AN ELECTRON MICROSCOPIC STUDY OF FERRITIN

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The protein ferritin, first isolated in crystalline form by LAUFBERGER[1] in 1937, is unique in that it contains iron up to 23% of its dry weight. Studies in the laboratories of MICHAELIS, WHIPPLE and others have shown that this large iron content is associated with the physiological function of iron storage.

The extensive literature on ferritin has been periodically reviewed by GRANICK[2,3] and MICHAELIS[4]. The earlier articles survey the experimental data which indicate that ferritin consists of a homogeneous protein portion, known as apoferritin, of molecular weight 465,000, associated with micelles of ferric hydroxide-phosphate of approximate composition $(FeOOH)_8(FeOPO_3H_2)$. Indirect methods have been inadequate to ascertain whether the micelles lie on the surfaces of the protein molecules or are contained within them. GRANICK[3] recently summarized those experimental data which apparently favour one or other of these two alternatives.

The facts which favour the view that the micelles are situated outside the apoferritin molecules are:

1. The iron of ferritin can be removed by reducing agents such as cysteine[5] without denaturing the apoferritin. GRANICK considers cysteine molecules to be too large to penetrate between the polypeptide layers of apoferritin.

2. Ferritin solutions coagulate reversibly at 60° C[6], whereas apoferritin does not coagulate at this temperature[7]. This suggests that the coagulation is due to the presence of the iron hydroxide micelles on the surface and does not depend on the surface properties of apoferritin.

GRANICK believes these arguments to be more conclusive than those which he lists in support of the alternative model, namely:

1. The electrophoretic mobilities of ferritin and apoferritin are identical[8,9].

2. The viscosities of ferritin and apoferritin solutions are identical[10].

3. Quantitative precipitin studies show that ferritin antibody precipitates identical amounts of nitrogen from either ferritin or apoferritin solutions[9]. Presumably the sites to which the antibody adsorbs are located only on those areas of the protein molecule not covered by the micelle or else the micelle is inside the protein molecule.

Electron microscopic studies reported in this paper indicate that the iron is concentrated in micelles which exhibit internal structure and which lie within the apoferritin molecules.

MATERIALS AND METHODS

Materials

The ferritin used was isolated from the spleens of healthy horses by GRANICK's modification[6] of the original method of LAUFBERGER[1], and recrystallized five times from 6% $CdSO_4$ solution.

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So-called "non-crystallizable ferritin" was obtained from the brown mother liquor remaining from the second recrystallization after it had stood for some months over ferritin crystals in the presence of 6% $CdSO_4$ solution.

Apoferritin was prepared by reducing the iron of acidified ferritin solution to the ferrous state and then removing it by dialysis as described by GRANICK AND MICHAELIS[7]. It was obtained as almost colourless crystals after repeated crystallization from 6% $CdSO_4$ solution.

Methods

To avoid obscuration of the protein molecules it was necessary to use solutions which were relatively free from $CdSO_4$ and these were obtained by washing the crystals in distilled water several times in the case of ferritin and apoferritin and by dialysis in the case of "non-crystallizable ferritin". Specimens for electron microscopy were prepared by allowing droplets of dilute solutions of the proteins (0.001 to 0.0001%) to dry on the usual collodion supporting membranes. Some of the specimens were examined unshadowed while others were shadow-cast with uranium.

The electron micrographs were taken with an R.C.A. model EMU microscope adjusted to minimize all instrumental factors known to detract from resolution. It was necessary to employ Eastman Kodak Type 548-o emulsion to take full advantage of the resolution available at the maximum instrumental magnification of 21,000 diameters. This emulsion is rather insensitive and normally requires exposures which are inconveniently long if resolution is not to be forfeited to image movement. The effective speed was increased by the use of the energetic developer Kodak D-8.

RESULTS

Fig. 1 shows a field of dispersed ferritin molecules and reveals that the iron is concentrated in angular micelles up to 55 A in diameter. The micelles exhibit internal structure which varies presumably according to the orientation of the molecule; the various appearances being compatible with a square planar arrangement of four particles each $\sim$ 27 A in diameter. The arrows indicate micelles in which the four particles are particularly well defined. Micelles of less perfect symmetry are numerous; indeed some seem to consist of a single small particle at the limit of detectability. This agrees with the ultracentrifuge studies made by ROTHEN[8], which indicate that the micelles are non-uniform and that about 25% of ferritin molecules contain little or no iron.

Despite the employment of a 15-micron aperture immediately below the back focal plane of the objective lens and the use of the high-contrast developer D-8, the contrast obtained is insufficient to reveal the organic part of the molecules, but its presence can be inferred from the fact that micelle centres rarely approach one another more closely than 90 A. Cases where the micelle centres are apparently separated by smaller distances are probably due to superposition of the molecules. Micrographs of fields which had been shadow-cast after having been photographed previously revealed that quite short exposures to the electron beam were sufficient to bury the molecules under a contaminating layer which no doubt contributed largely to the inability to image directly the protein part of the molecules.

Specimens shadow-cast with uranium in the conventional manner (5–7 A at artan 1/4) yielded no useful information as the micelles were no longer visible through the uranium layer and the thickness of uranium deposited on one side of the molecules distorted their shape and exaggerated their size. This difficulty was overcome by shadow-casting with much thinner layers of uranium ($\sim$ 1 A at artan 1/10) deposited successively from two opposite directions. Under these conditions the uranium layer is too thin to cast well defined shadows, but a thin wall of uranium built up around each molecule delineates the molecule yet still allows the iron hydroxide micelle to be seen at its centre as shown in Fig. 2. Out-of-focus micrographs of unshadowed ferritin molecules show similar rings arising from diffraction. Fig. 2, however, is the "in-focus" micrograph from

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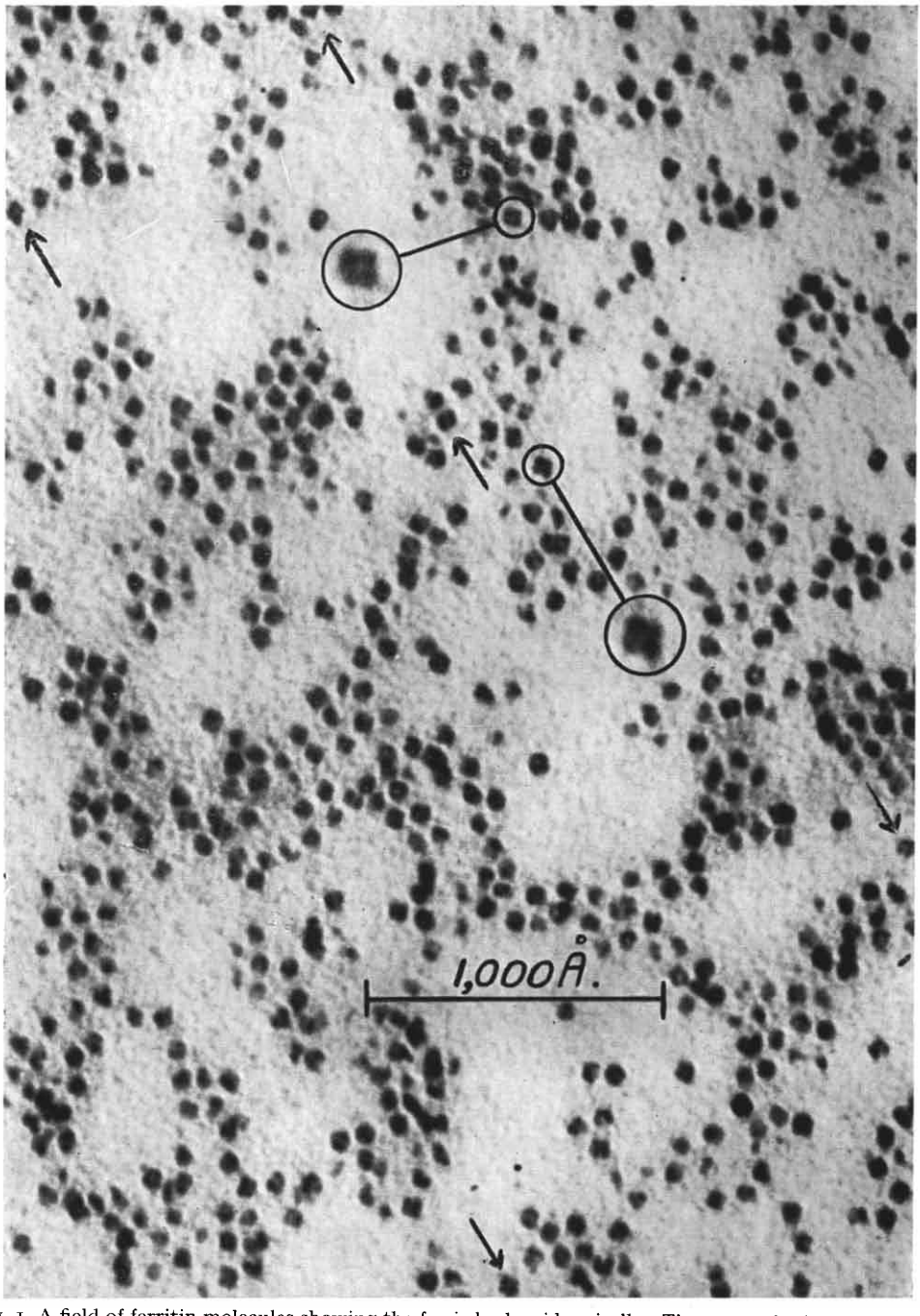

Fig. 1. A field of ferritin molecules showing the ferric hydroxide micelles. The arrows indicate micelles which exhibit fourfold symmetry. Magnification 415,000 ×. Magnification of inset micelles 900,000 ×.

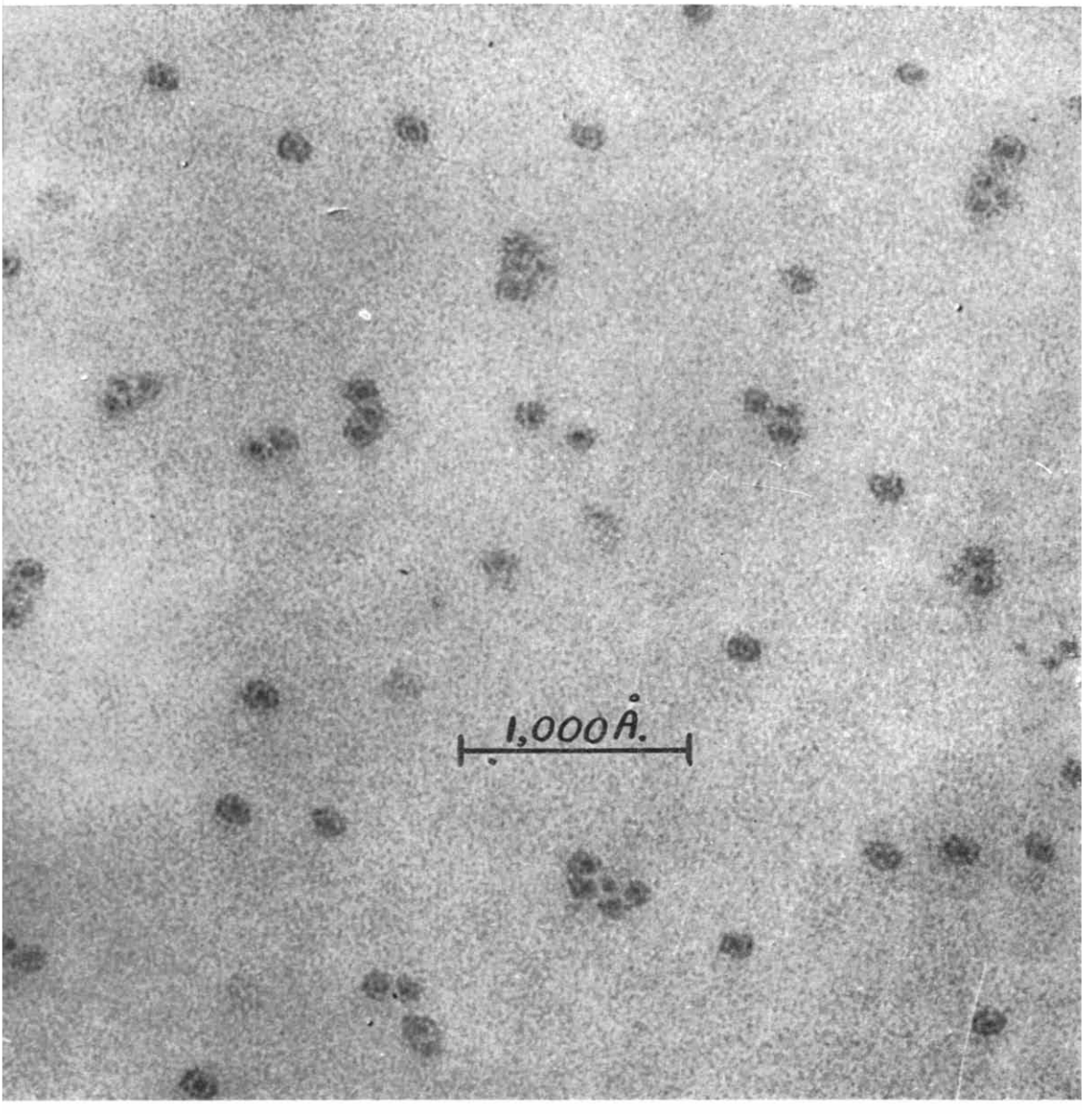

Fig. 2. Ferritin molecules lightly shadow-cast with uranium deposited from two opposite directions. Note that the micelles lie inside the protein molecules delineated by the "walls" of uranium. 320,000 ×.

a through focus series. Specimens shadow-cast from one direction only reveal the micelles lying at the centres of semicircular arcs of uranium so there is no possibility that the rings are a diffraction effect. If the micelle lay on the surface of the protein, it would then appear in the centre of the uranium enclosure only for special orientations of the protein molecule. The inner diameters of the uranium rings indicate the mean molecular diameter to be about 94 A. Apoferritin molecules when similarly shadowed exhibit rings

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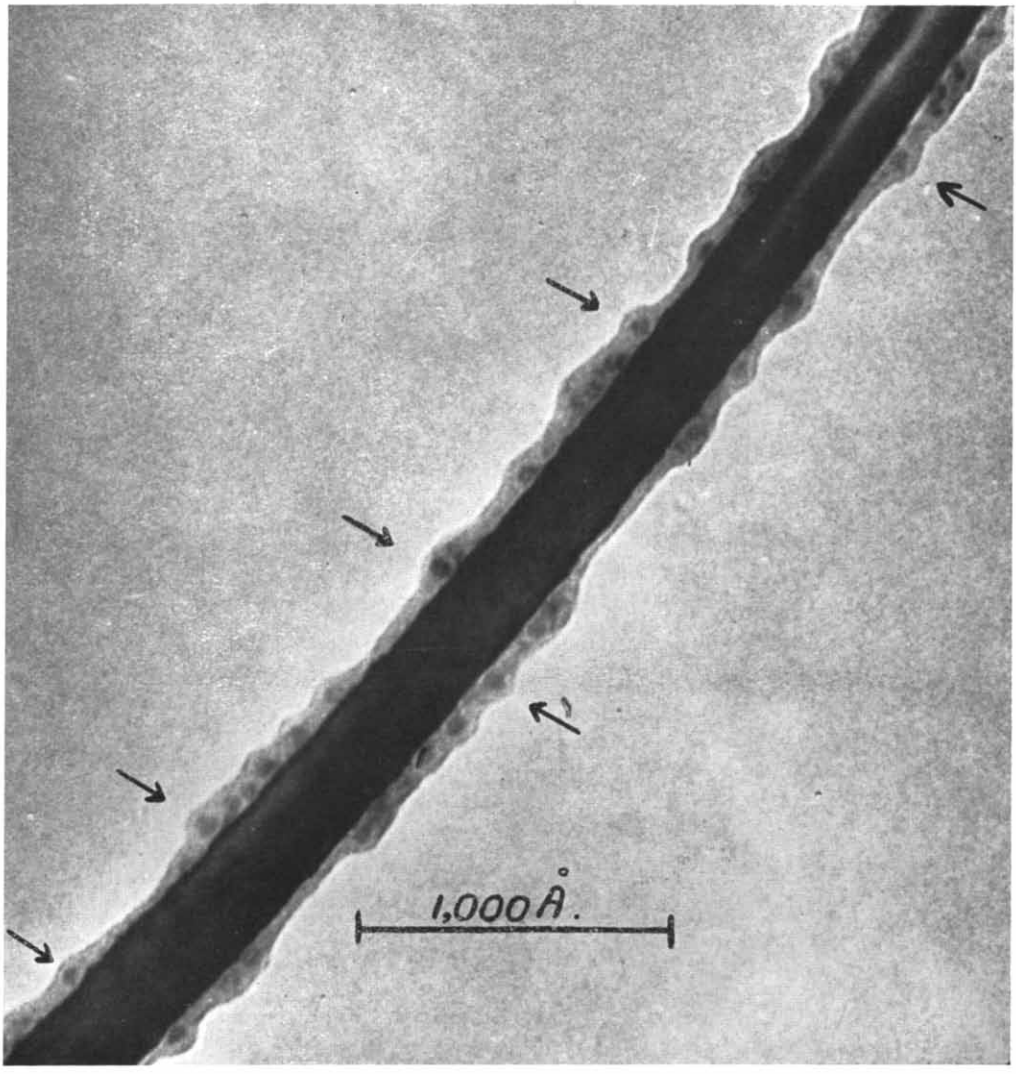

Fig. 3. Ferritin molecules supported on a zinc oxide smoke spine. The arrows indicate micelles held clear of the spine edges. 415,000 ×.

of the same mean diameter which lack the central body. This confirms the interpretation of these bodies as iron hydroxide micelles.

Similar results were obtained from specimens prepared from dilute ferritin solutions containing small amounts of $CdSO_4$. In places the salt formed a continuous layer on the collodion supporting film. The presence of the protein molecules was revealed by circular holes ~ 95 A in diameter in the $CdSO_4$ layer with the micelles lying at the centres of these holes.

It could be argued that the appearance of the micelles at the centres of the uranium rings is due to their adherence to the collodion film, so that in the microscope the micelles are viewed through the organic part of the molecules. This possibility was tested by

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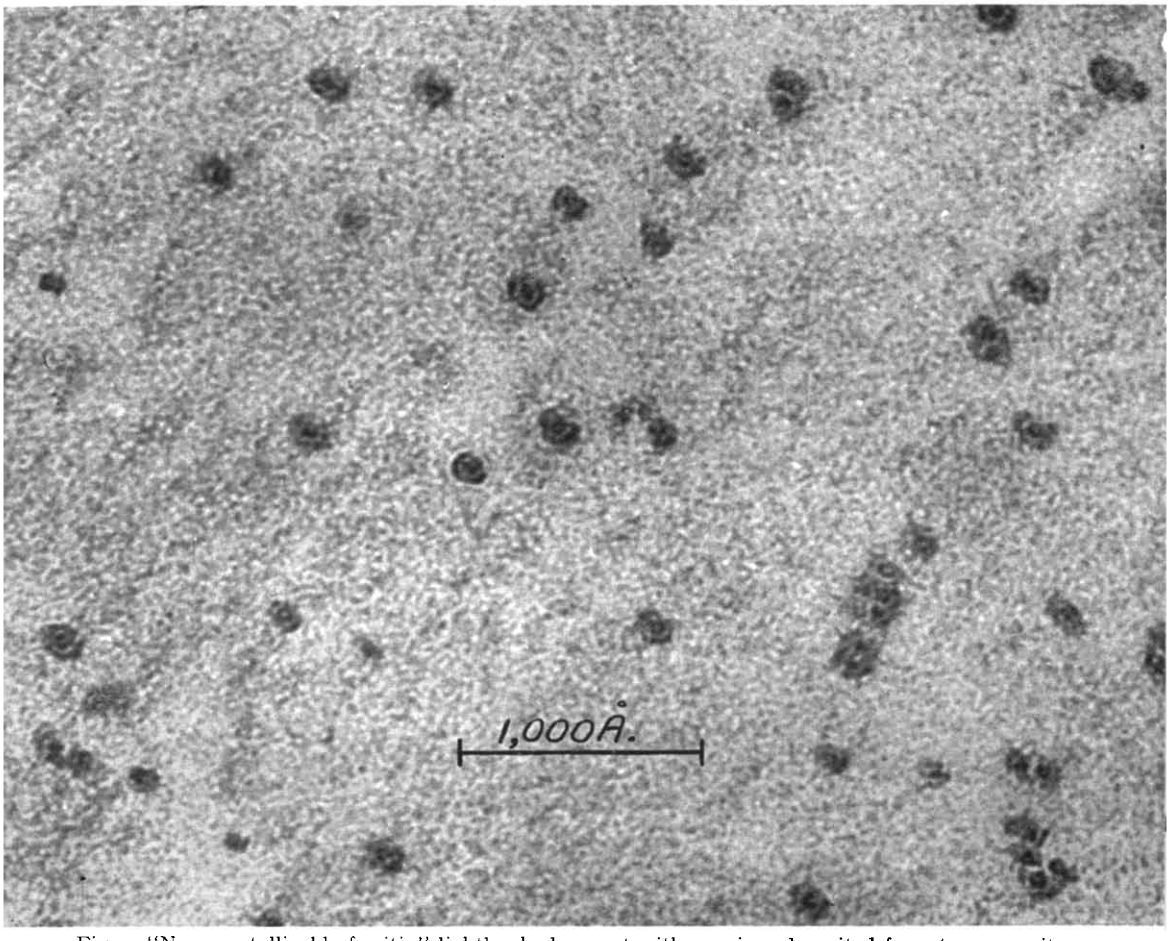

Fig. 4. "Non-crystallizable ferritin" lightly shadow-cast with uranium deposited from two opposite directions. Note that the molecules are indistinguishable from the ferritin molecules of Fig. 2. 320,000 ×.

taking micrographs of ferritin molecules supported on zinc oxide smoke spines as shown in Fig. 3. The arrows indicate micelles which are held clear of the spine edges. Unfortunately, contaminating layers build up so rapidly in the electron beam that it has not been possible to secure pictures showing the perimeters of the apoferritin molecules. Numerous similar micrographs were taken, but in no case was a micelle observed with its centre more than 50 A from the edge of the spine. If the micelles lie on the surfaces of the protein molecules it would be expected that in some cases the micelle centres would be seen at distances greater than the radius of the molecules from the edges of the spines.

Comparison of Figs. 2 and 4 shows that micrographs of fields of shadow-cast "non-crystallizable ferritin" are indistinguishable from those of shadow-cast ferritin.

DISCUSSION

The evidence that the micelles are located at the centres of the ferritin molecules must be reconciled with the facts advanced in favour of the alternative view.

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It appears possible that in ferritin the ferric ions of the micelles are in equilibrium with ferric ions lying between the polypeptide chains of the protein and on the surface of the molecules. If this is the case it is unnecessary for the large molecules of reducing agents such as cysteine to penetrate the polypeptide layers in order to remove centrally situated micelles. The reduction of the ferric ions lying on the surface of the protein molecule would displace the equilibrium so that the micelle would be removed by the diffusion of its ferric ions to the outside of the protein molecule.

The fact that ferritin solutions coagulate reversibly at 60° C, whereas apoferritin does not, suggests that the surface proporties of the two proteins differ. Here again the difference may be explained by the hypothesis that the surface of the ferritin molecules contain ferric ions in equilibrium with the iron of the micelles and that these ions are absent in apoferritin prepared by the action of reducing agents. In this connection, it is noteworthy that the addition of ferric salts to apoferritin results in the coagulation of the protein[7]. Alternatively, it is possible that the reducing agents used to prepare apoferritin may effect other changes in the surface properties of the molecules sufficient to cause the observed differences in coagulation. ROTHEN[8] found by ultracentrifugation of ferritin solutions that 25% of the material consists of apoferritin molecules containing little or no iron. It might be illuminating to determine whether apoferritin separated by this means exhibits reversible coagulation at 60° C.

The three facts which GRANICK advances[3] in support of the micelles being inside the protein molecules are, as he points out, inconclusive. It is not known to what degree the presence of micelles on the surfaces of the protein molecules would affect viscosity or electrophoretic mobility. As GRANICK indicates, the quantitative precipitin studies are also inconclusive, as the sites of antibody adsorption may lie on areas of the ferritin molecule surface not covered by the micelles even though the latter are on the surface. The complexes between ferritin and apoferritin and their antibodies might well repay electron microscopic investigation.

The identity of electron micrographs of ferritin and "non-crystallizable ferritin" is in accord with earlier findings. Chemical analysis of "non-crystallizable ferritin"[6] and the positive precipitin reaction of "non-crystallizable ferritin" with anti-ferritin serum[11] show that "non-crystallizable ferritin" does not consist of free micelles but rather of micelles associated with apoferritin molecules. GRANICK found[7] that when apoferritin is dissolved in solutions of "non-crystallizable ferritin", brown crystals of ferritin are formed when $CdSO_4$ is added. All these observations can be explained by the hypothesis that "non-crystallizable ferritin" consists of micelles associated with apoferritin molecules which are too imperfect to crystallize with one another in the presence of $CdSO_4$ but at least a certain proportion of them can be accommodated in a lattice of crystallizable apoferritin molecules.

Early X-ray diffraction studies[12] of ferritin and apoferritin crystals by the powder method indicated that both apparently consisted of face-centred cubic crystals having identical lattice parameters (a = 186 A for wet crystals and a = 163 A for air-dried crystals). More recent studies by HODGKIN[13,14] of single crystals of ferritin show that the unit cell is orthorhombic and has the dimensions 131.5 × 131.5 × 186 A when wet and 112 × 112 × 158.5 A when air-dried. Hodgkin's initial Patterson projection on the (010) plane shows peaks indicating that the micelles lie 93 A apart in the wet crystal. If the molecules are approximately spherical with the micelles at their centres as the micrographs indicate, then the molecular diameter is also 93 A. However, the dimensions

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of the unit cell of the air-dried crystals suggest that the molecular diameter is somewhat smaller than this.

Ultracentrifuge studies by ROTHEN[8] gave the molecular weight of apoferritin as 465,000. When combined with ROTHEN's value of 0.747 for the specific volume, this molecular weight yields 103 A as the molecular diameter on the basis of a spherical molecule. If the molecules are polyhedra filling space to a greater degree than spheres the molecular diameter would be less than 103 A. Thus the X-ray and ultra-centrifuge values agree reasonably well with the value of 94 ± 5 A for the molecular diameter measured from micrographs such as those of Figs. 2 and 4.

It is to be expected that the characteristic shape of the micelles should facilitate the location of ferritin in suitably thin tissue sections.

SUMMARY

Electron microscope studies of ferritin show that the ferric-hydroxide-phosphate complex which constitutes up to 40% of the dry weight of the protein exists as micelles about 55 A in diameter consisting of sub-units about 27 A in diameter and that the micelles are located within the protein molecules. Certain indirect evidence which has previously been interpreted as showing that the micelles are located on the surfaces of the protein molecules can be reconciled with the electron microscope evidence. The electron microscope value for the molecular diameter of ferritin and apoferritin is in fair agreement with the values obtained by X-ray diffraction and ultra-centrifuge studies.

RÉSUMÉ

L'examen au microscope électronique de la ferritine révèle que le complexe hydroxyde ferrique phosphate qui représente jusqu' à 40% du poids sec de la protéine se présente sous forme de micelles de 55 A de diamètre environ, formées de sous unités d'environ 27 A de diamètre. Ces micelles sont situées à l'intérieur des molécules protéiques.

Les indications indirectes selon lesquelles les micelles seraient situées à la surface des molécules protéiques sont conciliables avec les indications fournies par le microscope électronique; la valeur du diamètre moléculaire de la ferritine et de l'apoferritine, fournie par le microscope électronique, est en bon accord avec les valeurs obtenues par la diffraction des rayons X et l'ultracentrifugation.

ZUSAMMENFASSUNG

Elektronenmikroskopische Untersuchungen von Ferritin zeigen, dass der bis zu 40% des Trockengewichts des Proteins ausmachende Ferrihydroxydkomplex in Micellen von ungefähr 55 A Durchmesser mit Untereinheiten von ungefähr 27 A Durchmesser besteht und dass die Micellen im Proteinmolekül gelegen sind. Gewisse indirekte Beweise, die früher interpretiert wurden und zeigten, dass die Micellen an der Oberfläche der Proteinmoleküle liegen, können mit den elektronenmikroskopischen Beweisen in Einklang gebracht werden. Der elektronenmikroskopische Wert für den molekularen Durchmesser von Ferritin und Apoferritin ist in guter Übereinstimmung mit den aus Röntgenstrahlstreuungsuntersuchungen und Untersuchungen mit der Ultrazentrifuge erhaltenen Werten.

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