

**Internal Structure of Apoferritin as Revealed by the "Negative Staining Technique."\*** By  
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It has been surmised that the apoferritin molecule has a hollow center which communicates with the outer surface through some sort of channels or slots (1-2). This inference is based upon the following findings:

1. As determined by means of electron microscopy, the ferric hydroxide (FeOOH) micelles of ferritin molecules are located within spheroids of apoferritin (1);
2. In reducing media, such as dithionite solutions, iron can be removed with ease from ferritin, intact apoferritin being left behind (4);
3. Apoferritin, prepared following removal of iron from ferritin, has the immunologic specificity of ferritin, crystallizes in the same habit as ferritin, and has the molecular crystal lattice parameters of ferritin (3-8).

If the ferric hydroxide micelles in the ferritin molecule were tightly enclosed by a shell of the protein, apoferritin, their removal would require some kind of unfolding of the protein—*i.e.* denaturation. Such denaturation would be inconsistent with the facts just summarized.

On the other hand, if there are communications between the outside and the inside of the apoferritin shell, then various solvents and solutions should penetrate readily into the interior of ferritin molecules. It follows that with suitable solutions the reduction of the ferric hydroxide micelles to ferrous ions could be brought about in the interior of ferritin molecules; ferrous ions would then diffuse outward. This hypothesis is consistent with the facts summarized above, and with the findings reported by Bielig and Bayer (9) and by Loewus and Fineberg (10); *viz.* that ferritin can be reconstituted from apoferritin and ionic iron *in vitro*.

Like the molecules of many proteins, those of apoferritin are not sufficiently dense to electrons to be visible *directly* in the electron microscope. However, with the negative staining technique of Brenner and Horne (11), we have been able to demonstrate the contours of apoferritin molecules. In this technique phosphotungstic acid is used to

outline the profiles of material that has low density to electrons. The average diameter of phosphotungstate particles or "ions" is below 10 Å, and hence phosphotungstate should penetrate through openings larger than this.

#### *Preparation of Material*

Our material was prepared as follows. Using methods described by Granick (4), ferritin was extracted from human livers obtained at autopsies, was crystallized, and recrystallized. Then the iron was removed, and the remaining apoferritin crystallized with CdSO<sub>4</sub>, as also described by Granick (4). Subsequently, the CdSO<sub>4</sub> was removed from the crystals by dialysis. The final product was a gray precipitate. Portions of this precipitate were analyzed for iron, using the method of Lorber (13), a modified *o*-phenanthroline method, and a modified Wong procedure. No residual iron was detected in these tests. Under the light microscope the precipitate appeared to be partly amorphous, partly crystalline.

#### OBSERVATIONS

As a control, some of the apoferritin precipitate was dissolved in distilled water and sprayed onto carbon-coated specimen grids, which were then dried at room temperature for 24 hours and examined in the electron microscope. These preparations were devoid of the characteristic ferric hydroxide micelles of ferritin (1), and prolonged search of several preparations failed to reveal such micelles. By contrast, preparations of ferritin from which the apoferritin had been derived displayed these micelles in profusion. As a further control, apoferritin precipitates were dehydrated in graded concentrations of ethyl alcohol, embedded in methacrylate, and sectioned for electron microscopy. In these sections no ferric hydroxide micelles were found. Thus, no iron was detected in the apoferritin by means of the indicated chemical tests, and no ferric hydroxide micelles were found by means of electron microscopy.

To determine whether phosphotungstic acid or phosphotungstate (PTA) ions would penetrate into apoferritin molecules, dilute suspensions of the apoferritin precipitate (in distilled water) were mixed with equal volumes of 2 per cent phosphotungstic acid that had been brought to pH 7.0 with N KOH. Twenty minutes later the resultant suspensions were sprayed onto carbon-coated speci-

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men grids with an atomizer. These grids were dried at room temperature for at least 24 hours and then examined in the electron microscope.

The results are shown in Figs. 1 and 2. It can be seen that there are innumerable circular profiles, about 105 Å in diameter, which are outlined by a surrounding film of phosphotungstate. The diameters of these profiles agree with those of ferritin molecules in electron micrographs as determined by Farrant (1), Labaw and Wyckoff (6), Kuff and Dalton (12), and Richter (14). It can also be seen that in many of the "profiles" a peripheral shell of low density surrounds a denser center. As already described, nothing of this sort was found in control preparations in which apoferritin had been dissolved in distilled water. Specimen grids upon which such control apoferritin had been sprayed, displayed only scattered amorphous films that had a low density to electrons.

There are three possible explanations for the findings on apoferritin treated with PTA (Figs. 1 and 2):

- (a) PTA penetrated the apoferritin molecules.
- (b) PTA was deposited on the surfaces of apoferritin molecules.
- (c) There was penetration as well as surface deposition of PTA.

There is a manifest geometrical similarity between many profiles shown in Figs. 1 and 2 and the images of doubly "shadowed" ferritin molecules shown in Fig. 3. As first determined by Farrant (1), the center of the ferritin molecule is occupied by dense particles (most often four) that represent the ferric hydroxide micelles. The protein shell of the ferritin molecule is not directly visible in the electron microscope, but can be outlined by evaporating a metal upon its surface from two opposite directions. Topographical considerations indicate that many of the apoferritin molecules depicted in Figs. 1 and 2 were penetrated by PTA; that is, the dense centers within halos represent PTA. Apparently, PTA has occupied spaces in which FeOOH micelles were situated prior to extraction (in the preparation of the apoferritin). This inference is confirmed by the finding (Figs. 1 and 2) that in some apoferritin molecules the PTA has outlined three or four spaces. In other instances, however, there are strands of PTA within the halos. While such strands suggest the presence of channels through which PTA may have reached the hollow centers of apoferritin molecules, the images in Figs. 1 and 2 do not warrant definite conclusions on this

point. Certainly, one cannot rule out the presence of PTA on the surfaces of some of the apoferritin molecules. The average diameter of phosphotungstate particles (or ions) may be about 8 Å (11). One would expect, therefore, that phosphotungstate reaches the interior of apoferritin molecules through channels with cross-sectional diameters above 8 Å. Further work on this point is in progress.

In other experiments solutions containing both ferritin and apoferritin were briefly mixed with equal volumes of neutralized 2 per cent PTA solution. Five minutes later the mixtures were sprayed on carbon-coated specimen grids which were then dried at room temperature during 24 hours. In electron micrographs of such mixtures different particles can be distinguished from each other provided the PTA films on the specimen grids are sufficiently thin. Fig. 4 is a representative picture of a ferritin-apoferritin-PTA mixture. In the region shown, the PTA background was considerably lighter than the background in Figs. 1 and 2.

Images of three types of particles can be seen in Fig. 4:

- (a) Particles with central cores that measure 50 to 60 Å in diameter and that are considerably more opaque than the PTA film that surrounds the particles.
- (b) Particles that lack central cores.
- (c) Particles with cores that have approximately the density of the surrounding PTA film.

It is suggested that particles of type *a* represent ferritin molecules. Particles of type *b* cannot be ferritin molecules since they lack cores; presumably they represent apoferritin molecules that have not been filled with PTA. Particles of type *c* may represent apoferritin molecules filled with PTA, or ferritin molecules that are only partly charged with ferric hydroxide micelles.

It is of interest that close-packing of apoferritin molecules was also observed (Figs. 1, 2, and 4). Measurements of molecular distances and diameters in regions of close-packing agree well with those reported by Labaw and Wyckoff for surface replicas of crystalline ferritin (6).

One may assume that phosphotungstate particles in the interior of apoferritin molecules, as shown in Figs. 1 and 2, fill spaces without being bound to the apoferritin by covalent bonds. One would expect, however, that electrostatic forces play a part in the localization of phosphotungstate within apoferritin. The confinement of phospho-

tungstate within apoferritin molecules may differ from that of typical (FeOOH) micelles in that the latter are more firmly bound to apoferritin (4, 9, 10). The apoferritin shells that surround the FeOOH micelles in ferritin molecules can be viewed as "protective colloid." In their absence, ferric hydroxide (or hydrous ferric oxide) would precipitate at physiologic pH, while their presence facilitates its colloidal dispersion and transport.

It is reasonable to suppose that other poorly soluble hydroxides or hydrous oxides of metals might be inserted into apoferritin molecules. The work of Bielig and Bayer (9) and of Loewus and Fineberg (10) on the artificial formation of ferric hydroxide micelles in apoferritin makes this likely. Thus it may prove possible to produce various metal-apoferritin complexes that are useful in investigations concerning trace metals or metallic poisons.

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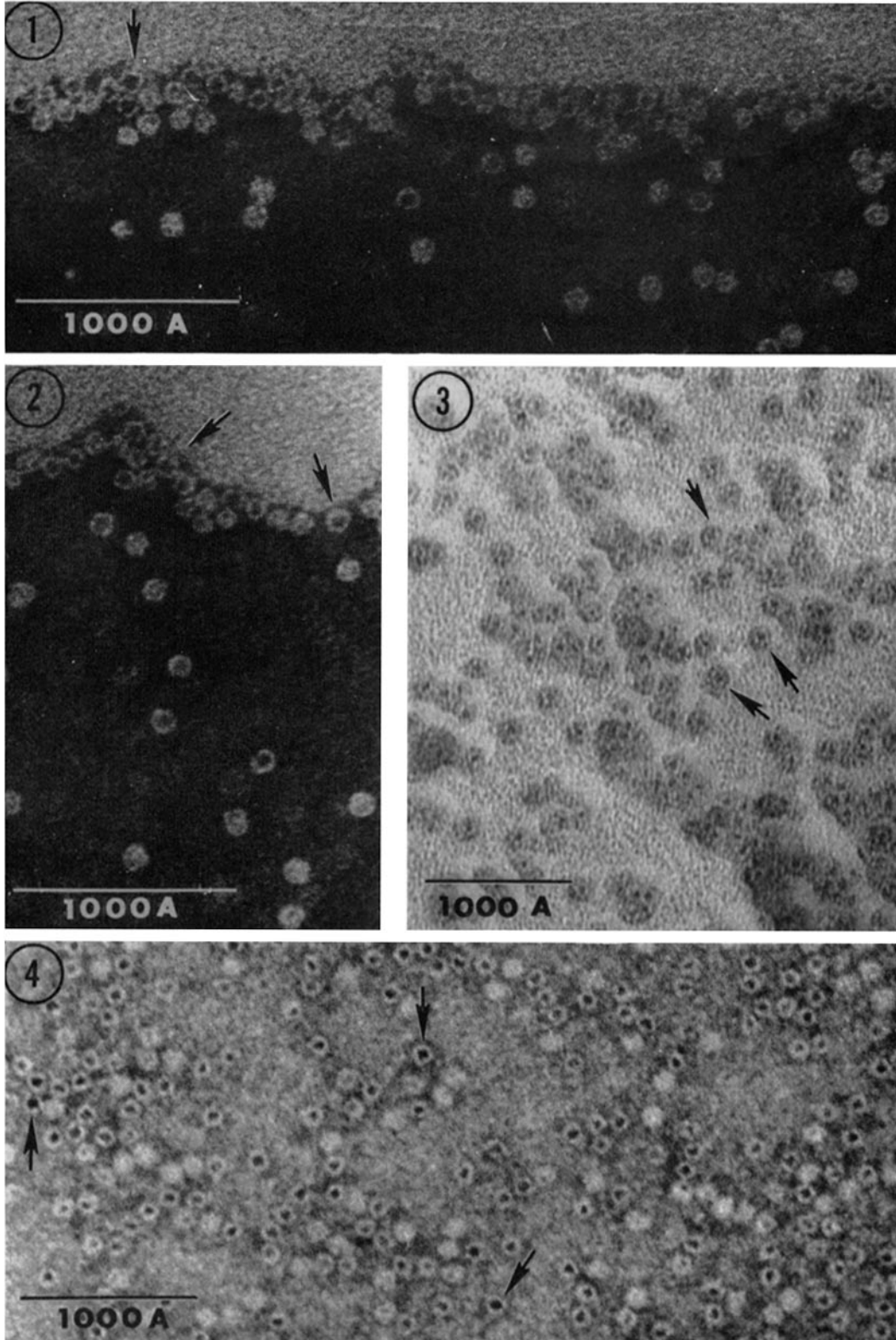
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## EXPLANATION OF PLATE 255

FIGS. 1 and 2. Apoferritin-phosphotungstate spray preparation. Circular profiles of particles, 105 Å in diameter, are outlined by the opaque phosphotungstate that surrounds them. The size, shape, and relative uniformity of the circular profiles correspond to those of apoferritin molecules as previously found (see text). Many profiles have opaque cores—presumably phosphotungstate which has penetrated the interior of the particles (apoferritin molecules). The configuration of some cores (arrows) resembles that of ferric hydroxide micelles in ferritin molecules (see Fig. 3). As seen in Figs. 1 and 2, the diameters of the cores vary, but where the cores resemble ferric hydroxide micelles, their diameters are between 50 and 70 Å. Compare with Fig. 3. Magnification (Figs. 1 and 2),  $\times 350,000$ .

FIG. 3. Images of ferritin molecules, doubly “shadowed” with chromium. Rings of evaporated chromium outline surfaces of apoferritin spheroids that contain cores iron hydroxide micelles. The latter appear as dense particles, often four, that are located within the spheroids (see arrows). When seen in square array, the cores measure 50 to 60 Å in diameter. The diameters of the circular profiles outlined by the chromium measure approximately 105 Å. Compare with Figs. 1, 2, and 4. Magnification,  $\times 230,000$ .

FIG. 4. Spray preparation made with a mixture of ferritin, apoferritin, and neutralized phosphotungstic acid (PTA). In the area shown, the PTA film is much thinner than the PTA film seen in Figs. 1 and 2. Three types of profiles with diameters of approximately 105 Å may be seen: (A) Profiles with cores that are much denser than the PTA background (arrows); (B) Profiles without apparent cores; and (C) Profiles with cores of approximately the density of the PTA background. Profiles of type A are thought to represent ferritin molecules. Profiles of type B presumably represent apoferritin molecules that have *not* been filled with PTA. Profiles of type C may represent either apoferritin molecules filled with PTA, or ferritin molecules that contain less than the average amount of ferric hydroxide. Magnification,  $\times 275,000$ .



(Richter: Structure of apoferritin)