Factors Influencing the Lipid Composition and Fluidity of Red Cell Membranes in Vitro: Production of Red Cells Possessing More Than Two Cholesterols per Phospholipid†

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ABSTRACT: The cholesterol/phospholipid mole ratio (*C/P*) of normal human red cell membranes is 0.9–1.0. In disease it may increase to 1.0–1.6, and we have reported experimental values of up to 2.0. This report describes red cell membranes which have *C/P* values of >2.0. Red cells were incubated with sonicated dispersions of cholesterol, cholesteryl linoleate, lecithin, and sphingomyelin. Dispersion *C/P* varied from 0 to 3.0. Red cell membrane *C/P* following incubation ranged from 0.4 to 2.7 and correlated strongly with dispersion *C/P*. This was not influenced by the class of phospholipid, by the phospholipid's fatty acid composition, or by the amount of cholesteryl ester present within the dispersion. Membrane fluidity was measured as the rotational diffusion of the fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) and expressed as the viscosity ($\bar{\eta}$) of an isotropic medium permitting equivalent rotational diffusion. Changes in membrane $\bar{\eta}$ correlated with membrane *C/P* without regard to the specific characteristics of the lipid dispersion. Membrane $\bar{\eta}$ was increased from 2.8 P in normal red cells to 6.0 P in red cells with a *C/P* of 2.0. Further increases in membrane *C/P* above 2.0 caused no further increase in $\bar{\eta}$. Thus, dispersions of cholesterol and phospholipid can be prepared which have *C/P* values of up to 3.0, and biologic membranes can incorporate cholesterol to reach *C/P* values of this magnitude. Dispersion *C/P*, as compared with other physical and chemical characteristics of lipid dispersions, is of primary importance in determining both the *C/P* and the fluidity of red cell membranes. However, the manner in which cholesterol is incorporated into membranes with *C/P* of <2.0 appears to differ from its incorporation at *C/P* levels >2.0.

Two processes appear to govern the movement of cholesterol between plasma lipoproteins and cell membranes. The first is a relatively rapid process of cholesterol exchange, which can be demonstrated by the equilibration of isotopically labeled cholesterol between red cells and lipoproteins over the course of several hours in vitro and in vivo (Hagerman & Gould, 1951). No net change in the amount of cholesterol within either membranes or lipoproteins accompanies this process.

A second, slower process affects the equilibrium partition of cholesterol between lipoproteins and cell membranes (Cooper & Jandl, 1969). Unlike isotopic exchange, equilibrium partition involves a net transfer of cholesterol between membranes and lipoproteins. This movement of cholesterol appears to depend on the mole ratio of cholesterol to phospholipid (*C/P*)[1] within membranes relative to the *C/P* in lipoproteins (Cooper et al., 1972, 1975). Changes in the cholesterol content of membranes affect the fluidity of membrane lipids. Therefore, factors governing the partition of cholesterol between lipoproteins and membranes have an important influence on the molecular dynamics within membranes. This has been demonstrated for red cells and platelets both in vitro and in vivo (Kroes et al., 1972; Vanderkooi et al., 1974; Shattil & Cooper, 1976).

In previous studies we have observed increases in red cell membrane *C/P* from normal values of 0.9–1.0 to values of 1.0–1.6 in the red cells of patients with liver disease (Cooper et al., 1972) and up to 2.0 in red cells incubated with sonicated dispersions of cholesterol and L-α-dipalmitoyllecithin (Cooper et al., 1975). These increases in membrane *C/P* were associated with a decrease in membrane fluidity (Vanderkooi et al., 1974). In further studies of this process, we have observed that red cell membranes are capable of incorporating more than two cholesterols per phospholipid. It is the purpose of this report to describe the conditions under which such cholesterol enrichment occurs and to define the effect which changes in membrane *C/P* of this magnitude have on membrane fluidity.

Materials and Methods

Preparation of Lipid Dispersions. Five phospholipids were utilized: the synthetic phospholipids, L-α-dipalmitoyllecithin (L-DPL) (Grand Island Biological Corp., Grand Island, N.Y.), L-α-dimyristoyllecithin (L-DML) (La Motte Chem. Products Co., Chestertown, Md.), and D,L-dipalmitoyllecithin (D,L-DPL) (Sigma Chemical Co., St. Louis, Mo.); and the natural phospholipids, egg lecithin, and bovine brain sphingomyelin (Lipid Products Ltd., South Nutfield, U.K.). All phospholipids were analyzed by thin-layer chromatography and found to have 1% or less contamination with other lipids. Lipid dispersions were prepared by sonifying 40 mg of the appropriate phospholipid with various amounts of cholesterol (Sigma Chemical Co., St. Louis, Mo.), and, in some experiments, with cholesteryl linoleate (Sigma Chemical Co., St. Louis, Mo.) in 10 mL of 0.155 M NaCl in a fluted metal container placed in a constant temperature bath maintained at 45 °C, unless otherwise noted. Sonication was conducted for 50 min at 85 W with a Branson Sonifier (Heat Systems-Ultrasonics, Inc., Plainview, L.I., N.Y.) with a standard tip. Dispersions of synthetic lecithins and brain sphingomyelin with cholesterol were made after crystalline lipids were added directly to the NaCl solution, except as discussed in Results. Dispersions including egg lec-

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[1] Abbreviations used are: *C/P*, cholesterol-to-phospholipid mole ratio; DPH, 1,6-diphenyl-1,3,5-hexatriene; $\bar{\eta}$, viscosity; ΔE, flow activation energy for viscosity.

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ithin or cholesteryl linoleate were made following dissolution of all lipids in chloroform:methanol, 3:2 (v/v), and recrystallization of lipids by solvent evaporation under vacuum. Cholesterol was added in amounts up to 80 mg (C/P mole ratio of 4.0) in order to obtain values of C/P in dispersions as high as 3.0. Cholesteryl linoleate was added in amounts up to 120 mg.

That cholesterol was totally dispersed under the conditions of these studies is indicated by the absence of cholesterol crystals in L-DPL–cholesterol dispersions (C/P = 2.8) as viewed by polarized light microscopy and by x-ray diffraction. When viewed by electron microscopy, these dispersions contained mixtures of unilamellar and multilamellar vesicles plus amorphous lipid structures. Cholesterol-rich dispersions were stable for only 24–36 h, as judged by the failure of lipid to precipitate, and stability for longer periods required the presence of small amounts of serum. Therefore, following sonication, 10 mL of the lipid dispersion was added to 4.0 mL of normal human serum, previously heated to 56 °C for 30 min, and the serum-sonicate mixture was centrifuged at 21 800g for 30 min to sediment undispersed lipid. In dispersions containing cholesteryl linoleate, undispersed lipid also accumulated at the air–water interface.

Preparation and Incubation of Red Cells and Red Cell Ghosts. For incubation, freshly obtained normal human red cells were washed three times with Hank's balanced salt solution and resuspended to a hematocrit of 10% in Hank's containing penicillin (1000 U/mL). Red cell suspensions were incubated with an equal volume of the appropriate serum-sonicate mixture in stoppered 16 × 150 mm test tubes at 37 °C in a shaking water bath for periods up to 40 h. Red cell ghosts were prepared according to Dodge et al. (1963).

Lipid Analyses. For measurement of cholesterol and phospholipid, washed red cells were extracted with 2-propanol:chloroform 10:6 (v/v) (Rose & Oklander, 1965), and lipid dispersions were extracted with acetone:ethanol 1:1 (v/v). Extracts were analyzed for total cholesterol (Zlatkis et al., 1953), unesterified cholesterol (Brown et al., 1954), and total lipid phosphorus (Bartlett, 1959). Phospholipids obtained commercially, as well as those present in red cells, were analyzed by thin-layer chromatography using chloroform:methanol:glacial acetic acid:water, 25:15:4:2 (Skipski et al., 1964).

Analysis of the Fluidity of Red Cell Membranes and Lipid Dispersions. The fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) (Aldrich Chemical Co., Milwaukee, Wisc.), was used to label red cell ghosts and lipid dispersions (free of serum). Measurements of fluorescence polarization and fluorescence intensity were performed with an Elscint MV-1 microviscosimeter (Elscint Corp., Hackensack, N.J.). This instrument is equipped with a 200-W mercury arc which generates a 366-nm band, an emission cut-off filter for wavelengths below 390 nm (aqueous 2 M $NaNO_2$), Glan Thompson Polarizer, dual photomultipliers to simultaneously record emission intensities parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the plane of excitation and a thermoregulated sample chamber. Temperature was measured with an electronic thermistor (Cole-Palmer Instrument Co., Chicago, Ill.).

DPH was kept as a stock solution in tetrahydrofuran at a concentration of 2×10^{-3} M. Immediately prior to use, it was diluted 1:2000 in 0.155 M NaCl with vigorous mixing. One volume of the dilute DPH dispersion was added to 1 volume of red cell ghosts suspended in 0.155 M NaCl at a ghost concentration of 1×10^8 per mL, and the mixture was incubated at 37 °C for 30 min.

The microviscosimeter simultaneously analyzes $I_\parallel$ and $I_\perp$ and calculates and displays the degree of fluorescence polarization (P) according to the formula (Seber, 1953):

$$P = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp} \quad (1)$$

Fluidity is expressed in terms of viscosity, calculated according to the Perrin equation (Perrin, 1934):

$$\frac{r_0}{r} = 1 + C(r)\frac{T\tau}{\bar{\eta}} \quad (2)$$

where r is the fluorescence anisotropy which is obtained from P by the relationship:

$$r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} \quad (3)$$

τ is the lifetime of the excited state, T is the absolute temperature, $\bar{\eta}$ is the effective viscosity, and r is the upper limit of r. For DPH, r_0 = 0.362 (Shinitzky & Barenholz, 1974). $C(r)$ is a structural parameter of the probe, which varies slightly with r and was calibrated for DPH by using paraffin oil as a reference solvent (Shinitzky & Inbar, 1974). The effective viscosity so obtained represents the harmonic mean of the effective viscosity opposing the rotational diffusion of the probe in all possible directions, other than that around an axis along the dipole, and it is expressed in macroscopic units (poise). The flow activation energy for viscosity (ΔE) was calculated from the slope of the plot, $\log \bar{\eta}$ vs. $1/T$, fit by the method of least squares (Shinitzky & Inbar, 1976).

Excited state lifetimes (τ) were estimated from the relative fluorescence intensities at each temperature ($I_\parallel + 2I_\perp$), assuming an upper limit value for τ of 11.4 ns (Shinitzky & Barenholz, 1974). These corresponded to independent lifetime measurements made at 24–25 °C using an Ortec photon-counting fluorescent lifetime instrument equipped with an air spark-gap type flash lamp and appropriate filters, as reported previously (Vanderkooi et al., 1974). The decay of fluorescence, which appeared to follow a single exponential, was defined by 265 data points which were analyzed by computer.

It should be noted that the calculation of $\bar{\eta}$ from a direct measure of $I_\parallel/I_\perp$ converts the arbitrary units of fluorescence anisotropy to an expression which defines the viscosity of an isotropic medium in which probe motion is similarly constrained. In the studies reported herein, the calculated $\bar{\eta}$ was directly proportional to r, since the calculation (eq. 2) utilizes two constants [r_0 and $C(r)$] and since the additional two terms (τ and T) were also constant throughout these experiments. Interpretation of $\bar{\eta}$ in terms of absolute viscosity is limited by the lack of a standard which more exactly represents the three-dimensional structure of the membrane's hydrophobic core than is represented by an isotropic medium such as paraffin oil. Therefore, in the present study, these measurements are used to compare the same membrane under different conditions.

To measure the amount of DPH taken up by ghosts, labeled ghosts were washed free of excess DPH, and the DPH present in both ghosts and in the supernatant was extracted with heptanol and measured fluorimetrically. Greater than 95% of the added DPH was taken up by ghosts over the course of 60 min at 37 °C up to the highest DPH per ghost ratio studies (10^{-6} M DPH and 10^5 ghosts/mL).

Results

Effect of Phospholipid Dispersions on Red Cell C/P. Sonicated dispersions which had a C/P of up to 3.0 could be prepared with sphingomyelin and with the various synthetic lecithins, and red cells incubated with these dispersions attained

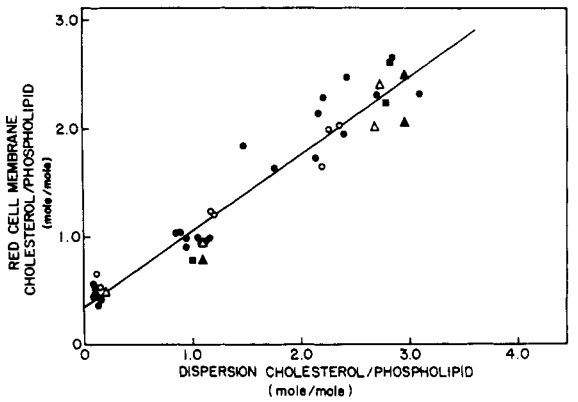

FIGURE 1: Relationship between the cholesterol/phospholipid mole ratio (*C/P*) of red cell membranes and the *C/P* of dispersions containing various phospholipids cosonicated with cholesterol. (○) Egg lecithin; (●) L-dipalmitoyllecithin; (▲) DL-dipalmitoyllecithin; (■) L-dimyristoyllecithin; and (△) bovine brain sphingomyelin.

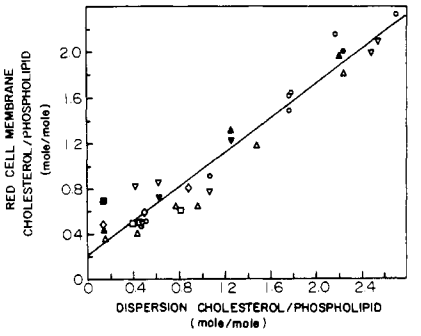

FIGURE 2: Equilibrium between red cells and cholesteryl ester containing cholesterol-phospholipid dispersions. Open symbols represent egg lecithin; closed symbols represent L-dipalmitoyllecithin. (○, ●) represent 0–9 wt % cholesteryl ester; (△, ▲) 10–19 wt %; (□, ■) 20–29 wt %; (▽, ▼) 30–39 wt %; and (◇, ◆) 40–49 wt %.

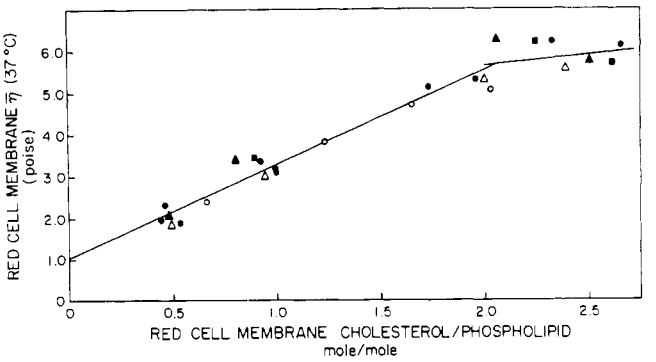

FIGURE 3: Effect of red cell membrane *C/P* on effective membrane viscosity ($\bar{\eta}$) at 37 °C. Red cells were incubated with dispersions of cholesterol and various phospholipids. Symbols as in Figure 1.

membrane *C/P* values of greater than 2.0 (Figure 1). In contrast, egg lecithin–cholesterol dispersions rarely exceeded *C/P* values of 2.4, and red cells incubated with cholesterol-rich egg lecithin dispersions never attained a *C/P* of greater than 2.0. The time course of the transfer of cholesterol from sonicated lipid dispersions with a *C/P* of >2.0 to red cell membranes was similar for egg lecithin, brain sphingomyelin, and synthetic lecithins, with incorporation maximal by approximately 24 h.

In the absence of added cholesterol, all phospholipids studied induced a similar degree of cholesterol loss by red cells during the course of 24 h of incubation in vitro (Figure 1). As noted previously (Cooper et al., 1972, 1974), red cells retained approximately 40% of their cholesterol under conditions of maximal cholesterol depletion. In the presence of equimolar cholesterol with each of the five phospholipids tested, the *C/P* of red cells did not change significantly from its normal value of 0.95. With all phospholipids studied, the absolute values of *C/P* attained by red cells were directly related to the *C/P* of the sonicated dispersions in a linear fashion (correlation coefficient = 0.96) throughout the range of membrane *C/P* values observed, from 0.4 to 2.7, with no apparent differences among the various phospholipids studied.

Because of data indicating that solvent (Freeman & Finean, 1975) and temperature (Horowitz et al., 1971) are important in preparing cholesterol-rich phospholipid dispersions, cholesterol–L-DPL dispersions were prepared under three conditions. First, crystalline cholesterol and crystalline L-DPL were dispersed in 0.155 M NaCl with sonication at 10 °C, as reported previously (Cooper et al., 1975). It was unusual under these conditions to achieve a *C/P* significantly greater than 2.0. Second, sonication of crystalline lipids was carried out at 45 °C, a temperature above the phase transition for L-DPL. This resulted in dispersions with a *C/P* approaching 3.0. Third, cholesterol and L-DPL were cocrystallized from chloroform–methanol prior to sonication at 45 °C, and this resulted in dispersions similar to those obtained with dry mixtures of cholesterol and L-DPL. The *C/P* of red cells incubated with these various cholesterol-rich L-DPL dispersions was directly proportional to the *C/P* of the dispersions, and it did not depend on the manner in which the dispersions were prepared. Under no condition described above was there a significant change in the total content of phospholipid in red cells following incubation, nor was there a change in the amount of lecithin or sphingomyelin, even after incubation for 40 h at 37 °C.

Effect of Cholesteryl Esters. Plasma lipoproteins carry three- to four-fold more cholesteryl esters than cholesterol. Previous studies have indicated that cholesteryl esters are solubilized to only a small degree by phospholipids (Janiak et al., 1974). To test the influence of cholesteryl esters on the *C/P* equilibrium between cell membranes and lipoproteins, cholesteryl linoleate was cosonicated with cholesterol and either egg lecithin or L-DPL. The amount of cholesterol linoleate dispersed represented between 5 and 50 wt % of the lipid dispersion (Figure 2). Nonetheless, the equilibrium partition of cholesterol between red cells and lipid dispersion correlated only with the *C/P* of the dispersion. Identical results were obtained for the two phospholipids studied. No modifying effect of cholesteryl esters was observed.

Effect of Lipid Dispersions on Membrane Fluidity. Under the conditions used for labeling ghosts (5×10^{-7} M DPH and 5×10^7 ghosts/mL), approximately 6×10^6 molecules of DPH were incorporated per ghost. This is equivalent to approximately 1 molecule of DPH per 100 molecules of ghost lipid. The $\bar{\eta}$ was found to remain constant over a range of DPH per ghost from one molecule of DPH for 1000 molecules of lipid (1.25×10^{-7} M DPH and 1×10^8 ghost per mL) to 1 molecule of DPH per 25 molecules of lipid (10^{-6} M DPH and 2.5×10^7 ghosts per mL). The excited state fluorescence lifetime of DPH in red cell ghosts was not influenced by the red cell *C/P*. Moreover, fluorescence decay appeared to follow a single exponential curve throughout the range of membrane *C/P*, and there was no evidence of a second exponential decay as has been observed in liposomal membranes (Chen et al., 1977).

The effect of various dispersions on the $\bar{\eta}$ of red cell membrane ghosts at 37 °C was examined (Figure 3). A close relation between *C/P* and $\bar{\eta}$ was seen through the range of red cell

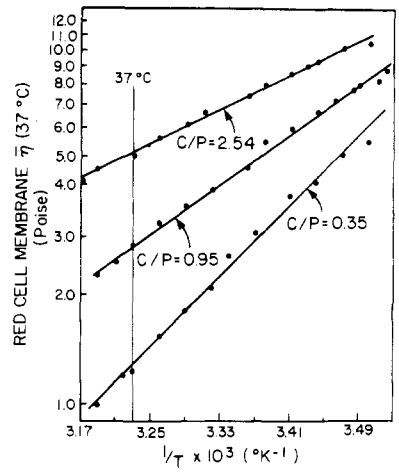

FIGURE 4: Influence of red cell membrane *C/P* on effective membrane viscosity ($\bar{\eta}$). Red cells were incubated with dispersions of cholesterol and L-dipalmitoyllecithin.

C/P values extending from 0.5 to 2.0. However, further increases in membrane *C/P* to values between 2.0 and 2.7 did not cause a further increase in $\bar{\eta}$. Values for membrane $\bar{\eta}$ ranged from 2.0 poise at a membrane *C/P* of 0.5 to approximately 6.0 P at *C/P* values of 2.0 to 2.7. No phase transition was apparent over the temperature range of 10–40 °C with red cell membranes at any *C/P* studied (Figure 4). ΔE was decreased from 8.3 kcal/mol in normal membranes to 5.8 kcal/mol in cholesterol-rich membranes (*C/P* = 2.54) (Figure 4), indicative of a high degree of order. Conversely, cholesterol depletion increased ΔE to 11.8 kcal/mol at a membrane *C/P* of 0.35.

Discussion

These studies demonstrate that sonicated dispersions of cholesterol and phospholipid can be prepared which have a *C/P* mole ratio of between 2.0 and 3.0, and it is the first indication that biologic membranes can incorporate cholesterol to reach *C/P* values of this magnitude. While fusion between cell membranes and phospholipid vesicles has been reported (Korn et al., 1974; Pagano & Huang, 1975; Martin & McDonald, 1976), the mechanism of cholesterol transfer from dispersions to red cell membranes in the present study appears to be by a net transfer of cholesterol, since no increase in the red cell content of lecithin or sphingomyelin was demonstrated. From the behavior of DPH in these cholesterol-rich red cell membranes, it appears that the manner in which cholesterol is incorporated in the membranes which have less than two cholesterols per phospholipid differs from the manner in which cholesterol enters the membrane to achieve more than two cholesterols per phospholipid.

These studies also demonstrate the overriding importance of the amount of cholesterol relative to the amount of phospholipid in sonified lipid dispersions and red cell membranes. Neither the class nor fatty acid composition of dispersed phospholipids affected this interrelationship despite marked differences in the fluidity of these phospholipids (Shinitzky & Barenholz, 1974; Shinitzky & Inbar, 1976; Cooper et al., 1977). Thus, when assessed in terms of exchange equilibrium at 37 °C, cholesterol demonstrates no preferential interaction with sphingomyelin or any of the lecithins studied as compared with its interaction with membranes. In contrast, Demel and his co-workers have found that, in mixed lipid systems in the region of the phase transitions, cholesterol has a higher affinity for sphingomyelin than for lecithin, and its affinity is least for phosphatidylethanolamine (VanDijck et al., 1976; Demel et al., 1977).

Earlier studies concluded that lecithin in water could accommodate no more than equimolar amounts of cholesterol (Bourges et al., 1967; Ladbrook et al., 1968). However, in dry films, cholesterol and lecithin can associate at a mole ratio of up to 2:1 (Zull et al., 1968). Moreover, several laboratories have obtained hydrated mixtures of cholesterol and egg lecithin in mole ratios of up to 2:1 by means of sonication (Freeman & Finean, 1975; Horowitz et al., 1971; Green & Green, 1973), and we have obtained similar results with cholesterol-L-DPL mixtures (Cooper et al., 1975). Lipid concentration and temperature appear to be critical in this process, with a maximum of 6–8 mg of total lipid dispersed per mL of water at 22 °C and 12 mg per mL at 50 °C (Horowitz et al., 1971; Green & Green, 1973). Excessive lipid concentrations and low temperature may underlie, in part, the failure of some workers to obtain sonicated mixtures of cholesterol and egg lecithin above a mole ratio 1:1 (Bourges et al., 1967; Hoyes & Saunders, 1966). Although *C/P* values greater than 2.0 could be obtained with all five phospholipids studied, values obtained with egg lecithin tended to be lower than with other phospholipids, and we have rarely observed cholesterol–egg lecithin mixtures with a *C/P* of greater than 2.4. The importance of temperature is seen in comparing the *C/P* values for cholesterol-L-DPL dispersion of up to 3.0 obtained at 45 °C in the present study with values that did not exceed 2.0 when otherwise identical dispersions were sonified at 10 °C (Cooper et al., 1975).

The fact that cholesteryl esters did not affect the interrelationship between the *C/P* of membranes and dispersions is consistent with information concerning cholesteryl esters in lecithin dispersions and in lipoproteins. For example, only small amounts (2–4 mol %) of cholesteryl esters can be incorporated into lecithin bilayers in aqueous mixtures (Janiak et al., 1974). In equimolar lecithin–cholesteryl oleate dispersions, most of the cholesteryl oleate is found in structures independent of those containing lecithin (Brecher et al., 1976). Recent studies also suggest that cholesteryl esters are in a separate phase in LDL, the major cholesteryl ester transport system in man (Deckelbaum et al., 1975). These considerations are consistent with the close correlation which we have observed between the *C/P* of LDL and that of red cell membranes in patients with liver disease, despite wide variations in the amount of cholesteryl esters relative to cholesterol in LDL obtained from these patients (Cooper et al., 1972).

Although a linear relationship existed between the *C/P* of dispersions and the *C/P* of membranes throughout the broad range of membrane *C/P* observed, the influence of *C/P* on membrane fluidity was continuous only to a membrane *C/P* value of 2.0. No further change in fluidity was apparent at membrane *C/P* values of greater than 2.0. This upper limit of 2.0 in terms of membrane *C/P* represents a 1:1 interaction of cholesterol molecules with the acyl chains of phospholipids. It is possible that membranes with *C/P* values in excess of 2.0 have domains which include cholesterol in a form that fails to influence membrane fluidity. Alternatively, such domains may exclude DPH and therefore the dynamics within these domains may not be represented by the rotational diffusion of this probe.

It is not clear whether changes in bulk fluidity, the formation of distinct lipid domains or some other effect of cholesterol on membrane structure is responsible for the physiologic abnormalities which have been observed in cholesterol-enriched membranes. For example, we have demonstrated differences in the transport properties (Wiley & Cooper, 1975) and morphologic appearance (Cooper et al., 1975) of human red

cells enriched with cholesterol as described herein. Cholesterol enrichment of human platelets under similar conditions affects the activity of the membrane enzyme, adenylate cyclase (Sinha et al., 1977), and alters the response of platelets to aggregating agents (Shattil et al., 1975). The present study defines the equilibrium conditions under which a broad range of changes in membrane *C/P* can be induced, and it provides insight into the lipid–lipid interactions which may underlie some of the alterations in membrane function which result.

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