Binding of Human Serum High Density Lipoprotein Apoprotein with Aqueous Dispersions of Phospholipids*

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SUMMARY

The apoprotein separated from human serum lipoproteins of density 1.063 to 1.21 (HDL) by extraction with ethanol-ethyl ether (3:2) at -10° was shown to recombine in definite stoichiometric ratios either with the whole phospholipide of HDL or with the lecithin, phosphatidylethanolamine, and sphingomyelin fractions separated from the same lipoproteins. For this reaction, aqueous dispersions of phospholipids were obtained by sonic oscillation. HDL apoprotein maintained its binding property for phospholipids after acetylation, succinylation, reduction and alkylation, and treatment with neuraminidase, as well as in the presence of urea. The reconstituted protein-phospholipid complex acted as a substrate activator for the enzyme lipoprotein lipase obtained from chicken adipose tissue. This activity, previously reported for whole HDL, was not observed when either HDL apoprotein or phospholipids were used alone. The results are considered compatible with the concept that HDL is made of apoprotein subunits held by lipid bridges.

The human serum high density lipoprotein of density between 1.063 and 1.21 g per ml can be deprived of essentially all of its lipid complement by treatment with organic solvents at low temperature to yield an apoprotein which retains solubility in aqueous media (1-4). A similar observation has been reported for the two HDL subclasses, 1.063 to 1.125, HDL₂ (5, 6); and 1.125 to 1.210, HDL₃ (6). Apo-HDL has been shown to exhibit a high affinity for lipids (7, 8) and, when mixed with either whole serum (7, 8) or a mixture of ultracentrifugally separated serum lipoproteins (7), to associate preferentially with its own parent lipoprotein. The observation that apo-HDL may reacquire lipids in vitro is of interest since it provides a means for studying the nature of the protein-lipid interactions in HDL and for possibly reconstituting the lipoprotein from its isolated components. The present data deal with studies on the interaction between apo-HDL and phospholipids dispersed in aqueous sols by sonic oscillation. The results indicate that apo-HDL and phospholipids combine in the absence of a coupled energy source to form a complex with definite physicalchemical and functional properties. 2, 3

EXPERIMENTAL PROCEDURE

Preparation and Purification of HDL—Lipoproteins of density 1.063 to 1.21 were prepared and purified by ultracentrifugal flotation (3) from fresh (less than 24 hours at 4°) sera of healthy, fasted (18 hours) human Caucasian male donors, aged 22 to 30, Group A, Rh negative. The studies were limited to a selected group of subjects to minimize possible genetic variations. Purity of each preparation was checked by both starch gel electrophoresis (3) and immunoelectrophoresis in agar gel with the use of antiserum produced in the rabbit against whole human serum, low density lipoproteins of density 1.006 to 1.063, HDL, apo-HDL, albumin, or γ-globulin (3, 9). The preparations of HDL selected for the study were those which reacted specifically with anti-HDL sera and maintained such a specificity after removal of their lipid moiety (see below). No further purification was achieved by either gel filtration (Sephadex G-25) or addition to HDL of heparin and divalent cations known to form a complex with the low density lipoproteins (10). After the purification steps, all HDL preparations gave similar patterns on starch gel and immunoelectrophoresis.

Preparation of Apo-HDL—Apo-HDL was obtained by extraction of HDL with ethanol-ethyl ether (3:2) at -10° according to a modification (3) of the method of Scanu, Lewis and Bumpus (2). Under these conditions the final product contains less than 1% lipid and is soluble in aqueous buffers (3).

Chemical Modifications of Apo-HDL—Apo-HDL was acetylated at 0° by reaction with acetic anhydride according to Fraenkel-Conrat (12). The pH was continuously monitored by a single

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1 The abbreviations used are: HDL, serum high density lipoprotein of density between 1.063 and 1.21 g per ml; apo-HDL, protein moiety of HDL obtained by extraction with ethanol-ethyl ether (3:2). The term is used here instead of the previously proposed one, αP (2).

2 A preliminary report of these findings has appeared (SCANU, A., Circulation, 22, Suppl. 2, 20 (1965)).

glass electrode and kept below 9 by addition of 0.1 N NaOH. The acetylated specimens were dialyzed against Tris buffer, pH 8.6, ionic strength 0.1. Succinylation was conducted by reaction with succinic anhydride at 25°C according to Hass (13). During the reaction the pH was kept below 8.0 by additions of 0.1 N NaOH. The highest degree of succinylation was obtained by using a molar ratio of succinic anhydride to lysine of 60:1. Dialysis was conducted against Tris buffer, pH 8.6, ionic strength 0.1, that was 0.5 M with respect to KCl, to increase the concentration of counterions in solution. The extent of either acetylation or succinylation was assessed by the ninhydrin reaction (14) and found to be in the order of 75 to 80%.

Apo-HDL was reduced at ambient temperature (27°C) with freshly redistilled 2-mercaptoethanol (Eastman), 0.1 to 0.3 M, either in the presence of 8 M urea or after heating at 60°C for 10 min. After various time intervals (10 to 60 min), the reduced apo-HDL was alkylated with 0.1 to 0.3 M iodoacetic acid for 1 to 3 hours at 4°C. Completeness of the reaction was assessed by quantitative amino acid analysis (3) of 6 N HCl hydrolysates of the reduced and alkylated apo-HDL with the use of L-carboxymethylcysteine (Nutritional Biochemicals) as a standard.

In another series of experiments, apo-HDL was either reduced, alkylated, and acetylated, or acetylated, reduced, and then alkylated. The products were ultimately dialyzed against Tris buffer, pH 8.6, ionic strength 0.1.

The knowledge that apo-HDL contains sialic acid (3) prompted treatment of the protein with neuraminidase (Calbiochem), 20 units per milliliter of protein, on the assumption that sialic acid is terminal in the polysaccharide chain. Following enzymatic treatment, the protein was extensively dialyzed against Tris buffer, pH 8.6, ionic strength 0.1. In contrast with untreated apo-HDL, the enzyme-treated specimen had no sialic acid as determined by the method of Svennerholm (15).

In some of the experiments on lipid binding, apo-HDL was dissolved (2 to 4 mg per ml) in Tris, pH 8.6, ionic strength 0.1, made 8 M with respect to urea. Under these conditions apo-HDL undergoes marked conformational changes as indicated by optical rotatory dispersion analysis (16).

**Preparation of Aqueous Dispersion of HDL Phospholipids—**

The ethanol ethyl ether (3:2) extracts collected during the delipidation of HDL (3) were brought to dryness under N₂ and redissolved in chloroform, and the phospholipids were separated from the neutral lipids by silicic acid chromatography (3). By thin layer chromatography (3) the phospholipids were found to be made of a mixture of lecithin, sphingomyelin, and phosphatidylethanolamine in the ratio of 70:20:10; lyssolecithin was present in trace amounts. In some of the experiments lecithin, sphingomyelin, and phosphatidylethanolamine were each separated from the phospholipid mixture by silicic acid chromatography and further purified by thin layer chromatography (3). Either the phospholipid mixture or fractions were dried under N₂ and redissolved in ethyl ether; to this solution, deionized water was added to ensure a final concentration of phospholipids of 2 to 4 mg per ml. The ether was evaporated under N₂ and the aqueous suspension was subjected to sonic oscillation in a Branson Sonifier, model S-110, at 20 kc per sec at 0°C for either 10 or 60 min (see "Results"). Any precipitate was removed by centrifugation at 10,000 X g for 20 min. The aqueous sols were stored at 4°C under N₂ and used not later than 48 hours after their preparation.

**Binding of Apo-HDL with Phospholipids—**

Apo-HDL and phospholipid sols in varying weight ratios were incubated at 25°C in Tris buffer, pH 8.6, ionic strength 0.1, under N₂ for different periods of time. In some experiments the protein of HDL was labeled with 125I (6), and phospholipid sols with 14C, by mixing, before sonic dispersion, nonradioactive HDL phospholipids with purified 14C-lecithin obtained from livers of rats that had been treated by injection with 14C-choline. Following incubation the mixtures were separated by preparative ultracentrifugation (No. 10.8 rotor, 114,000 X g, 24 hours, 16°C) at d 1.063 and 1.24. These two densities were found to separate unbound phospholipids from the protein-phospholipid complex (see "Results"). In each instance six 1-ml aliquots were aspirated from top to bottom of each tube and protein, phospholipid, and specific radioactivity were determined.

Incubated mixtures of apo-HDL and phospholipid were also separated by column chromatography on Sephadex G-100 (2 X 50 cm) equilibrated with Tris buffer, pH 8.6, ionic strength 0.1, at a flow rate of 1 ml/10 min. In each fraction, the presence of protein was assessed by absorbance readings at 280 mp. Elution peaks were concentrated by ultrafiltration and analyzed for protein and lipid content by analytical ultracentrifugation and starch gel electrophoresis.

**Analytical Techniques—**

Sedimentation velocities were determined in a Spinco model E analytical ultracentrifuge, with a single sector aluminum centerpiece and 12-mm cells, at 52,460 rpm. Each specimen was run with a minimum of two dilutions to permit calculation of d₂₀,₅₀. Pictures were taken at 16-min intervals with a bar angle of 65° and enlarged in a Nikon micro comparator. Corrections for solvent density and viscosity were made according to Schachman (17). Molecular weights were determined according to the Archibald method as outlined by Schachman (17). Density measurements were made in a calibrated 5-ml pycnometer at 25°C, and viscosity was measured in a capillary Ostwald viscometer having a flow time for water of 180 sec. Values of partial specific volume, β, were obtained from the equation

$$
\beta = \frac{1}{d_2} - \frac{1}{x} \left( \frac{d - d_2}{d_0} \right)
$$

when d = density of solution, d₀ = density of solvent, and x = protein concentration in grams per ml.

The techniques of microimmunoelctrophoresis, starch gel electrophoresis, and preparation of anti-HDL sera in the rabbit were described before (3, 9). Protein was determined by the method of Lowry et al. (18). In chloroform-methanol (2:1) extracts (19), lipid phosphorus was determined by the method of Stewart and Hendry (20), total cholesterol according to Abell et al. (21), and triglycerides according to Van Handel and Zilverstre (22). Radioactive specimens were dissolved in Bray's solution (23) and counted in a Packard Tri-Carb liquid scintillation spectrometer.

Optical rotatory dispersion measurements of solutions of apo-HDL in phosphate buffer, pH 8.4, ionic strength 0.1, were carried out in a Cary model 60 spectropolarimeter between 600 and 190 mp at 27°C by the technique previously described (16). Infrared studies in the frequency range of 1800 to 1450 cm⁻¹ were conducted in a Perkin-Elmer model 21 recording spectrophotometer at 25°C. Solutions (0.1 M KCl, pH 8.6) of apo-HDL

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4 Kindly provided by Dr. Godfrey Getz, Department of Pathology, University of Chicago.
in D₂O were studied in special jacketed silver chloride cells. For solid state analysis, lipoprotein preparations which had been dialyzed against D₂O were brought to dryness under N₂ and then ground in a mortar with 300 mg of solid KBr. Pellets were made in a Carver laboratory press (Fred S. Carver, Inc., Summit, New Jersey) and analyzed immediately.

Assay for Lipoprotein Lipase—Lipoprotein lipase was prepared from commercial frozen chicken adipose tissue by the method of Korn (24) and used without further purification. Several preparations were analyzed during the course of the study because of the tendency of the enzyme to lose its activity upon standing at 4° (24). The results were reproducible with each preparation. For assay, the enzyme was dissolved in NH₄Cl buffer, pH 8.6, to a concentration of 7 to 8 mg of protein per ml. Conditions for enzyme assay were essentially those reported by Korn (24); as a substrate activator, preparations of either HDL or apo-HDL were used. All products were dialyzed against NH₄Cl buffer before use. Enzyme activity was assessed by measuring glycerol released during lipolysis of the substrate, a coconut oil emulsion (Ediol, Schenley Laboratories).

RESULTS

Properties of "Modified" Preparations of Apo-HDL

As described previously (3), apo-HDL exhibits a solubility curve characterized by formation of a gel between pH 4 and 6, partial solubility between pH 6 and 8.6, and total solubility above pH 8.6. Upon either acetylation or succinylation, apo-HDL was completely soluble between pH 7 and 11 and, in contrast to the untreated product, sedimented as a single, symmetrical component in the analytical ultracentrifuge (Fig. 1). Similarly, acetylated or succinylated apo-HDL exhibited a major, single band on agar gel and starch gel electrophoresis and gave one line of precipitation with either anti-HDL or anti

![Fig. 1](http://www.jbc.org/) Analytical ultracentrifugal patterns of apo-HDL before (A) and after acetylation (B) and succinylation (C). The buffer was Tris, pH 8.6, ionic strength 0.1; protein concentration, 4 to 5 mg per ml; speed, 52,640 rpm; 20°; bar angle, 65°. Schlieren patterns were taken at 10-min intervals after rotor reached full speed.
Fig. 2. Agar immunoelectrophoresis and starch gel electrophoretic patterns of apo-HDL before and after acetylation. Immunoelectrophoresis was performed in Veronal buffer, pH 8.6, at 6 volts per cm and 40 ma, at 27°, for 90 min; the antiserum was anti-HDL; the stain was Amido black. Starch gel electrophoresis was conducted in a discontinuous Tris-boric acid buffer, pH 8.6, at 7.3 volts per cm and 14 ma, for 20 hours, at 4°; the stain was Amido black. Letters indicate position of bands.

TABLE I

Properties of apo-HDL before and after reaction with acetic and succinic anhydride

<table>
<thead>
<tr>
<th>Material</th>
<th>Solvent</th>
<th>( r/2 )</th>
<th>Mol wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo-HDL</td>
<td>Tris, pH 8.6, ( r/2 = 0.1 )</td>
<td>2.4-12.3*</td>
<td></td>
</tr>
<tr>
<td>Acetylated apo-HDL</td>
<td>Tris, pH 8.6, ( r/2 = 0.1 )</td>
<td>2.1</td>
<td>23,400 ± 2,000*</td>
</tr>
<tr>
<td>Succinylated apo-HDL</td>
<td>Tris, pH 8.6, ( r/2 = 0.1 ), + 0.5 M KCl</td>
<td>2.0</td>
<td>24,000 ± 2,400</td>
</tr>
</tbody>
</table>

* Data refer to the two sedimenting components.
* Standard error of four determinations.

apo-HDL sera (Fig. 2). By the Archibald method, the molecular weight of acetylated apo-HDL was slightly lower than that of the succinylated product (Table I). The determined values of partial specific volume were 0.723 for apo-HDL or acetylated apo-HDL and 0.704 for the succinylated product. The molecular weight estimated from the amino acid and carbohydrate analysis (3) was 23,710.

In the presence of 8 M urea the solubility of apo-HDL in aqueous media was markedly increased. In accordance with previous results (3), it sedimented in the analytical ultracentrifuge as a single component with an \( s_{20,w} \) value of 3.4 and had a molecular weight (uncorrected) of 48,000. This product probably represents a dimer of the succinylated apo-HDL.

Based on the yields of carboxymethylcysteine, conditions which led to complete reduction and alkylation of apo-HDL were treatment of the protein with 0.1 M 2-mercaptoethanol for 1 hour at 25° in the presence of 8 M urea, followed by reaction with 0.1 M iodoacetic acid for 3 hours at 4°. Upon extensive dialysis, this material was found to be heterogeneous in the analytical ultracentrifuge, with components of \( s_{20,w} \) of 2.6 and 4.8. When reduced, alkylated apo-HDL was either acetylated or succinylated, its properties in the analytical ultracentrifuge and starch gel electrophoresis were the same as those of apo-HDL preparations directly treated with acetic or succinic anhydride.

The sialic acid-free apo-HDL had solubility and ultracentrifugal properties similar to those of untreated apo-HDL, with the exception of a slight variation in electrophoretic mobility in agar or starch gel.

Properties of Aqueous Sol of Phospholipids

In the early part of the experiments a number of factors capable of influencing the dispersion of HDL phospholipid mixtures in aqueous solution were studied: phospholipid concentration, pH and ionic strength of the medium, and length of
sonic oscillation. The clearest solutions were obtained at phospholipid concentrations between 2 and 3 mg per ml in distilled water and by sonic oscillation at 20 kc per sec for 60 min. Since, however, sonic oscillation for 10 min was equally effective in producing phospholipid sols capable of binding apo-HDL, most of the experiments were conducted with preparations after sonic treatment for 10 min. Some of the properties of the two types of phospholipid dispersion are compared in Table II. For the estimation of average particle weight by the Archibald method, the partial specific volume used, 0.862, was obtained from density measurements in 5-ml pycnometers. This value was reproducible from preparation to preparation, and on 10 repeated determinations it varied less than 1%. Preparations kept at 4°C for more than 1 week had a tendency to aggregate into larger particles. In the experiments on recombination with apo-HDL, phospholipid sols were used less than 24 hours after their preparation. Before use, they were analyzed in the analytical ultracentrifuge for the presence of a sedimenting component (Fig. 3.1). Satisfactory preparations gave an average S value of 2.1. Pure preparations of lecithin, sphingomyelin, and phosphatidylethanolamine were treated in the same way as the mixture of HDL phospholipids. Satisfactory aqueous dispersions were obtained with phospholipid concentration in water of 1 to 2 mg per ml.

Combination of Apo-HDL with Phospholipids

Separation and Characterization of Complex—In early experiments, conditions were arbitrarily chosen whereby 1 part (by weight) of apo-HDL and 2 parts (by weight) of phospholipids, both in Tris buffer, pH 8.6, ionic strength 0.1, were incubated under nitrogen at 37°C for 16 hours. The products either were unlabeled or were tagged with 32p (apo-HDL) or 3H (phospholipids). Each constituent of the mixture, studied alone, was unaffected by the conditions of the incubation. Formation of a protein-phospholipid complex was first verified by preparative ultracentrifugation on the basis of the differential density of the products centrifuged either alone or after mixing. This permitted separation of unbound from apo-HDL-bound phospholipid sols (Table III). The protein-phospholipid complex in the top milliliter of density 1.24 (obtained by addition of solid NaCl) had a weight ratio of approximately 2:1. When apo-HDL and phospholipids were incubated at varying weight ratios and the mixtures were centrifuged at d 1.063 and 118,000 x g for 48 hours at 16°C, the apo-HDL in the bottom milliliter had an increasing percentage of bound phospholipids up to a maximum of 0.5 mg per mg of protein, even in the presence of a large phospholipid excess (Table IV). The complex in the d 1.063 bottom milliliter could be floated by adjusting the density to 1.24. Only in one instance (ratio of apo-HDL to phospholipids, 10:1) was a further density increase to 1.29 found necessary for flotation.

Evidence for formation of a complex between apo-HDL and phospholipid sols was also obtained by gel filtration. As shown

![Fig. 3. Analytical ultracentrifugal patterns of an aqueous dispersion of phospholipids from HDL (A) and a phospholipid-apo-HDL complex (B). Phospholipid concentration in deionized water was 2 mg per ml. The complex was isolated by ultracentrifugal flotation at density 1.24 and contained 28% lipid by weight. Conditions for analysis were the same as in Fig. 1.](http://www.jbc.org/)

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**Table II**

Properties of aqueous dispersions of whole phospholipids from HDL

<table>
<thead>
<tr>
<th>Length of sonic oscillation</th>
<th>[a]</th>
<th>$\alpha_{w, e}$</th>
<th>Average particle weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>4.2</td>
<td>3.8</td>
<td>$3.1 \times 10^6$</td>
</tr>
<tr>
<td>60</td>
<td>3.8</td>
<td>3.2</td>
<td>$3.2 \times 10^6$</td>
</tr>
</tbody>
</table>
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The mixtures were centrifuged in a Spinco No. 40.3 rotor at 114,000 \( \times g \) for 24 hours at 16°C. The experiments were conducted with either unlabeled or labeled (\(^{32} \)P-phospholipids; \( ^{125} \)I-apo-HDL) products. After ultracentrifugation, six 1-ml aliquots were aspirated from top to bottom of each tube and analyzed for protein and phospholipid content and radioactivity.

**TABLE III**

<table>
<thead>
<tr>
<th>Products</th>
<th>Density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.006</td>
</tr>
<tr>
<td>Apo-HDL</td>
<td>S</td>
</tr>
<tr>
<td>Phospholipid sols</td>
<td>S</td>
</tr>
<tr>
<td>Apo-HDL-phospholipid complex</td>
<td>S</td>
</tr>
</tbody>
</table>

* S, sedimentation; F, flotation.

b Sedimentation was only partial.

**TABLE IV**

Recombination of apo-HDL with varying amounts of whole phospholipids from HDL

In these experiments, protein and phospholipid in the stated proportions were centrifuged at \( d = 1.063 \) (118,000 \( \times g \) for 24 hours at 16°C) and the bottom milliliter, anticipated to contain the complex, was analyzed for protein and phospholipid content.

<table>
<thead>
<tr>
<th>Products in incubation mixture</th>
<th>Amount of phospholipid in resulting complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo-HDL</td>
<td>mg/mg protein</td>
</tr>
<tr>
<td>10 mg/ml</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>0.09</td>
</tr>
<tr>
<td>10 mg/ml</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>0.20</td>
</tr>
<tr>
<td>10 mg/ml</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>0.42</td>
</tr>
<tr>
<td>10 mg/ml</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>0.48</td>
</tr>
<tr>
<td>10 mg/ml</td>
<td>20</td>
</tr>
<tr>
<td>20</td>
<td>0.48</td>
</tr>
</tbody>
</table>

* In Fig. 4, the unbound phospholipids were completely excluded, whereas the complex was eluted in the effluent volume between 110 and 150 ml. This component had a protein to phospholipid ratio of approximately 2:1. When aqueous phospholipid dispersions alone were studied, they were all recovered in the void volume. Their properties did not appear to be affected by the separation procedure.

Following the demonstration that a complex forms upon mixing apo-HDL with phospholipids, studies were carried out on the influence of incubation time, temperature, pH, and ionic strength on the reaction. No significant differences were noted when the temperature was varied between 37°C and 4°C and the pH between 7 and 10 (for pH greater than 8.6, only the partially solubilized apo-HDL was used). That formation of the complex was independent of ionic strength was indicated by the fact that it could be separated in the ultracentrifuge in media of high salt concentration (density 1.063 and 1.24). The results were not affected by changes in times of incubation between 10 min and 16 hours. However, it could not be ruled out that interactions between apo-HDL and phospholipids were operative during fractionation procedures by either preparative ultracentrifugation or gel filtration.

The reconstituted protein-phospholipid complex which was separated by ultracentrifugal flotation at density 1.24 was highly soluble in aqueous buffers at pH 7 to 11, sedimented as a single component (\( d_{20,w} = 3.6 \)) in the analytical ultracentrifuge (Fig. 3B), and reacted with either anti-HDL or anti-apo-HDL sera. The partial specific volume of a complex containing 24% lipid was 0.780, and its molecular weight was 94,300 ± 1,000. Its optical properties (optical rotatory dispersion and infrared spectra) and intrinsic viscosity were similar to those of HDL (Table V).

Apo-HDL also reacted with aqueous dispersions of either HDL lecithin, phosphatidylethanolamine, or sphingomyelin. In each case, complexes formed with a protein to phospholipid ratio in the range of that observed in systems containing whole HDL phospholipids (0.40 to 0.46 mg of phospholipid per mg of protein).

Apo-HDL maintained phospholipid binding after acetylation; succinylation; reduction and alklylation; reduction, alklylation, and succinylation; and treatment with neuraminidase. Com-
FIG. 5. Role of substrate activator on the activity of lipoprotein lipase from chicken adipose tissue. In I, the complete system contained enzyme, 0.2 ml; Ediol (25 μmoles of triglycerides per ml), 0.2 ml; HDL, 0.4 mg of protein; albumin, 10%, 0.2 ml; CaCl$_2$, 1 M, 0.02 ml; and NH$_4$Cl-NH$_3$ buffer, pH 8.6, to a final volume of 1.2 ml. In II, HDL was replaced by apo-HDL and phospholipids; in III, HDL was replaced by apo-HDL; in IV, HDL was replaced by phospholipids; in V, 0.2 ml of 1 M NaCl was added to the complete system; in VI, the complete system was used with heated enzyme (60°, 10 min). Values for I and III represent the mean of three determinations ± S.E. The other values were below the sensitivity of the method (10 μmoles per ml).

pared with untreated apo-HDL the degree of lipid binding was slightly (10 to 15%) reduced in the acetylated and succinylated products. Binding between apo-HDL and phospholipids could also be demonstrated after their incubation in media that were 4 to 8 M with respect to urea. The complex present in the top fraction of density 1.24 exhibited, after extensive dialysis to remove urea, the same properties as the one obtained by mixing untreated apo-HDL and phospholipids.

Effect of Apo-HDL-Phospholipid Complex on Activity of Chicken Fat Lipoprotein Lipase—The complete system contained enzyme (6 to 8 mg of protein per ml), 0.2 ml; Ediol (diluted to a concentration of 24 μmoles of triglycerides per ml), 0.2 ml; HDL (2 mg of protein per ml), 0.1 ml; CaCl$_2$, 1 M, 0.02 ml; bovine serum albumin, 10%, 0.2 ml; and NH$_4$Cl-NH$_3$ buffer (pH 8.4, ionic strength 0.1) to a final volume of 1.2 ml. All components of the mixture were either dissolved in or dialyzed against NH$_4$Cl-NH$_3$ buffer before use. Substrate (Ediol) and activator (HDL or HDL products) were preincubated at 37° for 30 min. As shown in Fig. 5, the presence of HDL in the system proved necessary for enzymatic activity, as did that of HDL extracted with ethyl ether according to Avigan (1). Neither apo-HDL nor the phospholipid sols alone exhibited an

**FIG. 6.** Dependence of lipolytic activity of lipoprotein lipase on concentration of apo-HDL in the system. Experimental conditions were the same as in Fig. 4. Values refer to glycerol released after 120 min of incubation. Results are the means of triplicate determinations.

“activating” property toward the substrate. This property was restored, however, when apo-HDL and phospholipids (either whole HDL phospholipids or the lecithin, phosphatidyl-ethanolamine, and sphingomyelin fractions) were incubated at 37° for 16 hours before addition to the substrate (Ediol). Similarly active was the apo-HDL-phospholipid complex isolated in the density 1.24 top fraction and extensively dialyzed against NH$_4$Cl-NH$_3$ buffer. Substrate activation by the reconstituted apo-HDL-phospholipid complex was concentration-dependent (Fig. 6). Substrate activation was also exhibited by partially saturated complexes and by protein-phospholipid complexes containing apo-HDL modified chemically (acylation, succinylation, reduction and alkylation, or treatment with neuraminidase or with 8 M urea). No lipolysis was noted when the system contained either heated (60°, 10 min) enzyme or 1 M NaCl, a known inhibitor of lipoprotein lipase (94).

**DISCUSSION**

The present data indicate that the apoprotein of human serum high density lipoprotein, deprived of most of its lipid complement by treatment with organic solvents at low temperature, recombines spontaneously with phospholipids of the parent lipoprotein to form a complex with definite physicochemical and functional properties. Crucial for the results was the preparation by sonic irradiation of phospholipid sols which could interact with HDL apoprotein in a monophasic aqueous system. The readiness of the reactions in an energy-free system strongly suggests a noncovalent nature of the protein-phospholipid bonds in HDL, a conclusion which supports previous observations by this laboratory (7, 8). The nature of the noncovalent linkage between protein and lipid cannot be clearly defined by the present

5 A preliminary account of these findings has appeared (SCANZ, A., Science, 153, 640 (1966)).
results. They do suggest, however, that nonpolar interactions are of significance, and perhaps are predominant, since protein-phospholipid binding persisted in spite of changes in surface charge of the protein (acetylation and succinylation; reduction, alkylation, and succinylation), or variations of H+ concentration and ionic strength of the medium or of the polar moeity of the phospholipids (note similarity in binding of apo-HDL for lectin, phosphatidylethanolamine, and sphingomyelin).

A most intriguing question (and this may apply to other lipoproteins as well) is why HDL apoprotein is so avid for lipids. The fact that phospholipid binding is observed in the presence of urea, a condition which causes complete unfolding of the α-helical structure of the apoprotein of HDL (16), suggests that the information to bind lipids is contained in the primary structure and may only be unravelled by the definition of the amino acid sequence of the protein. As for the carbohydrate moiety, the production of sialic acid-free apo-HDL by neuraminidase and the persistence in this product of phospholipid binding indicate that the terminal end of the polysaccharide chain is not directly involved in carbohydrate-lipid interaction, evidence which has also been obtained for human serum β-lipoprotein (25). The role of the carbohydrate moiety in HDL is not yet defined; it may be considered as contributing to the stability of the peptide backbone and perhaps to the antigenic specificity of the molecule.

It has been previously postulated on the basis of physical and chemical studies (3, 6) that HDL is made of subunits held together by lipid bridges. Such a view, illustrated in Fig. 7A, is now supported by the findings of optical rotatory dispersion and infrared spectroscopy summarized in Table V. Removal of lipids would promote association of the protein subunits, with consequent formation of polymeric forms with poor solubility in aqueous media (Fig. 7B). In turn, relipidation would favor partial (Fig. 7C) to total (Fig. 7D) disruption of protein-protein interactions and consequent dissociation of the apoprotein subunits. Such a view is also supported by the finding that addition of phospholipids to apo-HDL increased its solubility in aqueous media, decreased its intrinsic viscosity (Table V), and markedly changed its sedimentation pattern in the analytical ultracentrifuge (see Figs. 1A and 3F).

According to Lucy and Glauert (26), aqueous dispersions of phospholipids may form globular subunits. Binding of phospholipids to protein may then be the resultant of a series of interactions between subunits of protein and lipids, the reactions being dependent on the nature of the solvent medium controlling the state of dispersion of these subunits and on the relative density of polar and nonpolar groups in each of them. It is conceivable that although lipid binding fundamentally depends on the special sequence of amino acids in the peptide backbone of HDL, the quaternary structure of the lipoprotein (i.e. number and dispersion of the various apoprotein subunits) controls the overall amount of lipid in the lipoprotein. In this respect, it is of interest that the amount of phospholipids taken up by apo-HDL was close to that in native HDL even in the presence of lipid excess.

It is appreciated, however, that conclusions on lipoprotein structure based on techniques of disassembly and reassembly in vitro may not necessarily apply to native products. This is particularly true for the present observations, in which only the phospholipid moiety was used for lipid enrichment of apo-HDL.

If one, for instance, computes the number of apoprotein subunits (weight average, 24,000) contained in the apo-HDL-phospholipid complex having a molecular weight of 95,000 and a lipid content of 28% (see “Results”), the number obtained (three) is less than that previously estimated for whole HDL (see Reference 6), five to seven depending upon the HDL subclass. Although indirect and based on the assumption of a close similarity among subunits, such comparative estimates suggest that phospholipids alone are not sufficient for restoring the structural arrangement of the apoprotein subunits in HDL, and that other lipids (cholesterol and glycerides) may be contributing as well.

Studies are now in progress in which relipidation of apo-HDL is being attempted with lipid mixtures from HDL containing cholesterol and triglycerides in addition to phospholipids. It is anticipated that such studies may provide an insight into the structural arrangement of lipids in this lipoprotein. Additional information is also likely to be obtained from reaction of apo-HDL with “unnatural” phospholipids, either synthetic or from biological sources, having fatty acid esters of varying chain length and saturation. A limiting step in these studies may be that of obtaining aqueous dispersions suitable for interaction with apo-HDL.

Chemical modification with succinic anhydride has been shown to cause complete dissociation of noncovalently linked subunits in hemerythrin (27), aldolase (13), and light and heavy chains of γ-globulin (28). This has been attributed to electrostatic repulsions attributable to the replacement of the positively charged ε-NH3+ groups of lysine by negatively charged succinyl residues. The phenomenon now applies to apo-HDL, the succinylation of which produced subunits having an average molecular weight close to the minimum computed from the amino acid analysis. Similar results have been reported with acetylation (3, 6) and have been confirmed by the present experiments. The findings, however, do not provide an answer to the question whether apo-HDL is made of isomeric units or of components with different primary structures. If the second hypothesis is correct, separation of the various subunits may become a difficult task owing to their similar behavior on electrophoresis, immunoelectrophoresis, and ultracentrifugation. Studies to elucidate the problem are in progress in this laboratory.

Initially through the studies by Korn (24, 28), a characteristic lipase has been recognized which requires for activity preincubation of the substrate with serum lipoproteins. Because of this requirement, the enzyme capable of cleaving glycerides into glycerol and fatty acids has been named, perhaps improperly, lipoprotein lipase. In the present studies, the enzyme isolated from chicken adipose tissue (29) responded to the definition of lipoprotein lipase in that lipolysis was dependent on the presence in the medium of serum HDL. The enzyme system proved use-
ful in distinguishing apo-HDL (enzyme activity absent) from preparations in which the apoprotein had been relipidated either with the entire phospholipid mixture from HDL or with the lecithin or phosphatidylethanolamine fraction (enzyme activity present). The results are of significance in that they clearly establish a lipoprotein requirement for lipoprotein lipase activity and indicate that addition of phospholipids to apo-HDL restores a function which had been recognized for whole HDL. The lipoprotein lipase system can distinguish, therefore, between preparations of apo-HDL with lipid and those greatly impoverished of lipid, an observation which may prove useful in studies of serum HDL formation in biological systems. As to the mechanism of substrate “activation” by either HDL- or apo-HDL-phospholipid complex, we may only surmise that a lipoprotein is required for the interaction between the enzyme and the fatty substrate. Elucidation of this interesting problem must await preparation of lipoprotein lipase in pure form. Isolation and properties of the enzyme are the object of inquiry.

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