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Kinetics of Lipid–Protein Interactions: Interaction of Apolipoprotein A-I from Human Plasma High Density Lipoproteins with Phosphatidylcholines†

Henry J. Pownall,*,‡ John B. Massey,§ Steven K. Kusserow, and Antonio M. Gotto, Jr.

ABSTRACT: We have studied the interaction of liposomes of dipalmitoyl- and dimyristoylphosphatidylcholine (DPPC and DMPC, respectively) with apolipoprotein A-I (apoA-I) from human plasma high density lipoproteins (HDL) by chromatography on Sepharose 4B and kinetic turbidimetric methods. The incubation and chromatography of apoA-I and highly turbid DMPC mixtures at the phospholipid gel→liquid crystalline transition temperature, T_c, revealed the complete incorporation of DMPC into a lipid–protein complex which scattered very little light. Similar experiments conducted above (30 °C) and below (22 °C) T_c showed much less complex formation even when incubated for much longer times. Over similar time periods DPPC failed to associate completely with apoA-I even at its T_c (41.5 °C). We have determined the rates of association of DPPC and DMPC with apoA-I as a function of temperature by measuring the rate of disappearance of liposomal turbidity. Below and above the T_c of DMPC, the rate of its association with apoA-I was slow, but increased by a factor of 500 to 1000 at T_c. A similar set of experiments substituting DPPC for DMPC showed very slow reaction rates even at the T_c of the former lipid. The relatively high rate of interaction of apoA-I and DMPC at T_c was assigned to the increased permeability of the DMPC matrix produced by a high percentage of boundary lipid which we viewed as a lattice defect between coexisting gel and liquid crystalline phases. The slow decrease in rate between T_c and T_c + 15 °C was assigned to the retention lattice defects composed of ordered and disordered populations of DMPC. The absence of a fast reaction between DPPC and apoA-I was probably due to its lower permeability even at its T_c. These results were suggested to be important in predicting the rate of transfer of apoA-I from HDL to phospholipids and may be important in regulating the activity of the enzyme, lecithin:cholesterol acyltransferase.

Apolipoprotein A-I (apoA-I), the major protein of the human plasma high density lipoproteins (HDL)[1] (Jackson et al., 1976), has been the subject of numerous structural studies in several laboratories. Its amino acid sequence has been reported by Baker et al. (1974). ApoA-I readily self-associates (Vitello & Scanu, 1976) and undergoes a helix→random coil transition upon heating (Tall et al., 1976; Gwynne et al., 1975), solute perturbations (Gwynne et al., 1974; Reynolds, 1976), or pH shifts (Gwynne et al., 1974, 1975). Apo-I interacts, presumably via the hydrophobic effect, with alkanes (Stone & Reynolds, 1975), lysophosphatides (Verdery & Nichols, 1974; Haberland & Reynolds, 1975), sodium dodecyl sulfate (Reynolds & Simon, 1974), and certain phosphatidylcholines. Assman & Brewer (1974) observed negligible interaction of apoA-I with egg phosphatidylcholine whereas Middelhoff et al. (1976) isolated substantial quantities of lipid–protein complexes from mixtures of apoA-I and DMPC or DPPC. However, they found that a large fraction of apoA-I did not associate with the lipids. Rosseneu et al. (1976) found a large negative enthalpy of interaction of apoA-I with DMPC and lysolecithin which they assigned to the enthalpy of binding;

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‡ H.J.P. is an Established Investigator of the American Heart Association.

§ J.B.M. is a Trainee of the National Institutes of Health, 1977–1979.

[1] Abbreviations used: PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; T_c, gel → liquid crystalline transition temperature; apoA-I, apolipoprotein A-I, the major protein of human high density lipoproteins; HDL, high density lipoprotein; Ans, 8-anilino-1-naphthalenesulfonate; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy.

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however, not all of the apoA-I was complexed with the lipid confirming earlier studies of Kruski & Scanu (1974) on apoHDL. Tall et al. (1975) have found that apoA-I thermally dissociates from HDL and that DMPC is a good acceptor of the released apoA-I (Tall & Small, 1977). However, they reported that apoA-I in the thermally unfolded state does not bind DMPC (Tall et al., 1975).

We have studied the kinetics of apoA-I association with DMPC and DPPC. These two lipids were selected since their physical properties are better understood than those of other PCs and because DMPC and DPPC exhibit gel→liquid crystalline transitions within an experimentally accessible temperature range; these transitions appear at 23.9 and 41.4 °C, respectively (Mabry & Sturtevant, 1976). The present study suggests that the rate of lipid–apoA-I interaction is determined more by the disorder within the phospholipid matrix than by changes in the protein structure.

Experimental Procedures

Materials. Human plasma high density lipoproteins (HDL) from normal subjects were isolated by the density flotation method of Scanu (1967). After removal of the KBr by dialysis and lyophilization, the HDL were delipidated at 4 °C by multiple extractions with diethyl ether:ethanol (3:1). After drying, the apoHDL (~250 mg) was solubilized in 6 M guanidine hydrochloride and applied to a 2.5 × 200 cm column of Sephadex G-150. The column was eluted with a buffer of 3.0 M guanidine hydrochloride, 0.1 M Tris, pH 8.6, 0.01% EDTA. The central portions of apoA-I fractions from two purifications were pooled, concentrated, and rechromatographed. The central portion of the apoA-I peak was combined; these fractions were devoid of other protein components as verified by amino acid analysis and the absence of reactivity of antibodies against human serum albumin, low density lipoprotein, and apoA-II. Only one band appeared in sodium dodecyl sulfate–polyacrylamide gel electrophoresis even when the gels were overloaded. Immediately prior to the kinetic experiments the required quantity of apoA-I was dissolved in 6 M guanidine hydrochloride and eluted on a Bio-Rad P-2 desalting column; this procedure provided us with a protein whose kinetic properties were highly reproducible. ^{125}I-labeled apoA-I, a gift from Dr. James Shepherd, coeluted with apoA-I on Sephadex G-150 in 3.0 M guanidine hydrochloride. This protein contained an average of 0.9 ^{125}I labels per apoA-I molecule. DMPC and DPPC were obtained from Sigma Chemical Co. and purified by high pressure liquid chromatography by Dr. James T. Sparrow on a Waters LC 500 system. ^{3}H-labeled DMPC and DPPC were synthesized from [^{3}H]myristic and [^{3}H]palmitic acids, respectively, by the method of Cubero Robles & van den Berg (1969) and were applied to a silicic acid column which was eluted with a 2–40% methanol/chloroform gradient. The phospholipid was collected in the fractions containing 35–40% methanol. These were further purified by high pressure liquid chromatography. Thin-layer chromatography of all phospholipids on silica gel plates eluted with chloroform:methanol:water (65:25:4) gave only one spot as visualized by a phospholipid specific spray (Dittmer & Lester, 1964) and by charring. Gas chromatographic analysis of the methyl esters obtained by trans-esterification of DMPC and DPPC showed the ester composition to be >99% methyl myristoate and methyl palmitate respectively. All buffer salts were obtained from Fisher Scientific.

Methods. DPPC or DMPC (20 mg) was vortexed briefly above its T_c in 1 mL of buffer composed of 8.5% KBr, 0.01% azide, 0.01% EDTA, 0.01 M Tris, pH 7.4. Addition of the KBr gave the buffer a density of 1.06 g/mL which was sufficient to prevent settling of the PC multi-bilayers in experiments which lasted as long as 24 h.[2] DMPC or DPPC (3 mL, 0.5 mg/ml) and apoA-I (2.65 mg/mL) were preincubated in quartz spectrophotometer cells (1-cm path length), in a thermostated cell compartment of a Beckman Acta V spectrophotometer for 10 min. A 0.3-mg sample of apoA-I (113 μL) was transferred to the cell containing the PC giving a PC/apoA-I ratio of 5/1 (w/w). The rate of lipid–protein association in a total volume of 3.11 mL was followed by monitoring the rate of clearing of lipid turbidity measured at 325 nm with the speetrometer operated in the absorbance mode. The rate constants were determined from a plot of log A vs. time and the $t_{1/2}$ was defined as the time required for a 50% decrease in the initial turbidity. The temperature dependence of the turbidity of these mixtures was determined by a similar method except that the precooled PC and apoA-I were mixed at 10 °C and the absorbance of the mixture measured as a function of increasing temperature (12 °C/h).

Chromatography of apoA-I, DPPC, DMPC, and DMPC/apoA-I and DPPC/apoA-I complexes over Sepharose 4B in a 90 × 1.6 cm water-jacketed column was performed with the same buffer system used in the kinetic experiments. The jacket temperature was maintained by a Lauda K/2R bath. The column was monitored by scintillation counting of [^{3}H]DMPC or [^{3}H]DPPC; apoA-I concentrations were measured by ^{125}I γ counting or intrinsic tryptophan fluorescence. A correction (~5%) was made for spillover of ^{125}I into the tritium channel.

Results

Chromatography of ApoA-I Complexes at Various Temperatures. The association of [^{3}H]DMPC and ^{125}I-labeled apoA-I was studied at several temperatures by chromatography over Sepharose 4B. Solutions of the lipid and protein (w/w = 5/1) were mixed, incubated at a given temperature for a fixed time interval, and transferred to a column at the same temperature. All of these operations were performed at the same temperature to obviate the effects of temperature changes on the rate or equilibrium of apoA-I association.

When DMPC and apoA-I were chromatographed separately their elution volumes were significantly different with the former eluting in the void volume (peak I) and the latter (peak III) appearing close to the salt peak (Figure 1A). Mixing apoA-I and DMPC can give a complex; after 5-min incubation of DMPC and apoA-I at 23.6 °C (T_c), all the DMPC coeluted (peak II) with the apoA-I at an elution volume different from those of the lipid and protein alone. A second small peak (III) of unassociated protein appeared at the same volume as that of the free protein. For short incubation intervals at 22 and 30 °C, little DMPC/apoA-I complex formation was observed. When the DMPC and the apoA-I were mixed at 22 or 30 °C and immediately eluted at 22 and 30 °C, respectively, 90% of the eluted PC appeared in the void volume and 80% of the apoA-I eluted as the free protein at the same position as peak III in Figure 1A. Similar experiments were conducted at 22 and 30 °C (Figures 1C and 1D, respectively) except that the incubation times were for 48 h instead of <5 min. At both of these temperatures we observed the elution of a complex (peak II) with a composition and elution volume similar to that of the complex show in Figure 1B. In spite of the longer incubation times at 22 and 30 °C not all of the DMPC was incorporated

[2] The kinetic experiments conducted in the absence of KBr also showed a maximal rate of binding at the T_c. This was only 25% of the rate determined in 8.5% KBr. We conducted most of the experiments in KBr solutions so that a wider temperature range could be explored. Without KBr, the liposomes settle significantly within 1 h.

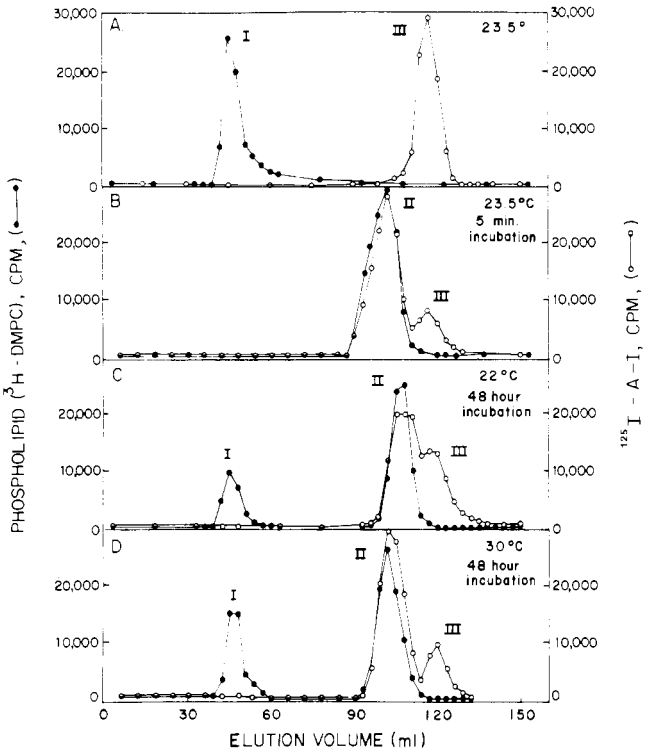

FIGURE 1: Gel filtration of (A) DMPC liposomes (3 mL of 0.5 mg/mL) or apoA-I (3 mL of 0.1 mg/mL). Each sample was run separately in a water-jacketed column at 23.5 °C. (B) DMPC–apoA-I complexes formed after 5-min incubation at 23.5 °C of DMPC (1.5 mg) and apoA-I (0.3 mg) in a final volume of 3 mL. The column was run at 23.5 °C. (C) DMPC–apoA-I complexes formed after a 48-h incubation of DMPC (1.5 mg) and apoA-I (0.3 mg) at 20 °C in a final volume of 3 mL. The column was run at 20 °C. (D) DMPC–apoA-I complexes formed after a 48-h incubation of DMPC (1.5 mg) and apoA-I (0.3 mg) at 30 °C in a final volume of 3 mL. The column was run at 30 °C. Conditions: 1.6 × 90 cm column of Sepharose 4B, 8 mL/h; 4.4 mL/fraction. DMPC and apoA-I concentrations were measured by scintillation counting of [^{3}H]DMPC and γ counting [^{125}I]apoA-I, respectively. Recovery of liposomes alone was only 50%. Recovery of PC with apoA-I complexes was >90%. Protein recovery was quantitative, ±5%.

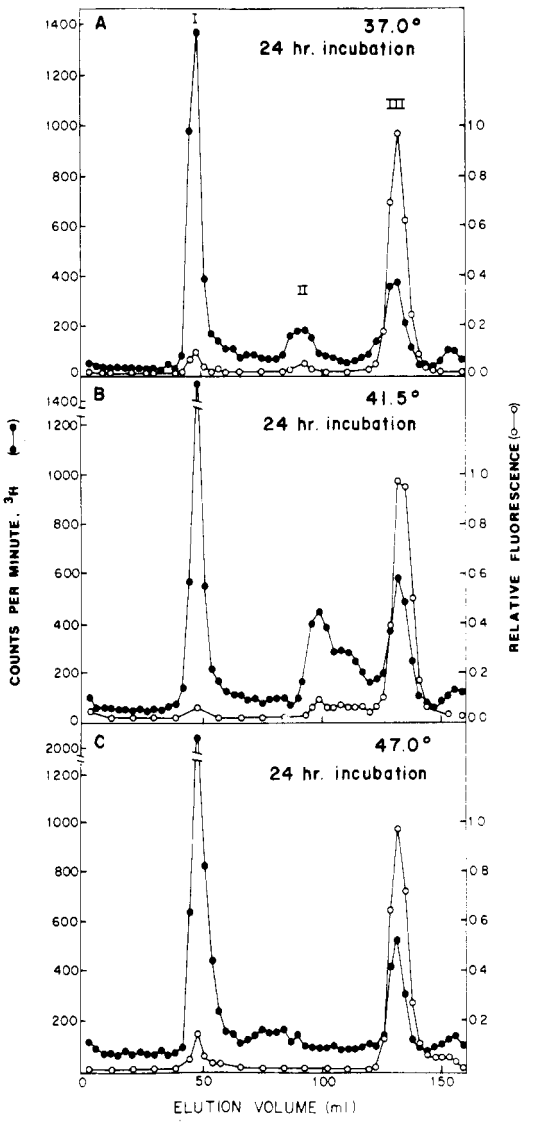

FIGURE 2: Gel filtration of apoA-I and DPPC. ApoA-I (0.3 mg) and DPPC (1.5 mg) in a final volume of 3 mL were incubated for 24 h at either 37, 42, or 47 °C. The samples were incubated and eluted at the same temperature. The elution profiles are for: (A) 37 °C; (B) 42 °C; and (C) 47 °C. Conditions: 1.6 × 90 cm Sepharose 4B, 8 mL/h. (The column used in this experiment was similar but not identical with the one used in Figure 1.) Recovery of DPPC was 40–50% with and without apoA-I. All apoA-I (±5%) was recovered.

into the lipid–protein complex; moreover, relative to the experiment at T_c, a greater amount of unassociated apoA-I eluted as a separate peak at 22 and 30 °C.

Similar experiments were conducted with DPPC and apoA-I in which the protein and lipid were mixed and incubated below (37 °C), at (41.5 °C), and above (47 °C) the T_c. The mixtures were eluted on a Sepharose 4B column which was maintained at the same temperature as the incubation. Three notable features emerged from these experiments (Figure 2). First, although the incubation was for a much longer period of time than for the same experiment conducted with apoA-I and DMPC, the majority of the lipid appeared in the void volume (peak I) coeluting with only a nominal amount of protein. This result obtained even at T_c, whereas with the corresponding DMPC–apoA-I experiment no free lipid or protein was observed. Second, at 37 and 42 °C a complex having an elution volume similar to that of the major lipid–protein complex produced by the association of apoA-I and DMPC was observed. Finally, at 47 °C no other prominent elution peaks were observed but at T_c a second complex having an elution volume between that of the major complex and that of apoA-I was observed. The amount of this complex at 47 °C was nil and that observed at 42 °C was five times that observed at 37 °C.

Temperature Dependence of Light Scattering by PC and apoA-I/PC Complexes. The light scattering properties of the PC liposomes and those of apoA-I and apoA-I/PC complexes differed greatly. The liposomes were highly turbid, but the apoA-I and apoA-I/PC complexes were clear at 325 nm. This suggested that the formation of a lipid–protein complex could be monitored by measuring the decrease in the liposome turbidity which occurred upon association of PC with apoA-I. Figure 3 shows the temperature dependence of this process. The light scattering of apoA-I mixed with DMPC or DPPC 15 °C below their respective T_c's was measured as a function of increasing temperature. As shown in Figure 3A the light scattering of the apoA-I/DMPC mixture decreased to practically nil when the transition temperature (arrow) was reached. No further change in light scattering intensity was observed up to 45 °C and as the sample was cooled to 10 °C little change in the light scattering was observed. This suggested that a thermally stable (between 10 and 45 °C) lipid–protein complex had been formed. Only a small change in the light scattering of DMPC liposomes (Figure 3B) without apoA-I present was observed over the same temperature range.

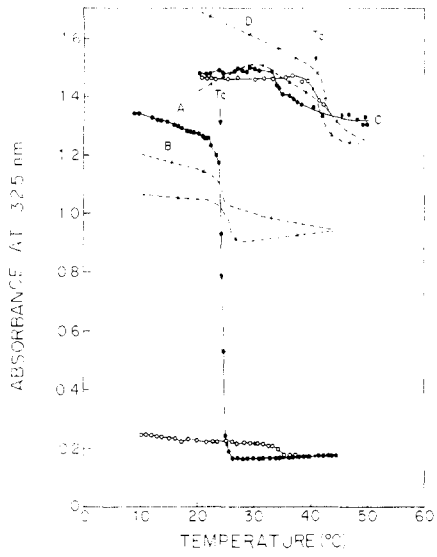

FIGURE 3: Turbidity vs. temperature profiles of apoA-I with PC. Profiles of apoA-I (0.3 mg) with phospholipid (1.5 mg) in 3 mL. The phospholipids and protein were combined at 10 °C and the turbidity at 325 nm measured as a function of linearly increasing temperature at a rate of 12 °C/h. (A) DMPC + apoA-I; (B) DMPC alone; (C) DPPC + apoA-I; (D) DPPC alone. Arrows indicate the calorimetrically determined T_c of DPPC and DMPC (Mabrey & Sturtevant, 1976).

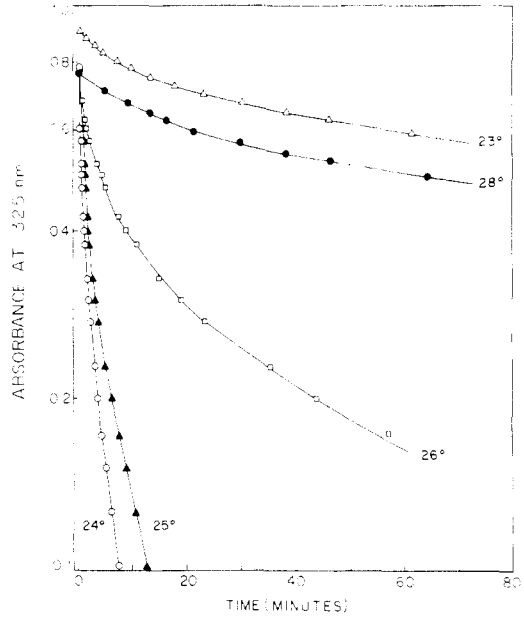

FIGURE 4: The log of DMPC turbidity at 325 nm plotted vs. time when DMPC liposomes and apoA-I were combined as described in Methods. The temperatures were (△) 23 °C; (○) 24 °C; (▲) 25 °C; (□) 26 °C; (●) 28 °C.

A similar experiment substituting DPPC for DMPC correlates with the large amount of highly turbid liposomes observed in the gel filtration experiments. At T_c there was only a modest change in the light scattering properties of this mixture (Figure 3C); this change, however, was at T_c (41.4 °C) (Mabrey & Sturtevant, 1976) and was little different from the same experiment conducted without added apoA-I (Figure 3D).

Kinetics of ApoA-I Interactions with DMPC and DPPC. We have followed the rates of association of apoA-I with phosphatidylcholines by measuring the rate of clearance of liposomal turbidity as a function of temperature. In Figure 4 we have plotted some representative curves of the absorbance

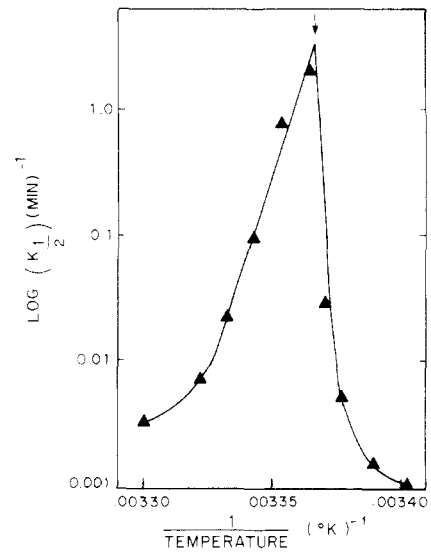

FIGURE 5: An Arrhenius plot of the temperature dependence of the rate of disappearance of turbidity. The arrow is at 23.9 °C.

at 325 nm due to DMPC liposomal turbidity as a function of time; these have been evaluated by plotting according to first-order kinetics. In each case the plots are clearly nonlinear; for the sake of comparison we have used the disappearance of the turbidity to one-half of its initial value to determine the $\tau_{1/2}$ of the reaction and define a rate constant $k_{1/2} = \tau_{1/2}^{-1}$. The temperature dependence of the rate of disappearance of turbidity is shown in Figure 5. At 20 °C the reaction was very slow and had $\tau_{1/2} \geq 1000$ min. As the temperature was raised to T_c, $\tau_{1/2}$ decreased to its minimal value of 0.5 min. Above T_c the reaction became slower until at 30 °C a $\tau_{1/2}$ of 300 min was obtained. The temperature range of rapid disappearance of liposome turbidity was less than 2 °C. We noted that the maximal rate was at the T_c of DMPC and that the temperature profile for binding was asymmetric; the rate of increase in the binding rate was much faster as the temperature approached T_c from $T < T_c$ than from $T > T_c$. These data show, by extrapolation, that the maximum binding rate was at 23.9 °C. The activation energies calculated from this plot were 290 and −2700 kcal/mol below and above T_c, respectively. We attempted similar kinetic experiments with DPPC but the much smaller changes in turbidity upon addition of apoA-I precluded quantification of the activation energies above and below T_c.

Discussion

The chromatography of apoA-I/DMPC mixtures over Sepharose 4B at different temperatures clearly establishes that the apoA-I and DMPC associate forming similar complexes at each temperature. More lipid–protein complex was formed at 23.5 °C than was formed at either 22 or 30 °C; this obtained in spite of the much longer incubation time in the latter two instances. Only at 23.5 °C did the chromatography show complete incorporation of DMPC into a lipid–protein complex. This finding is in qualitative agreement with the gel filtration data of Jonas et al. (1977) who performed similar experiments using apoA-I and DMPC single-bilayer vesicles instead of the liposomal substrate used in this study. In either instance, the chromatographic evidence suggests that the greater quantity of complex formed at T_c was due to a faster reaction rate relative to those reactions conducted at $T \neq T_c$.

We have quantified these rates by measuring the rate of disappearance of turbidity of DMPC resulting from its asso-

ciation with apoA-I. It is known that apoA-I reacts with phospholipids giving small (~75 Å radius) particles (Ritter & Scanu, 1977; Middelhoff et al., 1976; Jonas et al., 1977a,b) whose light scattering properties should be very different from those of the multilamellar liposomes which have particle radii much greater than 1000 Å. Our chromatographic data in Figure 1 clearly confirms that there are large differences in the size of DMPC liposomes and apoA-I/DMPC complexes. Other investigators have found that changes in the turbidity of DMPC liposomes are reliable for measuring the rates of DMPC melting (Tsong, 1974; Tsong & Kanehisa, 1977) and DMPC transfer to DPPC (Martin & McDonald, 1976); moreover, Tall et al. (1975) and Träuble et al. (1974) have shown that the clearing of DMPC turbidity by the addition of an apolipoprotein is characteristic of DMPC/apolipoprotein associations.

However, turbidity data must be considered with some caution; the liposome mixtures used in this and the latter studies were polydisperse and the total light scattering intensity from these mixtures represents different contributions from each size of liposome weighted according to their relative abundance. The relationship between liposome size and light scattering cross section is nonlinear so that the changes in light scattering intensity with time would not be expected to be exponential even for a first order reaction. Herein we have treated the data according to first-order kinetics only for the sake of comparing reaction rates; we do not suggest that this is the reaction order. This should not affect our interpretation in any important way.

Reaction of DMPC and ApoA-I at T_c. The rates of association of apoA-I with the gel and liquid crystalline phases of DMPC were those observed below and above T_c, respectively. The reaction at T_c is several orders of magnitude faster and, presumably, arises through the formation of a structural determinant associated with coexisting gel and liquid crystalline phases. This determinant could be either hole or channel defects in the lipid matrix at the borders of coexisting gel and liquid crystalline phases. Marsh et al. (1976) have suggested that the latter is the site of selective passive transport of Tempo-choline out of DMPC vesicles at T_c.

Reaction of DMPC with ApoA-I at $T \neq T_c$. As Figure 5 indicates the rate vs. temperature profile of DMPC–apoA-I interaction is asymmetric about T_c; at $T_c + 2$ °C the rate is still measurable but at $T_c - 2$ °C, the rate is practically nil. The gel phase reacts very slowly, perhaps due to the crystallinity of the bilayer; a stronger association of the acyl chains by an amount proportional to the enthalpy of DMPC melting should increase the activation energy for any process which requires formation of a hole through the lipid matrix. It is interesting that the rate at $T_c + 2$ °C is still appreciable; this temperature is well above the T_c and cannot be due to a defect at the border of coexisting gel and liquid crystalline phases. The gradual decrease in the rate of DMPC–apoA-I interaction at $T_c + \Delta T$ has a rate vs. temperature profile similar to that observed for permeation of DMPC by Ans (Tsong et al., 1975), Tempocholine (Marsh et al., 1976), and alkali chlorides (Blok et al., 1975). We suggest that the gradual decrease is due to the presence of a small number of clusters or "ordered" lipid molecules which do not pack perfectly with the disordered liquid crystalline phase. These clusters may be produced well above T_c as pointed out by Ubbelhode (1965) who discussed this effect in simple liquids and Lee et al. (1974) who extended the argument to phosphatidylcholines. In our model the decreased rate as $T_c + \Delta T$ increases would be due to the decrease in the number of lipid clusters and their requisite hole defects at their boundary with disordered lipid. It is thus not unexpected that a negative activation energy for DMPC/apoA-I association is observed above T_c since this would reflect the decrease in the number of reactive sites in the lipid matrix, i.e., cluster-disorder interfacial lipid defects.

Reaction of DPPC with ApoA-I. The chromatographic data indicated little apoA-I and DPPC association at all temperatures investigated. The kinetics, where measurable, also showed that the rate of apoA-I/DPPC interaction was quite slow even at T_c (41.5 °C). This decreased reactivity of DPPC relative to DMPC is probably not due to any major structural changes in apoA-I since Tall et al. (1975) have shown that the onset of the apoA-I thermal denaturation does not occur until 43 °C. Even up to the midpoint of the apoA-I denaturation (54 °C) there should be enough apoprotein (>50%) in the native conformation to interact with DPPC. We suggest that the decreased reactivity is due to the lower permeability of DPPC and the requirement for a permeable lipid substrate for binding by apoA-I. This view is supported by the data of Blok et al. (1975) who found DMPC liposomes to be highly permeable to K^+ ions at its T_c; in contrast they found only a small increase in the permeability of DPPC in the vicinity of T_c.

Biological Implications. Our results show that the association of apoA-I and DMPC is kinetically controlled; the variable results obtained by other investigators may be, in part, due to difference in the incubation temperature with respect to T_c. We found that the activation energy of the lipid–protein interaction was low enough for rapid association to occur only near T_c. In our model this corresponds to providing the maximal number of hole defects in the phospholipid matrix by introduction of molecules which create defects or by using an incubation temperature near T_c where hole defects exist at the boundaries of gel and liquid crystalline phases. Thus the rate of binding of apoA-I to phospholipids is increased under these conditions where the lipid is highly permeable. This behavior is similar to that of procine phospholipase A_2 which preferentially associates with phospholipid liposomes which are at their T_c's (op den Kamp et al., 1975).

The rate of apoA-I phospholipid association may be an important term in understanding the rate of apoA-I transfer or exchange among HDL and, perhaps, in regulating the activation of lecithin:cholesterol acyltransferase.

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Purification of Synaptic Vesicles from Elasmobranch Electric Organ and the Use of Biophysical Criteria to Demonstrate Purity†

Steven S. Carlson, John A. Wagner, and Regis B. Kelly*

ABSTRACT: We have purified cholinergic synaptic vesicles from the electric organs of two related marine elasmobranchs, *Torpedo californica* and *Narcine brasiliensis*, to a specific activity higher than had previously been obtained. We have demonstrated the homogeneity of the vesicles by biophysical criteria. The purification scheme consisted of differential centrifugation, flotation equilibrium in sucrose density gradients, and permeation chromatography on glass bead columns of average pore size 3000 Å. Our criteria for purity were that bound acetylcholine, bound nucleotide triphosphate, protein, and lipid-phosphorus behave identically when vesicles were analyzed by procedures which depend on vesicle size, density, and charge. Contaminants were not detected when vesicles were fractionated by preparative and analytical velocity sedimentation, by preparative equilibrium sedimentation using glycerol density gradients, or by electrophoresis in Ficoll density gradients. Pure synaptic vesicles, which have been purified 290-fold from the initial homogenate, contain per mg of protein: 8 μmol of acetylcholine, 3 μmol of ATP, and 7 μmol of lipid phosphorus. These procedures may be of general value in the purification of membrane vesicles.

In many secretory tissues the molecules intended for secretion are packaged inside approximately spherical membrane organelles. Release of the organelle contents is probably exocytotic and so requires fusion of the organelle membrane with the plasma membrane of the cell. Isolation of the membrane of the secretory organelle is an obvious first step in the investigation of the molecular mechanism of membrane fusion. Isolation of chromaffin granules from adrenal medulla (Johnson & Scarpa, 1976; Blaschko et al., 1956), of zymogen granules from the pancreas (Greene et al., 1963; Meldolesi et al., 1971), of secretory granules from the parotid gland (Amsterdam et al., 1971), and of synaptic vesicles from nerve terminals (Whittaker et al., 1972; Nagy et al., 1976) has been reported. In general, either electron microscopy or the absence of contaminating enzyme markers has been used to establish the purity of the final preparation.

In the purification of macromolecules or virus particles, purity is accepted when the macromolecule or particle is shown to be a single species by size, charge, and density and when the biological activity coincides with the mass of the material. We have sought to develop comparable criteria for assessing the homogeneity of organelle preparations, and to use those criteria to establish the purity of one of the above preparations, the synaptic vesicle. The criteria we have found most useful are sedimentation velocity, equilibrium centrifugation in a density gradient made of a membrane permeable substance, and mobility in an electric field. Use of these criteria in the preparation of synaptic vesicles from the electric organ of elasmobranchs showed the necessity of improving the purification procedure. Using the improved procedure we have obtained synaptic vesicles from both *Torpedo californica* and *Narcine brasili-*

† From the Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California 94143. *Received October 4, 1977.* R.B.K. was supported by the National Institutes of Health (NS 09878). J.A.W. and S.S.C. are National Institutes of Health Postdoctoral Fellows.

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