Electron-Microscopic and Chemical Studies of Oligomers in Horse Ferritin

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Horse ferritin was fractionated both by starch-gel electrophoresis and by gel filtration on Sephadex G-200. Monomer fractions contained up to 98% of monomer and oligomer fractions up to 76% of oligomers as determined by quantitative electron microscopy. Percentages obtained from electron micrographs correlated well with analytical starch-gel electrophoretograms and ultracentrifuge patterns. Amino acid analyses of monomer- and oligomer-enriched fractions showed no significant differences. Ferritin oligomers did not apparently dissociate on dilution for electron microscopy or on storage. Apoferritin dimers were stable in 0.01M-phosphate buffer at dilutions down to 0.19mg./ml. as shown by ultracentrifugation. Chemical studies indicated that the intermolecular bonds in oligomers are resistant to a variety of reagents and conditions, including those that would be expected to attack disulphide, peptide and ester linkages respectively. Partial disaggregation was achieved at high pH values and in 67% (v/v) acetic acid. Centre-to-centre intermolecular distances in dimers were found to be about 100Å. Three main types of trimer configuration were found and a variety of tetramers and pentamers. These configurations are described and discussed.

The heterogeneity of horse spleen ferritin has been studied from several aspects. In the ultracentrifuge ferritin gives a 17s peak corresponding to iron-free protein (apoferritin) and a broad diffuse boundary of average sedimentation coefficient about 65s, corresponding to a mixture of molecules of different iron contents (Rothen, 1944). Equilibrium density-gradient centrifugation shows that it consists of a continuous mixture of molecules, which may contain up to a maximum of 5000 iron atoms/mol. (Fischbach & Anderegg, 1965). Heterogeneity in the protein has also been observed. Apoferritin obtained by removal of the ferritin iron with reducing agents (Granick & Michaelis, 1943) gives two peaks in the ultracentrifuge (Rothen, 1944), a major 17s peak and a minor 25s peak. Both ferritin and apoferritin show three (or occasionally more) components on gel electrophoresis in starch or polyacrylamide (Saddi, 1962; Kopp, Vogt & Maass, 1963, 1964; Theron, Hawtrey & Schirren, 1963; Fine & Harris, 1963; Richter, 1963; Carnevali & Tecce, 1964). Chromatography of ferritin on DEAE-cellulose gives a complex elution pattern (Theron *et al.* 1963; Suran & Tarver, 1965) consisting of three peaks, which are not correlated in a straightforward manner with the

gel-electrophoresis components. Amino acid compositions of various chromatographic fractions were found to be identical (Suran & Tarver, 1965). Apoferritin is eluted as a single peak from DEAE-cellulose (Suran & Tarver, 1965).

In previous work (Harrison & Gregory, 1965) an attempt was made to establish the nature of the components of apoferritin giving the α -, β - and γ -bands on gel electrophoresis. These components could differ in size or charge or both. The fact that only one component was observed on free-boundary electrophoresis (Mazur & Shorr, 1950) indicates that the molecules are homogeneous with respect to surface charge. A combination of Sephadex G-200 gel filtration, starch-gel electrophoresis and ultracentrifugation showed that the α -, β - and γ -bands could be correlated with ultracentrifuge peaks having sedimentation coefficients respectively 17s, 24s and 33s. The magnitudes of these coefficients suggested that they corresponded to apoferritin monomer, dimer and trimer. A similar conclusion was reached by Suran & Tarver (1965) and is supported indirectly by the observation that the β -band becomes more pronounced after treatment with the bifunctional linking reagent bis-diazotized benzidine (Gregory &

Williams, 1967). Apoferritin was used in the earlier work because its ultracentrifuge patterns are not complicated by heterogeneity of iron content. In the present study we made use of the fact that the iron-containing cores of ferritin could be observed directly in the electron microscope (Farrant, 1954). This has enabled us to confirm the presence of monomer, dimer, trimer, tetramer, pentamer and higher oligomers in ferritin (reported briefly in Williams, Gregory & Harrison, 1966) and to show that a variety of shapes occurred in the aggregated forms.

We have obtained fractions, by recycling gel filtration on Sephadex G-200, that were shown by starch-gel electrophoresis to be substantially enriched with regard to either monomer or oligomers. These fractions were used for amino acid analysis, electron microscopy and other studies. The α -band-enriched and β -band-enriched fractions were shown by electron microscopy to be largely monomer and largely oligomers respectively. These results differ from those reported by Richter & Walker (1967), who found 'similar frequency distributions of monomers, dimers and trimers in α -, β - and γ -ferritins'. Richter & Walker (1967) concluded from their observations that dimers and trimers disaggregate on dilution. Our results indicate that the dimers and trimers present in our preparations can be purified, stored and subsequently diluted without disaggregation.

MATERIALS AND METHODS

Materials. The ferritin used in the experiments either was isolated from horse spleen by the method of Granick (1942) and crystallized three times from 5% CdSO₄ solution or was a commercial product obtained from Pentex Inc., Kankakee, Ill., U.S.A. (prepared from horse spleen and twice crystallized).

Apoferritin was prepared from ferritin by reduction with Na₂S₂O₄ in O₂-free 0.1 M-sodium acetate buffer, pH 4.8–5.0, until a colourless solution was obtained. The ferrous iron was removed by dialysis.

The starch used for gel electrophoresis was 'Starch-hydrolysed', obtained from Connaught Medical Research Laboratories, Toronto, Ont., Canada.

Gel filtration was carried out with Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden), superfine and standard grades being used as specified below.

Other special reagents used were Diazyme (Miles Chemical Co., Elkhart, Ind., U.S.A.), Pronase (Kaken; distributed by V. A. Howe and Co. Ltd., London, W. 11), dithiothreitol (Calbiochem, Los Angeles, Calif., U.S.A.), sodium dodecyl sulphate ('specially pure'; British Drug Houses Ltd., Poole, Dorset) and desferrioxamine (a gift from Ciba Ltd., Horsham, Sussex). Other reagents were A.R. grade.

Sephadex-gel filtration. Sephadex G-200 was swollen in buffer or saline in the cold for periods ranging from 1 to 7 weeks before use. The columns were packed and equilibrated in the cold (0–4°) and experiments were carried out at the same temperature. Columns of various sizes and

shapes were used as described in Harrison & Gregory (1965) and under the experimental conditions of two gel-filtration experiments reported below.

Gel electrophoresis. Electrophoresis in starch gels was used both as a method of preparing ferritin components and as a means of analysing them.

In preparative gel electrophoresis initial separation of ferritin into α -, β - and γ -bands was carried out either in the vertical starch-gel-electrophoresis apparatus as described by Smithies (1959) or in an extra-long horizontal tray (48 cm. $\times$ 12.2 cm. $\times$ 0.6 cm.) made by Otto Hiller, Madison, Wis., U.S.A. The starch gels were made up in sodium borate buffer by the procedure of Smithies (1955, 1959) and the sample was applied respectively either by pipetting into a preformed slot in the gel (approx. 100 mg. sample in a slot 1.8 mm. $\times$ 6.0 mm. $\times$ 108 mm.), or by inserting a strip of Whatman 3MM paper (6.0 mm. $\times$ 110 mm.), soaked in 20% ferritin solution, into a slit in the gel. Electrophoresis was carried out with a voltage gradient of 4 v/cm. for a period of 40–48 hr. with Ag/AgCl electrodes. The central strips (12.0 cm. long $\times$ 0.5 cm. wide) of the α - and β -bands were then cut out separately with double-mounted slicing blades and placed in slots made with the same blades in a second gel, and electrophoresis was carried out until the band had moved 5–12 cm. from its starting position. This procedure could be repeated a further one or two times. A small strip of gel was cut out and stained with Amido Black after the final electrophoresis. Only one band could be detected. The γ -band, which was only just visible without staining after the initial vertical separation, was also cut out, but not subjected to further electrophoresis. To find a suitable procedure for the extraction of the band from the starch gel, a test portion of gel was homogenized and spun in a bench centrifuge to remove the bulk of the starch. The opalescent supernatant was then centrifuged for 10 min. at 160 000 *g*_{av.} in an MSE Superspeed 50 centrifuge. After the second spin the supernatant was completely clear, but gave a positive reaction for starch with iodine in KI solution even after dialysis against glass-distilled water. A portion was then incubated overnight in a dialysis bag with a small amount of Diazyme at 36°, and simultaneously dialysed against water. After this treatment the starch-iodide test was negative. This procedure was subsequently adopted to free most ferritin bands from starch. However, a slower centrifuge speed (30 000 *g*_{max.}) was used to prevent coprecipitation of some of the ferritin with the starch. In some cases an alternative method was used. The extract was spun for 1 hr. at 160 000 *g*_{av.}, the colourless supernatant discarded, and the brown precipitate resuspended in a small volume of water and spun again to remove the starch.

Analytical starch-gel electrophoresis was in most cases carried out in horizontal gels in the Shandon apparatus, but smaller gel trays were normally used (10.5 cm. $\times$ 8.25 cm. $\times$ 0.3 cm.) instead of the usual (18.4 cm. $\times$ 10.1 cm. $\times$ 0.55 cm.). The discontinuous buffer system of Poulik (1957) was normally used (gels made in tris-citrate buffer, borate buffer in the electrode compartments), unless otherwise stated in the Results section. Electrophoresis was carried out at a constant current of 18 mA (100–140 v) at room temperature for 1–1½ hr. Under these conditions the front band had reached a distance of about 1–3 cm. from the origin and good separation was achieved.

Analytical ultracentrifugation. This was carried out in a Spinco model E ultracentrifuge equipped with a phase

plate. Ferritin solutions were centrifuged at 33450 rev./min., apoferritin solutions at 50740 or 59780 rev./min. Where areas of peaks were to be measured, centrifugation was carried out either in a Kel F-coated aluminium double-sector cell at 59780 rev./min. (solutions in sodium phosphate buffer, pH 7.6, or in sodium acetate buffer, pH 3.65), or in a 4° single-sector Kel F cell or a 4° single-sector aluminium cell with a 30 mm. centre-piece at 50740 rev./min. (solutions in sodium borate buffer, pH 11.2-12.4, or in sodium phosphate buffer, pH 7.0, respectively). Separate solvent runs were carried out in the latter cases. The plates were enlarged five times and peak areas measured with a planimeter. Areas were corrected for radial dilution.

Amino acid analyses. Amino acid analyses were carried out by Dr W. Ferdinand and Mr G. Atkin on an EEL amino acid analyser by the method of Spackman, Stein & Moore (1958). To prepare the samples for analysis the iron was removed in either of two ways: (1) samples prefixed AF were reduced with $\text{Na}_2\text{S}_2\text{O}_4$ as described above; (2) samples prefixed F were treated with 5N-HCl for 10 min. in acid-washed tubes; this precipitates the protein and solubilizes the iron; the precipitated protein was washed with N-HCl and glass-distilled water.

Protein samples (1-2 mg.) were hydrolysed in sealed tubes with 6N-HCl at 110°. Three hydrolyses (24, 48 and 72 hr.) and two cysteine acid determinations (Moore, 1963) were made for each fraction. Threonine and serine estimations were extrapolated to zero time. For other amino acids the results were averaged. The four final sets of amino acid analyses thus obtained for the different fractions were scaled together graphically. This method was preferred to the more usual one of scaling to a single amino acid, since the latter method can introduce bias if the reference amino acid is incorrectly determined.

Electron microscopy. Solutions of ferritin fractions were diluted to a concentration of about 30 $\mu\text{g./ml.}$ or less.

Solutions were spread on to 'holey' Formvar or carbon-coated 200-mesh copper grids by lightly touching the grid on to the surface of the solution, draining off the excess with filter paper and drying the preparation in a stream of warm air. BSA* was added to some ferritin solutions to act as a spreading agent. In early experiments at least 100 moles of BSA were added/mole of ferritin. This was subsequently increased to 1000 moles of BSA/mole of ferritin. A volatile salt (ammonium acetate, 1-5%, w/v) was added in early experiments, but was found to have no influence on the results and was later omitted. When required, specimens, prepared as described, were shadowed with platinum at angles between 10° and 45°. Negatively stained preparations were obtained by mixing ferritin solutions (with BSA added) with an equal volume of 2% potassium phosphotungstate (Brenner & Horne, 1959) or uranyl acetate-EDTA (van Bruggen & Wiebenga, 1962). These mixtures were dried down on to coated grids as before.

Electron micrographs were taken at plate magnifications of $\times 30000$ to $\times 250000$ with either an AEI EM6B or a Phillips EM200 electron microscope. In some cases micrographs were deliberately under-focused to enhance contrast. The two electron microscopes gave measurements agreeing within $\pm 2\%$ when operated at similar magnifications. One instrument was calibrated by using copper phthalocyanine crystals.

Measurements of intermolecular distances were made on electron micrographs with a travelling microscope. Molecules were judged to be in contact when the distance between their centres was one molecular diameter, about 120 Å, or less. Counts of molecules occurring singly, as pairs, triplets etc. were made. The configurations of triplets, quadruplets and quintuplets were studied by constructing drawings to scale (5 cm./100 Å). These were compared with various models by projecting the latter on to the drawings with a parallel beam of light.

RESULTS

The starch-gel electrophoresis of commercially obtained horse spleen ferritin and material prepared in this Laboratory showed three components termed α -, β and γ as described above (see Plate 1a). Preliminary experiments indicated (Harrison & Gregory, 1965; Williams *et al.* 1966) that the individual components could be partially purified by this technique (see Plates 1b and 1c), and that the β - and γ -bands represent dimer and trimer respectively. These results are described in detail below, together with further separations obtained by gel filtration.

The fractions obtained by these two methods were examined by electron microscopy for counting of monomers, pairs, triplets etc. Ferritin solutions, when dried on to Formvar or carbon films, tend to form close-packed monolayers. This tendency is diminished by decreasing the total number of molecules applied to the grid. This can be conveniently achieved only by diluting the ferritin solution to a low concentration (30 $\mu\text{g./ml.}$ or less). Even at such low dilutions ferritin tends to form close-packed monolayers over some of the grid on drying. Only those areas over which the molecules are well spread can be used for counting. Thus of necessity much of the preparation has to be ignored, and, in the absence of a spreading agent, it is possible that some pairs and triplets may be formed accidentally on drying.

We have found that the addition of BSA as a spreading agent overcomes these problems. Packed monolayers are then prevented from forming and regions of specimen may be selected at random for micrograph preparation and subsequent counting. Experiments were performed to test the effect of adding BSA on the proportion of monomers, pairs, triplets etc. counted in various preparations. The results for three such experiments are given in Table 1.

It can be seen that the addition of BSA has only a small effect on the counts of smaller oligomers and a more marked effect on the counts of quintuplets or larger oligomers. It evidently did not increase the proportions of pairs, triplets etc. (as suggested by Richter & Walker, 1967), and, if anything, slightly decreased their proportions. All

*Abbreviation; BSA, bovine serum albumin.

Table 1. *Effect of excess of BSA on counts of monomers, pairs, triplets etc. in three different ferritin preparations*

The ferritin used in these experiments was obtained from Pentex Inc. Counts were made in the electron microscope.

Expt. no.	Presence or absence of BSA	No. of molecules counted	% of molecules occurring as					Total aggregates
			Monomers	Pairs	Triplets	Quadruplets	Quintuplets or greater	
SGF I (tube 1)	—	817	23.0	43.9	19.2	8.3	5.6	77.0
	+	1061	32.8	43.1	15.5	7.2	1.4	67.2
SGF II (fraction D31-40)	—	657	88.9	10.4	0.0	0.0	0.7	11.1
	+	3279	89.6	8.7	1.5	0.2	0.0	10.4
SGF II (fraction A20-24)	—	565	22.7	42.5	17.5	10.6	6.7	77.3
	+	2472	25.5	55.3	10.1	6.8	2.3	74.5

Table 2. *Percentages of monomer and oligomers present in bands separated by starch-gel electrophoresis*

The ferritin used in these experiments was obtained from Pentex Inc. Counts were made in the electron microscope.

Expt. no.	Band	% of molecules occurring as					Total aggregates
		Monomer	Dimer	Trimer	Tetramer	Pentamer	
SGE I	α	89.4	10.6	0.0	0.0	0.0	10.6
	β	41.1	41.1	15.4	2.4	0.0	58.9
SGE II	α	97.6	2.4	0.0	0.0	0.0	2.4
	β	45.2	39.7	12.7	1.8	0.6	54.8
	γ	50.2	30.4	13.8	3.9	1.7	49.8
SGE III	γ	44.2	29.1	23.2	3.4	0.1	55.8
SGE IV	α	84.2	10.1	5.7	0.0	0.0	15.8
	β	28.6	68.5	2.9	0.0	0.0	71.4
	γ	38.6	30.8	27.6	1.8	1.2	61.4
Unfractionated ferritin A		82.3	14.0	3.4	0.0	0.3	17.7
Unfractionated ferritin B		88.5	10.1	1.4	0.0	0.0	11.5

electron-microscope counts of ferritin preparations given in subsequent sections relate to the preparations diluted with excess of BSA. In succeeding sections of this paper pairs, triplets etc. of molecules are referred to as dimers, trimers etc. The use of this terminology is justified in view of the experimental evidence discussed below.

Starch-gel electrophoresis

Attempts were made to purify the α -, β - and γ -components by repeated electrophoresis in starch gels as described in the Materials and Methods section. All three bands were brown, owing to the presence of ferric iron, and even the γ -band, though very pale, could be seen without staining if sufficiently large amounts of samples were applied. After three or four successive electrophoretic separations in the case of the α - and β -bands, and one (Expts. SGE II and III) or two (Expt. SGE IV) in the case of the γ -band, the bands were cut out,

eluted, treated to remove starch and examined in the electron microscope. Counts of monomers and oligomers for each band are shown in Table 2 with those of two samples of unfractionated ferritin for comparison. The β - and γ -fractions have been purified respectively by between two- and seven-fold and by between four- and 20-fold. Although further purifications might in principle have been achieved by this method, in practice it did not seem to be a very satisfactory one in view of the relatively small amounts of sample that could be used, the low recovery of bands from the gels and the presence of starch in the eluted products. Gel electrophoresis was therefore abandoned as a preparative method.

Gel filtration on Sephadex G-200

Gel filtration is well known as a means of separating molecules of different sizes and the use of Sephadex G-200 for this purpose has been studied in some detail by Andrews (1965). In Andrews's

(1965) experiments proteins of molecular weights close to those of apoferritin monomer (460 000) and dimer were eluted at volumes that, although close to the void volume, differed slightly, but significantly, from one another. Gel filtration of apoferritin on Sephadex G-200 was attempted and partial fractionation into α -, β - and γ -components was achieved, as shown by analytical starch-gel electrophoresis (Harrison & Gregory, 1965). Ferritin, although heterogeneous with respect to iron content, and having molecular weights of up to twice that of apoferritin, has the same molecular diameter as the latter (Harrison, 1959), and therefore would be expected to show a similar elution behaviour. This was indeed found to be the case (Andrews, 1965). [The amount of sample used in Andrews's (1965) experiments was presumably too small for the presence of the minor components to be detected.]

Since the β - and γ -components represent only

Fig. 1. Elution diagram of ferritin from Sephadex G-200 column (Expt. SGF I). Stippled areas represent proportions of fractions (1, 2, 4, 7 and 12) occurring as oligomers, as counted in the electron microscope. See also Plates 1(d)-1(g) and Table 3.

small proportions of native ferritin, rather large samples must be used to obtain adequate amounts of oligomer-enriched fractions. Experiments were carried out with different columns to find conditions in which adequate separation at reasonable flow rate could be achieved. Fractions were monitored by analytical starch-gel electrophoresis and electron microscopy, and in some cases the total amount of iron in each fraction was estimated from its extinction at 540 mμ. Results of two experiments are shown below.

Expt. SGF I. In this experiment a column of 2.8 cm. internal diam. $\times$ 50 cm. long (bed volume 300 ml.) was packed with Sephadex G-200 (superfine grade), which had previously been swollen for 7 weeks in 0.1 M-phosphate buffer, pH 7.5 (containing 0.02% sodium azide to prevent bacterial growth). After equilibration of the column, 3 ml. samples containing ferritin (250 mg.) and sucrose (500 mg.) were applied in a narrow band under the liquid at the top of the column. Effluent fractions (1.2 ml.) were collected at a flow rate of 4 ml./hr. The bulk of the ferritin appeared in 20 effluent fractions, which formed a single nearly symmetrical peak with a slight extension on the trailing edge. Peak fractions 5 and 15 were concentrated by ultrafiltration and monitored by starch-gel electrophoresis. Fraction 5 showed enrichment of the β -band and fraction 15 enrichment of the α -band.

The gel-filtration procedure was repeated for a further three 250 mg. ferritin samples, and the front five fractions of all four peaks (20 tubes) were combined. The pooled fractions were concentrated and reappplied to the column and 1.2 ml. fractions were collected as before. Extinctions of these fractions measured at 540 mμ are plotted in Fig. 1. A single asymmetrical peak was obtained as shown. Although resolution into separate components was not achieved, the front fractions showed considerable enrichment of dimer and trimer by electron microscopy (Table 3) and β - and γ -bands by starch-gel electrophoresis (Plates 1d-1g).

Table 3. Percentages of monomer and oligomers present in ferritin fractions obtained after two cycles of gel filtration through Sephadex G-200 (Expt. SGF I)

The ferritin used in this experiment was extracted in this Laboratory from horse spleen and crystallized three times from 5% CdSO₄. Counts were made in the electron microscope.

Tube no.	% of molecules occurring as					Total aggregates
	Monomer	Dimer	Trimer	Tetramer	Pentamer	
1	32.8	43.1	15.5	7.2	1.4	67.2
2	44.7	42.3	13.0	0.0	0.0	55.3
4	41.7	37.8	19.6	0.9	0.0	58.3
7	70.2	21.4	6.9	1.5	0.0	29.8
12	84.9	14.2	0.9	0.0	0.0	15.1

Table 4. Percentages of monomer and oligomers present in fractions obtained after two cycles of gel filtration through Sephadex G-200 (Expt. SGF II)

Counts were made in the electron microscope.

Tube nos.	% of molecules occurring as					Total aggregates
	Monomer	Dimer	Trimer	Tetramer	Pentamer	
A12-19*	24.0	54.8	13.5	5.9	1.8	76.0
D31-40*	94.7	4.8	0.5	0.0	0.0	5.3
D31-40†	89.6	8.7	1.5	0.2	0.0	10.4
A20-24†	25.5	55.3	10.1	6.8	2.3	74.5

*Freshly eluted samples.

†After storage for 9 months as a precipitate in 30% (w/v) $(\text{NH}_4)_2\text{SO}_4$.

Expt. SGF II. In Expt. SGF II a column of dimensions 2.5 cm. internal diam. $\times$ 100 cm. long (bed volume 485 ml.) was used. This column, purchased from Pharmacia Fine Chemicals, was fitted with an adaptor for upward-flow elution. The column was packed in the cold with Sephadex G-200 (standard grade), previously swollen in 0.1 M-sodium chloride for several days, and equilibrated with 0.1 M-sodium chloride before use. A 10 ml. sample containing 1 g. of ferritin was applied to the bottom of the column. Fractions (32 drops, about 2 ml.) were collected, the flow rate being approx. 25 ml./hr. The eluted sample was contained in 51 tubes. Fractions 12, 24, 41 and 47 were examined by starch-gel electrophoresis. Fraction 12 contained a high proportion of β -component, whereas in fractions 41 and 47 only the α -band was visible (Plates 1h and 1i).

Four more ferritin samples (each 1 g.) were applied to the column in succession and 64-drop fractions were collected. The front fractions, A (one-third of peak volumes), of all five samples were combined, as were the back fractions, D (one-quarter of peak volumes). The middle fractions were divided into two lots, B and C. The ferritin in the pooled fractions was then precipitated with 30% (w/v) ammonium sulphate. Precipitates A and D were centrifuged, suspended in small amounts of water, dialysed and then applied again in succession to the Sephadex column. Fractions (32 drops) were collected and samples through the peaks (about 50 tubes) were examined by starch-gel electrophoresis. Results are shown in Plates 1(j)-1(p). From these it was found that fractions A1-A24 were predominantly β -component, fraction A28 had α - and β -components in the approximate ratio 2:1, whereas in fraction A44 only the α -band was detected. The fractions were pooled as follows, A1-11, A12-19, A20-24, A25-29, A30-36 and A37-50, and the ferritin precipitated with 30% ammonium sulphate for storage until required.

Fractions D12, D18, D25 and D33 were examined by starch-gel electrophoresis. Only α -component was detected in these fractions (Plate 1q). The fractions were then pooled as follows, D1-18, D19-30, D31-40 and D41-52, and precipitated with 30% ammonium sulphate for storage. Counts of monomer, dimer, trimer, tetramer and pentamer were made on various pooled fractions with the electron microscope. Fraction A12-19 was largely composed of aggregated molecules. Fractions D31-40 and D41-52 were almost entirely composed of monomer. Counts for fractions A12-19 and D31-40 are shown in Table 4. Starch-gel-electrophoresis patterns of the same pooled fractions appear in good agreement with these results (see Plates 5c and 5f). Fractions A20-24 and D31-40 were also subsequently examined by electron microscopy after 9 months' storage as a precipitate under 30% ammonium sulphate (see Table 4).

Evaluation of Expts. SGE I-IV and SGF I and dII

From the gel-filtration behaviour and electron-microscopic appearance of the partially purified α -, β - and γ -components, and from previous work (Suran & Tarver, 1965; Harrison & Gregory, 1965), the conclusion now seems inescapable that α -, β - and γ -components represent monomeric, dimeric and trimeric forms of ferritin molecules respectively.

The above conclusion rests substantially on the electron-microscopic appearance of the various fractions. Plates 2 and 3 show electron-microscope fields from monomer-enriched and monomer-depleted preparations respectively. Plate 4(a) shows an area taken from a preparation of Expt. SGF II fraction A12-19, which demonstrates the presence of trimers as well as dimers in the preparation. The counts of monomer, dimer, trimer etc. for any given preparation are highly reproducible. Provided that large enough counts are made, different micrographs and grids give the same

EXPLANATION OF PLATE I

Starch-gel electrophoresis patterns of fractionated and unfractionated ferritin. All photographs show the start line at the bottom and migration in a vertical direction towards the anode. (a) Unfractionated ferritin showing α -, β - and γ -bands. (b) β -Band eluted from starch gel after electrophoretic separation and re-run; it shows contamination with α -band. (c) α -Band eluted from starch gel and re-run. (d)–(g) Ferritin fractions eluted from Sephadex G-200 (Expt. SGF I): (d) fraction 2; (e) fraction 4; (f) fraction 7; (g) fraction 12; the increased proportions of β - and γ -bands in (d) and (e) should be noted (see also Fig. 1 and Table 3). (h)–(q) Ferritin fractions eluted from Sephadex G-200 (Expt. SGF II): (h) and (i) leading and trailing fractions 12 and 47 respectively from the first separation; (j)–(p) second gel filtration of leading fractions, A, from first separation, pooled and rechromatographed; (j) fraction A8; (k) fraction A15; (l) fraction A21; (m) fraction A24; (n) fraction A28; (o) fraction A36; (p) fraction D33; (q) fraction A44 from rechromatography of trailing fractions, D, of first separation; the enrichment of β -band in leading fractions shown in (h), (j), (k), (l), (m) and (n) and of α -band in trailing fractions (i), (o), (p) and (q) should be noted.

EXPLANATION OF PLATE 2

Electron micrographs of purified ferritin monomer preparations. (a) By starch-gel electrophoresis (Expt. SGE II). Magnification $\times 280000$. (b) By Sephadex G-200 gel filtration (Expt. SGF II, fraction D31-40). Magnification $\times 280000$.

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EXPLANATION OF PLATE 3

Electron micrograph of an oligomer-enriched ferritin preparation obtained from Expt. SGF II fraction A12-19. Magnification $\times 235\,000$. The high proportion of paired molecules (dimers) should be noted.

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EXPLANATION OF PLATE 4

(a) Electron micrograph of an oligomer-enriched fraction (Expt. SGF II fraction A12-19). Magnification $\times 176\,000$. The presence of trimers should be noted. (b) Electron micrograph taken from a dimer-enriched preparation obtained by starch-gel electrophoresis (Expt. SGE IV). Magnification $\times 210\,000$. (c)–(f) Examples of trimers of various configurations: (c) linear; (d) triangle with apical angle approx. 120° ; (e) approximately equilateral triangle; (f) trimer (equilateral triangle) demonstrating marked tilting. (g)–(k) Examples of tetramers: (h), (j) and (k) contain approximately equilateral triangles; (j) and (k) might fit hexagonal close packing; (g) roughly trigonal arrangement probably not planar. (l)–(o) Examples of pentamers: (m) approximates to an hexagonal close-packed array; (n) pentagonal arrangement. Magnifications: (c), (d) and (f) $\times 300\,000$; (e), (g), (h), (i), (j), (k), (l), (m) and (o) $\times 352\,000$; (n) $\times 476\,000$.

EXPLANATION OF PLATE 5

(a)–(g) Starch-gel-electrophoretic patterns of ferritin fractions obtained in Expt. SGF II. The start line is at the bottom and migration in a vertical direction towards the anode. (c) Ferritin fraction A12-19; (a) fraction AF A12-19 (fraction F A12-19 reduced to apoferritin); (b) unfractionated ferritin; (d) ferritin fraction F D41-52; (e) fraction AF D41-52 (fraction F D41-52 reduced to apoferritin). (f) Ferritin fraction F D31-40 after exhaustive dialysis against glass-distilled water; (g) fraction F D31-40 after dialysis for 6 days against 0.1 M-sodium acetate buffer, pH 5.0. (h)–(k) Ultracentrifuge schlieren patterns of fraction AF A12-19 (apoferritin). The protein concentration was 1.41 mg./ml. Sedimentation is from right to left. The bar angle was 40° . (h) In 0.1 M-sodium acetate buffer, pH 5.0, at 19.5° , after 10 min. at 59780 rev./min. in a cell with a double-sector centrepiece (aluminium coated with Kel F). (i) In 0.02 M-sodium borate buffer, pH 11.2, at 20.0° , after 16 min. at 50740 rev./min. in a cell with 4° -sector Kel F centrepiece. (j) and (k) In sodium borate buffer, pH 12.0 and 12.4 respectively, otherwise as (i). The decrease in the relative proportion of fast component as pH is increased should be noted.

EXPLANATION OF PLATE 6

Ultracentrifuge schlieren patterns of dimer-enriched apoferritin (fraction AF A25-29). Sedimentation is from right to left. A cell with 30 mm. centrepiece was used. The temperature was 19.0°. (a)–(c) 20 min. and (d) 44 min. after reaching speed of 50740 rev./min. (a) Concn. of apoferritin 3.35 mg./ml. in 0.01 M-sodium phosphate buffer, pH 7.0; bar angle 60°. (b) Concn. of apoferritin 0.45 mg./ml. in 0.01 M-sodium phosphate buffer, pH 7.0, diluted 18 hr. before centrifugation; bar angle 50°. (c) Concn. of apoferritin 0.19 mg./ml. in 0.01 M-sodium phosphate buffer, pH 7.0, diluted 1 week before ultracentrifugation; bar angle 40°. (d) Concn. of apoferritin 0.45 mg./ml. in 0.01 M-sodium phosphate buffer containing dioxan (44%, v/v) and incubated for 3 hr. before ultracentrifugation; bar angle 50°. The relative proportions of the two major components are unaffected by changes in concentration or solvent.

Table 5. *Statistical analysis of the distribution of molecules on an electron micrograph*

In the first column all molecules were considered as separate individuals irrespective of their apparent state of aggregation; in succeeding columns only monomers, dimers or trimers respectively were counted. The observed values are compared with those expected for a Poisson distribution. The fraction studied was D31-40 from Expt. SGF II. The total number of squares was 1312. The mean molecules/square was 0.6242 (if all molecules were considered separately irrespective of their state of aggregation).

No. of molecules/square	All molecules		'Monomers' only		'Dimers' only		'Trimers' only	
	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected
0	720	702.8250	734	722.9268	1295	1295.1839	1311	1310.9508
1	419	438.7034	421	430.8644	17	16.8161	1	1.0492
2	132	136.9193	120	128.3976				
3	32	28.4883	30	25.5083				
4	5	4.4456	4	3.8007				
5 or more	4	0.6184	3	0.5022				
	χ^2 4.9735*		χ^2 2.6781*					

*Not significant at 5% level.

answer for a given solution, as was shown by the χ^2 test. Despite the high degree of reproducibility for any given sample, we considered it desirable to perform a statistical analysis of the distribution of ferritin on two representative micrographs. Plates of preparations of Expt. SGF II fractions D31-40 and A12-19 were enlarged to $\times 150000$. The micrographs were divided into squares of equal size (approx. $10^6 \text{ \AA}^2$). The numbers of molecules occurring in each square were counted in each of the following ways: (a) all molecules considered as separate individuals irrespective of their state of aggregation; (b) monomers only; (c) dimers only; (d) trimers only. The frequencies of squares with 0, 1, 2 etc. of (a) all molecules, (b) monomers, (c) dimers and (d) trimers were counted. The frequency distributions obtained, shown in Tables 5 and 6, were compared with those expected for a random (Poisson) distribution.

The molecules present in fraction D31-40 are distributed on a membrane in a pattern that closely approximates to random. The distribution of dimers was not detectably non-random. The monomers alone approximated closely to a random distribution. This result is consistent with the view that fraction D31-40 is composed almost entirely of monomer. This agrees with the result shown in Table 4 (fraction D31-40, 94.7% monomer). If all the molecules in fraction A12-19 are counted as individuals irrespective of whether they occur in ones, twos or threes, it is clear that the overall distribution deviates greatly from a random one. Thus the molecules show marked aggregation. On the other hand, if only molecules occurring singly are considered, these are seen to be random with respect to each other. A similar result is obtained for pairs and triplets. Thus pairs of molecules (dimers) and triplets of molecules

(trimers) behave on a Formvar surface as if they were joined firmly together, i.e. a pair of ferritin molecules behaves as a single unit.

Configurations of oligomers as seen in the electron microscope

Dimers. In this study dimers are taken to be pairs of iron cores, the centres of which are separated by not more than one molecular diameter ($120 \text{ \AA}$). The protein shells, which have diameters about twice those of the iron cores, are not directly visible in the electron microscope (see Fig. 2). Numerous examples of dimers are shown in Plates 3, 4(a) and 4(b), taken from fraction A12-19 obtained in Expt. SGF II. The intermolecular distances measured were mainly within the range $90\text{--}110 \text{ \AA}$. Measurements from one plate varied less than pooled measurements obtained from several plates. The average intermolecular distance was $100 \text{ \AA}$ (average of 64 dimers). Occasionally (12% of dimers measured) distances less than $90 \text{ \AA}$ were observed (see Fig. 3). It was thought that these could be attributed to tilting or possibly to distortion of the protein shells due to excessive shrinkage on drying.

Trimers. Measurements of intermolecular distances were made on 58 trimers and scale drawings ($5 \text{ cm.} \equiv 100 \text{ \AA}$) were constructed. The configurations of the trimers were examined in detail. Molecules were assumed to be bonded if the intermolecular distances fell within the range observed for dimers (i.e. less than $120 \text{ \AA}$). Of the 58 trimers examined, 27 were close to equilateral triangles, with all sides less than $120 \text{ \AA}$ (Plates 4e). Most of these, however, had one side in the range $110\text{--}120 \text{ \AA}$, i.e. larger than the average intermolecular distance in dimers. It is not clear whether these trimers had been distorted

Table 6. *Statistical analysis of the distribution of molecules on an electron micrograph*

In the first column all molecules were considered as separate individuals irrespective of their apparent state of aggregation; in succeeding columns only monomers, dimer or trimers respectively were counted. The observed values are compared with those expected for a Poisson distribution. The fraction studied was A12-19 from Expt. SGF II. The total number of squares was 926. The mean numbers of molecules/square was 0.4827 (if all molecules were considered separately irrespective of their state of aggregation).

No. of molecules/square	All molecules			Monomers only			Dimers only			Trimers only		
	Observed	Expected	No. of molecules/square	Observed	Expected	No. of molecules/square	Observed	Expected	No. of molecules/square	Observed	Expected	No. of molecules/square
0	703	571.4484	0	823	822.3567	0	809	803.0157	0	907	906.3035	0
1	82	275.8381	1	96	97.6137	1	105	114.4297	1	18	19.4855	1
2	96	66.5735	2 or more	7	6.0296	2	9	8.1531	2 or more	1	0.2110	2 or more
3	23	10.7117				3 or more	3	0.4015				
4	13	1.2926										
5	5	0.1248										
6	1	0.0100										
7 or more	3	0.0009										

$\chi^2 = 268.45^*$

$\chi^2 = 0.1834^\dagger$

$\chi^2 = 1.811^\dagger$

*Significant at 0.1% level.

†Not significant at 5% level.

Fig. 2. Diagrammatic representation of a ferritin dimer. The stippled areas represent the iron-containing core and the surrounding annuli represent protein shells. The protein shells are not visible in the electron micrographs (see Plates 2-4). Molecules in contact are taken to be iron-containing cores separated by the distance shown (one molecular diameter).

Fig. 3. Histogram of frequency distribution of intermolecular distances in dimers as seen in the electron microscope.

from equilateral on drying or whether the sides were unequal in the native state. It seems probable that in these trimers each molecule is in contact with two others, although the nature of the contacts may vary. In a further 20 trimers the configuration was triangular with two sides within the observed range of contact distances, while the third side was considerably more than the contact distance. The apical angle in these cases was clearly more than 90° and less than 180°, angles in the range 120-140° being usual (Plate 4d). In eight trimers the molecules were co-linear (Plate 4c). In seven of these the observed intermolecular distances were

approximately equal and within the acceptable limits. In the remaining linear trimer, the distances were unequal and the trimer could conceivably have been a triangle viewed edge on. Finally, a group of three trimers appeared to be triangles with one side larger than the contact distance and the apical angle about 90° . An example of an acutely tilted trimer was found (Plate 4f).

Tetramers. About 20 tetramers were studied and scale drawings were made of 13. Several configurations were observed, examples of which are shown in Plates 4(g)–4(k). Most of the tetramers were found to contain approximately equilateral triangles similar to those found in the trimers, e.g. Plates 4(h), 4(j) and 4(k). A fourth molecule was situated at various positions with respect to these triangles.

Of the remaining examples, one contained a right-angled triangle and one contained three

molecules disposed in a roughly trigonal arrangement around a central molecule (Plate 4g). No linear tetramers were observed, but examples similar to Plate 4(i) were occasionally seen.

Pentamers. Examples of pentamers are shown in Plates 4(l)–4(o). Of these configurations, those shown in Plates 4(l) and 4(m) were the most common. Equilateral triangles were again a feature of most of the pentamers. Occasional examples not including an equilateral triangle, such as the pentagon shown in Plate 4(n), were seen.

Amino acid analyses

Plots of the amino acid compositions of fraction AFA12–19 versus fraction FA12–19 and of fraction AFD41–52 versus fraction FD41–52 are shown in Figs. 4(a) and 4(b) respectively. In most cases each point represents the mean of five analyses for

Fig. 4. Plots of amino acid compositions of fractions: (a) fraction F A12–19 versus fraction AF A12–19; (b) fraction F D41–52 versus fraction AF D41–52; (c) fraction AF D41–52 versus fraction AF A12–19; (d) fraction F D41–52 versus fraction F A12–19.

all amino acids except serine, threonine and cysteic acid. These plots demonstrate the reproducibility of the analyses and show that differences in the methods of iron removal have a negligible effect on the results. Figs. 4(c) and 4(d) show plots of the amino acid compositions of fraction AF D41-52 versus fraction AF A12-19 and of fraction F

D41-52 versus fraction F A12-19. From these it is clear that the amino acid compositions of all the samples are the same within the experimental error. The average amino acid compositions for fractions D41-52 (F and AF) and A12-19 (F and AF) are plotted in Fig. 5 and listed as numbers of μ moles in Table 7, columns 1 and 2.

Differences between the values in columns 1 and 2 of Table 7 correspond at most to just under half a residue/sub-unit of mol.wt. 23000 and are usually very much less than this. The small differences between the mean values for any particular amino acid in fraction D41-52 compared with fraction A12-19 were tested for significance by Student's *t* test. In no case was the difference significant at the 5% level.

The standard deviations of the determinations allow estimates to be made of the minimal detectable changes (greater than 1.96 s.d.) in individual amino acids. Such calculations indicate that a change of one residue of any amino acid in every sub-unit would have been detected. In favourable cases single residue changes might have been detected if they occurred in as few as six sub-units out of 20. Changes as small as one residue in one sub-unit would not have been detected.

Within the limitations of this technique these results support the view that only one species of ferritin occurs in monomers, dimers and trimers.

Fig. 5. Plot of average amino acid compositions for fraction D41-52 (F and AF) versus fraction A12-19 (F and AF). The close agreement is obvious.

Table 7. *Amino acid composition of monomer-enriched (D41-52) and oligomer-enriched (A12-19) fractions*

Results are expressed as μ moles of amino acid, scaled together as explained in the text. Tryptophan was not determined. Results for monomer (fraction D41-52) are the average of those obtained for fractions F D41-52 and AF D41-52; results for oligomer (fraction A12-19) are the average of those obtained for fractions F A12-19 and AF A12-19.

Amino acid	Monomer (fraction D41-52)	Oligomer (fraction A12-19)	Mean of all samples $\pm$ s.d.	Total no. of determinations
Ala	3.889	3.838	3.865 ± 0.081	19
Arg	2.705	2.665	2.683 ± 0.082	15
Asp	4.693	4.724	4.708 ± 0.096	20
Cys*	0.521	0.549	0.535 ± 0.034	8
Glu	6.534	6.538	6.536 ± 0.110	19
Gly	2.678	2.662	2.670 ± 0.047	19
His	1.581	1.613	1.598 ± 0.072	13
Ile	1.037	1.007	1.022 ± 0.035	20
Leu	6.867	6.799	6.833 ± 0.096	20
Lys	2.412	2.461	2.440 ± 0.108	16
Met†	0.816	0.793	0.804 ± 0.046	17
Phe	1.975	1.998	1.986 ± 0.110	19
Pro	0.575	0.534	0.563 ± 0.050	14
Ser	2.565‡	2.605‡	2.585 ± 0.047	4
Thr	1.535‡	1.465‡	1.500 ± 0.047	4
Tyr	1.431	1.407	1.418 ± 0.059	13
Val	1.953	1.890	1.923 ± 0.119	19

*Determined as cysteic acid.

†Determined as methionine and methionine sulphone.

‡Extrapolated to zero time.

The average amino acid analysis is given in Table 7, column 3. With the exception of serine, threonine and half-cystine, these values represent the mean of 13 or more determinations.

Studies on the stability of ferritin oligomers

Effects of sample preparation and storage. It was found that the proportions of dimer and trimer in ferritin and apoferritin solutions (in water or in acetate buffer, pH 5.0) containing different relative and absolute amounts of aggregated molecules remained unchanged after storage in the cold for 2 months, as judged by starch-gel electrophoresis. Exhaustive dialysis against glass-distilled water was equally without effect. After storage as a precipitate in 30% ammonium sulphate, samples of ferritin, D31-40 and D41-52 (Expt. SGF II), which had previously appeared to be pure α -band, now contained traces of β -band on starch-gel electrophoresis (compare Plates 1*q* and 5*f*). This increase in dimer content was confirmed by electron microscopy (see Table 4). The amount of β -band was barely increased, however, by ten successive precipitations with 30% ammonium sulphate.

As previously reported (Harrison & Gregory, 1965) heating solutions of unfractionated ferritin for 1 hr. at temperatures up to 74° or for 35 min. at 77.5° did not affect the proportions of the various bands. Freeze-drying of apoferritin produced a product that dissolved with difficulty in 0.4M-sodium hydrogen carbonate, pH 8.3, and gave an ultracentrifuge pattern showing increased proportions of material having sedimentation coefficients close to those of apoferritin dimers and trimers (Hofmann & Harrison, 1963).

Reduction of ferritin to apoferritin with sodium dithionite (Granick & Michaelis, 1943) occasionally altered the band pattern obtained by starch-gel electrophoresis. This has also been noted by Suran & Tarver (1965). Plates 5(*a*), 5(*c*), 5(*d*) and 5(*e*) show samples of ferritin before and after reduction. In fraction A12-19 reduction has slightly modified the band pattern. Nevertheless quantitative measurements of the components of fractions F A12-19 (by electron microscopy; Table 4) and AF A12-19 (by ultracentrifugation; Table 11) show good agreement. On the other hand fraction F D41-52, which originally approached 100% monomer, on reduction by dithionite in sodium acetate buffer, pH 5.0, was found to contain dimers and trimers in proportions similar to those of native ferritin (Plates 5*d* and 5*e*). Reduction of a second fraction F D31-40 under similar conditions did not show this effect, although the same fraction dialysed against acetate buffer, pH 5.0, appeared to show a very slightly increased proportion of β -band (Plates 5*f* and 5*g*).

Table 8. *Percentages of monomer and oligomers in a mixture of fractions D31-40 and A20-24 obtained in Expt. SGF II*

Counts were made in the electron microscope.

	% of molecules occurring as			
	Monomer	Dimer	Trimer	Tetramer and higher oligomers
Predicted	52.60	35.37	6.43	5.40
Obtained	55.30	33.87	7.46	3.37

Effects of dilution and the mixing of monomer and dimer fractions. Samples used in starch-gel electrophoresis had an original concentration of 1-2% ferritin. Samples used in electron microscopy were about 30 μ g./ml. or less, i.e. a dilution of 300-fold or more, as compared with starch-gel samples. Starch-gel electrophoresis is not amenable to precise quantitative determination and no such determinations were attempted. The effect of dilution on the proportion of oligomers present was tested by studying the effects of mixing monomer- and oligomer-enriched fractions F A20-24 and F D31-40. Electron-microscope counts of the proportions of monomer and oligomers agreed closely with those predicted for the mixture (see Table 8). The electron-microscope counts on ferritin also agree quite closely with ultracentrifuge analyses of apoferritin derived from the same sample, e.g. compare count of 76.0% aggregates for sample F A12-19 (Table 4) with 70.5-72.3% aggregates for sample AF A12-19 (Table 11) (protein concentration 1.4 mg./ml.).

To test the effect of dilution on the percentage of dimer in dimer-enriched apoferritin, fraction AF A25-29 was examined by ultracentrifugation in a cell with a 30 mm. centre-piece (Plate 6). Solutions of the following concentrations were examined: 3.35, 0.66, 0.45, 0.33 and 0.19 mg./ml. The relative proportion of dimer as determined from peak areas did not decrease over this concentration range. (A small, but probably insignificant, increase in dimer from 35% at concentrations 0.45-3.35 mg./ml. to 40% at concentrations 0.19-0.33 mg./ml. was observed.)

Studies of the nature of the intermolecular linkage in ferritin and apoferritin aggregates

Attempts to split dimers and trimers. The effects of a number of reagents on the intermolecular linkages were examined under a wide variety of conditions. These included reagents specific for various covalent bonds and others that were less

Table 9. *Attempts to split dimer and trimer with non-specific reagents*

Reagent	System	Test condition	Comments
8 M-Urea	Dimer- and trimer-enriched ferritin, unfractionated apoferritin and ferritin	Urea-borate starch-gel electrophoresis	No effect
2-6 M-Guanidine hydrochloride, 20°	Apoferritin and ferritin with or without subsequent dialysis	Urea-borate starch-gel electrophoresis, ultracentrifuge	Some sub-units released, little effect on proportions of oligomers
3 M-NaCl	Dimer- and trimer-enriched apoferritin	Ultracentrifuge	No effect
2 M-Ammonium acetate	Unfractionated apoferritin	Ultracentrifuge	No effect
3% Ammonium acetate	Dimer- and trimer-enriched ferritin, unfractionated ferritin	Electron microscope	No effect
1% Sodium dodecyl sulphate, pH 7.5, 33°, 48 hr.	Unfractionated apoferritin	Electron microscope	No effect
0.1 M-Sodium acetate buffer pH 3.65, 20°, 16 hr.	Dimer- and trimer-enriched apoferritin	Ultracentrifuge	No effect
Up to 50% dioxan in 0.01 M-phosphate buffer, pH 7.0	Dimer- and trimer-enriched apoferritin, unfractionated ferritin	Ultracentrifuge, starch-gel electrophoresis	No effect
Acetic acid-water (2:1, v/v)	Unfractionated ferritin and apoferritin	Starch-gel electrophoresis	Sub-units released, some intact monomer, little or no oligomers left

Table 10. *Attempts to split dimer and trimer with specific reagents*

Reagent	Bond type studied	Analysis	Analysis conditions	Comments
Mercaptoethanol-8 M-urea in borate, pH 8.9, 20°, 2 hr.-7 days	S-S	Unfractionated apoferritin and ferritin	Starch-gel electrophoresis in borate containing 8 M-urea and mercaptoethanol	No effect
Mercaptoethanol in borate, pH 8.0, 20°, 2 hr.	S-S	Unfractionated apoferritin and ferritin	Starch-gel electrophoresis in borate containing mercaptoethanol	No effect
Sulphite-bisulphite (Creeth & Nicol, 1960)	S-S	Unfractionated apoferritin	Ultracentrifuge, starch-gel electrophoresis	No effect
Sulphite-bisulphite + 2.4 M-guanidine hydrochloride	S-S	Unfractionated apoferritin	Ultracentrifuge	No effect
Dithiothreitol (0.8 mg./ml.), pH 8	S-S	Unfractionated apoferritin and ferritin	Starch-gel electrophoresis in borate (dithiothreitol added)	No effect
Pronase (Nomoto, Narahashi & Murakami, 1960), 35°, 24 hr.	Peptide	Unfractionated apoferritin and ferritin	Starch-gel electrophoresis in tris-citrate	No effect
Hydroxylamine (Blumenfeld, Rojkind & Gallop, 1965)	Ester	Dimer- and trimer-enriched ferritin and apoferritin	Starch-gel electrophoresis in borate	No effect
EDTA, various concns. and times	Bivalent cations	Unfractionated ferritin and apoferritin	Starch-gel electrophoresis, ultracentrifuge	No effect
Desferrioxamine in acetate, pH 4.4	Fe ³⁺	Unfractionated ferritin and apoferritin	Starch-gel electrophoresis in borate	No effect

specific in their effect. Reagents and conditions are summarized in Tables 9, 10 and 11. Incubation at low pH in acetic acid-water mixtures or at high pH (Table 11) were the only treatments effective in splitting significant proportions of oligomers.

The results listed in Table 11 (see also Plates 5*h*-5*k*) were obtained from solutions of the same concentration of dimer-enriched apoferritin (frac-

tion AF A12-19). Table 11 shows that at pH 3.65 and 7.6 the proportions of monomer and dimer are similar (a small amount of trimer was measured at pH 3.65, but at pH 7.6 it was not sufficiently well resolved from the dimer peak for a trimer measurement to be made). At high pH values two effects were observed. The total amount of protein appearing as monomer and dimer fell progressively

Table 11. *Effect of pH on the proportion of aggregated apoferritin molecules*

The results were obtained from ultracentrifuge schlieren traces.

pH	Buffer	% of total protein				Peak 2 Peak 1
		Peak 1 (17s) Monomer	Peak 2 (24s) Dimer	Peak 3 (32s) Trimer	Sub-units	
3.65	0.1 M-Acetate	29.5	62.8	7.7	0	2.13
7.6	0.1 M-Phosphate	27.7	72.3*	—	0	2.61
11.2	0.02 M-Borate	42.1	56.9	0	1.0	1.35
11.7	0.02 M-Borate	41.2	49.0	0	9.8	1.19
12.0	0.02 M-Borate	35.0	25.9	0	39.1	0.74
12.4	0.02 M-Borate	30.8	12.2	0	57.0	0.40

*Contains peak 3.

as the pH increased. This was paralleled by the appearance and progressive increase of a slow-moving peak, which was taken to be apoferritin sub-units (Hofmann & Harrison, 1963). At the same time the amount of dimer fell dramatically, as did the ratio of dimer to monomer. At pH 11.2 and 11.7 it appeared that some of the dimer had been converted into monomer. It is not clear from the values at pH 12.0 and 12.4 whether the dimer was converted into monomer before splitting into sub-units, or whether some of it had been split into sub-units directly.

DISCUSSION

The experiments described here confirm that horse spleen ferritin contains monomer, dimer, trimer and higher oligomers and that partial fractionation of these components can be achieved.

In our hands the most successful method of fractionation on a preparative scale was gel filtration on Sephadex G-200. This technique allows ferritin monomers to be separated from oligomers. The oligomers are eluted too close to the void volume to permit further separation into dimer, trimers, tetramers etc. By this method enrichment of monomer from 85% up to 95% was achieved and of dimer and higher oligomers from 15% to 76%. Kopp, Vogt & Sund (1966) have achieved similar enrichments by repeated ammonium sulphate precipitation. Richter & Walker (1967) achieved an oligomer enrichment to 45% by repeated absorption on to cellulose, elution with ammonium sulphate solutions and subsequent gel filtration on Sephadex G-200. Starch-gel electrophoresis is less satisfactory as a preparative technique for oligomers, but small amounts of partially purified material could be prepared (see also Richter & Walker, 1967). Our failure to obtain pure β -band preparations, as judged by electron-microscope counts, might suggest a reversible association. However, in view of the stability exhibited by the dimer under

various conditions this explanation seems unlikely, though not impossible. Richter & Walker (1967) claim enrichment of α , β - and γ -bands to 100%, 95% and 85% of the α -, β - and γ -components respectively by polyacrylamide-gel electrophoresis.

The amino acid analyses and electron-microscopic evidence strongly support the view that the β - and γ -components consist of dimers and trimers of molecules similar to those present as monomers in the α -component. The amino acid analyses for fractionated ferritin resembled those obtained previously for unfractionated apoferritin (Harrison, Hofmann & Mainwaring, 1962) and for monomer-enriched fractions (Suran & Tarver, 1965). Shinjyo (1967), however, obtained significantly different analyses for unfractionated apoferritin and α -apoferritin, in conflict with our results.

Most of our electron-microscope observations were made on ferritin, in which the electron-dense iron-containing cores can be viewed directly and in which dimers can be identified as pairs of cores, the centres of which are about 100 Å apart. That such pairs of iron cores represent molecules that are in contact was confirmed by negative staining and shadowing techniques. Negative staining was not used extensively in this work because of the possibility of causing disaggregation. Our use of BSA as a spreading agent has been criticized by Richter & Walker (1967) on the grounds that its effects 'could be several and are unknown'. At the same time these authors suggest that differences in counts of monomer and oligomers for different preparations are a result of a failure to achieve adequate randomization of molecules on the grids, since they have obtained 'similar frequency distributions of monomers, dimers and trimers in α -, β - and γ -ferritins'. Our results show that BSA is without aggregative effect on monomers, whereas on oligomers its effect is, if anything, a dissociative one. The close agreement between electron-microscope, starch-gel-electrophoresis and ultracentrifuge observations, together with the statistical

evidence provided, demonstrates that our 'monomer' and 'oligomer' preparations clearly differ in their state of aggregation and not in their degree of randomization. Richter & Walker (1967) explain their data by suggesting that dilution of the samples before electron-microscope viewing causes disaggregation of oligomers followed by partial random association on drying. Our results could be explained in either of two ways: (1) the oligomers dissociated on dilution and reassociated on drying on the grid whereas the monomer did not associate; in this case two types of monomer ferritin molecule must be postulated, one which is capable of reversible association, and one which is not; (2) the oligomers did not dissociate. In the former case it is difficult to see how reassociation could occur in the presence of excess of BSA. The simpler explanation would be that the oligomers did not dissociate.

In our experiments ferritin oligomers appear to be stable in the concentration range 30 $\mu\text{g.}$ –10 mg./ml. It is possible that ferritin aggregates are unstable below 30 $\mu\text{g.}$ /ml. Richter & Walker (1967) may have diluted their ferritin samples for electron microscopy below this level, but they do not quote the concentrations they use. In their light-scattering experiments apoferritin appears to have dissociated at much higher concentrations (between 1.0 and 0.1 mg./ml.). It is possible that apoferritin behaves differently from ferritin, but in our experiments estimates of the proportions of monomer, dimer and trimer from apoferritin preparation AF A12–19 in the ultracentrifuge and ferritin preparation F A12–19 in the electron microscope agree closely. Good correlations were also obtained in previous work between components seen in the ultracentrifuge and on starch-gel electrophoresis (Harrison & Gregory, 1965; Suran & Tarver, 1965). Richter & Walker (1967) obtained 'no obvious correlation between the degree of heterogeneity detected by electrophoresis and the light-scattering curves'. Unfortunately, ultracentrifuge measurements were not made on those samples, which according to their light-scattering data contained high proportions of oligomers. We have not conducted light-scattering experiments, but in our ultracentrifuge observations dimer-enriched apoferritin preparations failed to show dissociation over a concentration range 3.35–0.19 mg./ml. As previously reported (Harrison & Gregory, 1965) monomer preparations showed a single peak in the ultracentrifuge. This result has been confirmed for apoferritin monomer solutions at concentrations up to 10 mg./ml. We have thus failed to detect evidence for reversible association-dissociation in ferritin or apoferritin solutions.

The nature of the intermolecular linkages in ferritin and apoferritin oligomers is as yet undeter-

mined. The evidence that we have obtained has largely been negative in character. From the failure to detect any of the more common covalent intermolecular linkages [e.g. disulphide bridges as in urease (Creeth & Nichol, 1960) and thietin homocysteine methyltransferase (Durell, Anderson & Cantoni, 1957), ester linkages as in collagen (Blumenfeld *et al.* 1965) or peptide bonds], together with the tendency to aggregation observed in 30% (w/v) ammonium sulphate and on freeze-drying (Hofmann & Harrison, 1963), it is improbable that the oligomers are bonded covalently. This is also suggested by the splitting observed in 67% acetic acid at 0° [which causes the disaggregation of a number of multi-sub-unit proteins in which the sub-units are not covalently bonded together, e.g. tobacco-mosaic virus (Fraenkel-Conrat, 1957)]. Predominantly polar interactions also seem unlikely since splitting was not detected in high concentrations of urea, guanidine hydrochloride or various salts. The possibility that Fe^{2+} ions were in some way implicated in aggregation was raised by Richter & Walker (1967); however, both they and we have found EDTA to be without effect. Desferrioxamine, which strongly binds Fe^{3+} (Schubert, 1964), was also ineffective in breaking both ferritin and apoferritin oligomers. The apparent absence of covalent intermolecular bonds and the stability of the oligomers in high salt concentrations, in which hydrophobic interactions would tend to be stabilized (Kauzmann, 1959), suggest that the latter may play an important role in ferritin aggregation. The fact that high pH values (pH 11–12 in solutions of low ionic strength) cause some disaggregation is not inconsistent with this supposition, since the molecules at these pH values bear a high net negative charge and would therefore be expected to repel one another. Conditions that cause disaggregation of oligomers (high or low pH at low ionic strength) also give rise to some disassembly of monomers into sub-units. This suggests that the interactions involved may be similar in character. The sub-units are known not to be joined together covalently (Hofmann & Harrison, 1963). If the association was primarily due to hydrophobic interactions, dissociation in organic solvents might have been expected. Solution in 44–50% dioxan, however, did not apparently disrupt either the dimer into monomers, or monomers into sub-units. On some occasions oligomers were formed during the reduction of ferritin to apoferritin with sodium dithionite, but this did not happen consistently (see also Suran & Tarver, 1965). More usually the proportions of oligomers in a given sample of ferritin remained unchanged on reduction to apoferritin. The formation of aggregates was also noted to occur on freeze-drying of apoferritin (Hofmann & Harrison, 1963), but the mode of

aggregation was not necessarily the same as in native preparations. Sodium dodecyl sulphate (approx. 1% solution) does not break down native ferritin or apoferritin oligomers to monomers, nor monomers to sub-units, though it degrades both monomers and aggregates in frozen-dried apoferritin into sub-units.

The centre-to-centre distances of about 100 Å in the dimers are close to the value, 94 ± 5 Å, for the diameters of ferritin measured as edge-to-edge distances in doubly shadowed molecules (Farrant, 1954). They are, however, appreciably smaller than measurements made by Kuff & Dalton (1957) (95–115 Å centre-to-centre distance in close-packed layers, 105–110 Å in shadowed single molecules) and Labaw & Wyckoff (1957) (109 ± 4 Å separation in crystals). Recently, however, R. Luftig (personal communication) has obtained a diameter of 106 ± 3 Å by edge-to-edge measurements of single negatively stained molecules (this is a revised value of that reported by Luftig, 1967). The intermolecular distance we have observed in dimers may not be significantly different from this diameter, but the lower value could arise from some interpenetration of the protein shells. It is appreciably smaller than the intermolecular distances between ferritin and monomers in air-dried crystals, 112 Å, measured by X-ray diffraction (Harrison, 1959). Both interpenetration of the protein shells and molecular shrinkage may be responsible for the shortened distance. Intermolecular distances in wet crystals, 130 Å (Harrison, 1959), are even larger and it would be of interest to compare this distance with intermolecular distances in oligomers in solution. Unfortunately, the latter are unknown. They might be expected to be smaller than the distances between monomers in wet crystals. The forces holding the molecules together in wet crystals are clearly much weaker than those in oligomers, since the monomer molecules can be separated by solution. We have found that, although small percentages of oligomers will readily co-crystallize with monomer (and crystallization could not therefore be used as a means of purification; Harrison & Gregory, 1965), oligomer-enriched fractions F and AF A12–19 failed to crystallize at all under similar conditions (1–5% cadmium sulphate solutions). This may well be a result of differences in relative molecular orientations, or of intermolecular packing distances, or both.

The configurations of the trimers analysed appear to be restricted in the main to a limited number of types. Equilateral or nearly equilateral triangles make up about half of the total and most of the remainder were isosceles triangles with apical angle about 120–144°. In such triangles each molecule could make two equal interactions with its neighbours. In non-equilateral triangles the

interactions would not all be the same and might be limited to two sides. Equilateral triangles might therefore have been expected to be the favoured form. At most, however, they only account for 50% of the trimers studied. The individual protein molecules are known to be composed of about 20 polypeptide chains or sub-units, probably arranged at the apices of a pentagonal dodecahedron. If interactions between molecules having this quaternary structure are considered, various possible geometrical arrangements suggest themselves. These include isosceles triangles with apical angles of 72°, 108° or 144°, an isosceles triangle with apical angle 117° and arrangements with angles of 60° or 120° as found in the crystal structure. Such angles may occur in some of the oligomers, but the measurements are not sufficiently precise for any of these alternatives to be clearly distinguished. The pentamer shown in Plate 4(n) could be a distorted view of a regular pentagon (angle 108°), but most of the remaining tetramers and pentamers seem to be composed of at least three molecules in a trigonal system. Again, in the larger oligomers the distance between molecules cannot be measured sufficiently precisely for the configuration of the oligomer to be clearly established. In several of them the arrangement appeared to be a co-planar one or only slightly distorted from co-planar. In others the electron micrograph could better be fitted with a non-planar grouping composed of one 'equilateral' triangle and one or more additional molecules not in the plane of this triangle. An equilateral tetrahedral arrangement was, perhaps surprisingly, never observed. The conclusion that many of the tetramers and pentamers are probably planar or near-planar again raises the possibility that some or all of them are artifacts formed from dimers and trimers by drying on the grid of oligomer-enriched fractions. The likelihood of formation of such artifacts in the presence of such a large excess of spreading agent is probably, however, small. Further, independent evidence for the presence of these higher oligomers in horse ferritin is suggested by the observations of δ - and ϵ -bands on gel electrophoresis of oligomer-enriched fractions. Drying on the electron-microscope grid may have caused distortion from the original oligomer configuration to a more nearly co-planar one.

The fact that several ferritins from horse and other species, which have been isolated by a variety of different procedures, have been found to exhibit heterogeneity on gel electrophoresis suggests that the α -, β -, γ -components etc. may well have been present within the cells. For horse ferritin, at least, these components are now clearly seen to be polymeric forms. This conclusion is supported for human ferritin by the electron-microscope observations by Zamiri & Mason (1968). If these

polymers are indeed present in the cell, this raises the questions of their mode of formation and biological significance. From gel electrophoresis and electron microscopy it is clear that monomers and polymers alike contain iron, and there does not therefore seem to be any obvious functional difference between them. Possibly the polymers are formed during an aging process in which minor alterations occur on the protein surface that favour polymer formation. Conceivably they are intermediates between ferritin monomer and haemosiderin.

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