Optical-Rotatory-Dispersion Studies of Compounds Related to Cholesterol in Liposomes and the Membranes of Erythrocyte 'Ghosts'

By JONATHAN R. GREEN, PETER A. EDWARDS and COLIN GREEN
Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

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1. Steroid molecules containing the α,β -unsaturated oxo group in various positions were incorporated with egg phosphatidylcholine into liposomes and into human erythrocyte membranes. 2. The liposomes formed contained 0.3–0.94 mol of steroid/mol of phospholipid and the steroids replaced 19–76% of the erythrocyte membrane sterol. 3. The optical rotatory dispersion (o.r.d.) spectra of the steroids in these structures were compared with those obtained in solvents of different polarity. 4. The o.r.d. spectra of cholesta-4,6-dien-3-one and 3-hydroxycholest-3-en-2-one in liposomes resembled those obtained with polar solvents such as ethanol or triethyl phosphate–water (1:1, v/v). 5. The o.r.d. spectra of 3-hydroxycholest-7-en-6-one and 3-hydroxycholest-5-en-7-one in liposomes resembled those obtained with moderately polar solvents such as dioxan. 6. The o.r.d. spectrum of 3-hydroxycholest-8(14)-en-15-one in liposomes resembled those obtained with non-polar solvents such as cyclohexane. 7. 3-Hydroxycholest-3-en-2-one did not exchange with erythrocyte membrane cholesterol, but the other steroids did do so and the o.r.d. spectra of the membranes containing them closely resembled those obtained with liposomes. 8. From the results, the position of sterol molecules with respect to the phospholipid molecules in liposomes and membranes of human erythrocyte 'ghosts' can be deduced.

The natural cholesterol of the erythrocyte membrane can be replaced to a considerable extent by other steroids by allowing an exchange to occur between the membrane and liposomes containing the steroid (Bruckdorfer *et al.*, 1968, 1969). This process has now been used to introduce steroids, containing a 'probe group' in different parts of the molecule, into the membrane. The α,β -unsaturated oxo group was chosen as the probe because it can be introduced at various points in the steroid skeleton with relatively little effect on the overall shape and size. The o.r.d.* spectrum of this chromophore shows a Cotton effect at about 350 nm, which is solvent-sensitive (Djerassi *et al.*, 1956) and which can be used to assess the nature of the environment of the chromophore. The o.r.d. spectra of such steroids in a number of solvents of different polarity have been compared with those obtained in liposomes and erythrocyte membranes. The formulae of these compounds are shown in Fig. 1. The chemical basis of the solvent shifts is not considered here but has been discussed fully by Kirk *et al.* (1970).

The probes used with other physical techniques, such as fluorescence and electron paramagnetic resonance, tend to be bulky and relatively few molecules are incorporated into the liposome or membrane. They may perturb the basic structure and give

information only about restricted regions of it (Wallach *et al.*, 1970; Oldfield & Chapman, 1972). The compounds used in this study closely resemble the native sterol and replace it by the natural exchange process. A large proportion of the cholesterol can be replaced, so that information is probably obtained about the membrane structure as a whole. A preliminary account of some of this work has been given (Green *et al.*, 1971).

Materials and Methods

Materials

Egg phosphatidylcholine was prepared by the method of Pangborn (1951) and purified (Lea *et al.*, 1955) until only one spot was obtained on t.l.c. Lysophosphatidylcholine was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. 3-Hydroxycholest-3-en-2-one was prepared as described by Stiller & Rosenheim (1938), 3-hydroxycholest-7-en-6-one as described by Barton & Robinson (1954) and 3-hydroxycholest-8(14)-en-15-one as described by Woodward *et al.* (1957). These compounds were purified by column chromatography or t.l.c. or both and repeated crystallization and their purity was confirmed by g.l.c., mass spectrometry and determination of their melting points and u.v. and i.r. spectra. Cholesta-4,6-dien-3-one was

* Abbreviation: o.r.d., optical rotatory dispersion.

Fig. 1. Formulae of steroids used

obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and 3-hydroxycholest-5-en-7-one was from Steraloids Ltd., Croydon, U.K. Both were recrystallized from methanol.

Solvents were from BDH Chemicals Ltd., Triethyl phosphate and ethyl acetate contained no detectable free acid. All other solvents were of the special standard for spectroscopy.

Liposomes

These were prepared as described by Stevens & Green (1972) but were centrifuged at 100000g for 25 min and then passed through a filter of 450 nm pore diameter [Millipore (U.K.) Ltd., Wembley, Middx., U.K.] to make the dispersion optically clear. They were examined (a) spectrophotometrically (Klein, 1970) to ensure that peroxidation of the fatty acids was kept below 2% and (b) by t.l.c. to check that no significant breakdown to lysophosphatidylcholine had occurred (Hauser, 1971).

If a steroid would not disperse with phosphatidylcholine in equimolar proportions, then sufficient cholesterol was added before sonication to give a 1:1 molar ratio of total steroid to phosphatidylcholine in the liposomes used for incubation with the erythrocyte 'ghosts'. This is to prevent loss of cholesterol from the membrane during the subsequent incubation (Bruckdorfer *et al.*, 1968). The liposomes so formed contain slightly lower proportions of the test steroids than those prepared without cholesterol

(Table 1) and so the percentages of membrane sterol replaced (Table 1) are not maximum values.

Erythrocyte membranes

'Ghosts' were prepared from human erythrocytes by the method of Dodge *et al.* (1963). Their cholesterol was partially replaced by the test steroid by incubation overnight with liposomes containing the steroid (Bruckdorfer *et al.*, 1968). A 2–5-fold excess of the steroid over the membrane cholesterol was used.

After incubation, the 'ghosts' were centrifuged at 25000g for 30 min and washed five times, the last two washings being free of steroid. The 'ghosts' were suspended in water to give 0.3–0.8 mg of total steroid/ml and clarified either by sonication or by disruption in a French pressure cell (Cauldwell & Schumaker, 1970). The o.r.d. spectra did not differ, but the latter disruption procedure proved more satisfactory, and all spectra given here are from 'ghosts' treated in this way. The suspension of particles was further clarified by filtration through glass-fibre and membrane filters if necessary.

Lipid extraction and determination of protein, phosphatidylcholine, cholesterol and cholesta-4,6-dien-3-one were done as previously described (Bruckdorfer *et al.*, 1968). Other steroids were assayed by measurement of the u.v.-absorption maximum in ethanol. Where mixtures of steroids were present, they were separated from other lipids by t.l.c. (Cuzner & Davison, 1967) and then fractionated

by g.l.c. (Rees *et al.*, 1968). The values used were: 3-hydroxycholest-3-en-2-one, $\lambda_{\max}$ 271 nm, $\epsilon 6.4 \times 10^3$; 3-hydroxycholest-7-en-6-one, $\lambda_{\max}$ 244 nm, $\epsilon 16.0 \times 10^3$; 3-hydroxycholest-5-en-7-one, $\lambda_{\max}$ 237.5 nm, $\epsilon 12.5 \times 10^3$; 3-hydroxycholest-8(14)-en-15-one, $\lambda_{\max}$ 260 nm, $\epsilon 15.4 \times 10^3$.

Measurement of o.r.d.

All measurements were made with a Bellingham and Stanley Polarmatic 62 spectropolarimeter fitted with a 450 W lamp with the cell compartment water-jacketed at 25°C. As the spectra obtained can be affected by the adjustment of the machine (Kirk *et al.*, 1970) constant conditions were employed. The spectra given here were recorded by using a cell of 10 mm light-path, with entrance and exit slits set at 1 mm and by using the 100-millidegree expansion range. Measurements were made at a concentration of 0.1–0.5 mg of test steroid/ml, depending on the magnitude of its Cotton effect, over the range 290–520 nm. The machine is calibrated in wavenumbers but for convenience the results are given as wavelengths, even though this gives a non-linear scale. Light-transmission was monitored throughout and recordings were stopped if this fell below 30%. At the extrema studied here, light-transmission was about 50% for 'ghosts' and 70–80% for solutions and dispersions. At least four different preparations of liposomes and two of erythrocyte 'ghosts' containing each steroid were examined and spectra were recorded several times at different scanning speeds to get a true assessment of the signal/noise ratio.

Except with 3-hydroxycholest-8(14)-en-15-one, only the first extremum of the Cotton effect could be measured because of low light-transmission and merging of the effect with the much larger one associated with the $\pi-\pi^*$ transition at lower wavelengths. Hence the amplitude could not be determined. Because the o.r.d. curves of steroids in lipos-

omes and membranes are superimposed on the negative plain curve of the structures themselves (Fig. 2), measurements of absolute rotation would also be misleading. It was therefore decided to express

Fig. 2. Derivation of $[\frac{1}{2}a]$ value and control o.r.d. spectra

(a) Spectrum of cholesta-4,6-dien-3-one in cyclohexane (148 $\mu\text{g/ml}$); (b) base-line obtained with water in the sample cell; (c) spectrum of liposomes containing equimolar proportions of cholesterol and phospholipid (about 1.5 mg of lipid/ml); (d) spectrum of erythrocyte membranes treated as described in the text.

Table 1. Incorporation of test steroids into liposomes and replacement of erythrocyte cholesterol by these compounds

Steroid and phosphatidylcholine were sonicated together to form liposomes and then these were incubated overnight at 37°C with a suspension of erythrocyte 'ghosts' to allow exchange of the steroid with the membrane cholesterol. Results are the means $\pm$ S.E.M. of four experiments.

Steroid	Steroid/phosphatidylcholine ratio in liposomes (mol/mol)	Replacement of erythrocyte membrane cholesterol (%)
Cholesta-4,6-dien-3-one	0.66 ± 0.10	46.7 ± 2.3
3-Hydroxycholest-3-en-2-one	0.42 ± 0.04	0
3-Hydroxycholest-7-en-6-one	0.30 ± 0.02	19.5*
3-Hydroxycholest-5-en-7-one	0.94 ± 0.03	76.0 ± 2.3
3-Hydroxycholest-8(14)-en-15-one	0.53 ± 0.02	53.5 ± 6.0

* Mean of two results.

the magnitude as $[\frac{1}{2}a]$, which is obtained by measuring the first extremum of the Cotton effect (x), as illustrated in Fig. 2, and calculating the value in millidegrees that would be given by a concentration of 1 mg of steroid/ml in a 10mm cell at 25°C.

Results

Dispersion and incorporation into membranes of test steroids

When cholesterol is sonicated with phosphatidylcholine in water under the conditions described here, liposomes containing equimolar amounts of the two lipids are formed. Table 1 shows the extent to which the other steroids are incorporated into liposomes. Although only 3-hydroxycholest-5-en-7-one disperses as well as cholesterol, all disperse readily with the phospholipid.

When liposomes containing most of these steroids were incubated with erythrocyte 'ghosts', the steroid exchanged with the membrane cholesterol since the steroid was found in the 'ghosts' and cholesterol appeared in the liposomes. The extent to which the steroids replaced the native sterol in the membrane preparations used for o.r.d. measurements is also shown in Table 1. All appeared to enter the membrane readily by exchange with the erythrocyte cholesterol except for 3-hydroxycholest-3-en-2-one, which did not exchange at all. The fact that no 3-hydroxycholest-3-en-2-one could be detected in the 'ghosts' after incubation shows that the washing procedure was effective and that the o.r.d. spectra obtained were not from adsorbed liposomes.

Control o.r.d. studies

Erythrocyte-'ghost' preparations and liposomes made from phosphatidylcholine with or without cholesterol always gave plain negative o.r.d. spectra over the range 290–520nm (Fig. 2). Problems caused by light-scattering have arisen in optical studies of membrane proteins (Urry *et al.*, 1971; Glaser & Singer, 1971). Many control experiments were carried out, including measurements made with different concentrations, cell sizes and slit widths, the use of tandem cells (one containing a solution of steroid, the other containing liposomes), studies of liposomes of different sizes (and degrees of opacity) produced by altering the sonication time, and studies of steroidal dienes, such as 7-dehydrocholesterol, whose o.r.d. spectra are not solvent-sensitive. These all indicated that light-scattering decreased the amplitudes but did not significantly shift the positions of the spectra. There was no problem with the liposomes because the $[\frac{1}{2}a]$ values obtained for the steroids whose o.r.d. spectra show no marked change of amplitude with solvent were the same for liposomes and true solutions. Hence no obvious flattening occurs as a result

Table 2. *Characteristics of o.r.d. spectra of test steroids in solvents of different polarity*

	Cyclohexane		Dioxan		Ethanol	
	Extrema (nm)	$[\frac{1}{2}a]$ (millidegrees)	Extrema (nm)	$[\frac{1}{2}a]$ (millidegrees)	Extremum (nm)	$[\frac{1}{2}a]$ (millidegrees)
Cholesta-4,6-dien-3-one	388, 406, 372	138	384 [400]	138	370	161
3-Hydroxycholest-3-en-2-one	339, 354	-66	344 (355)	-59	345	-59
3-Hydroxycholest-7-en-6-one	364, 349	148	358 (346)	154	344	143
3-Hydroxycholest-5-en-7-one	357, 370	55	352 [364]	53	350	53
3-Hydroxycholest-8(14)-en-15-one	372 [384]	76	371	91	366	85

The magnitude is expressed in terms of the $[\frac{1}{2}a]$ value whose derivation is described in the text. This is given for the major extremum (listed first) where there is fine structure. Brackets denote a distinct shoulder and parentheses a slight shoulder.

Fig. 3. *O.r.d. spectra of steroids in liposomes and erythrocyte membranes*

(a) Cholesta-4,6-dien-3-one: (1) in liposomes (0.11 mg/ml) and (2) in erythrocyte membranes. (b) 3-Hydroxycholest-3-en-2-one: (1) in liposomes (0.20 mg/ml), (2) in dioxan (0.5 mg/ml) and (3) in cyclohexane (0.67 mg/ml). (c) 3-Hydroxycholest-5-en-7-one: (1) in liposomes (0.55 mg/ml) and (2) in erythrocyte membranes.

of light-scattering. The 'ghost' preparations did scatter more light, but with light-transmission above 50% any effects should be small (Schneider *et al.*, 1970; Glaser & Singer, 1971).

O.r.d. of steroids in solution

The o.r.d. curves have three characteristics that were assessed: (1) the shape, i.e. the presence or absence of fine structure; (2) the position of the extremum; (3) the $[\lambda]_D$ value. Table 2 shows the general features of the spectra. In polar solvents such as ethanol, all give a simple curve with no fine structure. As the solvent polarity decreases, fine structure appears in the spectrum and the wavelength of the extremum changes. This is a bathochromic shift in all cases except for the 3-hydroxycholest-3-en-2-one, where the shift is hypsochromic. This compound also differs from the others in having a negative, not a positive, Cotton effect, but it is a much more complicated molecule than the others as it is an enol form of the 2,3-diketone.

The extent of the solvent shift varies, being about 18 nm for the cholesta-4,6-dien-3-one, which has an extra conjugated double bond in the chromophore, and 6–7 nm for the others. In most cases secondary peaks appear in non-polar solvents, and a distinct shoulder in the moderately polar ones. However, with 3-hydroxycholest-8(14)-en-15-one the shoulder

Table 3. *Characteristics of o.r.d. spectra of 4,6-cholestadien-3-one in solvents of different polarity*

The conventions used are the same as in Table 2. All solvent mixtures are given as % (v/v) in water.

Solvent	Extrema (nm)	$[\lambda]_D$ (millidegrees)
Trifluoroethanol	343	328
Ethanol (50%)	364	205
Ethanol (60%)	364	201
Ethanol (80%)	367	197
Triethyl phosphate (50%)	370	182
Triethyl phosphate (65%)	370	163
Triethyl phosphate (90%)	380 [400]	152
Triethyl phosphate	382 [400]	135

only becomes distinct in non-polar solvents. Some of these points are illustrated in Figs. 2 and 3.

Only the 3-oxo and 2-oxo compounds have o.r.d. curves whose $[\lambda]_D$ values show marked variation with solvent polarity and only those of the 3-oxo compound vary regularly. These are discussed below.

Liposome studies

The o.r.d. spectrum of liposomes containing cholesta-4,6-dien-3-one is shown in Fig. 3(a). The extremum is at 370 nm and there is no fine structure, so

the spectrum resembles that of an ethanolic solution. However, the $[\lambda]_D$ value of 176 is greater than that of such a solution, so a further series of solvents was studied (Table 3). This confirmed that the $[\lambda]_D$ value was directly related to solvent polarity. The ethanol-water mixtures all provide more polar environments for the probe group than do the liposomes as judged by the position and magnitude of the extremum. Esters such as methyl acetate and triethyl phosphate are similar in polarity to dioxan, but a mixture of triethyl phosphate and water (1:1, v/v) appears to provide an environment very similar to that obtaining in the liposomes. This mixture contains 9-10 molecules of water per molecule of triethyl phosphate.

The o.r.d. spectrum of 3-hydroxycholest-3-en-2-one in liposomes is shown in Fig. 3(b). It closely resembles that seen in polar solvents such as ethanol, with the extremum at 346 nm, but the $[\lambda]_D$ value (117) is much higher than expected. However, addition of alkali to an ethanolic solution of the compound greatly increases the optical rotation and causes a further bathochromic shift. In the very polar solvent trifluoroethanol the $[\lambda]_D$ value is also high (104). This compound has its carbonyl and hydroxyl (enol) groups very close together and not only can both form hydrogen bonds with solvents like ethanol or water, but intramolecular hydrogen bonding seems possible and even isomerization cannot be excluded. Thus there could be several reasons for the increased rotation seen with the liposomes, but the A ring of the steroid does appear to be in a region that strongly promotes electrostatic bonding.

Fig. 3(c) shows the o.r.d. spectrum of 3-hydroxycholest-5-en-7-one in liposomes. The extremum is at 354 nm with a shoulder at 365 nm and $[\lambda]_D$ value of 53,

corresponding most closely to the spectrum seen in a moderately polar solvent such as dioxan. Further studies with solvents revealed that in triethyl phosphate or methyl acetate the spectrum was similar to that of a dioxan solution but the shoulder was replaced by a clear secondary peak. Addition of small amounts of water (enough to saturate the methyl acetate or about 5% by volume to the triethyl phosphate) produced a spectrum very similar to that of the liposomes. The latter mixture contained roughly 1 molecule of water for every 2 of triethyl phosphate. Addition of more water (to 10% by vol.) caused the shoulder at 365 nm to be replaced by an inflexion.

The ability of the probe molecules to discriminate between different forms of lipid dispersion was also tested with the 7-oxo compound. It is known that lysophosphatidylcholine disperses in water with equimolar amounts of cholesterol to form a lamellar phase instead of the normal micellar solution (Dervichian, 1966). 3-Hydroxycholest-5-en-7-one was dispersed in water with lysophosphatidylcholine in the molar proportions 1:1 and 1:20 and the o.r.d. spectra were recorded (Fig. 4a). The equimolar mixture gives essentially the same spectrum as the liposome preparations, whereas the spectrum of the other preparation is like that of an ethanolic solution and indicates that the chromophore is in a more polar environment. This presumably reflects the looser organization of the micelle with wider spacing of the surface polar groups and deeper penetration of water (Dodd *et al.*, 1970).

3-Hydroxycholest-7-en-6-one gave very similar results to those from the 7-oxo compound, even although the incorporation of this steroid is lower. In

Fig. 4. O.r.d. spectra of steroids in lipid dispersions and in erythrocyte membranes

(a) 3-Hydroxycholest-5-en-7-one dispersed in water with lysophosphatidylcholine; (1) 0.46 mg of steroid and 12.1 mg of lysophosphatidylcholine/ml and (2) 0.5 mg of steroid and 0.65 mg of lysophosphatidylcholine/ml. (b) 3-Hydroxycholest-7-en-6-one: (1) in liposomes (0.15 mg/ml) and (2) in erythrocyte membranes. (c) 3-Hydroxycholest-8(14)-en-15-one: (1) in liposomes (0.28 mg/ml) and (2) in erythrocyte membranes.

liposomes, the spectrum showed an extremum at 357nm and a shoulder at 346nm (Fig. 4b) as in dioxan solution. Thus the region of C-6 and C-7 of these compounds is in a moderately polar environment in liposomes.

With the probe group moved towards the tail of the molecule, as in 3-hydroxycholest-8(14)-en-15-one, the o.r.d. spectrum indicates that the chromophore is in a non-polar environment as it is in cyclohexane solution (Fig. 4c). The extremum is at 371 nm with a shoulder at 384 nm and $[\lambda]_D$ is 91. In moderately polar solvents, including esters such as methyl acetate, there is not even a clearly discernible inflexion present in the spectrum so that although this compound does not show as much fine structure as the other compounds in non-polar solutions the spectrum is still distinctive.

Erythrocyte membranes

The spectra of all compounds in erythrocyte membranes are given with those for liposomes. In all cases, the results are qualitatively the same. The $[\lambda]_D$ values were not known precisely because the final steps of filtration and of dilution inside the cell itself slightly altered the final concentration of the membrane dispersions. However, the shapes of the curves, which are the most important criteria of environment, are clearly shown. These and the positions of the peaks closely resemble those for liposomes, although in some cases there appeared to be a slight shift of about 1 nm towards the red end of the spectrum. Such a slight shift could be an artifact caused by some feature of the membrane preparations not reproduced in the control experiments. Alternatively, sterol molecules in the membranes could be in a slightly more hydrophobic environment, perhaps because the lipids are more tightly packed than in liposomes.

Effects of probe molecules on erythrocyte permeability

The effects of 3-hydroxycholest-5-en-7-one and 3-hydroxycholest-8(14)-en-15-one on the permeability of the intact erythrocyte membrane to water were studied by incubating samples of human erythrocytes with liposomes containing the appropriate steroid at 37°C and measuring the degree of haemolysis with time, as the steroid exchanged with the membrane sterol (Fig. 5). It can be seen that neither compound causes marked disruption of the membrane structure, since cells containing either steroid haemolyse more slowly than do those that have exchanged their sterol for liposomal cholesterol. Control cells incubated in buffer alone showed only slight (5%) haemolysis in 6h. Thus the liposomes containing cholesterol may not have been fully saturated with the sterol, causing a net loss of membrane cholesterol during the incubation and hence haemolysis (Bruckdorfer *et al.*, 1968).

Fig. 5. Haemolysis of erythrocytes during incubation with liposomes

Washed erythrocytes (2ml) were incubated with liposomes containing the steroid under investigation (about 10mg of phosphatidylcholine) in 12ml of 0.15M-sodium phosphate buffer (pH 7.4) containing 0.1% glucose. Samples (1ml) were diluted with buffer and centrifuged at 1000g for 30min. The E_{576} of the liberated haemoglobin was compared with that of a similar sample diluted with water (taken as 100% haemolysis). ○, Liposomes containing cholesterol; ●, liposomes containing 3-hydroxycholest-8(14)-en-15-one; □, liposomes containing 3-hydroxycholest-5-en-7-one.

Discussion

In the erythrocyte 'ghosts' studied 45–75% of the natural sterol was replaced by the incorporated molecular probes (except for the 6-oxo compound), so the spectra obtained yielded information about the structure of the membrane as a whole, not just about discrete regions. The extent to which the probe molecules are incorporated into liposomes does not necessarily reflect their miscibility with the phosphatidylcholine molecules as it would if partition from true solution could be used. It also depends on the lattice energies of their crystals. This is illustrated by a previous finding (Bruckdorfer *et al.*, 1968) that 7-dehydrocholesterol was not incorporated into liposomes as readily as cholesterol under the same conditions, although it enters cell membranes readily *in vivo* and *in vitro* and exchanges between them and plasma lipoproteins. Hence information derived from a poorly incorporated sterol is not necessarily of less value than that from the others. However, three probe molecules were well incorporated into the liposomes and appeared to exchange readily with the erythrocyte-membrane cholesterol.

There is some evidence that the membranes of 'ghost' preparations are not identical with those of the whole erythrocyte (Roelofsen *et al.*, 1971; Woodward & Zwaal, 1972), but Landsberger *et al.* (1972) could find no differences between the lipid phases of the

two preparations. Sonication or use of the French pressure cell can also cause some changes in the properties of erythrocyte membranes (Godin & Schrier, 1972; Sheetz & Chan, 1972), but there do not appear to be any major alterations of the basic structure (Cauldwell & Schumaker, 1970; Kamat *et al.*, 1972).

The extent to which the probe molecules modify the liposome or membrane structure when they replace cholesterol must also be considered. Cholesta-4,6-dien-3-one has little effect on the osmotic fragility of erythrocytes but it does markedly alter the permeability of the cells or of liposomes to solutes like glycerol or glucose and packs with phosphatidylcholine in monolayers differently from cholesterol (Bruckdorfer *et al.*, 1969; Demel *et al.*, 1972a,b). However, the work of Kamel *et al.* (1971) indicates that 3-hydroxycholest-5-en-7-one has physical properties very similar to those of cholesterol in monolayers and bilayers of lipid. It is noteworthy that this compound, probably the most discriminating of the probes used, is incorporated best into liposomes and erythrocytes and causes least haemolysis.

It does not seem that the average position of each probe molecule within the bilayer is altered significantly by the changes in polarity caused by the extra polar group. Thus cholesta-4,6-dien-3-one is much less polar than 3-hydroxycholest-3-en-2-one and yet the results indicate that the A rings of both are located in the same region of the liposome structure.

The results are most easily explained if the sterol molecules in liposomes and erythrocyte membranes occupy an average position like that shown in Plate 1. This allows C-2 and C-3 of the skeleton to be in the hydrated region of the phosphate groups, C-6 and C-7 to be in the glycerol ester region, probably at the limit of hydration, and C-15 to be in the interior of the bilayer. This arrangement is consistent with all of the spectroscopic data. Although hydrated, the region of the phospholipid head groups is so closely packed that the physical properties will differ from those of pure water. For example, the head-group region in micelles has a dielectric constant much lower than that of bulk water (Mukerjee & Desai, 1969).

The proposed arrangement also allows for hydrogen bonding between the sterol hydroxyl group and the phospholipid phosphate group, as previously suggested (Bruckdorfer *et al.*, 1968) on the basis of steroid-exchange experiments. This type of arrangement has also been put forward by Darke *et al.* (1972) Demel *et al.* (1972a) and Hsia & Boggs (1972) from studies using other physical techniques to investigate the properties of the phospholipid molecules. The proposed arrangement is consistent with the X-ray-diffraction data (Rand & Luzzati, 1968).

Bonding between the sterol molecules and the phospholipid head groups is supported by the finding that 3-hydroxycholest-3-en-2-one does not exchange

between liposomes and erythrocyte membranes at all whereas cholesta-4,6-dien-3-one exchanges much more readily than cholesterol (Bruckdorfer *et al.*, 1968). Unlike cholesterol, the 3-oxo compound cannot form a hydrogen bond with the phosphate oxygen atoms, whereas the 2-oxo compound is more strongly bound than cholesterol. The high $[\eta]$ value of the 2-oxo compound in liposomes could simply result from the more ionic character of the hydrogen bonds formed with the electronegative phosphate oxygen atoms but in the presence of water multiple bonding could also occur. From i.r.-spectral studies of dry films of 2 molecules of cholesterol per molecule of phosphatidylcholine, Zull *et al.* (1968) proposed bonding between the cholesterol hydroxyl group and the phosphate ester oxygen atoms, rather than the non-esterified oxygen proposed by Bruckdorfer *et al.* (1968). The o.r.d. results are consistent with this and with the arrangement predicted on theoretical grounds by Vandenheuvel (1963), but the sterol-exchange results are not then so simply explained. The arrangements proposed by Roels *et al.* (1969), Caspar & Kirschner (1971) and Rothman & Engelman (1972) do not conform so well with the o.r.d. results, as in these the sterol molecule lies deeper within the bilayer with C-2, C-3, C-6 and C-7 in less-polar environments than the spectra of the probe molecules suggest.

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EXPLANATION OF PLATE I

Proposed arrangement of cholesterol and phosphatidylcholine molecules in liposomes and erythrocyte membranes

Models of the two molecules are superimposed in (b) with the atoms attached to C-1, C-2, C-3, C-6, C-7, C-11 and C-15 of the sterol numbered. Aligned with them (a) is a model of the phospholipid, which has been twisted to show more clearly the regions associated with the different probe groups.

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