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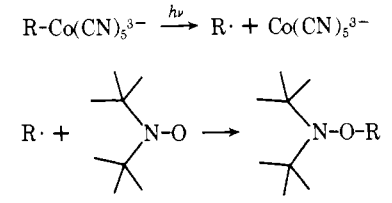
Surface Areas of Lipid Membranes†

Martin A. Schwartz and Harden M. McConnell*

ABSTRACT: Upon photolysis, alkyl pentacyanocobaltate complexes generate alkyl radicals which react rapidly and specifically with nitroxide radicals, and which do not penetrate phospholipid bilayers. By measuring the loss of paramagnetic resonance signal intensity when multilamellar liposomes containing a small amount of spin-labeled lipid are exposed to these radicals, we have measured the proportion of lipid on the external surface of liposomes. We have shown that liposomes prepared under specified conditions from dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine, and binary mixtures of dipalmitoylphosphatidylcholine and cholesterol all have the same proportion of external lipid.

In recent work we have studied antibody binding to lipid hapten-sensitized liposomal membranes as a function of host lipid composition (Brûlet et al., 1977; Brûlet & McConnell, 1976, 1977; Humphries & McConnell, 1977). Analogous studies were made of antibody-dependent complement fixation as a function of host lipid composition (Humphries & McConnell, 1975, 1977; Brûlet & McConnell, 1976, 1977). The quantitative interpretation of these experiments has been hampered by uncertainties concerning the number of haptens exposed to antibody on the external surface of these liposomal structures. This type of problem is in fact encountered in a wide variety of quantitative biophysical and biochemical studies of liposomes and lipid vesicles.

In recent work it has been shown that photo-chemical fragments of alkyl pentacyanocobaltates react rapidly and essentially specifically with the free radical nitroxide group of nitroxide spin labels according to (Sheats & McConnell, 1977):



As shown in the present paper, this affords an accurate, sensitive, and rapid spectroscopic method for determining the number of spin-label haptens on the external surfaces of liposomes. The number of externally exposed haptens is a measure of the external surface area of the liposomes.

†From the Stauffer Laboratory for Physical Chemistry, Stanford University, Stanford, California 94305. *Received September 29, 1977.* This work was supported by the National Science Foundation Grant No. PCM 75-02381 A02 and the National Institutes of Health Grant No. 5R01 AI13587-02.

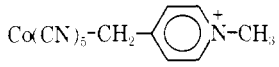
0006-2960/78/0417-0837$01.00/0

Materials and Methods

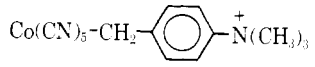
Alkyl Pentacyanocobaltates. Four different ligands were used in the alkyl pentacyanocobaltates. Carboxymethyl pentacyanocobaltate (III) was a gift from James Sheats (Sheats & McConnell, 1977). Iodomethylsulfonate was prepared by the method of Palmer (1954). (*N*-Methyl)pyridylbromomethane was prepared by dissolving 5.0 g of 4-pyridylcarbinol (0.0439 mol) (Aldrich) in about 20 mL of methanol with

$Co(CN)_5\text{-}CH_2COO^-$
carboxymethyl pentacyanocobaltate (I)

$Co(CN)_5\text{-}CH_2SO_3^-$
sulfonylmethyl pentacyanocobaltate (II)



4-(*N*-methyl)pyridylmethyl pentacyanocobaltate (III)



4-(*N,N,N*-trimethylamino)benzyl pentacyanocobaltate (IV)

equimolar iodomethane and stirring for 4 h at room temperature under argon. Thirty milliliters of diethyl ether was added, and the product was collected by suction filtration and then vacuum desiccated to dryness. The bromide was prepared by treating this alcohol with HBr, according to the method of Bixler & Niemann (1958).

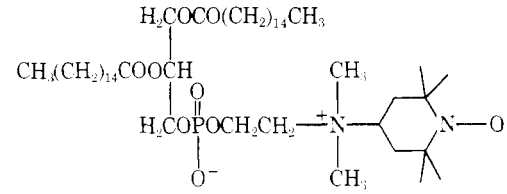
N,N,N-Trimethylaminobenzyl iodide was prepared by dissolving 0.5 g of 4-aminobenzyl alcohol (0.0041 mol) (Aldrich) in 25 mL of acetone, with fourfold excess of iodomethane and 4 mL of 2,6-lutidine, and stirring for 36 h at room temperature under argon. The white crystals were collected by suction filtration and recrystallized twice from methanol/NaOH/ether, keeping the product under argon during all operations. The iodide was prepared by treating this alcohol with HI, by the method of Hass & Bender (1949).

In each case, the alkyl halide was reacted with pentacyanocobaltate II according to the procedure of Halpern & Mayer (1964, 1965). The crude product was precipitated from aqueous solution by adding excess acetone, redissolved in water, and ethanol slowly added until white precipitate no longer formed. The colored oil was then precipitated with excess ether, dissolved in water, and used without further purification. In each case the product was assayed by its ability to abolish the paramagnetic resonance signal in a solution of 4-hydroxyl-2,2,6,6-tetramethylpiperadine-1-oxyl (Tempol)[1] upon photolysis. A stock solution of 12 mM alkyl pentacyanocobaltate in water was prepared.

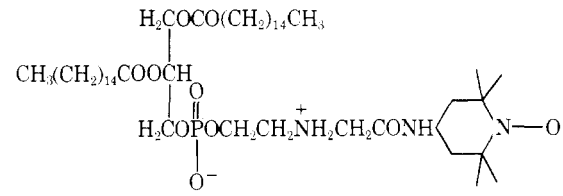
Lipids. Dipalmitoylphosphatidylcholine (DPPC) (Sigma, 98%), dimyristoylphosphatidylcholine (DMPC) (Sigma, 98%), and dicetyl phosphate (Sigma) all ran as one spot in thin-layer chromatography and were used without further purification. They were stored as ethanol solutions. Cholesterol was recrystallized from ethanol and vacuum desiccated. It was found to be pure by thin-layer chromatography and was stored as a chloroform solution.

Spin Labels. Two phospholipid head group spin labels were employed. Spin label V was a gift from James Sheats. Spin label VI was a gift from W. Parce. These two spin labels gave identical results and were used interchangeably.

Liposomes. Stock solutions of lipid containing 0.5% spin label and 2.0 μmol total lipid were evaporated to dryness to coat



V



VI

a 5-mL round-bottomed flask. For samples containing cholesterol, the lipid was redissolved in freshly distilled chloroform and the solvent evaporated again. The flasks were placed under vacuum for at least 1 h to remove any traces of solvent, 0.1 mL of phosphate buffer (0.1 M, 7.0 pH) was added, and the lipid was hydrated at about 15 °C above the chain-melting transition temperature and dispersed in the buffer.

For pure DPPC and DMPC liposomes, a measured amount of alkyl cobaltate stock solution was added to the lipid preparations, and the mixture was transferred to a 50-μL pipet (sealed at one end) which was used for paramagnetic resonance.

For liposomes containing cholesterol, the cobalt complexes caused agglutination which made transfer to the 50-μL pipet difficult. Instead, 50 μL of the lipid dispersion was transferred to the pipet, and 5—10 μL of alkyl cobaltate solution was added with a drawn out Pasteur pipet, distributing the cobalt solution as evenly as possible. Mixing was completed by shaking the liposomes to the sealed end of the tube.

Paramagnetic Resonance Spectra. A Varian E-4 spectrometer with the sample holder described by Gaffney (1974) was used. For experiments in which the sample was photolyzed in the resonance cavity, a quartz tube replaced the Dewar. To control the temperature in the experiments with DMPC, nitrogen which had been passed through dry ice–acetone was blown through the cavity.

Photolysis. For the experiments with pure DPPC, a mercury arc lamp was used to photolyze the samples. After the initial measurement of resonance signal intensity, the tube was removed from the cavity, briefly cooled in an ice bath, and placed several inches from the lamp for the desired length of time. For the experiments with DMPC and with DPPC–cholesterol, the sample was inserted into the resonance cavity from the back and the beam from a Spectra Physics 164-05 argon laser operating at 300 mW, at 351.1 and 353.8 nm, and was directed into the cavity from the front, down the axis of the sample tube.

Results

Properties of Alkyl Pentacyanocobaltates. Carboxymethyl pentacyanocobaltate (I) was found to be unsuitable for three reasons. First, to ensure that the protonated form of the radical did not penetrate the membrane, the pH had to be kept above 9. Second, a large excess of the cobalt complex had to be used, which resulted in nonspecific reactions between the radical species and the phospholipids. Third, the reactions with nitroxides occur only in the absence of oxygen. Sulfonylmethyl pentacyanocobaltate (II) was found to be unsuitable because,

[1] Abbreviations used: Tempol, 4-hydroxyl-2,2,6,6-tetramethylpiperadine-1-oxyl; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine.

like the carboxy compound, nonspecific reactions occur between the radical and phospholipids, and with oxygen.

To ensure high specificity for the nitroxide group, a benzyl compound, 4-(*N*-methyl)pyridylmethyl pentacyanocobaltate (III) was synthesized. The benzyl radicals reacted with neither phospholipids nor oxygen, even when cobalt complex was present in great excess. However, the radical readily penetrated phospholipid bilayers (presumably due to the highly delocalized charge of the pyridinium ring). Also, this complex photolyzed in laboratory light.

4-(*N,N,N*-Trimethylamino)benzyl pentacyanocobaltate (IV) does not penetrate phospholipid bilayers, is stable in air and light for several days, and is highly specific for nitroxides in the presence of air and phospholipids. This compound was used in all of the following experiments.

Reduction of Nitroxides. When an excess of cobalt complex IV is added to multilamellar liposome preparations and photolyzed, a plot of signal destroyed vs. time of photolysis shows an initial sharp decrease in signal intensity which then asymptotes to a much lower rate of reduction (Figure 1). We interpret this as rapid destruction of the nitroxides on the surface of the liposomes, followed by slow leakage of radicals through the membrane, destroying internal nitroxides. Assuming the rate of leakage is linear over the range of photolysis times, the proportion of nitroxides on the surface can be determined by extrapolating the slow rate of signal decrease back to zero time. This is done graphically in Figure 1.

To test the precision of this technique, pure DPPC dispersions were prepared seven times by hydrating for 1 min at about 55 °C and gently vortexing to disperse the lipid in buffer. The external label was found to be 8.0% and never varied from this by more than 0.5%. To what extent these variations are due to differences in the liposome preparations or to error in the measurement is not clear. It is clear, however, that the results are reproducible to within ±0.5% of the total signal intensity, and to within ±5% of the external signal.

DPPC Liposomes. Liposomes of pure DPPC with 0.5% spin label were prepared by a variety of methods. (1) Hydration time was varied from 1 to 60 min. Gentle vortexing was needed to detach the lipid from the glass surfaces for all hydration times. In all cases, 8.0 ± 0.5% of the signal was on the surface. (2) Liposomes dispersed by vigorous vortexing, either initially to detach from the glass or after gently vortexing, always had 5.5 ± 1% external signal. (3) Lipid was dispersed by adding a glass bead and shaking by hand; 8.5 ± 0.5% of the signal was external, but those liposomes showed very high permeability to the radicals. (4) Lipid hydrated 11 h readily formed liposomes when gently shaken by hand. External signal equalled 5.5%. (5) Using higher or lower concentrations of cobalt complex, employing larger flasks so that the lipid was spread over larger surface area, or hydrating and dispersing at higher or lower temperatures (all above the chain-melting transition temperature, 41 °C) had no effect upon the percent of external spin label. (The lipid coating the larger flasks was always at least several hundred layers thick, so our results are not directly comparable to those of Reeves & Dowben (1969).)

DMPC Liposomes. DMPC was hydrated at 37 °C, roughly the same elevation above the chain-melting transition temperature as DPPC. When hydrated for 1 min and vortexed gently, the external signal was 7–7.5%. When dispersed by vigorous vortexing, external signal averaged 9%, but results were so irregular that there is no way to estimate the significance of this number. When hydrated 12 h and dispersed by gently shaking by hand, the external signal was between 5 and 5.5%.

Cholesterol-Containing Liposomes. When liposomes contained 10–50% cholesterol, hydration became significantly slower, especially for high concentrations of cholesterol. Hydration times of up to 1 h and gentle vortexing gave very irregular results. When the lipid was hydrated for 11 h and dispersed by gently shaking by hand, *the external signal was always between 5 and 5.5%, independent of cholesterol concentration.*

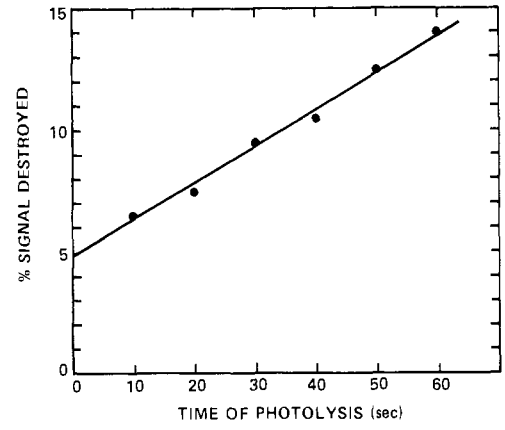


FIGURE 1: Rate of signal reduction for 90% DPPC: 10% cholesterol, prepared by hydrating lipid films 11 h and gently shaking by hand, shows an initial sharp decrease (complete in 10 s) followed by slow reduction (presumably due to leakage of radicals through the membrane). The slow rate is extrapolated back to $t = 0$; the intercept gives the percent of spin label on the external liposomal surface.

Dicetyl Phosphate-Containing Liposomes. Ten percent dicetyl phosphate, 90% DPPC liposomes prepared by gently vortexing gives 11% exposed signal.

Discussion

We have shown that 4-(*N,N,N*-trimethyl)aminobenzyl pentacyanocobaltate (IV) produces a radical upon photolysis which reacts rapidly with nitroxides and crosses lipid bilayers only slowly. We have employed this compound to measure the proportion of exposed spin label in multilamellar liposomes. We have found that for pure DPPC liposomes short hydration times and gentle vortexing yields 8% exposed spin label while vigorous vortexing or long hydration times with gentle hand shaking yields 5.5% exposed spin label. For DMPC and DPPC–cholesterol liposomes, long hydration times and gentle hand shaking yield 5–5.5% exposed spin label, independent of composition.

If we assume that the spin-labeled lipid is distributed randomly between the many lamellae of the liposomes, and that the diameter of the liposomes is much greater than the total thickness of the bilayers (as we have observed in the optical microscopy), we can calculate the average number of lamellae per liposome from the percent exposed signal. This is done in Figure 2. Eight percent exposed signal corresponds to 6.25 bilayers, while 5% exposed spin label corresponds to 10.0 bilayers.

The spontaneous formation of multilamellar liposomes implies that cohesive forces exist between layers. Thus the fact that vigorous vortexing or long hydration favor liposomes with more lamellae can be understood if these conditions favor thermodynamic equilibrium. It is not clear why liposomes averaging 10 lamellae form so consistently for all compositions, and why more than 10 lamellae are not found under any conditions. Kinetic factors may inhibit the formation of thicker liposomes, or forces may exist which favor few bilayers so that true thermodynamic equilibrium exists in our preparation; our results do not distinguish between these possibilities.

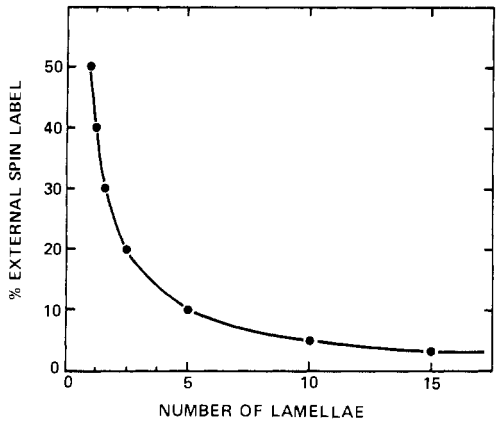


FIGURE 2: Calculated average number of lamellae per liposome as a function of exposed spin label.

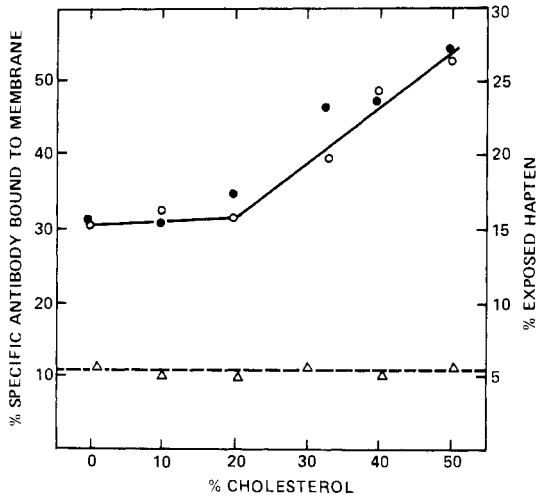


FIGURE 3: The solid line represents binding of specific IgG to DPPC-cholesterol membranes containing 0.5% spin label IV, taken from Brûlet & McConnell. Points O give IgG binding and points ● give corresponding Fab binding. The broken line represents the percent of hapten exposed on the outer surface for similarly prepared membranes.

These results bear upon the work of Brûlet & McConnell (1976, 1977) who measured the binding of anti-nitroxide antibodies to head group spin-label haptens in multilamellar liposomes. Their results are displayed in Figure 3. Their discovery that antibody binding increased with higher cholesterol content could have been due either to a higher proportion of exposed haptens or to enhanced binding to the same amount of hapten. Superimposed on their results are our results for the percentage of exposed spin label as a function of cholesterol content. It is clear that enhanced antibody binding is due to changes in mobility or availability of the same number of haptens, rather than the presence of a greater number of haptens.

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