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THE INTERACTION OF 3,3′,4′,5-TETRACHLOROSALICYLANILIDE WITH PHOSPHATIDYLCHOLINE BILAYERS

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Summary

1. The interaction of the germicide 3,3′,4′,5-tetrachlorosalicylanilide (T_4CS) with vesicles and dispersions of egg phosphatidylcholine has been studied by gel permeation chromatography, electron microscopy, electron spin resonance spin labelling and ion permeability measurements.

2. Incorporation of T_4CS into vesicles of egg phosphatidylcholine gives rise to a large increase in the permeability rate of the paramagnetic cation *N,N*-dimethyl-*N*-(1′-oxyl-2′,2′,6′,6′-tetramethyl-4′-piperidyl)-2-hydroxyethylammonium chloride through the lipid bilayer but has no significant effect on the vesicle sizes as measured by gel permeation chromatography or electron microscopy.

3. ESR studies using a spin-labelled fatty acid have demonstrated the presence of two different environments for the spin label when T_4CS is incorporated into phosphatidylcholine bilayers. These two environments are identified as (a) highly ordered areas of the bilayer, rich in T_4CS and (b) areas with very similar ordering to that in pure egg phosphatidylcholine.

4. The effectiveness of very low concentrations of the germicide in increasing vesicle permeability is explained in terms of its clustering to give rigid patches, rich in T_4CS, rather than being evenly distributed throughout the bilayer. It is proposed that the increased ion permeability arises from leakage at the interfaces between the rigid and flexible regions of the lipid bilayer.

5. Comparisons between the effective levels of T_4CS in phosphatidylcholine vesicles and its minimum inhibitory concentration with a Gram-positive bacterium confirm the validity of phospholipid vesicles as a model for studies of germicidal activity.

Abbreviations: T_4CS, 3,3′,4′,5-tetrachlorosalicylanilide; tempocholine chloride, *N,N*-dimethyl-*N*-(1′-oxyl-2′,2′,6′,6′-tetramethyl-4′-piperidyl)-2-hydroxyethylammonium chloride; 12NS, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinoxyl; PC/T_4CS, dispersion of egg phosphatidylcholine with T_4CS.

Introduction

3,3′,4′,5-Tetrachlorosalicylanilide (T_4CS) is a powerful bactericidal agent particularly against Gram-positive bacteria. Its commercial use has been discontinued on account of its light-mediated reaction with human skin [1].

T_4CS has previously been shown to be an uncoupler of oxidative phosphorylation [2] and to cause increases in the permeability of bacterial membranes to nitrate ions [3] and to protons [4]. The exact nature of the interaction between T_4CS and the bacterial membrane, however, has not been clearly defined.

In this paper, we describe the effect of T_4CS on the structure and organisation of vesicles, formed by the controlled sonication of aqueous dispersions of egg phosphatidylcholine. Systems of this type have previously been used as models for the interaction of antibiotics with the phospholipid bilayer regions of bacterial and other biological membranes [5—7].

The techniques employed are gel permeation chromatography, electron microscopy, electron spin resonance (ESR) spin labelling and ion permeability measurements.

Materials and Methods

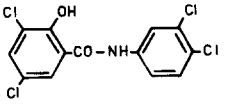
Egg phosphatidylcholine was purchased from Lipid Products, South Nutfield, U.K., and was used without further purification.

3,3′,4′,5-Tetrachlorosalicylanilide (T_4CS) was purchased from Ciba-Geigy Ltd. and recrystallised from isopropanol/dioxan. It was found to be pure by thin-layer chromatography.

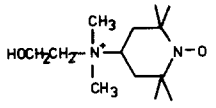
N,*N*-Dimethyl-*N*-(1′-oxyl-2′,2′,6′,6′-tetramethyl-4′-piperidyl)-2-hydroxyethylammonium chloride (tempocholine chloride) was prepared by the method of Kornberg and McConnell [8].

2-(10-Carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinoxyl (12NS) was synthesised by the method of Waggoner et al. [9].

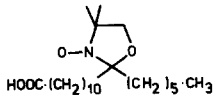
The chemical structures of these compounds are shown below.



T_4CS



Tempocholine



12NS

Dispersions of egg phosphatidylcholine or PC/T_4CS were prepared by mixing the appropriate chloroform/methanol solutions, evaporating to dryness and shaking in buffer solution (0.1 M NaCl, 0.01 M Tris, 0.001 M EDTA, 0.02% NaN_3, pH 8.6). Sonication was carried out using a Kerry Vibrason 150 (Kerry Ultrasonics Ltd., Hitchin) at low power, tuned to give maximum cavitation. The samples (1 ml) were sonicated for five 2-min periods in a polypropylene centrifuge tube surrounded by crushed ice and flushed with nitrogen [10].

For the gel permeation and leakage experiments, 70 mM and 1 mM tempocholine chloride, respectively, was added to the buffer prior to sonication. For the ESR spin probe work the spin-labelled fatty acid (12NS) was added in chloroform/methanol at the initial mixing stage to give a final probe concentration of 10^{-4} M, in 5% phosphatidylcholine (lipid/probe molar ratio = 670 : 1), and sonication was omitted.

Gel permeation chromatography. Gel permeation chromatography was carried out at room temperature using a column of Sepharose 4B (45 cm × 5 cm^2) in the buffer described above. The column eluate was monitored using an LKB Uvicord absorptiometer operating at 280 nm.

The column was calibrated by plotting the penetration coefficients, K_d of marker proteins against their Stokes' radii:

$$K_d = \frac{V_e - V_0}{V_t - V_0}$$

where V_e is the elution volume and V_0 and V_t are the column void and total volumes, respectively. The column calibration was in good agreement with published data for Sepharose 4B in an identical buffer system [11, 12].

Electron microscopy. Electron microscopy of vesicle preparations was performed using a JEOL JEM 7 instrument.

Samples were prepared by placing one drop of a diluted (about 0.1%) vesicle peparation on a carbon-coated copper grid. One drop of 1% potassium phosphotungstate solution (pH 7.0) was added and the grid was blotted immediately with filter paper.

Permeability measurements. A dispersion of egg phosphatidylcholine (1 ml, 5%) sonicated in buffer containing 1 mM tempocholine chloride, was placed in an open-ended dialysis bag (Visking 8/32 inch) and dialysed against 4.5 l of distilled water with rapid stirring. The stirring was sufficiently rapid to cause some agitation of the dialysis bag, the contents of which were mixed during the sampling procedure. The tempocholine chloride concentration in the dialysis bag was measured by ESR at 30-min intervals.

Electron spin resonance spectroscopy. ESR spectra were measured on a Varian E-4 spectrometer equippped with an E-257-9 variable temperature accessory.

For the measurement of tempocholine concentrations, spectra were run under identical conditions of microwave power and modulation amplitude. The concentrations were then calculated, relative to the initial concentration (1 mM) from the respective spectral peak amplitudes and spectrometer gains.

When fatty acid spin labels such as 12NS are dissolved in phospholipid dispersions, they undergo rapid anisotropic rotation about an axis perpendicular to the surface of the lipid bilayer [13]. If we ascribe a coordinate system to the

nitroxide radical such that the N-O bond is along the x-axis and the 2p-type orbital is along the z-axis, then the mean square deviation of the z-axis during rotation about the axis perpendicular to the bilayer surface is given by $\cos^2\theta$ where θ is the angle between the two axes. The degree of order of the z-axis with respect to the bilayer is given by

$$S_3 = \tfrac{1}{2}(3\langle\cos^2\theta\rangle - 1)$$

(using the notation of Seelig [13].)

The order parameter S_3 may be determined experimentally from the expression

$$S_3 = \frac{T_\parallel - T_\perp}{T_{zz} - T_{xx}}$$

where T_{zz} and T_{xx} are the values of the hyperfine coupling tensor in the z- and x-directions (assuming axial symmetry) and the spectral parameters $T_\parallel$ and $T_\perp$ (the observed hyperfine couplings parallel and perpendicular to the magnetic field) are measured as indicated in Fig. 1.

In some spectra, particularly those arising from several different nitroxide environments, it is only possible to measure $T_\parallel$.

As $a_N = 1/3\,(T_\parallel + 2\,T_\perp)$ where a_N is the isotropic hyperfine coupling constant, $T_\parallel$ may be used directly as a measure of the order parameter, S_3.

Results

Gel permeation chromatography

Fig. 2 shows the elution profile on Sepharose 4B of a 5% dispersion of egg phosphatidylcholine containing T_4CS (10/1, molar ratio) (1 ml) sonicated in the presence of tempocholine chloride (73 mM). The peak at V_e = 220 ml (V_t) arose from the excess of tempocholine chloride in the system. The small peak at the void volume (71 ml) indicated that some multilamellar phospholipid remained in the system. The bulk of the material was eluted as vesicles at an elution volume of 130 ml corresponding to a Stokes' radius of 102 Å. The concentrations of tempocholine chloride remaining inside the vesicles were mea-

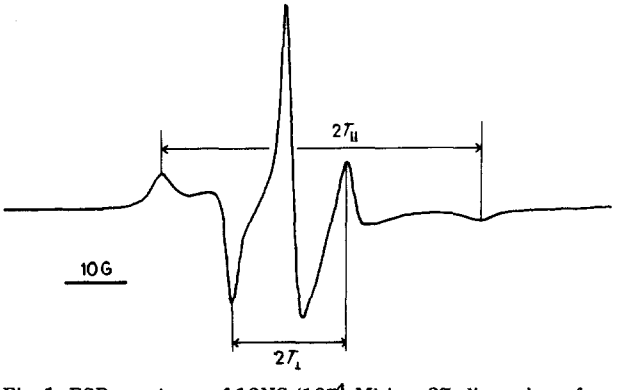


Fig. 1. ESR spectrum of 12NS (10^{-4} M) in a 2% dispersion of egg phosphatidylcholine, pH 7.5.

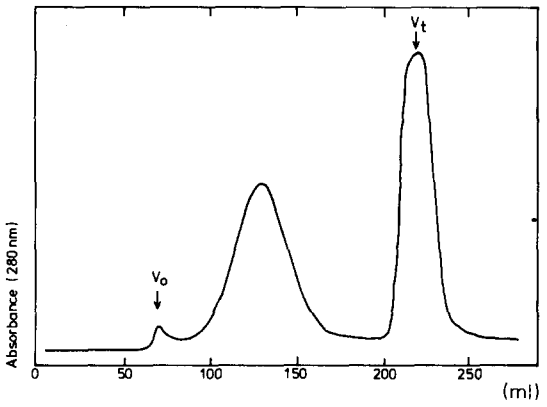


Fig. 2. Elution profile of a sonicated dispersion of egg PC/T_4CS (molar ratio, 10/1) from Sepharose 4B.

sured by ESR for the fraction of the peak maximum for 1 ml samples containing phosphatidylcholine (5%) run through the column under identical conditions.

The measured Stokes' radii and tempocholine concentrations for sonicated egg phosphatidylcholine dispersions with several ratios of T_4CS are shown in Table I. The incorporation of T_4CS into vesicles of phosphatidylcholine resulted in a loss of tempocholine chloride from inside the vesicles. There was no significant change in either the Stokes' radius or the elution profile of the vesicles on incorporating T_4CS. The average value for all the samples was 107 ± 6 Å.

Electron microscopy

Fig. 3 shows electron micrographs of negatively stained sonicated dispersions of egg phosphatidylcholine and PC/T_4CS (10/1). The vesicles of PC/T_4CS show penetration of the stain into the internal compartment whereas those of the pure phosphatidylcholine do not.

TABLE I

STOKES' RADII AND TEMPOCHOLINE CONCENTRATIONS IN VESICLE FRACTIONS

Egg PC/T_4CS (molar ratio)	Stokes' radius (Å)	Tempocholine concn. (M)
Pure phosphatidylcholine	102, 104, 112 106	$2.3 \cdot 10^{-5}$
160	102	$9.4 \cdot 10^{-6}$
80	102	$2.0 \cdot 10^{-6}$
40	102	$0.8 \cdot 10^{-6}$
20	115	$<2 \cdot 10^{-7}$
10	112, 102	$<2 \cdot 10^{-7}$
Average:	107 ± 6	

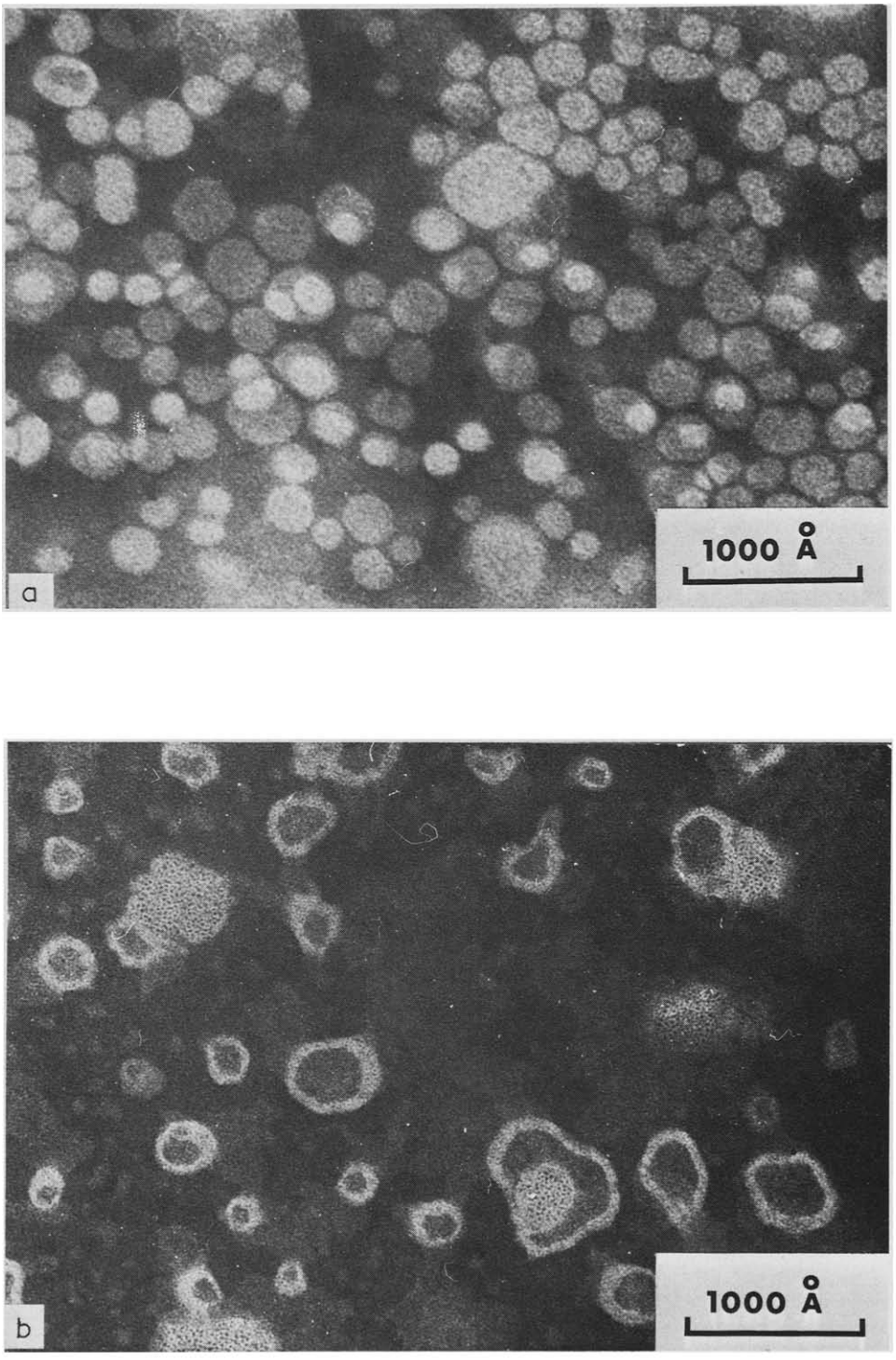


Fig. 3. Electron micrographs of negatively stained vesicles of (a) egg phosphatidylcholine, and (b) egg PC/T_4CS (molar ratio, 10/1).

Vesicle permeability measurements

Graphs of log[tempocholine] versus time are shown in Fig. 4 for sonicated dispersions of egg phosphatidylcholine and PC/T_4CS mixtures. In all cases there was a rapid initial decrease in tempocholine concentration in the dialysis bag, due to loss of tempocholine from outside the vesicles. This was usually followed by a more gradual linear decrease when loss of tempocholine through the vesicle walls became the rate-limiting step.

The gradients of the linear portions give the first-order rate constants for vesicle permeability. The ratio of the extrapolated intercept on the concentration axis to the initial concentration gives the fraction of the total volume of the dispersion which is enclosed by the vesicles. This varied from 0.9 to 1.6% probably due to variations in the conditions of sonication.

The permeability rate constants for egg phosphatidylcholine and a series of PC/T_4CS mixtures are shown in Table II. The permeability rate constant for tempocholine alone through the dialysis tubing was typically $2—3 \cdot 10^{-4}$ s^{-1}.

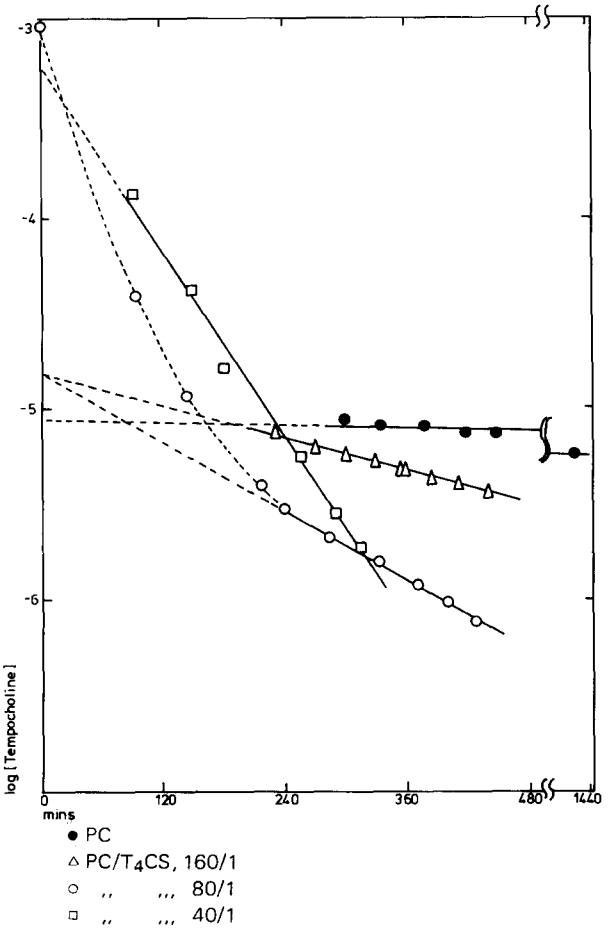


Fig. 4. Log[tempocholine] versus time of dialysis for sonicated dispersions of egg phosphatidylcholine and PC/T_4CS. PC, phosphatidylcholine.

TABLE II

PERMEABILITY RATE CONSTANTS FOR TEMPOCHOLINE

Egg PC/T_4CS (molar ratio)	Molecules of T_4CS/vesicle *	$k(s^{-1})$
∞	0	$3.3 \cdot 10^{-6}$
640	6.3	$1.6 \cdot 10^{-5}$
320	12.5	$2.9 \cdot 10^{-5}$
160 **	25	$5.6 \cdot 10^{-5}$
80	50	$1.2 \cdot 10^{-4}$
60	75	$>3.6 \cdot 10^{-4}$ ***
40	100	$>3.1 \cdot 10^{-4}$ ***

* Assuming 4000 phosphatidylcholine molecules/vesicle [15].

** Identical rate constant obtained with and without ultraviolet irradiation in presence of air.

*** These two values almost certainly reflect the permeability of the dialysis tubing rather than the vesicles.

The rate constants increase with increasing incorporating of T_4CS into the vesicles.

A graph of k/k_0 versus n (where k and k_0, respectively, are the permeability rate constants with and without T_4CS and n is the average number of T_4CS molecules/vesicle) shows that the permeability rate increases linearly with the T_4CS concentration up to about 30 molecules/vesicle (Fig. 5); at higher levels of T_4CS it increases more rapidly. This is confirmed by a plot of $\log k$ versus $\log n$ (not illustrated) which has a slope of unity for low levels ($n < 30$) of T_4CS and increases for higher levels.

The permeability rate constants were not corrected for the permeability of the dialysis tubing. At low levels of T_4CS, the correction is probably insignificant; where the permeability rate constants for the vesicles approach that of the dialysis tubing, the observed values are lower than the true rate constants.

A graph such as Fig. 5 using the true rate constant would therefore show a greater deviation from linearity than is obtained from the observed values.

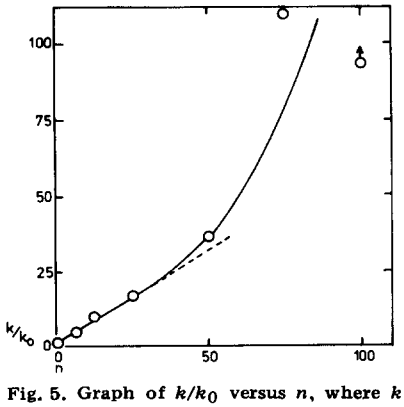


Fig. 5. Graph of k/k_0 versus n, where k and k_0, respectively, are the permeability rate constants with and without T_4CS, and n is the average number of T_4CS molecules/vesicle.

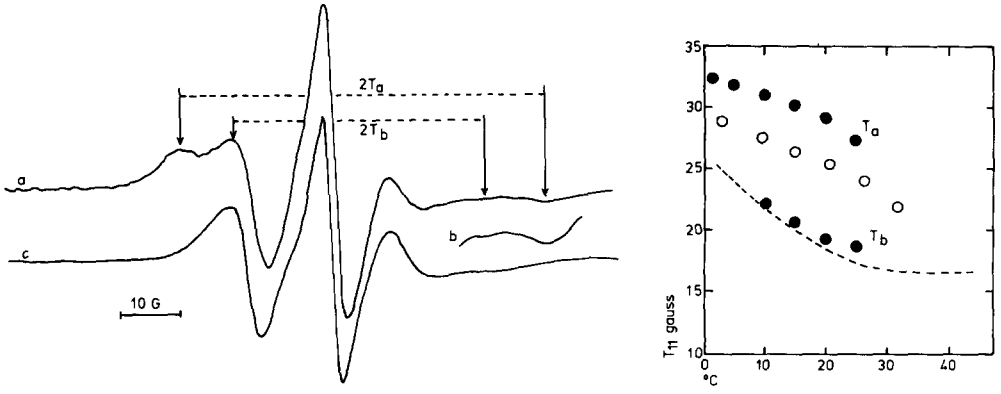


Fig. 6. ESR spectrum of 10^{-4} M 12NS in (a) a dispersion of egg PC/T_4CS (molar ratio, 2/1), pH 8.6, showing two different $T_{\parallel}$ values. (b) As (a), gain X 10. (c) In egg phosphatidylcholine (5%). Temperature 15°C, modulation amplitude, 1 G.

Fig. 7. $T_{\parallel}$ versus temperature for 12NS in dispersion of egg phosphatidylcholine (- - - - -), PC/T_4CS (molar ratio, 2/1) (●) and dimyristoyl phosphatidylcholine/cholesterol (molar ratio, 1/1) (○). T_a and T_b were measured as indicated in Fig. 6.

Electron spin resonance

Plots of order parameter S_3 versus temperature for 12NS in egg phosphatidylcholine and in PC/T_4CS (10/1) were identical within experimental error. On increasing the level of T_4CS to PC/T_4CS (2/1) it becomes clear that the ESR spectrum is a superimposition of two separate spectra (Fig. 6a and b) which appear from the low-field part of Fig. 6a to be present in roughly equal proportions.

A spectrum of 12NS in 5% egg phosphatidylcholine is also shown for comparison (Fig. 6c).

The $T_{\parallel}$ values for these two environments are shown in Fig. 7 as a function of temperature along with $T_{\parallel}$ values for 12NS in egg phosphatidylcholine and in dimyristoyl phosphatidylcholine/cholesterol (molar ratio, 1/1) for comparison.

The two components of the PC/T_4CS spectrum arise from spin probe molecules (a) in a highly ordered environment and (b) in a much less ordered environment very similar to that in pure egg phosphatidylcholine.

Discussion

Vesicles formed by sonication of aqueous dispersions of egg phosphatidylcholine are inhomogeneous with a particle size range of 95—200 Å radius [10]. The particle size range depends heavily on the exact conditions of sonication used [10].

No difference in Stokes' radius was observed by gel filtration on Sepharose 4B between vesicles of pure egg phosphatidylcholine and those containing various ratios of T_4CS. The average observed Stokes' radius of 107 ± 6 Å compares well with the values reported by other workers, e.g., 95 Å [6], 115 Å [10], 125 Å [14].

The lack of size change observed when T_4CS is incorporated into phosphatidylcholine vesicles suggests that the vesicle structure is retained to a large extent. This is confirmed by electron micrographs of negatively stained (potassium phosphotungstate) dispersions of PC/T_4CS (10/1) which show vesicle structures identical in size to those of pure phosphatidylcholine. The negatively stained vesicles of PC/T_4CS differ from those of pure phosphatidylcholine in that a large number of them show penetration of the stain into the internal compartment. Similar effects were observed when the peptide antibiotic alamethicin was incorporated into vesicles of phosphatidylcholine [15].

The permeability rate constant for tempocholine out of egg phosphatidylcholine vesicles was $3.3 \cdot 10^{-6}$ s^{-1} at room temperature (21—22°C). Comparable permeability rates are $1.6 \cdot 10^{-6}$ s^{-1} for K^+ out of egg phosphatidylcholine/4% phosphatidic acid at 37°C [16] and $8 \cdot 10^{-8}$ s^{-1} for Na^+ out of egg phosphatidylcholine vesicles at 4°C [15].

Using the activation energy for the ion permeability through phospholipid vesicles (15.4 kcal/mol [17]), the Na^+ permeability value becomes about $5 \cdot 10^{-7}$ s^{-1} at 21°C. The permeability rate for tempocholine out of phosphatidylcholine vesicles is somewhat higher than these values. This may be due to the rather more hydrophobic nature of tempocholine compared with Na^+ or K^+.

The incorporation of low levels of T_4CS into phosphatidylcholine vesicles caused an initial increase in the tempocholine permeability rate, which was linearly dependent on the concentration of T_4CS. The addition of higher levels (above about 30 molecules of T_4CS/vesicle) resulted in a disproportionately large increase in the permeability rate. The increase in the gradient of the log k versus log n plot above unity suggests that the T_4CS molecules behave cooperatively in the bilayer, i.e. they form clusters.

There was no difference in the permeability rate of a sonicated dispersion of PC/T_4CS (160/1) which had been irradiated at 360 nm (the absorption maximum for T_4CS) for 25 min in the presence of air.

It has previously been reported that T_4CS increases the photohaemolysis of red cells [18]. The lack of any observable effect of ultraviolet light on the permeability rate of PC/T_4CS vesicles suggests that the photohaemolytic effect on red cells may be a result of the interaction of T_4CS with the membrane protein rather than with the lipid. (T_4CS has been shown to react photochemically with proteins, e.g. [19].)

The results obtained by incorporating a spin-labelled fatty acid into dispersions of PC/T_4CS also give a good indication of the nature of the interaction between phosphatidylcholine and T_4CS.

The ESR results demonstrate the presence of two different motional environments, one highly ordered and the other with approximately the same fluidity as that of pure phosphatidylcholine. This supports the permeability rate results in indicating that the T_4CS molecules are clustered together, rather than being distributed evenly throughout the bilayer. The highly ordered environment has an even greater rigidity than that of a phosphatidylcholine cholesterol (1/1) mixture. The clusters probably take the form of areas rich in T_4CS rather than of areas of pure T_4CS, which would not be expected to solubilise the spin-labelled fatty acid.

The enhanced permeability of the PC/T_4CS bilayer due to clustering

probably occurs at the interface between the ordered pathces of clustered T_4CS molecules rather than through them.

The permeability of $^{22}Na^+$ through vesicles of dipalmitoyl phosphatidylcholine has been shown to reach a maximum at the mid-point of the lipid chain-melting transition [20]. Similarly, the interfaces between the rigid boundary lipid and the fluid membrane lipid have been proposed as the sites of fragility of red cell membranes [21].

How good a model is the phospholipid vesicle for the bacterial membrane? The Gram-positive bacterium *Staphylococcus aureus* has a diameter of about 1 μm and therefore a surface area of about $3.14 \cdot 10^8$ Å^2 ($6.3 \cdot 10^8$ Å^2 for both sides of the bilayer). A phosphatidylcholine bilayer of that area would contain $9 \cdot 10^6$ molecules (assuming about 70 Å^2/molecule for the area occupied by a phosphatidylcholine molecule [22]). At the minimum inhibitory concentration, the number of T_4CS molecules/bacterium is $7.5 \cdot 10^4$ [23]. The minimum inhibitory level of T_4CS is therefore one molecule/120 phosphatidylcholine molecules (assuming the bacterial membrane is made up entirely of phosphatidylcholine). Up to 80% of the membrane may be occupied by protein, therefore the minimum inhibitory level of T_4CS ranges from between 1 to 120 and 1 to 24 molecules/molecule of phosphatidylcholine. These ratios are the same as those for which we observe large increases in ion permeability in phosphatidylcholine vesicles, and appear to confirm the validity of phospholipid vesicles as a model for studies of germicidal activity.

Acknowledgement

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