresh, sometimes from Difco or Miles) was titrated as follows after absorption with sheep red blood cells: x μL of complement (1:250) was added to a series of tubes (x = 0, 10, 20, . . .) and each tube was filled to a volume of 300 μL with VBS. The tubes were incubated for a specified time (e.g., 2 h) at 32 °C. The tubes were then cooled to ice temperature. To each tube was added 1.2 mL of cold standard cell preparation and the tubes were then incubated at 37 °C for 1 h. The tubes were centrifuged, the supernatant was removed, and the optical density of the supernatant was measured in a 1-cm light path at 413 nm. A plot of this optical density at 413 nm is a function of x, the μL of complement supplied, and is also a function of the hemolysin "activity" (i.e., concentrations and nature of the anti-sheep red blood cell antibodies in the hemolysin solution). Plots of the optical density of the released hemoglobin at 413 nm vs. x (μL of complement solution supplied) depend on the activity of the hemolysin used in the standard cell preparation, and this activity can be adjusted by varying the concentration and source of the hemolysin used in the preparation of the standard cell preparation. As shown in the work of Wasserman and Levine (1961), the composition of the standard cell preparation can be adjusted so that the percent of cell hemolysis is a strong linear function of the concentration of x (the amount of complement added), when the percent of hemolysis varied between 10 and 70%. In most of our studies the composition of the amount of complement supplied in 100 μL was such as to lyse about 90% of the sheep red blood cells.

After having adjusted the hemolysin composition in the standard cell preparation to give a strong and nearly linear red cell lysis as a function of complement supplied, complement depletion by the hapten-sensitized membrane was assayed as follows.

Solutions were prepared containing a specified concentration of IgG in 100 μL, a given concentration of hapten and liposomes in 100 μL together with 100 μL of complement, to form a total volume of 300 μL. Controls were always included which contained the following: (a) complement plus 200 μL of VBS, (b) complement plus liposomes plus 100 μL of VBS. The final mixtures were then incubated at a given temperature for a given period of time. Thereafter 1.2 mL of standard cell

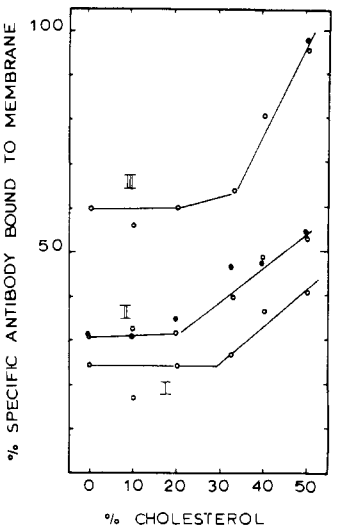


FIGURE 1: Binding of specific IgG* to DPPC-cholesterol membranes containing 0.5 mol % of I, II, and III (lower, intermediate, and upper curves, respectively). Points ○ give IgG* binding and points ● give specific Fab* binding (see Materials and Methods section). Separate experiments (see text) indicate all solutions contain antibody (or Fab) excess relative to exposed haptens, with the possible exception of the 50 mol % cholesterol liposomes containing III where exposed haptens and bound IgG (or Fab) may be close to equivalence. Temperature is equal to 32 °C. The nonspecific binding of IgG* to hapten-free liposomes is typically in the range of 10–20% and no strong dependence of this nonspecific binding on cholesterol has been detected. In the assay mixture the lipid concentration is 0.9×10^{-2} M. The total hapten in the reaction mixture is 4.5×10^{-5} M, of which we estimate approximately 10% is on the exposed surface. A typical specific IgG* concentration in the reaction mixture is 7.3×10^{-6} M.

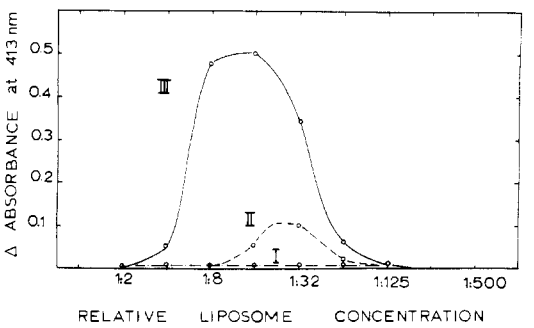


FIGURE 2: Complement depletion by DPPC liposomes together with an estimated concentration of specific IgG of 3.3×10^{-4} mg/mL (2.2×10^{-9} M), and a total IgG concentration of 6.6×10^{-3} mg/mL (4.4×10^{-8} M). The liposome concentration (1:1) is 0.3×10^{-3} M in lipids and the lipid haptens are present at a concentration [c] = 0.5 mol %. At a liposome dilution of 1:16, the total hapten concentration is about 10^{-7} M. Upper, middle, and lower curves refer to spin-label haptens III, II, and I, respectively. Complement supplied is sufficient to lyse about 90% of the sheep red blood cells and control lysis has an optical absorbance at 413 nm of $A_{413} = 0.59$. Temperature is 32 °C.

preparation was added on ice and the resultant sample incubated for 1 h at 37 °C. The sample was then centrifuged and the absorbance of the supernatant at 413 nm measured. This optical density is then a measure of the complement depletion and is a linear measure of complement depletion under most of the experimental conditions used in the present work. However, small derivations from linearity in complement fixation are not considered significant for the present studies and, therefore, our results are not reported in terms of the conventional percent complement depletion, but merely as depletion of cell lysis due to fixation of complement by hapten-sensitized liposomes and antibodies. This depletion is simply indicated by ΔA_{413}, the absolute value of the difference in absorbance (A) of samples relative to controls, at a wavelength of 413 nm. Complement depletion measured by ΔA_{413} or the more conventional "percent complement depletion in units of CH_{50}" provides no information on which components are limiting in a given experiment, or even whether the depletion is a simple monotonic function of the degree of C1 activation. The complement depletion measured by ΔA_{413} *is* dependent on the simultaneous presence of lipid haptens and specific IgG antibodies.

The above assay was originally devised by Dr. G. M. K. Humphries and differs slightly from that of Wasserman and Levine (1961).

Magnetic Resonance Methods. ^{13}C nuclear resonance spectra of [^{13}C]choline-enriched dipalmitoylphosphatidylcholine were obtained with a Bruker instrument. This instrumentation, typical ^{13}C resonance spectra, and the preparation of the ^{13}C-enriched lipid are described elsewhere (Brûlet and McConnell, 1976b, c).

Resonance spectra of the spin-labeled lipids were obtained with Varian E4 and E12 spectrometers, equipped for variable-temperature studies. Resonance spectra were obtained on liposomal preparations. For extensive discussions of the analysis of spin-label resonance spectra, see Berliner (1976).

Results

Figure 1 shows the binding of specific IgG* to DPPC-cholesterol membranes containing 0.5 mol % I, II, and III in the plane of the membrane. It will be seen that the IgG* binding depends strongly on the cholesterol concentration, and also on the structure of the lipid hapten itself. A longer chain connecting the haptenic group to the polar head group (i.e., the phosphodiester linkage) evidently facilitates antibody binding. Figure 1 also gives the binding of specific antinitroxide Fab* fragments to DPPC-cholesterol membranes containing 0.5 mol % II. As noted previously (Brûlet and McConnell, 1976a), the bindings of specific IgG* and Fab* to DPPC-cholesterol membranes containing 0.5 mol % II are equal.

No studies of Fab* biding to I in membranes were carried out. A few determinations of Fab* binding to III in DPPC-cholesterol liposomes were made. It is clear that Fab* binding to 0.5 mol % III in DPPC-cholesterol membranes is always stronger than Fab* binding to the corresponding liposome containing II. It was also established that at 50 mol % cholesterol the depletion of Fab* binding sites in solution by III was equal to the depletion of a corresponding concentration of binding sites in IgG* molecules by III.

Figure 2 illustrates the relative efficiencies for complement depletion by DPPC liposomes containing 0.5 mol % I, II, and III, at 32 °C. Under the conditions of antibody concentration given in the legend to Figure 2, complement fixation by I is negligible and essentially equal to the control value. The complement fixation is biphasic as a function of liposome dilution. At high liposome dilution (e.g., 1:125), complement fixation diminishes. At low liposome dilution (e.g., 1:2), complement depletion is small.

Figure 3 shows the results of a series of studies of complement fixation by liposomal membranes containing DPPC and various mole fractions of cholesterol together with 0.5 mol % I. For experimental details, see the legend to Figure 3. The enhancement of complement fixation by increasing cholesterol concentration parallels at least in a qualitative way the enhancement of antibody binding to liposomes with increasing cholesterol concentration.

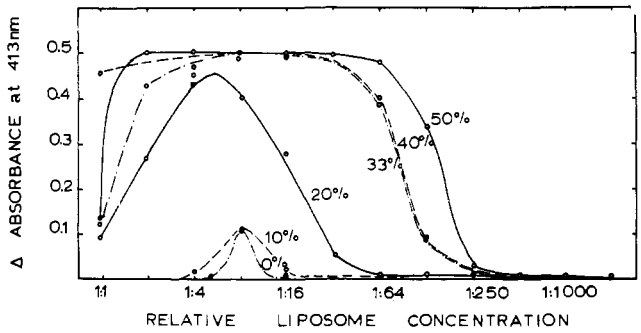


FIGURE 3: Complement depletion by liposomal membranes composed of DPPC and various indicated mole fractions of cholesterol, and containing 0.5 mol % I. Sepharose-separated IgG was employed and contained 30×10^{-3} mg/mL (20×10^{-8} M) and an estimated concentration of specific IgG of 15×10^{-4} mg/mL (10×10^{-9} M). The degree of complement fixation is related to ΔA_{413} (see text). These experiments involve a slight excess of complement. Temperature is 6 °C. The 1:1 lipid concentration is 0.66×10^{-3} M. Data when both lipid concentration is high (e.g., 1:1) and cholesterol concentration is high (e.g., 50 mol %) may be unreliable because of lipid-induced lysis of the red blood cells. Note that the maximum complement depletion that can be detected in this set of experiments corresponds to $\Delta A_{413} \simeq 0.5$, and the relative complement-fixing activity of the liposomes with high cholesterol concentrations may be grossly underestimated, particularly in the region of hapten-antibody equivalence.

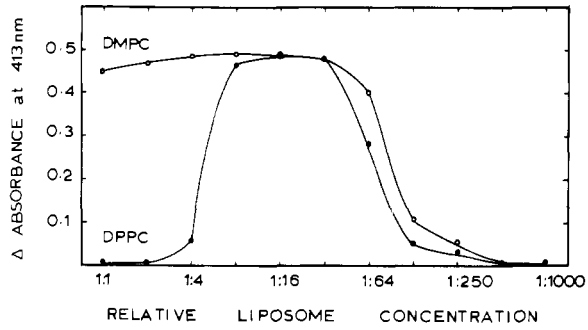


FIGURE 4: Complement fixation by DPPC and DMPC liposomes containing 0.5% lipid hapten II at 32 °C, as a function of lipid dilution. The 1:1 lipid dilution corresponds to a concentration of 0.3×10^{-3} M. The estimated concentration of specific IgG is 6×10^{-4} mg/mL (4×10^{-9} M) and the total IgG concentration is 12×10^{-3} mg/mL (8×10^{-8} M).

Figure 4 compares complement fixation by DPPC and DMPC liposomes containing 0.5 mol % haptens II (or II′) at 32 °C, as a function of lipid dilution. In these liposomal membranes the fixation of complement by the biradical lipid hapten II′ is not detectable. (In liposomes containing 50 mol % cholesterol, complement depletion by the biradical II′ is detectable but very small.) One striking feature of the data in Figure 4 is the marked difference in complement fixation between high liposomal concentrations of DMPC and DPPC, each containing 0.5 mol % II. The lipid concentration at which this effect takes place clearly depends on the concentration of IgG supplied in the reaction mixture, and probably also on the hapten concentration in the plane of the membrane, and finally, of course, on the affinity of the antibodies. To illustrate this point we mention other experiments, not described here in detail, in which complement depletions by DMPC or DPPC liposomes (0.15×10^{-3} M lipids, 0.1 mol % II) are nearly equal for some IgG* concentrations, and are not equal for other IgG* concentrations. In fact, the range of maximal difference between DMPC and DPPC is critical, being small at low IgG* concentrations, and also small at high IgG* concentrations.

Figure 6 shows complement fixation by DPPC liposomes containing 50 mol % cholesterol, and 0.5 mol % II, as a function

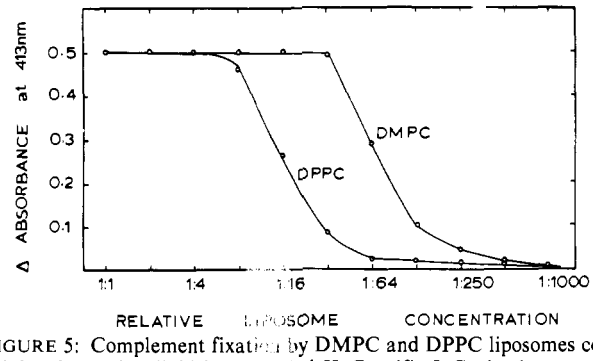


FIGURE 5: Complement fixation by DMPC and DPPC liposomes containing 0.1 mol % lipid hapten label II. Specific IgG* in the reaction mixture of 300 μL was 8.3×10^{-3} mg/mL (5.5×10^{-8} M). The 1:1 liposome concentration is 0.3×10^{-3} M. Temperature is equal to 32 °C. Control lysis has an optical absorbance at 413 nm of $A_{413} = 0.570$. About 10% of the sheep red blood cells are left unlysed; total lysis, $A_{413} = 0.61$.

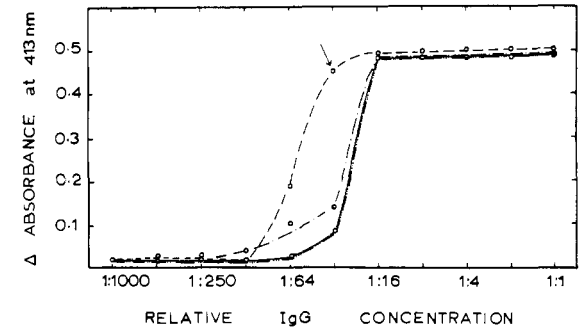


FIGURE 6: Complement fixation by liposomes containing DPPC and 50% cholesterol, and 0.5 mol % II. The specific 1:1 IgG* antibody concentration is 0.0133 mg/mL (8.9×10^{-8} M). The lipid concentrations are 0.066 μmol/mL (6.6×10^{-5} M) (—), 0.33 μmol/mL (3.3×10^{-5} M) (- · · · · · ·), 0.0165 μmol/mL (1.65×10^{-5} M) (- - -), and 0.00825 μmol/mL (0.825×10^{-5} M) (- · · · · · ·). Temperature is equal to 32 °C. Complement fixation 2 h. Control lysis, $A_{413} = 0.595$ (about 10% of cells left unlysed).

of IgG* concentration. This is a typical fixation curve as a function of antibody concentration (Kabat and Mayer, 1971). The point indicated by the arrow is taken as an "equivalence point". At this point it is likely that the exposed haptens and antibodies are present in roughly equivalent amounts; at this IgG concentration complement depletion decreases most rapidly when the lipid concentration is increased, *or* decreased. This conclusion that this represents an equivalence point is supported in a separate titration (Tempo binding assay) in which the sample is saturated with increasing amounts of specific IgG* and a relatively sharp break is observed in the titration curve. In both cases the (IgG*)/(total hapten ratio) is the same, namely 0.1. In still another experiment not reported here in detail, complement fixation was studied as a function of liposome dilution for these same liposomes using a nonaffinity purified IgG preparation. The lipid concentration yielding maximal complement fixation was 1.65×10^{-5} M lipids, essentially the same concentration yielding maximal complement fixation for the IgG* concentration in Figure 4.

The paramagnetic resonance spectra of II at a concentration of 0.25 mol % in "fluid" (DMPC), "solid" (DPPC) membranes and 50:50 DPPC-cholesterol membranes are shown in Figure 7.

Similar results are found for labels I and III. At higher spin label concentrations (e.g., 2.5 mol % III), one can detect the

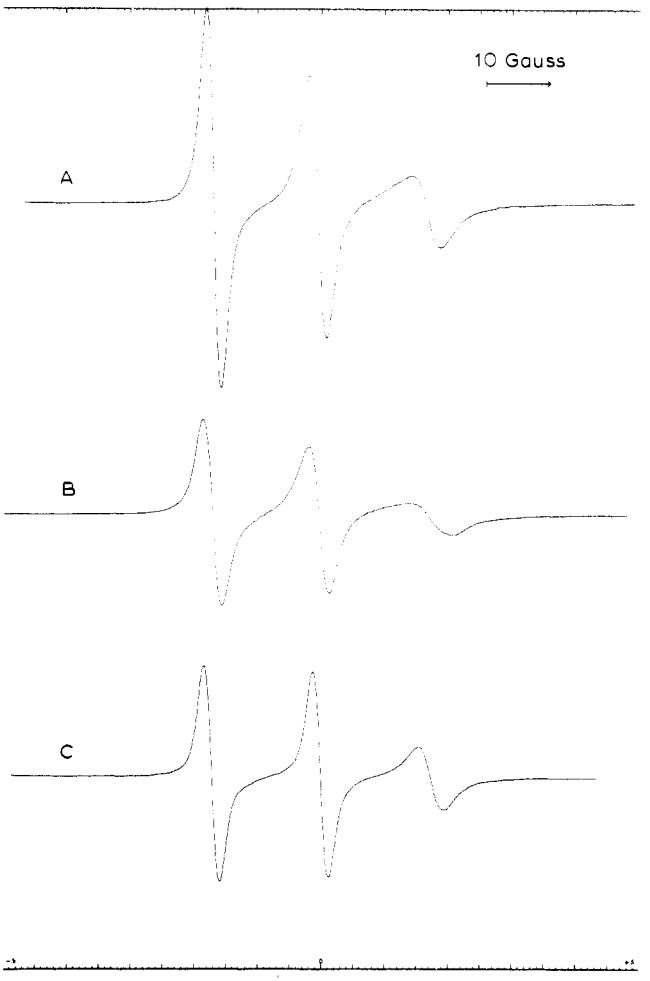


FIGURE 7: Paramagnetic resonance spectra of spin label II at 0.25 mol % in the plane of the membrane. (A) A "fluid membrane" (DMPC above its chain melting temperature, 23 °C); (B) a "rigid membrane" (DPPC below its chain melting transition temperature, 42 °C); (C) a 50:50 mol % cholesterol DPPC membrane. None of the resonance spectra show any indication of strong spin exchange of spin–spin dipolar broadening; therefore there is no detectable clustering of these haptens in the plane of the membrane. Spectrum C corresponds most closely to effectively itotropic motion. Temperature is 32 °C.

onset of spin exchange collisions between labels that is related to the known rates of lipid lateral diffusion (Devaux and McConnell, 1972; Sackmann and Träuble, 1972; Devaux et al., 1973).

^{13}C nuclear magnetic resonance spectra of proportions of ^{13}C-labeled choline methyl enriched DPPC with various proportions of cholesterol were also measured in an effort to determine the effects of cholesterol on molecular motion in this mixture of lipids. Typical ^{13}C resonance spectra of [^{13}C]-choline-enriched phosphatidylcholine are given elsewhere (Brûlet and McConnell, 1975, 1976b,c). Figure 8 gives resonance shifts as a function of cholesterol concentration and temperature.

Agglutination and Cross-Linking of Liposomes. While performing the Tempo binding assay with the liposomes containing different amounts of cholesterol, we noticed that a very fast and extensive agglutination of the liposomes containing DPPC and 50 mol % cholesterol and 0.5 mol % III occurred upon adding IgG*. No such extensive agglutination was noticed with DPPC containing 0.5 mol % III. As a control, no visible agglutination was observed using Fab* or either sample. Agglutination was also much less extensive when using II under the same conditions as above. Some agglutination of the

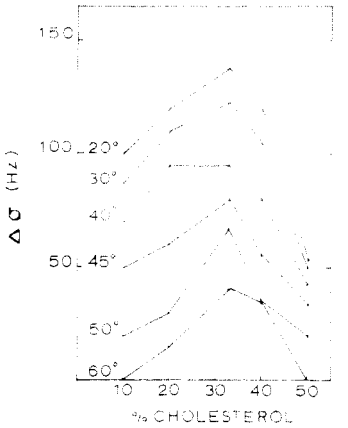


FIGURE 8: The upfield chemical shift (at 90.5 MHz) of ^{13}C in liposomes containing a binary mixture of cholesterol and DPPC enriched the ^{13}C-labeled choline methyl group. The composition region 20–40% cholesterol is presumed to be one in which there is the onset of the formation of an ordered lipid phase.

liposomes could be noticed but only after a much longer incubation.

The same results were obtained at different IgG* concentrations. Only III and 50% cholesterol membranes always showed agglutination; the kinetics of this process is affected by the concentration of IgG. The specific effect of cross-linking and agglutination on complement fixation is not known; however, it is known that for water-soluble multideterminant antigens, agglutination curves often parallel complement depletion curves as a function of antigen added (Davis et al., 1973).

Discussion

The activation of complement by cell surface membranes and specific antibodies against cell surface components may be looked upon as a special type of cell surface recognition. Even though the complement reactions leading to target cell lysis by the classical pathway have been studied extensively, and are notoriously complex, it is possible that the initial critical events involving recognition of "nonself" by the antibody–complement system will eventually be understood at the molecular level (Porter, 1975; Müller-Eberhard, 1975). In the present and previous work (Brûlet et al., 1977; Brûlet and McConnell, 1976a) we have tried to infer something about the initial events in complement fixation, and the role of hapten mobility, from measurements of overall complement depletion ("fixation"). This is admittedly a hazardous effort, when the complexities of the complement reaction are combined with our lack of complete knowledge concerning the size, shape, and surface area of the target membranes, namely, liposomes. Even so, the present and earlier results do provide encouragement for further pursuit of related, more definitive studies.

The following discussion is divided into several sections, dealing with distinct aspects of the present work.

Antibody Binding as a Function of Hapten Structure—Accessibility of the Hapten to the Antibody Combining Site. A comparison of antibody binding data in Figure 1 for lipid haptens I, II, and III with the chemical structures for these haptens immediately suggests that extending the length of the chain connecting the haptenic nitroxide group from the "surface" of the membrane (e.g., the plane containing the phosphate groups) enhances the number of antibody molecules bound to the membrane. We suggest that, for the particular haptens and antibodies used in the present work, the *major* source of the difference in antibody binding to I, II, and III (at

any given cholesterol concentration) arises from differences in steric constraints, that allow, or prohibit, the haptens from reaching the antibody binding sites without energetically large changes in antibody, or hapten, or membrane structures from their normal structures. There are two factors that bear on the above discussion. (i) In comparing the binding of antibodies to I, II, and III in liposomes, one must be mindful of the fact that their differing chemical compositions might lead to different intrinsic binding affinities, particularly, if one of the haptenic groups had a chemical structure that is closely related to the chemical groups present in the immunogen. (ii) With increasing intrinsic antibody–hapten binding affinity, clearly larger and larger membrane, hapten, and antibody structural distortions might be tolerated, leading to an enhancement of the number of antibodies bound to the hapten-sensitized liposome.

Six et al. (1973) have compared the degree of binding of both high and low affinity IgG antibodies to sphingomyelin–cholesterol–dicetyl phosphate (2:1.5:0.2) liposomes containing 10 mol % Dnp-Cap-PE, or 10 mol % Dnp-PE. In each case the binding to the "longer" hapten Dnp-Cap-PE was more extensive. Six et al. (1973) suggested that this enhanced binding to Dnp-Cap-PE relative to Dnp-PE liposomes may be related to an effect analogous to i mentioned above. Their observation that intrinsically higher affinity antibodies (against ϵ-Dnp-lysine) bind more extensively to sensitized liposomes than do the low affinity antibodies may be due in part to effect ii mentioned above. We do not believe effect i is very important in our experiments since our antibody preparations bind to a wide variety of water-soluble nitroxide haptens containing the six-membered nitroxide group with very similar binding constants (Rey and McConnell, 1976). Thus, our view that the depth of the antibody combining site is most directly related to the relative bindings seen in Figure 1 should not be viewed as being in conflict with the results or conclusions of Six et al. (1973).

Comparison of IgG and Fab* Binding.* A comparison of IgG* and Fab* binding to lipid hapten II in Figure 1 shows that the bindings are equal to one another, in the following sense. A solution containing x mol/L of IgG* loses xf mol/L of IgG* when exposed to a given liposomal preparation sensitized with II. A solution containing $2x$ mol/L of Fab* loses $2xf$ mol/L of Fab* when exposed to an identical liposomal preparation sensitized with II. Thus, a given liposomal preparation removes a certain number of hapten combining sites from a given solution, irrespective of whether these hapten combining sites are present at a given concentration in the form of IgG* or Fab molecules.

Our interpretation of this equivalence of the binding of IgG* and Fab* to II (and also to III at 50 mol % cholesterol) is the following. For a given hapten, and a given cholesterol concentration, the antibody molecules can be divided quite sharply into two groups: in one group the hapten can reach the combining site, and in the other group the hapten cannot reach the combining site. In the first group, essentially all the IgG* and Fab* molecules bind and are removed from solution; in the second group none of the IgG* and Fab* molecules are removed from solution. The reader will note that the observed results are inconsistent with a number of otherwise plausible possibilities, such as the IgG* molecules binding with only one binding site, or the IgG* molecules binding much more strongly than the Fab* molecules. Under other experimental conditions (e.g., more dilute solutions), it can be anticipated that the differences in Fab* and IgG* binding will be observable.

Cholesterol Dependence of IgG Binding to Liposomes Sensitized with I, II, and III.* The binding of IgG* to I, II, or III DPPC-cholesterol liposomes at 32 °C increases markedly as the concentration of cholesterol is increased above 20–40%, and in every case reaches a maximum at a concentration of 50 mol % cholesterol in the range of 0–50 mol %. A number of physical and biochemical properties of phosphatidylcholine–cholesterol-containing membranes change at cholesterol concentrations in the range 20–40%. (See Kleemann and McConnell, 1976, and further references contained therein.) As suggested by Figure 3, and shown earlier by Humphries and McConnell (1975), complement fixation can also increase rapidly at cholesterol concentrations above 20–40%. An important feature of the change in lipid properties that takes place in the 20–40% cholesterol composition range is that this change occurs over a wide temperature range. This result is clearly shown by the ^{13}C nuclear resonance line-shift data seen in Figure 8.

There are two plausible explanations of this enhancement of antibody binding with increasing cholesterol concentration. A higher cholesterol concentration may modify the structure of the liposomes so as to enhance the area of the outer surface and, thus, enhance the number of haptens available for antibody binding. There are several publications indicating that this enhancement of area does take place with *small* (150–200 Å) diameter single compartment lipid vesicles (Newman and Huang, 1975; Gent and Prestegard, 1974; de Kruijff et al., 1976). Alternatively, higher cholesterol concentrations may modify the bilayer structure so as to facilitate hapten exposure for antibody binding, e.g., increase the accessibility of haptens to antibody binding sites. At present we have no proof for either explanation, but consider one (or both) of these effects to be very likely. Evidence for this second possibility is summarized below.

Two spectral results indicate that cholesterol enhances the local motional freedom of phosphatidylcholine-related haptens, and, by inference, their accessibility to antibody combining sites. The ^{13}C resonance line widths of choline methyl groups in DPPC exhibit a sharp minimum at 50 mol % cholesterol, showing maximal motional freedom of the choline methyl group at 50 mol % cholesterol (Brûlet and McConnell, unpublished). Further, the paramagnetic resonance spectrum of II is more nearly isotropic in 50:50 DPPC–cholesterol at 32 °C than it is in *fluid* DMPC or *solid* DPPC at this temperature. See Figure 7.

Note that these resonance spectra were obtained at relatively low hapten concentrations (0.25 mol %) and show no evidence whatsoever for line broadening due to clustering of the labels. (This should not be regarded as a trivial result; in separate work by Rey and McConnell (1977), it is shown that certain amphiphilic nitroxide haptens can undergo specific, strongly host-lipid dependent and hapten-concentration dependent clustering at hapten concentrations in the range 0.01–0.05 mol %.) The spectra of Figure 7 also show no evidence for line broadening brought about by diffusion-controlled spin-exchange collisions. (For comparison, the incipient line broadening of III at 2.5 mol % in DMPC can be seen from the paramagnetic resonance spectra as this lipid undergoes its chain melting phase transition; see Brûlet et al., 1977.) The spectra in Figure 7, then, have shapes and line widths that are determined exclusively by local motion. For a detailed discussion of line shapes, see Berliner (1976) or Gaffney and McConnell (1974).

Complement Fixation for Haptens I, II, and III. Under the experimental conditions set forth in Figure 2, haptens I, II, and

III differ markedly in their effectiveness in complement fixation, the effectiveness again increasing with increasing potential extension of the haptenic group from the plane of the bilayer surface.

The maxima in the complement fixation curves in Figure 2 may be assumed to represent an approximate stoichiometric equivalence between specific complement-fixing antibodies and surface haptens. We believe these curves exhibit maxima because, at liposome dilutions beyond the equivalence point, the number of hapten-antibody complexes necessarily decreases, and complement fixation decreases. At liposome (hapten) concentrations *above* the equivalence point (hapten excess), the bound IgG* molecules become separated from one another in the rigid membrane (DPPC at 32 °C), and complement fixation again decreases, since adjacent pairs of larger aggregates of IgG* molecules are necessary to activate the complement system (by the classical pathway).

Note that there is a qualitative parallel between IgG* binding to liposomes containing I, II, and III and the degree of complement fixation. However, it is difficult to make any direct comparison between these results since, as is evident from the data given in the figure legends, the concentrations of both haptens and antibodies used for the complement fixation experiments are orders of magnitude less than those used in the binding studies.

If we pursue the argument that a larger number of IgG molecules are available for binding to III than are available for binding to II because of the depth of the antibody combining site, then the apparent relative displacements of the peaks for II and III in Figure 2 are understandable. A greater dilution of liposomes containing II is required for equivalence, since fewer IgG* molecules can bind to II, relative to III. (However, the displacement in Figure 2 is not much greater than possible experimental errors.)

Effect of Cholesterol on Complement Fixation. The data in Figure 3 illustrate the marked enhancement of complement fixation by increasing cholesterol concentrations in DPPC–cholesterol mixtures containing 0.5 mol % I. We interpret the enhancement of complement fixation with increasing cholesterol concentration as due to an enhancement of antibody binding, in turn due to enhanced accessibility of hapten to the antibody combining sites (due either to a local molecular structure change, or due to an increased outer membrane surface). Data taken at the highest liposome and cholesterol concentrations may not be reliable, due to interactions between these lipids and the sheep erythrocytes used for the complement fixation assay.

The marked enhancement of complement fixation by cholesterol appears to be a general effect for the monovalent haptens studied thus far, for various temperatures (e.g., 6–37 °C) and for relatively large hapten concentrations. As pointed out below, however, cholesterol may have a second effect at low hapten concentrations (i.e., $\lesssim$0.1 mol %) and at temperatures where cholesterol may significantly affect the lateral mobility of haptens.

It should be noted that Inoue et al. (1971) have also observed a marked effect of lipid composition on complement mediated glucose release from liposomes; this may in some cases be limited by the ease of interaction of the late-acting components of the complement sequence, and thus their results are not directly comparable to ours. For a recent investigation of the interaction of the late-acting components of complement with bilayer membranes, see Michaels et al. (1976).

Effect of Membrane Fluidity on Complement Fixation. In a separate paper we have described the results of a set of experiments specifically designed to show that complement fixation by hapten-sensitized liposomes depends on the lateral mobility of the haptens when the mole percent of lipid hapten is below some critical value, [c*] $\lesssim$ 0.1% for II, for example (Brûlet and McConnell, 1976a). This paper also provides a theoretical interpretation of this value for [c*] in terms of the distance between IgG combining sites. We limit the present discussion to the new results reported here that bear on this subject. For example, it will be noted in Figure 4 that at 32 °C, when DMPC is "fluid" and DPPC is "solid", complement depletion is much larger *in the region of hapten excess* in the fluid membrane (DMPC) than in the solid (DPPC) membrane. This result is understandable from the point of view that in the fluid membrane the IgG*–lipid hapten complexes can come together by diffusion and thus activate the fixation of complement by the classical pathway.

The data in Figure 4 also suggest that the fluid DMPC liposomes are in addition slightly more efficient for complement fixation in the region of antibody excess. Under the experimental conditions shown in Figure 5 this difference is even more apparent. Since at high hapten dilution (small *c;* antibody excess), complement depletion is limited by the number of "effective" IgG–hapten pairs on the membrane surface, one can readily imagine that because of the higher mobility of the IgG–hapten complexes in the fluid membrane there is more opportunity for associations between the subclasses of IgG that fix complement in the fluid membrane.

Studies by other investigators also support the idea that [c*] $\simeq$ 0.1 mol % may represent a critical concentration for complement fixation by lipid haptens. For example, Six et al. (1973) have observed a very strong enhancement in the amount of glucose released from sphingomyelin–cholesterol–dicetyl phosphate (2:1.5:0.2 mol ratios) liposomes sensitized with varying concentrations of Dnp-Cap-PE, or Dnp-PE, the enhancement being extremely steep when the concentration of Dnp-Cap-PE is increased above 0.1 mol % in the presence of high affinity IgG antibodies.

Also, Alving et al. (1974b) have carried out a similar study of glucose release by complement-mediated damage to liposomes (phospholipid–cholesterol–dicetyl phosphate, 2:1.5:0.22 mol ratio) containing relatively low concentrations of Forssman antigen, in the presence of IgM hemolysin, IgG hemolysin (and also Waldenstrom macroglobulin). These investigators also report a marked *increase* in complement-mediated lysis for Forssman hapten concentrations of the order of 0.1 mol %.

Although the above results of Six et al. (1973) and Alving et al. (1974a,b) seem to be in excellent accord with the spin-label hapten data, the agreement between all the results would be more convincing if the lateral mobilities and distributions of the haptens or antigens in the liposomes used by Six et al. (1973) and Alving et al. (1974a,b) were known. Assuming these membranes are essentially rigid under the experimental conditions of these authors, the results of Alving et al. (1974b) are of special interest since they would then provide evidence that activation of complement by IgM antibodies requires binding by at least two binding sites.

Obviously a *sharp* increase in the degree of complement fixation at hapten concentrations [c*] $\gtrsim$ 0.1 mol % requires antibodies of sufficiently high affinity; with sufficiently low affinity antibodies one can anticipate that, under comparable experimental conditions, lipid hapten concentrations above 0.1 mol % would be required in order to obtain observable enhancements of complement fixation; an example of this sort has been given by Six et al. (1973).

Alving et al. (1974b) have also observed an enhancement

in glucose release phospholipid–cholesterol–dicetyl phosphate (2:1.5:0.22) from liposomes containing galactosyl haptens when the phospholipid is changed from DSPC to DPPC to DMPC. This change may be related to changes in lateral mobility, but, as pointed out by these authors, it may also be due to increases in the antigen (hapten) exposure for the shorter chain lipid hapten.

The Number of Haptens Exposed to Antibodies in Liposomes. One of the obvious drawbacks in the use of liposomes for quantitative studies of immunochemistry is the difficulty of knowing the number of haptens that are available for antibody binding. (This is a doubly difficult problem in the presence of activated complement.) The maxima seen in the complement depletion vs. liposome dilution experiments exhibited in Figures 2–4 show one approach to estimating a stoichiometry of hapten–antibody binding, but the above-discussed role of membrane fluidity at low hapten concentrations indicates only one of the pitfalls that this approach is subject to. An additional attempt to determine the number of reactive haptens in the presence of complement is shown by the data in Figure 6, in which the concentration of liposomes (haptens) and antibodies are both varied so as to achieve optimum conditions for complement depletion: one finds the lowest possible concentration of IgG* that is required for optimal complement depletion. This presumably represents the condition under which there is an equivalence between hapten combining sites and complement-fixing antibodies. As pointed out in the Results section, the arrow point in Figure 6 may be taken as essentially an equivalence point, and at this point the ratio of (IgG*)/(total haptens) is approximately 0.034. If the IgG* binds with both binding sites, this indicates approximately 6.7% of the hapten is exposed on the outer surface. (As indicated earlier, a titration experiment under conditions similar to those in Figure 1 indicated approximately 10% of the haptens is exposed on the outer surface in these liposomes.) The concentration of haptens in the plane of the membrane in this experiment (c = 0.5 mol %) is sufficiently high that we expect no first-order effect of hapten lateral mobility on the complement fixation.

(After the work described in this paper was completed, J. Sheats and M. Schwartz in this laboratory developed photosensitive, membrane-impermeable compounds that can be activated to eliminate nitroxide groups on the outer surface of liposomal membranes. Preliminary experiments by Schwartz indicate ~4.5–6.5% externally exposed nitroxide haptens in liposomes similar to those used in the present work.)

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