Reversible Association of Apoferritin Molecules. Comparison of Light-Scattering and Other Data*

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ABSTRACT: The heterogeneity of horse spleen apoferritin previously reported was investigated concurrently by light scattering, polyacrylamide gel electrophoresis, ultracentrifugation, and diffusion; the corresponding horse spleen ferritin was studied by polyacrylamide gel electrophoresis and by electron microscopy. Light-scattering data indicate that reversible association of apoferritin molecules, dependent on concentration, takes place in solution. The monomer is approximately spherical. As determined by light scattering, the molecular weight of the monomer lies between 430,000 and 470,000. The following association constants (25°) were obtained: k_2 (monomers to dimers) = 1.67×10^6 and k_3 (dimers to trimers) = 3.63×10^5. The corresponding changes in free energy are $\Delta F^\circ_2 = -7.72$ kcal/mole and $\Delta F^\circ_3 = -7.59$ kcal/mole. Sedimentation velocity diagrams revealed 18S, 25S, and 33S peaks. Gel electrophoresis demonstrated the presence of α, β, γ, δ, and ϵ fractions in the apoferritin. Diffusion experiments confirmed polydispersity and indicated that the value for $D_{20,w}$ used in the past to calculate monomer molecular weight of apoferritin by the Svedberg equation must be too low. Hence, the monomer molecular weight should be less than 465,000, a value frequently cited. Statistical evaluation of electron micrographs revealed similar frequency distributions of monomers, dimers, and trimers in α-, β-, and γ-ferritins. This result is consistent with the occurrence of reversible association in solution. We conclude that reversible association of molecules, involving noncovalent bonds, accounts for the heterogeneity of apoferritin and ferritin. Possibly, dissociable Fe^{2+}, located on the external surface of the monomer, may be implicated in the process of association.

It has been shown that both ferritin and apoferritin, isolated from any one of a number of mammalian species, are heterogeneous by gel electrophoresis (Saddi, 1962; Richter, 1963a,b, 1964; Kopp *et al.*, 1963; Theron *et al.*, 1963; Suran and Tarver, 1965). By ultracentrifugation, three components have been found in horse spleen apoferritin, and these correspond to the three major fractions identified by gel electrophoresis (Suran and Tarver, 1965; Harrison and Gregory, 1965; Kopp *et al.*, 1966). The electrophoretic components have been termed α-, β-, γ-, δ-, and ϵ-ferritins and -apoferritins (Richter, 1963a,b; Harrison and Gregory, 1965; Kopp *et al.*, 1966). The mobilities of each of these fractions of horse spleen apoferritin in starch or polyacrylamide gels are the same as those of the corresponding fractions of horse spleen ferritin (Kopp *et al.*, 1963; Richter, 1964; Suran and Tarver, 1965; Harrison and Gregory, 1965; Kopp *et al.*, 1966). Evidence obtained in three laboratories (Suran and Tarver, 1965; Harrison and Gregory, 1965; Kopp *et al.*, 1966) has made it appear likely that this heterogeneity results from the presence of dimers, trimers, tetramers, and higher polymers of a protein monomer which, in turn, is characteristic for the animal species or cell line from which it is obtained. It has been proposed (Harrison and Gregory, 1965) that in the polymers, monomer units are firmly and stably linked since the sedimentation coefficients of heterogeneous apoferritin were not changed by treatment with 1 M NaCl or sodium dodecyl sulfate or thioglycolate, and since electrophoretic patterns of horse spleen ferritin and apoferritin in starch or polyacrylamide gels were not altered by treatment with 7–8 M urea, 2–6 M guanidine hydrochloride, or with 5% mercaptoethanol. Harrison and Gregory (1965) have stated that monomers are linked by covalent bonds while Suran and Tarver (1965) have left the question open. From the biologist's point of view, the existence of covalent linkages between monomer units would have deeper and, we believe, more significant implications than would the occurrence of more loosely associated complexes, for the latter might well be produced *in vitro* as is true of many proteins; but proof of covalently linked dimers, tetramers, etc., in simple cell extracts would imply the existence of an intracellular polymerization reaction. Beyond this, the problem has a bearing on the finding that ferritins synthesized *in vitro* by several malignant human and rat cell lines differ in their absolute electrophoretic mobilities from ferritins occurring in nonmalignant cell lines, and in livers, spleens, or kidneys (Richter, 1963b, 1964, 1965). For these reasons, we have investigated solutions of horse spleen apoferritin by means of light-scattering

* From the Department of Pathology, Cornell University Medical College, New York, New York 10021. *Received May 2, 1967.* This investigation was supported by Research Grants AM 00823 and GM 12023 from the National Institutes of Health, U. S. Public Health Service, and by an award (I-137) from the Health Research Council of the City of New York.

techniques, and concurrently by gel electrophoresis, ultracentrifugation, and diffusion methods. The results were compared with data on ferritin obtained by electron microscopy.

Materials and Methods

Preparation of Ferritin and Apoferritin. Samples of ferritin from horse spleen were obtained: (1) from Mann Research Laboratories, Inc., New York, N. Y.; (2) from Nutritional Biochemicals Inc., Cleveland, Ohio; and (3) by extraction of horse spleens freshly taken at a slaughter house.

The commercial samples had been previously crystallized twice by means of $CdSO_4$, then redissolved in 0.9% aqueous NaCl solution. Such material was recrystallized three or four times with 5–10% aqueous $CdSO_4$ solution, essentially by the method of Granick (1946), with interposed solubilizations in 1–2% aqueous solutions of $(NH_4)_2SO_4$, care being taken to eliminate, by centrifugation, any insoluble amorphous material. The extraction and preparation of crystalline ferritin from the horse spleens was done according to the method described by Behrens and Taubert (1952).

Apoferritin was prepared from recrystallized ferritin as described by Bielig and Bayer (1960), with only minor modifications. The purified, recrystallized samples of apoferritin were dialyzed against the buffer or solution chosen for the analytical procedure to be performed and listed further on. One batch was dialyzed against a sodium phosphate buffer at pH 6.8, $\Gamma/2$ of 0.1 (Long, 1961), diluted 1:1 (v/v) with 2% aqueous NaCl solution, and then put through a column of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden).

Enrichment Procedures. Elution from polyacrylamide gel columns yielded purified α-, β-, and γ-ferritins after repeated cycles of electrophoresis, done as described further on. The gel particles were separated from the eluates by repeated centrifugation, including a 2-hr run at 10,000 rpm in the type 40 rotor of a Spinco Model L ultracentrifuge.

Absorption elution with *uncharged* cellulose, followed by filtration through Sephadex G-200, was also tried since DEAE-cellulose chromatography had in the hands of others produced fractions that could not be directly related to the electrophoretic α, β, and γ fractions (Suran and Tarver, 1965). The procedure was as follows. Four-times-crystallized horse spleen apoferritin in aqueous 0.5% $(NH_4)_2SO_4$ solution was adsorbed onto powdered cellulose. Enough aqueous 4 M $(NH_4)_2SO_4$ solution was added to obtain a concentration of 2 M $(NH_4)_2SO_4$. The suspension was packed into a column. Elution was started with an aqueous solution of 1.1 M $(NH_4)_2SO_4$, followed by 0.8 and 0.5 M $(NH_4)_2SO_4$, and finally with distilled water. The 1.1, 0.8, and 0.5 M eluates were found to be enriched in β-apoferritin (as determined by gel electrophoresis). They were pooled and adsorbed on cellulose. This was followed by another elution, using 1.1, 0.8, and 0.5 M aqueous $(NH_4)_2SO_4$. The resulting eluates were pooled, and apoferritin was precipitated by addition of solid $(NH_4)_2SO_4$ (to 30 g/100 ml). The precipitates were dissolved in and dialyzed against phosphate buffer at pH of 6.8, $\Gamma/2$ of 0.1 (Long, 1961). This material was run through a Sephadex G-200 column, using the same buffer for washout. Fractions collected gave a non-Gaussian peak, owing to tailing. The ascending portion of the elution curve was found to correspond on disc gel electrophoresis to fractions enriched from 2 to 2.5 times in β-apoferritin. Therefore, these fractions were pooled and the pool was sedimented by preparative ultracentrifugation at 145,000*g* (18 hr). The sediment was redissolved in the minimum necessary volume of phosphate buffer at pH 7.0, $\Gamma/2$ of 0.1 (Long, 1961), to effect solution.

By using only filtration through Sephadex G-200 in phosphate buffer at pH 7.0, $\Gamma/2$ of 0.1, and then recycling the fractions indicated by ascending limbs of elution curves, enrichment of β-apoferritin from approximately 11.5 to 19.8% of total apoferritin content was effected.

Analyses for Iron, Nitrogen, and Protein. Quantitative analyses for residual iron in *apoferritin* were carried out by the bathophenanthroline method (Diehl and Smith, 1960).[1] This method was also used on very dilute samples of *ferritin*, but for more concentrated ones (>1% iron by weight) Lorber's (1927) method was used. Nitrogen was determined by micro-Kjeldahl procedure (Kabat and Mayer, 1961) and protein (apoferritin only) by the method of Lowry *et al.* (1951), and also refractometrically.

Light Scattering and Refractometry. A Brice-Phoenix dual-channel photometer, Model 2000 DM, was used. The instrument was calibrated with an opal glass diffusor furnished by the manufacturer, and was checked with standard sample no. 705, polystyrene, obtained from the National Bureau of Standards, Washington, D. C., and subsequently dispersed in cyclohexane. The weight-average molecular weight found by the National Bureau of Standards was 179,300 with a standard deviation of ±740. Two determinations by us gave 180,180 and 177,030, respectively.

Scattering ratios were determined at 25° with incident light from a mercury vapor lamp at 436 or 546 mμ, using appropriate filters. The reproducibility of results obtained at either of these wavelengths in sequential repeat determinations was within ±2%. Rayleigh–Debye scattering ratios were obtained from measurements in square cells and in semioctagonal cells, dissymetry ratios were determined with semioctagonal cells, and a cylindrical cell was used for measurements over a wide range of angles.

Solutions of apoferritin were clarified by preliminary centrifugation at speeds up to 8000 rpm, followed by repeated filtration through filters, directly into the scattering cell. Filters used were Millipore, types GS and HA (Millipore Filter Corp., Bedford, Mass.), and Gelman types GA-9 or GA-10 (Gelman Instru-

[1] The iron reagent bathophenanthroline was prepared by G. Frederick Smith Chemical Co.

TABLE I: Data Obtained by Light Scattering from Solutions of Apoferritin.

Sample	Residual Fe (%)	Fe:N	Solvent (aq) (M)	pH	Γ/2	Hc/τ at Infinite Diln	Mol Wt	Z at Infinite Diln
2	0.2	0.0145	NaCl (0.34)	5.50	0.34	2.18	459,000	
3	0.2	0.0145	NaCl (0.34)	5.50	0.34	2.18	459,000	
5a	0.2	0.0144	NaCl (0.34)	5.48	0.34	2.12	472,000	
5b	0.2	0.0144	NaCl (0.34)	5.50	0.34	2.14	467,000	
5c	0.2	0.0144	Borate buffer	8.37	0.06	2.14	467,000	
5e	0.2	0.0144	Borate buffer	8.32	0.11	2.38	420,000	1.10
6f[a]	0.1		Phosphate buffer	7.00	0.10	2.32	431,000	1.03
7b-1			Phosphate buffer	7.00	0.10	2.32	431,000	
7a			Phosphate buffer	7.00	0.10	2.32	431,000	1.06

[a] Preparation 6f was used before and after treatment with Versene (Na-EDTA) in order to check for possible effects of residual cadmium ions. However, the scattering curves obtained were not significantly different.

ment Co., Ann Arbor, Mich.). Mean pore sizes ranged between 4500 and 500 A. Two samples were filtered through 4500-A filters, four samples through 2200-A filters. Three other samples were filtered through 500-A filters. One sample was first filtered through 1000-A filters, then light-scattering ratios were obtained on various dilutions of this sample; afterwards the same material was filtered through 500-A Gelman filters and light-scattering ratios were determined once more. Analyses by gel electrophoresis were also done before and after filtration through the various filters. It was determined by this means that even a 500-A filter did not significantly influence the electrophoretic patterns; in particular, the relative proportions of α-, β-, and γ-apoferritins remained unchanged.

Refractive index increments of solutions were determined at 25° with a Brice-Phoenix differential refractometer, Model BP-2000-V. The instrument was calibrated with solutions of KCl of known concentrations. Data of Kruis (1936) were used for reference. Concentrations of apoferritin were calculated from micro-Kjeldahl analyses for nitrogen, choosing a working factor of 6.25 for converting nitrogen to protein.

Ultracentrifugation and Diffusion. Sedimentation velocity studies were done with Spinco Model E analytical ultracentrifuges, using single- or double-sector wedge cells. Sedimentation diagrams were obtained with schlieren optics. The ultracentrifuges were run at either 44,770 or 37,020 rpm. Concentrations of apoferritin varied from 1.0 to 0.1%. Buffers in which apoferritin was dissolved, and pH and ionic strengths are listed in Table I. The method of Schachman (1957) was used in calculating sedimentation constants.

The diffusion coefficient of apoferritin was determined in a Tiselius cell with a Perkin-Elmer electrophoresis diffusion apparatus (Model 238) equipped with Rayleigh interference optics. Measurements and calculations were made by the integral fringe method, as described by Schachman (1957).

Electrophoresis. Columns 0.5 or 1.0 cm in diameter and containing single polyacrylamide gels were used, with the concentration of gels at 5.6%. The formulation of the gels was essentially in proportions recommended by Ornstein (1964), from three components: (A) glycine, KOH, and tetraethylmethylenediamine; (B) ammonium persulfate; and (C) acrylamide dissolved in distilled water, *N,N*-methylenebisacrylamide, and $K_3Fe(CN)_6$.

Apoferritin (20–40 μl) in the solutions employed was added to the tops of the gel columns. Electrophoresis was done vertically as described by Ornstein (1964), through a glycine–2,6-lutidine buffer at pH 8.3, at constant currents of 4 ma/tube, though at the start of each run the current was raised to 8 ma/tube for 5 min to allow rapid penetration of apoferritin into the gel. Generally, good separation was obtained in 35–45 min. Protein was stained with Amido-Schwarz. The Prussian blue reaction for ferric iron was used to check for presence or absence of iron in apoferritin and to compare ferritin with apoferritin. The distribution of proteins in the gels was determined with a densitometer manufactured by Canal Industrial Corp. (Rockville, Md.) for use with disc gel columns.

Electron Microscopy. Ferritin, rather than apoferritin, was used for electron microscopy since molecules of ferritin can be directly visualized by virtue of the dense FeOOH micelles that, in effect, serve as natural markers (Farrant, 1954). To visualize *apoferritin*, complicating factors such as stains or metal-shadowing procedures would have to be introduced and these might influence the state of association of molecules. Therefore, electron microscopy was done on samples of *ferritin*,

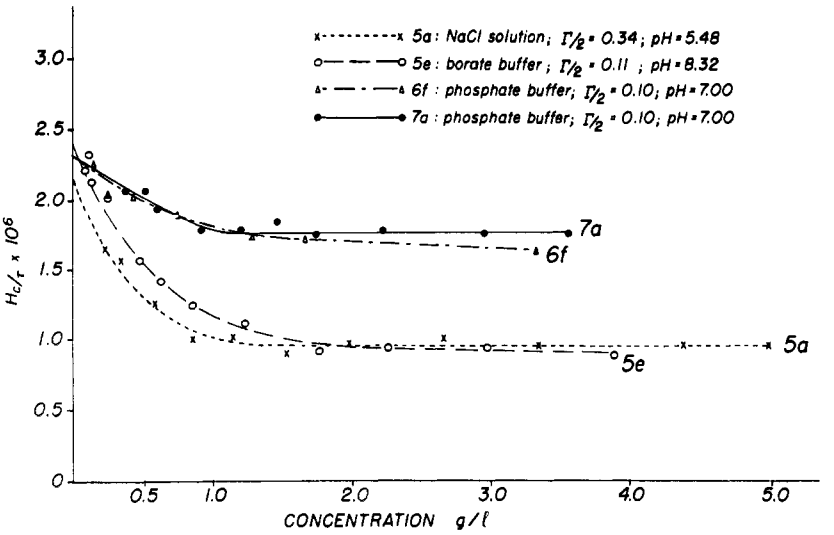

FIGURE 1: Light-scattering curves obtained on apoferritin. Additional data are given in Table I.

containing α, β, and γ fractions. Single droplets of a chosen solution were dropped onto formvar-coated copper-mesh specimen grids. Any excess liquid was drained gently off the grid by the touch of a piece of filter paper. Frequency distributions of single ferritin molecules, doublets, triplets, etc., were determined for very dilute solutions, using distilled water as diluent of the initial solution of 0.9%, aqueous NaCl. In each case, the degree of dilution chosen was that which produced wide and random separation of particles (*i.e.*, of single molecules, doublets, or triplets, etc.) to be counted.

A Siemens Elmiskop I electron microscope was used. The iron hydroxide micelles of ferritin, which average about 60 A in over-all diameter (as seen by electron microscopy), were used to check magnifications.

Results

Light Scattering. Results obtained on several samples of horse spleen apoferritin are shown in Figure 1 and Tables I–III. The following relations were used to make calculations

$$\frac{Hc}{\tau} = \frac{1}{M} + 2Bc + \ldots. \qquad (1)$$

where τ is the turbidity, c the concentration, M the weight-average molecular weight, B an interaction constant, and H a factor derived from the Rayleigh-scattering law (*cf.* Stacey, 1956; Geiduschek and Holtzer, 1958; Peterlin, 1959; Tanford, 1961). At infinite dilution, and also when the slope of the scattering curve is zero, this equation reduces to

$$\frac{Hc}{\tau} = \frac{1}{M} \qquad (2)$$

Plots of Hc/τ against c, derived from the light-scattering measurements of four different preparations of horse spleen apoferritin, are shown in Figure 1. The four intercepts with the ordinate were in reasonable agreement (within the expected error of the light-scattering method). As shown in Table I, large changes in pH affected the location of Hc/τ at infinite dilution relatively little, nor did variation in ionic strength of the solutions from 0.06 to 0.34 have any definite effect on the value of $(1/M)$ at the ordinate. On the other hand, the location of segments of the curves for which $2B$ approximates 0, and for which Hc/τ remains constant, varied in different samples of apoferritin. Although the data in Figure 1 might be interpreted as indicating that dissociation takes place more readily at pH 7 than at more acid or more alkaline pH, our experience to date suggests that dissociability of apoferritin varied somewhat from sample to sample. The isoelectric point of horse spleen apoferritin is in the vicinity of pH 4.6; hence all of the light scattering was done well above the isoelectric point.

Since it was thought possible that cadmium ions, retained in apoferritin even after thorough dialysis, might have affected the results obtained, an apoferritin solution in phosphate buffer was treated with Na-EDTA, then dialyzed against phosphate buffer, filtered through a 500-A Gelman filter, and reanalyzed by the light-scattering method. The results were almost identical with those obtained on the same batch of apoferritin (sample 6f, Table I; Figure 1) prior to treatment with Na-EDTA.

Dissymmetry ratios (Z) were also determined, with results as shown in Table I. Since the apoferritin monomer is nearly spherical (Fischbach and Anderegg, 1965; Bielig *et al.*, 1966), measuring about 120 A in diameter when hydrated, the deviations of Z from unity (Table I) can be assumed to be based on experimental error, probably due to minute amounts of residual dust in the solutions. As a check, light-scattering

TABLE II: Comparison of Data Obtained by Disc Gel Electrophoresis and by Light Scattering.

Sample	Electrophoretic Components (%)					Light Scattering	
	α	β	γ	δ	ϵ	Mol Wt at Infinite Diln	Mol Wt at $2B \simeq 0$ Extrapolated
5e	76.5	14.6	5.0	2.1	1.8	420,000	1,050,000
6f	89.7	8.1	1.8	0.4		431,000	580,000
7a	95.3	4.7				431,000	560,000
7b-1	84.5	11.5	4.0			431,000	

ratios were determined for a series of angle pairs, θ and $(180 - \theta)$. The result was a straight-line function in good agreement with the relation $R(\theta) = K(1 + \cos^2 \theta)$, as expected for Rayleigh scattering. We conclude from this that at infinite dilution, dissymmetry was absent or insignificant.

There was no obvious correlation between the degree of heterogeneity detected by electrophoresis (*i.e.*, the percentages of α-apoferritin and non-α-apoferritin) and the light-scattering curves (Table II). In all cases, the light-scattering curves were similar in that with decreasing concentration of apoferritin they turned upwards from slopes close to zero until they approached the ordinate at the level corresponding approximately to the molecular weight of monomer molecules of apoferritin. Steiner (1952, 1953) developed a theoretical treatment of reversible association of protein molecules in relation to light scattering. In his treatment the simplifying assumption of ideal solution behavior was made, and was utilized by Doty and Myers (1953) and by Townend and Timasheff (1960) in further work. In treating our data, we have adopted the assumption of ideal solution behavior. It should be noted, however, that Adams and Filmer (1966) have developed a treatment for nonideality based on sedimentation equilibrium and on osmotic pressure and have suggested that light-scattering data might be analyzed for nonideality in a similar way. A satisfactory theoretical basis for this extension of Adams' and Filmer's work to light scattering has yet to be developed, however.

According to Steiner (1952, 1953), at any concentration (c) of protein in solution, the weight fraction (X_1) of monomer is given by

$$\ln X_1 = \int_0^c \frac{((M/m) - 1)}{c} dc \qquad (3)$$

where X_1 = monomer weight fraction, M = weight-average molecular weight of polymer, m = weight-average molecular weight of monomer, and c = concentration of apoferritin.

A series of measurements of M plus knowledge of the monomer molecular weight (m) from light-scattering or other data can thus be used to determine values of X_1 by graphic integration of eq 3. The following equation can be used to obtain association constants k_2, k_3, k_4, etc., of the polymers

$$\frac{M}{mX_1} = 1 + 4k_2(X_1c/m) + 9k_2k_3(X_1c/m)^2 + 16k_2k_3k_4(X_1c/m)^3 + \ldots . \qquad (4)$$

The values of X_1 from eq 3 are substituted into eq 4; from the limiting slope of a plot of M/mX_1 as a function of X_1c/m, k_2 may be obtained. From the limiting slope of $(M/mX_1) - (4k_2X_1c/m)$ as a function of $(X_1c/m)^2$, k_3 can be obtained.

By these methods, X_1 was determined over a wide range of concentrations for sample 5e at pH 8.32 and $\Gamma/2$ of 0.11 (sodium borate buffer). It can be seen by inspection of Figure 1 that sample 5a (pH 5.48, $\Gamma/2$ of 0.34, NaCl solution) gave a scattering curve approximating that of sample 5e; hence, values for X_1 at various concentrations would also be approximately the same, as would calculations based on X_1. Association constants k_2 (monomer to dimer) and k_3 (dimer to trimer) were calculated, and with these constants the changes in free energy (ΔF°_2 and ΔF°_3) were obtained by use of the relation

$$\Delta F^\circ = -RT \ln k \qquad (5)$$

Results of these calculations are shown in Table III. k_2 was found to be 4.5×10^5 moles/l. and $k_3 = 3.6 \times 10^5$ moles/l. The two values for ΔF° at 25° were of the same order of magnitude, *viz.*, $\Delta F^\circ_2 = -7719$ cal/mole and $\Delta F^\circ_3 = -7592$ cal/mole. These association energies appear to be too low for the formation of covalent bonds between apoferritin molecules. A study of association in relation to elapsed time has not yet been done.

Results of Ultracentrifugation and Diffusion in Comparison with Light Scattering. Table IV shows the results obtained in sedimentation velocity runs. In agreement with previous work by others (Rothen, 1944; Hofmann and Harrison, 1963; Suran and Tarver, 1965; Harrison and Gregory, 1965), the principal component of apoferritin has an $s_{20,w}$ in the range 17–18 S. Components with coefficients of about 25–26 S were detected in three samples that had been enriched

TABLE III: Data on the Derivation of Association Constants and ΔF° from Scattering Curve for Sample 5e.

c (g/l.)	M/m	X_1	
0.10	1.082	0.9287	
0.20	1.190	0.8617	$k_2 = \frac{\text{initial slope of } M/X_1m \text{ vs. } X_1c/m}{4} = 4.50 \times 10^5$ moles/l.
0.48	1.515	0.6974	
0.63	1.700	0.6295	$k_3 = \frac{\text{slope of } (M/X_1m) - (4k_2X_1c/m) \text{ vs. } (X_1c/m)^2}{9k_2} = 3.63 \times 10^5$ moles/l.
0.85	1.904	0.5516	
1.20	2.164	0.4629	
1.77	2.506	0.3703	
2.26	2.506	0.3196	$\Delta F^\circ{}_2{}^{25} = -RT \ln k_2 = -7719$ cal/mole
2.97	2.506	0.2714	
3.90	2.506	0.2274	$\Delta F^\circ{}_3{}^{25} = -RT \ln k_3 = -7592$ cal/mole

TABLE IV: Comparison of Sedimentation Coefficients with Data from Disc Gel Electrophoresis.

	Ultracentrifugation			Gel Electrophoresis (%)			
Sample	Concn of Apoferritin (%) (w/v)	Buffer (pH 7.0)	$s_{20,w}$[a]	α	β	γ	δ
6a, f	0.3	Phosphate	a, 17.5	89.7	8.1	1.8	0.4
7b-2, β enriched[b]	0.3	Phosphate	a, 17.5; b, 26.5; c, 32.0; d, 33.1	75.9	19.8	4.3	
8, β enriched[b]	0.1	Phosphate	a, 17.3; b, 25.2	20.9	73.4	3.2	2.5
9, β enriched[b]	0.1	Phosphate	a, 17.3; b, 24.5	54.9	37.6	6.0	1.5

[a] Individual components are termed a–d. [b] Sample 7b-2 was enriched by filtration through Sephadex G-200 as explained in text. Sample 8 was enriched by several cycles of electrophoresis and elution of β-ferritin, followed by removal of iron. Sample 9 was enriched by adsorption on cellulose, elution with 1.1–0.5 M $(NH_4)_2SO_4$ solution, and differential filtration through Sephadex G-200.

in β-apoferritin, and in one of these samples 32S and 33S peaks were also found.

The results of light scattering on those preparations of apoferritin on which sedimentation velocity determinations were also done, were similar to one another. The monomer molecular weight determined by light scattering for samples 6f, 7a, and 7b-1 was 431,000 (Tables I and IV), the light-scattering curves sweeping upwards at concentrations of apoferritin below 1.5 g/l. (Figure 1).

Diffusion experiments further substantiated the presence of polydispersity. Attempts were made to obtain a monomer diffusion coefficient of apoferritin for use in the Svedberg equation. As shown in Figure 2, the apparent diffusion coefficient was both concentration dependent and time dependent. A uniform straight-line relation between D_{app} and 1/time was not obtained. This indicates polydispersity and, therefore, a reliable diffusion coefficient for apoferritin monomer cannot be determined from the data. It is of interest to note, however, that extrapolation of the quasi-straight-line segment of the curve obtained for D_{app} from a 0.54% solution of apoferritin to infinite time gives a D_{app} of 1.87×10^{-7} cm²/sec. When corrected, this gives $D_{1,w} = 1.78 \times 10^{-7}$ cm²/sec. Rothen (1944) found $D_{1,w} = 1.91 \times 10^{-7}$ cm²/sec.

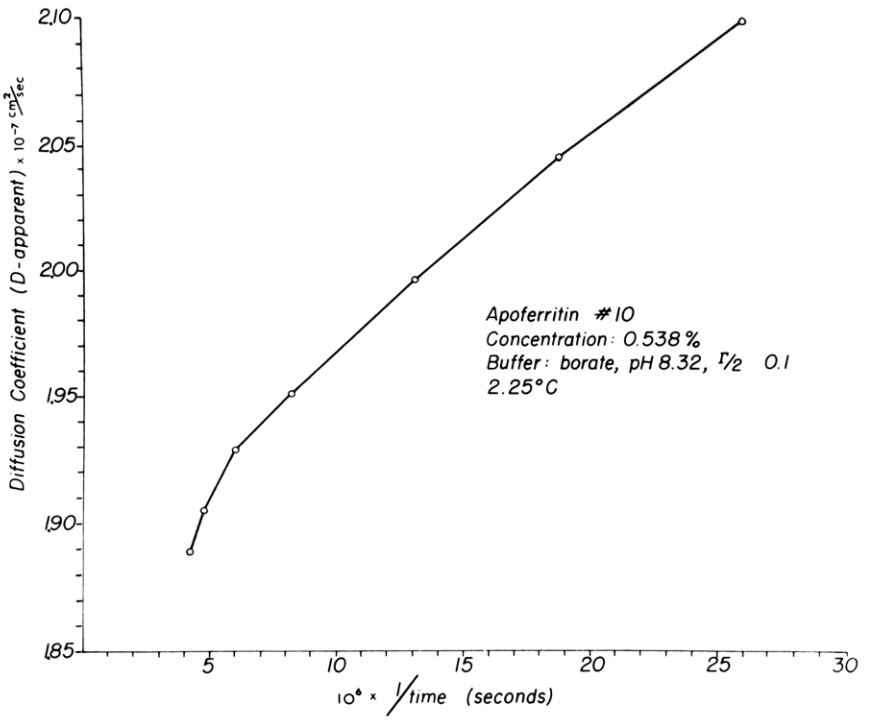

FIGURE 2: Plot of diffusion coefficients *vs.* reciprocal of time. Initial concentration of apoferritin was 0.538% (w/v).

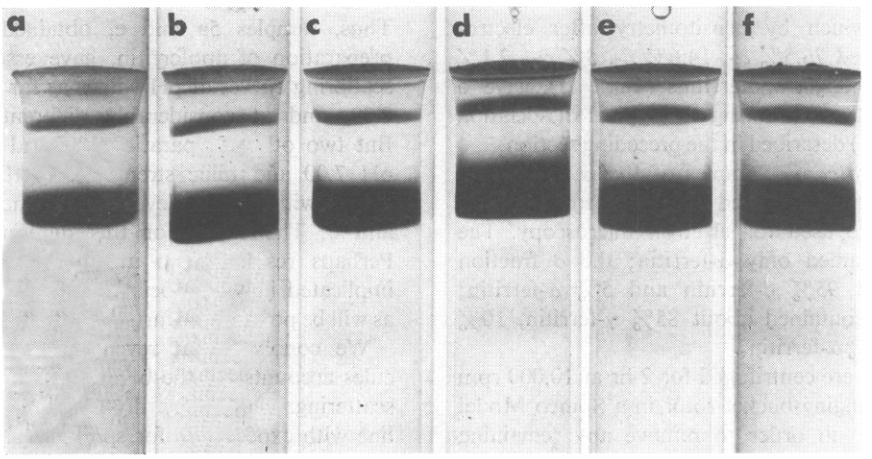

FIGURE 3: Comparison of horse spleen apoferritin (sample 5e) with ferritin from which it was prepared. Disc gel electrophoresis at pH 8.3. Stained with Amido-schwarz. (a) Ferritin; (b) apoferritin; (c) apoferritin; (d) apoferritin, twice concentration in b and c; (e) ferritin and apoferritin (1:1 mixture); (f) ferritin and apoferritin (1:1 mixture).

In view of the evident polydispersity, both of these values must be lower than the true diffusion coefficient of apoferritin monomer. As a consequence, the monomer molecular weight of apoferritin calculated with the Svedberg equation by Rothen (1944), *i.e.*, m = 465,000, may be too high. Some of the light-scattering data we obtained (Table I, Figure 1) indicate a monomer molecular weight of close to 430,000.

Results of Electrophoresis in Comparison with Other Data. As shown in Figure 3, the α, β, γ, δ, and ϵ fractions of apoferritin samples had mobilities that were identical with those of the corresponding fractions of ferritin from horse spleen. Percentages of these components varied; some are listed in Tables II and IV and are in agreement with previous reports (Richter, 1964; Suran and Tarver, 1965; Harrison and Gregory, 1965; Kopp *et al.*, 1966). It can be seen in Figure 3 that reproducibility of separations was good. Samples 6f and 7a were heterogeneous on gel electrophoresis and gave light-scattering curves indicating dissociation.

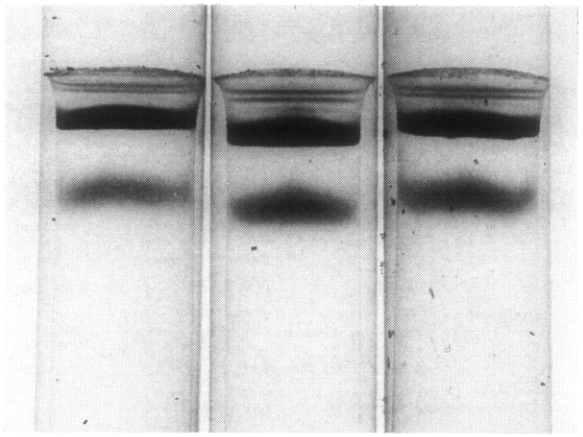

FIGURE 4: Sample 8 (apoferritin) after disc gel electrophoresis and staining with Amido-Schwarz. See Table IV for densitometric and sedimentation velocity data. Note enrichment of β fraction.

Samples 7b-2, 8, and 9, which had been enriched in β-apoferritin, as demonstrated by gel electrophoresis (Table IV and Figure 4), were found to contain the 25–26S component on ultracentrifugation in spite of the low total concentration of apoferritin in the sample.

Sample 5e, which by densitometry after electrophoresis contained 76.5% α-, 14.6% β-, 5% γ-, 2.1% δ-, and about 1.8% ϵ-apoferritins (Table II), gave a light-scattering curve showing the typical dissociation pattern (Figure 1) described in the preceding section.

Electron Microscopy. Samples of the α, β, and γ fractions of ferritin, isolated as described earlier in this report, were used for electron microscopy. The α fraction contained only α-ferritin; the β fraction contained about 95% β-ferritin and 5% α-ferritin; the γ fraction contained about 85% γ-ferritin, 10% β-ferritin, and 5% α-ferritin.

The samples were centrifuged for 2 hr at 10,000 rpm in an SW-39 swinging-bucket rotor in a Spinco Model L ultracentrifuge in order to remove any remaining gel particles. Aliquots of the final supernatants were diluted empirically with distilled water until a dilution was reached that gave good separation of ferritin particles on examination in the electron microscope by the methods described earlier in this report. Table V gives the frequency distributions of single particles, doublets, triplets, etc., for α, β, and γ fractions. These distributions were quite similar. Statistical analysis revealed almost identical standard errors of sampling and showed no significant differences between the three frequency distributions (Table V). We conclude that by electron microscopy, singlets, doublets, and triplets of molecules were equally frequent in these preparations, obtained from α, β, and γ fractions. These findings may indicate that there was considerable dissociation of doublets, triplets, and other multiplets when samples were diluted prior to spreading on the specimen grids. Judging from results obtained with the α fraction, some association of molecules had taken place. The data shown in Table V are in substantial agreement with the light-scattering data for apoferritin, which indicate reversible association of molecules, dependent on concentration.

Discussion

Our data raise a number of questions that will now be considered. The light-scattering curves indicate clearly that reversible association of apoferritin molecules, dependent on concentration, takes place in solution at pH ranging from 5.5 to 8.4, and ionic strengths ranging from 0.06 to 0.34. These results are comparable to findings reported for other proteins, for example, mercury mercaptalbumin (Stacey, 1956), insulin (Steiner, 1952, 1953; Doty and Myers, 1953), and α-chymotrypsin (Steiner, 1954).

At concentrations of apoferritin above 2 g/l., the scattering curves have slopes (2B) equal to (or approximately equal to) zero. Thus, above 2 g/l. the apparent molecular weight remains approximately constant over a wide range (up to at least 5 g/l.). As concentrations decrease progressively from 2 to 0 g/l., the apparent molecular weight also decreases, until the monomer weight of apoferritin is approached. The plateau values of molecular weight at high concentrations (Figure 1) varied somewhat in different preparations. Thus, samples 5a and e, obtained from the same preparation of apoferritin, gave essentially the same scattering curves at pH values as far apart as 5.48 and 8.32, and at considerably different ionic strengths. But two other preparations (6f and 7a), examined at pH 7.00 and ionic strength of 0.10, gave scattering curves with plateau levels above those of samples 5a and e. The reason for this difference is not clear. Perhaps residual iron atoms on the molecules are implicated in the association of apoferritin molecules, as will be pointed out further on.

We conclude that reversible association of molecules accounts for the behavior of apoferritin on light scattering. Checks for dissymmetry were negative, in line with expectation for spherical particles of the size of apoferritin monomer (diameter $< \lambda/20$). However, the largest dimension of nondissociable dimers would be at the borderline (240 A) for production of significant dissymmetry at the lower wavelength of light employed for this study (λ = 4360 A). The lowest values for dissymmetry (Table I) are somewhat above the ideal value of 1.00, but minute traces of residual dust in the test solutions usually account for such deviations. Moreover, a check of the angular dependence of scattering in a cylindrical cell revealed a regular, periodic distribution of scattering envelopes, according to the $(1 + \cos^2\theta)$ term in Rayleigh's equation.

Several of the samples examined by light scattering contained appreciable quantities of β- and γ-apoferritins, as determined by gel electrophoresis and by ultracentrifugation (Tables II and IV). Nondissociable β and γ polymers, if present, should have a detect-

able influence on the apparent molecular weight at infinite dilution or near it since the intensity of scattered light is proportional to the square of the molecular weight of the scattering particles. However, the molecular weights found at and near $c = 0$ approximate the expected monomer molecular weight (m) with adequate precision. It is necessary, therefore, to consider whether the β, γ, δ, and ϵ fractions found by gel electrophoresis or by ultracentrifugation are dissociable polymers, held together by noncovalent bonds.

The values of k_2 and k_3 and the corresponding ΔF°_2 and ΔF°_3 (Table III) calculated for sample 5e (and roughly applicable also to sample 5a) are in the range of weak intermolecular forces. While temperature dependence of association has not yet been investigated for this system (and values for ΔH and ΔS are therefore not available) the values obtained for ΔF°_2 and ΔF°_3 indicate bond energies in the range between 5 and 10 kcal/mole, characteristic of weak intermolecular bonds. ΔF° and ΔH for association of insulin molecules (Doty and Myers, 1953; Steiner, 1953), for α-chymotrypsin molecules (Steiner, 1954), and for β-lactoglobulin molecules (Townend and Timasheff, 1960) are within this range.

As to the behavior during sedimentation, there is adequate basis for assuming reversible association. It has been shown by Rao and Kegeles (1958), Fujita (1962), Gilbert (1959), and others that the presence of multiple peaks can be compatible with reversible association of molecules. The nature of this process has been reviewed by Nichol *et al.* (1964).

Work in several laboratories has demonstrated the presence not only of 17–18S, but also of 24–26S and 34–36S components in horse spleen apoferritin (Suran and Tarver, 1965; Harrison and Gregory, 1965; Kopp *et al.*, 1966). Suran and Tarver (1965), Harrison and Gregory (1965), and Kopp *et al.* (1966) have related these three sedimentation peaks to the α, β, and γ fractions, respectively. The observation, reported by Harrison and Gregory (1965) and Suran and Tarver (1965), that 6–7 M urea and 2–6 M guanidine hydrochloride do not affect the behavior of β-ferritin during gel electrophoresis, does not argue against reversibility of association. The forces holding the molecules together are unknown. They may not be hydrogen bonds, but covalency is not the sole alternative to hydrogen bonding. Residual divalent atoms of iron on the outer surfaces of molecules, as indicated by the work of Green and Mazur *et al.* (1957, 1958), might stabilize dimers and higher polymers, rendering them relatively insusceptible to the action of urea or guanidine hydrochloride. This would imply the presence of at least one iron(II) atom per pair of protein molecules in the polymer.

It is not clear how all the data obtained by gel electrophoresis can be fitted together with the light-scattering data. For example, after several cycles of electrophoresis and elution, relatively pure α-apoferritin or α-ferritin were isolated, and these fractions retained their mobilities on repeated reelectrophoresis. Since the observed molecular association is dependent on con-

TABLE V: Statistical Summary of Frequency Distributions of Singlets, Doublets, and Higher Multiplets of Ferritin Molecules in Samples of α, β, γ Fractions.[a]

Eluate or Sample	Expt	Classes (n = 1–6)	Total Count	Sample Mean	Mean Square of Sample	Std Error	% Error	Mean Square of Sample Means $(\sigma_1)^2$	Mean Square of Combined Samples $(\sigma_2)^2$	F $(\sigma_1)^2/(\sigma_2)^2$	Significance of F
α	1	6	450	75	7,245	±35	±7.8				Not significant. At 25% level, F = 1.51 for applicable degrees of freedom, *viz.*, 2 and 17.
β	1	6	569	95	11,609	±44	±7.7	16,037	11,786	1.36	
γ	1	6	790	132	19,233	±57	±7.2				
Unseparated ($\alpha + \beta + \gamma$)	1	18	1,809	101	11,786						

[a] Isolated and purified by gel electrophoresis. The counts were obtained from electron micrographs of greatly diluted samples.

centration, conditions at the beginning of a disc gel electrophoresis run should promote association because the stacking process at the top of polyacrylamide columns greatly concentrates proteins. On the other hand, stacking should prevent dissociation of complexes previously formed. The procedures thus far employed for purification of α-, β-, and γ-apoferritins (or -ferritins) are insufficient to permit a rigorous test by gel electrophoresis for reversibility of association in individual fractions. However, disc gel electrophoresis, because it is a discontinuous method of separation in which proteins are concentrated into relatively narrow disks, would be a questionable means of testing for reversibility of association.

The evidence obtained by electron microscopy confirms that provided by light scattering. In this approach to the problem, it was essential that samples of eluted fractions were greatly diluted prior to spreading on specimen grids. When this precaution was taken, the statistics of particle association were the same for α-, β-, and γ-ferritins. While drying from solution on formvar or carbon substrates, ferritin has a strong tendency to form monolayers or aggregates. Williams *et al.* (1966) have reported results of electron microscopy of eluted α, β, and γ fractions of horse spleen ferritin. In the β fraction, they found 42% monomer, 43.8% dimer, and 12.1% trimer; the γ fraction was thought to contain 44.8% monomer, 29.8% dimer, and 21% trimer. Statistical evaluations were not detailed in their report, but the authors raised the possibility of dissociation. Judging from our experience, it seems likely that the concentrations of ferritin in the samples evaluated by Williams *et al.* (1966) were still too high to provide good randomization of particles. Moreover, 100 moles of bovine serum albumin/mole of ferritin was used to help spread the ferritin molecules; but the effects of albumin on single ferritin molecules, dimers, and trimers could be several, and are unknown. Our observation that doublets and triplets of molecules were present in spreads of material from the α fraction is best explained on the basis of random association during drying on specimen grids.

Acknowledgments

We are greatly indebted to Drs. Esther Breslow and Leonhard Korngold of Cornell University Medical College, and to Drs. Dan Moore and Dominic Dziewiatkowski of the Rockefeller University for permitting us to use their equipment and for much helpful advice.

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Magnetic Resonance Studies of Macromolecules. I. Aromatic–Methyl Interactions and Helical Structure Effects in Lysozyme*

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ABSTRACT: Lysozyme nuclear magnetic resonance spectra display a number of upfield resonances (some occur above tetramethylsilane) which change in a striking manner as the enzyme denatures. These resonances arise primarily from methyl–aromatic magnetic interactions. A comparison of optical rotatory dispersion measurements with the changes in these upfield resonances during thermal denaturation indicates that helical region unwinding occurs simultaneously with the opening up of the protein. Furthermore, a preliminary reconstruction of the upfield resonances based on the X-ray crystallographic coordinates and aromatic ring current data gives results which are in good agreement with the observed spectra. Barring the possibility of a fortuitous agreement, this is highly suggestive that the enzyme structure in solution and in the crystal state are very similar, if not identical. Some efforts at direct experimental identification of the upfield resonances are also briefly reported. The lysozyme spectra show changes in the α-hydrogen region upon folding. Electric field effects, which arise from the permanent dipoles of the peptide units in a helix configuration, and, probably, magnetic anisotropy contributions from the carbonyls are important. Resonances from α-hydrogens in infinite right-handed helices are predicted to occur $\sim$0.5 ppm downfield of the corresponding resonance for infinite left polypeptides. The P_E resonance (text) in the α-hydrogen region grows as the enzyme folds. This resonance may be associated with shifts arising from helical ordering.

Magnetic resonance as a technique to study protein conformation has been explored by a number of investigators over the past decade (Kowalsky and Cohn, 1964; Kowalsky, 1962; Mandel, 1965). Recently high-resolution proton resonance studies at 220 Mcycles of a number of enzymes have been reported by Phillips and collaborators (McDonald and Phillips, 1967). These studies clearly establish the magnetic resonance technique as a powerful tool for protein investigations. A few of the areas where significant contributions from magnetic resonance are imminent follow. (1) (protein structure) The first X-ray crystal structure of an enzyme (lysozyme) has recently been determined by Phillips and collaborators (Phillips, 1966; Blake *et al.*, 1965). Questions have been raised concerning the nature of the enzyme structure when in solution. M. A. Raftery (private communication) has indirect evidence of the similarity of structures from spectrophotometric and magnetic resonance studies of substrate and inhibitor binding to lysozyme in solution. It is our intention in this paper to present more direct experimental verification of the similarity of crystal and solution structures; (2) protein folding and hydrophobic interactions (Schachman, 1963); and (3) practical calculations of protein conformation. Within the past few years, considerable progress has been made in understanding the stereochemical principles of polypeptide chain formation (Ramakishnan and Ramachandran, 1965; Brant and Flory, 1965; Leach *et al.*, 1966a,b; Vanderkooi *et al.*, 1966; Liquori, 1966). Efforts are now being made to "predict" protein conformation (Leach *et al.*, 1966a,b; Vanderkooi *et al.*, 1966; Levinthal, 1966). The results can be checked against nuclear magnetic resonance observations, where practical.

In this laboratory, over the past year, we have been concerned with nuclear magnetic resonance studies in proteins. In particular we have been interested in spectral changes that occur as a consequence of the tertiary protein structure. We have limited ourselves primarily to lysozyme since any conclusions reached by magnetic resonnace could ultimately, in principle, be checked against Phillip's (1966) X-ray determination. Our own study at 100 Mcycles has benefited considerably from the parallel studies done at 220 Mcycles (McDonald and Phillips, 1967). In this paper we discuss two aspects of tertiary protein structure: hydrophobic interactions and helical structure. By

* From the Department of Chemistry, University of California, Berkeley, California 94720. *Received April 3, 1967.* This study was aided by Grant No. GM-14313-01 from the National Institutes of Health.