Human serum apolipoprotein A-I (apo-A-I), the major protein component of the human serum high density lipoproteins (HDL), was studied in aqueous solutions of differing ionic strengths and pH by the techniques of sedimentation equilibrium ultracentrifugation and frontal analysis gel chromatography. The ultracentrifugal studies indicate that apo-A-I is a self-associating system that is dependent upon protein concentration, but relatively independent of the nature of the medium. The apparent weight average molecular weights obtained from solutions of initial apo-A-I concentration between 0.2 and 0.9 mg/ml were in the range of 3.0 to 16.7 x 10^6 (monomer molecular weight = 28,014). Of the several models of self-association examined, that which gave the best theoretical fit was for the monomer-dimer-tetramer-octamer model. The self-association of apo-A-I in aqueous solutions was further documented by frontal analysis gel chromatography, which not only corroborated the ultracentrifugal results, but also indicated that the multiple species of apo-A-I in solution attain equilibrium rather rapidly.

Besides having intrinsic importance, these results indicate that the solution properties of apo-A-I must be established before ligand binding studies are conducted and interpreted.
The partial specific volume of apo-A-I, calculated from its amino acid composition (2, 3), was found to be 0.736 ml/g. Solvent densities were measured at 20° in a Met&-Paar (Hightstown, N. J.) mechanical oscillator density meter.

Frontal Elution Column Chromatography—Frontal elution chromatography was performed at 20° in a Sephadex G-75 (Superfine) column (1 x 9 cm), equilibrated, and eluted in 0.02 m EDTA, pH 8.6. The flow rate of 6 ml/hour was kept constant by means of a Chromatronix of protein in the plateau region of the elution profile equaled that of the greater than the void volume of the column, so that the concentration of protein in the plateau region of the elution profile equaled that of the sample. The apo-A-I solution was introduced by means of a Chromatronix sample injection valve, with no interruption of the flow. The elution patterns were monitored at 280 nm, and the integral elution profile was recorded directly (9). The column was calibrated against the following standards: xanthine oxidase, catalase, bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin, and ribonuclease. The exclusion volume, V,, was determined from the relation V, = V,,,,,, where R is the gas constant, T is the absolute temperature, U is the partial specific volume of the solute, ρ is the density of the solvent, and ω is the angular velocity.

\[ M_{\text{app}} = \frac{2RT}{(1 - \beta)} \frac{d \ln c}{d(r^2)} \]  

where \( M_{\text{app}} \) is the weight average molecular weight, \( M_{\text{app}} \) according to the expression

The range of \( M_{\text{app}} \), obtained from several experiments at different initial concentrations of apo-A-I and speeds, varied from about 30,000 to 167,000. The observed steady decrease of the slope, \( d \ln c/d(r^2) \), and thus of the molecular weight of apo-A-I, could be assigned to self-association, to impurities of high molecular weight, or to irreversible aggregation. The second possibility appeared to be ruled out by the results of polyacrylamide gel electrophoresis in the presence of Na dodecyl-SO₄, which showed no high molecular weight components. The presence of an irreversible aggregate could also be ruled out by the observation that a decrease in protein concentration or ultracentrifugal speed, or both, was accompanied by changes in molecular weight distribution toward lower limiting values, indicating the existence of an equilibrium among the interacting species of apo-A-I in solution. Furthermore, the results were not influenced by the life history of the sample, particularly whether or not it had been subjected to lyophilization or concentration by dialysis. Thus it became apparent that the ultracentrifugal behavior observed with solutions of apo-A-I was due to self-association of the protein.

In order to determine whether the self-association of apo-A-I was influenced by the nature of the solvent, we also carried out sedimentation equilibrium studies on apo-A-I dissolved in 0.02 m EDTA, pH 10.1. No differences in the aggregation patterns were observed at this higher pH. Additional experiments were conducted in 0.01 m sodium phosphate (pH 7.1, \( \mu = 0.024 \)) and 0.15 m KCl/0.01 m Tris (pH 8.6, \( \mu = 0.10 \)). Again, the behavior of apo-A-I was similar to that observed in 0.02 m EDTA, pH 8.6.

Since most of the studies were carried out in 0.02 m EDTA, pH 8.6, the following treatment of the results pertains to this aqueous system. It should be noted that the monomer molecular weight of apo-A-I is 28,014, by sequence data (4), and that similar values can be obtained by several experimental methods in the presence of dissociating agents such as sodium dodecyl sulfate, urea, or guanidinium hydrochloride (2, 3).

The concentration dependence of the apparent weight average molecular weight, \( M_{\text{app}} \), of apo-A-I in 0.02 m EDTA, pH 8.6, is shown in Fig. 2, where each point represents an average of at least three determinations. One of the characteristics of this plot is the very fast increase in \( M_{\text{app}} \), as a function of concentration, indicating a strong self-association.

The treatment of self-associating systems requires several basic assumptions (12-15): (a) the refractive index increment, dn/dc, is the same for all associating species; (b) the partial specific volume, \( \bar{\epsilon} \), of all associating species is equal; and (c) the logarithm of the activity coefficient, \( y_i \), for each species is ln \( y_i = iBM/c \), where \( i \) is the number of species in solution, \( B \) is the second virial coefficient, higher order terms in \( c \) are negligible, and \( M_1 \) is the molecular weight of the monomer.

The concentration dependence of the apparent weight average molecular weight is given by

\[ M_1 \frac{M_1}{M_{\text{app}}} = \frac{M_1}{M_1 + BM/c} \]  

where \( M_1 \) is the molecular weight of the monomer and \( M_1 \) is the weight average molecular weight corrected for the nonideal term defined as

\[ M_1 = \sum c_i M_i/c_i \]  

The total concentration of an associating system can be expressed in terms of the sum of the concentrations of each species.
Fig. 2. Dependence of the apparent weight average molecular weight on concentration for apo A-I in 0.02 M EDTA, pH 8.6. Filled circles are experimental values. The smooth curve was obtained from the calculated values for the monomer-dimer-tetramer-octamer model (see text for details).

The concentration of the monomer is given by

\[ c = \sum c_i. \]  

(4)

The value of the apparent number average molecular weight \( M_{\text{app}} \), which includes data obtained at several initial protein concentrations and at various ultracentrifugal speeds. Several theoretical models are outlined below.

**Monomer-n-mer Associations**—Associations of this type may be represented by

\[ nP \leftrightarrow P_n, \]

where the association constants on a weight per volume basis are given by

\[ K = \frac{c_n}{(c_i)^n}. \]  

(8)

On the basis of the value of the apparent weight average molecular weight at the highest concentration, 167,000, the degree of association must be at least 6. We attempted to fit the data to several degrees of association, \( n = 6, 7, \) or 8. By proper combination of the relationships given, an equation was obtained in which the weight average molecular weight is expressed as a function of the concentration of the monomer, the equilibrium constant, and the nonideal term

\[ \frac{1}{c_{M_{\text{app}}}} = \alpha \exp(-BM,c) + nK_n\left[\alpha \exp(-BM,c)\right]^n. \]  

(9)

Of the two unknowns, \( K_n \) and \( BM \), the former is eliminated if Equation 4 is written for two species, monomer and n-mer, and then combined with Equations 5 and 8 to give:

\[ n c = (n - 1)\alpha \exp(-BM,c) + \frac{1}{c_{M_{\text{app}}}} - BM. \]  

(10)

By solving Equation 10 for \( BM \), we found that, in all three cases \( (n = 6, 7, \) or 8), \( BM \) decreased systematically as the protein concentration increased. The average values and their standard deviations were 1.2 ± 2.0 \times 10^2 ml/g for \( n = 6, 1.6 ± 2.4 \times 10^2 ml/g for n = 7, \) and 1.6 ± 2.8 \times 10^2 ml/g for \( n = 8 ), indicating that \( BM \) was equal to zero within experimental error in all three cases. The values of \( K_n \) were obtained from Equation 9.

We used a nonlinear least squares analysis to obtain the best fit of the experimental data to the given association model. The values of \( K_n \) and the sum of the squares of the deviations are given in Table I. In Fig. 3, the experimental values of \( M_{\text{app}}/M_{\text{app}} \) are compared to those found by use of the calculated \( K_n \). Table II lists the experimental and calculated values of \( M_{\text{app}}/M_{\text{app}} \) for the monomer-heptamer system. From these results it is apparent that the aggregation of apo-A-I does not follow the models shown in Fig. 3 and may require intermediate steps in the association in order to fit the experimental results.

**Indefinite Self-association**—This solute-solute interaction may be described by associations of the type

\[ 2P \leftrightarrow P_2, K_{2P} = \frac{[P]}{[P]}; P_2 \leftrightarrow P_3, K_{3P} = \frac{[P]}{[P]}[P]; \text{etc}. \]

which indicate that the self-associations continue without limit. By setting the equilibrium constants in this system equal to each other and by the use of concentration units in grams per ml, we can, according to Adams et al. (13–16), define the intrinsic equilibrium constant, \( k \), as 1000 \( K/M \), which is the value of \( M_{\text{app}} \), being obtained from the area of the plot \( M_{\text{app}}/M_{\text{app}} \) versus \( c \) and the number average molecular weight from the relation \( M_{\text{app}} = c\sum c_i/M_i \). Quantities of the form \( M_{\text{app}}^n \) \( \sum c_i/M_i \) have been developed (13, 15) and are also used in the analysis of associating systems.

The concentration dependence of the apparent weight average molecular weight of apo-A-I in 0.02 M EDTA, pH 8.6, is shown in Fig. 2, which includes data obtained at several initial protein concentrations and at various ultracentrifugal speeds. Several theoretical models are outlined below.

**Monomer-dimer Association**—Associations of this type may be represented by

\[ nP \leftrightarrow P_n, \]

where the association constants on a weight per volume basis are given by

\[ K = \frac{c_n}{(c_i)^n}. \]  

Solving Equation 13 for \( BM \), at each protein concentration by successive approximation, we found the value of \( BM \) for

\[ \frac{M_{\text{app}}}{M_{\text{app}}} = \frac{1}{c_{M_{\text{app}}} - BM,c} \]  

(13)
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TABLE I

<table>
<thead>
<tr>
<th>$K_n$</th>
<th>$n = 1, 6$</th>
<th>$n = 1, 3, 6$</th>
<th>$n = 1, 2, 4, 6$</th>
<th>$n = 1, 7$</th>
<th>$n = 1, 8$</th>
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<th>$n = 1, 4, 8$</th>
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<tr>
<td>$K_1$</td>
<td>$1.6 \pm 0.5$</td>
<td>$4.0 \pm 0.1$</td>
<td>$3.3 \pm 0.2$</td>
<td>$3.3 \pm 0.2$</td>
<td>$3.3 \pm 0.2$</td>
<td>$3.3 \pm 0.2$</td>
<td>$3.3 \pm 0.2$</td>
<td>$3.3 \pm 0.2$</td>
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</tr>
<tr>
<td>$K_2$</td>
<td>$(8.4 \pm 3.4) \times 10^3$</td>
<td>$(1.5 \pm 0.2) \times 10^9$</td>
<td>$(1.5 \pm 0.2) \times 10^9$</td>
<td>$(1.5 \pm 0.2) \times 10^9$</td>
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<td>$(1.5 \pm 0.2) \times 10^9$</td>
</tr>
<tr>
<td>$K_3$</td>
<td>$(9.2 \pm 0.3) \times 10^2$</td>
<td>$(8.4 \pm 0.3) \times 10^2$</td>
<td>$(8.4 \pm 0.3) \times 10^2$</td>
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</tr>
<tr>
<td>$K_4$</td>
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<tr>
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<tr>
<td>$K_6$</td>
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<td>$(9.2 \pm 0.3) \times 10^2$</td>
<td>$(9.2 \pm 0.3) \times 10^2$</td>
</tr>
</tbody>
</table>

$$\sum \delta^{10} = 5.701 \quad 4.916 \quad 3.928 \quad 9.035 \quad 12.717 \quad 0.206 \quad 1.736 \quad 0.072 \quad 0.072 \quad 0.077$$

$n$ designates a given n-mer (i.e., $n = 1, 2, 4, 6$, monomer, dimer, tetramer, hexamer).

*Sum of the squares of the deviations.

---

apo-A-I to be $-3.6 \pm 1.2 \times 10^{-1}$ ml/g. The corresponding value of $k$ was evaluated according to Equation 13

$$\frac{M_1}{M_{app}} = \frac{1 - k c_1 + BMc}{2} \quad (14)$$

and found to be $13.85 \times 10^9$ ml/g. The ratio of $M_1/M_{app}$ for the indefinite self-association is calculated from

$$\frac{M_1}{M_{app}} = \frac{1 - k c_1}{1 + k c_1} + BMc \quad (15)$$

The results obtained show no correspondence between predicted and experimental values (Fig. 4), indicating that this model does not describe the experimental data.

Discrete Self-associations—Self-associations of the discrete type are represented by

$$2P_1 \rightleftharpoons P_2; 3P_1 \rightleftharpoons P_3; \cdots, \text{etc.},$$

where the association constants on a weight per volume basis are given by $K_2 = (c_2/c_1)^2; K_3 = (c_3/c_1)^3; \text{etc.}$ Adams (13, 14) has described in detail the methods for establishing the relationships among the equilibrium constants, the nonideal term, and the molecular weight of discrete self-associating systems. The procedure requires the elimination of each unknown until an equation is obtained having only one unknown, $BM_c$. The necessary steps are similar to those described for the monomer-$n$-mer associations, which are, in

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![Fig. 3 (left). Experimental and calculated values of $M_1/M_{app}$; O, experimental; ---, monomer-hexamer; - - -, monomer-heptamer; O--O, monomer-octamer.](http://www.jbc.org/)

![Fig. 4 (right). Experimental and calculated values of $M_1/M_{app}$; O, experimental; ---, monomer-tetramer-octamer; - - -, monomer-hexamer-octamer; O, definite self-association.](http://www.jbc.org/)
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Comparison of experimental values of $M/M_{\text{app}}$ with results from different self-associating models (Apo A-I in 0.02 M EDTA, pH 8.6)

<table>
<thead>
<tr>
<th>$c$ (fringes)</th>
<th>Experiments</th>
<th>$n=1, 7^a$</th>
<th>$n=1, 2, 8^a$</th>
<th>$n=1, 2, 4, 8^a$</th>
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<tbody>
<tr>
<td>2</td>
<td>0.492</td>
<td>0.625</td>
<td>0.507</td>
<td>0.489</td>
</tr>
<tr>
<td>4</td>
<td>0.281</td>
<td>0.273</td>
<td>0.288</td>
<td>0.283</td>
</tr>
<tr>
<td>6</td>
<td>0.225</td>
<td>0.210</td>
<td>0.223</td>
<td>0.224</td>
</tr>
<tr>
<td>8</td>
<td>0.199</td>
<td>0.186</td>
<td>0.195</td>
<td>0.199</td>
</tr>
<tr>
<td>10</td>
<td>0.182</td>
<td>0.174</td>
<td>0.179</td>
<td>0.184</td>
</tr>
</tbody>
</table>

$^a M/M_{\text{app}}$ calculated by use of $K_a$ values in Table I (see text for details).

As noted above, Equation 16 may be reduced to represent different types of associations if some of the equilibrium constants are set equal to zero. In addition to the interactions represented by Equation 16, the data for apo-A-I were analyzed in terms of: (a) sequential dimerizations ($K_2 = K_3 = K_4 = 0$ and $K_1 = K_0 = K_3 = 0$); (b) formation of the monomer-dimer-tetramer-octamer model, (c) sequential tetramerization ($K_2 = K_3 = K_4 = K_5 = 0$); (d) trimer formation ($K_2 = K_4 = K_6 = 0$); and (e) monomer-dimer-tetramer association.

Frontal Analysis Gel Filtration Chromatography—This technique was utilized as an independent method of analyzing the self-association of apo-A-I.

The elution profile for apo-A-I in 0.02 M EDTA (pH 8.6), $c_0 = 0.94$ mg/ml, is shown in Fig. 5A. The profile is characterized by unimodal leading and trailing edges, the former being characteristically distinctive of relatively fast associations, the latter representing a system in which several associating species are present (16). The unimodal nature of these boundaries is clearly shown in the differential elution profile at this concentration (Fig. 5B). These results confirm the presence of more than two species in solution and indicate that the equilibrium among species is attained rapidly compared to the time of passage through the column as shown by the steep increase of the leading edge.

DISCUSSION

Our studies have shown that, in aqueous solutions, apo-A-I self-associates under a variety of conditions of pH and ionic strength. In the range of protein concentrations used, the solute-solute interactions may best be described as an ideal discrete self-association of monomer-dimer-tetramer-octamer. Our data, obtained mainly by sedimentation equilibrium, are supported by the results of frontal analysis gel filtration chromatography, which indicate that equilibrium among the aggregating species is maintained during elution through the column. In addition, the relative insensitivity of the self-association of apo-A-I to changes in pH and ionic strength indicates that the interactions among the monomeric units are primarily nonionic. Our results are in keeping with a recent report by Stone and Reynolds (17), but do not support the observations of Gwynne et al. (18) who found that solutions of apo-A-I were monomeric at concentrations in the range of those used in this work. At this time we are not able to reconcile this discrepancy. It is of interest to point out that apo-A-II, the second major apolipoprotein of HDL, also self-associates in aqueous solution (26). A comparison of the associating characteristics of apo-A-I and apo-A-II (26), under identical conditions, showed that the equilibrium constant for the formation of the dimer ($K_2$) is an order of magnitude greater for apo-A-I, indicating that the solute-solute interactions are stronger for the latter apoprotein.

The properties of A-I to self-associate offer a possible explanation for the difficulty in obtaining complete relipidation of apo-A-I with the high density lipoprotein lipids under the usual conditions of reconstitution (1), and also suggest

$^a$ M. C. Ritter and A. M. Scanu, unpublished results.
that apo-A-I has to be in its monomeric form (protein concentration \( \leq 10^{-7} \) M) to bind lipids readily. In this regard, it is of interest to note that apo-A-II, in contrast to apo-A-I, has no such a requirement and binds lipids even at concentrations at which dimerization of the protein occurs. In accord with the interpretation of our results the author considered the possibility that the self-association of apo-A-I and apo-A-II play in determining the overall structure of human HDL cannot be assessed from the present data, or from those available in the literature. It has been reported that, in their lipid-free form, these apoproteins form a 1:1 molar complex at specific concentrations. Studies in our laboratory have shown that the amount of apo-A-I recovered as a protein-lipid complex increases when mixtures of apo-A-I and apo-A-II are present in solution. Before further conclusions are drawn, however, the capability of C-peptides to form protein-protein complexes with apo-A-I or apo-A-II, or both, must be assessed.

Furthermore, even though studies on binding of apo-HDL or its individual apoproteins with HDL lipids (20-22) or other ligands (19) have been reported, our current findings indicate that it is necessary to determine the solution properties of these apoproteins before such interactions are examined and interpreted. Studies of the properties of apo-A-I from animal species other than man, may also provide further insights into the mechanism of lipid binding and the overall structure of HDL. Recently, Jonas (23, 24) reported on the solution properties of bovine apo-A-I. The observations derived from studies on fluorescence polarization and ultracentrifugal techniques indicate that bovine apo-A-I forms stable tetramers within a wide range of pH and ionic strength. In accord with the interpretation of our results the author considered the possibility that the strong protein-protein interactions (\( K_a < 10^{-7} \) M) could compete with the capacity of bovine apo-A-I to associate with lipids.

Barbeau and Scanu (25) have conducted studies on apo-A-I isolated from the serum of rhesus monkey (Macaca mulatta). In contrast to human and bovine apo-A-I, this apoprotein was found to be monomeric over a wide range of concentrations. Perhaps more significantly, rhesus apo-A-I bound more lipids than human apo-A-I under comparable conditions of relipidation.

From the above it becomes apparent that the state of association of a protein in solution plays a major role in determining its capacity to bind lipids or other ligands in vitro. According to our present work, apo-A-I is a good example of this.

Acknowledgments—The authors wish to thank Dr. C. Lim and Mr. D. Barbeau for preparing pure samples of apo-A-I and Mr. L. Lusk for his assistance in the purification of human HDL. They also thank Dr. A. Frankfater of Loyola University School of Medicine, Chicago, Ill., in whose laboratory the gel filtration chromatography was performed and who provided his guidance and advice. The authors also acknowledge the valuable comments provided by Drs. F. Kézdy and J. Erman.

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L B Vitello and A M Scanu


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