# Composition–structure–function correlations in the binding of an apolipoprotein to phosphatidylcholine bilayer mixtures

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**Abstract** We have studied the lipid binding of apoC-III with two types of mixed vesicles of DMPC (dimyristoyl phosphatidylcholine) and DPPC (dipalmitoyl phosphatidylcholine). DMPC vesicles mixed with those of DPPC produce a macroscopic mixture in which the DMPC and DPPC vesicles remain intact. The circular dichroism and fluorescence spectra of apoC-III in the presence of this macroscopic mixture exhibit major changes near the transition temperature of each of the pure lipids, confirming the independent existence of the two PC's. Combining DMPC:DPPC macroscopic mixtures with apoC-III above the transition temperature, $T_c$ 23°C, of DMPC produces an isolatable complex consisting of 4:1 DMPC:DPPC. If the DMPC and DPPC are within the same vesicle, this microscopic lipid mixture has properties that are functions of the temperature and lipid composition. Spectral analysis of apoC-III in the presence of the micromixtures reveals a single transition, which occurs between the respective thermal transitions of DMPC (23°C) and DPPC (41°C). The lipid:protein complexes isolated from the microscopic mixtures have a DMPC:DPPC ratio identical to that of the vesicle with which the apoprotein was mixed; the formation of these complexes is most efficient above the temperature range where these microscopic mixtures induce major structural changes in the apoprotein. The absence of the preferential binding to DMPC observed in the macroscopic mixtures suggests that apoC-III interacts with large lipid domains within a vesicle and does not selectively bind the low melting DMPC. The temperature dependence of the binding of apoC-III to the microscopic mixtures may be due to phase separation or to cocrystallization; our data support the latter process.

**Supplementary key words** phospholipids · lipoproteins · lipid-protein association · transition temperature · circular dichroism · fluorescence

The gel → liquid crystalline transition temperature, $T_c$, of phospholipids has been characterized by a variety of spectral (1–6) and calorimetric (7–10) techniques. Analogous transitions found in membranes (11–16) and lipoproteins (2, 17–21) have been correlated with the activation energies of various biological and physical processes. In contrast to studies on model systems containing pure synthetic lipids, real membranes and lipoproteins are composed of mixtures of lipids, so it is crucial to identify the composition–structure–function relationships that are important in more complex lipid–protein systems. Many different phospholipid pools are available for binding to an apolipoprotein between its site of synthesis and the point of catabolism; the rate and course of interaction of these various lipid pools with the protein will depend, in part, upon the composition and physical form of the lipid.

In model systems, the plasma apolipoproteins have been shown to interact preferentially with phospholipids near or above the melting or transition temperature of the phospholipid (17, 18, 20). There is no available information concerning such interactions in a mixed system of phospholipids. Furthermore, the interaction between an apoprotein and phospholipid may be regulated by either the micromolecular properties of the individual phospholipid molecules or a macroscopic or bulk phase property of a phospholipid system, such as its fluidity.

In the experiments reported in this paper, we have studied the interaction of apoC-III with pure DMPC and DPPC and with mixtures of DMPC and DPPC in which these two lipids are in the same vesicle (microscopic mixtures) or in separate vesicles (macroscopic mixture). We have studied the interaction of apoC-III with the phospholipids as a function of temperature, concentration, and the physical state of the lipids. We have correlated our results with those obtained using electron paramagnetic resonance (3, 22, 23) and calorimetry (24–28). We expected

Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-3-glycerophosphorylcholine; DPPC, 1,2-dipalmitoyl-*sn*-3-glycerophosphorylcholine; CD, circular dichroic; PC, *sn*-3-glycerophosphorylcholine; apoC-III, apolipoprotein-C-III$_1$, a major protein of the human very low density plasma lipoproteins; TEMPO, 2,2,6,6-tetramethylpiperidine-l-oxyl; $T_c$, transition temperature; SDS, sodium docecyl sulfate; EPR, electron paramagnetic resonance.

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that our approach would answer the fundamental question of whether apoC-III binds equivalently to all phospholipids in a binary lipid mixture that is fluid and above the transition temperature of DMPC but below that of DPPC.

Since many of these phenomena exhibit profound changes as the system traverses its transition temperature, we believe that the conclusions obtained in these studies may be relevant to studies of membrane functions such as transport, enzymic activity (11, 12, 29, 30), and permeability (31–39) and to the composition–structure–activity relationships of lipid metabolizing enzymes (2, 40).

## EXPERIMENTAL PROCEDURE

ApoC-III$_1$ isolated from fasting subjects with type IV or V hyperlipoproteinemia by the method of Brown, Levy, and Fredrickson (41) as modified by Morrisett et al. (42) was used in all of these studies.

1,2 Di[1-$^{14}$C]myristoyl-*sn*-3-glycerophosphorylcholine was custom synthesized by Applied Science Laboratories, State College, PA. 1-Palmitoyl,2-[9,10-$^3H_2$]palmitoyl-*sn*-3-glycerophosphorylcholine was synthesized from 1-palmitoyl-*sn*-3-glycerophosphorylcholine and [9,10-$^3H_2$] palmitic anhydride as described previously (43). Both phospholipids were $\geq 99\%$ pure as determined by thin-layer chromatography (44) and gas–liquid chromatography of their fatty acid methyl esters.

The fluorescence, circular dichroic, and ultracentrifugal methods were similar to those described previously (17, 42). The $\alpha$-helical content was determined from

$$\%\ \alpha\text{-helix} = \frac{[\theta_{222} + 3000]}{39{,}000} \times 100\%$$

where $\theta_{222}$ represents the mean residue ellipticity at 222 nm (42).

Phosphorus analyses were obtained by the method of Bartlett (45). Protein was determined by measuring the optical density at 280 nm except in highly turbid samples. Such samples were clarified by dissolving an aliquot in a 10 mg/ml solution of SDS (sodium dodecyl sulfate) at pH 7.4. Their concentrations were obtained by comparison of their fluorescence intensities to those of standard solutions of apoC-III recorded under the same conditions.

Micro- and macromolecularly mixed liposome mixtures of DMPC and DPPC were prepared by a modification of the method of Batzri and Korn (46). The micromolecular mixtures were prepared by dissolving the two lipids in ethanol at the desired weight ratio and injecting the resulting solution (20–30 $\mu$l, 50–60 mg/ml) into ~1.0 ml of buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM sodium azide, and 100 mM sodium chloride) at ~60°C while vortexing (Method 1). Both multilamellar and single bilayer vesicles are formed as determined by chromatography on Sepharose 6B; each vesicle has the same lipid composition as the injected ethanolic solution (see below).

The macromolecularly mixed liposomes were prepared similarly (Method 2) except that the ethanolic solutions of each phospholipid were injected into separate buffer solutions. Each solution was then cooled to ~0°C and an appropriate volume of each was mixed at ~0°C to produce the desired macromolecular mixture of DMPC and DPPC liposomes. Both the micro- and macromolecularly mixed liposomes were used within 30 min after their preparation. Experimental details of the thermal studies of the binding of apoC-III to phospholipids measured by spectral and ultracentrifugal methods, were similar to those described previously (17). The densities given here were determined from refractive index measured at ~22°C, whereas the density gradients were spun at ~5°C. Therefore, the density values were useful only for the comparison of one gradient profile with another.

## RESULTS

### Thermocircular dichoric spectra

The CD spectrum of apoC-III in the presence of the phospholipid micro- and macroscopic mixtures (all were 50:1 lipid:protein molar ratios and were mixed with the protein at 5°C) were measured as a function of increasing temperature. An increase in the ellipticity of the protein occurred at a critical temperature, $T_c$. The characteristics of these curves depended on the composition and the method of preparing the liposomes. When the liposomes contained only pure DMPC or DPPC (**Fig. 1A, B**) the $T_c$ was sharp and had an upper boundary (23.5 and 41°C, respectively) that coincided with the transition temperature of the lipid as determined by calorimetric methods (23.7 and 41.8°C, respectively) (8). This behavior was in contrast to that of apoC-III alone, which showed little change in its CD spectrum over the same temperature range (17). The calculated maximum helical contents of apoC-III developed in the presence of DMPC (70%) and DPPC (50%) were substantially different.

The thermal behavior of the macromolecular mixtures of PC, i.e., the DMPC and DPPC were in

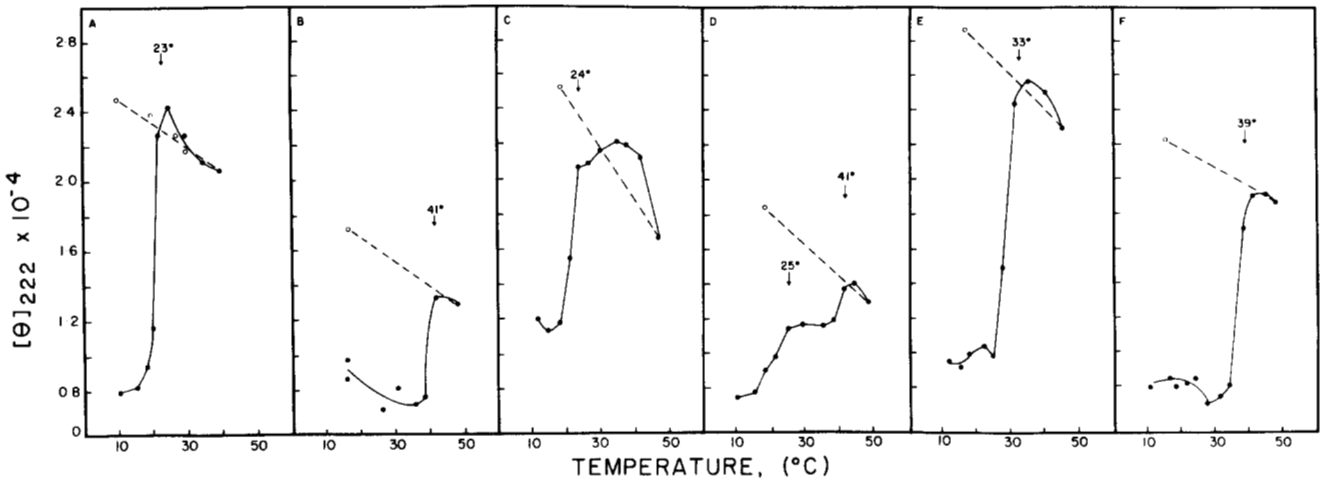


**Fig. 1.** Ellipticity vs. temperature profiles of apoC-III with a total phospholipid:protein ratio of 50:1. The phospholipids and apoprotein were combined at ~5°C and the ellipticity measured as a function of increasing temperature. The lipid component contained DMPC and DPPC in the following ratios and types of phospholipid preparations: (A) pure DMPC; (B) pure DPPC; (C) 1:1 DMPC:DPPC macroscopic mixture; (D) 1:5 DMPC:DPPC macroscopic mixture; (E) 1:1 DMPC:DPPC microscopic mixture; (F) 1:5 DMPC:DPPC microscopic mixture. The arrows indicate the approximate temperature at which melting of the lipids was completed. Filled and unfilled circles represent heating and cooling of the mixtures, respectively.

separate vesicles, with apoC-III (Fig. 1C) qualitatively resembled superposed behavior of the two separate lipid:protein systems shown in Fig. 1A, B. For a 1:1 macroscopic lipid mixture of DMPC/DPPC with apoC-III, a lower $T_c$ appeared at about 23°C and a second smaller one occurred at approximately 35°C. The two transitions were more distinct in the circular dichroic thermogram of apoC-III plus a 1:5 DMPC:DPPC macroscopic mixture (Fig. 1D, prepared by Method 2). The lower and upper $T_c$ values were 25 and 41.5°C; the latter was better defined than the upper $T_c$ for the equimolar lipid mixtures. The helical content increased during the cooling portion of the curve and was increased with increasing mole fractions of DMPC in the lipid mixture.

TABLE 1. Thermo-circular dichroic properties of apoC-III with various DMPC:DPPC mixtures

| Phospholipid Mixed with the ApoC-III[a] | Maximum α-Helicity (~5°)[b] | Melting Range[c] |
|---|---|---|
| | | °C |
| DMPC | 71% | 18.5–23° (23.6[d]) |
| DPPC | 52% | 37.5–41° (41.6[d]) |
| 1:1 DMPC:DPPC (micromixture) | 81% | 25.5–32° |
| 1:1 DMPC:DPPC (macromixture) | 73% | 18–24° |
| 1:5 DMPC:DPPC (micromixture) | 65% | 34–39° |
| 1:5 DMPC:DPPC (macromixture) | 55% | 15–25° 36–41° |

[a] Initial lipid:protein molar ratio was 50:1; lipid and protein were mixed at 5°C and a heating and cooling curve obtained according to Fig. 1.

[b] Obtained from cooling portion of α-helix vs. temperature curves.

[c] Defined by the onset and completion of changes in α-helicity shown in Fig. 1. (The calorimetrically determined values were identical to the completion of the melting of pure DMPC and DPPC in our data).

[d] Calorimetric values (8–11).

The CD thermograms of the microscopically mixed liposomes of DMPC and DPPC plus apoC-III (Fig. 1E–F) were quite different from those of the pure and the macroscopically mixed lipids. These transitions appeared to be unique for a given lipid mixture, occurring at 25.5–34°C and 34–39°C for the 1:1 and 1:5 DMPC:DPPC microscopic mixtures, respectively. After cooling to 5°C, the estimated helical content of the apoC-III in these respective mixtures, 81% and 65%, was significantly greater than for the corresponding macroscopic mixtures, 73% and 55%. The results of the CD thermograms are summarized in **Table 1**.

## Thermofluorimetric analysis of lipid:protein interaction

The decrease in wavelength of maximal fluorescence ($\lambda_{max}$) of apoC-III that accompanies its binding to phospholipids (17, 42) at a critical temperature, $T_c$, provided evidence complementary to that obtained in the CD studies. The experiments shown in Fig. 2A–F were performed under the same conditions as those used in the CD experiments of Fig. 1A–F. The results are given in **Table 2**. The major findings were as follows. *1*) The wavelength vs. temperature plots of apoC-III in the presence of pure DMPC or DPPC

TABLE 2. Thermo-fluorimetric properties of apoC-III with various DMPC and DPPC mixtures[a]

| | Δλ, nm[b] | Melting Range[d] |
|---|---|---|
| | | °C |
| DMPC | 9.5 | 19–24° (23.6[c]) |
| DPPC | 4.5 | 39–41° (41.6[c]) |
| 1:1 DMPC:DPPC (micromixture) | 5 | 25.5–33° |
| 1:1 DMPC:DPPC (macromixture) | 3.5 | 18.5–22° 39°–(shoulder) |
| 1:5 DMPC:DPPC (micromixture) | 7.5 | 34.5–38° |
| 1:5 DMPC:DPPC (macromixture) | 5.0 | 17–23° 35–40° |

[a] Lipid:protein molar ratio was 50:1. Data obtained according to Fig. 2.
[b] Total wavelength shift obtained after mixing protein and lipid at 5°C and heating to ~45°C and cooling to 10°C.
[c] Calorimetrically determined values (8–11).
[d] Defined by the onset and completion of changes in the wavelength shifts given in Fig. 2. Note that the completion of melting in the pure PC's is the same as the calorimetrically determined value.

were minimal at the transition temperature of the lipid (24° and 41°C, respectively), corresponding to total wavelength shifts of 9.5 and 5 nm, respectively (Fig. 2A,B). *2*) In the macromolecular mixtures, two $\lambda_{max}$ shifts appeared at temperatures (Fig. 2C,D) that were, qualitatively, the same as those of the pure constituent lipids; the upper transition is much better defined in the 1:5 DMPC:DPPC macromolecular mixtures (Fig. 2D) than in the 1:1 macromolecular mixture (Fig. 2C). *3*) The $T_c$ values for the micromolecular mixtures of DMPC and DPPC, defined by the minima in the wavelength vs. temperature plots of the fluorescence maxima (**Fig. 2E,F**), were unique for a given DMPC and DPPC composition and were intermediate between those of the pure lipids (Fig. 2A,B).

### Ultracentrifugal behavior of DMPC:DPPC–apoC-III mixtures

The observation of irreversible thermally induced changes in the spectral properties of apoC-III with various lipid preparations suggested that a stable lipid:protein complex might be isolated from these lipid mixtures. Knowledge of the composition of the complex might clarify the structural and compositional requirements of the mixed lipid systems that optimally bind apoC-III.

The ultracentrifugal behavior of micro- and macromolecular mixtures of DMPC and DPPC without protein at two different temperatures is shown in **Fig. 3**. The macromolecular mixtures, which were prepared at two different temperatures (Fig. 3A,B), behaved similarly and were different from the micromolecular mixtures (Fig. 3C,D) prepared at the same two temperatures. Another sample, in which the two lipids were incubated at 35°C, behaved similarly. We, therefore,

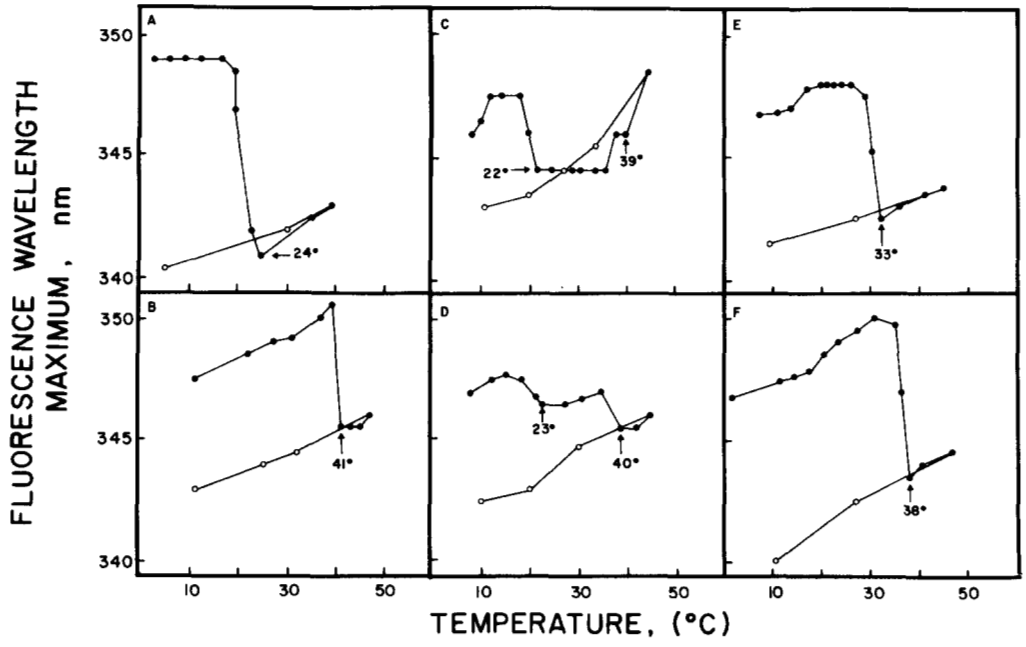


**Fig. 2.** Wavelength of fluorescence maximum of apoC-III vs. temperature. See Fig. 1 for details. (A) pure DMPC: (B) pure DPPC; (C) 1:1 DMPC:DPPC macroscopic mixture; (D) 1:5 DMPC:DPPC macroscopic mixture; (E) 1:1 DMPC:DPPC microscopic mixture; (F) 1:5 DMPC:DPPC microscopic mixture.

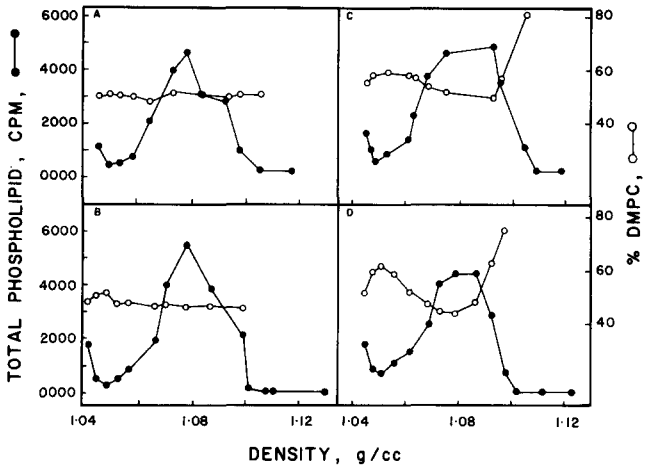


**Fig. 3.** Composition vs. density profiles resulting from the centrifugation of DMPC and DPPC in the absence of protein (A) 1:1 microscopic mixture incubated at 10°C; (B) 1:1 microscopic mixture incubated at 50°C; (C) 1:1 macroscopic mixture incubated at 10°C; (D) 1:1 macroscopic mixture incubated at 50°C[2].

conclude that macromolecular mixtures of DMPC and DPPC have similar centrifugal properties between 10 and 50°C. The two microscopic mixtures also behaved similarly between 10 and 50°C but differed from the macroscopic lipid mixtures. There was overlap of the DMPC and DPPC into the density region of the other lipid. Significantly, the composition of each gradient fraction in the microscopic lipid mixtures was invariant with the density, whereas in the macroscopic lipid mixtures the composition was variable. These results are consistent with those of Rothman and Dawidowicz (47) and Martin and MacDonald (48) who have shown that under similar conditions the separate DMPC and DPPC vesicles remain intact during a 10 min incubation period at each temperature. Our incubation periods did not exceed 10 min unless specified otherwise.

The ultracentrifugal behavior of complexes formed by apoC-III with pure DMPC (**Fig. 4A**), DPPC (Fig. 4B), and the 1:1 macroscopically mixed DMPC:DPPC (Fig. 4C) was compared. At a DMPC:apoC-III molar ratio of 120:1 ($T > T_c = 23°C$), 95% of the total apoC-III in the incubation mixture appeared in the complex (Fig. 4A). The DMPC:protein molar ratio in the isolated complex was about 105:1. With DPPC ($T > T_c = 41°C$), on the other hand, the phospholipid:protein molar ratio of the complex was about 75:1; only 55% of the total apoC-III that was incubated with the DPPC appeared in the complex (Fig. 4B). Note that these results are the same at incubation times of 5, 20, and 90 min (**Table 3**). A 1:1 DMPC:DPPC macroscopic mixture incubated with apoC-III (120:1 lipid:protein molar ratio) did not form a detectable amount of complex below the transition temperature of the DMPC. However, significant quantities of a phospholipid–apoprotein complex were formed when the incubation temperature was between 22 and 50°C; incubation of apoC-III with a 1:1 macroscopic mixture of DMPC and DPPC in a lipid to protein molar ratio of 120:1 at 50° (Fig. 4C) formed a complex. The principal lipid component in the complex was DMPC (nearly 90%) and only 70% of the total protein included in the incubation mixture appeared in the lipid–protein complex.

At a lipid to protein molar ratio of 120:1 the 1:1 DMPC:DPPC microscopic mixtures (prepared by Method 1) behaved differently from the corresponding macroscopic lipid mixture. Below 25°C little or no complex was formed (**Fig. 5A**) but at 28°C and above (Table 3), an appreciable amount of complex was formed. At 28°C 90% of the apoC-III in the incubation mixture appeared in a complex having a DMPC:DPPC ratio of 1:1 and a lipid:protein molar ratio of 97:1 (Fig. 5B). An increased association of apoC-III and the 1:1 DMPC:DPPC microscopic

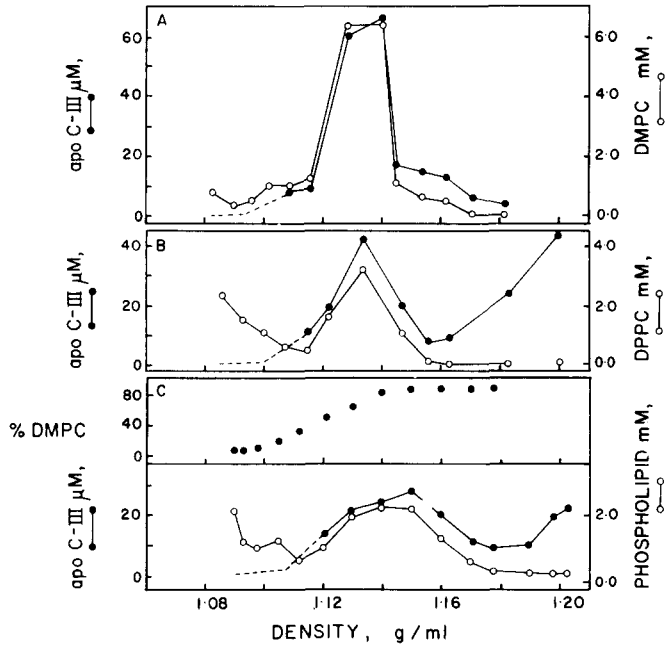


**Fig. 4.** Linear density gradient ultracentrifugal distribution of (A) DMPC and apoC-III after incubation together for 10 minutes at 28°C; (B) DPPC and apoC-III after incubation together for 10 min at 45°C; (C) 1:1 DMPC:DPPC macroscopic mixture and apoC-III after incubation together for 10 min at 50°C. Total lipid to apoprotein ratio in the incubation was 120:1. Nearly all of the DMPC appears in a complex, whereas neither DPPC nor the 1:1 DMPC:DPPC macroscopic mixtures are totally incorporated into the complex. Note that the complex contains largely DMPC as the lipid component and that the unbound lipid is composed of about 80% DPPC. Similar results were observed between 22°C and 50°C. (The apparent high lipid concentrations in the low density portion of the gradients are artifactual and are attributed to the retention of unbound phospholipid by the polyallomer centrifuge tube.)

TABLE 3. Properties of apoC-III:phospholipid complexes isolated by density gradient ultracentrifugation

| DMPC/DPPC Ratio of PC's Mixed with ApoC-III | Total PC/ApoC-III Contained in the Incubation Mixture | Incubation Temperature[a] | Phospholipid/ Protein Molar Ratio in Complex | DMPC/DPPC Ratio in Complex | % of Total ApoC-III in Mixture Appearing in Complex |
|---|---|---|---|---|---|
| | | °C | | | |
| 100% DMPC | 120 | 28° (5 min) | 108 | | ~95 |
| | | 28° (20 min) | 110 | | ~95 |
| | | 28° (90 min) | 102 | | ~95 |
| 100% DPPC | 120 | 45° (5 min) | 75 | | 53 |
| | | 45° (20 min) | 73 | | 50 |
| | | 45° (90 min) | 72 | | 57 |
| 1:1 DMPC:DPPC (micromixture) | 120 | 20° | | | nil |
| | | 25° | | | nil |
| | | 28° | 97 | 1:1 | 90 |
| | | 35° | 105 | 1:1 | 93 |
| | | 50° | 113 | 1:1 | 85 |
| 1:1 DMPC:DPPC (macromixture) | 120 | 10° | | | nil |
| | | 50° | 80 | 6.7:1 | 70 |
| 1:5 DMPC:DPPC (micromixture) | 120 | 31° | | | nil |
| | | 34° | 72 | 1:5 | 44 |
| | | 36° | 85 | 1:5 | 82 |
| | | 41° | 96 | 1:5 | 70 |
| | | 50° | 100 | 1:5 | 80 |
| 5:1 DMPC:DPPC (micromixture) | 120 | 35° | 107 | 5:1 | 95 |
| | | 50° | 99 | 5:1 | 90 |
| 1:1 DMPC:DPPC (micromixture) | 80 | 32° | 60 | 1:1 | 95 |
| | | 50° | 64 | 1:1 | 90 |
| 1:5 DMPC:DPPC (micromixture) | 50 | 36° | 50 | 1:5 | 60 |
| 1:1 DMPC:DPPC (micromixture) | 50 | 13° | | | nil |
| | | 32° | 42 | 1:1 | 54 |
| | | 50° | 40 | 1:1 | 52 |
| 1:1 DMPC:DPPC (macromixture) | 50 | 13° | | | nil |
| | | 32° | b | 6:1 | b |
| | | 50° | b | 6:1 | b |

[a] All incubations at the given temperature were for 10 min unless specified otherwise. All experiments used 0.5 mg of apoC-III with the specified molar ratio of phospholipid.
[b] A lipid–protein complex cosedimented with the protein so these values were unavailable in these experiments.

mixture apparently occurs between 25 and 28°C. It should be noted that the DMPC:DPPC ratio was 1:1 throughout the entire density gradient; i.e., the same ratio as that of the mixture incubated with the apoC-III to form the complex.

The experiment shown in Fig. 5 was repeated with a 1:5 DMPC:DPPC micromixture (prepared by Method 1) that had been incubated with apoC-III (lipid:protein molar ratio of 120:1) for 10 min at 31°C, 34°C, and 36°C. Although the temperature range was small, significant differences among the three experiments were observed (**Fig. 6**). Substantially more complex was formed at the higher temperatures, beginning at about 34°C. In spite of the differences in the amount of complex formed, the ratio of DMPC to DPPC in all three experiments was the same in every gradient fraction, i.e., 1:5. Although little complex was formed at 31°C under similar conditions, an increasing fraction of the total apoC-III incubated with the 1:5 DMPC:DPPC microscopic mixture appeared in the complex as the incubation temperature was increased to 36°C (Fig. 5A–C). Again this was the same DMPC:DPPC molar ratio as that of the initial mixture. Additionally, as Table 3 indicates, in the 1:1 microscopically mixed PC's a decrease in the lipid to protein ratio of the resulting complex was observed when the starting lipid to protein ratio of the incubation mixture was decreased. Mixing the phospholipids and the protein in ratios of 120:1, 80:1, and 50:1 led to complexes having lipid to protein ratios of 105:1, 60:1, and 42:1, respectively. In each instance, the DMPC:DPPC ratio of the complex was that of the starting mixture (1:1) that was incubated with the apoC-III. Finally, com-

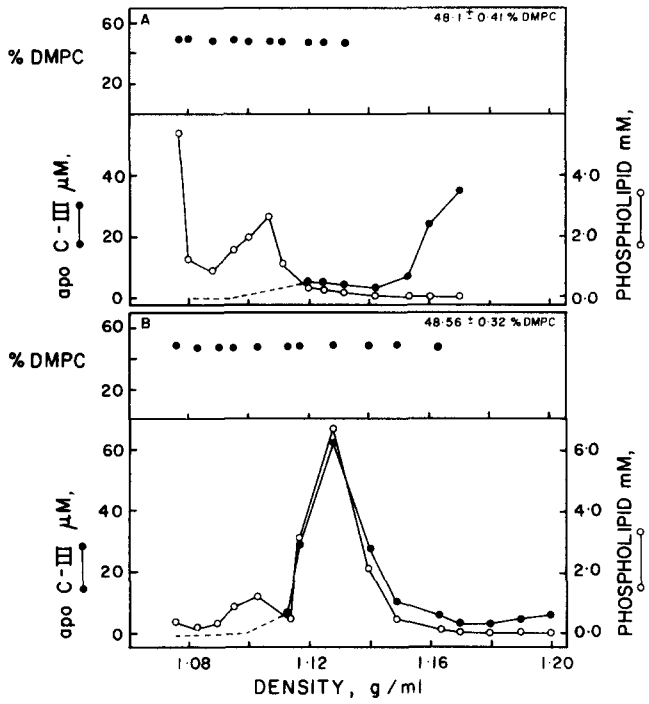


**Fig. 5.** Composition vs. density profiles obtained from the linear density gradient ultracentrifugation of apoC-III with a DMPC: DPPC 1:1 microscopic mixture as a function of temperature. (A) 25°C; (B) 28°C; the total lipid to protein ratio was 120:1. The dashed lines represent the protein concentration as determined by fluorescence intensity. The average DMPC composition and average deviation are given in the upper right hand corner of each panel. (The apparent high lipid concentrations in the low density portion of the gradients are artifactual and are attributed to the retention of unbound phospholipid by the polyallomer centrifuge tube.) The percent DMPC given for each fraction refers to the lipid composition.

parison of the data in Tables 1–3 shows that the incubation temperature at which the apoC-III and the microscopically mixed PC's exhibit maximal complex formation corresponds to that temperature where the onset of major changes in CD and fluorescence spectra occur; significantly, the spectroscopic changes observed in the pure PC's begin at temperatures significantly below those of their calorimetrically determined transition temperatures.

## DISCUSSION

The interpretation of our results is based upon the hypothesis that apolipoproteins preferentially interact with fluid domains of phospholipids (17, 18, 20). The binding data on the macroscopically mixed DMPC:DPPC are consistent with this view since preferential binding to DMPC (the lower melting PC) is observed.

Although transfer of DMPC to DPPC in a macroscopic mixture of these two lipids is predicted (48), the very small decrease in the upper transition of these mixtures suggests that only a small amount of transfer has occurred under our experimental conditions.

One would expect DMPC to bind more efficiently for two reasons. First, apoC-III forms a complex more efficiently with DMPC than with DPPC, as indicated by the higher lipid:protein ratio (105:1) and the greater percent of apoC-III incorporated by the former PC (95%). Second, in each of these studies, the lipid:protein mixtures, which were prepared at icewater temperatures, reached the transition temperature of DMPC (23°C) before that of DPPC (41°C). It is then not surprising that the composition of the resulting complex reflects that of the lipid that is the most fluid and reactive at lower temperatures. Particularly, this would obtain in cases where lipid is in great excess, since all of the more reactive lipid pools will bind the protein before those having a lower binding affinity.

A different interpretation is given for the binding to the microscopic mixtures. It has been reported that apoC-III binds to DMPC forming small particles with a diameter of about 80–100 Å compared with the original liposomes which, in that study, ranged in size

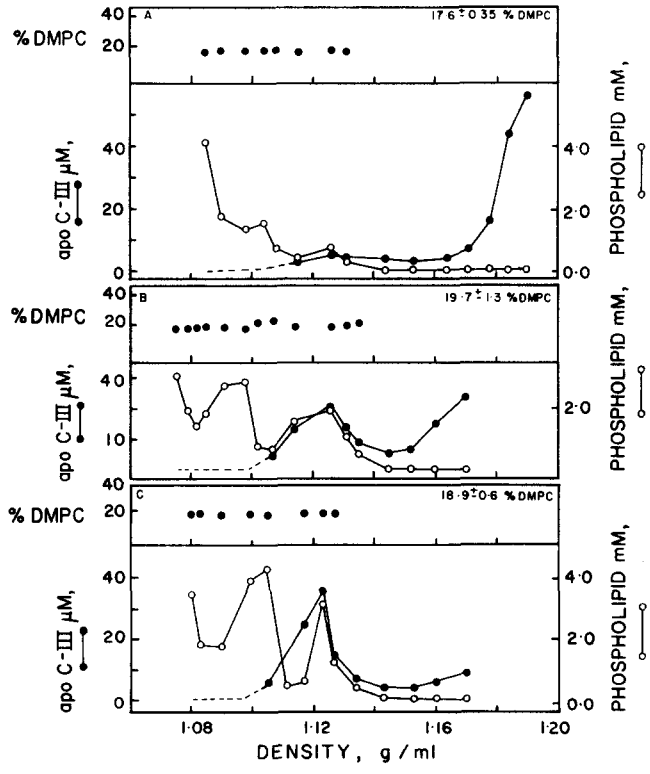


**Fig. 6.** Composition vs. density profiles obtained from the linear density gradient ultracentrifugation of apoC-III with a DMPC: DPPC 1:5 microscopic mixture as a function of temperature. The total lipid to protein ratio was 120:1. (A) 31°C; (B) 34°C; (C) 36°C. See Fig. 5 for other details.

from 250–2000 Å (20). If DMPC–DPPC mixtures undergo phase separation and form two domains, one would, on the basis of the preferential binding of apoC-III to fluid lipids, predict the formation of a lipid:protein complex whose lipid composition is the same as that of the fluid phospholipid domain. At incubation temperatures corresponding to the onset of the melting of the 1:1 microscopic mixtures, one would expect the lipid:protein complex that is formed to be composed mostly of DMPC, since the first fluid phase formed should be mostly the low melting component, DMPC. Our results, however, show that although binding did occur at the midpoint of that temperature range where acyl chain melting of phospholipids occurs (3) the lipid composition of the complex is identical to that of the lipid mixture with which the apoprotein was incubated. A similar result was observed with other microscopic mixtures of DMPC:DPPC having different DMPC:DPPC ratios. In contrast the EPR data of Shimshick and McConnell (3) showed that DMPC and DPPC undergo phase separation into fluid (liquid crystalline) and solid (gel) domains having compositions that are a function of temperature and the DMPC:DPPC ratio. The observation of a DMPC:DPPC composition in the complex identical to the starting DMPC:DPPC ratio regardless of temperature suggests that there are important molecular differences between apoC-III binding to PC's and TEMPO fluidity[2] measurements of mixed PC's.

Accepting that apoC-III preferentially binds to fluid lipids and that phase separation (3) occurs in this mixed lipid system, one might speculate upon the inferred differences between the data obtained in our studies and those of the EPR studies. First, the relative sizes of the probes used in these two studies must be considered. The fluidity of a mixed lipid system, determined by EPR methods, usually employs a small probe such as TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) (3) or di-tertiarybutyl-nitroxide (49). The utility of these two probes is derived from their partitioning between fluid lipid phases and the aqueous compartment of a mixed lipid system. A protein, on the other hand, such as apoC-III (mol wt 9300), is much larger and would not necessarily mimic the behavior of such a small probe as TEMPO. This is particularly true in instances where small (relative to apoC-III), solid and fluid phases exist as separate domains on the surface of a lipid vesicle (50). Whereas TEMPO is sufficiently small to interact with one phase and not the other, apoC-III may recognize only the average properties of an extended bilayer structure consisting of alternate fluid and solid lipid regions.

A second important consideration is the time scale on which these two measurements are made. The partitioning of TEMPO between the fluid lipid phase and the aqueous phase is on the order of $10^{-7}$ sec (51) and is at equilibrium shortly after the components of the system are mixed. On the other hand, a mixture of apoC-III and micromolecular vesicle mixture of DMPC and DPPC would be expected to react on a much slower time scale (1.00 sec).[3] During the time of this interaction between apoC-III and the vesicles, a distribution of the lipids in the fluid phase into a solid phase and a corresponding redistribution of some of the lipids in the solid phase into the liquid phase may occur such that the apoprotein only recognizes, again, a time-averaged property of the vesicle and not those of a small isolated region of the vesicle. The binding of apoC-III to the microscopic mixtures becomes maximal at the lower end of the melting curve measured by TEMPO, suggesting that only a small fraction of fluid lipid is needed to activate the binding to the entire phospholipid system including both fluid and solid phospholipid domains.

These results also show that a mixed lipid system can be highly fluid, even if only a small fraction (ca. 20%) of the total lipid present is the lower melting component of the mixture, e.g., the 1:1 DMPC:DPPC micromixture has a $T_c$ only slightly higher than that of pure DMPC (Tables 1–3). This implies that small amounts of a low melting phospholipid can have a profound effect on the physical behavior and, perhaps, biological function of a membrane or a lipoprotein.

Another important conclusion that may be drawn from the data in Table 3 is that an increase in the lipid:protein ratio in the starting incubation mixture will be reflected in the stoichiometry of the product as an increase in its lipid:protein molar ratio. In these experiments, a 50:1 molar ratio of lipids produced a product with a lipid:protein molar ratio of about 40:1. Increasing the starting lipid:protein ratio to 120:1 raised the product complex molar ratio to about 110:1. Novosad, et al. (52) have shown that this complex may have either a vesicle structure at low protein to lipid ratios (< 1:120) and a micellar structure at higher protein to lipid ratios. The latter

[2] The utility of TEMPO to measure fluidity is derived from its preferential partitioning into fluid lipid phases. By measuring the relative amounts of TEMPO dissolved in PC and the aqueous phase at various temperatures, one can measure both transition temperatures and phase separation of PC's.

[3] Pownall, H. J., J. D. Morrisett, and A. M. Gotto, Jr. Unpublished results.

type of structure has also been reported by Träuble, Middelhof, and Brown (20).

In summary, these results show that a temperature-dependent composition–structure–function relationship is involved in the binding of apoC-III to DMPC: DPPC bilayer mixtures. The generality of this relationship remains to be shown; however, such a mechanism may be important in lipoprotein formation and in the interaction of proteins with membranes.

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