Payne, P. I., and Loening, U. E. (1970), *Biochim. Biophys. Acta 224*, 128.
Peacock, A., and Dingman, C. W. (1967), *Biochemistry 6*, 1818.
Peacock, A., and Dingman, C. W. (1968), *Biochemistry 7*, 668.
Petermann, M. L. (1964), The Physical and Chemical Properties of Ribosomes, Amsterdam, Elsevier.
Petermann, M. L., and Pavlovec, A. (1963a), *J. Biol. Chem. 238*, 318.
Petermann, M. L., and Pavlovec, A. (1963b), *J. Biol. Chem 238*, 3717.
Petermann, M. L., and Pavlovec, A. (1966), *Biochim. Biophys. Acta 114*, 264.
Spirin, A. S. (1969), *Progr. Biophys. Mol. Biol. 19*, 135.
Stevens, A. R., and Pachler, P. F. (1972), *J. Mol. Biol. 66*, 225.
Takagi, M., Inove, T., and Umemura, Y. (1971), *J. Biochem. 70*, 451.
Wunner, W. H., Bell, J., and Munro, H. N. (1966), *Biochem. J. 101*, 417.

# Interaction of an Apolipoprotein (ApoLP-Alanine) with Phosphatidylcholine†

Joel D. Morrisett,* Joshua S. K. David, Henry J. Pownall, and Antonio M. Gotto, Jr.

ABSTRACT: The apolipoprotein containing C-terminal alanine (apoLP-Ala) from very low density lipoprotein and phosphatidylcholine were used as a prototype to study lipid–protein interactions in human plasma lipoproteins. ApoLP-Ala strongly inhibited the reactivation of delipidated mitochondrial β-hydroxybutyrate dehydrogenase, an enzyme which requires phosphatidylcholine for biological activity. When apoLP-Ala was sonicated with a 100-fold molar excess of phosphatidylcholine, a lipid–protein complex resulted which could be isolated free of excess lipid by ultracentrifugal flotation in potassium bromide solution at a density of 1.063–1.21 g/ml. The complex contained an average of 38 ± 5 phosphatidylcholine molecules for every 1 apoLP-Ala molecule. Further experiments showed that ultracentrifugation on a sucrose density gradient afforded a heterogeneous population of complexes whose average stoichiometry was 46 phosphatidylcholine molecules per apoLP-Ala molecule. When a saline gradient was used, the average stoichiometry was reduced to 30:1. When the titration of apoLP-Ala with a sonicated dispersion of phosphatidylcholine was observed by circular dichroism, the calculated α-helical content of the protein increased from 22 to 54%. At maximal helicity, the average stoichiometry of the complex was about 50 phosphatidylcholine molecules to 1 apoLP-Ala molecule. When the titration experiment was monitored by intrinsic fluorescence of the tryptophan residues, the maximum at 352 nm was gradually blue-shifted until a level of about 80 phosphatidylcholine molecules to 1 apoLP-Ala molecule was reached. These studies indicate that apoLP-Ala can bind up to a saturating level of 50–80 phosphatidylcholine molecules. The binding of phosphatidylcholine induces a shift from a disordered to a helical secondary structure and shifts one or more of the three tryptophan residues from a more polar to a more hydrophobic environment. These results show that highly lipidated species of apoLP-Ala may be formed which can be partially dissociated at high salt concentrations and suggest that ionic associations of lipid and apolipoproteins may play at least a minor role in the formation of plasma lipoprotein complexes. Our experiments are discussed in terms of their relationship to possible lipid–protein interactions in membranes.

The very low density lipoproteins (VLDL)[1] of human plasma represent the major vehicle for the transport of endogenously synthesized triglycerides in blood. By weight, the VLDL particles have an approximate composition of 51% triglyceride, 20% cholesterol, 19% phospholipid, and 8% protein (Oncley and Harvie, 1969). The protein constituents of VLDL are heterogeneous (Brown *et al.*, 1969; Shore and Shore, 1969; Pearlstein and Aladjem, 1972; Albers and Scanu, 1972). There are several relatively small protein components comprising 40–50% of the total protein which have been referred to collectively as the "D peptides" (Brown *et al.*, 1970b), the "C proteins" (Alaupovic, 1971), or "fraction V" (Scanu *et al.*, 1969). The other major protein component of VLDL, comprising 40–50% by weight, is the β protein or apoLDL (Brown *et al.*, 1970b). This protein component(s) has been recently shown by Gotto *et al.* (1972) to be immunochemically identical with and indistinguishable in amino acid composition and circular dichroism (CD) from the major protein constituent of LDL. Bilheimer *et al.* (1972) have shown that the larger, triglyceride-rich VLDL particles have a relatively high ratio of "C proteins" to apoLDL. The ratio is reversed in the smaller VLDL particles. Furthermore, the "C proteins" have been found to exchange rapidly *in vitro* and *in vivo* between VLDL and HDL (Eisenberg *et al.*, 1972). It is not known at this time whether the exchange involves the transfer of phospholipid and possibly other lipids as well.

† From the Departments of Medicine and Biochemistry, The Methodist Hospital, Baylor College of Medicine, Houston, Texas 77025. *Received November 3, 1972.* This research was supported in part by Department of Health, Education, and Welfare Grants No. HL-14194 and HL-05435/34, by National Institutes of Health Contract 71-2156, and by a grant from the John A. Hartford Foundation, Inc., H. J. P. was supported by Grant No. 1162 from the Damon Runyan Foundation.

[1] Abbreviations used are: apoLP-Ala, alanine apolipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; LDL, low density lipoprotein; $NAD^+$ and NADH, oxidized and reduced nicotinamide adenine dinucleotide.

Because of the potential physiological and biological importance of the interactions between the "C proteins" and lipids, we have undertaken an investigation of the binding of phosphatidylcholine to the best characterized of the "C proteins," the one with a carboxyl-terminal alanine (Brown *et al.*, 1970a) or apoLP-Ala. This protein contains 79 amino acid residues and its amino acid sequence has been reported recently by Brewer *et al.* (1972b). The protein elutes from DEAE-cellulose in two peaks, apoLP-Ala I and -II, which contain, respectively, 1 and 2 mol of sialic acid per mol of protein (Brown *et al.*, 1970a). The lipid-free apoLP-Ala has the spectral characteristics of a disordered peptide. In a preliminary communication, Shulman *et al.* (1971) reported that apoLP-Ala can achieve a helical secondary structure in the presence of high concentrations of 2-chloroethanol and that the tryptophan residues are shifted to a more nonpolar environment under these conditions.

We now report a study of the binding of phosphatidylcholine to apoLP-Ala, using four different techniques to assess the interaction: (1) inhibition of the reactivation of defatted mitochondrial β-hydroxybutyrate dehydrogenase; (2) ultracentrifugal isolation and chemical analysis of the phospholipid–protein complexes; (3) circular dichroism; and (4) intrinsic fluorescence of the apolipoprotein during its titration with phosphatidylcholine.

## Experimental Procedures

*General Methods and Materials.* Guanidine hydrochloride was purchased from Schwarz-Mann, Trizma Base from Sigma, and all other buffer salts from Fisher. Water was doubly deionized. For amino acid analysis, proteins were hydrolyzed either in constant boiling hydrochloric acid (5.7 M) for 22 hr at 110°, or in 3 N toluenesulfonic acid with 0.2% tryptamine for 24 hr at 110° as described by Liu and Chang (1971). Amino acid analyses were carried out on a Beckman Model 117 analyzer equipped with an Infotronics Integrator and an automatic sample injection accessory. The pH was routinely determined by a Radiometer TTT2 pH meter equipped with a combined microelectrode type GK2321 C. Absorption spectroscopic measurements were done on a Beckman Acta V spectrophotometer. Centrifugation was performed in a Beckman L2-65 ultracentrifuge using a 60 Ti rotor unless stated otherwise.

*Isolation of Apolipoproteins.* Plasma was obtained from fasting subjects with types IV or V hyperlipoproteinemia by plasmapheresis. The plasma was collected in 0.01% disodium EDTA, pH 7.5, and used within 48 hr for the isolation of VLDL. After an initial centrifugation to remove chylomicrons, VLDL was isolated by ultracentrifugal flotation at 5°, dialyzed against 0.01% disodium EDTA, pH 7.5, lyophilized, and delipidated as previously described (Brown *et al.*, 1970a). In similar delipidation experiments, ether–ethanol (3:1) was replaced with chloroform–methanol (2:1). The method of delipidation did not significantly alter the findings in the subsequent experiments to be described.

*Purification of ApoLP-Ala.* The proteins of VLDL were fractionated by a modification of the procedure of Brown *et al.* (1969). A column (2.5 × 90 cm) of Sephadex G-100 was prepared and equilibrated with 0.001 M sodium decyl sulfate and 0.20 M Tris-HCl, pH 8.5. Lyophilized, lipid-free VLDL proteins (100 mg) were dissolved in a minimal volume of 0.1 M sodium decyl sulfate and 0.20 M Tris-HCl buffer at pH 8.25, and applied to the column at room temperature. The column was eluted with 0.001 M sodium decyl sulfate and 0.20 M Tris-HCl, pH 8.25, at a flow rate of approximately 20 ml/hr. An initial peak contained apoLDL and a second peak contained a mixture of the "C proteins." Tubes from the second peak were pooled, lyophilized, solubilized in 0.10 M $NH_4HCO_3$, pH 7.8, and desalted on a 2.5 × 30 cm column of Bio-Rad P-2. The desalted mixture of apolipoproteins was lyophilized, dissolved in 0.01 M Tris-HCl, pH 8.5, containing 6 M urea, and applied to a Bio-Rad Cellex-D column (1.6 × 30 cm) equilibrated with the Tris–urea buffer. The column was eluted with 0.01 M Tris-HCl, pH 8.25, and 6 M urea, containing a linear gradient of sodium chloride from 0 to 0.125 M. In a typical experiment, apoLP-Ala-I and -II were eluted at respective conductivities of 5–6 and 6–7 mmhos. The fractions associated with these separate peaks were pooled and concentrated to approximatly 15 ml with an Amicon ultrafiltration cell equipped with a UM-2 membrane. The concentrates were desalted on Bio-Rad P-2 in a 2.5 × 30 cm column equilibrated with 0.10 M $NH_4HCO_3$, pH 7.8. All chromatographic experiments were performed at 22–23°. After lyophilization the apolipoproteins were dissolved in 0.01% disodium EDTA, pH 7.5. Comparisons were made between samples stored at 4° and stored in a frozen state at −40°. A single freezing and thawing did not appear to affect significantly the results of the experiments undertaken with the apoproteins. ApoLP-Ala-I and -II each gave a single immunoprecipitin line with rabbit antibodies prepared against either apolipoprotein. Each had an amino acid composition consistent with that reported by Brown *et al.* (1970a). The sialic acid content of apoLP-Ala-I was 0.84 mol/mol of protein and that of apoLP-Ala-II was 1.48 mol/mol of protein as determined by the method of Warren (1959) after hydrolysis in 0.10 M $H_2SO_4$.

*Phospholipid Dispersions.* Phosphatidylcholine was prepared from hen egg yolks by the method of Singleton *et al.* (1965), purified further by rechromatography over Unisil (Clarkson Chemical Co.) as described by Rouser *et al.* (1963), and stored in absolute ethanol under argon at 4° until use. This material exhibited a single spot on thin-layer chromatography as visualized either by charring or with a phospholipid spray (Dittmer and Lester, 1964). An aliquot (100 mg of phospholipid) of the ethanolic stock solution was evaporated to dryness under a stream of dry argon. To the dry phospholipid was added 5 ml of 20 mM Tris-HCl buffer containing 1 mM sodium EDTA, pH 8.0. This mixture was initially dispersed on a Vortex mixer, then subjected to sonication with a Bronwill Biosonicator equipped with a microtip probe. During the period of sonication, the sample was cooled with ice water (which maintained it at a temperature of 15°) and was flushed with a stream of nitrogen. Sonication was carried out at a power setting of 35 for 15-sec bursts separated by intervals of 15 sec. The procedure was continued for a total time of 30 min. The resulting opalescent dispersion contained no degradation products of phosphatidylcholine (Hauser, 1971) as determined by thin-layer chromatography. The dispersion contained vesicles of 250–1750-Å diameter as determined by electron microscopy for preparations negatively stained with phosphotungstic acid. Before experimental use, the dispersion was centrifuged to sediment any titanium particles released from the probe during sonication. The concentration of phosphaitidylcholine was estimated from phosphorus determinations on aliquots of the dispersion by the procedure of Bartlett (1959).

*Ultracentrifugal Flotation of Phospholipid–ApoLP-Ala Complexes.* Egg lecithin dispersed by sonication in 20 mM Tris–1 mM EDTA, pH 7.4, was added to a solution of apoLP-Ala

TABLE I[a]

| *d* 1.063 Salt | *d* 1.210 Salt | *d* 1.063 Sucrose | *d* 1.210 Sucrose |
|---|---|---|---|
| 0.483 M NaCl | 1.58 M NaCl | 16% sucrose | 46% sucrose |
| 0.54 M KBr | 1.80 M KBr | 10 mM NaCl | 10 mM NaCl |

[a] All tubes contained 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA.

in the same buffer. The mixture was either incubated or sonicated and the resulting complexes were isolated by ultracentrifugal flotation. The solution containing the complex was adjusted to a density of 1.063 g/ml by the addition of solid KBr, and was spun for 16 hr in a 60 Ti rotor at 55,000 rpm. The supernatant fraction contained excess, unbound phospholipid while the infranatant contained the phospholipid–apoLP-Ala complex and any unbound protein. The infranatant was adjusted to a density of 1.210 g/ml by the addition of solid KBr and was spun for 34 hr at 55,000 rpm. The lipid–protein complex was found in the supernatant while residual unbound protein was in the infranatant. Supernatant and infranatant fractions were analyzed for phospholipid by determination of radioactivity or by the Bartlett procedure (1959). Protein was determined by the procedure of Lowry *et al.* (1951) using a bovine serum albumin standard.

*Density Gradient Centrifugation of Apolipoprotein Complexes.* Linear density gradients (ranging from $d = 1.063$ to $d = 1.210$) of sucrose or salt were prepared in 5-ml nitrocellulose tubes at 23° with a Buchler peristaltic pump and a 20-ml gradient maker. These tubes were filled simultaneously to a level of 4.6 ml. Solutions for generation of the gradients were kept at pH 7.4 and had the composition shown in Table I. After equilibration of the tubes for 24 hr at 5°, an aliquot (approximately 0.3 ml) of the solution containing the lipid–apoprotein mixture in 20 mM Tris–1 mM EDTA, pH 7.4, was applied to each one. This mixture (1.3 ml) contained 50 mg of phosphatidylcholine (23,000 cpm/mg) and 4.25 mg of apoLP-Ala-II. The protein–phospholipid mixture was subjected to sonication for 4 min at 20° before application to the top of each gradient. The tubes were spun in a Beckman SW 50.1 rotor at 50,000 rpm (234,000*g*) for 48 hr at 4°. The contents of each tube were fractionated with an ISCO fractionator using 56% sucrose to drive out the gradient. Ten fractions of 0.5 ml each were collected. Densities of each fraction were determined on a Bausch and Lomb refractometer. Each fraction was analyzed for protein by the procedure of Lowry *et al.* (1951) and for phospholipid by counting the radioactivity.

*Circular Dichroism.* Circular dichroism (CD) was measured at 23° on a Cary 61 spectropolarimeter. The instrument was calibrated with *d*-10-camphorsulfonic acid at 290 nm, assuming a CD peak of 0.31-deg ellipticity for a 0.1% aqueous solution in a cell of 1.0-cm light path. Spectra were run in duplicate on solutions whose protein concentrations varied from 0.1 to 2.0 mg/ml. The buffer solution used was 0.02 M Tris–0.001 M EDTA, pH 8.0. After each pair of duplicate spectra, a blank was run in which the protein was omitted. The mean residue ellipticity $[\theta]_\lambda$ was calculated from the equation

$$[\theta]_\lambda = \frac{\theta^\circ_\lambda MRW}{10lc}$$

The mean residue weight (*MRW*) was 111 from amino acid analysis. In the equation above, $l$ is the optical path length of the cell in centimeters, $\theta_\lambda$ is the measured ellipticity angle in degrees at wavelength $\lambda$, and $c$ is the concentration of protein in grams/milliliter. Spectra were obtained at light paths of 0.1 and 0.5 mm. The observed spectra were digitized with a Hewlett-Packard calculator Model 9100-B equipped with a 9107-A digitizer; $\theta_\lambda$ was calculated and plotted with an X–Y recorder.

*Fluorescence.* Fluorescence spectra were measured at 23° on an Aminco-Bowman spectrophotofluorimeter equipped with an off-axis ellipsoidal condensing system. The spectra were calibrated against mercury lines and were not corrected for the spectral response of the analyzing system. Quantum efficiencies were determined using tryptophan ($Q_f = 0.2$) as a reference (Teale and Weber, 1950). The emission properties of free tryptophan provide a useful comparison for the tryptophan residues of the polypeptide chain of apoLP-Ala. All spectra were obtained with excitation at 280 nm. Slit widths for the excitation and analyzing monochrometer were set at 2.0 and 1.0 mm, respectively. The fluorescence maxima were accurate to within 0.5 nm of the quoted values.

*Binding Assay Using a Lipid-Requiring Enzyme.* An assessment of the binding of phosphatidylcholine by apoLP-Ala was made with delipidated mitochondrial β-hydroxybutyrate dehydrogenase. The apodehydrogenase is catalytically active only if phosphatidylcholine is present in the assay mixture. No other phospholipid will suffice. Binding of phosphatidylcholine by apoLP-Ala renders the lipid unavailable to the apodehydrogenase with a consequent loss of enzymic activity. The apodehydrogenase was isolated from beef heart mitochondria by the procedure of Sekuzu *et al.* (1963) in the absence of detergent. Fraction 1D of this procedure was used in our assays. Sonicated phospholipid dispersions were prepared as described above. Assay conditions were modified from those described by Fleischer *et al.* (1967). ApoLP-Ala was incubated for 10 min at 38° in a reaction mixture that contained a total volume of 0.90 ml. The assay mixture contained approximately 140 μg of phosphatidylcholine, 50 μmol of Tris-HCl (pH 8.1), 0.5 μmol of EDTA, 0.3 μmol of dithiothreitol, 40 μg of bovine serum albumin, 2 μmol of $NAD^-$, and 3% ethanol (v/v). The apodehydrogenase (approximately 25 μg of protein) was then added. This mixture was incubated an additional 15 min at 38°. The assay reaction was initiated by the addition of 20 μmol of sodium DL-β-hydroxybutyrate in 0.1 ml. The reduction of $NAD^+$ to NADH was followed by monitoring the absorbance at 340 nm. A standard curve of apodehydrogenase activity *vs.* phospholipid concentration was run each day on which lipid binding experiments were performed. For the assays, the quantity of phospholipid added was calculated to give approximately 50% of the maximal activity of the preparation of β-hydroxybutyrate dehydrogenase.

## Results

*Characterization of ApoLP-Ala.* The purified preparation of apoLP-Ala-I and apoLP-Ala-II prepared as described in the experimental Methods section each had an amino acid composition consistent with published values, including the complete absence of cystine. There was a very small amount of isoleucine present (Table II). A more sensitive test for the exclusion of apoLP-serine and apoLP-glutamic acid was the failure of the apolipoprotein preparations to activate lipoprotein lipase from either human postheparin plasma or from milk (Smith, 1972).

TABLE II: Amino Acid Content of ApoLP-Ala-I and ApoLP-Ala-II.[a]

| | ApoLP-Ala-I (Exptl Values) | ApoLP-Ala-II (Exptl Values) | ApoLP-Ala Brown *et al.* (1970a) |
|---|---|---|---|
| Asp | 6.99 (7) | 6.94 (7) | 6.5 |
| Thr | 5.16 (5) | 4.79 (5) | 4.8 |
| Ser | 9.77 (11)[b] | 9.84 (11)[b] | 10.1 |
| Glu | 10.02 (10) | 9.67 (10) | 10.6 |
| Pro | 1.96 (2) | 2.02 (2) | 2.2 |
| Gly | 3.17 (3) | 3.03 (3) | 3.0 |
| Ala | 9.82 (10) | 10.01 (10) | 10.4 |
| Cys/2 | 0 (0) | 0 (0) | 0.0 |
| Val | 6.20 (6) | 5.95 (6) | 6.0 |
| Met | 1.90 (2) | 1.79 (2) | 2.0 |
| Ile | 0.13 (0) | 0.11 (0) | 0.0 |
| Leu | 4.76 (5) | 5.07 (5) | 5.3 |
| Tyr | 2.00 (2) | 1.98 (2) | 1.7 |
| Phe | 3.90 (4) | 4.13 (4) | 4.0 |
| Lys | 6.13 (6) | 6.34 (6) | 7.6 |
| His | 0.90 (1) | 0.96 (1) | 1.0 |
| Arg | 2.04 (2) | 2.04 (2) | 2.4 |
| Trp | 2.49 (3) | 2.97 (3) | 2.8 |
| Total | (79) | (79) | |

[a] Values are given in moles of amino acid per mole of apoprotein. Each value represents the average of two determinations except for the single determination for tryptophan. Hydrolysis was carried out in 6 N HCl or in 3 N toluenesulfonic acid (with tryptamine) for 24 hr at 110°. All values are uncorrected for destruction during hydrolysis. [b] When corrected for destruction during hydrolysis, this value is raised to about 11.

*Inhibition of Activation of β-Hydroxybutyrate Apodehydrogenase.* The delipidated mitochondrial enzyme, β-hydroxybutyrate apodehydrogenase, has an absolute requirement for phosphatidylcholine for reactivation. Phosphatidylcholine dispersions were preincubated for 10 min with various quantities of apoLP-Ala. Subsequent inhibition of the reactivation of apodehydrogenase was used to assess the binding of phosphatidylcholine by apoLP-Ala. Significant binding was observed at the nanomole level of apoLP-Ala (Figure 1). Other nonlipoproteins, including lysozyme and ribonuclease, did not bind phospholipid in this assay. The inhibition of the reactivation of the apodehydrogenase was dependent upon the amount of added apoLP-Ala. Small differences were seen between apoLP-Ala-I and -II. It is not presently possible to assess the significance of these differences, which may have been due to experimental error.

*Ultracentrifugal Isolation of Complexes of ApoLP-Ala and Phosphatidylcholine.* ApoLP-Ala-II (1 mg) and dispersed phosphatidylcholine (8 mg), each in 20 mM Tris–1 mM EDTA, pH 7.4, were mixed under the following conditions: (1) 37° for 2 hr without sonication; (2) sonication for 1 min at 20°; (3) sonication for 5 min at 20°; and (4) sonication for 10 min at 20°. In control experiments either (5) apoLP-Ala-II or (6) phosphatidylcholine was omitted. The lipid–protein complex was isolated as described under Experimental Procedures.

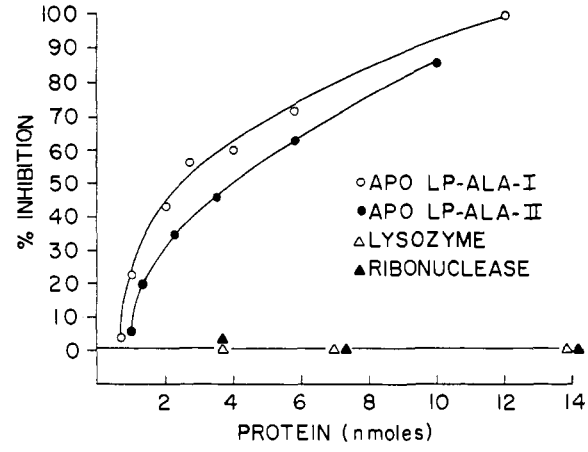


FIGURE 1: Assay for lipid-binding ability of apoLP-Ala-I and apoLP-Ala-II. The apoprotein competes with β-hydroxybutyrate dehydrogenase for the phosphatidylcholine. The enzyme exhibits a specific requirement for this lipid. Its activity is determined by measuring the increase in optical absorption of generated NADH at 340 mμ as described by Fleischer *et al.* (1967). The per cent inhibition of the enzyme gives a qualitative indication of the phospholipid-binding capacity of the apoprotein.

The average composition of the isolated complexes of apoLP-Ala and phosphatidylcholine for all four experimental conditions was 34.8 ± 3.5 (standard deviation) mol of phospholipid/mol of protein (Table III). No significant amount of phospholipid was found in the supernatant of the *d* 1.210 spin in the absence of apoLP-Ala and no protein was isolated under these conditions in the absence of phospholipid. Formation of the lipid–protein complex was not dependent upon sonication of apoLP-Ala in the presence of the previously dispersed phospholipid. The ratio of phospholipid to protein in the complex was not significantly affected by either the method of mixing or the length of sonication.

In a separate set of experiments, the ratio of the initial concentration of phosphatidylcholine to apoLP-Ala was progressively increased. Mixing was by 1-min sonication. The ratio of lipid to protein in the complex isolated ultracentrifugally between densities 1.063 and 1.210 g/ml was not strongly dependent upon the initial ratio of lipid to protein. The average molar ratio of phosphatidylcholine to apoLP-Ala was 38.8 ± 5.2 (standard deviation) (Table IV).

In order to determine if the complexes of phosphatidylcholine and apoLP-Ala were of uniform composition and to evaluate the effect of high salt concentration, separation was undertaken with density gradient centrifugation employing both sodium chloride and sucrose. The complexes were shown to be quite heterogeneous with respect to lipid and protein compositions (Table V). The ratio of phospholipid to protein varied from 17:1 to 51:1 in the sodium chloride gradient with a weighted average composition of 30:1. In the sucrose gradient, the ratio of phospholipid to protein varied from 24:1 to 76:1 with an average of 46:1. These differences were reproducible and most likely reflected a partial dissociation of the phospholipid under conditions of high salt concentration.

*Circular Dichroism Experiments.* Similar to earlier studies by Brown *et al.* (1970a) the CD spectra of apoLP-Ala showed primarily a strongly negative trough near 204 nm indicating a largely disordered conformation (Figure 2). When apoLP-Ala was reconstituted with phosphatidylcholine and isolated by ultracentrifugation in KBr, a complex was isolated with a

TABLE III: Ultracentrifugal Flotation of ApoLP-Ala-II Phosphatidylcholine Complex.[a]

| Expt No. | Tube Contents[b] | Mixing Conditions | PC[b] (mg) | Protein (mg) | mg of PC/ mg of Protein | mol of PC/ mol of Protein |
|---|---|---|---|---|---|---|
| 1 | PC + apoLP-Ala-II | Incubation 2 hr, 37° | 1.056 | 0.329 | 3.2 | 37.1 |
| 2 | PC + apoLP-Ala-II | Sonication 1 min, 20° | 0.797 | 0.300 | 2.7 | 31.3 |
| 3 | PC + apoLP-Ala-II | Sonication 5 min, 20° | 0.551 | 0.175 | 3.1 | 36.0 |
| 4 | PC + apoLP-Ala-II | Sonication 10 min, 20° | 1.051 | 0.345 | 3.0 | 34.8 |
| 5 | PC | None | 0 | 0 | | |
| 6 | apoLP-Ala-II | None | 0 | 0 | | |

[a] A sonicated dispersion of phospholipid (8 mg) was mixed with apoLP-Ala-II (1 mg) under different conditions. The complexes were isolated by ultracentrifugation and analyzed by methods described under Experimental Procedures. The composition of these isolated complexes is shown below. [b] PC, phosphatidylcholine.

protein to phospholipid average ratio of 1:35. In contrast to the spectrum of lipid-free apolipoprotein, the relipidated complex exhibited a CD spectrum with prominent negative troughs at 220 and 208 nm, characteristic of the $\alpha$-helical conformation (Figure 2).

A given quantity of apoLP-Ala-II (0.70 mg/ml in 1 mM EDTA–20 mM Tris-HCl, pH 8.0) was titrated against increasing quantities of the sonicated dispersion of phosphatidylcholine. At each step, 100 $\mu$l of the phospholipid dispersion (8.0 mg of phospholipid/ml of Tris-EDTA buffer) was added. The mixture was sonicated for 1 min at 20°. With each increment of phospholipid, there was a progressive increase in the negative ellipticity at 222 nm until a value of $-17{,}000$ deg cm$^2$/dmol was reached at an approximate molar ratio of phospholipid to protein of 54:1 (Figures 3 and 4). At the end of the experiment, where the ratio of phospholipid to protein was 64.3:1, the lipid–protein complex was isolated by ultracentrifugal flotation in KBr as described for Tables III and IV. The complex isolated between densities of 1.063 and 1.210 g/ml had a phospholipid to protein molar ratio of 35:1 and a CD spectrum indistinguishable from that of the 35:1 complex shown in Figure 2. Because of the marked increase in helicity of apoLP-Ala upon relipidation, the apolipoprotein was dissolved in 2-chloroethanol as described in the Experimental Methods section in order to determine the maximal amount of $\alpha$ helix that could be induced. In chloroethanol, apoLP-Ala-I and apoLP-Ala-II had helicities[2] of ~90 and ~85%, respectively, based on the ellipticity at 222 nm (Figure 5). The titration experiment was repeated with a buffer system containing 10 mM sodium chloride, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4, in order to make a comparison with the fluorescence experiments (described below) under identical experimental conditions. The results of these experiments were indistinguishable from those obtained from the titration experiments employing the Tris-EDTA buffer.

It was not necessary to sonicate apoLP-Ala in the presence of previously sonicated dispersions of phosphatidylcholine in order to induce an increased helicity. However, the magnitude of the increase after stirring of the protein and phospholipid dispersions at 20° for 1 min was approximately 75% of the helicity induced by sonication under the same conditions.

*Fluorescence Experiments.* The intrinsic fluorescence spectrum of apoLP-Ala-II in 10 mM sodium chloride, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4, exhibits a maximum at 350.4 ± 0.5 nm (Figure 6, curve C). When the apolipoprotein is unfolded in the presence of 4 M guanidine-HCl the maximum is red-shifted only 1.2 nm to 351.6 nm (Figure 6, curve E). These findings indicate that the three tryptophan residues of apoLP-Ala-II are highly exposed to the polar solvent since guanidine-HCl produces such a slight effect. When apoLP-Ala-II is titrated with increments of phosphatidylcholine, the fluorescence maximum is progressively shifted toward shorter wavelengths until a molar ratio of phosphatidylcholine to protein of approximately 90:1 is reached. At this ratio, the spectral maximum is 345.2 nm (Figure 6, curve B, and Figure 7). The fluorescence quantum efficiency of apoLP-Ala is reduced from 17 to 12% as a consequence of relipidation. While the fluorescence maximum of free tryptophan is not dependent on the concentration of phosphatidylcholine (Figure 6, curve D) the intensity is diminished owing to light scattering from the phospholipid dispersion. Fluorescence maxima of apoLP-Ala-II and tryptophan under varied experimental conditions are given in Table VI.

TABLE IV: Ultracentrifugal Flotation of ApoLP-Ala-II–Phosphatidylcholine Complex.[a]

| Initial Mixture | | Isolated Complex | |
|---|---|---|---|
| mg of PC[b]/ mg of ApoLP-Ala-II | mol of PC/ mol of ApoLP-Ala-II | mg of PC/ mg of ApoLP-Ala-II | mol of PC/ mol of ApoLP-Ala-II |
| 2.0 | 23.2 | 3.7 | 42.9 |
| 5.5 | 64.3 | 2.9 | 33.6 |
| 8.0 | 92.8 | 3.4 | 39.4 |
| 32.0 | 371.0 | 3.4 | 39.4 |

[a] Mixtures of various ratios of lipid to protein were sonicated 1 min. Excess component and the lipid–protein complex were each isolated by ultracentrifugation and analyzed by methods described under Experimental Procedures. [b] PC, phosphatidylcholine.

[2] The per cent helicity of the peptide was estimated from an empirical relationship derived from the CD spectra of completely random and completely helical poly-L-lysine. The equation below is similar to that used by Greenfield and Fasman (1969).

$$\% \alpha \text{ helix} = ([\theta]_{222} + 3000)/(36{,}000 + 3000).$$

TABLE V: Salt and Sucrose Density Gradient Centrifugation Experiments.[a]

| Fraction | Protein (mg/ml) | Phospholipid (mg/ml) | mol of Phospholipid/mol of ApoLP-Ala-II | Wtd Av |
|---|---|---|---|---|
| | | Salt Gradient | | |
| 1 | 0.221 | 0.889 | 51.0 | |
| 2 | 0.210 | 0.722 | 41.7 | |
| 3 | 0.234 | 0.716 | 37.2 | |
| 4 | 0.223 | 0.592 | 32.2 | 30.5 |
| 5 | 0.281 | 0.555 | 24.0 | |
| 6 | 0.322 | 0.511 | 19.3 | |
| 7 | 0.229 | 0.325 | 17.2 | |
| 8 | 0.062 | 0.154 | 2.9 | |
| 9 | 0.038 | 0.138 | 4.5 | |
| 10 | 0.008 | 0.083 | 1.2 | |
| | | Sucrose Gradient | | |
| 1 | 0.219 | 1.380 | 76.5 | |
| 2 | 0.144 | 0.679 | 57.3 | |
| 3 | 0.213 | 0.526 | 30.0 | 46.5 |
| 4 | 0.198 | 0.395 | 24.3 | |
| 5 | 0.033 | 0.180 | 6.5 | |
| 6 | 0.013 | 0.120 | 11.6 | |
| 7 | 0.009 | 0.143 | 18.0 | |
| 8 | 0.008 | 0.123 | 1.7 | |
| 9 | 0.004 | 0.116 | 3.7 | |
| 10 | 0.004 | 0.079 | 2.5 | |

[a] To the top of each gradient (4.6 ml) was applied 0.3 ml of a mixture containing dispersed phosphatidylcholine (38.4 mg/ml) and apoLP-Ala-II (3.3 mg/ml). Gradients were spun at 50,000 rpm in a Beckman SW 50.1 rotor (234,000*g*) for 48 hr. Gradients were fractionated and densities determined by refractometry. Each fraction (0.5 ml) was dialyzed to remove salt or sucrose. Dialysis was attended by some loss of unbound phospholipid also. Phospholipid was determined by counting $^{14}C$ (23,000 cpm/mg of phospholipid) and protein was estimated by the method of Lowry *et al.* (1951). Numbers represent the average of two experiments. The weighted average of moles of phospholipid per moles of apoLP-Ala-II was obtained by summing the contributions of the individual ratios to the total average. For example, the amount of complex in fraction 1 of the saline gradient can be represented by the amount of protein (0.211 mg/ml) and its fraction of the total (1.710 from fractions 1–7) is given by 0.221/1.710 = 0.123. Hence, the ratio contribution of this particular fraction to a mixture of all the complexes is given by 0.123 × 51.0 = 6.2. When each of the ratios for fractions 1–7 is treated in this manner, and these are then summed, the weighted average is obtained. This calculated number corresponds to that value one would expect to obtain if the complete, unfractionated mixture were isolated by ultracentrifugal flotation as in Tables III and IV.

## Discussion

The interaction of phosphatidylcholine and apoLP-Ala has been studied by several experimental approaches. The assay with β-hydroxybutyrate apodehydrogenase provides qualitative evidence for the binding of phosphatidylcholine by apoLP-Ala at nanomole levels of the protein. The apodehydrogenase prepared from mitochondria has an absolute requirement for phosphatidylcholine for activation. This assay procedure is particularly useful when only microgram quantities of apolipoproteins or purified peptides are available. The assay is rapid and is useful for obtaining a qualitative assessment of phospholipid binding in multiple samples. While the assay provides a qualitative indication of binding capacity, we could not use the procedure to determine the stoichiometry of the lipid–protein complex, since a quantitative relationship

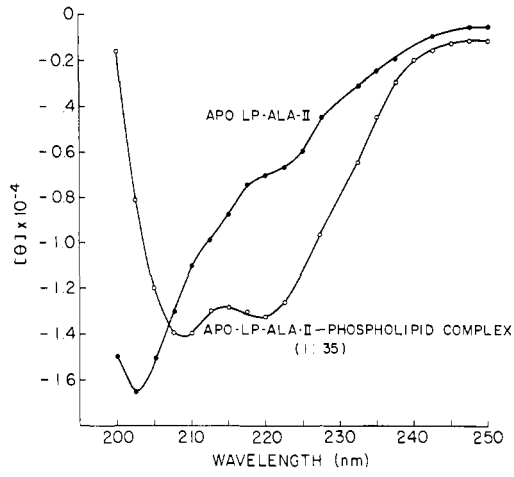


FIGURE 2: Circular dichroic spectra of apoLP-Ala-II alone and in the lipid–protein complex isolated by ultracentrifugation as described under Experimental Procedures. The mean residue ellipticity at 222 nm of the complex is the same as that observed for 34.8:1 phosphatidylcholine: apoLP-Ala-II in the titration study illustrated in Figure 3.

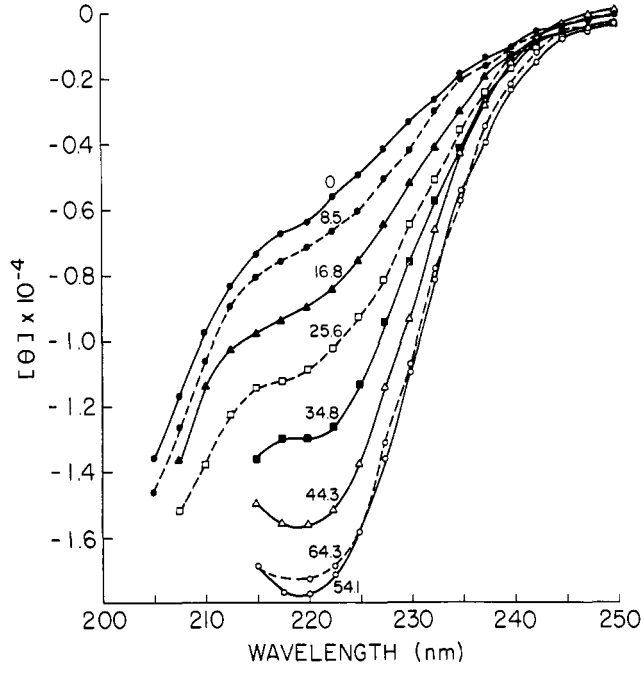


FIGURE 3: Titration of apoLP-Ala-II (0.70 mg/ml in 1 mM EDTA–20 mM Tris, pH 7.4) with a sonicated dispersion of phosphatidylcholine (same buffer) as monitored by circular dichroism. The numbers adjacent to each curve represent the molar ratio of phospholipid to apoprotein.

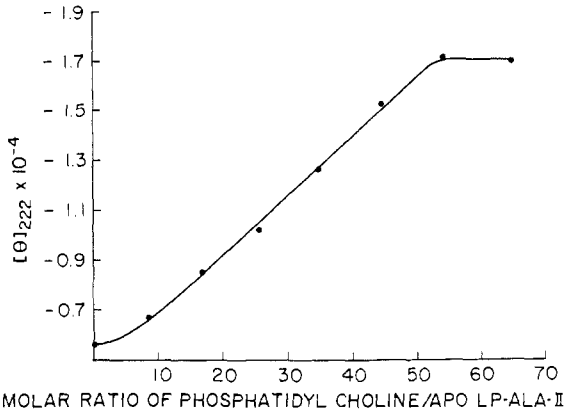


FIGURE 4: Plot of mean residue ellipticity against the molar ratio of phosphatidylcholine to apoLP-Ala-II for the titration experiment illustrated in Figure 3. Full experimental details are given in the text.

TABLE VI: Fluorescence Maxima of Tryptophan and ApoLP-Ala-II.

| Solution[d] | Fluorescence Max (nm) | Spectrum[b] |
|---|---|---|
| Trp in buffer[a] | 351.6 | C |
| Trp in buffered 4 M Gdn-HCl | 351.6 | N.S.[c] |
| Trp in buffer with 40-fold molar excess of phospholipid | 351.6 | D |
| ApoLP-Ala-II in buffer | 350.2 | A |
| ApoLP-Ala-II in buffered 4 M Gdn-HCl | 351.6 | E |
| ApoLP-Ala-II in buffer with 109-fold molar excess of phospholipid | 345.2 | B |

[a] Buffer = 1 mM EDTA–10 mM NaCl–10 mM sodium phosphate, pH 7.4. [b] Spectra are shown in Figure 6. [c] N.S. = not shown. [d] The absorption at 280 nm was 1.31 ± 0.03 for each solution.

has not been established between the degree of inhibition and the quantity of phospholipid bound to the inhibiting protein or peptide.

The results with the apodehydrogenase system were corroborated by reconstitution experiments employing ultracentrifugal flotation, the traditional method for separating and classifying the plasma lipoproteins. The occurrence of phosphatidylcholine and apoLP-Ala at a density where neither was found if the other was omitted indicates interaction of the two molecules. This interaction was also confirmed by CD and fluorescence experiments. The helicity of apoLP-Ala-II can be increased 2.5-fold (from 22 to 54%) by the addition of about 50 mol of phosphatidylcholine/mol of protein. Above this ratio, no further increase in helicity was observed. While apoLP-Ala-I reproducibly contained about 5% more helicity than did apoLP-Ala-II, each preparation increased by roughly the same percentage upon relipidation. Much higher degrees of ellipticity were obtainable in the presence of 2-chloroethanol, consistent with the previous finding of Shulman *et al.* (1971). Lux *et al.* (1972) have shown that apoHDL and its major proteins, apoLP-Gln-I and apoLP-Gln-II, also exhibit increases in helicity upon relipidation. However, the magnitude of these effects are significantly less than those observed with apoLP-Ala-II. Reconstitution with cholesteryl ester in addition to phospholipid led to a further increase in the helicity of the HDL apolipoproteins. Experiments are currently in progress to examine the effects of introducing neutral lipid to the complexes of apoLP-Ala and phosphatidylcholine. It must be emphasized that at the present time the mechanism of these effects of phospholipid on increasing the helicity of the plasma apolipoproteins is not well understood. Significantly, all of the apolipoproteins (or fragments derived therefrom) which bind phosphatidylcholine also exhibit an increase in helicity. Also, a number of apolipoproteins isolated from membranes are predominantly $\alpha$ helical.

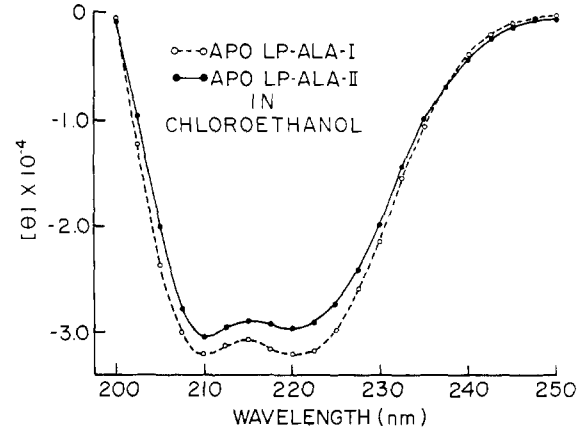


FIGURE 5: Circular dichroic spectra of apoLP-Ala-I and apoLP-Ala-II in chloroethanol.

The presence of three tryptophan residues in apoLP-Ala renders this protein amenable to study by fluorescence. The quantum yield and spectral position of the fluorescence in apoLP-Ala-II indicated that the lowest $\pi \rightarrow \pi^*$ transitions of the indole moieties are the source of this emission (Shore and Pardee, 1956). It has been shown that polar environments shift $\pi \rightarrow \pi^*$ transitions to longer wavelengths, and nonpolar or hydrophobic environments cause blue shifts to shorter wavelengths (Parker, 1968). Based on the above assignment and these empirical observations, the fluorescence data of apoLP-Ala-II can be interpreted in terms of the various polar or nonpolar perturbations to which the tryptophan residues are sensitive. The fluorescence spectrum of tryptophan in buffer (Figure 6, spectrum C) serves as a convenient point of reference. The maximum of the spectrum (351.6 nm) was not shifted by the presence of 4 M guanidine hydrochloride nor by a 40-fold molar excess of phospholipid (Figure 6 and Table VI). The spectrum of apoLP-Ala-II in buffer exhibited a maximum at 350.2 nm (Figure 6, curve A). The maximum of the protein in buffered 4 M guanidine hydrochloride was shifted only 1.4 nm to 351.6. Taken together these data suggested that the tryptophan residues of the protein are extensively exposed to the polar solvent. It is possible that there are insufficient amino acid residues in this molecule (mol wt 9600) to give a chain long enough to create a hydrophobic pocket in which the indole rings can reside. The stepwise relipidation of apoLP-Ala-II was accompanied by concomitant blue shifts in the wavelength of emission maximum. This titration experiment (Figure 7) was similar to the one monitored by CD (Figure 3). The maximum was blue-shifted a total of 5.2 nm, indicating that the interaction with phospholipids has placed the tryptophan residues in a less polar and more hydrophobic environ-

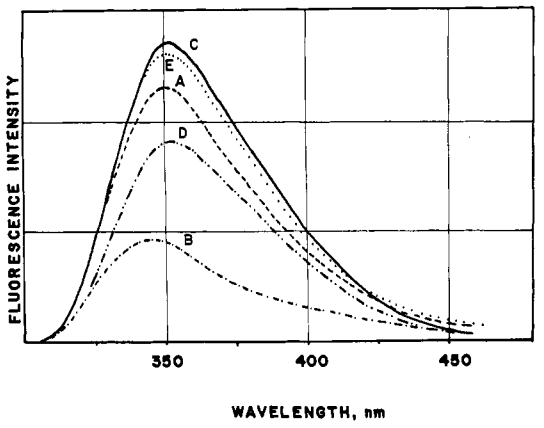


FIGURE 6: Fluorescence spectra of: tryptophan in buffer (C), apoLP-Ala-II in buffer containing 4 M guanidine hydrochloride (E), apoLP-Ala-II in buffer alone (A), tryptophan in 40-fold molar excess of phosphatidylcholine (D), apoLP-Ala-II in 109-fold molar excess of phosphatidylcholine (B). The optical density of each sample at 280 nm was 1.31 ± 0.03. The buffer was 1 mM EDTA–10 mM NaCl–10 mM sodium phosphate, pH 7.4, in all cases. The wavelength of the exciting light was 280 nm. Full details are given under Experimental Procedures.

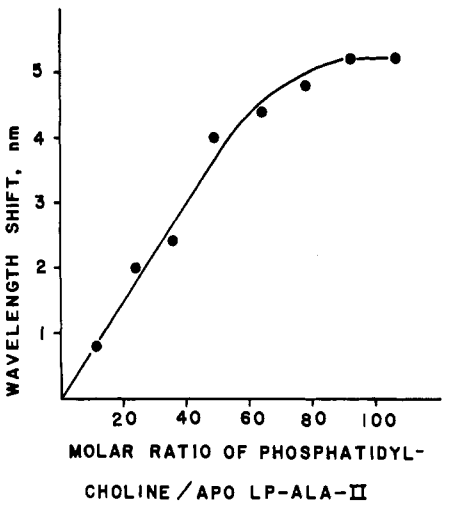


FIGURE 7: Plot of fluorescence wavelength shift against molar ratio of phosphatidylcholine to apoLP-Ala-II for the titration of the apoprotein (0.7 mg/ml) with a sonicated dispersion of egg lecithin (8 mg/ml).

ment. The effect represents a specific interaction of the protein with phospholipid since the fluorescence maximum of tryptophan as the free amino acid was not affected by the presence of phospholipid. The blue shift was linearly related to the phosphatidylcholine:apoLP-Ala-II ratio only up to a level of about 50–60:1, after which point the slope began to decrease until a ratio of about 90:1 was reached (Figure 7). By contrast, when the titration was monitored by CD the change in ellipticity leveled off sharply at about 50:1 phosphatidylcholine:apoLP-Ala-II (Figure 4). It must be borne in mind that two different properties of the protein were being assessed, one involving mainly changes in secondary structure and the other measuring primarily a tertiary structural feature, the exposure of tryptophan. It is possible that CD reflected the lipidation process only to a point where maximum helicity was induced (phosphatidylcholine:protein = 50:1) while fluorescence detected higher degrees of lipidation. Although titration curves for the relipidation experiments as monitored by CD (Figure 4) and fluorescence (Figure 7) did not plateau at the same ratio of phosphatidylcholine:apoLP-Ala-II, the curves did change slope at about the same ratio level (50–60:1). Hence the CD and fluorescence data may be mutually consistent.

*Structural Implications.* As discussed above, the results from the CD experiments show that the binding of phosphatidylcholine to apoLP-Ala is attended by a significant change from disordered structure to $\alpha$ helix. The induction of helicity in proteins by surface-active molecules has been observed previously by others. For example, Jirgensons (1961) found that detergent-treated $\gamma$-globulins, pepsin, and soybean trypsin inhibitor exhibited limited formation of $\alpha$ helices as determined by optical rotatory dispersion (ORD). It was also observed that the detergents were more active the longer their aliphatic chains, suggesting that hydrophobic binding is an important aspect of this detergent-induced helicity (Jirgensons, 1967). In a CD titration study similar to the one reported by us in this paper, Schneider and Edelhoch (1972) observed stepwise increases in the helicity of glucagon upon the addition of aliquots of lysolecithin.

The $\alpha$-helical conformation also appears to be an important factor in the interaction of the constituent lipids and proteins within the red cell membrane. Based on CD and ORD experiments, Lenard and Singer (1966) have estimated that between one-fourth and one-third of the polypeptide backbone of the membrane protein is helical. These investigators have proposed that the more nonpolar helical sections of the membrane proteins interact with the hydrophobic fatty chains while traversing the entire phospholipid bilayer. Other studies on the conformation of membrane proteins indicate that significant amounts of $\beta$ structure may also be present in the red cell membrane (Singer and Morrison, 1972).

A potential objection to the specificity of the interaction between phosphatidylcholine and apoLP-Ala in the experiments presented in this article is the relatively high level of phospholipid required to saturate the protein in the CD and fluorescence experiments. These experiments suggest that changes in secondary (tertiary) structure continue up to phospholipid:apoLP-Ala ratios of about 50:1 (90:1). These ratios are higher than those found in HDL isolated by ultracentrifugal flotation in salt solutions, in which case the molar ratio of phospholipid to protein is approximately 16:1. Nonetheless, high ratios of phospholipid to protein are required for the specific activation of membrane $Na-K^+$-ATPase by phosphatidylserine (Kimelberg and Papahadjopoulos, 1972). Approximately 3000 molecules of phosphatidylserine are required for maximal activation of one molecule of ATPase. Since the molecular weight of ATPase is approximately 300,000 while that of apoLP-Ala is less than 10,000, the quantities of phospholipid bound per residue of amino acid are quite similar in the ATPase and apoLP-Ala systems.

Our results provide evidence that highly lipidated complexes of apoLP-Ala may be formed with phospholipid which are partially dissociated by concentrated salt solutions. From our data it is not possible to determine whether the primary effect of salt is to disrupt lipid–lipid or lipid–protein interactions in the complexes. The role of ionic binding in the plasma lipoproteins has been thought to be of minor importance. In quantitative terms, this probably is a correct view since the plasma lipoproteins have traditionally been isolated by ultracentrifugal flotation in concentrated salt solutions. However,

ApoLP-Ala

```
1                5                10               15               20
Ser- Glu-Ala- Glu-Asp-Ala- Ser- Leu-Leu-Ser- Phe-Met-Gln-Gly-Tyr-Met-Lys-His- Ala-Thr-
                 25               30               35               40
Lys-Thr-Ala- Lys-Asp-Ala-Leu-Ser- Ser- Val- Gln-Ser- Gln-Gln-Val- Ala- Ala-Gln-Gln- Arg-
                 45               50               55               60
Gly-Trp-Val- Thr-Asp-Gly- Phe-Ser- Ser- Leu-Lys- Asp-Tyr-Trp-Ser- Thr- Val- Lys-Asp-Lys-
                 65               70               75
Phe-Ser -Glu-Phe-Trp-Asp- Leu-Asp-Pro- Glu-Val- Arg-Pro- Thr-Ser- Ala- Val- Ala-Ala
                                                      |
                                                     CHO
```

FIGURE 8: Amino acid sequence of the apoLP-alanine protein from apoVLDL (Brewer *et al.*, 1971).

it is known that the major apolipoprotein of the high density lipoproteins, apoLP-Gln-I, may be completely dissociated from the HDL complex by ultracentrifugation in salt solutions (Albers and Aladjem, 1971). As far as we are aware, experiments have not been done to differentiate between the effects of ultracentrifugation *per se* and that of exposure to high ionic strength. Our results clearly establish the effects of salt apart from exposure to ultracentrifugal forces. In view of this finding and because of the recent evolution of knowledge concerning the amino acid sequence of apolipoproteins, it may be worthwhile to reconsider the potential importance of ionic bonding.

Brewer *et al.* (1972a) have observed that apoLP-Ala contains three pairs of sequentially adjacent amino acids whose side chains are oppositely charged, *i.e.*, Lys-24,Asp-25, Lys-51,Asp-52, and Lys-58,Asp-59. A similar feature has been observed in apoLP-Gln-II and parathyroid hormone (Brewer *et al.*, 1972). While ionic binding may be of minor importance as compared to hydrophobic binding, ionic interactions could serve to initiate helix formation in the bipolar regions of the protein. This sequence of events might then be followed by hydrophobic binding of phospholipid to the generated helix.

A careful study of the helix-forming ability of each amino acid residue (Robson and Pain, 1972) in apoLP-Ala (see Figure 8) reveals that the N-terminal decapeptide ($Ser_1$-$Ser_{10}$) can form an $\alpha$ helix in the carboxyl-terminal direction. The sequence between this peptide and the first Lys-Asp pair is comprised primarily of amino acids which either resist helix formation or promote helix formation in the opposite direction. Similarly, the tetradecapeptide $Ala_{26}$-$Gln_{39}$ can assume helical structure in the carboxyl direction but is interrupted by a probably disordered region from Arg-40 to the next Lys-Asp pair at positions 51 and 52. The remainder of the sequence is not likely to contain significant lengths of helix, except for the C-terminal pentapeptide. Interestingly, there is a serine residue located three residues after each of the Lys-Asp pairs. Serine is the amino acid most frequently found in $\beta$ bends in ribonuclease, lysozyme, and chymotrypsin (Lewis *et al.*, 1971). The total intrinsic helicity of apoLP-Ala based on the above analysis would be about 29%. This compares to 22% as determined experimentally by CD. Clearly, phospholipid binding to the protein provides those forces necessary to increase this helicity 2.5-fold. It is possible that this increase occurs in those regions of disordered structure between the Lys-Asp pairs and the helical segments.

While we may have emphasized the potential role of the $\alpha$ helix in lipid binding in the discussion above, it is quite possible that other conformations or mixtures of conformations are also involved. Previous studies have suggested a relatively high content of $\beta$ structure in low density lipoproteins (Gotto *et al.*, 1968).

Studies on synthetic analogs by apoLP-Ala which contain substitutions at the Lys-Asp position and other regions will allow testing of the model described above. The incorporation of a spin-labeled amino acid for tyrosine or phenylalanine at a single site may permit location of the hydrophobic regions of the molecule involved in lipid binding. Experiments of this kind are currently in progress in our laboratory.

## Acknowledgments

The authors are grateful to Mrs. Diane Dukes and Mrs. Nancy Yen for technical assistance, to Mr. Richard Plumlee for recording many of the CD spectra, and to Dr. Tom Snow for developing the computer method for rapid normalization of CD data.

## References

Alaupovic, P. (1971), *Atherosclerosis 13*, 141.
Albers, J. J., and Aladjem, F. (1971), *Biochemistry 10*, 3436.
Albers, J. A., and Scanu, A. M. (1972), *Biochim. Biophys. Acta 236*, 29.
Bartlett, G. R. (1959), *J. Biol. Chem. 234*, 466.
Bilheimer, D., Eisenberg, S., and Levy, R. I. (1972), *Biochim. Biophys. Acta 260*, 212.
Brewer, H. B., Lux, S. E., Ronan, R., and John, K. M. (1972a), *Proc. Nat. Acad. Sci. U. S. 67*, 1304.
Brewer, H. B., Shulman, R., Herbert, P., Ronan, R., and Wehrly, K. (1972b), *Advan. Exp. Med. Biol. 5*, 280.
Brown, W. V., Levy, R. I., and Fredrickson, D. S. (1969), *J. Biol. Chem. 244*, 5687.
Brown, W. V., Levy, R. I., and Fredrickson, D. S. (1970a), *J. Biol. Chem. 245*, 6588.
Brown, W. V., Levy, R. I., and Fredrickson, D. S. (1970b), *Biochim. Biophys. Acta 280*, 573.
Dittmer, J. C., and Lester, R. L. (1964), *J. Lipid Res. 5*, 126.
Eisenberg, S., Bilheimer, D., Lindgren, F., and Levy, R. I. (1972), *Biochim. Biophys. Acta 260*, 329.
Fleischer, B., Sekuzu, I., and Fleischer, S. (1967), *Biochim. Biophys. Acta 147*, 552.
Gotto, A. M., Brown, W. V., Levy, R. I., Birnbaumer, M. E., and Fredrickson, D. S. (1972), *J. Clin. Invest. 51*, 1486.
Gotto, A. M., Levy, R. I., and Fredrickson, D. S. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 1436.
Greenfield, N., and Fasman, G. C. (1969), *Biochemistry 8*, 4108.
Hauser, H. O. (1971), *Biochem. Biophys. Res. Commun. 45*, 1049.
Jirgensons, B. (1961), *Arch. Biochem. Biophys. 94*, 59.
Jirgensons, B. (1967), *J. Biol. Chem. 242*, 912.

Kimelberg, H., and Papahadjopoulos, D. (1972), *Biochim. Biophys. Acta 282*, 277.
Lenard, J., and Singer, S. J. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1828.
Lewis, P. N., Momany, F. A., and Scheraga, H. A. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2293.
Liu, T. Y., and Chang, Y. H. (1971), *J. Biol. Chem. 246*, 2842.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 173*, 265.
Lux, S. E., Hirz, R., Shrager, R. I., and Gotto, A. M. (1972), *J. Biol. Chem. 247*, 2598.
Oncley, J. L., and Harvie, N. R. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 112.
Parker, C. A. (1968), Photoluminescence of Solutions, New York, N. Y., Elsevier.
Pearlstein, E., and Aladjem, F. (1972), *Biochemistry 11*, 2553.
Robson, B., and Pain, R. H. (1972), *Nature (London), New Biol. 238*, 107.
Rouser, G., Kritchersky, C., Heller, D., and Lieber, E. (1963), *J. Amer. Oil Chem. Soc. 40*, 425.
Scanu, A., Toth, T., Edelstein, C., Koga, S., and Stiller, E. (1969), *Biochemistry 8*, 3309.
Schneider, A. B., and Edelhoch, H. (1972), *J. Biol. Chem. 247*, 4992.
Sekuzu, I., Jurtshuk, P., and Green, D. E. (1963), *J. Biol. Chem. 238*, 975.
Shore, B., and Shore, V. (1969), *Biochemistry 8*, 4510.
Shore, V., and Pardee, A. (1956), *Arch. Biochem. Biophys. 60*, 100.
Singer, J. A., and Morrison, M. (1972), *Biochim. Biophys. Acta 274*, 64.
Singleton, W. S., Gray, M. S., Brown, M. L., and White, J. L. (1965), *J. Amer. Oil Chem. Soc. 42*, 53.
Shulman, R., Herbert, P., and Brewer, H. B. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 787.
Smith, L. C. (1972), *J. Lipid Res. 13*, 769.
Teale, F., and Weber, G. (1950), *Biochem. J. 65*, 476.
Warren, L. (1959), *J. Biol. Chem. 234*, 1971.

# Hybridization Studies with Nucleic Acids from Murine Myelomas. Kinetics of the Reaction and Characterization of the Hybrid†

R. G. Krueger* and K. Kosky

ABSTRACT: The ability of pulse-labeled RNA from four different murine myelomas to hybridize with DNA isolated from homologous or heterologous tumors or mouse liver in the presence of formamide was examined. RNA from a given tumor reacted maximally with its homologous tumor DNA and to lesser extents with heterologous tumor or liver DNA. At low RNA/DNA ratios, a given tumor RNA reacted more rapidly with homologous DNA as compared to heterologous tumor or liver DNA. Homologous tumor RNA–DNA hybrids formed at low RNA/DNA ratios in the presence of 50% formamide have higher thermal stabilities than heterologous hybrids formed under the same conditions and are relatively resistant to RNase. At high RNA/DNA ratios, differences in the rates of formation and thermal stability of homologous and heterologous tumor hybrids were minimized, independent of the formamide concentration, and the hybrids were susceptible to RNase. These studies suggest differences among the different myeloma DNAs, possibly in their content of specific amplified genes.

It is thought that an individual is capable of synthesizing antibodies of $10^6$ different specificities (Jerne, 1967). It has been proposed that the ability to generate antibody variability occurs during somatic development (Smithies, 1967; Brenner and Milstein, 1969; Gally and Edelman, 1970) or is the result of evolution (Dreyer *et al.*, 1967; Hood and Talmage, 1970). Basically, these theories differ as to whether each clone of antibody forming cells has one set of genes coding for the light and heavy chains of its specific antibody or whether all clones have the same information content in their DNAs for the synthesis of all possible antibody molecules but with specific derepression of one set of genes. For example, according to the latter hypothesis, each cell would have to devote approximately 0.2% of genetic material per haploid genome ($2 \times 10^4$ variable genes) to immunoglobulin synthesis (Hood and Talmage, 1970). Storb (1972) has recently estimated that there are 6–14 $\times 10^3$ immunoglobulin genes the size of the variable region of the immunoglobulin peptide per mouse spleen haploid genome.

Murine myeloma represents a homogeneous, differentiated cell population committed to the synthesis of a large amount of homogeneous protein. These tumors apparently arise as a result of a malignant alternation in a plasma cell precursor committed to the synthesis of a specific immunoglobulin. It has been estimated that the myeloma globulin represents about 40% of the total cellular protein synthesized in a $\gamma$G producing tumor (Askonas, 1961). Both somatic recombina-

† From the Department of Microbiology, University of Washington, Seattle, Washington 98105, and the Department of Microbiology and Immunology, Mayo Medical School, Mayo Foundation, Rochester, Minnesota 55901. *Received June 22, 1972.* This research was supported by Grant E546 from the American Cancer Society, and U. S. Public Health Service Grants CA-12502 and CA-13333, from the National Cancer Institute.

* Address correspondence to this author at the Mayo Foundation.