Phase Transitions in Bilamellar Vesicles. Measurements by Pyrene Excimer Fluorescence and Effect on Transacylation by Lecithin:Cholesterol Acyltransferase†

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ABSTRACT: Pyrene excimer fluorescence has been employed as a probe of the microviscosity of the hydrocarbon interior of a variety of phospholipid dispersions. The transition temperature, T_t, at which the nonpolar regions of these molecules undergo phase changes, was found to be 11, 24, 30, 41, and −3° for dilauroyl-, dimyristoyl-, 1-palmitoyl-2-palmitoleyl-, dipalmitoyl-, and egg phosphatidylcholine, respectively. These values agree closely with values obtained by other methods. The activation energy for the transacylation by lecithin:cholesterol acyltransferase is changed at a temperature which coincides with the transition temperature of the phosphatidylcholine acyl donor in the phosphatidylcholine:cholesterol vesicle. This dependence is consistent with a preferential association of the enzyme and phosphatidylcholine.

Lecithin:cholesterol acyltransferase catalyzes the formation of the majority of cholesteryl esters found in human plasma (Glomset, 1962). The transfer of the fatty acyl group from the 2 position of phosphatidylcholine to the 3-hydroxyl group of cholesterol apparently takes place on or within the lipoprotein complexes, predominantly high density lipoproteins, HDL[1] (Glomset *et al.*, 1966; Akanuma and Glomset, 1968). Since the cholesteryl ester produced in the reaction exchanges with lipids of other lipoprotein classes, LCAT appears to be responsible in part for maintaining the distribution of lipids among the lipoprotein classes (Glomset, 1972). The specificity of the LCAT reaction has been studied with sonicated dispersions of phospholipid and cholesterol as substrates. Sgoutas (1972) demonstrated that the nature of the fatty acyl side chain of phosphatidylcholine is an important aspect of enzyme specificity in that unsaturated or short chain fatty acyl groups were transferred more readily than longer saturated fatty acyl groups of the various phospholipids. Phosphatidylcholine appears to be the only acyl donor (Nichols and Gong, 1971). The activity of partially purified LCAT toward substrates consisting of natural lipid mixtures has been shown to be dependent on the addition to the reaction mixture of apoHDL or the major protein species of HDL, apoLP-Gln-I (Fielding *et al.*, 1972). Enhancement of the transacylation reaction by apoLP-Ser from VLDL with didodecanoylphosphatidylcholine as acyl donor has been reported by this laboratory (Garner *et al.*, 1972). The extent of activation by the apolipoproteins from HDL and VLDL is dependent on the nature of the acyl donor, although the mechanism by which LCAT is activated by apolipoproteins or by various lipoprotein classes (Marcel and Vezina, 1973) is not yet known. We infer from our studies and those of Sgoutas (1972) that the physical state of the lipid substrate is an important determinant of LCAT activity.

The nature of the physical state of phospholipids has been studied by a variety of physical techniques. These investigations, recently summarized by Traüble (1972), have employed infrared spectroscopy, nuclear magnetic resonance (nmr), wide angle X-ray scattering (Chapman and Dodd, 1971), spin-labeling (Jost *et al.*, 1971), absorption and fluorescence spectroscopy (Stryer, 1968), light scattering (Ohki, 1970), volumetric dilatometry (Traüble and Haynes, 1971; Melchior and Morowitz, 1972), calorimetry (Chapman *et al.*, 1967; Ladbrooke and Chapman, 1969; Phillips *et al.*, 1969), and fluorescence polarization (Shinitzky *et al.*, 1971; Lussan and Faucon, 1971; Cogan *et al.*, 1973). These methods have been used to measure T_t, the transition temperature at which the hydrocarbon regions of lipids undergo phase changes, and a change in microscopic viscosity. The microscopic viscosity has been measured recently in micelles (Shinitzky *et al.*, 1971; Pownall and Smith, 1973) and lipid dispersions (Lussan and Faucon, 1971; Cogan *et al.*, 1973) by two fluorescence methods. One of these, fluorescence polarization, is based on the viscosity dependence of the rotational diffusion rate of the fluorophore in hydrocarbon regions (Shinitzky *et al.*, 1971; Lussan and Faucon, 1971; Cogan *et al.*, 1973). The other method, pyrene excimer fluorescence, is based on the viscosity dependence of the translational diffusion rate of the probe (Pownall and Smith, 1973; Birks, 1968).

At a given temperature, a series of phosphatidylcholines with homologous hydrocarbon chains differ more in their physical characteristics than in chemical properties. If there is a difference in the rate of enzymic reaction involving two adjacent phosphatidylcholines in a homologous series, it is difficult to identify the physical parameters responsible for the different rates of catalysis. This point was illustrated well by de Haas

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[1] Abbreviations used are: LCAT, lecithin:cholesterol acyltransferase (EC 2.3.1.43); HDL, high density lipoprotein; VLDL, very low density lipoprotein; DLPC, L-α-dilauroylphosphatidylcholine; DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; PPOPC, L-α-1-palmitoyl-2-palmitoleoylphosphatidylcholine. The system of nomenclature for the apoproteins from human plasma lipoproteins is based on the COOH-terminal amino acids (Lux *et al.*, 1972). ApoLP-Gln-I and apoLP-Gln-II are the major protein components of HDL present in a molar ratio of 3:1. ApoLP-Ser, apoLP-Glu, and apoLP-Ala are the protein species of VLDL which also occur as minor components of HDL.

and coworkers (de Haas *et al.*, 1971; Zografi *et al.*, 1971; Verger *et al.*, 1973) with studies of the action of phospholipase A on short-chain phosphatidylcholines. Although phospholipase A hydrolyzed monomeric phosphatidylcholine, micellar solutions were hydrolyzed at a much higher rate. The maximum rate of hydrolysis with dioctanoylphosphatidylcholine as substrate was about 20-fold higher than the rate with diheptanoylphosphatidylcholine. In contrast, vesicles of dinonanoylphosphatidylcholine were not degraded by the enzyme. High concentrations of NaCl, up to 3.9 M, gave an 80-fold stimulation of enzymic activity with the diheptanoylphosphatidylcholine as substrate. Small differences in the architecture of the lipid-water interfaces, such as the area per molecule, were thought to be responsible for these large effects on enzyme activity.

In this communication we describe the determination of T_t utilizing excimer fluorescence of pyrene incorporated into the hydrocarbon regions of a variety of phospholipid dispersions. The behavior of pyrene fluorescence in a liquid crystal has also been examined in order to clarify the results obtained with the lipid dispersions. Temperature variation was selected as a mild procedure to alter specifically the physical state of the lipid substrate dispersions for LCAT. The mechanism by which apoLP-Gln-I enhances the rate of transacylation by LCAT and the sequence of interactions among the two protein components and the two lipid reactants are unknown. In these studies we have found that the activation energy for the transacylation catalyzed by LCAT changes at a temperature which coincides with the transition temperature of the phospholipid. This dependence is consistent with a preferential association of the enzyme with the acyl donor in the fluid domain of the phospholipid:cholesterol vesicle. A preliminary report of this work has appeared (Parker *et al.*, 1973).

Experimental Section

Enzyme Preparation. LCAT was purified from normal human plasma from which the lipoproteins were first removed by ultracentrifugation. All operations were carried out at 4–6° unless otherwise stated. After addition of 1 ml of 0.5 M EDTA (pH 7.4) to 500 ml of human plasma (Blood Services of Houston), the density of the solution was adjusted to 1.21 g/ml by the addition of solid NaBr (0.25 g/ml). The plasma (35 ml/tube) was placed in polyallomer tubes (capacity 38.5 ml) and overlayed with 25% w/v sodium bromide solution. After centrifugation for 40 hr at 45,000 rpm in a fixed angle Beckman Type 60 Ti rotor in a Beckman L-350 preparative ultracentrifuge, the yellow supernatant layers (4 ml/tube) containing the lipoproteins were separated with a tube slicer and discarded. The central colorless zone contained most of the LCAT activity. The orange lower layer which contained much of the albumin was discarded. The central zones (20 ml/tube) were pooled and dialyzed overnight against two changes of 1 l. of the standard buffer, 50 mM Tris-chloride (pH 8.0), containing 10 mM 2-mercaptoethanol and 2 mM EDTA. Finely ground ammonium sulfate was added to the dialyzed enzyme solution to give 50% saturation (275 mg/ml). The pH was maintained at 8.1 by the addition of 1 M ammonium hydroxide. One-half volume of cold 1-butanol was then added slowly, and the resultant mixture was stirred briskly for an additional 30 min. After centrifugation for 15 min at 12,000*g*, the precipitate which formed at the interface was removed and dissolved in the original volume of standard buffer and dialyzed overnight against two changes of 1 l. of buffer. Any undissolved material was removed by centrifugation. To concentrate the enzyme solution for column chromatography the protein was precipitated with ammonium sulfate (70% saturation, 230 mg/ml). The precipitate was dissolved in 1–2 ml of standard buffer and dialyzed overnight against two changes of 100 ml of standard buffer. The dialyzed enzyme solution was applied to a column of G-100 Sephadex (2 × 90 cm) equilibrated in standard buffer. The flow rate (20 ml/hr) was maintained with a peristaltic pump. The 5-ml fractions were assayed for protein by determination of A_{280} and for LCAT activity by the assay procedure described below. Those fractions containing LCAT activity were pooled and concentrated using an Amicon ultrafiltration cell with a 43-mm PM-10 membrane. Protein concentration was determined from the A_{280}/A_{260} ratio (Warburg and Christian, 1941).

Enzyme Assay. LCAT activity was determined routinely by the method of Nichols and Gong (1971) except bilamellar vesicles prepared by the method of Batzri and Korn (1973) were substituted for sonicated dispersions. An ethanolic solution, 0.1 ml, of 12 mM phosphatidylcholine and 1.2 mM [^{3}H]cholesterol, specific activity 100 Ci/mol, was injected into 5.0 ml 0.05 M potassium phosphate (pH 7.4) containing 0.15 M sodium chloride and 1 mM dithiothreitol, above the transition temperature of the phospholipid. This solution was then concentrated fivefold at room temperature using an Amicon ultrafiltration device with a 23-mm XM-100A membrane under 2 psi N_2 gas pressure. The final solution contained 1.2 mM phosphatidylcholine and 0.12 mM [^{3}H]cholesterol. In some experiments pyrene, 1% by weight of phospholipid, was added to the ethanolic stock solution.

The incubation mixture contained 50 μl of the vesicle solution, the apolipoprotein when added, and sufficient buffer to give a final assay volume of 120 μl. The incubation buffer was 0.05 M potassium phosphate (pH 7.4) containing 0.15 M sodium chloride and 1 mM dithiothreitol. In the incubation mixture, the final concentrations of phospholipid were 0.35 mM and 0.035 mM cholesterol. This mixture was equilibrated at the desired temperature 15 min before the addition of the enzyme (5–50 μl). The routine assay temperature was 37°. After 60 min of incubation, 0.5 ml of methanol was added to each tube followed by 4.0 ml of hexane to extract the cholesterol and cholesteryl ester. The tubes were shaken and centrifuged briefly in a bench centrifuge and the hexane layer was quantitatively removed and evaporated to dryness under a stream of nitrogen. To each tube was added 0.1 ml of cholesteryl oleate (100 μg/ml) in hexane as carrier. The entire sample was applied to a 3.7 × 5.0 cm silica gel thin-layer chromatogram sheet (Eastman Kodak 6060). After development of the chromatogram in hexane–ether–glacial acetic acid (90:10:1) v/v), the cholesteryl ester was located with iodine vapor. The portion of the sheet containing the cholesteryl ester was placed in a scintillation vial containing 3 ml of Triton:toluene scintillation fluid (Moses, 1972) for determination of the radioactivity in a Beckman LS-30-D liquid scintillation counter. LCAT activity is expressed as pmoles of cholesteryl ester formed per hour. All assays were performed in triplicate. The enzyme solution was omitted from the incubation mixture in the control tubes.

Fluorimetric Methods. Preliminary studies were done with sonicated dispersions. A phospholipid dispersion (~15 mg/ml) in 50 mM Tris-chloride (pH 8.1) containing 200 mM sodium chloride was prepared by ultrasonic irradiation in ice under nitrogen for 15 min with a Biosonik IV operated at medium power. The phospholipid dispersion was sonicated a second time with a small amount of pyrene (~5 mg) previously evaporated from chloroform with dry nitrogen onto the inner surface of a thick-wall test tube. The sample was then centrifuged at 17,000 rpm for 10 min at 4° to remove titanium, particulate

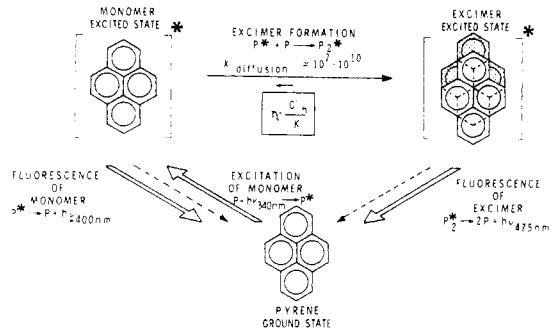

FIGURE 1: A schematic representation of the photophysical processes involved in the excimer and monomer fluorescence of pyrene.

pyrene, and large lipid aggregates. The lipid dispersion containing pyrene was used for spectral studies the same day of preparation. The final concentration of pyrene was about 10–30 μg/mg of lipid. Pyrene (5 mM) was dissolved in the liquid crystal at room temperature.

Except where noted, bilamellar vesicles were used for the experiments reported in this paper. The vesicles were prepared by injection of 50 μl of an ethanolic solution of phosphatidylcholine (22 mg/ml), pyrene (0.11 mg/ml), and various amounts of cholesterol into 5 ml of 0.5 M potassium phosphate (pH 7.4) containing 0.15 M sodium chloride above the transition temperature of the phospholipid. Examination by electron microscopy confirmed the observation of Batrzi and Korn (1973) that these preparations are bilamellar vesicles. To assess the effect of O_2 quenching on the I_E/I_M ratio, vesicles of DMPC and PPOPC were prepared under N_2 in a buffer solution through which N_2 had been bubbled for 20 min. The samples were maintained in a N_2 atmosphere during the temperature run. The temperature profiles were identical with those obtained with vesicles prepared without purging with nitrogen. A 1-ml aliquot of the phospholipid vesicle solution containing pyrene was transferred to a 1 × 1 × 3 cm quartz fluorescence cell contained in the thermostatically controlled cell compartment of an Aminco Bowman spectrofluorimeter. The slit width on both excitation and analyzing monochromators was 2.0 mm. The excitation wavelength was selected between 300 and 350 nm. The temperature was raised in 2–5° increments and the sample allowed to equilibrate for 15 min at each temperature before the fluorescence spectrum was recorded.

Materials. Tris (Trizma base), 2-mercaptoethanol, dithiothreitol, cholesterol, cholesteryl oleate, and DL-α-dipalmitoylphosphatidylcholine were obtained from Sigma. KH_2PO_4 and K_2HPO_4 were obtained by Baker; EDTA was supplied by Fisher. Reagent grade NaBr, NaCl, $(NH_4)_2SO_4$, 1-butanol, methanol, hexanes, and scintillation grade toluene were obtained from Matheson Coleman and Bell. Scintillation grade Triton was obtained from Packard. Generally labeled [^{3}H]cholesterol, specific activity 8.5 Ci/mmol, was obtained from Amersham-Searle. L-α-Dipalmitoylphosphatidylcholine, L-α-dimyristoylphosphatidylcholine, and L-α-dilauroylphosphatidylcholine were obtained from Supelco. Applied Science was the source of L-α-1-palmitoyl-2-palmitoleylphosphatidylcholine. The nominal fatty acid composition was that specified by the supplier. Egg phosphatidylcholine prepared by the method of Singleton *et al.* (1965) and purified as described by Rouser *et al.* (1963) was a gift from Dr. J. D. Morrisett. All lipids used in this study contained less than 1% impurity by thin-layer chromatography. Zone-refined pyrene (Aldrich) and *N*-(*p*-methoxybenzylidene)-*p*-butylaniline (Eastman) were used as received. ApoLP-Gln-I, purified and characterized as described by Baker *et al.* (1973), was provided by Drs. R. L. Jackson and H. N. Baker.

Results

The photophysical details of pyrene excimer fluorescence appear elsewhere (Förster, 1969; Birks, 1968). The following abbreviated kinetic scheme omits radiationless transitions and the processes involving the triplet state since these do not affect the results and conclusions. Pyrene in its ground state, the first excited singlet state, the excimer form, and the unstable dimer form are represented by P, P*, P_2*, and P_2, respectively. "Excimer" is derived from *exci*ted di*mer*. A schematic description of these processes is shown in Figure 1.

$$P + h\nu \xrightarrow{I} P^* \qquad \text{absorption} \qquad (1)$$

$$P^* \xrightarrow{k_f} P + h\nu' \qquad \text{monomer fluorescence} \qquad (2)$$

$$P + P^* \xrightarrow{k_a} P_2^* \qquad \text{excimer formation} \qquad (3)$$

$$P_2^* \xrightarrow{k'_f} P_2 + h\nu'' \qquad \text{excimer fluorescence} \qquad (4)$$

$$P_2 \xrightarrow{k_d} 2P \qquad \text{dimer association} \qquad (5)$$

Inspection of eq 1–5 reveals that, under steady-state illumination, the rate of excimer fluorescence $k'_f[P_2^*]$ is proportional to the rate of excimer formation, $k_a[P^*][P]$. The intensity of monomer fluorescence at 390 nm, I_M, is given by $I_M \propto k_f[P^*]$. The intensity of excimer fluorescence at 475 nm, I_E, is given by $I_E \propto k_f'[P_2^*] \propto k_a[P][P^*]$, so that $I_E/I_M \propto k_a[P]/k_f$. The rate constant, k_a, is given by the Einstein diffusion equation as $k_a = 8RT/3000\eta$, where R, T, and η indicate the gas constant, the absolute temperature, and the viscosity, respectively (Umberger and LaMer, 1945). Therefore, the ratio of intensities, I_E/I_M, can be described by the relationship $I_E/I_M = [P]TK/\eta$, where K incorporates both theoretical and instrumental parameters. Therefore, at a constant temperature and microscopic concentration of pyrene, the ratio I_E/I_M will increase as the viscosity of the hydrocarbon phase decreases. Measurement of I_E/I_M ratio as a function of T should then provide information about changes in the microscopic viscosity.

The total fluorescence spectrum of pyrene in 2-propanol at 23° is shown in Figure 2 at various concentrations of pyrene. With increasing pyrene concentration a broad band at 475 nm becomes more intense with a corresponding reduction of the emission at shorter wavelengths. These spectra have been normalized at 392.5 nm to demonstrate the linear increase in the excimer emission at 475 nm with pyrene concentration shown in the inset of the figure.

The effect of temperature on the fluorescence intensities at 475 nm and at 390 nm (I_E/I_M) is depicted in Figure 3 for pyrene dissolved in toluene, and in a liquid crystal. In toluene the I_E/I_M ratio gradually decreases with increasing temperature. In contrast, in the liquid crystal, *N*-(*p*-methoxybenzylidene)-*p*-butylaniline, no change in I_E/I_M is observed between 10 and 19°. Above 19°, I_E/I_M gradually increases until the temperature reached 43°. Above 43°, the I_E/I_M ratio is reduced by a factor of 10. Above 45°, the I_E/I_M values of the liquid crystal and toluene are comparable.

The sharply defined transition temperatures of aqueous dispersions of phosphatidylcholines shown in Figure 4 are dependent on both chain length and degree of unsaturation of fatty acyl groups. Dimyristoylphosphatidylcholine exhibits a phase transition at 24° (Figure 4A). Egg phosphatidylcholine (Figure 4B) exhibits no marked phase changes over the temperature range from 0 to 45° but does exhibit a poorly defined discontinuity at approximately −3°. The phase transition for di-

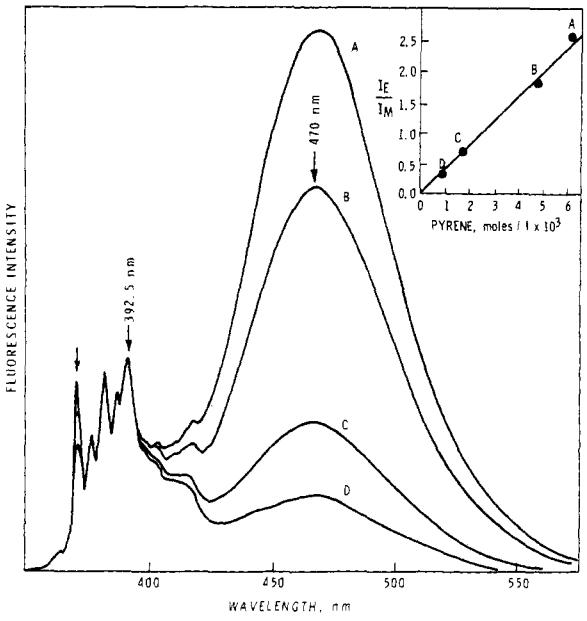

FIGURE 2: The pyrene fluorescence in 2-propanol at 22°, at various concentrations. Pyrene concentrations are: (A) 6.15×10^{-3} M; (B) 4.65×10^{-3} M; (C) 1.72×10^{-3} M; (D) 0.9×10^{-3} M. (The unlabeled arrow indicates the region of strong self-absorption.) Inset plot: I_E(475 nm)/I_M(392.5 nm) *vs.* pyrene concentration in 2-propanol. Concentrations are the same as those of the adjacent spectrum.

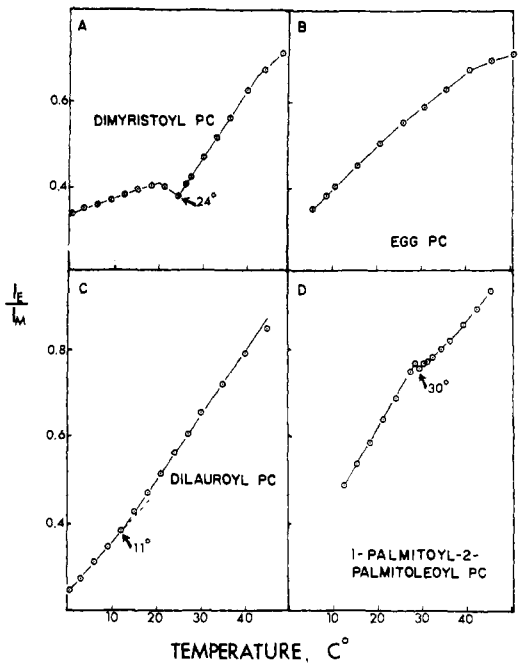

FIGURE 4: Effect of temperature on the relative magnitudes of pyrene excimer (I_E) and monomer (I_M) fluorescence in DMPC (A); in egg PC (B); in DLPC (C); in PPOPC (D). Experimental details are described in the Experimental Section.

lauroylphosphatidylcholine occurred at 11° (Figure 4C); 1-palmitoyl-2-palmitoleylphosphatidylcholine, at 30° (Figure 4D). The plot of temperature dependence of the excimer/monomer ratio of pyrene in sonicated dispersions of dipalmitoylphosphatidylcholine is not shown, but is similar to that observed for DMPC vesicles. Two discontinuities in the I_E/I_M *vs.* T plots for DMPC, DPPC, and PPOPC are noted where the slope of I_E/I_M changes from positive to negative, and then at 1–4° higher temperature the slope again becomes positive. The discontinuities in the temperature profiles which indicate the transition temperatures were the same with pyrene concentrations which ranged from 0.25 to 5% by weight of phospholipid. No hysteresis was observed in duplicate experiments with DMPC vesicles.

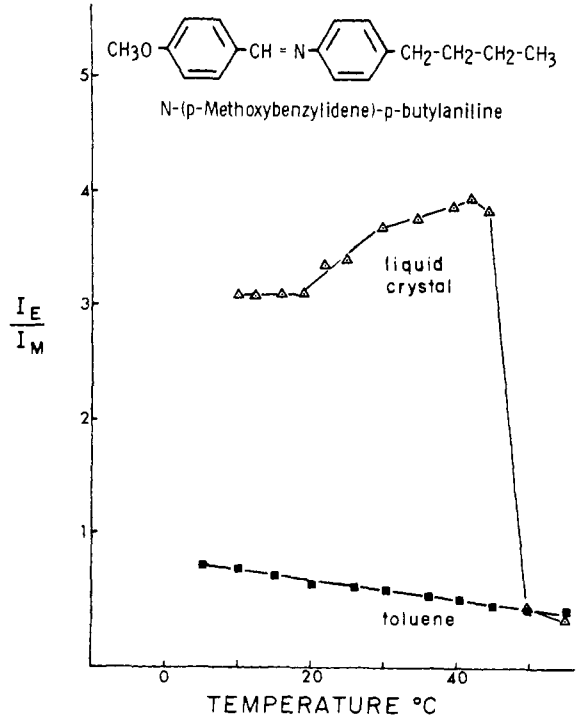

FIGURE 3: Effect of temperature on the relative magnitudes of pyrene excimer (I_E) and monomer (I_M) fluorescence, toluene, ■; and liquid crystal, *N*-(*p*-methoxybenzylidene)-*p*-butylaniline, Δ. Experimental details are described in the Experimental Section.

Inclusion of cholesterol in the DMPC liposomes decreases the excimer/monomer ratio over the entire temperature range (Figure 5). There is also a decrease in the I_E/I_M ratio with increasing cholesterol concentration, although the microscopic concentration of pyrene is constant. The phase transition at 24° observed for pure phospholipid in the vesicle containing only DMPC is also observed with vesicles containing 5 and 10 mol % cholesterol. This transition is no longer observed when the cholesterol concentration is increased to 20 mol %. A transition at 7.5° becomes more pronounced with the increase in cholesterol content. Addition of 5 μM apoLP-Gln-I to pyrene labeled DMPC vesicles did not change the value of I_E/I_M or the phase transition temperature of the phospholipid.

The purification for this LCAT preparation from human plasma is summarized in Table I. The enzyme preparation ob-

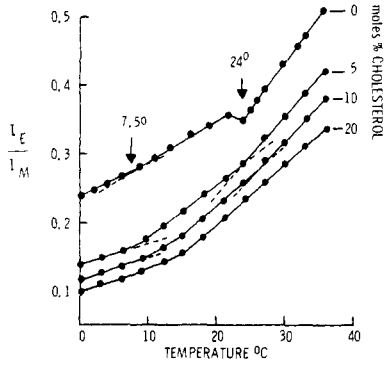

FIGURE 5: The relative intensity of the excimer/monomer fluorescence (I_E/I_M) of pyrene dissolved in DMPC vesicles containing variable amounts of cholesterol. The concentration of DMPC in the aqueous solution was 0.3 mM; pyrene was 0.5% by weight of the phospholipid.

TABLE I: Purification of LCAT from Human Plasma.

	Volume (ml)	Protein concn (mg/ml)	Total Activity (pmol/hr)	Specific Activity (pmol mg/hr)
I. Whole plasma	85	71.2	750	182[a]
II. Ultracentrifugal zones				
1. Lipoproteins	27			
2. Middle zone	75	5.34	560	1,400
3. Lower zone	48	74.6	210	59
III. Butanol–$(NH_4)_2SO_4$	5.5	4.35	198[b]	8,250
precipitate			1290[c]	54,000
IV. G-100 Sephadex	10	1.55	36[b]	2,320
			786[c]	50,500

[a] LCAT activity was determined with egg phosphatidylcholine vesicles as substrate and after dialysis. [b] Enzyme assayed for activity in absence of added apoLP-Gln-I. [c] Enzyme assayed in presence of 5 μM apoLP-Gln-I. There is no activation by apoLP-Gln-I before the ammonium sulfate precipitation. Experimental details are discussed in Methods of Procedure.

tained from the G-100 Sephadex column after concentration by ultrafiltration is stable for several weeks at −30°, in contrast to the previous report that LCAT is highly unstable when purified to any extent (Fielding and Fielding, 1971). Although this enzyme preparation is active with vesicles of synthetic lipid mixtures containing phosphatidylcholine and cholesterol in the absence of added apoprotein cofactor, there was no observable immunoprecipitin line with purified antibody to apoLP-Gln-I. Addition of 5 μM apoLP-Gln-I, with egg phosphatidylcholine as acyl donor, gives a three- to fourfold stimulation of enzyme activity at 37°.

Although other workers have frequently used sonicated dispersions of phosphatidylcholine and cholesterol as a substrate for LCAT, such preparations vary widely in their properties in that the particle size is nonuniform and irreproducible. In addition, there may be oxidative degradation of the lipids as well as contamination of the substrate preparation with metallic particles from the sonicator probe. For these reasons we have adopted the method of Batzri and Korn (1973) to prepare vesicles containing phosphatidylcholine and cholesterol as LCAT substrates. This procedure does not involve sonication and gives optically clear solutions of lipids in an aqueous buffer. The rate of transacylation with vesicles as substrate is slightly higher (~10%) than the rate observed with sonicated dispersions. Enzyme assays varied from 10 to 30% with different preparations of the same substrate dispersions prepared by sonication. In contrast, the variation in the assay employing vesicles as substrates is reduced to ±5%. Since the reaction is linear for at least 90 min with all phosphatidylcholines used in these studies, a single time point determination after 60 min of incubation was used for the assay of LCAT in all subsequent experiments.

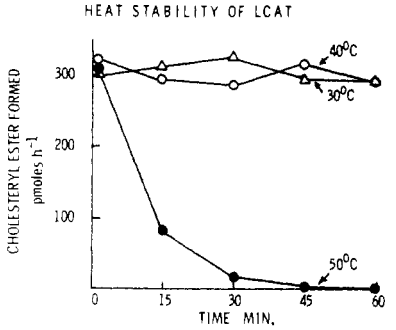

FIGURE 6: The temperature stability of purified LCAT. The enzyme was incubated at each temperature and samples were withdrawn for assay at 22° with egg phosphatidylcholine vesicles as the substrate at the time intervals shown. The amount of protein was 6 μg.

The stability of partially purified LCAT as a function of temperature is shown in Figure 6. Samples of the enzyme, 0.2 mg/ml of protein in 50 mM Tris-chloride (pH 8.1) containing 10 mM 2-mercaptoethanol and 2 mM EDTA, were incubated at 30, 40, and 50° with aliquots removed at 15-min intervals over a period of 1 hr. These samples were assayed immediately at 22° for LCAT activity with egg phosphatidylcholine vesicles as substrate. The enzyme retained essentially all of its activity at 30 and 40°, whereas at 50° the activity decreased rapidly with a half-life of approximately 10 min.

The effect of temperature on the activity of LCAT toward different phosphatidylcholine acyl donors is shown in Figure 7. The second graph in each panel is an Arrhenius plot. Each reaction mixture contains phosphatidylcholine and [^{3}H]cholesterol in a molar ratio of 10:1 and 5 μM apoLP-Gln-I. The acyl donors may be divided into two classes. Egg PC and DLPC (Figures 7A and 7B) show no phase transitions in the temperature range between 15 and 40°. The LCAT activity cannot be measured satisfactorily below 15°. The second group of acyl donors, DMPC and PPOPC (Figures 7C and 7D), have phase transitions at 24 and 30°, respectively. The activity of LCAT with DPPC as acyl donor was quite low and uninformative over the available temperature range. With egg phosphatidylcholine and DLPC as substrates for LCAT, the enzyme activity increases smoothly as a function of temperature. Although there are no distinct discontinuities in these curves, the Arrhenius plots derived from these data are not linear. With DMPC as acyl donor there is a discontinuity in the curve of activity with increasing temperature at 24°, the temperature at which the pure phosphatidylcholine vesicle exhibits a phase transition. There is an inflection in the activity curve at 30° with PPOPC vesicles as substrate which coincides with the phase transition temperature of the pure phospholipid dispersion. The Arrhenius plots illustrate more clearly the inflection points for these two substrate dispersions. In both cases, the slopes of the Arrhenius plots are greater below the transition temperature than above. The slope of the plot for DMPC is not linear above the transition temperature of this phospholipid.

The activity of LCAT at 37° with DMPC vesicles containing 5 and 10 mol % cholesterol (Figure 5) is the same and approximately twofold higher than with DMPC vesicles containing 20 mol % cholesterol. In DMPC vesicles containing 20 mol % cholesterol, the phase transition at 24° is not detectable, in agreement with calorimetric studies. With this substrate, the temperature dependence of the transacylation is identical with

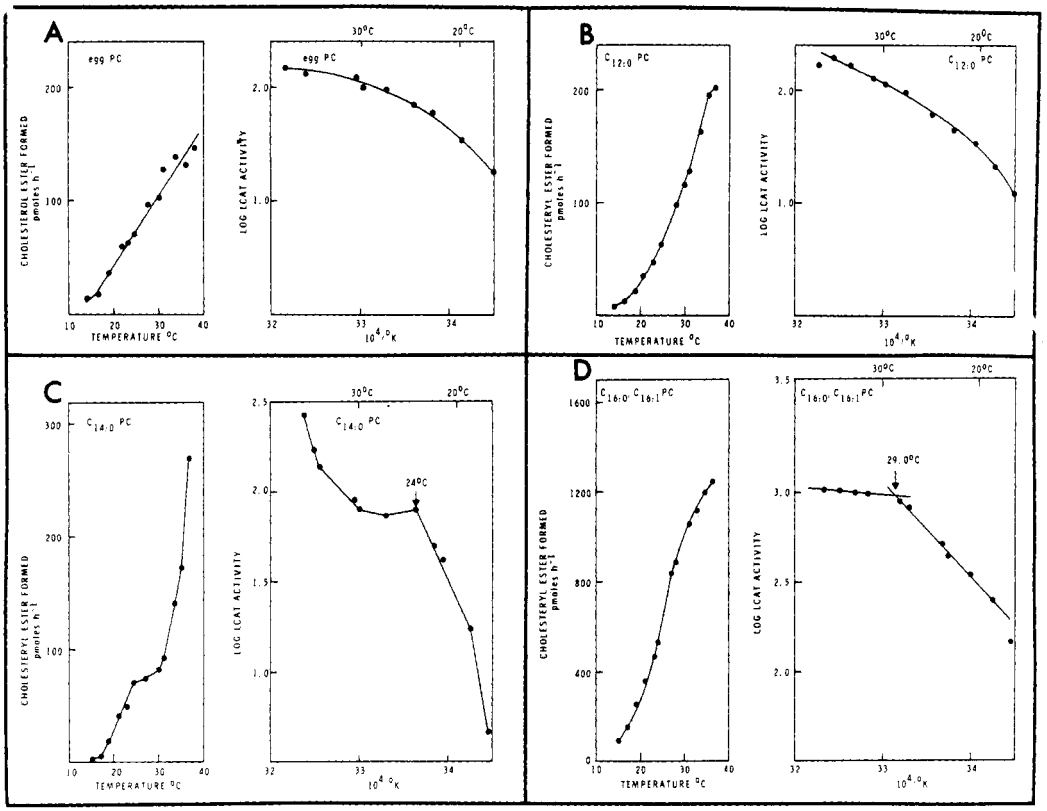

FIGURE 7: The effect of temperature on the rate of cholesteryl ester formation catalyzed by LCAT and the Arrhenius plot derived from the data. The assays were carried out as described in Methods. The vesicles contained 10 mol % cholesterol. The amount of protein in the final assay mixture was 3.3 μg.

that shown in Figure 7. Although there is no detectable change in the physical state of the substrate, the activation energy of the enzyme-catalyzed reaction changes at the temperature which coincides with the phase transition of the fluid phospholipid in the lipid mixture. This change in the rate of transacylation is also observed with an $(NH_4)_2SO_4$ precipitate of the middle zone from the density centrifugation and does not appear to be dependent on the extent of purification.

To ascertain that the addition of the fluorescent probe to the vesicles at the concentration required to measure the excimer/monomer fluorescence ratio does not grossly affect the structure and organization of the substrate dispersion, the temperature profiles of LCAT activity toward DMPC:cholesterol vesicles and vesicles containing in addition 1% pyrene by weight are compared in Figure 8. The transition temperature of the enzyme activity is identical, and below the transition temperature the curve is superimposed. Only above the transition temperature are there any differences in LCAT activity where the rate of production of cholesteryl ester is reduced 20–25% by this concentration of pyrene.

Discussion

The importance of the various lipid:protein interactions in the reaction catalyzed by LCAT has been difficult to assess because of the uncertainty about the physical state of the substrate dispersion. The acceleration of the LCAT catalyzed reaction occurs at a temperature which corresponds to the phase transition of the particular phosphatidylcholine in the vesicle substrate. One interpretation of these data is that an interaction of the enzyme with the phosphatidylcholine acyl donor in the phosphatidylcholine:cholesterol mixture influences the rate-determining step.

It has been shown by several different methods that with increasing temperature phospholipids undergo a change from a more ordered crystalline phase to a liquid crystalline phase in which the molecules have considerably greater freedom of movement (Sackmann and Traüble, 1972). To study changes in the organizational parameters of the hydrocarbon region of phospholipids, we have used the viscosity dependence of the translational diffusion rate of the fluorescent probe pyrene. The effect of temperature on pyrene excimer fluorescence has been studied in solvents with clearly defined physical states. In an isotropic liquid such as toluene, with increasing temperature, the decrease in viscosity will increase I_E/I_M. On the other hand, increasing thermal dissociation of the excimer (Förster, 1969; Birks, 1968) will decrease I_E/I_M. In toluene, which undergoes no major phase changes between 5 and 50°, the viscosity effect is relatively small (Riddick and Bunger, 1970) and I_E/I_M should decrease with temperature as was observed.

In phospholipids and liquid crystals, however, viscosity effects will be more important since major structural changes

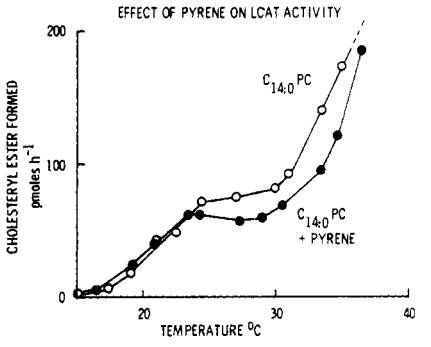

FIGURE 8: The effect of pyrene in the substrate vesicles on the temperature dependence of LCAT activity toward DMPC. The DMPC substrates prepared (○) without pyrene and those with pyrene (●), 1% by weight of the phospholipid. The amount of protein in the assay mixture was 3.3 μg.

TABLE II: Transition Temperatures of Phosphatidylcholine Bilamellar Vesicles Determined by Excimer Fluorescence.

Phosphatidylcholine	T_t, °C: Fluorescence[d]	T_t, °C: Calorimetry	Ref
Dilauroyl	11	~0	*a*
Dimyristoyl	24	23–24	*a–c*
Dipalmitoyl	41	41–43	*a–c*
1-Palmitoyl-2-palmitoleoyl	30		
Egg	~3	~−10	*a*

[a] Ladbrooke and Chapman (1969). [b] Abramson (1970). [c] Hinz and Sturtevant (1972). [d] Average deviation is ± 1.5° for at least three determinations.

occur in these systems between −10 and 50°. In the liquid crystal no change in I_E/I_M with temperature is observed below T_t, 19° (the crystal to liquid crystalline transition). Above T_t an increase in I_E/I_M occurs until the isotropic liquid phase region above 43° is reached (Chistyakov *et al.*, 1971). This increase in I_E/I_M in the liquid crystal is attributed to (a) increased kinetic energy with temperature which necessarily increases the collision rate, (b) a decrease in viscosity which also increases the rate of collision, and (c) the orienting effect of the liquid crystalline phase which produces an arrangement of the pyrene molecules that is favorable to excimer formation. Since a "sandwich" of two pyrene molecules has been assigned to the excimer structure the liquid crystal apparently orients pyrene so that the molecular planes are parallel. The complete orientation of molecules in liquid crystalline structures is a well-known technique for polarization spectroscopy (Wilson *et al.*, 1973). When the liquid crystalline material passes into its isotropic liquid phase the decrease in I_E/I_M may be attributed to the loss in orientation imparted by the liquid-crystalline phase. This occurs in spite of the increase in collision rate as the result of higher temperature and decreased viscosity.

In temperature studies with vesicles of the synthetic phosphatidylcholines DLPC, DMPC, PPOPC, and DPPC, we assign the points of discontinuity at 11, 24, 30, and 41°, respectively, to the phase changes which have been identified at these temperatures by calorimetric methods. Different slopes above and below T_t may result from differences in the temperature coefficient of viscosity of the two phases.

The phospholipid, like the liquid crystal, may be highly organized so that alterations in the organization of the hydrocarbon region may affect the alignment of the pyrene molecules in configurations which favor excimer formation and produce a change in I_E/I_M. The relative importance of these factors differs with each phospholipid. The minimum which precedes the transition temperature I_E/I_M *vs.* T curve measured in DMPC, PPOPC, and DPPC was not detected in the curves obtained with DLPC. The difference may be explicable by the dilatometric measurements on DMPC and DPPC (Traüble and Haynes, 1971; Melchior and Morowitz, 1972). For example, DPPC shows an increase in volume (2.2%) at the transition temperature which causes a decrease in the microscopic concentration of pyrene. Since the I_E/I_M ratio is also directly proportional to the microscopic pyrene concentration or inversely proportional to the microscopic volume confining the pyrene, a decrease in I_E/I_M at the transition temperature can be understood. The appearance of this minimum in DPPL and DMPC but not in DLPC may be due to a much larger change in η at

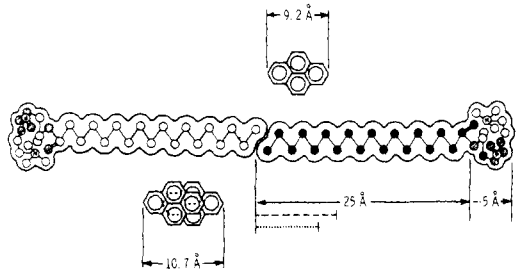

FIGURE 9: Molecular dimensions of pyrene, pyrene excimer, and a phospholipid bilayer.

T_t for DLPC. The large changes in I_E/I_M for DLPC are consistent with the fully expanded physical state found with monomolecular films of this phospholipid (Phillips and Chapman, 1968).

The changes observed in I_E/I_M *vs.* T plots with egg phosphatidylcholine are very broad; the transition at ~−3° can be identified with an expanded scale. This slow change in I_E/I_M with temperature is probably due to the heterogeneous composition of egg phosphatidylcholine which leads to a superposition of the changes of the individual components.

The similarity observed in the I_E/I_M *vs.* T plots of liquid crystals and synthetic phospholipids may be assigned to similar structural changes. Below T_t in the phospholipids the increase in I_E/I_M values is a consequence of increasing collision rates resulting from lower viscosity and higher temperature. Above T_t increased mobility and favorable orientation of pyrene produced an increase in I_E/I_M. For this reason we concur with previous suggestions (Traüble, 1972) that the phase changes in phospholipids are transitions from a gel or crystalline state. The gel designation of the lower temperature mesophase seems more appropriate since the crystalline form would not permit rapid diffusion; the observation of excimer fluorescence implies that diffusion does occur.

In spite of the various factors which can influence the I_E/I_M ratio, Table II shows an acceptable correlation of the T_t values obtained by this method and calorimetric methods. It is possible that changes in both localization and orientation of the probe within the bilayer might be occurring during phase transitions. Unequivocal separation of these effects requires further studies. However, on an empirical basis, this probe can be used to detect phase transition of lipids. An X-ray study of pyrene has shown that the molecule is 9 Å in the longest dimension (Robertson and White, 1947). Pyrene can be accommodated by the hydrocarbon region of the bilayer without greatly perturbing the structure. The relative sizes are compared in Figure 9. In addition, pyrene is slightly smaller than the probes used for fluorescence depolarization studies.

To determine with certainty that pyrene does not bind to protein, we have studied the interaction of pyrene with apoHDL, human serum albumin and erythrocyte membrane glycoprotein. The latter protein has a very distinct and well characterized hydrophobic region (Segrest *et al.*, 1973). Protein solutions were sonicated with pyrene before filtration through a 5 μ Millipore filter. Analysis of protein and pyrene shows that protein is in great excess, with molar ratios of 15:1, 20:1, and 100:1, respectively. In no instance is any excimer fluorescence observed.

It is now well accepted that cholesterol has an effect on the mobility of the hydrocarbon region of phospholipid bilayer (Chapman, 1973). This effect has been explained for binary mixtures of DMPC and cholesterol in terms of a phase diagram (Shimshick and McConnell, 1973). These workers have measured the fluidity of the hydrocarbon region by means of a

spin-label probe with a different solubility in gel and liquid crystalline regions. From the phase diagram constructed on the basis of the difference in solubility of the probe in the two phases, they suggest that lateral phase separations occur in DMPC:cholesterol mixtures and that gel and liquid crystalline regions are both present in the bilayer at a given temperature. As the concentration of cholesterol in the DMPC bilayer is gradually increased, the temperature at which all of the lipid mixture is in the gel phase remains constant until a critical molar ratio of 20 mol % cholesterol is reached. Above 20 mol % cholesterol, the temperature at which the liquid crystalline region is first detected increases as a function of cholesterol concentration.

These observations explain the apparent disappearance of the phase transition at 24° in the DMPC vesicles (Figure 4) containing 20 mol % cholesterol. The increase in I_E/I_M at a given temperature with increasing cholesterol concentration reflects the decrease in the fluidity of the hydrocarbon region of DMPC vesicles caused by cholesterol. Other studies are required to determine the appropriate characterization of the phase which exists below 7.5°.

Nichols and Gong (1971) have shown that LCAT is sensitive to the relative proportions of phosphatidylcholine and cholesterol in synthetic substrate mixtures in a way that is not readily explained in terms of the usual enzyme kinetics. Increased concentrations of cholesterol inhibited LCAT activity unless the phospholipid concentration was proportionally increased. We attribute the effect of cholesterol to reduction of the fluidity of the hydrocarbon region of the vesicle substrate. The properties of the lipid-water interface also change at the transition temperature (Traüble, 1972).

In the studies described here we have utilized a dispersion containing only one phosphatidylcholine species and cholesterol. The enzyme reaction has a different activation energy depending on whether the lipid mixture is in the gel or liquid crystalline phase. This difference strongly indicates that the activity of LCAT is sensitive to purely physical changes in the substrate and to the properties of lipid-water interface. Although the experiments of Sgoutas (1972) pointed to this conclusion, these studies were somewhat ambiguous in that the phosphatidylcholine acyl donors contained a variety of fatty acid moieties with one predominant fatty acid containing the radioactivity. The physical states of these substrate dispersions have not been studied, but are probably similar to the egg phosphatidylcholine:cholesterol dispersions.

It is becoming increasingly obvious that no two phosphatidylcholine species will behave identically in aqueous dispersions and that for each phosphatidylcholine, the properties of the hydrocarbon region and lipid-water interface are different. These experiments indicate that changes in these properties are important determinants of LCAT activity. The slopes of the Arrhenius plots illustrate that the activation energy of the reaction is greater below the transition temperature of the phospholipid. This difference suggests that more energy is required for the enzyme either to penetrate the lipid layer or possibly to extract the substrates from the vesicles when the phospholipids are in their more rigid crystalline phase. Furthermore, it appears that the rate-limiting step above the transition temperature involves the interaction of the phosphatidylcholine acyl donor with the enzyme. Below the transition temperature, it is possible that other unidentified factors are more important; for example, the rate of diffusion of the cholesteryl ester from the active site of the enzyme may be the rate-limiting step. Other studies are required to define precisely the kinetic mechanism.

It will be of interest to determine if the phospholipid acyl donor functions as a boundary lipid component similar to that described by Jost *et al.* (1973a, 1973b) for cytochrome oxidase. The importance of the fluid character of phospholipid-requiring enzymes has been documented by studies of the microsomal cytochrome P-450 reductase (Stier and Sackmann, 1973), the ($Na^+ + K^+$) adenosine triphosphatase (Kimelberg and Papahadjopoulos, 1972; Grisham and Barnett, 1973), and the *Escherichia coli* membrane enzymes—lactate oxidase, NADH oxidase, and succinic dichloroindophenol reductase (Overath *et al.*, 1970; Esfahani *et al.*, 1971, 1972). The inhibitory effect of cholesterol on the activity of the ($Na^+ + K^+$) ATPase dependent on phospholipid was related to inhibition of molecular motion within the phospholipid bilayer (Papahadjopoulos *et al.*, 1973).

The temperature transitions determined by pyrene excimer fluorescence in studies with *E. coli* membranes and human plasma lipoproteins correlate very well with results from spin-label studies (Keith *et al.*, 1973) and from wide angle X-ray analysis (Esfahani *et al.*, 1971; Morrisett *et al.*, 1974). The speed and experimental simplicity of the pyrene excimer fluorescence method and the unambiguous data which is obtained in a linear form (I_E/I_M *vs.* T) make its application in a variety of biological systems attractive.

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