Membrane Microviscosity and Human Platelet Function†

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ABSTRACT: An increased sensitivity to epinephrine-induced aggregation has been observed both in platelets obtained from patients with type IIa hyperlipoproteinemia and in normal platelets following incubation with cholesterol-rich lecithin dispersions. We have reported previously that the membrane fraction of platelets is enriched with cholesterol relative to phospholipid under each of these conditions. To further explore the effect of cholesterol on platelet membranes, we have examined the fluidity (microviscosity) of whole platelets and platelet subcellular fractions using a hydrophobic fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), under conditions in which the cholesterol-to-phospholipid mole ratio (C/PL) of platelets was varied by incubation with various cholesterol–lecithin sonicated dispersions. The C/PL of platelets directly influenced the rotational diffusion of DPH, as indicated by changes in fluorescence polarization. This was reflected in an increase in microviscosity at 37 °C ($\bar{\eta}_{37}$) from 2.84 P in normal platelets to 4.06 P in platelets with a 118% increase in C/PL. Conversely, platelets with a 43% decrease in C/PL had a 13% decrease in $\bar{\eta}_{37}$. A strong correlation ($r = 0.94$) existed between C/PL and $\bar{\eta}_{37}$ throughout this entire range. However, C/PL had no effect on the excited-state fluorescence lifetime of DPH. Both C/PL and $\bar{\eta}_{37}$ were lower in isolated platelet membranes than in the platelet granule fraction. When platelets were incubated for 20 h with cholesterol-rich dispersions, there was an increase in C/PL and $\bar{\eta}_{37}$ in both the membrane and granule fractions. However, this occurred more rapidly in membranes so that, at 5 h (a time when an increased sensitivity of whole platelets to epinephrine is evident), membrane C/PL had increased 55% and $\bar{\eta}_{37}$ had increased 42%, whereas granule C/PL and $\bar{\eta}_{37}$ had changed minimally. Cholesterol-rich platelets and subcellular fractions had a lower fusion (or flow) activation energy for viscosity (ΔE), reflecting a higher degree of order, and the converse was true in cholesterol-poor platelets. Moreover, a strong negative correlation existed between the percent change in ΔE and the percent change in $\bar{\eta}_{37}$ induced either by cholesterol incorporation or depletion. These data demonstrate that cholesterol influences the fluidity and the degree of order within the hydrophobic core of platelet membranes. Changes induced in these physical properties by an excess of cholesterol relative to phospholipid may underlie the abnormal reception or transmission of the aggregation stimulus in cholesterol-rich platelets.

Human platelets aggregate in vivo in response to vessel injury. While this serves an important role in the control of hemorrhage, the aggregation of platelets at the vessel wall may also lead to disease. Indeed, a pathogenetic role has been proposed for platelets in both thrombotic disorders (Harker and Slichter, 1972) and atherosclerosis (Ross et al., 1974; Roberts and Ferrans, 1976). The platelet surface appears to be a critical determinant of these normal and pathologic processes. Interactions between the platelet surface and either the vessel wall or small molecular weight substances such as adenosine diphosphate and epinephrine cause platelets to aggregate, release vasoactive substances, and make available procoagulant material.

Like all mammalian cell membranes, the surface membrane of platelets is composed of lipid and protein. Perturbation of the lipid composition of platelet membranes results in altered platelet function. In previous studies, we have demonstrated that platelets which have acquired an increase in the mole ratio of cholesterol to phospholipid (C/PL)[1] following exposure to cholesterol-rich phospholipid dispersions are more sensitive than normal to the aggregating agents, epinephrine and ADP (Shattil et al., 1975). These cholesterol-rich platelets also have an elevated basal activity of adenylate cyclase (Sinha et al., 1976), and they are resistant to the aggregation inhibitor, prostaglandin E_1 (Colman et al., 1975). The converse is true as well; i.e., platelets in which the membrane C/PL has been decreased by incubation with pure phospholipid dispersions have a decreased responsiveness to epinephrine and ADP (Shattil et al., 1975). These observations in vitro may have a counterpart in human disease since platelets from individuals with type IIa hyperlipoproteinemia have an increased membrane C/PL (Shattil et al., 1976), and they are more sensitive than normal to aggregating agents (Carvalho et al., 1974).

Many investigators have shown that the cholesterol content of membranes has a substantial effect on lipid–lipid interactions as assessed by physical measurements such as scanning calorimetry (Ladbrooke et al., 1968), nuclear magnetic resonance (Penkett et al., 1968), and membrane probes of both the electron spin (Hubbell and McConnell, 1971) and fluorescence polarization (Shinitzky and Inbar, 1976) types. Shinitzky and co-workers have described a fluorescent compound, 1,6-diphenyl-1,3,5-hexatriene (DPH), which dissolves in the hydrocarbon region of the membrane where it serves as a convenient probe of the fluidity (or microviscosity) of the lipid environment in which it resides (Shinitzky and Barenholz, 1974; Shinitzky and Inbar, 1974; Aloni et al., 1974). Using this technique we have shown a direct relationship between the C/PL and the microviscosity of red cell ghost membranes (Cooper and Leslie, 1976). We have selected DPH as a tool to examine the microviscosity of human platelets and of

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[1] Abbreviations used are: C/PL, cholesterol-to-phospholipid mole ratio; DPH, 1,6-diphenyl-1,3,5-hexatriene; PRP, platelet-rich plasma; P, fluorescence polarization; $\bar{\eta}$, microviscosity; ΔE, fusion activation energy for microviscosity; SD, standard deviation.

platelet subcellular fractions in an effort to clarify the mechanisms by which the cholesterol content of membranes influences membrane-associated platelet functions.

Materials and Methods

Platelet Preparation. Venous blood was obtained from healthy volunteers who had abstained from medications for at least 2 weeks. It was anticoagulated with either 1/9 volume of trisodium citrate (0.129 M) or with acid-citrate-dextrose (JA-25N Blood Pack, Fenwal Laboratories, Morton Grove, Ill.). Platelet-rich plasma (PRP) was obtained by centrifugation at 22 °C for 20 min at 100*g*. Platelet-poor plasma was obtained by centrifugation of blood at 1800*g* for 15 min. PRP was either used in incubation experiments with lipid dispersions or the platelets were washed three times in a platelet wash buffer (Gaintner et al., 1962) and processed for studies of microviscosity and lipid composition.

Platelet Incubation System. Unesterified cholesterol (Sigma Chemical Company, St. Louis, Mo.) and L-α-dipalmitoyllecithin (Grand Island Biological Company, Grand Island, N.Y.) were assayed for purity and sonicated at 45 °C in 10 ml of modified Tyrode's buffer (0.1369 M NaCl, 0.0027 M KCl, 0.0119 M $NaHCO_3$, 0.00042 M $NaH_2PO_4H_2O$, pH 7.4) to produce lipid dispersions as described previously (Shattil et al., 1975; Cooper et al., 1975). Three different dispersions were made, each differing in their free cholesterol-to-phospholipid mole ratio (C/PL). "Cholesterol-normal" dispersions, prepared in 10 ml of buffer from lecithin (40 mg) plus cholesterol (23 mg), had a C/PL of 1.0 and had been shown previously not to affect platelet cholesterol or phospholipid content during prolonged incubation (Shattil et al., 1975). "Cholesterol-rich" dispersions (lecithin (40 mg) plus cholesterol (80 mg)) had a C/PL of 2.0–2.2 and had been demonstrated to transfer cholesterol but not phospholipid to platelets (Shattil et al., 1975). "Cholesterol-poor" dispersions (lecithin, 40 mg) had a C/PL of zero and they decreased the cholesterol content of platelets. Immediately before use, dispersions were centrifuged at 21 800*g* for 30 min to sediment undispersed lipid.

In studies pertaining to whole platelets, PRP was adjusted with platelet-poor plasma to a platelet count of $3\text{–}4 \times 10^8$/ml and incubated with an equal volume of either Tyrode's buffer or one of the lipid dispersions in Tyrode's. Penicillin (200 units/ml final concentration) was added and mixtures were incubated for up to 20 h in a 37 °C water bath with gentle inversion of the incubation tubes hourly. Samples were removed at various times for analysis of platelet lipid composition and microviscosity. Incubations requiring large numbers of platelets for subsequent subcellular fractionation consisted of 1.9 volumes of PRP, 1 volume of Tyrode's buffer or lipid dispersions, and penicillin, 200 units/ml.

Platelet Lipid Analysis and Subcellular Fractionation. Platelets were washed three times in platelet wash buffer, and platelet cholesterol (Zlatkis et al., 1953) and lipid phosphorus contents (Bartlett, 1959) were measured in quadruplicate aliquots of washed platelets extracted with 80 volumes of isopropyl alcohol and chloroform (Rose and Oklander, 1965). Total phospholipid content was taken to equal lipid phosphorus times 25. Subcellular fractionation was carried out by the method of Marcus et al. (1966), and each fraction was washed twice with platelet wash buffer at 130 500*g* for 1 h. The resultant membrane and granule fractions had a pattern of enzyme activity as described previously (Shattil et al., 1975; Marcus et al., 1966). Isopropyl alcohol–chloroform extracts of membranes and granules were analyzed for cholesterol (Rudel and Morris, 1973) and phospholipid (Bartlett, 1959).

Analysis of Platelet Microviscosity. The fluorescent probe, DPH (Aldrich Chemical Co., Milwaukee, Wisc.), was used to label thrice-washed platelets, or twice-washed, isolated membranes and granules. The optimal conditions of labeling are described in Results. Measurements of fluorescence polarization and fluorescence intensity were performed with an Elscint MV-1 microviscosimeter (Elscint Corporation, Haifa, Israel) as described by Shinitzky and co-workers (Shinitzky and Barenholz, 1974; Cogan et al., 1973). 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 polarizers, dual photomultipliers to record emission intensities parallel and perpendicular to the plane of excitation, and a constant-temperature chamber. Temperature was measured with an electronic thermistor (Cole-Palmer Inst. Co., Chicago, Ill.).

DPH was dissolved in tetrahydrofuran at a concentration of 2×10^{-3} M. Immediately prior to use, it was diluted 1:2000 in platelet wash buffer, with vigorous mixing. One volume of the dilute DPH dispersion was added to 1 volume of platelets suspended in platelet wash buffer at a platelet concentration of 1×10^8/ml, and the mixture was incubated at 37 °C for 30 min. The output signals from platelets labeled with DPH were more than 50-fold greater than those obtained with unlabeled platelets.

The microviscosimeter simultaneously analyzes $I_\parallel$ and $I_\perp$, the fluorescence intensities detected through a polarizer oriented parallel and perpendicular, respectively, to the direction of the polarized excitation beam. From these measurements it calculates polarization (P) according to the formula:

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

Microviscosity is calculated according to the Perrin equation:

$$\frac{r_0}{r} = 1 + C(r)\frac{Tt}{\bar{\eta}}$$

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

$$r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} = \frac{(I_\parallel/I_\perp) - 1}{(I_\parallel/I_\perp) + 2} = \frac{2P}{3 - P}$$

r_0 is the upper theoretical limit of $I_\parallel/I_\perp$ ($r_0 = 0.362$) (Shinitzky and Barenholz, 1974), t is the lifetime of the excited state, T is the absolute temperature, and $\bar{\eta}$ is the microviscosity. $C(r)$ is a structural parameter of the probe which varies slightly with r and was calibrated for DPH by Shinitzky and Inbar (1974). The microviscosity 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 equal to the dipole, and it is expressed in poise. The fusion (or flow) activation energy for microviscosity (ΔE) was calculated from the slope of the plot, $\log \bar{\eta}$ vs. $1/T$, fit by the method of least squares.

Excited-state lifetimes (t) were estimated from the relative fluorescence intensities at each temperature ($I_\parallel + 2I_\perp$) and from a value of t_0 of 11.4 ns (Shinitzky and Barenholz, 1974). These corresponded to independent lifetime measurements made at 24–25 °C using an Ortec photon-counting fluorescence lifetime instrument equipped with an air spark-gap type flash lamp and appropriate filters. The decay of fluorescence was defined by 265 data points which were analyzed by com-

TABLE I: Excited-State Fluorescence Lifetimes of DPH in Platelets.

Platelets Incubated 20 h with:	Platelet Cholesterol/Phospholipid (mol/mol)	T (°C)	Fluorescence Lifetime[a] (ns)
Tyrode's buffer	0.55	24.2	8.8
Cholesterol-rich lecithin dispersions	1.07	25.0	9.0
Cholesterol-normal lecithin dispersions	0.55	25.0	9.2
Cholesterol-poor lecithin dispersions	0.34	24.2	9.0

[a] Obtained by direct lifetime measurements.

puter (Vanderkooi et al., 1974).

Results

Characteristics of DPH Fluorescence in Human Platelets. Preliminary studies were carried out to determine the optimal conditions for labeling human platelets with DPH. The degree of fluorescence polarization (P) of DPH was constant after incubation of platelets with an aqueous dispersion of DPH (final concentration, 5×10^{-7} M) in platelet wash buffer for 30 min at 37 °C over a range of 3×10^7 to 1.5×10^8 platelets/ml. When DPH was incubated with 5×10^7 platelets/ml for up to 3 h at 37 °C, the intensity of the fluorescence signal increased by first-order kinetics, and plateaued by 30 min. However, P remained constant over the entire period of the labeling process. Moreover, at this cell concentration, sequential dilutions of the DPH-labeled platelet dispersions with platelet wash buffer revealed a constant value of P, thus excluding depolarization due to light scattering. In the experiments to be described, platelets (5×10^7/ml) were labeled by incubation with DPH (5×10^{-7} M) in platelet wash buffer for 30 min at 37 °C. The amount of DPH presented to platelets was equivalent to approximately 1 molecule of DPH per 100 molecules of platelet phospholipid.

The P of platelets occasionally increased as much as 10% after incubation for up to 72 h in plasma, Tyrode's buffer, or platelet wash buffer. The extent of this incubation effect was variable, it occurred whether or not DPH was present in the medium for the entire incubation, and it was temperature dependent, occurring during incubation at 37 °C but not at 4 °C. Although the mechanism of this effect was not investigated further, it was eliminated as a source of error in the following experiments by comparing the P of platelets incubated with lipid dispersions with that of the same platelets incubated for the same period of time with plasma or buffer alone.

The excited-state fluorescence lifetime (t) of DPH in platelets, measured directly at 25 °C, was not influenced by the platelet cholesterol-to-phospholipid mole ratio (C/PL) (Table I) and a single exponential decay curve was obtained under all four conditions studied. Values obtained directly were very similar to values obtained by calculating fluorescence lifetime from a measure of fluorescence intensity assuming t_0 to equal 11.4 ns (Shinitzky and Barenholz, 1974). Therefore, fluorescence intensity was used routinely to compute fluorescence lifetimes for calculation of microviscosity

Effect of C/PL on Platelet Membrane Microviscosity. Whole platelets from 13 normal donors had a mean C/PL of 0.57 ± 0.04 (1 SD), a mean platelet microviscosity at 37 °C ($\bar{\eta}_{37}$) of 2.84 ± 0.24 P, and a mean microviscosity at 28 °C ($\bar{\eta}_{28}$) of 3.74 ± 0.26 P. To determine the effect of platelet C/PL on platelet microviscosity, platelets were incubated up to 20 h at 37 °C with various lipid dispersions. Platelets incubated with cholesterol-rich dispersions selectively gained cholesterol, resulting in an increase in their C/PL of up to 118%; this was associated with an increase in $\bar{\eta}_{37}$ of up to 43%. In contrast, platelets incubated with cholesterol-poor dispersions decreased their C/PL as much as 38% and their η_{37} by 13%. Platelets incubated with cholesterol-normal dispersions or with Tyrode's buffer alone underwent no change in either C/PL or microviscosity. There was a strong correlation between platelet C/PL and $\bar{\eta}_{37}$ ($r = 0.94$; $p < 0.001$) (Figure 1) as well as between C/PL and $\bar{\eta}_{28}$ ($r = 0.90$; $p < 0.001$).

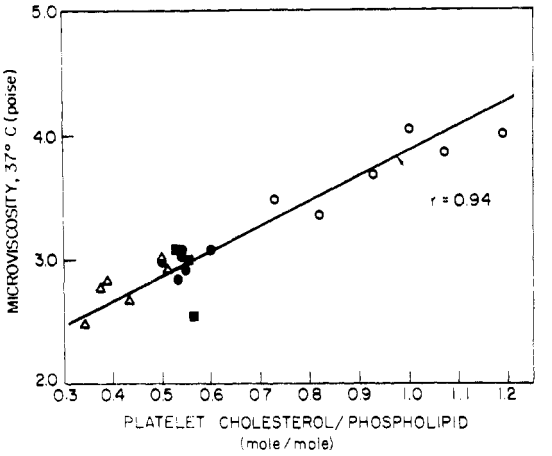

FIGURE 1: The relationship between platelet microviscosity at 37 °C and the platelet cholesterol-to-phospholipid mole ratio. Closed circles represent platelets in plasma incubated for variable periods of time with an equal volume of Tyrode's buffer. Open circles represent platelets incubated with cholesterol-rich phospholipid dispersions and open triangles, platelets incubated with cholesterol-poor phospholipid dispersions. Closed squares represent fresh, unincubated platelets.

The relationship between temperature and microviscosity in platelets which were normal, cholesterol-rich, and cholesterol-poor is shown in Figure 2. Characteristic of biomembranes, a plot of the logarithm of platelet microviscosity against the reciprocal of the temperature was linear over the temperature range studied (12.0–44.5 °C). The slope of this plot is a measure of ΔE, the fusion (or flow) activation energy for viscosity. This is an expression which characterizes the degree of order in lipid–lipid interactions, a lower value for ΔE indicating a higher degree of order. The addition of cholesterol to platelet membranes increased microviscosity at all temperatures and decreased ΔE from 9.4 to 9.0 kcal/mol. Thus, cholesterol simultaneously caused an increase in both the microviscosity and the degree of order within the membrane. Platelets poor in cholesterol exhibited the converse charac-

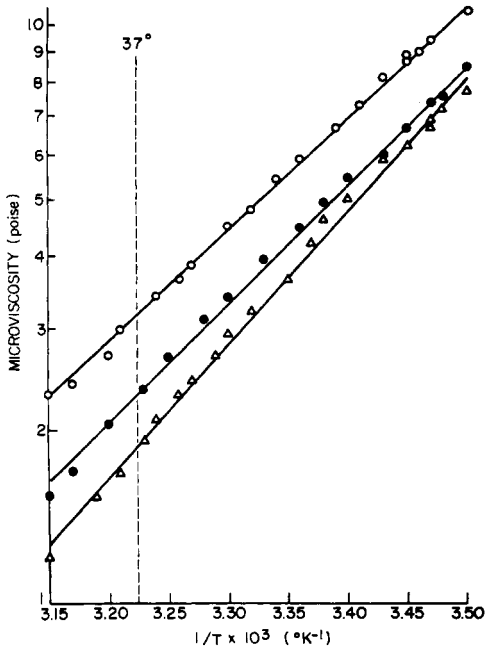

FIGURE 2: Logarithmic plot of microviscosity vs. the reciprocal of the absolute temperature for cholesterol-rich platelets (C/PL = 1.07) (open circles), normal platelets (C/PL = 0.55) (closed circles), and cholesterol-poor platelets (C/PL = 0.34) (open triangles). Fusion activation energy for microviscosity (ΔE) was 9.4 kcal/mol for normal platelets, 9.0 kcal/mol for cholesterol-rich platelets, and 10.8 kcal/mol for cholesterol-poor platelets.

TABLE II: Lipid Composition and Microviscosity of Normal Platelet Membranes and Granules.

	Membranes (5)[a]	Granules (5)
Cholesterol/phospholipid (mol/mol)	0.502 ± 0.04[b]	0.665 ± 0.05
Microviscosity (P)		
28 °C	3.25 ± 0.18	3.46 ± 0.24
37 °C	2.47 ± 0.21	2.78 ± 0.24

[a] Numbers in parentheses indicate the number of platelet donors studied. [b] One standard deviation.

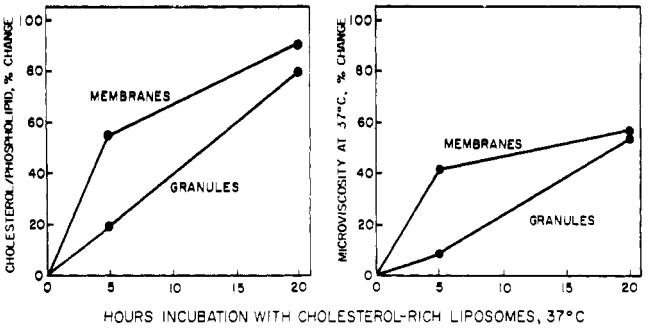

FIGURE 3: Changes in lipid composition and microviscosity at 37 °C of platelet membranes and granules following incubation of platelets with cholesterol-rich phospholipid dispersions. This experiment is representative of three so performed.

teristics, with a lower microviscosity but a lower degree of order, as evidenced by a ΔE of 10.8 kcal/mol.

More than 90% of the cholesterol in platelets can be recovered in two membrane-containing subfractions when platelets are homogenized and fractionated by sucrose density gradient centrifugation. One fraction (the "membrane" fraction) probably contains both plasma membranes and membranes of the platelet surface-connecting and dense tubular systems (Taylor and Crawford, 1974). A second, the "granule" fraction, consists of the membranous coat of α granules and dense granules. We examined the contribution of each of these platelet subfractions to microviscosity and the extent to which each was perturbed during incubation of whole platelets with cholesterol-rich lipid dispersions.

The C/PL of the membrane fraction prepared from normal human platelets was lower than that of the granule fraction and microviscosity was correspondingly lower in the membrane than the granule fraction (Table II). When platelets were incubated for 20 h with cholesterol-rich dispersions, cholesterol was acquired by both the membrane and granule fractions, and this was accompanied by an increase in the microviscosity of both subcellular fractions. However, there was a marked difference between membranes and granules with respect to the rate at which each fraction acquired cholesterol and underwent a change in microviscosity (Figure 3). Both the C/PL and the microviscosity of the membrane fraction increased rapidly, and approximately two-thirds of the increase in each observed at 20 h was present after 5 h of incubation. In contrast, only a small increase was observed in the C/PL of the granule fraction, and granule microviscosity was increased only slightly by 5 h (Figures 3 and 4A). Thus, at a time when platelet functional changes induced by cholesterol acquisition are apparent (5 h) (Shattil et al., 1975), the microviscosity of platelet membranes from cholesterol-rich platelets was markedly increased, whereas that of the granule fraction was similar to normal. However, at 20 h, the microviscosity of granules had increased as well (Figures 3 and 4B). These values for the microviscosity of platelet subcellular fractions bore a direct relationship to their C/PL, and this relationship in the subcellular fractions was similar to that which had been observed in whole platelets (Figure 1), although there was a tendency for the subcellular fractions to be somewhat more fluid at each C/PL than had been observed with whole platelets (Figure 5).

The mean ΔE of whole platelets from four donors was 9.4 kcal/mol, that of normal membranes was 8.5 kcal/mol, and the ΔE of the granule fraction of normal platelets was 7.1 kcal/mol. There was a close negative correlation between the percent change in ΔE and the percent change in the microviscosity of whole platelets and of platelet subcellular fractions following incubation with lipid dispersions ($r = -0.90$; $p < 0.001$; Figure 6). A similar correlation was observed between changes in ΔE and in platelet C/PL ($r = -0.76$; $p < 0.01$).

Discussion

The values obtained for the microviscosity and fusion activation energy for viscosity, ΔE, of normal platelets are similar to those reported for other human blood cells using DPH as a probe (Shinitzky and Inbar, 1976). Platelets which acquired or lost cholesterol underwent an increase or decrease in platelet microviscosity, respectively. Each percent change in cholesterol content was associated with a change of 0.02 P in microviscosity at 37 °C (Figure 1). These changes were prominent in platelet membranes after short term incubation (5 h) of platelets with cholesterol-rich lipid dispersions but were observed in granule lipids as well after prolonged incubation (20 h).

DPH has been employed in studies of the hydrocarbon core of membranes of relatively simple cells such as erythrocytes

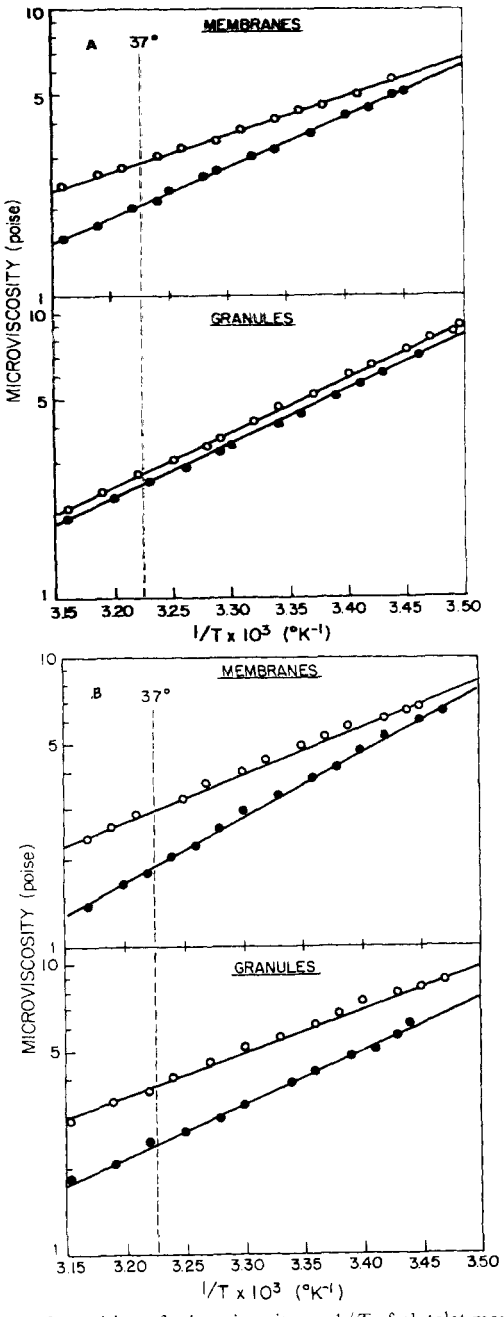

FIGURE 4: Logarithm of microviscosity vs. $1/T$ of platelet membranes and granules after incubation of platelets with cholesterol-rich phospholipid dispersions (open circles) or Tyrode's buffer (closed circles) for (A) 5 h and (B) 20 h.

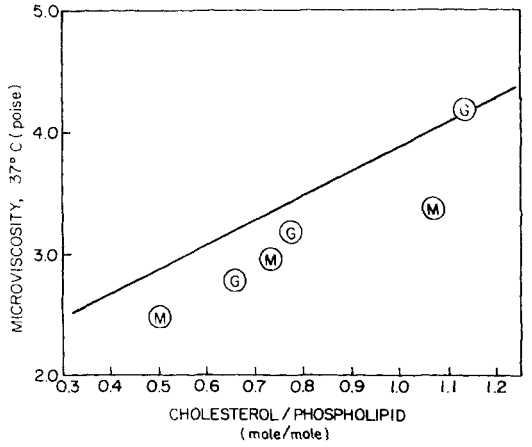

FIGURE 5: Relationship between microviscosity at 37 °C and cholesterol-to-phospholipid mole ratio of platelet membranes (M) and granules (G) following the incubation of platelets with either Tyrode's buffer or cholesterol-rich dispersions for variable periods of time at 37 °C. The line represents the regression line of the same relationship in whole platelets (Figure 1).

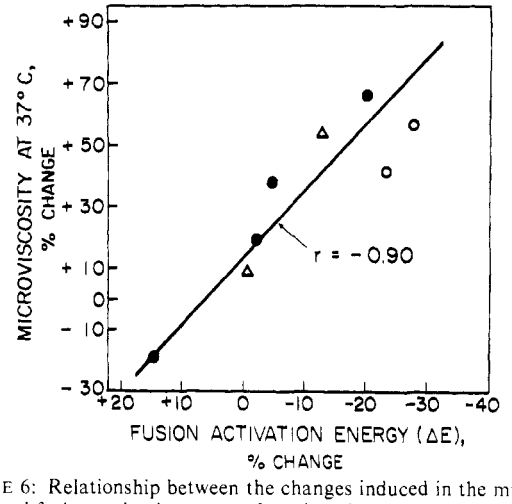

FIGURE 6: Relationship between the changes induced in the microviscosity and fusion activation energy for microviscosity (ΔE) of platelets, membranes, and granules as a result of incubation of platelets with cholesterol-rich or cholesterol-poor dispersions. Closed circles depict whole platelets, open circles, platelet membranes, and open triangles, platelet granules.

(Aloni et al., 1974) and of more complex nucleated cells such as mouse lymphocytes and lymphoma cells (Shinitzky and Inbar, 1974) and mouse 3T3 cells in culture (Fuchs et al., 1975). Certain limitations of the fluorescent probe technique must be considered when analyzing data from these studies as well as from the present study. *First,* a microviscosity value for a given membrane represents an average microviscosity. Therefore small, local variations of fluidity in a membrane would be overlooked despite their potential importance in the modulation of membrane function. *Second,* although the localization of DPH to the hydrophobic portion of the membrane bilayer is well documented (Shinitzky and Barenholz, 1974), the kinetics of probe distribution among various membranes is not known. Were DPH to label internal membranes as well as the surface membrane of a complex cell, values might not truly reflect the fluidity of surface membranes alone. The close correlation between whole platelet microviscosity and C/PL, whether variations in C/PL were due to selective changes in membrane C/PL (as at 5 h) or whether both granule and membrane C/PL were affected (as at 20 h), suggests that DPH gains entry into internal membranes and that an average value for all hydrophobic portions of the platelet is obtained. Thus, at least in platelets, DPH cannot be considered to be specific for the surface membrane. *Third,* the platelet "membrane" fraction consists of both surface membrane and membranes of the surface-connecting and dense tubular systems (Taylor and Crawford, 1974). Therefore, conclusions regarding the microviscosity of this fraction cannot be assumed to apply to the surface membrane alone. It should be noted that microviscosity values were calculated utilizing fluorescence intensity as a measure of fluorescence lifetime. This appears valid for platelet membranes since fluorescence decay followed a single exponential curve and since lifetime values so calculated agreed with values independently determined by photon counting.

The addition of cholesterol to phospholipid artificial bilayers

or to natural membranes has two important effects which are demonstrated in these studies. Both relate to the interposition of cholesterol between adjacent phospholipid hydrocarbon chains. On the one hand, cholesterol decreases the random rotational motion of these hydrocarbon chains within the hydrophobic core of the lipid bilayer. On the other hand, it increases the degree of order by preventing the fusion and dissociation of hydrocarbon chains as random motion changes with temperature. The effect on rotational motion is reflected by a change in microviscosity, and a close correlation was seen between the C/PL and microviscosity of platelets and platelet membranes over a broad range of C/PL. The effect on hydrocarbon chain order is seen as a progressive decrease in the temperature-related fusion of flowing segments of hydrocarbon chains. This was observed in both whole platelets and in isolated membrane and granule fractions in our studies. Thus, cholesterol decreases molecular motion and increases the degree of order in the hydrophobic core of platelet membranes.

Cholesterol is acquired by platelets through, what appears to be, a process of exchange rather than either phagocytosis or fusion. This conclusion is supported by the fact that there was no bulk increase in phospholipid content and also by the fact that "cholesterol-normal" dispersions did not alter either the cholesterol or phospholipid content of platelets. The slower time course of granule cholesterol acquisition compared with the acquisition of cholesterol by the membrane fraction is consistent with an initial exchange of dispersion cholesterol with surface membranes and a second exchange between surface membranes and organelle membranes. It is unknown whether this latter process involves a transport protein as has been demonstrated for the exchange of phospholipids between plasma membranes and internal membranes in several cell types (Helmkamp et al., 1974) or for the transport of sterols in hepatocytes (Scallen et al., 1974).

The increased microviscosity of platelet membranes at 5 h, when granule lipids are still normal, is coincident with a 35-fold increase in the sensitivity of platelets to epinephrine-induced aggregation and a 15-fold increase in sensitivity to ADP (Shattil et al., 1975). Cholesterol-induced changes in lipid fluidity have also been associated with modifications of membrane function in red cells (Wiley and Cooper, 1975) and in mycoplasma (Read and McElhaney, 1975). While variations in the fluid nature of the hydrophobic core of the membrane may directly affect these transmembrane events, other changes within the membrane, such as protein–protein spacing or receptor position within the membrane, may also be important.

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