Asymmetry of Apolipoprotein A-I in Solution as Assessed from Ultracentrifugal, Viscometric, and Fluorescence Polarization Studies†

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ABSTRACT: The solution properties of apolipoprotein A-I (apo A-I) isolated from human and rhesus monkey serum high-density lipoproteins were examined by the techniques of fluorescence polarization, sedimentation velocity, sedimentation equilibrium, and viscometry as a function of protein concentration. The ultracentrifugal studies confirmed previous reports that human and rhesus monkey apo A-I undergo a concentration-dependent, reversible self-association in aqueous solutions. The sedimentation velocity data, extrapolated to infinite dilution, indicated that the apo A-I monomer is asymmetric, with an axial ratio of about 6. The reduced viscosity of apo A-I solutions was also markedly affected by protein concentration; within the range of concentrations studied, the values for human apo A-I ranged from 11.19 to 21.24 mL/g and for rhesus apo A-I, from 8.26 to 16.83 mL/g. The intrinsic viscosity, 6.58 mL/g, of the apo A-I monomer was significantly higher than that of a typical globular protein (3–4 mL/g); the axial ratio, 5.5, was in agreement with that obtained from the ultracentrifugation analyses. In the fluorescence polarization studies, human and rhesus apo A-I were dansylated to the extent of 1.0–2.5 mol of dansyl group/mol of apo A-I. This chemical modification was attended by no appreciable change in the secondary structure of apo A-I, as assessed by circular dichroism spectra. The apparent sedimentation coefficient, molecular weight, and rotational relaxation time of each of the dansylated proteins indicated that both human and rhesus monkey apo A-I self-associated to a lesser extent than the unmodified apo A-I. At protein concentrations ranging from 0.002 to 3.0 g/L, the fluorescence polarization values of dansylated apo A-I remained essentially constant (0.185–0.192), although rotational relaxation times indicated self-association at the higher concentrations. After extrapolation to infinite dilution, the axial ratio of apo A-I was calculated to be 5.8. Taken together, these results indicate that the apo A-I monomer is asymmetric in solution. The model best representing the experimental results was found to be an elongated ellipsoid having a semimajor axis (*a*) of 75.6 Å and a semiminor axis (*b*) of 12.6 Å. Moreover, the substantial increase of the reduced viscosity of apo A-I as a function of protein concentration, coupled with the failure of fluorescence polarization to detect concentration-dependent changes in the dansylated apo A-I, suggests that the asymmetric monomers dimerize along their major axis and that these dimers elongate according to an end-to-end association. The decreased tendency of the dansylated apo A-I to self-associate as compared to unmodified apo A-I suggests that the functional groups which underwent modification (NH_2 terminus of the aspartic acid and the lysine residues) are involved in the oligomerization process.

Apolipoprotein A-I (apo A-I)[1] of plasma high-density lipoproteins has been the subject of numerous investigations in recent years as efforts were made to define its physical properties in solution and to relate them to its capacity to combine with lipids. Conformational studies (Jonas, 1973; Gwynne et al., 1974; Tall et al., 1975; Reynolds, 1976; Tall et al., 1976) have shown that, compared to typical globular proteins, apo A-I has a loosely folded tertiary structure and a high degree of exposure of its hydrophobic amino acid residues to solvent. Detergent binding studies (Reynolds & Simon, 1974; Makino et al., 1974; Stone & Reynolds, 1975a,b; Reynolds et al., 1977) have indicated that, unlike most water-soluble globular proteins which lack detergent binding sites in their native state, apo A-I in its native state contains four high-affinity hydrophobic binding sites. It has also been demonstrated by ultracentrifugal and chromatographic techniques that apo A-I has a marked tendency to self-associate in aqueous solution, primarily by hydrophobic interactions (Jonas, 1973; Stone & Reynolds, 1975a,b; Vitello & Scanu, 1976; Barbeau & Scanu, 1976; Teng et al., 1977).

In most of these studies, it was assumed that apo A-I possessed an approximately spherical shape similar to that of most water-soluble globular proteins, although its solution properties were inconsistent with this concept. In this investigation, we used viscometry, ultracentrifugal sedimentation techniques, and fluorescence polarization measurements to estimate the shape of human and rhesus monkey apo A-I in solution. Since these techniques are extremely sensitive to the axial ratio of the macromolecule, reliable estimates of the dimensions of apo A-I in solution were obtained. The results of these studies will be presented and discussed in the context of possible models of self-association of apo A-I in solution.

Materials and Methods

Isolation and Purification of apo A-I. The procedures used for the isolation and purification of apo A-I, from either human or rhesus monkey serum, have been reported (Edelstein et al., 1972; Edelstein et al., 1973). By polyacrylamide gel electrophoresis both in 0.01% sodium dodecyl sulfate, 10% acrylamide, and in 8 M urea, 7.5% acrylamide, apo A-I preparations used in this study showed a single band up to a loading of 200 μg of protein. The results concerning amino

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‡ Operated by The University of Chicago for the U.S. Department of Energy under Contract No. EY-76-C-02-0069.

[1] Abbreviations used: apo A-I, apolipoprotein A-I; Dns-Cl, dansyl chloride (5-dimethylamino-1-naphthalenesulfonyl chloride); apo A-I Dns, dansylated apo A-I; HDL, high-density lipoproteins; EDTA, ethylenediaminetetraacetic acid; $s_{20,w}$, sedimentation velocity coefficient corrected to water at 20 °C.

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acid composition were identical with those reported previously (Morrisett et al., 1975; Scanu et al., 1975). Since the polymorphic forms of apo A-I as obtained from DEAE ion exchange chromatography have been shown to possess similar self-associating properties (Barbeau & Scanu, unpublished observation; Osborne & Brewer, 1977), apo A-I containing the two recognized polymorphic forms were used in this study.

Preparation of Dns-Labeled apo A-I Proteins. The proteins were dissolved in 1% $NaHCO_3$, pH 8.0, to give solutions of 1.5–6.0 g/L. Dansyl chloride (Dns-Cl), dissolved in cold acetone at a concentration of 4.0 g/L, was added in small aliquots to cold protein solutions (4 °C) with constant stirring. Additions were completed over 1 h, and a total of 0.04 mg of Dns-Cl was added per mg of protein. After filtering through 0.45 μm Millipore filters, the solutions were extensively dialyzed against 0.02 M EDTA, 1 mM NaN_3, pH 8.6. Two intermediate dialysis steps were performed with a slurry of Dowex-2X resin in OH^- form to insure complete removal of any free label.

The analysis of the apo A-I Dns proteins by TLC on silica gel Eastman Kodak plates, with a solvent system of 60:40 (v/v) 0.2 M sodium acetate–ethanol, revealed only a covalently bound probe; no free fluorescent probe could be detected.

The extent of labeling varied somewhat from preparation to preparation but was always in the range from 1.0 to 2.5 Dns per apo A-I monomer.

Fluorescence Polarization Studies. For the fluorescence polarization measurements, we used the double-photomultiplier, Series-400 polarization spectrofluorimeter manufactured by SLM Instruments, Inc. Each experimental point was the average of at least 100 sets of data. The fluorescence excitation was 340 nm; the emission wavelength was selected with Corning glass 3-72 filters. The temperature was maintained at 25.0 ± 0.1 °C unless stated otherwise.

Rotational Relaxation Times and Axial Ratios of apo A-I Dns Proteins. The determination of rotational relaxation times was based on Perrin's equation (Perrin, 1926)

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho_h}\right) \quad (1)$$

where P is the observed polarization, P_0 is the limiting polarization in the absence of Brownian rotation, τ is the fluorescence lifetime, and ρ_h is the rotational relaxation time. The time ρ_h is related to molecular parameters by the equation $\rho_h = 3V\eta/kT$, where V is the hydrated volume of a spherical molecule, η is the viscosity of the medium, k is Boltzmann's constant, and T is the absolute temperature. From preparation to preparation the polarization varied by as much as 7%; the precision for each measurement, however, was better than 1%.

Fluorescence lifetimes were measured with the cross-correlation phase fluorimeter described by Spencer & Weber (1969), under the same conditions as those used in polarization measurements. Since the fluorescence lifetimes were found to be independent of concentration, the polarization values were a function of the rotational relaxation time (ρ) and thus reflected changes in the hydrodynamic volume of apo A-I.

Axial ratios (a/b) for prolate ellipsoids were obtained from rotational relaxation measurement values (Weber, 1953b); ρ_0 was calculated from known molecular weights and partial specific volumes, with the assumption of a spherical shape and a hydration of 0.3 g of H_2O/g of protein. In estimating the expected fluorescence polarization changes due to end-to-end or side-by-side self-association of elongated monomers (a/b = 6) into dimers, we assumed no major changes in monomer structure upon self-association, and we used the equation given by Weber (1953b) relating fluorescence polarization to the principal rotational relaxation times of ellipsoids of revolution. Those relaxation times were determined from $\rho_h = 2(1/\rho_a + 1/\rho_b)$ and $\rho_a/\rho_b = (a/b)^3$.

Ultracentrifugal Studies. All of the ultracentrifugal studies were performed in a Beckman-Spinco Model E analytical ultracentrifuge equipped with an RTIC temperature control unit and an electronic speed control system. Prior to use, all the apo A-I samples were dialyzed overnight against 0.02 M EDTA, pH 8.6, containing 1 mM NaN_3. Sedimentation velocity runs were conducted at 52000 to 60000 rpm in aluminum-filled Epon double-sector cells having 12-mm centerpieces with sapphire windows. For dilute protein samples (less than 1.0 g/L), the sedimentation profiles were monitored at either 280 or 340 nm with the Beckman split-beam photoelectric scanner. The sedimentation rate was calculated from the midpoint of the protein boundary. The schlieren optical system was used for samples with protein concentrations higher than 1.0 g/L. The sedimentation profiles were recorded on Kodak Mettalographic plates and analyzed on a Nikon 6C microcomparator. Next, the enlarged schlieren images were traced on paper, and the respective peak areas were measured with a Keuffel and Esser compensating polar planimeter. The radial dilution was corrected as described by Chervenka (1970).

The sedimentation equilibrium experiments were performed in a 12-mm aluminum-filled Epon double-sector cell or a charcoal-filled Epon 6-channel Yphantis cell with sapphire windows. The interference patterns were magnified 20-fold and measured on a Nikon 6C microcomparator. The molecular weight determinations were carried out by both the meniscus-depletion sedimentation equilibrium method of Yphantis (1964) and the conventional sedimentation equilibrium technique as modified by Richards et al. (1968). An initial overspeed period was used for the conventional sedimentation equilibrium, as described by Chervenka (1970). The initial concentration (c_0) was determined from a synthetic boundary diffusion run immediately following the equilibrium experiment. The conversion factor from fringes to apo A-I concentration was taken to be 4.0 fringes L g^{-1} for both the unlabeled and Dns-labeled apo A-I proteins (Barbeau & Scanu, 1976).

Axial ratios for monomeric proteins were obtained from the frictional coefficient ratio, f/f_0 (Schachman, 1959; Tanford, 1961). The observed f/f_0 ratio was calculated from

$$f/f_0 = \frac{M_r(1 - \bar{v}_2\rho)}{Ns_{20,w}{}^0 6\pi\eta\left(\frac{3M_r\bar{v}_2}{4\pi N}\right)^{1/3}} \quad (2)$$

where $s_{20,w}{}^0$ is the sedimentation velocity coefficient at infinite dilution, M_r is molecular weight, $\bar{v}_2$ is the partial specific volume of the protein, ρ is the density of the solution, N is Avogadro's number, and η is the viscosity of the medium. The observed frictional coefficient is related to both hydration and asymmetry by the following relation

$$(f/f_0)_{obsd} = (f/f_0)_{asym}\left(1 + \frac{\delta_1}{\bar{v}_2\rho_1}\right)^{1/3} \quad (3)$$

where δ_1 is the water of hydration in g/g of protein.

Viscosity. Viscosity measurements of apo A-I solutions were made with a Cannon-Manning No. 5 semimicrocapillary viscometer at 20.0 ± 0.005 °C. Flow time for the solvent was 288 s, measured with a Heuer 10-minute stopwatch. Six to ten replicate readings, reproducible to ±0.1 s, were made for

each protein solution obtained by serial dilution of the most concentrated sample. All solutions were dialyzed extensively against 0.02 M EDTA, pH 8.6, and filtered prior to use with a 0.45 μm Millipore filter (Millipore, Inc.). The intrinsic viscosity $[\eta]$ in milliliters per gram was evaluated by solving the following

$$\frac{\eta_{sp}}{c} = [\eta] + k'[\eta]^2 c \quad (4)$$

where c is protein concentration in grams per milliliter and specific viscosity $\eta_{sp} = (\eta - \eta_0)/\eta$, where η and η_0 are the viscosity of the solution and that of the solvent, respectively. The dimensionless Huggins' constant, k', is a measure of the concentration dependence and is usually of the order of 2 for spheres, 0.4 for rigid rods, and about 10 for associated rigid rods (Bradbury, 1970).

The shape of apo A-I in solution was estimated from the equation beow

$$[\eta] = \nu(\bar{v}_2 + \delta_1\bar{v}) \quad (5)$$

where ν is the Simha viscosity increment and $\bar{v}_1$ is the partial specific volume of the solvent (Bradbury, 1970). The axial ratio (a/b) of a prolate ellipsoid is related to ν according to the following equation of Simha (Doty et al., 1956):

$$\nu = \frac{(a/b)^2}{15\left(\ln\frac{2a}{b} - 1.5\right)} + \frac{(a/b)^2}{5\left(\ln\frac{2a}{b} - 0.5\right)} + \frac{14}{15} \quad (6)$$

Circular Dichroism. Circular-dichroism measurements were carried out with a Cary Model 6001 spectropolarimeter equipped with a circular dichroism unit and a cell of 1.0-mm path length. The instrument was calibrated before each use with *d*-camphorsulfonic acid, and spectra were recorded at room temperature between 200 and 260 nm at a chart speed of 1 Å/s.

Ultraviolet Difference Spectroscopy. Ultraviolet difference spectroscopy of unmodified and dansylated apo A-I was performed in a Cary Model 15 spectrophotometer (Varian, Inc.) at room temperature, with a cell having a 1.0-cm path length.

Other Analyses. The solvent densities were measured at 20.0 ± 0.005 °C in a Mettlar-Paar (Hightstown, NJ) mechanical oscillator. The partial specific volume of both human and rhesus monkey apo A-I was 0.736 mL/g, as calculated from their amino acid composition (Kratky et al., 1973). Protein concentrations were determined either by the method of Lowry (Lowry et al., 1951), with bovine serum albumin used as the standard and corrected as previously described from amino acid analysis (Barbeau & Scanu, 1976), or by absorbance at 280 nm. Absorption spectra were recorded with a Beckman Acta VI spectrophotometer. Analyses by radioimmunoassay were carried out as previously described (Karlin et al., 1976). Determination of dansyl amino acids was performed by thin-layer chromatography (Gray, 1972).

Reagents. All chemicals were reagent grade and were used without further purification except for urea, which was recrystallized from ethyl alcohol.

Results

A. Studies on Unmodified apo A-I. At protein concentrations ranging from 0.05 to 0.7 g/L, as monitored by UV tracings at A_{280nm}, human apo A-I in 0.02 M EDTA, pH 8.6, exhibited smooth sedimentation profiles with no evidence of boundary separation. The apparent weight-average sedimentation coefficient increased rapidly with protein concentration (Figure 1). By extrapolation to zero protein

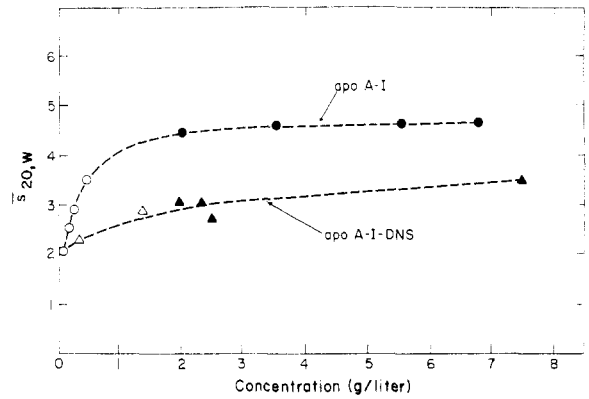

FIGURE 1: Concentration dependence of weight-average sedimentation coefficient of human apo A-I in 0.02 M EDTA, 1 mM NaN_3, pH 8.6, at 20 °C. (○ and △) Values obtained at a rotor speed of 52 000 rpm and monitored by scanner tracings at A_{280nm}; (● and ▲) values from runs at 60 000 rpm, obtained with schlieren optics.

concentration, the apo A-I monomer had an $s_{20,w}^0$ value of 2.0, which corresponds to that previously reported for the monomeric form of rhesus monkey apo A-I (Barbeau & Scanu, 1976). At protein concentrations higher than 1.5 g/L, however, human apo A-I consistently showed two well-separated schlieren peaks which were fairly symmetrical in shape. The $s_{20,w}^0$ values for the slow- and fast-sedimenting components were 4.1 and 6.0 *S*, respectively. Furthermore, the proportion of the fast-sedimenting peak increased with increasing protein concentration. These observations were consistent with previous reports on the self-association of apo A-I in solution (Vitello & Scanu, 1976; Teng et al., 1977).

Based on the knowledge of their molecular weight (Edelstein et al., 1972), partial specific volume, and $s_{20,w}^0$, we were able to calculate the general shape of both human and rhesus apo A-I monomers. The observed frictional ratios were 1.43 and 1.46 for human and rhesus monkey apo A-I, respectively. In both cases, assuming a hydration of 0.3 g of H_2O/g of protein, the axial ratios (a/b) due to asymmetry were estimated to be 5.4 and 5.9 for man and rhesus monkey, respectively, indicating that both apo A-I monomers are highly asymmetrical.

Viscosity Data. The reduced viscosity, η_{sp}/c, of human apo A-I solutions in 0.02 M EDTA, pH 8.6, increased linearly from 11.9 ± 0.62 to 21.24 ± 0.05 mL/g in the apo A-I concentration range of 0.85–3.67 g/L. In a similar manner, the reduced viscosity of rhesus monkey apo A-I solutions increased from 8.26 ± 0.66 to 16.83 ± 0.10 in the concentration range of 0.81–4.32 g/L (Figure 2). Linear extrapolation of these data to infinite dilution according to eq 4 (Bradbury, 1970) yielded a maximum value of 6.58 ± 0.24 mL/g for the intrinsic viscosity, $[\eta]$, of rhesus monkey apo A-I.

The intrinsic viscosity of human apo A-I solutions, obtained by linear extrapolation, was 9.2 ± 0.38 mL/g. This value was considered unreliable, however, since the protein is in considerably associated form at the lower concentrations (Vitello & Scanu, 1976; Stone & Reynolds, 1975a); measurements of such dilute solutions may lead to misleading results (Reisler & Eisenberg, 1970). According to eq 5, the viscosity increment for rhesus monkey apo A-I ($\delta_1 = 0.30$) was 6.36. This value was compatible with a prolate ellipsoid having an axial ratio of 5.5, comparable to that determined from the sedimentation velocity studies. By using an average axial ratio of $a/b = 6$ for both human and rhesus monkey apo A-I, and the following relationship for the volume of an ellipsoid

$$M_r(\bar{v}_2 + \delta_1\bar{v}_1) = \frac{4\pi N(a/b)b^3}{3} \quad (7)$$

Table 1: Calculated and Experimental Values of η_{sp}/c for apo A-I Oligomerization

apo A-I (g/L)	experimental η_{sp}/c^a (mL/g)		calculated η_{sp}/c (mL/g) models of association							
			human			rhesus monkey				
	human	rhesus	I	II[b]	III[b]	I	II[b]	III[b]	IV	V
0.5	10.78		16.76	7.53 (5.35)[c]	10.75 (7.64)[c]					
1.0	12.33	8.86	55.42	13.35 (9.48)	18.99 (13.48)	9.62	7.86	8.06	7.54	7.67
1.5	13.89		81.71	17.44 (12.38)	24.47 (17.48)					
2.0	15.44	11.13	97.87	19.97 (14.18)	27.83 (19.76)	21.81	9.50	10.89	7.21	8.15
2.5	17.00		108.64	21.66 (14.44)	30.06 (21.35)					
3.0		13.40				33.85	11.81	14.90	6.75	8.83
4.0		15.68				45.86	13.31	17.49	6.45	9.27
RMS[d]			73.63	4.08 (3.47)	10.96 (3.93)	21.94	1.98	1.44	6.99	4.91

[a] Values of η_{sp}/c were obtained from linear regression analysis of the experimental data. [b] An average value of 1.5 × diameter of monomer was assumed for diameter of dimer (Lowey & Holzter, 1959). [c] Calculated η_{sp}/c values, assuming no hydration ($\delta_1 = 0$), are given in parentheses to demonstrate the variation obtained with different values of b [e.g., b = 11.2 A ($\delta_1 = 0$), b = 12.6 A ($\delta_1 = 0.3$)]. [d] Root mean square deviation of experimental and calculated values.

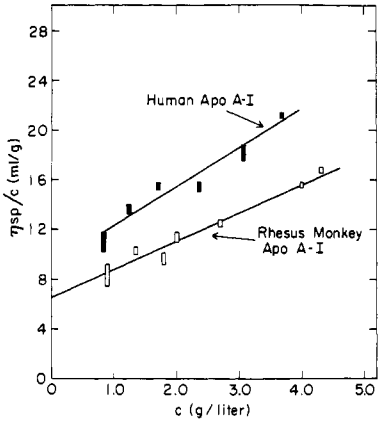

FIGURE 2: Concentration dependence of reduced viscosity of human and rhesus monkey apo A-I solutions in 0.02 M EDTA, 1 mM NaN_3, pH 8.6, at 20 °C. Lines drawn through the data points for extrapolation to infinite dilution were fitted by linear least-squares treatment, with η_{sp}/c values calculated for each experimental outflow time.

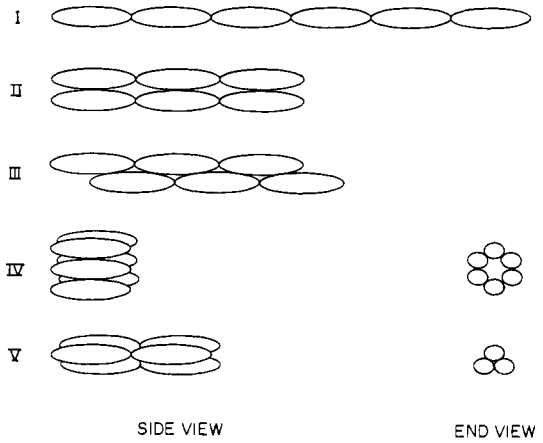

FIGURE 3: Proposed models for association of elongated monomers. This scheme illustrates the proposed structures for the monomer–hexamer association of rhesus monkey apo A-I. I, end-to-end association of monomers; II, end-to-end association of side-by-side dimers; III, end-to-end association of staggered side-by-side dimers; IV, cyclic hexamer; V, end-to-end association of cylindrical envelope trimers.

we calculated the dimensions of the apo A-I monomer. In this equation, $\bar{v}_1$ refers to the partial specific volume of the solvent, and all other symbols are described under Materials and Methods. Assuming a hydration of $\delta_1 = 0.3$, we obtained values of a = 75.6 Å and b = 12.6 Å, compatible with an overall length of the monomer, $2a$, of 151.2 Å.

The rapid increase observed for the reduced viscosity of the apo A-I solutions with increasing protein concentration is characteristic of associating rigid rods (Doty et al., 1956). The calculated values of k' from eq 4 were 36.7 for human apo A-I and 52.4 for rhesus monkey apo A-I. These values suggest that the association of elongated monomers results in further asymmetry as a consequence of an end-to-end process of oligomerization.

Representative models for the association of apo A-I monomer by end-to-end elongation and side-by-side interaction are shown in Figure 3. By assuming no significant shape changes of apo A-I upon association, by minimizing molecular overlap, and by assigning a mean diameter (d) of 1.5 × d (monomer) to protein dimers in models II and III, we calculated the expected η_{sp}/c values for both human and rhesus monkey apo A-I solutions at various protein concentrations according to the following equations (Cragg & Bigelow, 1955; Nichol et al., 1964)

$$\frac{\eta_{sp}}{c} = \sum_{i=1}^{i} \frac{\alpha_i \nu_i V_i}{M_{ri}} \tag{8}$$

where α_i is the weight fraction of the associated species i, V_i represents the equivalent volume of a prolate ellipsoid given by $V = 4\pi Nab^2/3$, and M_{ri} is the molecular weight of species i.

The weight fractions (α_i) of the oligomeric species of both human and rhesus monkey apo A-I (Figure 4) were estimated from the previously obtained equilibrium constants (Vitello & Scanu, 1976; Barbeau & Scanu, 1976). In each case, the Simha viscosity increment (ν_i) was estimated from the assumed axial ratio of the oligomeric apo A-I species according to eq 6. The expected η_{sp}/c values for both human and rhesus monkey apo A-I solutions, as estimated from eq 8, are given in Table I for each of the proposed models of association. In contrast to the extreme case of elongation (model I) and those suggesting formation of more spherical structures (models IV

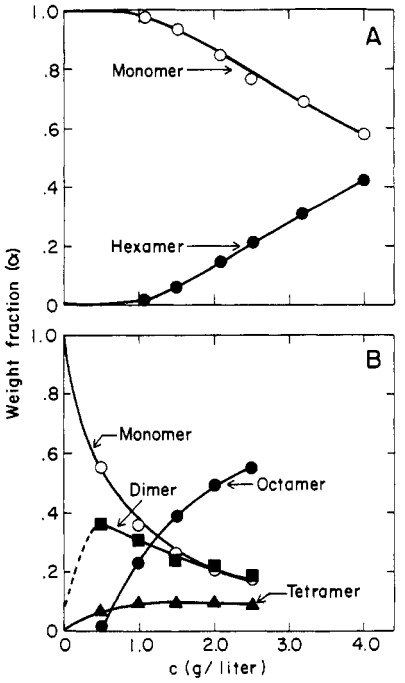

FIGURE 4: Estimated weight fraction of monomers and associated species of apo A-I, obtained from previously published data. (A) Rhesus monkey apo A-I: ○, monomers; ●, hexamers (from Barbeau & Scanu, 1976). (B) Human apo A-I: ○, monomers; ■, dimers; ▲, tetramers; ●, octamers (from Vitello & Scanu, 1976).

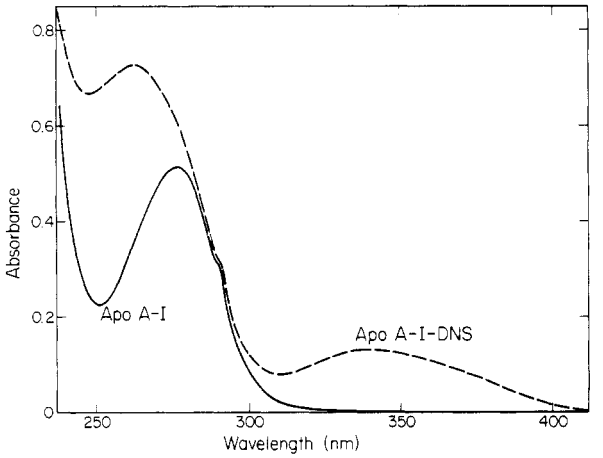

FIGURE 5: Absorption spectra of human apo A-I in unlabeled (—) and Dns-labeled (---) forms. Spectra are shown for identical protein concentrations, prepared in 0.02 M EDTA, 1 mM NaN_3, pH 8.6.

and V), the calculated η_{sp}/c values for both human and rhesus monkey apo A-I solutions were consistent with a process of end-to-end association intermediate between models II and III. This suggests that the self-association of apo A-I consists of a side-by-side dimerization of the asymmetric monomers and the elongation of these dimers through an end-to-end process.

B. Studies on Dansylated apo A-I. Chemical and Spectroscopic Data. The incorporation of covalent Dns labels into apo A-I was determined from absorption spectra of the labeled and unlabeled proteins (Figure 5). The amount of label bound per apo A-I molecule was assessed from protein determinations and from Dns absorbance at 340 nm; a molar extinction coefficient of 4.30×10^4 M^{-1} cm^{-1} was used at this wavelength (Weber, 1953a). Under the same experimental conditions, the incorporation of Dns labels was very similar, in terms of extent and spectral changes, for human and rhesus monkey apo A-I.

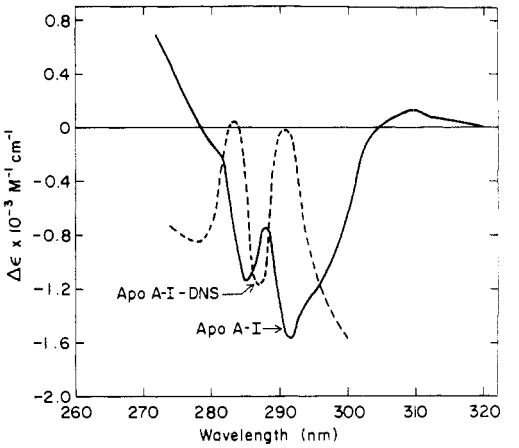

FIGURE 6: Ultraviolet difference spectra of human apo A-I (—) and Dns-labeled human apo A-I (---) in 0.005 M NH_4HCO_3, 1 mM NaN_3, pH 8.6, at a protein concentration of 0.27 g/L against the apoprotein in 6 M guanidine hydrochloride.

The amino acid analysis of the dansylated apo A-I showed a decrease of 2–3 lysine residues as compared to unmodified apo A-I (Edelstein et al., 1972). Thin-layer chromatography of the amino acids from dansylated apo A-I hydrolysates confirmed the existence of ε-dansyllysine and traces of dansylaspartate as the chemically modified residues in the protein. Thus, both the ε-amino groups of lysine and the NH_2 terminus (aspartic acid) were dansylated under our conditions. Conformational changes in apo A-I induced by dansylation proved to be minor and probably insignificant. The circular dichroic spectra of unmodified and dansylated human apo A-I showed only a minor increase in ellipticity of apo A-I occurring after dansylation, indicating that there were no significant alterations in the protein secondary structure. The ultraviolet difference spectra (Figure 6) of the unmodified and dansylated apo A-I also showed a great similarity in the exposure of hydrophobic groups to the solvent (Tall et al., 1975; Gwynne et al., 1974; Reynolds, 1976; Jonas, 1973).

The relative resistance of apo A-I to underlying conformational changes upon dansylation was also shown by the parallel binding observed between unmodified and dansylated protein with antibodies raised against apo A-I by means of radioimmunoassay techniques.

Ultracentrifugal Data. At protein concentrations comparable to those used for unmodified apo A-I, the dansylated human apo A-I preparations (1.4 mol of Dns/mol of monomer apo A-I) exhibited smaller weight-average sedimentation coefficients (Figure 1), suggesting that the proteins became less associated in solution after the introduction of the dansyl groups. Similarly, the unmodified rhesus monkey apo A-I showed a fairly symmetrical schlieren peak having a $s_{20,w}^0$ of 3.95 at 4.0 g/L. However, after dansylation (2.3 mol of Dns/mol of monomer), the protein solution displayed a highly heterogeneous schlieren profile and had a weight-average sedimentation coefficient of 3.3 S at the same concentration. At a protein concentration of 0.7 g/L, the dansylated rhesus apo A-I exhibited an $s_{20,w}$ of 1.96 S, which corresponds to that previously reported for the monomeric form of rhesus monkey apo A-I (Barbeau & Scanu, 1976). It is evident that the addition of 1–3 mol of dansyl group to apo A-I did not alter the shape of apo A-I. These results are summarized in Table II.

Sedimentation equilibrium experiments were carried out for dansylated apo A-I preparations from both human and rhesus monkey HDL. We monitored the concentration distribution at various radial distances by measuring either the refractive

Table II: Hydrodynamic Properties of Native and Dansylated apo A-I from Human and Rhesus Monkey HDL

apo A-I		monomers[a]			oligomers $\bar{s}_{20,w}$	
		$s_{20,w}^0$	f/f_0	a/b[b]	$c = 0.6$ g/L	$c = 4.0$ g/L
human	native	2.00	1.43	5.4	3.8	4.5
	dansylated	2.00	1.45	5.7	2.6	3.2
rhesus monkey	native	1.96	1.46	5.9	1.96[c]	3.9
	dansylated	1.96	1.48	6.1	1.96	3.4

[a] Monomer molecular weights, 28 000 for native apo A-I and 28 500 for dansylated apo A-I, were taken from amino acid composition data, assuming an average of 2 mol of DNS/mol of apo A-I monomer for the latter. In both cases, the partial specific volume was taken as 0.736. [b] A hydration of $\delta_1 = 0.3$ was assumed. [c] Extrapolated value of $s_{20,w}^0$ taken from Barbeau & Scanu (1976).

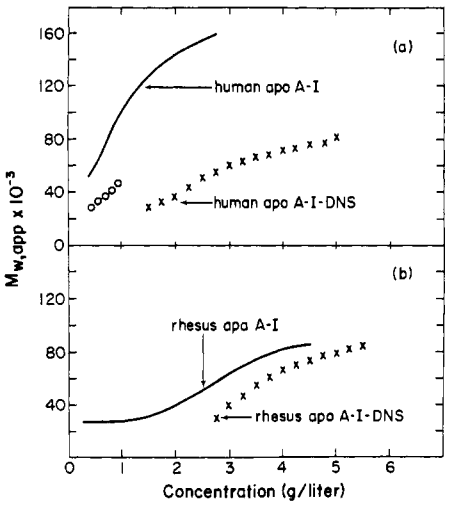

FIGURE 7: Molecular weight vs. concentration plots for dansylated apo A-I preparations from human and rhesus monkey HDL. Rotor speeds were 8000 rpm (X) and 30 000 rpm (O). (a) Human apo A-I Dns and (b) rhesus monkey apo A-I Dns. The smooth curves represent data obtained from native forms of human and rhesus apo A-I (taken from Vitello & Scanu, 1976; Barbeau & Scanu, 1976).

index increment or the absorption (at 280 nm for the protein, at 340 nm for the dansylated apo A-I). In all instances, the ln c vs. r^2 plots curved upward, indicating a similar behavior of unmodified and dansylated apo A-I. The $M_{w,app}$ vs. protein concentration plots obtained at various initial protein concentrations and rotor speeds exhibited no overlap (Figure 7). In addition, the values of the $M_{w,app}$ were found to be lower than those of the native protein at comparable concentrations, indicating a decreased tendency of dansylated apo A-I to self-associate. Similar results were obtained by estimation of molecular weights from sedimentation and diffusion coefficients (Schachman, 1959).

Rotational Relaxation Times and Axial Ratios of apo A-I Dns Proteins. Table III gives a summary of the fluorescence lifetimes and rotational relaxation times at 25 °C for the monomeric Dns-labeled human and rhesus apo A-I at 5×10^{-6} M (0.14 g/L) and for the self-associated Dns-labeled human apo A-I at 10^{-4} M (2.8 g/L). The table also gives values for the minimum rotational relaxation times (ρ_0) calculated from partial specific volumes, from known molecular weights, assuming a hydration of 0.3 g of H_2O/g of protein, and from the axial ratios (a/b) for prolate-ellipsoid shapes (Weber, 1953b). The estimated values of molecular asymmetry from fluorescence measurements are 6.5 for human apo A-I and 5.8 for rhesus apo A-I monomers; these values are consistent with those from sedimentation velocity and viscosity measurements.

Table III: Fluorescence Lifetimes, Rotational Relaxation Times, and Axial Ratios of Dansylated apo A-I Proteins

apo A-I	[apo A-I] (g/L)	τ (ns)	ρ_h (ns)	ρ_h/ρ_0	a/b[a]
human	0.14[b]	10.6 ± 0.1	66.8 ± 4.0	2.10	6.5
	0.28	10.6 ± 0.1	68.2 ± 4.0	2.14	6.7
	1.80	10.6 ± 0.1	79.0 ± 4.5	2.20	7.0
	2.80[c]	10.7 ± 0.1	142.0 ± 9.0	2.23	7.4
rhesus monkey	0.14	9.9 ± 0.1	64.5 ± 4.0	2.03	5.8

[a] A hydration of 0.3 g of H_2O/g of protein and a prolate ellipsoid model are assumed; a/b ratios were estimated from ρ_h/ρ_0 (Weber, 1953b). [b] At 0.14 g/L, the human and rhesus proteins are in the monomeric form. [c] At 2.80 g/L, the apparent weight-average molecular weight of dansylated human apo A-I is 57 000 (Figure 8).

Table IV: Fluorescence Polarization Values for Dansylated Human apo A-I and Self-Association Models

apo A-I	fluorescence polarization observed[a]	fluorescence polarization calculated[b]
monomer (0.0028 g/L)	0.185 ± 0.002	
dimer (2.8 g/L)	0.192 ± 0.002	
dimer (end-to-end)		0.186
(side-by-side)		0.212
(spherical)		0.249

[a] Fluorescence polarization was measured at 25 °C. [b] The observed polarization at maximum dilution was used as the monomer polarization in the calculations for dimer models. The end-to-end and side-by-side dimerization models assume an elongated monomer with axial ratio $a/b = 6$ and dimers with $a/b = 12$ and 4.2, respectively. The spherical model assumes a spherical monomer which doubles its volume upon dimerization.

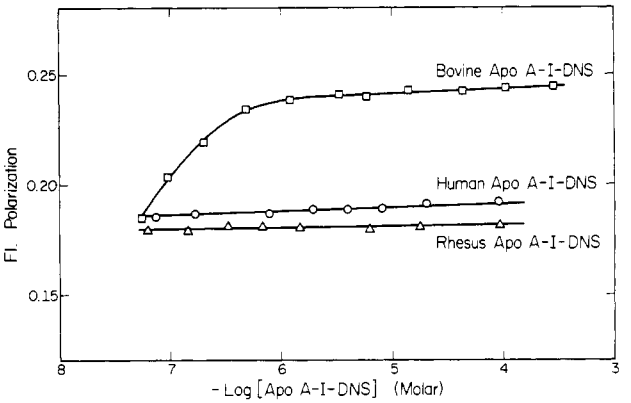

FIGURE 8: Concentration dependence of fluorescence polarization for Dns-labeled human (O) and rhesus (Δ) apo A-I proteins, in 0.02 M EDTA, 1 mM NaN_3, pH 8.6. Measurements were carried out at 25.0 °C and concentrations below 10^{-6} M. Results for Dns-labeled bovine apo A-I (□) were taken from Jonas (1975).

Concentration Dependence of Fluorescence Polarization of apo A-I Dns. Fluorescence polarization data for serial dilutions of Dns-labeled apo A-I, covering the concentration range from 3.0 to 0.002 g/L, are shown in Figure 8. At low protein concentrations (below 10^{-6} M), the polarization values were corrected by subtraction of the background contribution due to fluorescence intensity signals arising from buffer alone. The fluorescence polarization varied only minimally, from 0.192 to 0.185 for the human apo A-I and from 0.182 to 0.179 for the rhesus apo A-I, in the entire concentration range. The expected polarization values of asymmetric apo A-I monomers forming elongated end-to-end or side-by-side dimers are shown

in Table IV. The calculated polarization values which have greatest similarity to the experimental values are intermediate between the extremes for side-by-side and end-to-end dimers but more closely approach the end-to-end self-association model.

Discussion

The results of the present study with the techniques of analytical ultracentrifugation, viscometry, and fluorescence polarization show that both human and rhesus monkey apo A-I, in their monomeric form, are highly asymmetric in solution and that each monomer conforms to the shape of an elongated ellipsoid having an axial ratio of about 6.0 and dimensions of 25.2 × 151.2 Å. These dimensions, together with a value of 10.5 Å for the diameter of an α helix (Dickerson, 1964) and an α-helical content of 50% for apo A-I, suggest that this apoprotein in its monomeric form may be folded back upon itself through apposition of the hydrophobic sites of the helical segments. This molecular arrangement, which would be favored by the amphiphilic nature of the α-helical segments of apo A-I previously recognized by Segrest et al. (1974), would have a shape similar to that reported for diverse macromolecules such as myosin (Lowey & Holtzer, 1959), poly(γ-benzyl-L-glutamate) (Doty et al., 1956), and a series of protein–dodecyl sulfate complexes (Reynolds & Tanford, 1970). Moreover, this proposed folded structure of apo A-I is likely to be unstable in aqueous media and may explain the conformational changes readily exhibited by this apoprotein at protein concentrations less than 0.5 g/L.[2] Tall et al. (1975, 1976) have reported that apo A-I has a very low free energy of stabilization (ΔG = 2.4 kcal/mol), compared to that of other globular proteins (8–14 kcal/mol), and have attributed this property to a loosely folded tertiary structure which would minimize long-range interactions within the protein. The structure which we propose is consistent with their interpretation. In addition, Reynolds et al. (1977) have postulated the existence of an asymmetric apo A-I monomer from their amphiphile binding studies.

Our results have also shown that the reduced viscosity of apo A-I solutions is strongly dependent upon protein concentration. This observation suggests that strong protein–protein interactions exist (Cragg & Bigelow, 1955) and that the process of oligomerization of apo A-I is accompanied by an increase in molecular asymmetry. Of the several geometric arrangements of apo A-I considered for the association process, that most likely to describe the structure of this apoprotein in solution involves the formation of dimers through side-by-side association of the asymmetric monomers and elongation of the dimers through end-to-end association. The theoretical values of the reduced viscosity which make use of this model of association (Table I) closely approximate the values derived from our experimental data. Moreover, this mode of self-association is supported by the studies on dansylated apo A-I. It is particularly significant in this regard that dansylated apo A-I, in spite of the evidence for self-association provided by the rotational relaxation studies, gave fluorescence polarization values which were independent of protein concentration. Since the rotational diffusion of proteins is extremely sensitive to shape (Weber, 1953a,b), particularly to changes in length (Nichol et al., 1964), our inability to detect self-association in apo A-I by using fluorescence polarization reinforces our conclusion that the oligomerization of apo A-I occurs through an elongation process. An alternative explanation for the lack of correlation between fluorescence polarization and increase in protein concentration might be that the probe, upon self-association, underwent an increase in local rotation. However, the fluorescence spectra of dansyl labels at low (0.14 g/L) and high (2.8 g/L) protein concentrations had identical shapes and wavelength maxima, suggesting that the fluorophores had a similar local environment. Also, the rotational relaxation times determined at both of these concentrations indicated the presence of immobilized dansyl probes. The fact that the dansylation of apo A-I caused a block of the NH_2 terminus adds further support to the view that the self-association of apo A-I involves an end-to-end process. The effects of dansylation on proteins have been reported previously; the effects have been variable, however (Anderson, 1969; Gennis & Cantor, 1976). In the case of dansylated bovine apo A-I, Jonas (1975) observed that her modified apoprotein, contrary to human and rhesus monkey apo A-I, exhibited a marked dependence of the fluorescence polarization on protein concentration.

The molecular arrangement of apo A-I in solution proposed in this study, together with previous reports on the conformational instability of this apoprotein (Tall et al., 1975, 1976; Gwynne et al., 1974; Makino et al., 1974), its mode of self-association (Vitello & Scanu, 1976; Barbeau & Scanu, 1976; Stone & Reynolds, 1975a,b), and its detergent binding (Reynolds & Simon, 1974; Makino et al., 1974), indicates that the properties of apo A-I are quite distinct from those of most water-soluble globular proteins. Recent studies of apo A-I at the air–water surface interface support this conclusion.[3]

In human and rhesus monkey apo A-I, which have been shown to have a very similar amino acid composition, both monomers exhibit the same asymmetry and mode of self-association (i.e., side-by-side dimerization and end-to-end elongation). Previous studies have indicated, however, that the model of self-association for human apo A-I (monomer–dimer–tetramer–octamer) is different from that of rhesus monkey apo A-I (monomer–hexamer). The reasons for this difference in the elongation process are unclear at this time. They are likely to be due to subtle differences, which are still unknown. An explanation may emerge when the complete amino acid sequence of rhesus monkey apo A-I can be compared with that of the human product.

At this time we cannot establish whether the observed asymmetry of the apo A-I monomer in vitro and the elongated shape of its oligomers have relevance to the function of apo A-I in vivo. It is known that a water soluble apo A-I, essentially lipid free, can be generated during the ultracentrifugation of HDL (Osborne & Brewer, 1977); on the other hand, the occurrence of a lipid-free apo A-I in the circulation has not yet been documented. We may reasonably speculate that, if lipid-free apo A-I does occur in plasma, the existence of asymmetric monomers and oligomers with their readily accessible hydrophobic regions would favor interaction with lipophilic surfaces.

Acknowledgments

The authors thank Dr. Chang T. Lim for providing samples of human apo A-I and Dr. Linda M. Keefer for the dansyl amino acid analysis. They would like to acknowledge the services of the CORE Laboratories of the Specialized Center of Research in Atherosclerosis for the handling of the animals and preparation of the rhesus monkey plasma lipoproteins.

[2] Edelstein, C. E., Sakoda, N., & Scanu, A. M., manuscript in preparation.

[3] Shen, B. W., Scanu, A. M., & Kezdy, F. J., manuscript in preparation.

The excellent editing of this manuscript by Elisabeth Lanzl is also acknowledged.

References

Anderson, S. R. (1969) *Biochemistry 8*, 1394.
Barbeau, D. L., & Scanu, A. M. (1976) *J. Biol. Chem. 251*, 7437.
Bradbury, J. H. (1970) in *Physical Principles and Techniques of Protein Chemistry* (Leach, S. J., Ed.) pp 100–145, Academic Press, New York.
Cragg, L. H., & Bigelow, C. C. (1955) *J. Polym. Sci. 16*, 177.
Chervenka, C. H. (1970) *A Manual of Methods for the Analytical Ultracentrifuge*, pp 42–50, Beckman Instruments, Palo Alto, CA.
Dickerson, R. E. (1964) *Proteins, 2nd Ed. 2*, 603–778.
Doty, P., Bradbury, J. H., & Holtzer, A. M. (1956) *J. Am. Chem. Soc. 78*, 947.
Edelstein, C., Lim, C. T., & Scanu, A. M. (1972) *J. Biol. Chem. 247*, 5842.
Edelstein, C., Lim, C. T., & Scanu, A. M. (1973) *J. Biol. Chem. 248*, 7653.
Gennis, L. S., & Cantor, C. R. (1976) *J. Biol. Chem. 251*, 747.
Gray, W. R. (1972) *Methods Enzymol. 25* (part B), 333.
Gwynne, J., Brewer, B., & Edelhoch, H. (1974) *J. Biol. Chem. 249*, 2411.
Holtzer, A., & Lowey, S. (1959) *J. Am. Chem. Soc. 81*, 1370.
Jonas, A. (1973) *Biochemistry 12*, 4503.
Jonas, A. (1975) *Biochim. Biophys. Acta 393*, 471.
Karlin, J. B., Juhn, D. J., Starr, J. I., Scanu, A. M., & Rubenstein, A. H. (1976) *J. Lipid Res. 17*, 30.
Kratky, O., Leopold, H., & Stabinger, H. (1973) *Methods Enzymol. 25* (part D), 98.
Lowey, S., & Holtzer, A. (1959) *J. Am. Chem. Soc. 81*, 1378.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Makino, S., Tanford, C., & Reynolds, J. A. (1974) *J. Biol. Chem. 249*, 7379.
Morrisett, J. D., Jackson, R. L., & Gotto, A. M. (1975) *Annu. Rev. Biochem. 44*, 183.
Nichol, L. W., Bethune, J. L., Kegeles, G., & Hess, E. L. (1964) *Proteins, 2nd Ed. 2*, 389.
Osborne, J. C., Jr., & Brewer, H. B., Jr. (1977), *Adv. Protein Chem. 31*, 253.
Perrin, R. (1926) *J. Phys. Radium 7*, 390.
Reisler, E., & Eisenberg, H. (1970) *Biopolymers 9*, 877.
Reynolds, J. A. (1976) *J. Biol. Chem. 251*, 6013.
Reynolds, J. A., & Simon, R. H. (1974) *J. Biol. Chem. 249*, 3937.
Reynolds, J. A., & Tanford, C. (1970) *J. Biol. Chem. 245*, 5161.
Reynolds, J. A., Tanford, C., & Stone, W. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3796.
Richards, E. G., Teller, D. C., & Schachman, H. (1968) *Biochemistry 7*, 1054.
Scanu, A. M., Edelstein, C., & Keim, P. (1975) *Plasma Proteins, 2nd Ed.*, 317–391.
Schachman, H. (1959) *Ultracentrifugation in Biochemistry*, p 239, Academic Press, New York.
Segrest, J. R., Jackson, R. L., Morrisett, J. D., & Gotto, A. M., Jr. (1974) *FEBS Lett. 38*, 247.
Spencer, R. D., & Weber, G. (1969) *Ann. N. Y. Acad. Sci. 158*, 361.
Stone, W. L., & Reynolds, J. A. (1975a) *J. Biol. Chem. 250*, 8045.
Stone, W. L., & Reynolds, J. A. (1975b) *J. Biol. Chem. 250*, 3584.
Tall, A. R., Shipley, G. G., & Small, D. M. (1976) *J. Biol. Chem. 251*, 3749.
Tall, A. R., Small, D. M., Shipley, G. G., & Lees, R. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4940.
Tanford, C. (1961) *Physical Chemistry of Macromolecules*, Wiley, New York.
Teng, T. L., Edelstein, C., Barbeau, D. L., & Scanu, A. M. (1977) *J. Biol. Chem. 252*, 8634.
Vitello, L. B., & Scanu, A. M. (1976) *J. Biol. Chem. 251*, 1131.
Weber, G. (1953a) *Discuss. Faraday Soc. 14*, 33.
Weber, G. (1953b) *Adv. Protein Chem. 8*, 415.
Yphantis, D. A. (1964) *Biochemistry 3*, 297.