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THE ORGANISATION OF CHOLESTEROL AND ERGOSTEROL IN LIPID BILAYERS BASED ON STUDIES USING NON-PERTURBING FLUORESCENT STEROL PROBES

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Summary

The fluorescence properties of dehydroergosterol and cholesta-5,7,9-trien-3β-ol have been studied in organic solution, in aqueous dispersions and incorporated into aqueous lipid dispersions. The absorption spectra of aqueous dispersions of the probes are very different to those in organic solution, and aqueous dispersions are non-fluorescent. This can be attributed to micelle formation with dimerisation and/or aggregation in the micelles. Concentration quenching also occurs when sterols are incorporated into lipid bilayers, but relatively high fluorescence is observed even at a 1 : 1 steroid : lipid molar ratio. Further, the fluorescence is still polarized at these high molar ratios. We attribute this to the formation of ordered arrays of sterol molecules in the lipid bilayers. In these arrays the sterol molecules are organised in an end-to-end fashion, and face-to-face overlap of the sterols is prevented by the lipid molecules.

Possible structures for 1 : 1 mixtures are presented.

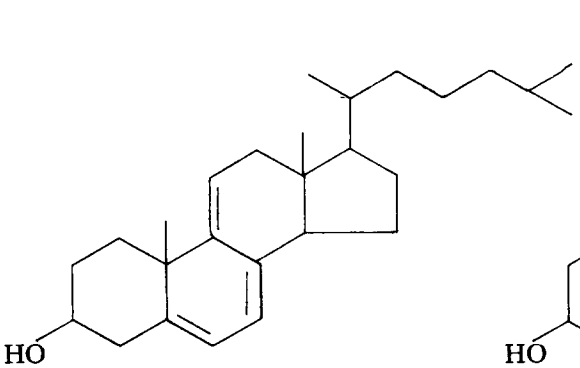
Introduction

The outer membranes of animal cells contain high proportions of cholesterol; in the case of erythrocytes, for example, the cholesterol : phospholipid molar ratio is 1 : 1 [1]. Cholesterol is not, however, a general requirement for membrane function, for the inner membranes of animal cells and the membranes of bacteria contain little or none. Related sterols are often found in membranes of other organisms: ergosterol, for example, is the predominant sterol in fungi and yeast. Many studies have shown that cholesterol has profound effects on phospholipid molecules in membranes, as it reduces the fluidity of lipids in the

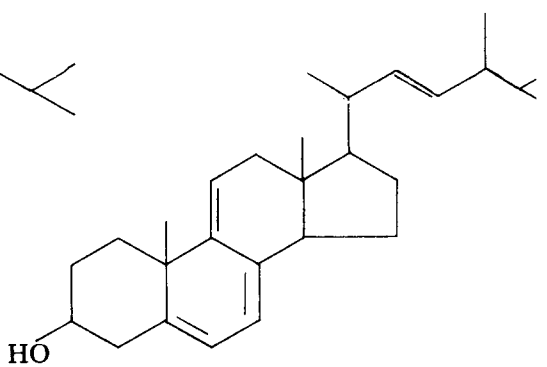
liquid crystalline state and increases the fluidity of lipids in the gel state [2,3]. Much less is known about the state of the cholesterol molecule itself.

In nuclear magnetic resonance experiments (^1H or ^{13}C) with lipid bilayers containing cholesterol, signals are generally not seen from nuclei in the sterol ring system suggesting that the sterol nucleus is immobilised in the bilayer [4—6] which is consistent with deuterium NMR studies with deuterated cholesterol [7]. Broad signals can, however, be observed for the isopropyl group of the cholesterol side chain, suggesting that this group has more mobility than the sterol nucleus [6]. These conclusions have been confirmed by more recent studies employing the techniques of proton-enhanced ^{13}C NMR [8]. However, since the ^{13}C nucleus in NMR experiments is relatively insensitive to intermolecular effects [5], this technique is unlikely to give much information about long-range organisation in the bilayer. A more suitable technique would be electron spin resonance and Trauble and Sackmann [9] have discussed the organisation of a spin-labelled derivative of 5β-androstan-17β-ol-3-one in lipid bilayers. The steroid was found to form small clusters at temperatures below the phase transition temperature of the lipid, but to form a homogeneous mixture above the phase transition. It is not clear how similar the packing of cholesterol and ergosterol will be to that of derivatives of steroid hormones, and it is also not clear how important a perturbation is caused by the bulky nitroxide spin-label group.

Since molecular fluorescence is known to be very sensitive to intermolecular interactions because of the possibility of excimer formation and concentration quenching [10] we decided to develop fluorescence techniques for studying the organisation of cholesterol and related sterols in lipid bilayers and biological membranes. Here we report on studies using two such probes, cholesta-5,7,9-trien-3β-ol (I) and ergosta-5,7,9,22-tetraen-3-β-ol (dehydroergosterol) (II), the first being a probe for cholesterol and the second for ergosterol.



(I)



(II)

Dehydroergosterol and cholesta-5,7,9-trien-3β-ol have been used previously to study the interaction between sterols and polyene antibiotics in lipid vesicles [11] and between sterols and plasma lipoproteins [12], respectively.

As shown using space-filling models (Fig. 1), the introduction of an extra double bond has virtually no effect on the three-dimensional structures of the

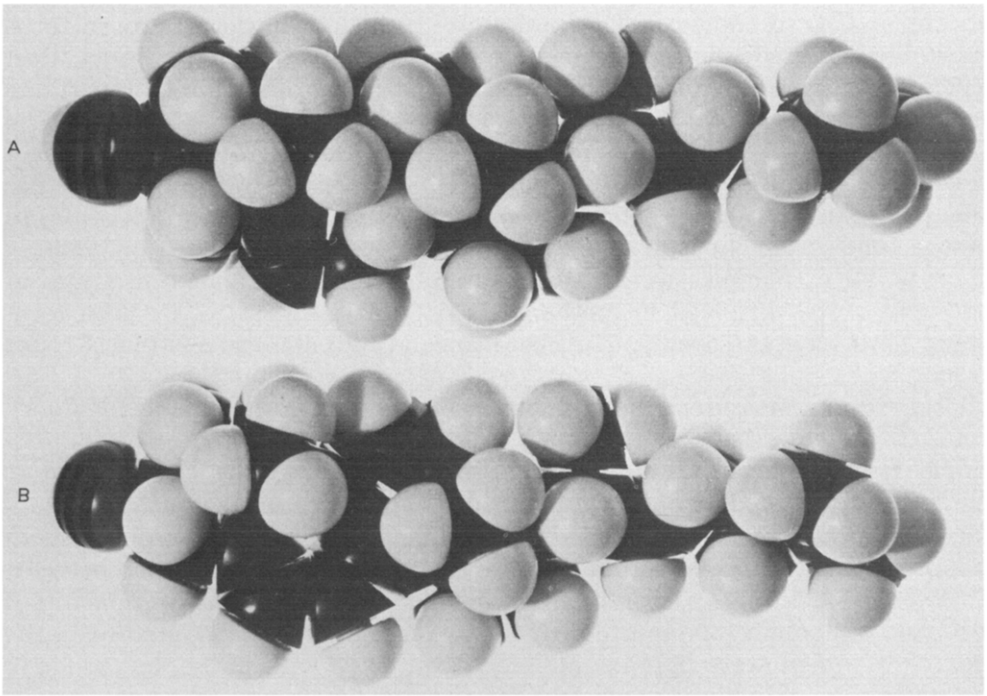


Fig. 1. Space-filling models of cholesterol (A) and cholesta-5,7,9-triene-3β-ol (B).

sterols. This was confirmed by comparison of the properties of the fluorescent probes with their parent sterols in both liposomes and biological membranes. Our results show that the fluorescence yields obtained with these non-perturbing compounds are very sensitive to inter-molecular effects and suggest the presence of extensive ordering of cholesterol molecules in lipid bilayers.

Experimental

Dipalmitoyl phosphatidylcholine was obtained from Koch-Light and egg-yolk lecithin from Lipid Products Ltd. Ergosterol (from Sigma) was recrystallised from diethyl ether/methol. 7-Dehydrocholesterol (Peboc Chemicals Ltd., Middlesex, England) was recrystallised from diethyl ether/methanol; cholesterol (from B.D.H.) was twice recrystallised from methanol.

Dehydroergosterol was prepared from ergosterol by oxidation with mercuric acetate as previously described [13]. Cholesta-5,7,9-trien-3β-ol was similarly prepared from 7-dehydrocholesterol. Both probes were recrystallised three times from diethyl ether/methanol. The mass spectrum for dehydroergosterol showed a molecular ion at 394 and non at 396 (ergosterol). The cholesterol probe showed a molecular ion of low intensity at 382 and no unchanged 7-dehydrocholesterol. After thin-layer chromatography on silica gel impregnated with 10% $AgNO_3$ each compound gave a single spot visualised with ultraviolet light, after development in chloroform/acetone/glacial acetic acid (194 : 4.6 : 1, v/v). Dehydroergosterol (II) was found to be stable when stored for many

months at 0°C in the dark. The cholesterol probe (I), however, proved to be less stable and gradually decomposed to an unidentified yellow material. Most experiments were therefore performed with dehydroergosterol. Liposomes were prepared by dissolving lipids plus fluorescent probe ($7.5 \cdot 10^{-9}$ mol) in chloroform in 10-ml stoppered flasks and evaporating to dryness under a stream of N_2. 4 ml 0.01 M sodium phosphate (pH 7.2)/0.1 M NaCl buffer was added and the flask was very briefly dipped into a sonicating bath (Megason) to remove solids from the side of the flask. The mixture was shaken on a vortex mixer at 50°C. Samples were also prepared by injecting 25 μl of mixtures of lipid and probe dissolved in methanol into 3 ml buffer at 50°C. Both techniques gave identical results. The absorbance at 325 nm was less than 0.2 for all samples.

Fluorescence measurements were made on an Aminco Bowman SPF fluorimeter, the sample temperature being continuously monitored with a thermocouple inserted into the sample. Fluorescence of the probes was excited at 325 nm and recorded at 385 nm. Corrections for scattering were made by subtracting the reading obtained for lipid samples either without probe or with cholesterol instead of the probe; there was no significant difference between the two methods. For samples containing high concentrations of dipalmitoyl phosphatidylcholine (of the order of $5 \cdot 10^{-3}$ M); the readings obtained in the absence of probe were approx. 50% less than those with probe, but at lower concentrations of lipid the correction for scattering was only approx. 10%.

Fluorescence polarization measurements were made on the Aminco Bowman fluorimeter using Quartz Polacoat filters. Values of the polarization were calculated using the standard equation:

$$P = \frac{I_{11} - cI_1}{I_{11} + cI_1}$$

Where I_{11} and I_1 are the fluorescence intensities observed with the analysing polarizer, parallel and perpendicular, respectively, to the polarized excitation beam and c is a constant to correct for instrument polarization. The constant c and corrections for blank scattering were determined as discussed by Shinitzky et al. [14].

Tetrahymena pyriformis strain W cultures (200 ml) were grown as described previously [15]. The harvested cells were suspended in 10 ml 10% methanolic KOH (w/v) and an internal standard of β-amyrin (0.7 μmol) was added. After saponification at 70°C for 1 h water (2 ml) was added and the neutral lipids were extracted twice with 5 ml of light petroleum (b.p. 40—60°C)/diethyl ether (1 : 1, v/v). The combined extracts were dried (anhydrous Na_2SO_4) evaporated and redissolved in ethyl acetate for analysis by gas-liquid chromatography (GLC) [16]. For experiments involving water permeation through liposomal membranes, multilamellar liposomes were prepared from dimyristoyl phosphatidylcholine (from Koch-Light) containing 4% myristic acid (from Sigma) together with the required amount of sterol. The liposomes were suspended in 0.01 M sodium phosphate buffer (pH 7.2) containing 3 mM EDTA and 10 mM glucose at a final concentration of 0.92 mM phospholipid. The liposomes were subjected to an osmotic shock with glucose (120 mM in buffer) and the rate and extent of shrinkage was monitored by the decrease in

absorbance at 400 nm after mixing in a 1 : 1 ratio using a stopped-flow spectrophotometer. The activation energies for water permeation at temperatures above the phase transition (>30° C) were calculated for each sterol according to the method described by Blok et al. [17].

Results

Fluorescence properties of the probes

Fig. 2 shows the uncorrected fluorescence excitation and emission spectra for the cholesterol probe (I) in chloroform. They are very similar to those previously published for these fluorescent sterols [11,12]. The excitation and emission maxima are rather close, which creates problems with light scattering, but the separation is comparable to that of other probes in membrane studies [18]. In chloroform solution, the fluorescence intensity of cholesterol probe (I) increases linearly with concentration up to approx. $3 \cdot 10^{-5}$ M, at which point the absorbance has reached 0.35 so that self-absorption becomes important. This is consistent with observations (using nuclear magnetic resonances) that only monomers and dimers are formed in solutions of cholesterol, with dimers appearing at relatively high concentrations [19]. In contrast, no fluorescence was observed from aqueous dispersions of cholesterol probe (I). Further (Fig. 3), the intensity of the absorption spectrum in water is very considerably less than that for the same amount of probe in chloroform. The loss of fluorescence and hypochromicity compares with that observed for many organic dyes in solution [20,21] and has been attributed to aggregation. These results, therefore, suggest the formation of micelles in aqueous solution, with strong quenching of fluorescence: the critical micelle concentration for cholesterol in aqueous solution is 25—40 nM [22].

The polarisation of the spectra were measured at 385 nm as a function of excitation in a variety of organic solvents and the polarisation varied from 0.15 in diethyl ether to 0.25 in *n*-hexadecane, exciting between 270 and 350 nm.

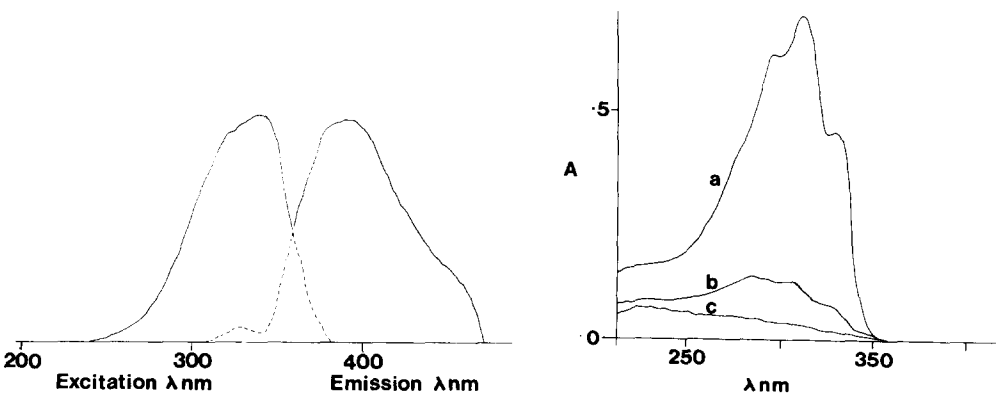


Fig. 2. Fluorescence excitation spectrum (emission recorded at 385 nm) and fluorescence emission spectrum (excitation at 325 nm) of cholesta-5,7,9-trien-3β-ol in chloroform solution.

Fig. 3. Absorption spectra of cholesta-5,7,9-trien-3β-ol. a, $5.24 \cdot 10^{-5}$ M in chloroform; b, $2.62 \cdot 10^{-5}$ M in a 1 : 1 molar ratio with dipalmitoyl phosphatidylcholine at 23° C; c, $5.24 \cdot 10^{-5}$ M in water.

The polarization measured in propylene glycol at about −50°C is 0.34. These values are comparable to those measured for other common fluorescent probes [14,23].

Fluorescence spectra in phospholipids

Figs. 4a and 4b show the variation in fluorescence intensity for dehydroergosterol incorporated into liposomes of egg phosphatidylcholine and dipalmitoyl phosphatidylcholine as the lipid probe molar ratio is changed, while keeping the amount of probe constant. Identical results were obtained with the two probes. The results shown are the averages of four determinations since some variation in absolute levels of fluorescence was observed between samples. In the absence of any concentration quenching, the fluorescence intensity would have remained constant, since the amount of fluorescent probe was constant. In fact, in liposomes of egg phosphatidylcholine the fluorescence intensity was much higher in those liposomes containing a high proportion of lipids. Fluorescence was consistently less in liposomes of dipalmitoyl phosphatidylcholine and showed less variation with varying concentration. An important observation is, however, that even at a 1 : 1 lipid/probe ratio, the fluorescence had not declined to zero. Fluorescence quenching for chlorophyll *a* in liposomes, for example, resulted in zero fluorescence at high chlorophyll/lipid molar ratios [24,25]. One possibility is that the fluorescence observed at high probe/lipid molar ratios is in fact not due to monomeric probe molecules but is due either to some aggregated species or to excimer fluorescence. This is unlikely, since the fluorescence spectrum appears not to change in shape with changing molar ratio of probe: the ratios of the fluorescence intensities measured at equal wavelengths for liposomes with lipid/probe molar ratios of 500 : 1 and 1 : 1 were constant over the whole excitation and emission spectra. We therefore conclude that the fluorescence observed was that due to monomeric probe molecules and the observed decrease in fluorescence was due to con-

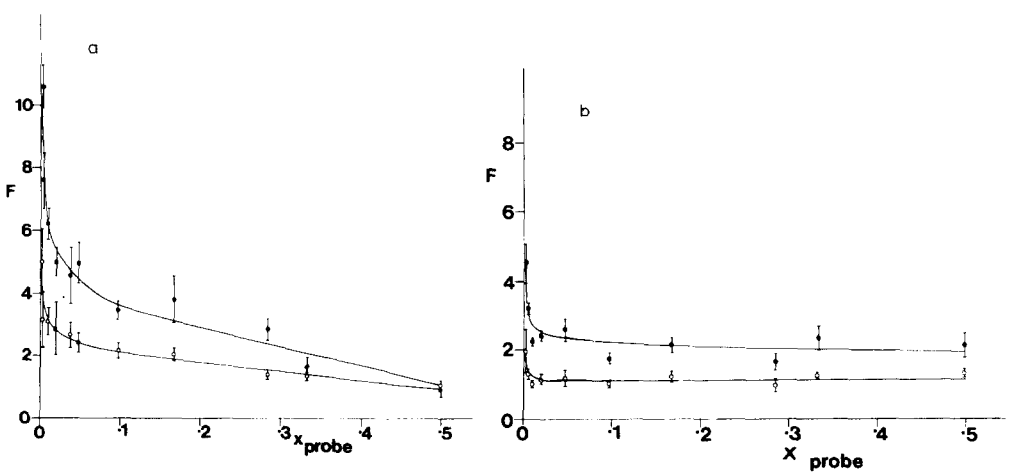


Fig. 4. (a) Plots of fluorescence intensity (arbitrary units) vs. mol ratio of dehydroergosterol in egg phosphatidylcholine containing a constant amount of probe ($7.5 \cdot 10^{-9}$ M), measured at 20°C (•) and 50°C (○). (b) Plots of fluorescence intensity (arbitrary units) vs. mol ratio of dehydroergosterol in dipalmitoyl phosphatidylcholine containing a constant amount of probe ($7.5 \cdot 10^{-9}$ M), measured at 20°C (•) and 50°C (○).

centration quenching as observed in aqueous dispersions. Consistent with this interpretation is the difference in absorption spectra for cholesterol probe (I) in liposomes and in chloroform solution (Fig. 3).

If the concentration quenching were due to deactivating collisions between excited molecules and molecules in the ground state, the fluorescence intensity, N, would follow a Stern-Volmer relation of the type:

$$\frac{N_0}{N} = 1 + \frac{C}{C^*}$$

where N_0 is the fluorescence intensity in the absence of quenching, C is the molar ratio of fluorophor and C^* is the molar ratio at which the fluorescence yield has fallen to one half [9]. To explain the decline in fluorescence intensity at low probe/lipid molar ratios, C^* would have to be, for example, 0.03 in egg lecithin at 20° C. However, this would predict that the fluorescence intensity at 1 : 1 probe/lipid molar ratio would be a factor of 24 smaller than that at a molar ratio of 1 : 500; in fact it is only a factor of 3 smaller. It is clear, therefore, that the Stern-Volmer relation does not explain the experimental data. Similar conclusions apply to the other concentration-fluorescence plots in Figs. 4a and 4b. Preliminary fluorescence lifetime measurements show that the fluorescence decay for dehydroergosterol in liposomes of dipalmitoyl phosphatidylcholine at 20° C cannot be fitted to a single exponential, so that the decay is complex. However, there is no significant difference between decay curves recorded at sterol/lipid molar ratios 1 : 100 and 1 : 1, so that again it appears that Stern-Volmer quenching (which would lead to a large decrease in fluorescence lifetime) cannot be important.

A further interesting observation is that the fluorescence intensity of dehydroergosterol in liposomes decreases with increasing temperature (Figs. 4 and 5). A decrease in fluorescence with increasing temperature has been observed for a large number of fluorescent dyes and attributed in the Stern-Volmer model, to an increased rate of collision between excited molecules and molecules in the ground state [10].

In bilayers of dipalmitoyl phosphatidylcholine containing dehydroergosterol at a probe/lipid molar ratio of 1 : 100, there is a marked drop in fluorescence intensity centred at 40° C which can be attributed to the presence of a phase transition in dipalmitoyl phosphatidylcholine observed calorimetrically at 41.75° C. The temperatures of onset and completion of the transition are clearly detectable for mixtures with low probe/lipid molar ratios, and these temperatures are plotted in Fig. 6. However, with increasing probe concentrations, the transition broadens and is no longer detectable at a molar ratio of 1 : 1. Identical results were obtained with cholesterol probe (I). Similar changes in transition temperature were observed when varying amounts of cholesterol were added to liposomes of dipalmitoyl phosphatidylcholine containing a fixed amount of dehydroergosterol at a total lipid : probe ratio of 100 : 1 (Fig. 6).

Energy transfer to perylene

To show that resonance energy transfer involving the probe molecules is possible in these bilayers, an experiment was performed to see whether resonance energy transfer could occur between probe and perylene. The excita-

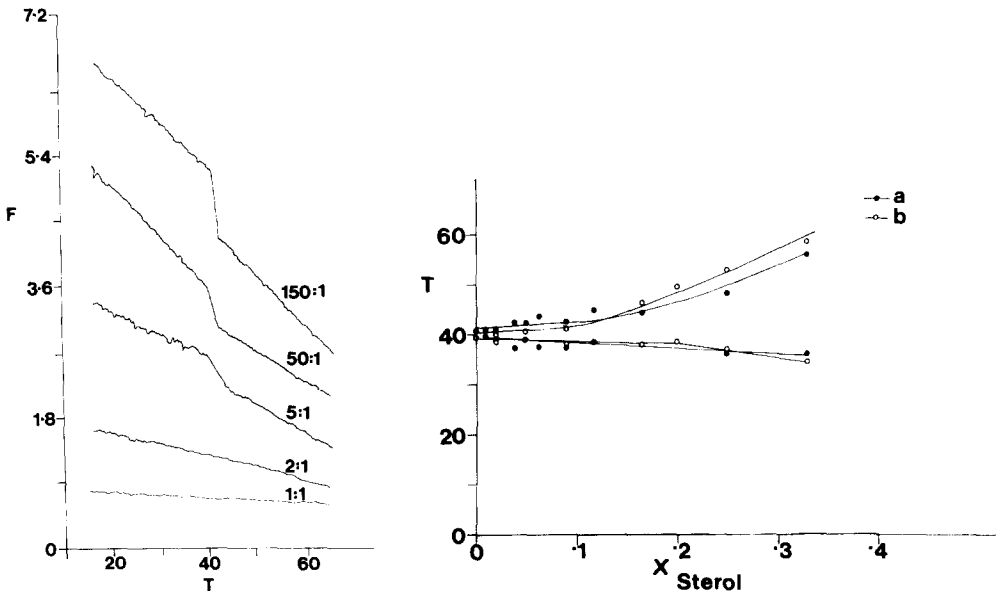


Fig. 5. Plots of fluorescence intensity vs. temperature for $7.5 \cdot 10^{-9}$ M dehydroergosterol in dipalmitoyl phosphatidylcholine at the given lipid/probe molar ratios.

Fig. 6. Plots of the temperature of onset and completion of the phase transition vs. mol ratio of sterol. (a) For a constant amount of probe ($7.5 \cdot 10^{-9}$ M dehydroergosterol) in dipalmitoyl phosphatidylcholine, varying the lipid/sterol molar ratio by varying the amount of lipid. (b) For a constant amount of probe ($7.5 \cdot 10^{-9}$ M dehydroergosterol) in liposomes comprising various ratios of dipalmitoyl phosphatidylcholine/cholesterol.

tion maximum of perylene is close to the emission maximum of dehydroergosterol. As shown in Fig. 7, perylene added to a lipid dispersion showed no fluorescence at 450 nm when excited at 325 nm. In the presence of dehydroergosterol, however, excitation at 325 nm did result in fluorescence at 450 nm and increasing the amount of perylene in the system caused an increase in the

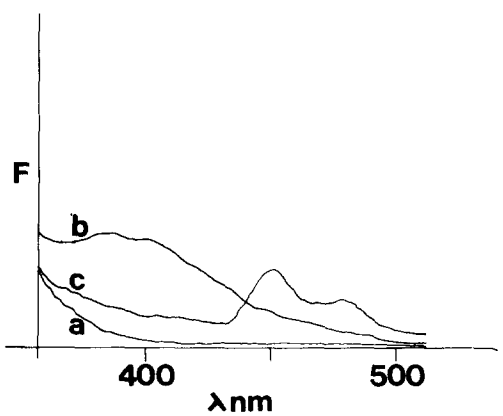


Fig. 7. Energy transfer from dehydroergosterol to perylene in liposomes of dipalmitoyl phosphatidylcholine, exciting fluorescence at 325 nm. a, Liposomes containing perylene; b, liposomes containing dehydroergosterol; c, liposomes containing dehydroergosterol plus perylene.

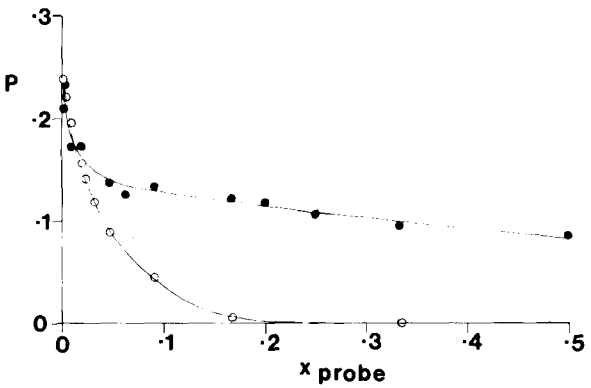


Fig. 8. Plots of fluorescence polarization vs. mol fraction of cholesta-5,7,9-trien-3β-ol in dipalmitoyl phosphatidylcholine at 20°C (●): calculated values using the Forster relationship with $P_0 = 0.25$ and $c_0 = 0.07$ (○).

fluorescence at 450 nm at the expense of that due to dehydroergosterol at 385 nm. That this is not a trivial reabsorption process is shown by the fact that the absorbance of the perylene solutions used was less than 0.01 at 285 nm and also by the fact that the observed effects took about 5—10 min to develop: this time presumably corresponding to the time taken for the added perylene to partition into the lipid phase [26].

Fluorescence polarization

Fig. 8 shows the measured fluorescence polarization for the cholesterol probe (I) in liposomes of dipalmitoyl phosphatidylcholine at 20°C as a function of sterol/lipid molar ratio. Again, although there is a decrease in polarization with increasing molar ratio of sterol, fluorescence is still polarized at a 1 : 1 molar ratio. Similar results were obtained at 50°C, with a fluorescence polarization of 0.05 at a 1 : 1 sterol/lipid molar ratio. Forster [27] suggested that depolarization can be brought about by energy transfer between like molecules, in the absence of molecular motion. The equation describing depolarization is

$$\frac{P}{P_0} = 1 - \pi^{1/2}\lambda \exp(\lambda^2)(1 - \operatorname{erf} \lambda)$$

where P and P_0 are the polarizations in the presence and absence of energy transfer and λ is equal to c/c_0, where c is the concentration of probe and c_0 is the concentration where $P/P_0 = 0.5$. The error integral is

$$\operatorname{erf} m = 2\pi^{-1/2} \int_0^m \exp(-x^2)dx$$

and is tabulated in, e.g. ref. 28. This equation describes well the fluorescence depolarization for chlorophyll *b* incorporated into lipid bilayers [29]. However, as illustrated in Fig. 8, although the data for the sterol probe at low mol fractions of sterol can be fitted with a value of c_0 of 0.07, the equation would predict complete depolarization at a mol fraction of 0.3 sterol, and this is not

observed experimentally. In these calculations, a value of 0.25 for P_0 was assumed: results calculated with P_0 equal to 0.35 are not significantly different.

Comparison of the properties of cholesta-5,7,9-trien-3β-ol (I) and dehydroergosterol (II) with their corresponding natural analogues cholesterol and ergosterol

The effect of sterols on water permeation through liposomal membranes prepared from dimyristoyl phosphatidylcholine. The effect of osmotic shock has been used to study the role of cholesterol on water permeation through liposomal membranes [17]. We have used this characteristic of membrane structure to compare the fluorescent probes I and II with their parent sterols and have found that for each parameter that was investigated the fluorescent sterol behaved identically to its non-fluorescent analogue. The results may be summarised by comparing the activation energy for water permeation through the liposomal membrane at temperatures above the transition temperature of dimyristoyl phosphatidylcholine. The values of the activation energy for water permeation were calculated for both a low (2 : 1) and a high (20 : 1) phospholipid to sterol ratio in the liposomes (Table I) and it can be seen that the activation energy is very similar for the fluorescent probe and its parent sterol. This similarity in values indicate that the parent sterol and the fluorescent probe must be behaving in the same way in the liposomal membrane.

Utilisation of fluorescent sterols by T. pyriformis. It is well established that the protozoan *T. pyriformis* is able to utilize both cholesterol and ergosterol when these sterols are added to the growth medium while there is a parallel inhibition of tetrahymanol synthesis in the organism [30]. The fluorescent probes were compared with cholesterol and ergosterol in terms of their ability to replace tetrahymanol in the membranes of this organism. The results (Table II) show very clearly that the fluorescent analogues behaved identically to the parent sterol both in terms of uptake by the organism and inhibition of tetrahymanol biosynthesis. Moreover, the growth rate of the organism was the same for cultures containing cholesterol and the fluorescent probe cholesta-

TABLE I

COMPARISON OF THE EFFECT OF THE FLUORESCENT PROBES AND THEIR PARENT STEROLS ON THE ACTIVATION ENERGY OF WATER PERMEATION THROUGH THE MEMBRANES OF DIMYRISTOYL PHOSPHATIDYLCHOLINE LIPOSOMES ABOVE THE PHASE TRANSITION TEMPERATURE (>30°C)

Sterol	Phospholipid/sterol (molar ratio)	Activation energy (kcal/mol)
None	—	11.92
Ergosterol	20 : 1	13.91
	2 : 1	21.06
Dehydroergosterol	20 : 1	13.91
	2 : 1	20.37
Cholesterol	20 : 1	11.92
	2 : 1	19.87
Cholesta-5,7,9-trien-3β-ol (I)	20 : 1	10.92
	2 : 1	19.87

TABLE II

THE EFFECT OF ADDED STEROLS IN *TETRAHYMENA PYRIFORMIS*

Cultures of *T. pyriformis* (200 ml) were incubated aerobically for 24 h at 28°C in the presence or absence of added sterols. The sterols (3 mg) were added in ethanol (0.4 ml) while the control without sterol received ethanol alone. After extraction the sterol and tetrahymanol content was analysed by GLC using β-amyrin as an internal standard. The cell numbers were determined after 24 h using a Coulter Counter.

Sterol added to the growth medium	Sterol content in mol per 10^8 cells	Sterol uptake from medium in percent	Tetrahymanol content in mol per 10^8 cells	Inhibition of tetrahymanol biosynthesis in percent	Number of cells per ml of culture
None	—	—	$9.1 \cdot 10^{-7}$	—	$4.0 \cdot 10^{-4}$
Ergosterol	$8.13 \cdot 10^{-5}$	99	n.d.	100	$5.5 \cdot 10^{-4}$
Dehydroergosterol (II)	$3.10 \cdot 10^{-5}$	102	n.d.	100	$1.2 \cdot 10^{-5}$
Cholesterol	$1.80 \cdot 10^{-5}$	55	n.d.	100	$1.1 \cdot 10^{-5}$
Cholesta-5,7,9-trien-3β-ol (I)	$1.33 \cdot 10^{-5}$	43	n.d.	100	$1.1 \cdot 10^{-5}$

n.d., not detectable.

5,7,9-trien-3β-ol while in the ergosterol series the fluorescent probe dehydroergosterol resulted in a faster growth rate than with the parent sterol. In all cases the culture of the protozoan grew better with added sterol than in the absence of sterol. These results clearly demonstrate that the fluorescent probes I and II are able to completely replace the natural sterols in the membranes of *T. pyriformis* without any deleterious effect on the growth of the organism. Therefore these probes must be biologically viable as replacements for the natural sterols in this organism.

Discussion

Fluorescence properties

The key to an interpretation of the data presented in this paper lies in the fluorescence quenching properties of dehydroergosterol and the cholesterol probe (I). It is known that many dyes in solution at high concentrations form 'ground state dimers' and that this dimerisation is responsible for changes in the shape of the absorption spectrum and for fluorescence quenching, since such dimers are often non-fluorescent [20]. It is also known that planarity is a feature common to many compounds subject to concentration quenching or excimer formation [21]. Further, theoretical studies predict that quenching and excimer formation for planer molecules such as pyrene will occur when a pair of molecules adopt a sandwich configuration and not when they adopt an end-to-end arrangement [21].

Molecular models of the fluorescent sterols suggest that 'side-ways' overlap of the triene system of two adjacent molecules will be possible in the bilayer when the hydroxyl groups are localised at the bilayer surface. The X-ray crystal structure of cholesteryl myristate shows a 'sandwich' type overlap for two steroid molecules in the unit cell, with the β-sides of the rings in contact [31]. Overlap of the ring systems also occurs in crystals of cholesteryl bromide although in this case alternate steroid molecules are rotated through 180° [22].

Both dehydroergosterol and cholesterol probe (I) have been observed here to be non-fluorescent in aqueous suspension and to have a very different absorption spectra in aqueous suspension and in organic solutions. Since the critical micelle concentration of cholesterol is known to be very low [22] the altered fluorescence and absorption properties can be attributed to micelle formation and the formation of dimers or aggregates of the steroids in these micelles. Clearly, the sterol probes can exhibit concentration quenching. To test whether such quenching was also likely to occur in lipid bilayers we studied energy transfer between dehydroergosterol and perylene in lipid bilayers. As shown by the data in Fig. 7, energy transfer can occur between dehydroergosterol and perylene.

We therefore conclude that sideways overlap of the triene systems of the steroid bilayers is possible, and will lead to the formation of non-fluorescent dimers or larger aggregates. Concentration quenching then follows because of a reduction in the number of monomeric molecules and probably also because of energy transfer from the fluorescent monomers to the non-fluorescent dimers: an explanation of this general type has been proposed by Beddard et al. [25] to account for concentration quenching for chlorophyll *a* in lipid bilayers. The important difference between the results reported here for the steroid probes and those for chlorophyll *a* is that whereas in the latter system the fluorescence intensity has decreased by a factor of 50 at a chlorophyll/lipid molar ratio of 1 : 10 [24,25] in the former system the fluorescence has decreased by only a factor of 3 at a sterol/lipid molar ratio of 1 : 1. The most reasonable explanation of this difference is that the sterol-lipid system is ordered in such a way as to prevent sideways overlap of adjacent sterol molecules. This is further confirmed by the significant fluorescence polarization present at a sterol/lipid molar ratio of 1 : 1 (Fig. 8). If energy transfer were occurring freely between randomly distributed sterol molecules in the lipid bilayer then the fluorescence would be expected to be completely depolarized at these very high molar concentrations.

In egg lecithin up to a sterol/lipid ratio of approx. 1 : 100, the sterol would appear to be fairly homogeneously dispersed. At a sterol/lipid molar ratio of greater than approx. 1 : 50 however, the sterol would be ordered in the membrane, perhaps, as suggested below, into rows, the rows being separated by at least one row of phospholipid molecules (see Fig. 9). Clearly, the lifetime of this arrangement would not be infinite, and there would always be the possibility of the structure momentarily breaking down to allow two sterol molecules to move to a sandwich arrangement within the fluorescent lifetime of an excited molecule: this would produce further fluorescence quenching. The breakdown of the structure would be more frequent at higher temperatures, leading to a reduced fluorescence intensity in egg lecithin at higher temperatures (Fig. 4a). In bilayers of dipalmitoyl phosphatidylcholine, it would be expected that the effects of temperature would be more marked, because of the presence of the lipid crystalline to gel phase transition at 41°C. It is observed that the fluorescence intensity for dehydroergosterol in bilayers of dipalmitoyl phosphatidylcholine is higher at 20°C than it is in the liquid crystalline phase (Fig. 4b), consistent with decreased motion and increased order in the gel phase.

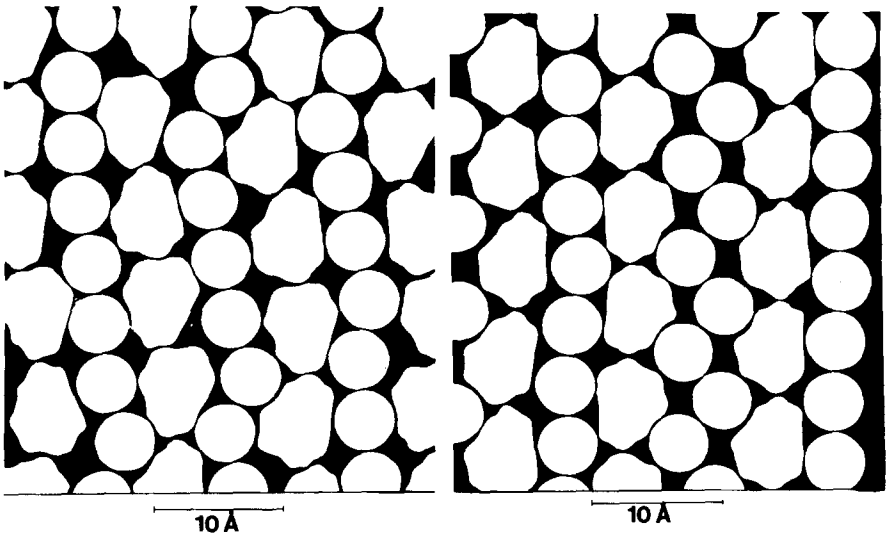


Fig. 9. Two possible models for a lipid/cholesterol mixed phase of approx. 1 : 1 molar ratio, viewed perpendicular to the plane of the lamellar phase. The circles represent the lipid fatty acyl chains and the irregular shapes represent cholesterol molecules as projected down their long axes.

The organisation of cholesterol in lipid bilayers

The facts that a relatively high fluorescence intensity and polarization are observed at a 1 : 1 sterol/lipid molar ratio, and that these change relatively little up to a 1 : 50 sterol/lipid molar ratio, suggest that within these concentration ranges, regions of ordered 1 : 1 sterol/lipid 'complexes' are formed. As illustrated in Fig. 9, we suggest that within these regions the sterol molecules are arranged in an end-to-end manner, with rows of phospholipid molecules running parallel to rows of sterol. At a sterol/lipid molar ratio less than approx. 1 : 50, the distribution of sterol molecules is probably more homogeneous.

Using the projection of lipid and cholesterol molecules taken from CPK models by Engelman and Rothman [33] it is not possible to construct any one uniquely favourable arrangement of steroids and lipids at exactely a 1 : 1 molar ratio. Fig. 9 shows two possible arrangements with cholesterol/lipid molar ratios of 1 : 0.9 and 1 : 0.8. The available information does not provide any information on the orientation of the cholesterol molecules. However, we note that the proton-enhanced ^{13}C nuclear magnetic resonance studies of Opella et al. [8] suggest the presence of two types of cholesterol molecule in mixtures of cholesterol and dipalmitoyl phosphatidylcholine at a molar ratio of 1 : 1 at 32°C. These two types of cholesterol molecule appear to be present in slightly different amounts and probably correspond to molecules with differing motional properties. Further, up to approx. 20 mol% cholesterol, only a single type of cholesterol molecule is detected in the nuclear magnetic resonance experiments [8]. It is therefore possible that in a 1 : 1 lipid/cholesterol mixture, more than one type of molecular organisation is possible. Also, of course, although it is usual to say that lipid bilayers take up cholesterol to a

1 : 1 molar ratio, in fact experimentally a range of values has been reported between 0.98 : 1 and 1.07 : 1 [34—36].

A number of previous studies have been suggested that in lipid/cholesterol mixtures containing less than 33 mol% cholesterol, two phases are present: free lipid and lipid/cholesterol 1 : 1 'complex'. We believe that the approx. 1 : 1 stoichiometry reflects the appropriate geometrical arrangement for optimal packing, as described above, and does not represent the formation of a chemical complex. For simple steric reasons, the fatty acyl chains of lipid molecules adjacent to cholesterol molecules will be immobilized relative to those of lipid molecules in the liquid crystalline state [2]. This will lead to broadening of the signals in proton NMR spectra which have been interpreted in terms of a 1 : 1 interaction [37]. It will also lead to a reduction in solubility for hydrophobic molecules in the lipid, and explain the increased aggregation of chlorophyll *a* observed in lipid bilayers following addition of cholesterol: these experiments were also interpreted in terms of a 1 : 1 lipid/cholesterol stoichiometry [3].

In the gel phase, decreased mobility will tend to create greater order in the membrane, and as shown in Fig. 5, results in a higher fluorescence for dehydroergosterol. As shown in Fig. 6 addition of increasing amounts of dehydroergosterol both broadens the temperature range of the phase transition in dipalmitoyl phosphatidylcholine and increases the temperature for the onset of gel phase formation. The temperature-composition plot (Fig. 6) is very similar to that derived previously from the studies of fluorescence changes for chlorophyll *a* incorporated into bilayers of cholesterol and dipalmitoyl phosphatidylcholine [3].

This interpretation is also consistent with the studies of Hui and Parsons [38,39] using electron microscopy. In mixtures of dipalmitoyl phosphatidylcholine and cholesterol at 11°C an electron diffraction ring characteristic of crystalline fatty acyl chains was seen up to a molar cholesterol ratio of 1 : 1. Further, in mixtures containing less than 50 mol% cholesterol, a ribbon-like structure was seen, each ribbon being less than 600 nm wide. It was suggested that these separate domains could correspond to areas of pure lipid and lipid/cholesterol 'complex'. On raising the temperature above the transition temperature, both the diffraction ring due to the fatty acyl chains and the ribbon pattern disappeared [38].

Cholesterol in biological membranes

Since many biological membranes contain high proportions of cholesterol, we suggest that structures of the sort proposed here must also be important in biological membranes. It has been suggested by Bieri and Wallach [40] and Warren et al. [41] that cholesterol and other steroids may be excluded from the annulus surrounding membrane proteins. This will then serve to increase the proportion of cholesterol in the non-annular lipid.

We have also shown that dehydroergosterol is taken up by *T. pyriformis* and incorporated into the ciliary membrane. Therefore it should be possible to use isolated cilia containing the fluorescent probe to study the organisation of sterols and their possible interaction with membrane proteins, in a biological membrane which is not perturbed by artificial probes.

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