Use of fluorescent probes in the study of phospholipid–sterol bilayers

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(Received 22 May 1980/Accepted 23 July 1980)

1. The transfer of excitation energy between the fluorescent probes 1,6-diphenylhexa-1,3,5-triene and 12-(9-anthroyl)stearic acid and the cholesterol analogue cholesta-4,6-dien-3-one in phosphatidylcholine liposomes has been investigated. 2. The results indicate that probes and steroid are randomly distributed in the bilayer at steroid concentrations up to 35 mol%. 3. The degree of polarization of diphenylhexatriene fluorescence increases with increasing cholesterol content. Other sterols, differing in structure in the region of the polar group or in the side chain at position-17, produce similar but not identical effects. 4. The results are consistent with the proposal that diphenylhexatriene gives a general picture of the state of the bilayer and that there is no segregation of sterols in liquid-crystalline phosphatidylcholine bilayers.

Although many physical techniques have been used to study cholesterol–phospholipid interactions (Lee, 1975; Green, 1977), the arrangement of the two molecules in bilayers at temperatures above the gel–liquid-crystalline transition of the phospholipid is still unknown. The cholesterol could be (a) randomly dispersed (Cornell *et al.*, 1979), (b) present in discrete regions composed of complexes of phospholipid and cholesterol (Phillips & Finer, 1974), or (c) arranged in ordered arrays (Rogers *et al.*, 1979). It has been postulated that in small vesicles where there is a high degree of curvature, cholesterol and phospholipid are not equally distributed between the two leaflets of the bilayer (Huang *et al.*, 1974; de Kruijff *et al.*, 1976) so that each leaflet may have a different organization.

Sterols other than cholesterol can be readily incorporated into phospholipid bilayers. Such molecules are of interest as components of bilayers both because they can be used as structural probes (Green *et al.*, 1973) and because they may be able to modify the properties of liposomes injected *in vivo* (Wharton & Green, 1978).

In many studies of membrane cholesterol, use has been made of the fluorescent probe 1,6-diphenylhexa-1,3,5-triene (Shinitzky & Inbar, 1976; Cooper *et al.*, 1978). Some of the conclusions drawn from previous experiments with this probe have been

questioned (Dale *et al.*, 1977; Hildenbrand & Nicolau, 1979) and with any probe molecule there are uncertainties as to its distribution within the system under investigation and as to the degree to which the region on which it reports is representative of the whole (Bieri & Wallach, 1975).

We have therefore investigated the distribution of the cholesterol analogue cholesta-4,6-dien-3-one together with that of diphenylhexatriene [and another fluorescent probe, 12-(9-anthroyl)stearic acid] in phosphatidylcholine bilayers. We have then used the former probe to compare the effects of various sterols on the hydrocarbon regions of such bilayers.

Materials and methods

Materials

Chemicals were obtained from the following sources. Dimyristoylglycerophosphocholine [(dimyristoyl)phosphatidylcholine] and cholest-4-en-3-one were from Koch–Light Laboratories, Colnbrook, Bucks., U.K.; stigmasterol and 3-hydroxycholest-5-en-7-one were from Steraloids, Croydon, U.K.; cholesterol and cholesta-4,6-dien-3-one were from BDH Chemicals, Poole, Dorset, U.K.; 1,6-diphenylhexa-1,3,5-triene was from Aldrich Chemical Co., Gillingham, Dorset, U.K.; 12-(9-anthroyl)stearic acid was from Sigma (London) Chemical Co., Kingston-upon-Thames, U.K.

Egg phosphatidylcholine, 3-hydroxycholest-3-en-2-one and 1-methyl-10-norcholesta-1,3,5(10)-trien-3-ol were made as before (Green *et al.*, 1973; Smith & Green, 1974).

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Liposomes were prepared by sonication (McCabe & Green, 1977) and consisted mainly of unilamellar vesicles of less than 50 nm diameter.

Methods

1,6-Diphenylhexa-1,3,5-triene and 12-(9-anthroyl)stearic acid were estimated from their fluorescence spectra in cyclohexane solution. Phospholipid was determined by the method of Cox & Gökçen (1973) and stigmasterol by the same method as cholesterol, but by using a standard curve obtained with the former sterol. Lipid extraction and estimation of cholesterol and other steroids were as previously described (Graham & Green, 1970; Green *et al.*, 1973; Smith & Green, 1974).

Fluorescence measurements. All measurements were made at 20–21°C in a 1 cm cell in a Perkin-Elmer-Hitachi MPF 2A spectrofluorimeter fitted with a high-sensitivity sample holder and polarization accessory. Excitation and emission wavelengths for diphenylhexatriene were 366 and 425 nm respectively and, for anthroylstearic acid, 362 and 434 nm respectively. To avoid photoisomerization, excitation never exceeded 15 s at any one time and an interval of 30 s was left between measurements.

Quenching experiments. Liposomes were prepared from 10 mg of phosphatidylcholine, 12.5 µg of diphenylhexatriene or 20 µg of anthroylstearic acid and the appropriate amount of cholesta-4,6-dien-3-one in 10 ml of water. After samples were taken for phospholipid and steroid determination, the preparation was divided. One portion was kept in the dark for at least 15 min before the fluorescence (quenched) was measured. To the other portion, excess sodium borohydride was added and after 30 min, when spectroscopic examination showed that the chromophoric conjugated oxo group of the steroid had been reduced to the hydroxy group, the unquenched fluorescence was measured.

In some experiments, where low concentrations of steroid were required, liposomes containing only phosphatidylcholine and diphenylhexatriene were prepared. The required amount of cholesta-4,6-dien-3-one was added by rapidly injecting a solution of the steroid in 25 µl of ethanol through a fine needle into 3 ml of the liposome preparation. The sample was shaken for 60 min before the fluorescence was measured.

The efficiency of fluorescence energy transfer $[1 - (F/F_0)]$ was plotted against the surface density of the acceptor (mol of acceptor/mol of phospholipid), where F_0 is the unquenched fluorescence and F is the quenched fluorescence (Fung & Stryer, 1978).

Polarization measurements. Liposomes were prepared from 5 mg of egg phosphatidylcholine and 0–5 mg of test sterol in 10 ml of water. To a 3 ml

sample, 6.25 µg of diphenylhexatriene in 25 µl of ethanol was added. After at least 30 min in the dark, the degree of polarization of fluorescence (P) was determined (Shinitzky & Inbar, 1974; Shukla *et al.*, 1980). At least eight preparations with different steroid/phospholipid ratios were examined for each steroid studied. None of the steroids used quenched the fluorescence of diphenylhexatriene in concentrated solution or in liposomes.

Light scattering. Because the excitation and emission wavelengths for diphenylhexatriene are well separated, light scattering is not so much of a problem as it is with other probes (Shinitzky & Inbar, 1974). However, to obviate any effects of scattering, only samples with a transmittance of at least 93% over the range 366–425 nm were used. By using the method described by Johnson & Kramers (1978), it was confirmed that as long as transmittance was greater than 85–90%, light-scattering corrections were not necessary. In some experiments, a solution of 2 M-sodium nitrite was used as a filter to cut off light of wavelengths below 390 nm (Shinitzky & Inbar, 1976). This eliminated the Rayleigh scattering peak, but had no effect on the polarization value obtained and its use was discontinued.

Results

Fluorescence quenching

Incorporation of cholesta-4,6-dien-3-one into phosphatidylcholine bilayers containing either diphenylhexatriene or anthroylstearic acid resulted in a decrease in the fluorescence emission, which varied with the concentration of the steroid. The quenching of the fluorescence did not result in any change in the shape of the emission spectrum so it was not via reabsorption of emitted light. Nor could it be caused by a concentration-dependent self-quenching of the fluorophore such as would occur if the probe were forced into steroid-free regions as the steroid concentration increased (Lee, 1976), because the quenched and unquenched fluorescence were measured with the same amount of steroid (or sterol) in the bilayer. It probably occurs via the classic dipole-dipole non-radiative energy transfer described by Förster (1959), since cholesta-4,6-dien-3-one has a small absorption peak at about 375 nm that overlaps slightly with the emission peaks of the two probes. With conjugated carbonyl compounds such as the steroid, formation of a charge-transfer stabilized exciplex is also a possible mechanism (Busch *et al.*, 1977). In solutions of donor and acceptor, quenching by either mechanism will obey Stern-Volmer kinetics (Shinitzky & Rivnay, 1977).

When the efficiency of energy transfer is plotted against acceptor surface density (Figs. 1 and 2), the curves correspond to those given by Fung & Stryer

(1978) for randomly dispersed donor and acceptor molecules. The theoretical curves are derived assuming that R_0 , the distance between donor and acceptor at which the transfer efficiency is 50%, is 1.35 nm for diphenylhexatriene and 1.15 nm for anthroylstearic acid. Diphenylhexatriene fluorescence is more efficiently quenched by cholesta-4,6-dien-3-one than anthroylstearic acid fluorescence, but in both cases the quenching efficiency is low. This is desirable, because if the efficiency is high only low concentrations of steroid in the bilayer can be studied.

Fig. 1. *Transfer of excitation energy between diphenylhexatriene and cholesta-4,6-dien-3-one*

—, Theoretical curve for random distribution of donor and acceptor; O, acceptor steroid incorporated into lipid used to prepare liposomes; ●, acceptor steroid added to preformed liposomes. F_0 , unquenched fluorescence; F , quenched fluorescence.

Bieri & Wallach (1975), from the theory developed by Träuble & Sackmann (1972), established that if a fluorophore and quenching agent form clusters in the bilayer, the quenching of fluorescence should vary linearly with $[(1+c)/c]^{\frac{1}{2}}$ over a wide range of c , where c is the surface density of the quencher and the term $[(1+c)/c]^{\frac{1}{2}}$ is proportional to the average nearest-neighbour-distance between the interacting molecules. When the results obtained in the present study were plotted in this form (Fig. 3), the curves showed no linear regions, confirming that the quenching steroid was not present in clusters.

Fluorescence-polarization measurements

The effects of increasing the cholesterol content of liposomes on the polarization of diphenylhexatriene fluorescence are shown in Fig. 4(a). The increase in

Fig. 2. *Transfer of excitation energy between anthroylstearic acid and cholesta-4,6-dien-3-one*

—, Theoretical curve for random distribution of donor and acceptor.

Fig. 3. *Quenching of fluorescence of diphenylhexatriene and anthroylstearic acid by increasing concentrations of cholesta-4,6-dien-3-one*

(a) Diphenylhexatriene; (b) anthroylstearic acid. The results from Figs. 1 and 2 are replotted in the form proposed by Bieri & Wallach (1975). C is the surface density of the steroid in the bilayers of the liposomes.

Fig. 4. Effect of sterols on the polarization of diphenylhexatriene fluorescence

(a) Cholesterol; (b) ----, with cholesterol; O, 3-hydroxycholest-3-en-2-one; ●, 3-hydroxycholest-5-en-7-one; □, 1-methyl-10-norcholesta-1,3,5(10)-trien-3-ol; (c) ----, with cholesterol; O, cholest-4-en-3-one; ●, stigmasterol.

the value of P with increasing cholesterol concentration up to a sterol/phospholipid ratio of about 1:1 confirms the findings of other workers with liposomes and cell membranes (Shinitzky & Inbar, 1976; Cooper *et al.*, 1978).

Figs. 4(b) and 4(c) show the effects of other sterols on the polarization of diphenylhexatriene fluorescence. The maximum incorporations of these compounds, apart from 3-hydroxycholest-5-en-7-one, are lower than that of cholesterol, but the values obtained are similar to those previously reported (Green *et al.*, 1973). Within the limits of incorporation, all sterols, except the phenolic compound 1-methyl-10-norcholesta-1,3,5(10)-trien-3-ol at higher concentrations, give results similar to those obtained with cholesterol. All except cholest-4-en-3-one give slightly higher polarization values than those obtained with cholesterol at an equivalent concentration.

Discussion

Cholesta-4,6-dien-3-one, used here as a cholesterol analogue, is well incorporated into phospholipid bilayers and possesses the small polar group at position-3, the flat ring system and the side chain at position-17, which appear to be essential for membrane function. However, it does show some differences from cholesterol in its effects on bilayer permeability (Bruckdorfer *et al.*, 1969; Demel *et al.*, 1972) as a result of the replacement of the 3β -hydroxy group by a ketone function. Although it has often been stated that a 3β -hydroxy group is necessary for membrane sterols, Odriozola *et al.* (1978) have recently shown that this is not the case and that in some membranes at least it can be replaced by a methyl ether group.

The results of the quenching experiments demonstrate that at cholesta-4,6-dien-3-one/phospholipid molar ratios up to at least 0.35, steroid and probe molecules are randomly dispersed. This is con-

sistent with the findings of others (de Kruijff *et al.*, 1976; Lee, 1976; Huang *et al.*, 1974). The experiments do not establish whether the molecules are static or freely diffusing in the membrane, but as there is now a wealth of evidence that all lipid molecules in bilayers have high rates of lateral diffusion (see Green, 1977), it is probable that the random arrangement is the result of free diffusion of the probes and the quencher within the plane of the bilayer. The results are not dependent on the nature of the phosphatidylcholine used, since they are seen with a pure saturated phospholipid as well as the mixed unsaturated phosphatidylcholines from egg. The nature of the probe also does not affect the results, because similar results were obtained with the purely hydrophobic probe diphenylhexatriene and with anthroylstearic acid, which is anchored at the water/hydrocarbon interface by its carboxy group.

The findings do not agree with those of Rogers *et al.* (1979). These workers used fluorescent sterol molecules as cholesterol analogues and concluded from the variation of fluorescence with concentration that when the sterols were dispersed in water with phospholipids at concentrations greater than about 2 mol%, they formed linear arrays within the bilayers. The different results obtained in the two investigations may reflect the different molecular properties of the sterol probes used or a difference between the small sonicated vesicles used in the present work and the large multilamellar liposomes used by Rogers *et al.* (1979). The results of de Kruijff *et al.* (1976) suggest that the effects of curvature would be to make the sterol less uniformly distributed in the small vesicles than in the larger liposomes. As we found the distribution in the small liposomes to be essentially random, the latter explanation is unlikely. However, the sterol arrangement postulated by Rogers *et al.* (1979) is based on calculations in which it is assumed that all of the sterol in a shaken mixture of phospholipid and

sterol is incorporated into the bilayers and the validity of this is questionable. Indeed, incorporation of appreciable amounts of the parent sterols of the two probes used (7-dehydrocholesterol and ergosterol) into small sonicated liposomes, where the degree of incorporation can be established with more certainty, can only be achieved with difficulty (Green *et al.*, 1973). Thus the fluorescence signal from the sterol probes may be more a function of their partition between microcrystals, micelles and bilayers than of their organization within the bilayer.

The results also do not fit with the proposal (Phillips & Finer, 1974; Engelman & Rothman, 1972; Jain & White, 1977) that phosphatidylcholine and cholesterol molecules may separate into discrete regions within the otherwise fluid phospholipid bilayer. In phospholipid bilayers below the gel-liquid-crystalline transition temperature, on the other hand, steroid molecules are not randomly distributed but are segregated into clusters (Träuble & Sackmann, 1972; Bieri & Wallach, 1975; Lee, 1976). Above the transition temperature, spin-labelled sterols are randomly distributed at low concentrations, although they may cluster as the concentration is increased (Träuble & Sackmann, 1972; Bieri & Wallach, 1975). None of the discontinuities detected by other techniques at cholesterol/phospholipid molar ratios below 0.3 (Rubinstein *et al.*, 1980) was revealed in the present experiments by using probes that are known to report on the hydrocarbon region of the bilayer (Lesslauer *et al.*, 1972). These discontinuities appear to reflect changes in the freedom of motion of the phospholipid head groups as they are spaced out by the sterol molecules (Rubinstein *et al.*, 1980).

Several proposals have been made, based on both theoretical considerations and experimental results, that a change in the organization of cholesterol and phospholipid molecules occurs at molar ratios of about 0.33 (see Cornell *et al.*, 1979; Rubinstein *et al.*, 1980). Because this is at the upper end of the steroid/phospholipid ratios used to study fluorescence quenching, such a change might have escaped detection in our experiments. On the other hand, comparison of the results obtained by fluorescence quenching with those obtained in the fluorescence-polarization experiments provides no support for this proposal. If there were any abrupt change in sterol distribution, it should have been detected in the polarization-versus-cholesterol-concentration curve both in the present study (Fig. 4) and in those of other workers. Thus although bilayers above their transition temperature may contain short-lived clusters of various types (Lee *et al.*, 1974), our results lead us to support the general model of phospholipid-cholesterol bilayers put forward by Cornell *et al.* (1979), in which chole-

sterol and phospholipid molecules are uniformly distributed in bilayers at up to equimolar proportions.

The polarization values obtained with diphenylhexatriene in phospholipid-cholesterol bilayers have usually been correlated with the microviscosity of the hydrocarbon region, but recent results suggest that static orientational restriction is the major factor determining the change in the degree of polarization with increasing cholesterol rather than changes in the rates of diffusion (Hildenbrand & Nicolau, 1979; Heyn, 1979). The similarity of the results obtained with the other sterols to those obtained with cholesterol indicate that the main effects on the hydrocarbon region of the bilayer as measured by the fluorescence-polarization technique are determined by the ring system. Changes in the region of the polar group on ring A or stiffening and enlarging the side chain produced relatively small effects. On the other hand, probes that report more specifically on localized regions of the bilayer may show larger differences. Thus, although other workers have also reported that cholest-4-en-3-one orders bilayers less well than cholesterol, the differences seen by diphenylhexatriene fluorescence polarization are much less marked than those detected by spin probes that locate in the polar region (Hsia *et al.*, 1972) or by differential scanning calorimetry and permeability measurements (Demel *et al.*, 1972; Demel & de Kruijff, 1976). Very little difference is seen (Fig. 4) between the degree of ordering induced by cholesterol and that induced by stigmasterol, despite the presence of a larger and more rigid side chain in the latter. Again, clear differences are revealed by spin probes located specifically in the region of the side chain when cholesterol is replaced by sterols with modified side chains (Suckling & Boyd, 1976; Craig *et al.*, 1978).

The findings are consistent with the conclusions of Heyn (1979) that diphenylhexatriene gives a general average picture of the state of the bilayer and that the estimated degree of order correlates with the degree of order around the C₍₁₀₎-C₍₁₂₎ region of the fatty acid chains determined from n.m.r. measurements. That this average picture may be more closely related to the role of sterols in natural membranes than specific effects detected at either the head group or the side chain region is suggested by the variation in size and rigidity of the side chains found on sterols present in cell membranes (Green, 1977) and by the demonstration that the 3 β -hydroxy group can be replaced by the methyl ether in some membranes (Odrizola *et al.*, 1978).

We are indebted to the Medical Research Council (S. A. W.) and to the Charles Brotherton Trust (S. S. G. De M.) for financial support.

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