because a 5,5' dimer would have two chiral centers. Hence the analogous stereochemistry would be expected to be that found for tartaric acid, i.e., two enantiomers and a meso form. These three isomers presumably show two different ^{1}H NMR spectra because the enantiomers have the same chemical shifts and different from those of the meso form. The chemical shifts are as follows (dimer I): N(3)CH$_3$, 2.80, N(10)CH$_3$, 3.25, C(5)H, 4.10, ArH, 6.28–7.28 ppm; (dimer II) N(3)CH$_3$, 2.47, N(10)CH$_3$, 3.35, C(5), 4.03, ArH, 6.28–7.28 ppm.

Interconversion of the dimers is possible. If the irradiation is continued for 8 h before isolation, the ^{1}H NMR spectrum is almost pure dimer II. If, on the other hand, the mixture is incubated anaerobically in 6 N HCl, the ^{1}H NMR spectrum is almost pure dimer I. The two dimers have nearly identical absorption spectra, but they can be distinguished also on the basis of their rates of reoxidation with ferricyanide. At pH 8.6, 25 °C, dimer I is converted to the oxidized deazaflavin at a rate of 2.5×10^4 M^{-1} s^{-1}; the corresponding rate for dimer II is 7.2×10^3 M^{-1} s^{-1}. Because of the rapidity of this reoxidation, titration with K$_3$Fe(CN)$_6$ yields accurate information on the oxidation–reduction state of the isolated compounds. With both I and II exactly 1 equiv of K$_3$Fe(CN)$_6$ was consumed per equiv of oxidized deazaflavin formed, providing definitive evidence for their dimeric structure. This work will be reported in full elsewhere.

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Sedimentation Behavior of Native and Reduced Apolipoprotein A-II from Human High Density Lipoproteins[†]

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ABSTRACT: The solution properties of human serum apolipoprotein A-II, both in the native and in the reduced forms, were investigated by the technique of sedimentation equilibrium in the analytical ultracentrifuge. For both proteins, the apparent weight average molecular weights determined in neutral buffer systems were found to be dependent on protein concentration and invariant with the rotor speeds used (16 000 to 44 000 rpm) indicating a reversible self-association. These results were also found to be independent of temperature between 5 and 30 °C. The pattern of self-association of native apolipoprotein A-II could best be described by a monomer–dimer–trimer equilibrium, in agreement with previously reported data (Vitello, L. B., and Scanu, A. M. (1976), *Biochemistry 15*, 1161). The self-association pattern of apolipoprotein A-II reduced in the presence of 50 mM dithiothreitol conformed with a monomer–dimer–tetramer equilibrium similar to that reported for the native single chain apolipoprotein A-II of the rhesus monkey (Barbeau, D. L., et al. (1977), *J. Biol. Chem. 252*, 6745), but differing significantly from that reported for the reduced and carboxymethylated human product (Osborne, J. C., et al. (1975), *Biochemistry 14*, 3741).

Apolipoprotein A-II (apo-A-II),[1] one of the major protein components of human high density lipoprotein (HDL) (Morrisett et al., 1975; Scanu et al., 1975), contains two identical polypeptide chains linked together by a disulfide bond located in position 6 from the amino-terminal residue (Brewer et al., 1972). Recent reports from this and other laboratories have shown that human apo-A-II in its native unreduced form undergoes self-association in aqueous solutions. However, the reported modes of self-association show no agreement (Vitello and Scanu, 1976; Stone and Reynolds, 1975; Gwynne et al., 1975).

Recent studies from this laboratory (Barbeau et al., 1977) have indicated that the native single chain apo-A-II from the rhesus monkey undergoes self-association in solution, and that the mode of association differs from that reported for the reduced and carboxymethylated human apo-A-II (Cm-apo-A-II) (Osborne et al., 1975). These observations led us to suspect that the carboxymethyl group might influence the self-association of the single-chain apo-A-II. Based on the above information, we felt it appropriate to reinvestigate the solution properties of native apo-A-II in the ultracentrifuge and to compare the results with those of the corresponding single-chain protein in the presence of the reducing agent, dithiothreitol. In this report, we will present the results of sedimentation equilibrium studies which were carried out over a wide range of initial protein concentrations and temperatures.

Materials and Methods

Preparation and Purification of Apo-A-II. The HDL of density 1.063–1.21 g/mL were separated from fresh human sera by cumulative ultracentrifugal flotation as previously

[1] Abbreviations used: HDL, high density lipoproteins of density 1.063 to 1.210 g/mL; apo-HDL, delipidated HDL; apo-A-I, apolipoprotein A-I; apo-A-II, apolipoprotein A-II; Cm-apo-A-II, reduced and carboxymethylated apo-A-II; EDTA, ethylenediaminetetraacetic acid; DEAE diethylaminoethyl; RMS, root mean square.

TABLE I: Association Constants[a] for the Models of Self-Association of Native (Unreduced) Apo-A-II in 0.02 M EDTA, pH 7.0, 20 °C.

Mode of self-association	K_2	K_3	K_4	RMS-M_w[c]	RMS-c[d]
Isodesmic indefinite[b]	0.70×10^4	0.70×10^4	0.70×10^4	8084	1.461
Monomer-dimer	1.04×10^4			4477	0.511
Monomer-trimer		15.25×10^8		13028	1.570
Monomer-tetramer			70.29×10^{12}	94630	11.989
Monomer-dimer-trimer	6.05×10^4	12.80×10^8		333	0.029
Monomer-dimer-tetramer	8.34×10^4		18.20×10^{12}	737	0.024
Monomer-dimer-trimer-tetramer	7.48×10^4	5.72×10^8	9.88×10^{12}	667	0.032

[a] Units of M^{n-1}, where n is the degree of association. [b] Equilibrium constants are equal: $K_2 = K_3 = K_4 = \ldots K_i$ (in units of liter/mole). [c] Root-mean-square $[\Sigma[M_{w,app}(\text{exptl}) - M_{w,app}(\text{calcd})]^2/D]^{1/2}$, where D is the number of data points. [d] Root-mean-square $[\Sigma(\text{concn}_{\text{exptl}} - \text{concn}_{\text{calcd}})^2/D]^{1/2}$.

described (Scanu, 1966). The delipidated HDL, apo-HDL, was obtained by ethanol–ether (3/2, v/v) extraction at −10 °C (Scanu and Edelstein, 1971). Apo-A-II was purified from apo-HDL by gel filtration on Sephadex G-200 in 8 M urea, followed by DEAE-cellulose ion-exchange column chromatography also in 8 M urea (Scanu et al., 1972). The final preparation was extensively dialyzed against 0.01 M NH_4HCO_3, pH 8.2, lyophilized, and then stored at −10 °C under nitrogen gas. The preparations used in this study showed no detectable amount of lipids and exhibited a single band by polyacrylamide gel electrophoresis either in the presence of 0.1% sodium dodecyl sulfate or in 8 M urea up to a gel loading of 100 μg of protein. The amino acid composition was identical with that reported in the literature (Morrisett et al., 1975).

Sedimentation Equilibrium Studies. All of the ultracentrifugal experiments were performed in a Beckman Model E analytical ultracentrifuge equipped with an RTIC temperature control unit, an electronic speed control system, and Rayleigh interference optics. Meniscus-depletion sedimentation equilibrium experiments were carried out as described by Yphantis (1964), using a 12-mm charcoal-filled Epon 6 channel cell or aluminum-filled Epon double sector cell with sapphire windows. The conventional sedimentation equilibrium experiments were performed according to the method of Richards et al. (1968), using a 12-mm synthetic boundary cell. The initial protein concentration (c_0) was determined from a diffusion run immediately following the sedimentation equilibrium experiment. The interference patterns were recorded on Kodak Metallographic plates and analyzed on a Nikon 6C microcomparator with a 20-fold magnification. A fringe was measured to be 282 μm and the conversion factor from fringes to apo-A-II concentration was taken as 4.0 fringes L −g⁻¹ (Babul and Stellwagan, 1969). Prior to the sedimentation equilibrium studies, apo-A-II was dissolved in and extensively dialyzed against the appropriate buffer. A partial specific volume of 0.743 mL/g for both the native and reduced apo-A-II was calculated from the amino acid composition. The solvent density was measured at 20.0 ± 0.005 °C in a Mettler Paar density meter (Highstown, N.J.).

Reagents. All chemicals were reagent grade. Urea was recrystallized from ethyl alcohol. Prior to use, the urea solution was passed through a mixed-bed ion-exchange column of AG 501-X8 (D) (Bio-Rad Laboratories, Richmond, Calif.).

Calculation of Sedimentation Equilibrium Data. The apparent weight average molecular weight ($M_{w,app}$) was calculated according to the well-known equation

$$M_{w,app} = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \frac{d \ln c}{dr^2} \qquad (1)$$

where R is the gas constant, T is the absolute temperature, $\bar{v}$ is the partial specific volume of the protein, ρ is the density of

the solution, ω is the angular velocity, c is the protein concentration, and r is the radial distance from the center of the rotor. For the conventional sedimentation equilibrium experiments, ln c vs. r^2 data were fitted to a least-squares polynomial and values of d ln c/dr^2 were calculated at the desired radial distances. Alternatively, by meniscus-depletion sedimentation equilibria, the values of the d ln c/dr^2 were calculated by the sliding five-point least quadratic treatment (Yphantis, 1964).

Evaluation of Mode of Self-Association and Equilibrium Constants. Since the detailed methods of analysis used in this study were the same as those previously reported (Barbeau et al., 1977), only the essential aspects of them are described. For the discrete self-association, the models and the association constants were evaluated according to the equation

$$A = c_1 + 2K_2c_1^2 + 3K_3c_1^3 + 4K_4c_1^4 \qquad (2)$$

where $A = 1/[(M_1/cM_{w,app}) - BM_1]$; M_1 is the molecular weight of monomer, taken as 17 000 for native apo-A-II and 8700 for reduced apo-A-II (Brewer et al., 1972); B is the nonideality term; K_2, K_3, and K_4 are the association constants as defined by $K_n = c_n/c_1^n$ with $n = 2, 3$, and 4; c is the protein concentration in g/L, c_n and c_1 are the oligomer and monomer concentration, respectively. For the monomer–$n$mer type of self-association, the equilibrium constant was calculated pointwise by the relation $K_n = [(A/c_1) - 1]/nc_1^{n-1}$. To evaluate the monomer–dimer–trimer model, the quantities of $[(A/c_1) - 1]/c_1$ were plotted against c_1. The slope and the intercept of the resultant straight line determined the values of K_3 and K_2, respectively. For the monomer–dimer–tetramer model of association, K_4 and K_2 were evaluated from the $[(A/c_1) - 1]/c_1$ vs. c_1^2 plot. To analyze the monomer–dimer–trimer–tetramer model, the equilibrium constants were also calculated by evaluating eq 2 according to the method of the least-squares polynomial as previously reported (Barbeau et al., 1977).

For the isodesmic indefinite self-association, the intrinsic association constant (k) was calculated from the equation (Van Holde and Rossetti, 1967)

$$4k = \left[\frac{(M_{w,app}/M_1)^2}{(1 - cBM_{w,app})^2} - 1 \right] \frac{1}{c} \qquad (3)$$

Results

Studies on Native Apo-A-II. Native apo-A-II, dissolved in 0.02 M EDTA, pH 7, at 20 °C, was studied using both the conventional and meniscus-depletion sedimentation equilibrium methods. The rotor speeds used in these studies ranged from 16 000 to 44 000 rpm and the initial protein concentrations ranged between 0.3 to 2.0 g/L. When the data from these experiments were combined, the resulting $M_{w,app}$ vs. concen-
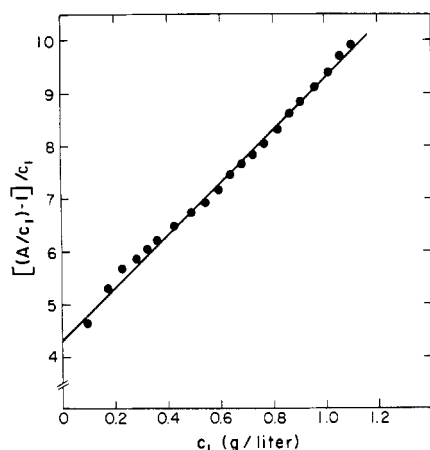
FIGURE 1: Plot of $[(A/c_1) - 1]/c_1$ vs. c_1 for native apo-A-II. The association constants K_2 and K_3 were obtained from the intercept and the slope of the straight line, respectively.

FIGURE 2: Weight fraction of monomer (f_1), dimer (f_2), and trimer (f_3) as a function of native apo-A-II concentration. The values were calculated by using the association constants $K_2 = 6.05 \times 10^4$ M^{-1}, and $K_3 = 12.80 \times 10^4$ M^{-2}.

FIGURE 3: Apparent weight average molecular weight of reduced apo-A-II as a function of protein concentration in 0.02 M EDTA, pH 7.4, containing 50 mM dithiothreitol. The smooth curve represents the average values of $M_{w,app}$ obtained from four sedimentation equilibrium experiments conducted at 20 °C. Other data are from experiments at 5 (●), 27 (△), and 30 °C (X).

tration plots were found to be overlapping. This observation indicated that the apolipoprotein was reversibly self-associating in solution and that the $M_{w,app}$ was not affected by hydrostatic pressure.

We then examined several possible models of self-association with values of BM_1 ranging from 0 to 0.03 L/g. The results indicated that the use of the nonideality term (BM_1) did not improve the fitting of the experimental data. Table I presents a list of the models examined, the equilibrium constants and the RMS deviations calculated at $BM_1 = 0$. Based on the large values of RMS, it is apparent that the monomer–$n$mer models and the isodesmic indefinite self-association did not describe the experimental data. On the other hand, the monomer–dimer–trimer, monomer–dimer–tetramer, and monomer–dimer–trimer–tetramer models exhibited reasonably small RMS deviations. Figure 1 shows that the plot of $[(A/c_1) - 1]/c_1$ vs. c_1 is linear, as would be expected for the monomer–dimer–trimer model. The lack of upward curvature at high protein concentration indicates the absence of molecular species larger than a trimer. This information, together with the smallest RMS deviations observed, suggests that the best fitting model is the monomer–dimer–trimer association. The equilibrium constants for this model are $K_2 = 6.05 \times 10^4$ M^{-1} and $K_3 = 12.8 \times 10^8$ M^{-2}. Figure 2 shows the distributions of the monomer, dimer, and trimer as a function of native apo-A-II concentration.

Studies on Reduced Apo-A-II. The reduction of the disulfide bond in apo-A-II was carried out by dialysis overnight of the protein in 0.02 M EDTA, pH 7.4, containing an excess amount of dithiothreitol (4 or 50 mM). The solution properties of the reduced apo-A-II were then examined by the method of meniscus-depletion sedimentation equilibrium. The ln c vs. r^2 plots showed pronounced upward curvature, indicating heterogeneity with respect to molecular weight. When the data from several experiments, which were conducted at different initial protein concentrations and rotor speeds, were combined, the $M_{w,app}$ vs. c plots were found to be overlapping with each other. Apo-A-II reduced in the presence of either 4 or 50 mM dithiothreitol gave identical $M_{w,app}$ vs. c plots. These results were taken to indicate that the reduction of the disulfide bond had reached completion and that the reduced apo-A-II was self-associating in a reversible manner.

In order to evaluate the mode of self-association of the reduced apo-A-II, we calculated the average values of $M_{w,app}$ as a function of protein concentration (Figure 3). The data were first examined for three types of monomer–$n$mer systems,
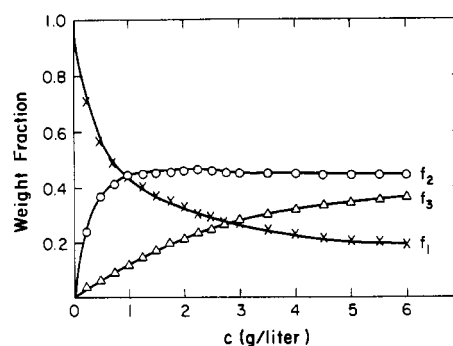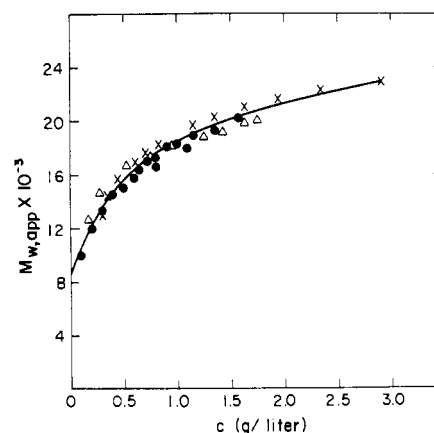
namely: monomer–dimer; monomer–trimer; and monomer–tetramer. The results showed that the ideal monomer–$n$mer models ($BM_1 = 0$) could not describe the self-association of reduced apo-A-II in solution, because of the large differences between the experimental and the calculated values, both in terms of M_w and protein concentration. In order to determine whether the same models could be applicable to nonideal cases, the association constants were calculated at BM_1 values from 0.001 to 0.06 L/g. No significant reduction of the values of RMS deviation was observed. This led to the conclusion that the self-association system involved more than two molecular species.

We then examined the ideal monomer–dimer–trimer model. For this purpose, we calculated the quantities of $[(A/c_1) - 1]/c_1$ at $BM_1 = 0$ as a function of the monomer concentration (Figure 4). From the intercept and slope of the straight line drawn through the experimental points, we calculated $K_2 = 0.90$ L/g and $K_3 = 12.43$ L^2/g^2, respectively. However, the plot exhibited a slight upward curvature, suggesting that species larger than a trimer were present. For a nonideal monomer–dimer–trimer model using values of BM_1 from 0.01 to 0.03 L/g, plots with pronounced upward curvature were observed.

We further considered the monomer–dimer–tetramer model at values of BM_1 ranging from 0 to 0.03 L/g. A linear relationship of $[(A/c_1) - 1]/c_1$ vs. c_1^2 was found only for the BM_1 term equal to or approaching zero. Thus, the self-association of reduced apo-A-II could be explained by an ideal mono-

TABLE II: Association Constants[a] for the Models of Self-Association of Reduced Apo-A-II in 0.02 M EDTA, 50 mM Dithiothreitol, pH 7.4, 20 °C.

Mode of self-association	K_2	K_3	K_4	RMS-M_w[b]	RMS-c[c]
Monomer–dimer	6.60×10^4			4717	0.379
Monomer–trimer		11.15×10^8		1259	0.096
Monomer–tetramer			28.46×10^{12}	14531	1.476
Monomer–dimer–trimer	0.78×10^4	9.41×10^8		561	0.043
Monomer–dimer–tetramer	3.22×10^4		8.80×10^{12}	331	0.038
Monomer–dimer–trimer–tetramer	3.48×10^4	0.60×10^8	7.92×10^{12}	816	0.038
Isodesmic indefinite[d]	0.74×10^4	0.74×10^4	0.74×10^4	2483	0.458

[a] Units of M^{n-1}, where n is the degree of association. [b] Root-mean-square deviation $[\Sigma[M_{w,app}(exptl) - M_{w,app}(calcd)]^2/D]^{1/2}$, where D is the number of data points. [c] Root-mean-square deviation $[\Sigma(concn_{exptl} - concn_{calcd})^2/D]^{1/2}$. [d] Equilibrium constants are equal: $K_2 = K_3 = K_4 = \ldots K_i$ (in units of liter/mole).
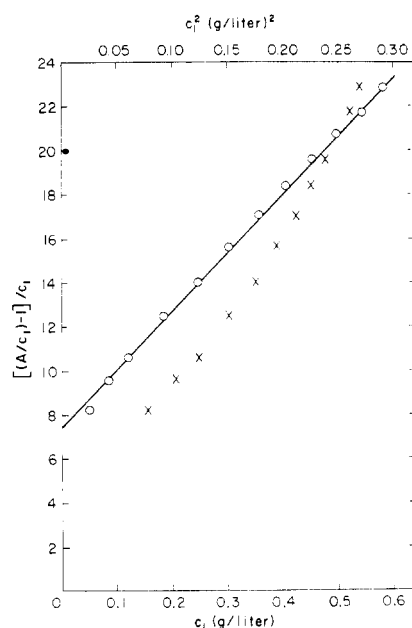

FIGURE 4: Plots to test the mode of self-association of reduced apo-A-II. The $[(A/c_1) - 1]/c_1$ vs. c_1 plot illustrates the monomer–dimer–trimer model (X) and the $[(A/c_1) - 1]/c_1$ vs. c_1^2 plot illustrates the monomer–dimer–tetramer association (O). The association constants were calculated from the intercepts and the slopes of these plots (see text for details).

FIGURE 5: Weight fraction of monomer (f_1), dimer (f_2), and tetramer (f_4) as a function of reduced apo-A-II concentration. The values were calculated by using the association constants $K_2 = 3.22 \times 10^4$ M^{-1}, and $K_4 = 8.80 \times 10^{12}$ M^{-3}.

mer–dimer–tetramer model, with $K_2 = 3.71$ L/g and $K_4 = 13.36$ L^3/g^3. Alternatively, we were able to fit eq 2 to a polynomial and to solve the various coefficients by the method of least squares after eliminating the $3K_3c_1^3$ term (Barbeau et al., 1977). The results of this calculation indicated that, at BM$_1$ = 0, the RMS deviations were at a minimum, and that the association constants so obtained were identical with those derived from the graphical presentation. We also considered the monomer–dimer–trimer–tetramer model at various BM$_1$ values. Again, the RMS deviations were found to be at a minimum for BM$_1$ = 0, with association constants of $K_2 = 4.00$ L/g, $K_3 = 0.78$ L/g, and $K_4 = 12.02$ L^3/g^3. This model gave a small value of K_3 indicating that the trimer did not play a significant role in determining the M_w of reduced apo-A-II.

We finally examined the data according to eq 3 in terms of the isodesmic indefinite self-association. Since the values of the RMS deviations were large for both BM$_1$ = 0 and BM$_1$ > 0, we concluded that this model did not describe the experimental data.

In Table II, we have listed the modes of self-association examined, the respective association constants in units of M^{n-1} (BM$_1$ = 0), and the values of RMS deviations. Based on the values of the RMS deviations, three models are plausible: the
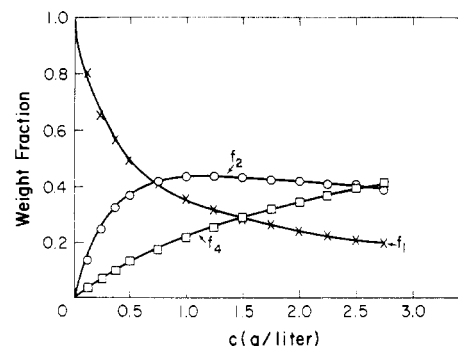
monomer–dimer–trimer; the monomer–dimer–tetramer; and the monomer–dimer–trimer–tetramer. Although a clear distinction among them cannot be established, two of these models appear to be of less importance due to the following reasons: First, the plot of $[(A/c_1) - 1]/c_1$ vs. c_1 showed an upward curvature indicating that species larger than a trimer were present. This would make the monomer–dimer–trimer model less likely. Second, when the monomer–dimer–trimer–tetramer model was examined, the calculated association constant K_3 was small suggesting that the presence of trimer was not essential for describing the experimental data. Therefore, the model of choice was considered to be the monomer–dimer–tetramer equilibrium having the association constants $K_2 = 3.22 \times 10^4$ M^{-1} (3.71 L/g) and $K_4 = 8.80 \times 10^{12}$ M^{-3} (13.36 L^3/g^3). The distribution of each species according to this model is given in Figure 5 where we have plotted the weight fractions as a function of protein concentration.

Self-Association of Native and Reduced Apo-A-II at Different Temperatures. It has been reported that the fluorescence polarization of tyrosine and the difference spectra of both the native apo-A-II and the reduced and carboxymethylated apo-A-II are dependent on temperature (Osborne et al., 1975). In the present study, we examined the possible effect of temperature on the self-association of both native and reduced apo-A-II utilizing sedimentation equilibrium results obtained at temperatures ranging from 5 to 30 °C. When the data from these experiments were combined, the $M_{w,app}$ vs. concentration plots for both native and reduced apo-A-II were found to be overlapping within the range of experimental error. As examples, Figure 3 shows the plots of $M_{w,app}$ vs. concentration for reduced apo-A-II obtained at 5, 27, and 30 °C. Similar plots were obtained for native apo-A-II at comparable temperatures. Therefore, we concluded that temperature had

no effect on the self-association of both forms of human apo-A-II.

Discussion

The results of the present study have shown that native unreduced apo-A-II self-associates in 0.02 M EDTA, pH 7.0, 20 °C, best conforming with a monomer–dimer–trimer association. This mode of self-association is in accord with the results obtained in the same buffer but at pH 8.6 by Vitello and Scanu (1976) who also considered the indefinite self-association. In the present study, by employing protein concentrations up to 6 g/L, we were able to rule out the latter mode of self-association. Since the monomer–dimer–trimer association best applies to native apo-A-II in 0.02 M EDTA, both at pH 7.0 and at pH 8.6, it appears that the pH of the solution has no significant effect on the self-association of this apolipoprotein. Recent sedimentation equilibrium data from this laboratory (unpublished) indicates an identical mode of self-association for native apo-A-II in 0.15 N NaCl, pH 7, at 20 °C. These observations suggest that the interactions among the apo-A-II monomers are not electrostatic in nature. Our proposed model of self-association is at variance with the recent report by Gwynne et al. (1975) who used a monomer–dimer equilibrium to fit their sedimentation equilibrium data obtained in 0.01 M phosphate, pH 7.4, at 20 °C. Our data are also at variance with those of Stone and Reynolds (1975) who described native human apo-A-II as a stable dimer in 0.02 M Tris-HCl, pH 8.3, at 20 °C. The reasons for the divergent conclusions are not readily apparent particularly because detailed plots of $M_{w,app}$ vs. concentration and RMS deviations were not given in those studies. However, it appears unlikely that the various buffers used in the various studies accounted for the differences in results. The failure in those reports to detect molecular species larger than a dimer could be explained by the relatively low protein concentrations used.

The present results have also shown that the single chain human apo-A-II, which was prepared in the reduced form in the presence of dithiothreitol, self-associates in aqueous solutions according to a monomer–dimer–tetramer equilibrium. Osborne et al. (1975) examined the solution properties of human Cm-apo-A-II and concluded that this chemically modified single chain polypeptide conformed with a monomer–dimer mode of self-association. It is likely that the introduction of the carboxymethyl group may have affected the self-association of the apoprotein. The mode of self-association observed in the present study is in agreement with that obtained for the naturally occurring single chain apo-A-II from rhesus monkey (Barbeau et al., 1977), which has a primary sequence very similar to that of human apo-A-II (Edelstein et al., 1976). Thus, the failure to detect tetramers of Cm-apo-II may be attributed to the low protein concentrations employed in the analysis (Osborne et al., 1975).

Our study has also demonstrated that temperature has no effect on the self-associations of both native and reduced apo-A-II, at least within the temperature range studied (5 to 30 °C). Gwynne et al. (1975) and Osborne et al. (1975) have suggested that self-association, at least in part, was responsible for the temperature-induced spectroscopic changes that they observed with both native and Cm-apo-A-II. In view of our results, their conclusion is in need of reevaluation. It is of interest that temperatures between 5 and 20 °C have been shown

not to affect the self-association pattern of canine apo-A-I[2] and β-lactoglobulin c (Sarquis and Adams, 1976).

To sum up, the results of this study have shown that the covalently linked double chain native apo-A-II and its reduced single chain polypeptide both self-associate in aqueous solutions and that the respective association constants are insensitive to temperature and hydrostatic pressure. The information obtained in this study should help in the clarification of controversial literature findings and also prove useful in the planning and interpretation of binding studies. A preliminary account on such studies has recently appeared (Ritter and Scanu, 1977).

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