Structure of an Apolipoprotein–Phospholipid Complex: ApoC-III Induced Changes in the Physical Properties of Dimyristoylphosphatidylcholine†

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ABSTRACT: The effect of ApoC-III, a major apoprotein constituent of human very low density lipoproteins, on the physical properties of dimyristoylphosphatidylcholine (DMPC) vesicles has been studied by magnetic resonance and fluorescence techniques. The sharp gel → liquid crystalline transition usually observed at 23 °C in DMPC is both broadened and elevated when ApoC-III is bound as determined (a) from measurements of microscopic viscosity by pyrene excimer fluorescence, (b) from the distribution of di-*tert*-butyl nitroxide between the bulk aqueous phase and the fluid lipid phase, and (c) from the motion of fatty acyl chains of spin-labeled phosphatidylcholine. Experiments involving the translocation of ascorbate and charged nitroxide ions and the movement of paramagnetic Eu^{3+} ions indicate that when ApoC-III binds to DMPC vesicles, it increases their permeability or destroys their original bilayer structure. These two possibilities were distinguishable by gel filtration of the DMPC–ApoC-III complex (~34 mol/mol) that indicated that the product particles were significantly smaller than the original vesicles. Taken together, the data indicate that ApoC-III binding to DMPC not only decreases the acyl chain motion of individual lipid molecules, but also induces breakdown of bilamellar vesicular structure to give significantly smaller complexes.

Previous studies from this laboratory have demonstrated the significant effects of phosphatidylcholine on the structure of the apolipoprotein, ApoC-III.[1] These effects include an increase in the α-helicity of the peptide backbone, an increase in the hydrophobicity of the environment around its tryptophan residues, and a decrease in the density at which the complexed apoprotein bands in a linear density gradient (Morrisett et al., 1973). Subsequent work has shown that the rate and magnitude of these structural changes in the apoprotein are significantly greater with phospholipids in the more fluid liquid crystalline state than in the less fluid gel state (Pownall et al., 1974, 1976).

At the same time, ApoC-III strongly influences the structure of phosphatidylcholine. This influence is especially evident at the *macro*molecular or vesicle level. Upon apoprotein binding, EYPC vesicles undergo changes in sedimentation and diffusion behavior (Morrisett et al., 1974), and in electron microscopic appearance (Hoff et al., 1973). In this report, we present evidence that ApoC-III also affects the structure of phosphatidylcholine at the *micro*molecular or cooperative unit level. This evidence includes apoprotein-induced changes in (a) steric accessibility of pyrene intercalated in the lipid, (b) thermotropic properties of the lipid, (c) motion of fatty acyl chains of spin-labeled phosphatidylcholine, and (d) permeability of the bilayer to a monovalent and trivalent cation.

Experimental Section

Materials. ApoC-III$_1$ was isolated from the very low density lipoproteins of patients with type IV or V hyperlipoproteinemia as described previously (Morrisett et al., 1973). Care was exercised to minimize aggregation of the apoprotein by avoiding lyophilization and by treating with 6 M guanidine hydrochloride immediately before use (Morrisett et al., 1976). Crystalline DMPC was obtained from Sigma; it migrated as a single species on a thin layer of silica gel ($CHCl_3$–CH_3OH–H_2O 65:25:4) and had a fatty acid composition of 99.47% myristic acid. 2,2-Dimethyloxazolidinyl-1-oxy (doxyl) derivatives of 16- and 12-ketostearate and 5-ketopalmitate were prepared by standard procedures (Gaffney, 1976). These spin-labeled fatty acids were used for acylating lysolecithin (Hubbell and McConnell, 1971) to obtain the respective phosphatidylcholines (16-DSPC, I; 12-DSPC, II; 5-DPPC, III). Di-*tert*-butyl nitroxide (DTBN, IV) was prepared from 5-nitrobutane by the method of Hoffman and Henderson (1961) as modified by Rozantsev (1970). 2,2,6,6-Tetramethylpiperidinyl-1-oxy-4-dimethylethanolamine (TC, V) was prepared by alkylation of 4-(*N,N*-dimethylamino)-2,2,6,6-tetramethylpiperidine with 2-bromoethyl acetate and with subsequent hydrolysis of the ester and oxidation of the amine (Kornberg and McConnell, 1971). Zone-refined pyrene (VI) was obtained from Aldrich.

Instrumentation. Fluorescence measurements were performed on 1-ml samples contained in a quartz fluorescence cuvette held in a thermostatted cell compartment (±0.5 °C) of an Aminco-Bowman spectrofluorimeter. Approximately 15 min was allowed for thermal equilibration of the samples, after which time the fluorescence spectrum was recorded and the temperature raised 3 °C. At each temperature, the inten-

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[1] Abbreviations used are: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; EYPC, egg yolk phosphatidylcholine; ApoC-III$_1$, an apolipoprotein component (mol wt ~10 000) that contains one residue of sialic acid and is isolated from human very low density lipoproteins; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; doxyl, 2,2-dimethyloxazolidinyl-1-oxy; DTBN, di-*tert*-butyl nitroxide; TC, Tempo-choline or 2,2,6,6-tetramethylpiperidinyl-1-oxy-4-dimethylethanolamine; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

sity of excimer fluorescence at 475 and monomer fluorescence at 390 nm was recorded. The excitation wavelength was 335 nm, the excitation and emission slit widths were each 1.0 mm, and the signal:noise ratio was about 400:1. Fluorescence lifetimes of pyrene were measured at ambient temperature without degassing using an Ortec photon fluorometer equipped with an RCA 8850 photomultiplier tube. Electron paramagnetic resonance spectra were recorded on a Varian E-12 spectrometer operated at a microwave frequency of 9.15 GHz and samples were contained in disposable pipets sealed at the small end. Nuclear magnetic resonance spectra were recorded on a Varian XL-100A-15 spectrometer interfaced to a Nicolet TT-100X Fourier transform accessory equipped for quadrature phase detection. The temperature of the EPR cavity and NMR probe was governed by a Varian temperature controller and measured to an accuracy of ±0.5 °C with a Tri-R electronic thermometer. Samples were allowed to equilibrate for 5 min at the desired temperature before their spectra were recorded.

Methods. A standard buffer consisting of 100 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM NaN_3, pH 7.40, was used throughout unless indicated otherwise. Linear salt density gradients (Pownall et al., 1976) were prepared by mixing the low-density solution (2.5 ml of standard buffer containing the sample) and high-density solution (2.5 ml of standard buffer containing 2.52 M KBr) in a Buchler gradient maker. Gradients were spun in a Beckman SW 50.1 rotor at 50 000 rpm (248 000*g*) and 5 °C for 72 h. Centrifuged samples were fractionated at 0 °C by collecting 0.3-ml portions from the bottom of the pierced tubes. The density of each fraction was estimated by refractometry[2] (Weast, 1969), the phospholipid concentration by phosphorus analysis (Bartlett, 1959), and the protein concentration by amino acid analysis or the procedure of Lowry et al. (1951).

Preparation of DMPC Vesicles. Bilamellar vesicles of DMPC were usually prepared by the method of Batzri and Korn (1973). About 5.5 μmol of DMPC dissolved in 50 μl of ethanol was injected from a Hamilton syringe into 4 ml of vigorously shaken buffer. When labeled vesicles were required, known amounts of pyrene or spin-labeled phosphatidylcholine were added to the ethanolic DMPC solution before injection into standard buffer. In a few cases, vesicles were isolated by chromatography over Sepharose 6B. For NMR experiments, DMPC was sonicated at 30 °C until the solution became transparent (~30 min). This dispersion typically contained >80% bilamellar vesicles (Figure 8A). Pyrene concentration in all samples was determined from absorbance at 338 nm after 1 → 2 dilution with sodium dodecyl sulfate (10 mg/ml) to clarify the samples.

Isolation of DMPC–ApoC-III Complexes. The apoprotein was added dropwise to freshly prepared vesicles until the desired lipid:protein ratio was reached. This mixture was usually incubated for 30 min at 28–30 °C before density gradient centrifugation. Gradient fractions containing the complex were pooled and concentrated by ultrafiltration (Amicon UM-10 membrane) for study.

Results

Effect of ApoC-III on the Thermotropic Properties of DMPC. (A) Pyrene Fluorescence. Basic assumptions regarding the behavior of the pyrene probe (VI, Figure 1) in natural and model membranes have been enumerated previously (Morrisett et al., 1975a). It is a water-insoluble fluorophore that dissolves only in the hydrophobic region(s) of lipid-containing systems, such as membranes or lipoproteins. It exhibits characteristic emission bands at about 470 and 392 nm (Figure 2A). The 392-nm band is due to monomer fluorescence and the 470-nm band to fluorescence of the *excited* state *dimer* (*excimer*). Because excimer formation is diffusion controlled, the intensity of the excimer fluorescence (I_E) varies inversely with the viscosity of its environment. The excimer:monomer intensity ratio, I_E/I_M, can, therefore, be used to monitor the fluidity of a lipid-containing system as a function of temperature (Soutar et al., 1974). Figure 3A illustrates the thermotropic behavior of DMPC as determined with pyrene. A change in the slope of the I_E/I_M vs. T plot occurs at about 24 °C that implies a sudden increase in lipid fluidity at this temperature and that corresponds to the calorimetrically determined gel → liquid crystalline phase transition for DMPC (23.70 °C) (Hinz and Sturtevant, 1972). This transition for the pure lipid is fully reversible. When pyrene-labeled DMPC is mixed at 3 °C with ApoC-III in a molar ratio of 53:1, a more complex thermotropic behavior of the lipid is observed (Figure 3B). Initially, in the range 0–20 °C, the plot of this mixture is

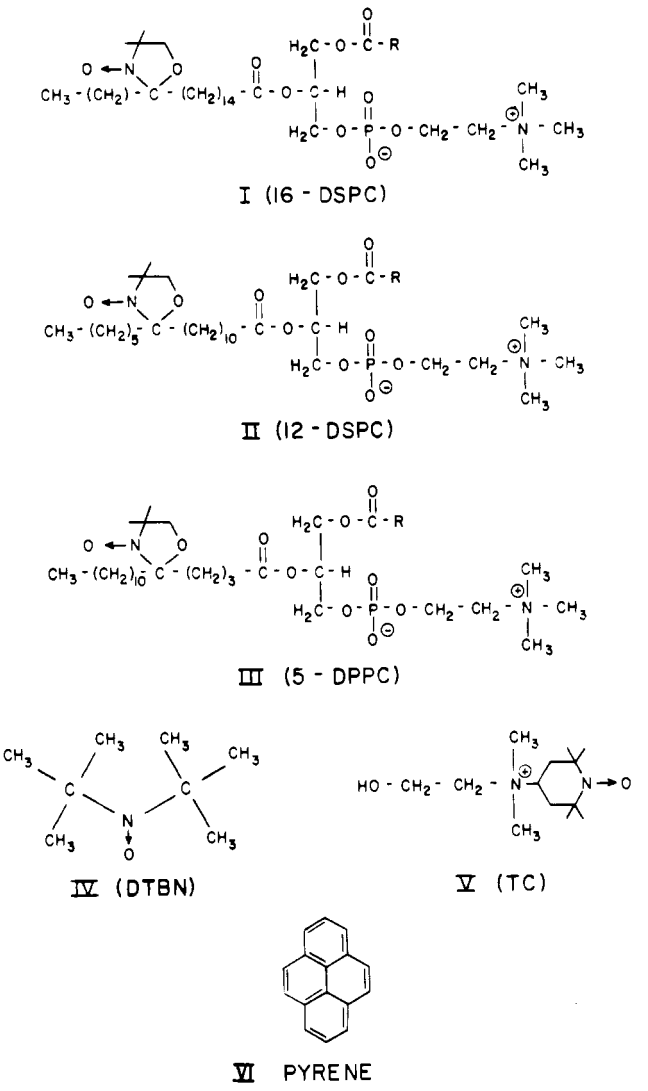

FIGURE 1: Chemical structures for reporter molecules used in this study. I, (16-DSPC) 16-doxylstearoylphosphatidylcholine; II, (12-DSPC) 12-doxylstearoylphosphatidylcholine; III, (5-DPPC) 5-doxylpalmitoylphosphatidylcholine; IV, (DTBN) di-*tert*-butyl nitroxide; V, (TC) Tempo-choline; VI, pyrene.

[2] This estimate is influenced by the presence of either protein and/or lipid at various locations in the gradient.

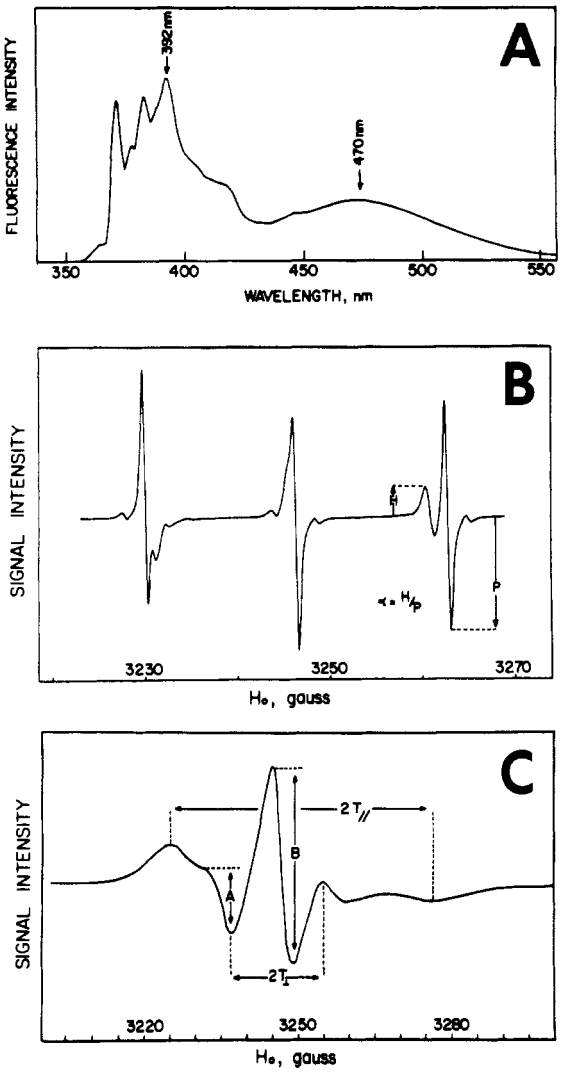

FIGURE 2: Methods used to study the thermotropic properties of DMPC in the absence and presence of apoC-III. (A) Pyrene fluorescence. Fluorescence spectrum of pyrene (0.5%) intercalated into DMPC (0.20 mg/ml, 20 °C). The peak at 392 nm (470 nm) is due to monomer (excimer) emission. The ratio of emission intensities, I_E/I_M, is directly related to the fluidity of the lipid in which it resides. (B) Di-*tert*-butyl nitroxide paramagnetic resonance. The EPR spectrum of di-*tert*-butyl nitroxide in a solution of DMPC vesicles (33 mg/ml, 26 °C). That portion of the high-field resonance line indicated by H (P) is due to the spin-label dissolved in the hydrophobic fluid phase (the polar aqueous phase). The ratio of these line intensities, H/P, is dependent on the relative concentrations of fluid lipid phase and polar aqueous phase (Griffith et al., 1974). (C) Spin-labeled phosphatidylcholine paramagnetic resonance. The EPR spectrum of 5-DPPC (I) in DMPC vesicles at 22 °C. Spectral splittings indicated as $2T_\perp$ and $2T_\parallel$ were used to calculate the order parameters S_5 and S_{12} for 12-DSPC and 5-DPPC. With 16-DSPC, changes in S_{16} were relatively small at ≥20°. The line height ratio, A/B, was considerably more sensitive to changes in acyl chain motion.

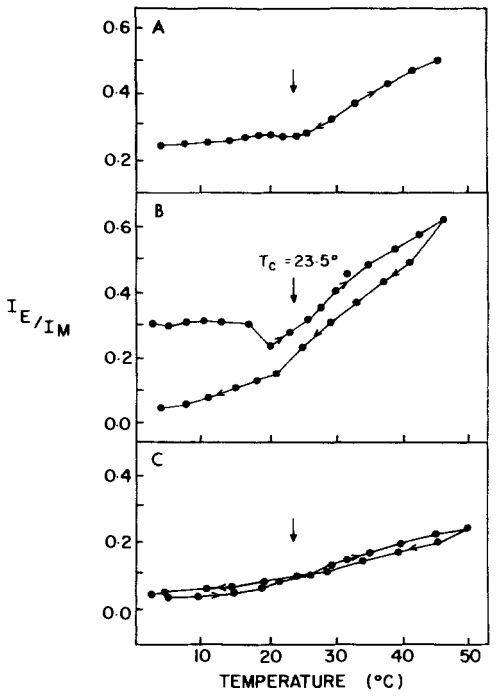

FIGURE 3: Thermotropic properties of (A) DMPC vesicles alone, (B) DMPC vesicles and ApoC-III (50:1) mixed at 3 °C, and (C) DMPC-ApoC-III complex isolated from a density gradient, as determined by pyrene fluorescence (470 nm). The excimer (470 nm) and monomer (392 nm) emission intensities of pyrene were measured and their ratio plotted as a function of temperature. The *micro*scopic pyrene concentration in experiments A, B, and C are not equal (*macro*scopic concentration ≃6 μg/ml), hence, absolute I_E/I_M values cannot be compared. The DMPC concentration ≃ 3 mg/ml.

comparable to that of the lipid alone. At 20 °C, a small decrease followed by a large increase in lipid fluidity is observed. As the temperature of this mixture is decreased from 45 °C, the average fluidity is consistently lower than that observed initially with increasing temperature. After this heating and cooling cycle, the thermotropic behavior of the mixture can be described by the cooling portion of the cycle. For a DMPC-ApoC-III complex isolated from a density gradient (Figure 3C), the abruptness of the slope change at 24 °C is highly attenuated and the fluidity change of the complexed lipid per temperature increment is less than that observed for the lipid alone. Measurements at both increasing and decreasing temperatures illustrate the reversibility of this thermal behavior.

When the fluorescence lifetime of pyrene in the lipid alone and in the lipid-apoprotein complex is known, the accessibility of intercalated pyrene may be estimated from eq 1

$$P = \frac{K_{sv}}{k_d \times \tau_f} \quad (1)$$

where P is the steric parameter, K_{sv} is the Stern-Volmer quenching constant, k_d is the diffusion-controlled rate constant for bimolecular reaction, and τ_f is the fluorescence lifetime of the excited state of pyrene monomer. To determine the effect of ApoC-III on the steric accessibility of pyrene intercalated within bound DMPC, a series of iodide quenching experiments was performed. Stern-Volmer plots (Birks, 1970) for the quenching by iodide of pyrene in DMPC alone and the DMPC-ApoC-III complex (50:1) exhibit small but reproducible slope differences. From these slopes can be determined the Stern-Volmer quenching constants for each of these systems (Table I). The larger quenching constant for pyrene monomer in the complex (14.3) suggests increased accessibility for iodide to pyrene in the system. However, this increase is probably due in part to increased fluorescence lifetime (142 ns).

(B) Di-*tert*-butyl nitroxide and Doxyl Fatty Acid Paramagnetic Resonance. The utility of small amphipathic nitroxyl molecules such as Tempo and DTBN (IV, Figure 1) has been previously demonstrated in several laboratories (Shimshick and McConnell, 1973; Lee et al., 1974; Griffith et al., 1974). The usefulness of these molecules derives primarily from the dependence of their hyperfine tensors (A_0) on solvent polarity (e.g., in hexane, DTBN has A_0 = 15.10; in water A_0 = 17.16)

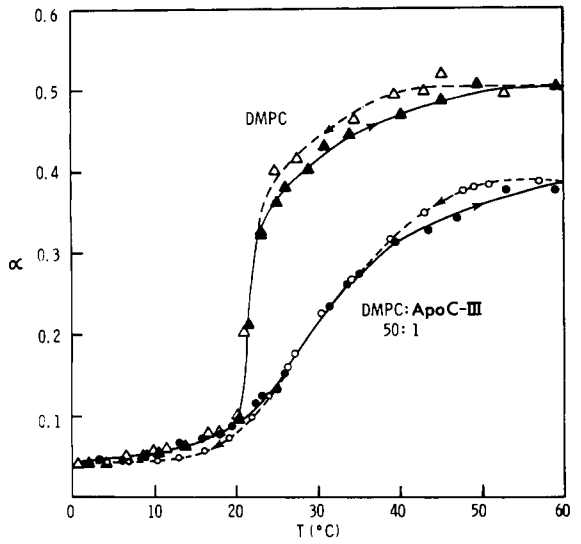

FIGURE 4: Thermotropic properties of DMPC vesicles alone (triangles) and DMPC vesicles mixed with ApoC-III (50:1) at 3 °C (circles) as determined by di-*tert*-butyl nitroxide (IV). Closed symbols (open symbols) indicate data collected during increasing (decreasing) temperatures. The parameter α is approximately equal to the ratio of label in the fluid lipid phase and the bulk aqueous phase and is a semiquantitative indicator of the degree of lipid fluidity. DMPC $\simeq$ 56 mg/ml.

(Griffith et al., 1974). Therefore, when this amphipathic spin-label distributes between the aqueous bulk phase and the fluid lipid phase, a mixed paramagnetic resonance spectrum is produced that contains two components (Figure 2B). The lower and higher field components of the high-field resonance line, M_{-1}, are almost completely resolved and correspond to nitroxide dissolved in the hydrophobic and hydrophilic parts of the system, respectively. The ratio of amplitudes of these two spectral components may be represented by the parameter α. For a lipid-containing system such as DMPC vesicles, α is approximately equal to the ratio of nitroxide in the fluid lipid phase and the bulk aqueous solution. The plot of α vs. temperature for DMPC exhibits a sharp increase at ~21 °C that correlates with the gel → liquid crystalline transition for this lipid (Figure 4). The slight hysteresis observed at temperatures above the major part of the transition is probably due to supercooling effects. When sufficient ApoC-III is added to the phospholipid to give a lipid:apoprotein molar ratio of 50:1, the midpoint of the transition is shifted to a significantly higher temperature ($\Delta T \simeq 10$ °C) and the transition is considerably broadened.

To evaluate the effect of ApoC-III on the acyl chain motion of DMPC, spin-labeled phosphatidylcholine with the β-acyl chain bearing the doxyl group at C-5 (5-DPPC), C-12 (12-DSPC), and C-16 (16-DSPC) were used (III, II, I; Figure 1). Each of these reporter molecules detected the phase transition of the DMPC bilayer: 16-DSPC at 23 °C, 12-DSPC at 22 °C, and 5-DPPC at 21 °C (Figure 5A,B,C). When ApoC-III was bound to the vesicles (50 mol/mol), a significant change in the thermotropic behavior of DMPC was observed by all three spin-labeled lipids. This effect was manifested in elevation of the transition temperature by ~5 °C. With 5-DPPC and 16-DSPC, the transition became significantly broader but with 12-DSPC the transition remained almost as sharp in the complex as in the pure lipid. 12-DSPC indicated virtually no difference in the transition of a complex with a lipid-protein molar ratio of 46:1 and of 23:1.

Effect of ApoC-III on DMPC Vesicle Permeability and Structure. (A) Tempo-choline EPR. To assess the effect of ApoC-III on the permeability of DMPC vesicles, efflux of a positively charged spin-label (Tempo-choline) and/or influx of negatively charged ascorbate was studied.[3] The relative flux rates for these two system was determined by measuring the time-dependent decrease in the Tempo-choline EPR line height. This diminution of signal amplitude is due to chemical reduction of the nitroxyl moiety by ascorbate, resulting in loss of paramagnetism. The time required to quench half the paramagnetism initially inside *bi*lamellar vesicles at 10 and 15 °C was 2750 and 360 s, respectively (Figure 6B). When ApoC-III was added in a 50:1 molar ratio to Tempo-choline labeled vesicles followed by addition of ascorbate at 3 °C, the loss of signal amplitude was too rapid to permit kinetic measurements. To obtain measurable flux rates, we resorted to the use of *multi*lamellar vesicles through which ion translocation is considerably slower. With these liposomes, the half-time for signal loss was 2000 s at 19 °C (Figure 6A). Upon addition of ApoC-III to attain a lipid-protein molar ratio of 50:1, the half-time dropped sharply to 160 s indicating markedly increased permeability. That kinetic flux experiments with DMPC *bi*lamellar vesicles were not possible after ApoC-III addition raised the question of whether these vesicles still retained their inside-outside structure after apoprotein binding. We attempted to answer this question with experiments utilizing a trivalent cation that should traverse the bilayer much more slowly than Tempo-choline or ascorbate ions. These experiments are described in the next section.

(B) ^{31}P NMR. The addition of Pr^{3+} to stable bilamellar vesicles allows differentiation between the inside and outside polar head groups (Bystrov et al., 1972). As long as a sufficiently large concentration gradient of this or certain other paramagnetic ions exists across the bilayer, resonances of those nuclei exposed to the higher concentration will be chemically shifted from those exposed to the lower concentration. A 20 mg/ml dispersion of DMPC containing greater than 80% bilamellar vesicles (Figure 8A) gives a ^{31}P-NMR spectrum similar to that shown in Figure 7a. The spectrum is not detectably affected by the addition of ApoC-III up to a level of 1370 mol of lipid/mol of apoprotein. The addition of para-

TABLE I: Quenching of Pyrene Fluorescence by Iodide Ion.[a]

	DMPC Vesicles	DMPC–ApoC-III Complex
k_{sv}	11.1	14.3
τ_f (ns)[b]	130	142
k_f (s^{-1})	7.7×10^6	7.0×10^6

[a] Accessibility of pyrene intercalated in DMPC vesicles and in DMPC–ApoC-III complexes was estimated from the ability of iodide to quench monomer (392 nm) fluorescence. Dimyristoylphosphatidylcholine vesicles (1.6 mg/ml) containing pyrene (8 μg/ml) were studied alone and when complexed with ApoC-III (50 mol/mol). The complex was isolated by ultracentrifugation and dialyzed exhaustively against standard buffer to remove quenching bromide ions. Emission was then measured at 20, 40, 60, 80, and 100 mM NaI. Quenching constants were calculated from the slopes of I_0/I vs. $[I^-]$ plots. Temperature = 25 °C. [b] We attribute the slightly longer lifetime of the excited state of pyrene monomer in the complex to the reduced diffusion rate of oxygen present in the lipid.

[3] At the pH of these experiments (pH 7.4), ascorbic acid carries a single negative charge (pK_{a_1} = 4.17 and pK_{a_2} = 11.57) (Kutsky, 1973). Since monovalent anions generally traverse phosphatidylcholine bilayers faster than monovalent cations (Bangham et al., 1974; Kornberg and McConnell, 1972), loss of paramagnetism is probably due mostly to ascorbate influx.

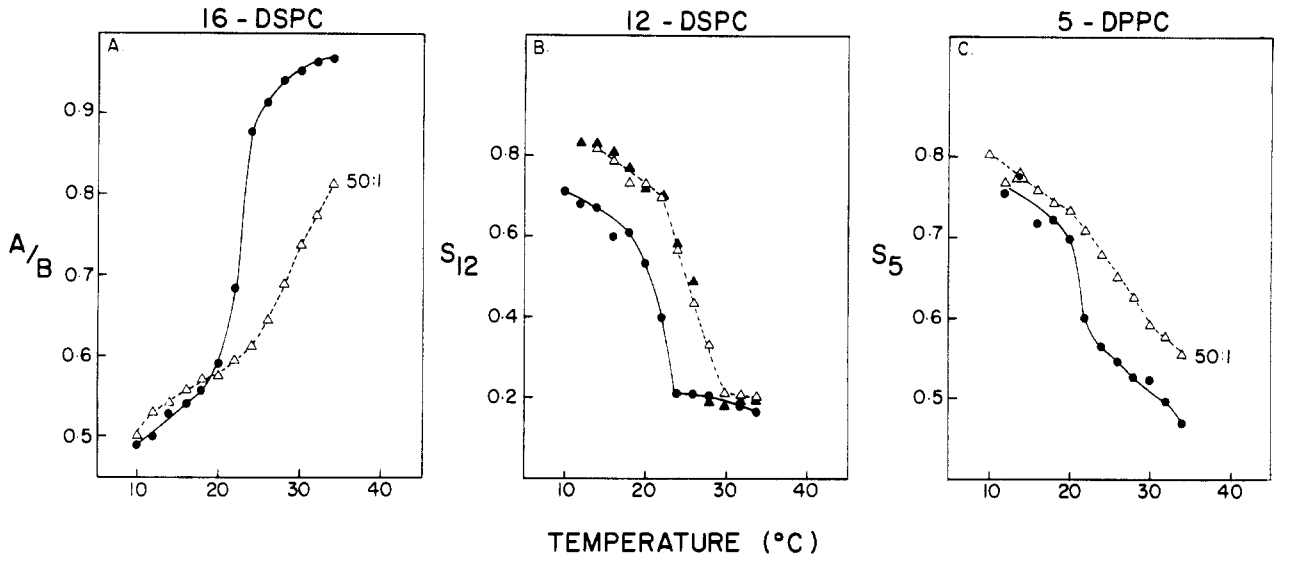

FIGURE 5: Thermotropic properties of DMPC alone (closed circles) and DMPC–ApoC-III complexes (triangles) as determined with spin-labeled phosphatidylcholine. A complex with lipid:protein molar ratio of 43:1 was studied by 16-DSPC, of 46:1 by 12-DSPC, and of 39:1 by 5-DPPC. A complex with 24:1 stoichiometry (▲) was studied by 12-DSPC only. DMPC vesicles (2 mg/ml) containing 3% by weight of spin-labeled PC (I, II, or III) was mixed with ApoC-III (0.41 mg/ml) to give lipid:protein ratios of ~25:1 or ~40:1. The mixture was incubated for 20 min at 37 °C, then subjected to density gradient centrifugation as described under the Experimental Section and illustrated elsewhere. Gradient fractions that contained complex were pooled and concentrated by ultrafiltration (Amicon XM-100) for study.

magnetic Eu^{3+} ions to this complex splits the spectrum into two components, the narrower low-field component being due to phospholipid polar head groups on the inside of the vesicles, and the broader high-field resonance being due to outside polar head groups exposed to the paramagnetic cations (Figure 7b). Maintaining the temperature of the mixture at 28 °C for 2 h results in a coalescence of the exposed and unexposed (external and internal) nuclei due to equilibration of Eu^{3+} across the bilayer (Figure 7c). Further addition of Eu^{3+} partially resolves the inner and outer resonances and also broadens them (Figure 7d). This broadening is attributable to faster relaxation caused by the increased paramagnetic ion concentration. A second set of experiments was begun by the addition of sufficient ApoC-III to DMPC vesicles to give a lipid–protein ratio of 685 mol/mol. At 10 °C, the spectrum of this complex is not significantly chemically shifted from that of the 1370:1 complex (Figure 7a). Addition of Eu^{3+} to this complex at 10 °C broadened the phosphorus resonance but did not split it into two components. However, raising the temperature to 23 °C did allow resolution of the inner (low field) and outer (high field) resonances (Figure 7g). This resolution is further enhanced by raising the temperature to 28 °C (Figure 7h). In a third series of experiments, a lipoprotein complex of ~55:1 was used. The resonance line of this complex in the absence of paramagnetic ions (Figure 7i) is only slightly broader than that of the 1380:1 complex (Figure 7a). The addition of Eu^{3+} to this complex at 9 °C did not split the spectrum into two separate resonances (Figure 7j) nor did increasing the temperature to 28 °C (Figure 7k,l), indicating an extremely porous bilayer *or* loss of original vesicle structure altogether.

Both the Tempo-choline and Eu^{3+} flux experiments established that at the level of ~55 mol of lipid/mol of protein, ApoC-III alters DMPC vesicles in such a way that inside–outside ion concentration gradients are either absent or undetectable by the methods employed. However, neither set of these experiments permitted unequivocal distinction between vesicles made highly permeable by apoprotein binding, and vesicles totally reorganized to a different type of structure (e.g., a monolamellar micelle). In the first case, one would not expect to observe a decrease in molecular weight, whereas in the latter case, this is a real possibility. This possibility led to the simple gel filtration experiment shown in Figure 8B. A DMPC–ApoC-III mixture (50 mol/mol) when chromatographed on Sepharose 6B elutes as a *sharp* symmetrical band at a volume of ~60 ml, whereas the starting vesicles elute as a much *broader* band centered at ~51 ml. A small amount of unbound apoprotein emerges from the column at about 87 ml. Chemical analysis of the eluted complex[5] revealed a stoichiometry of 34 mol of DMPC/mole of apoprotein. Hence, this complex[4] must result from ApoC-III induced breakdown of the original bilamellar vesicle.

Discussion

ApoC-III has profound effects on individual lipid molecules or groups of molecules that behave cooperatively within a DMPC bilamellar vesicle. In the region of 20–30 °C, acyl chain motion of the lipid molecules is markedly reduced by apoprotein binding (Figure 5). The apoprotein appears to exert this motional constraint along the entire length of the acyl chains. The ability of ApoC-III to reduce the liquidity of fluid domains in DMPC vesicles is also reflected in the reduced distribution of DTBN into the lipid phase when ApoC-III is present (Figure 4). Not only does the apoprotein reduce the motion of individual lipid molecules but also the apparent[6] extent of their cooperative interactions. This apparent loss of

[4] This complex has been rigorously characterized by sedimentation velocity ultracentrifugation, quasi-elastic light scattering, and low-angle x-ray scattering. A detailed description of its structure will be published later. For purposes of the present paper, suffice it to say that the sedimentation coefficient is smaller and the diffusion coefficient larger than that of the DMPC bilamellar vesicle. Furthermore, the x-ray scattering pattern characteristic of the original bilayer structure is absent. These results indicate breakdown of the vesicle to a smaller particle.

[5] The recovery of DMPC applied to this column was ~80%. Apparently some remained adsorbed to the gel. Nevertheless, apoC-III was in excess as indicated by the small peak emerging at ~85 ml (Figure 8B).

[6] This decrease in cooperativity must be considered apparent, since real changes in cooperativity can be measured only by calorimetric techniques that yield the enthalpy for the transition.

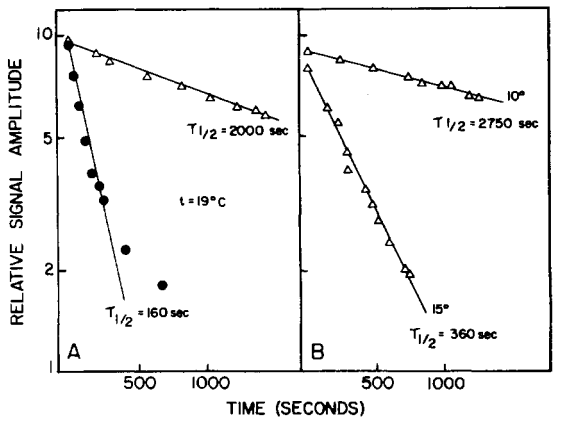

FIGURE 6: Relative permeabilities of DMPC vesicles alone and complexed to ApoC-III. The flux of Tempo-choline outward from the inside and/or of ascorbic acid inward from the outside was monitored by recording the low-field Tempo-choline resonance line (M_{+1}). Diminution of signal amplitude resulted from the quenching of paramagnetism by ascorbate. (A) *Multilamellar* vesicles alone (- Δ - Δ -) and complexed with ApoC-III (- ● - ● -) at 19 °C. To 1.9 ml of DMPC vesicles (8.0 mg/ml) was added 180 μl of 32 mM Tempo-choline iodide. After equilibration of the spin-label between the inside and outside of the bilayer (30 min at 35 °C), 15 μl of these labeled vesicles were mixed with 50 μl of ApoC-III in standard buffer (1.8 mg/ml) and incubated at 30 °C for 1 h. The mixture was cooled to 3 °C and 5 μl of cold 68 mM ascorbic acid was added to quench the paramagnetism of initially external spin label. The amount of ascorbic acid present represents an eightfold molar excess over that required to quench all Tempo-choline. The spectrum of the sample was then recorded at 60-s intervals for ~25 min. (B) *Bilamellar* vesicles alone at 10 and 15 °C. Vesicles were prepared by sonication, gel filtration (Sepharose 6B), and concentration (Amicon XM 100A membrane) to give a stock solution of 14.0 mg/ml. To 1 ml of this stock was added 75 μl of Tempo-choline (206 mM). To one 300-μl aliquot of this mixture was added 400 μl of ApoC-III (2.6 mg/ml) and to a second 300-μl aliquot was added 400 μl of standard buffer. Each mixture was vortexed, incubated at 35 °C for 10 min and 28 °C overnight, then cooled to 3 °C. A 100-μl aliquot was treated with 3 μl of 1.88 M ascorbic acid before transferring to a Corning micropipet sealed at one end. The amount of ascorbate represents an eightfold molar excess over that required to quench the paramagnetism of all Tempo-choline present. The spectrum of the sample was then recorded at 60-s intervals for about 25 min. The flux of Tempo-choline and/or ascorbic acid through the DMPC bilamellar vesicle-ApoC-III complex at 10 or 15 °C was too rapid to measure, i.e., no EPR signal could be detected immediately after addition of the ascorbic acid.

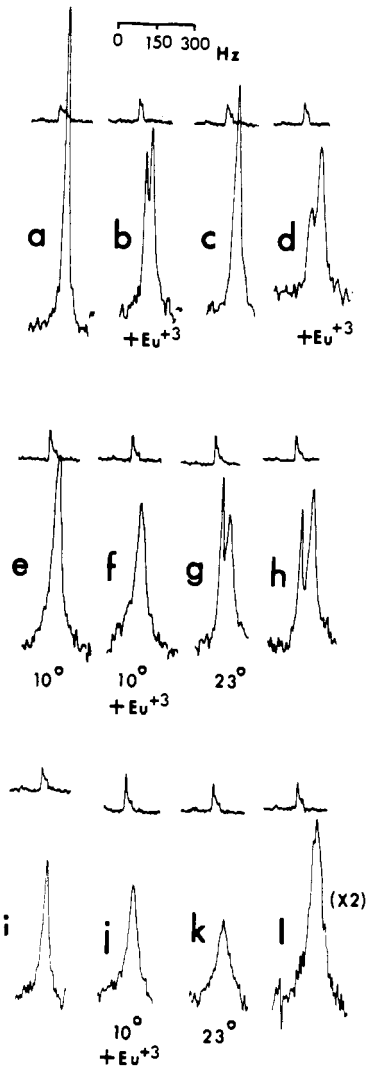

FIGURE 7: ^{31}P NMR spectra of DMPC vesicles in the presence and absence of ApoC-III and Eu^{3+}. All spectra were obtained at 40.5 MHz in the Fourier transform mode by applying 512 pulses of 12-μs duration and using an acquisition time of 2.048 s for a spectral width of 2 kHz. An ^{19}F external lock was employed and all tuning was done on 85% H_3PO_4 in the same geometry as the analytical samples. The reference signal (shown above each sample trace) was for 85% H_3PO_4 in a 2-mm capillary concentric within a 12-mm tube containing the sample. Since spinning the sample did not sharpen the resonance lines within experimental error, but did accelerate the rate at which sample turbidity developed, none of the samples were spun. The signal was exponentially filtered equivalent to 1 Hz broadening before transformation, and a 12-point smoothing function was applied to the final spectrum. Temperatures were controlled to the nearest degree by a Varian temperature controller. The sample temperature was determined before the start of each measurement with a TRI-R electronic thermometer. All temperatures were 28 °C except where indicated otherwise. The DMPC was sonically dispersed in 150 mM NaCl to give dispersions consisting of 80-90% bilamellar vesicles (Figure 8A). To form the lipid-protein complex, ApoC-III was added dropwise to the stirred DMPC dispersion at 28 °C and the mixture incubated for ≥30 min at that temperature before addition of Eu^{3+} and/or measurement. (a) DMPC-ApoC-III, 100:1 (w/w); (b) a plus 20 μl of 1 mM $Eu(NO_3)_3$; (c) b allowed to stand at 28 °C for 2 h; (d) c plus 20 μl of 1 mM $Eu(NO_3)_3$; (e) DMPC-ApoC-III, 50:1 (w/w) at 10 °C; (f) e plus 40 μl of 1 mM $Eu(NO_3)_3$ at 10 °C; (g) f at 23 °C; (h) f at 28 °C; (i) DMPC-ApoC-III, 5:1 (w/w); (j) i plus 60 μl of 1 mM $Eu(NO_3)_3$; (k) j at 24 °C; (l) j at 28 °C, twice normal scale.

cooperativity is reflected in the increased breadth of the thermotropic phase transition of the lipid within the complex (Figures 3, 4, and 5). If the cooperative unit within a DMPC vesicle contains 200 (Hinz and Sturtevant, 1972) to 250 (Tsong, 1974) molecules and if the apoprotein inserts into the bilayer uniformly over the complete outer surface of the vesicles, then for a DMPC-ApoC-III complex with a 50:1 stoichiometry one would expect the size of the initial cooperative unit to be decreased about four-fivefold. If those lipid molecules that bound each apoprotein molecule (i.e., the boundary lipids) are not part of a cooperative unit, then the size of that unit would be decreased even further. An expected consequence of the reduction in cooperative unit size is a decrease in lipid packing efficiency and an increase in the accessibility of ions on either side of the bilayer to the hydrophobic interior. While a difference in accessibility to pyrene in this hydrophobic interior was indicated by the Stern-Volmer quenching constants (Table I), this difference was too small for attributing to steric accessibility differences only. Results from Tempo-choline flux measurements were more conclusive. Ascorbic acid on the outside of the vesicle and Tempo-choline on the inside rapidly traverse the bilayer when ApoC-III binds (Figure 6). Initially, it appeared that the apoprotein created only local defects in lipid packing but with retention of bilayer structure. Attempts to support this view by demonstrating the presence of inside and outside polar head groups with paramagnetic Eu^{3+} ions were unsuccessful; results strongly suggested that ApoC-III destroys the original structure of DMPC

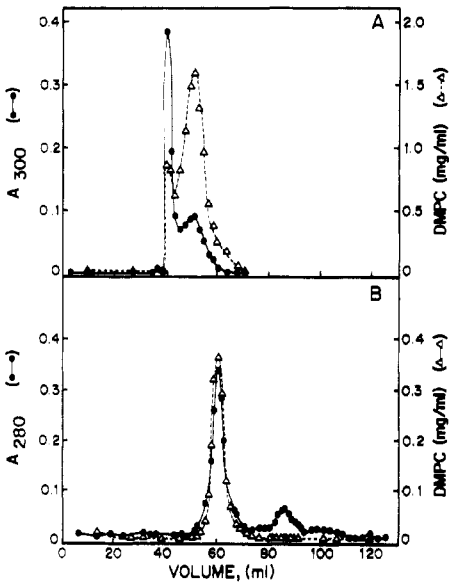

FIGURE 8: Gel filtration elution profiles of: (A) 40 mg of DMPC sonicated in 1 ml of standard buffer for 30 min at 30 °C (82% bilamellar vesicles) and (B) DMPC–ApoC-III (~50 mol/mol) complex; 0.25 ml of ApoC-III (1.75 mg/ml) added dropwise to 0.75 ml of a slowly stirred solution of DMPC (2.32 mg/ml). The mixture was incubated at 28 °C for 4 h before chromatography. Conditions: 0.9 × 180 cm column of Sepharose 6B equilibrated with 100 mM NaCl, 10 mM Tris, 1 mM NaN_3, 1 mM EDTA, pH 7.4, at 28 °C; flow rate ≃4 ml/h.

vesicles (Figure 7). This suggestion was, in fact, confirmed by a simple gel filtration experiment that provided a highly homogeneous population of lipid–apoprotein complexes that eluted at a volume greater than the initial DMPC vesicles, firmly establishing that the former were smaller than the latter (Figure 8).

The structure of the principal product resulting from the interaction of ApoC-III with an egg yolk phosphatidylcholine vesicle (Morrisett et al., 1974) is decidedly different from that resulting from dimyristoylphosphatidylcholine vesicle.[4] In the former case, the apoprotein binds to the vesicle in such a fashion that a portion of it (probably the carboxyl-terminal half according to Shulman et al., 1973 and Segrest et al., 1974) extends into the hydrophobic bilayer, while the remaining part occupies space in the polar head group region originally occupied by water. There is complete retention of structural integrity with the egg yolk phosphatidylcholine (EYPC) vesicle. In contrast, the interaction of ApoC-III with a DMPC vesicle produces a particle that has no demonstrable bilayer structure and is distinctly smaller than the original vesicle. This difference in structure of the lipid–apoprotein complexes is undoubtedly due to differences in the physical properties of the original vesicles. At 20 °C DMPC vesicles are much more permeable to $^{22}Na^+$ (J. D. Morrisett, unpublished results) and glucose (Inoue, 1974) than EYPC vesicles. EYPC vesicles do not undergo fusion (Kornberg et al., 1972; Taupin et al., 1975), whereas the fusion of DMPC vesicles (Prestegard and Fellmeth, 1974) as well as other saturated diacylphosphatidylcholine vesicles (Taupin et al., 1975; Papahadjopoulos et al., 1974) is well documented.

Melchior and Morowitz (1972) have observed a 0.023 ml/g volume increase associated with the gel → liquid crystalline phase transition of DMPC. Volume expansion of DMPC at or near its transition temperature should make the bilayer laterally compressible (Linden et al., 1973; Phillips et al., 1975) so that a phospholipid binding protein such as ApoC-III could penetrate to a depth not easily accessible in a EYPC bilayer. The breakdown of the resulting DMPC vesicle–ApoC-III complex but not of the complex with EYPC is probably due in part to this difference in penetration depth (as well as to a difference in intrinsic vesicle stability resulting from a different average length of the acyl chains). Deeper penetration enhances lipid–protein interaction and diminishes lipid–lipid interaction. We have considerable indirect evidence and some direct evidence[4] that the breakdown product is probably a micellar-type structure. Conversion from the vesicular to a micellar complex would be irreversible (see Figures 3, 4, and 8) because of the lower free energy of the latter which, as a monolamellar structure, should allow more extensive interaction with the apoprotein than the bilamellar vesicle. This is reflected in the different stoichiometries of the two lipid–protein complexes (~55:1 for the vesicular complex (Morrisett et al., 1974) and ~34:1 for that obtained in the present study). A detailed description of this product is the subject of a future report.

Acknowledgments

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References

Bangham, A. D., Hill, M. W., and Miller, N. G. A. (1974), in Methods of Membrane Biology, Korn, E. D., Ed., New York, N.Y., Academic Press.

Bartlett, G. R. (1959), *J. Biol. Chem. 234,* 466.

Batzri, S., and Korn, E. (1973), *Biochim. Biophys. Acta 298,* 1015.

Birks, J. B. (1970), Photophysics of Aromatic Molecules, Chapter 10, London, Wiley-Interscience.

Blok, M. C., Van der Neut-Kok, E. C. M., Van Deenen, L. L. M., and De Gier, J. (1975), *Biochim. Biophys. Acta 406,* 187.

Bystrov, V. F., Shapiro, Y. E., Viktorov, A. V., Barsukov, L. I., and Bergelson, L. D. (1972), *FEBS Lett. 25,* 337.

Fischkoff, S., and Vanderkooi, J. M. (1975), *J. Gen. Physiol. 65,* 663.

Gaffney, B. J. (1976), in Spin Labeling: Theory and Applications, Chapter 5, Berliner, L. J., Ed., New York, N.Y., Academic Press.

Griffith, O. H., Dehlinger, P. J., and Van, S. P. (1974), *J. Membr. Biol. 15,* 159.

Hinz, H.-J., and Sturtevant, J. M. (1972), *J. Biol. Chem. 247,* 6071.

Hoff, H. F., Morrisett, J. D., and Gotto, A. M. (1973), *Biochim. Biophys. Acta 296,* 653.

Hoffman, A., and Henderson, A. (1961), *J. Am. Chem. Soc. 83,* 4671.

Hubbell, W. L., and McConnell, H. M. (1971), *J. Am. Chem. Soc. 93,* 314.

Inoue, K. (1974), *Biochim. Biophys. Acta 339,* 390.

Kornberg, R. D., and McConnell, H. M. (1971), *Biochemistry 10,* 1111.

Kornberg, R. D., McNamee, M. G., and McConnell, H. M.

(1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1508.
Kutsky, R. J. (1973), Handbook of Vitamins and Hormones, New York, N.Y., Van Nostrand Reinhold Co., p 73.
Lee, A. G., Birdsall, N. J. M., Metcalfe, J. C., Toon, P. A., and Warren, G. B. (1974), *Biochemistry 13,* 3699.
Linden, C. D., Wright, K. L., McConnell, H. M., and Fox, C. F. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2271.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 173,* 265.
Melchoir, D. L., and Morowitz, H. J. (1972), *Biochemistry 11,* 4558.
Morrisett, J. D., David, J. S. K., Pownall, H. J., and Gotto, A. M. (1973), *Biochemistry 12,* 1290.
Morrisett, J. D., Gallagher, J. G., Aune, K. C., and Gotto, A. M. (1974), *Biochemistry 13,* 4765.
Morrisett, J. D., Pownall, H. J., Plumlee, R. T., Smith, L. C., Zehner, Z. E., Esfahani, M., and Wakil, S. J. (1975a), *J. Biol. Chem. 250,* 6969.
Morrisett, J. D., Pownall, H. J., Sparrow, J. T., Jackson, R. L., and Gotto, A. M. (1975b), *Adv. Exp. Med. Biol. 63,* 7.
Morrisett, J. D., Pownall, H. J., and Gotto, A. M. (1976) (submitted for publication).
Papahadjopoulos, D., Poste, G., Schaeffer, B. E., and Vail, W. T. (1974), *Biochim. Biophys. Acta 352,* 10.
Phillips, M. C., Graham, D. E., and Hauser, H. (1975), *Nature (London) 254,* 154.
Pownall, H. J., Morrisett, J. D., Sparrow, J. T., and Gotto, A. M. (1974), *Biochem. Biophys. Res. Commun. 60,* 779.
Pownall, H. J., Morrisett, J. D., and Gotto, A. M. (1976) (submitted for publication).
Prestegard, J. H., and Fellmeth, B. (1974), *Biochemistry 13,* 1122.
Rozantsev, E. G. (1970), Free Nitroxyl Radicals, New York, N.Y., Plenum Press, p 232.
Segrest, J. P., Jackson, R. L., Morrisett, J. D., and Gotto, A. M. (1974), *FEBS Lett. 38,* 247.
Shimshick, E. J., and McConnell, H. M. (1973), *Biochem. Biophys. Res. Commun. 53,* 446.
Shulman, R. S., Herbert, P. N., Witters, L. A., Quicker, T., Wehrly, K. A., Fredrickson, D. S., and Levy, R. I. (1973), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32,* Abst. 1858.
Soutar, A. K., Pownall, H. J., Hu, A., and Smith, L. C. (1974), *Biochemistry 13,* 2828.
Taupin, C., Dvolaitzky, M., and Sauterey, C. (1975), *Biochemistry 14,* 4771.
Trudell, J. R., Payan, D. G., Chin, J. H., and Cohen, E. N. (1974), *Biochim. Biophys. Acta 373,* 141.
Tsong, T. Y. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2684.
Weast, R. C. (1969), Chemical Rubber Handbook, Cleveland, Ohio, CRC Publishing Co., p D-192.

Intramitochondrial Localization and Proposed Metabolic Significance of Serine Transhydroxymethylase†

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ABSTRACT: Serine transhydroxymethylase is a latent enzyme of intact rat liver mitochondria. The enzyme is neither solubilized by the selective removal of the outer membrane with digitonin, nor inactivated by concentrations of diazobenzenesulfonate that do not penetrate the inner membrane, but that do inhibit solubilized serine transhydroxymethylase. Swelling of mitochondria was studied in isoosmotic solutions of substrates under conditions that would define transport as neutral uniport, anion–hydroxyl exchange, anion–anion exchange, or electrophoretic. L-Serine and glycine appear to be rapidly taken up by a nonelectrophoretic uniport mechanism, while folate and tetrahydrofolate are not transported. The results localize the enzyme in the matrix and indicate that the latent activity results from a lack of tetrahydrofolate transport across the inner membrane. Based on these results, the dual localization of serine transhydroxymethylase in the mitochondria and the cytosol is proposed to provide a one-carbon shuttle system to link one-carbon metabolism in the two-cellular compartments.

Serine transhydroxymethylase (L-serine-tetrahydrofolate 5,10-hydroxymethyltransferase, EC 2.1.2.1), which catalyzes the reaction shown in eq 1, has been found to exist in approximately equal proportions as cytosolic and mitochondrial isozymes in rat liver (Nakano et al., 1968; Motokawa and Kikuchi, 1971; Palekar et al., 1973).

L-serine + *d*-L-tetrahydrofolate ⇌ glycine + *l*-L-5,10-methylenetetrahydrofolate + H_2O (1)

Yoshida and Kikuchi (1973) have suggested that serine transhydroxymethylase functions in the major pathway of serine catabolism in mammalian liver. The reaction may, therefore, be an important source of one-carbon units required for the biosynthesis of purines, thymidylate, and methionine. The isolation of a glycine-dependent mutant of Chinese hamster ovarian cells (Kao et al., 1969; Chasin et al., 1974) that is deficient in mitochondrial, but not cytosolic serine transhydroxymethylase, indicates the necessity of this isozyme for normal growth.

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