FERRITIN IN THE FUNGUS PHYCOMYCES

Thesis by

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ABSTRACT

The iron storage protein ferritin has been purified from mycelium, sporangiophores, and spores of the fungus Phycomyces blakesleeanus. Its morphology in the electron microscope and the electron diffraction pattern of its iron core are almost indistinguishable from those of horse ferritin. Its protein/iron ratio is 4.7. It has a sedimentation coefficient of 55S and a buoyant density in CsCl density gradients of 1.82 g/cm^3.

In the cytoplasm of Phycomyces, ferritin is located on the surface of lipid droplets (0.5-2.0 μ diameter) where it forms crystalline monolayers which are conspicuous in electron micrographs of sporangiophore thin sections. The ferritin-lipid complex can be isolated from sporangiophore lysates. Treatment of the complex with detergent separates ferritin from the lipid.

The level of ferritin iron in Phycomyces is regulated by the iron level in the growth medium. A 20-40 fold increase in ferritin has been achieved by supplementing the medium with iron.

Ferritin and iron are concentrated in spores. In lysates of sporangiophores grown on low-iron growth medium about 80% of the iron is recoverable in the spores. At least one half of this spore iron can be extracted and is shown to be ferritin. The remaining iron is unextractable. There is no extractable low molecular weight iron in ungerminated spores.

When ferritin is present in "limiting" amounts in spores, it disappears rapidly upon germination and simultaneously a low molecular weight form of iron becomes extractable. When present in large amounts

in spores, ferritin does not disappear following germination although low molecular weight iron does become extractable. These results suggest that ferritin in Phycomyces spores functions as a source of iron in the early stages of germination.

Because the initial intracellular ferritin concentration and the external iron concentration of the germination medium can be varied, Phycomyces spores are potentially a very useful system in which to study the mechanism and control of ferritin iron metabolism.

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CHAPTER I

GENERAL INTRODUCTION

This thesis concerns an iron-containing protein, ferritin, which has been isolated from the fungus Phycomyces blakesleeanus. Chapters 2 and 3 describe the purification and properties of the protein and its intracellular location. The control of its biosynthesis by external iron concentrations and its metabolism following spore germination will also be discussed. To set the stage for the experimental results and conclusions with Phycomyces ferritin, Chapter 1 will review what is known about the structure and function of ferritin as it occurs in other organisms. Although ferritin has now been shown to occur in widely different organisms, most of the experimental work on the structure of the molecule, its biosynthesis, and its function has been done on mammalian ferritin, particularly from horse spleen and rat liver (for Reviews see Granick, 1946; Bothwell and Finch, 1962; Harrison, 1964).

Isolation

Mammalian ferritin is easily isolated from tissue because of its unusual heat stability and the ease with which it crystallizes from cadmium sulfate solutions (Granick, 1942). Plant ferritin can be isolated by a similar procedure (Hyde et al., 1963). Prepared in this way ferritin is not a uniform molecular species, but rather consists of a mixture of ferritin and apoferritin (the iron-free protein moiety). Rothen (1944) resolved this mixture in the ultracentrifuge by observing a sharply defined, colorless component (17.6S; molecular weight 465,000) and a broad band of colored material sedimenting much faster and of heterogeneous S-value.

The heterogeneity of the fast sedimenting material has been shown by Fischbach and Anderegg (1965) to be due to varying amounts of iron per ferritin molecule. Following sedimentation to equilibrium in CsCl density gradients, ferritin fractions homogeneous in density and S-value can be obtained. Fractions of higher density have higher S-values, and the protein/iron ratio is inversely related to the S-value. Ferritin maximally loaded with iron has a buoyant density of about 1.8 g/cm^3 and sediments between 65-70S.

Although heterogeneous in iron content ferritin is homogeneous by moving boundary electrophoresis, which indicates that the iron does not contribute to the effective charge of the molecule and, therefore, is located inside the protein. In gel electrophoresis two or three bands are observed (Kopp, et al., 1963; Fine and Harris, 1963; Richter, 1963; Cornerali and Tecce, 1964). These are the monomer, dimer, and trimer of ferritin or apoferritin (Harrison and Gregory, 1965).

Structure — Electron Microscopy

Farrant (1954) was the first to elucidate the relationship of protein and iron in ferritin. Examination in electron microscope reveals that the iron is concentrated in a central core surrounded by a shell of protein which can be revealed in shadow cast or negatively stained preparations. The size of the iron micelle is thus limited by the space inside the protein. In electron micrographs the core has a diameter of 55-60 Å and the protein shell a diameter of 94-110 Å (Farrant, 1954; Kuff and Dalton, 1957; Labaw and Wyckoff, 1957; Richter, 1959; Kerr and Muir, 1960; Haggis, 1965). Controversy has developed over the structure

of the micelle. As visualized in the electron microscope it quite frequently appears to consist of subunits although their number and arrangement are not clear.

Structure — Low Angle X-Ray Diffraction

The low angle X-ray diffraction pattern of apoferritin agrees well with that of a uniform density, 0.6 hollow sphere of 122 Å diameter (Fischbach and Anderegg, 1965). A Fourier transform of the scattering data yielded a single sharp peak of electron density between the radii 37 Å and 61 Å. This is in good agreement with the structure of apoferritin observed in electron microscope studies.

To study scattering from the ferritin micelle, apoferritin scattering was eliminated by placing ferritin in a solvent having the same electron density as the protein (53% sucrose). Ferritin fractionated by density gradient centrifugation in CsCl was used — i.e. molecules having a uniform iron content. The results for "full" ferritin agree well with the theoretical scattering curve for a uniform sphere of 73 Å in diameter. Theoretical scattering curves for the subunit models proposed from electron microscope studies do not agree with the experimental scattering curve for full ferritin.

Structure — X-ray Diffraction and Chemical Composition of Apoferritin

X-ray diffraction studies on crystalline horse apoferritin suggest that the molecule consists of subunits - probably 20 located at the vertices of a pentagonal dodecahedron (Harrison, 1959; 1963). The sub-

unit hypothesis has been confirmed in studies of the amino acid composition and of the number of tryptic peptides (Harrison and Hofmann, 196.2; Harrison, et al., 1962). Based on the amino acid composition, 409 tryptic peptides would be expected and twenty-one should contain tryptophane if apoferritin were a single polypeptide chain. Twenty-one major peptides were identified in an Ingram-type fingerprint, only one of which contained tryptophane. These results strongly support the conclusion that apoferritin consists of identical subunits — probably 20 in number. Subsequently Mainwaring (1963) found 19.7 acetyl residues per mole of apoferritin (no N-terminal amino acid was found) and 19 moles of arginine per mole of apoferritin have been isolated from the C-terminal end using carboxypeptidase B.

Hofmann and Harrison (1963) have obtained a subunit species by degradation of apoferritin. Dilute alkali (pH 12.6) yielded 50% degradation to a 2.1S subunit. Complete degradation was achieved by dehydrating apoferritin in alcohol or lyophilization followed by solubilization in sodium dodecyl sulfate. This procedure yielded a 2.5 S subunit which contained detergent complexed with protein. Taking into account the detergent bound, sedimentation and diffusion data for the detergent-protein complex at infinite dilution gave a molecular weight of 25,000 for the apoferritin subunit. This implies 18-19 subunits per apoferritin in agreement with other determinations.

Micelle Composition and Structure

The chemical composition of the ferritin core is $(FeOOH)_8(FeOOPO_3H_2)$. (Granick and Hahn, 1944). Treatment of ferritin with 1M NaOH releases the

iron micelles from the protein and precipitates them. Although all the ferritin iron is recovered in this precipitate, it contains only 25% of the phosphorus. The remaining phosphorus is found as soluble orthophosphate (Granick, 1942). Since the precipitated micelles are identical to native ferritin micelles (Michaelis, et al., 1943), the phosphorus may not be a true micelle constituent but simply stuck on the micelle surface. A similar conclusion is suggested by the finding that phosphorus-free ferritin can be reconstituted from ferrous ammonium sulfate and apoferritin (Bielig and Bayer, 1955b; Harrison, et al., 1967).

Two approaches have been taken to elucidate the structure of the iron micelle: investigation of the magnetic properties of the iron and determination of the X-ray and electron diffraction pattern of the micelles. Neither method yields the structure directly but comparison with known iron compounds has led to a tentative structure (Harrison, et al., 1967; Towe and Bradley, 1967).

Michaelis, et al. (1943) investigated the magnetic susceptibility of ferritin and of several preparations of colloidal ferric hydroxide. The result with ferritin was 3.9 Bohr magnetons indicating iron with three unpaired electrons - an unusual state for iron. Of the colloidal ferric hydroxides two preparations made from ferric nitrate solutions at high temperatures had similar magnetic properties.

Haggis (1965) and Harrison et al. (1967) have examined native ferritin and isolated cores in both wet and dry states by X-ray and electron diffraction. Both ferritin and cores give the same diffraction pattern in intensity and line width. The pattern, however, does not correspond to the pattern given by any of the known oxides or oxyhydroxides of iron.

Harrison et al. (1967) have also shown that ferritin can be reconstituted when the oxidation of ferrous ammonium sulfate solutions is carried out in the presence of apoferritin. The product has the sedimentation properties and morphology of ferritin and yields a ferritin-like diffraction pattern. If the oxidation is not carried out in the presence of apoferritin, then several of the known oxyhydroxides of iron are obtained which yield different diffraction patterns from those of the ferritin core. The conclusion from these experiments is that although the exact structure of the core is as yet unknown, it can be synthesized by apoferritin upon oxidation of ferrous iron. This result agrees well with experiments on the biosynthesis of ferritin to be discussed below.

Control and Mechanism of Ferritin Biosynthesis

The biosynthesis of apoferritin in mammals can be stimulated by injection of iron. Using guinea pigs Fineberg and Greenberg (1955a) demonstrated that injection of iron into the animals prior to injection of ^{14}C-glycine lead to a 5-fold increase in the rate of incorporation of glycine into apoferritin. This increased rate of incorporation was specific for apoferritin; the rate of incorporation of ^{14}C-glycine into general liver proteins was unaffected by the injection of iron. A similar result has been demonstrated in rat liver (Loftfield and Eigner, 1958; Saddi and von der Decken, 1964, 1965; Drysdale and Munro, 1965, 1966; Yu and Fineberg, 1965; Yoshino et al, 1966). These authors also investigated the effect of actinomycin-D — a potent inhibitor of DNA-

dependent RNA synthesis (Reich et al., 1961) — on the iron stimulated apoferritin synthesis. Saddi and von der Decken (1965) and Drysdale and Munro (1965, 1966) reported no inhibition by actinomycin-D (70-80 ug/100 g body weight) in experiments including controls for effects of the drug on general protein synthesis. Yu and Fineberg (1965) and Yoshino et al. (1966) used higher concentrations of the drug (up to 1800 ug/100 g body weight) and reported inhibition of iron-stimulated apoferritin synthesis. However, no controls were presented regarding the effects of these high levels of actinomycin-D on general protein synthesis and Drysdale and Munro have indicated that such levels are markedly inhibitory in a non-specific way. These experiments suggest that the iron effect on apoferritin biosynthesis is not at the level of genetic transcription. In further experiments, Drysdale and Munro showed that repeated iron injections also stabilized newly synthesized apoferritin against breakdown. It is tempting to imagine that this is the mechanism by which iron affects the apoferritin level.

The mechanism of iron incorporation into ferritin was investigated by Fineberg and Greenberg (1955b) and by Drysdale and Munro (1966). Rats were injected with iron followed by labeled amino acids and the ferritin was purified at various intervals. In both experiments apoferritin and ferritin containing various amounts of iron were separated centrifugally. It was shown that the apoferritin and ferritin of very low iron content were labeled initially and that with time the labeled molecules became more heavily loaded with iron.

Recently Pape et al. (1968a) have proposed that ferritin is synthesized by condensation of apoferritin subunits around a preformed polynuclear iron core. Such a mechanism makes ferritin which initially has a high iron content. This disagrees with the in vivo biosynthesis data above and also the observations of Harrison, et al. (1967) that the particular polynuclear iron complex in ferritin has an unusual structure which is only formed in the presence of the apoferritin molecule.

Mechanism of Iron Release from Ferritin

Granick and Michaelis (1943) showed that iron could be removed from ferritin by reduction of the Fe^{+++} to Fe^{++} with sodium dithionite at pH 4.6 in the presence of a ferrous iron chelating agent. The apoferritin thus produced is identical to apoferritin present in native ferritin preparations from horse liver or spleen. Mazur et al. (1955) and Bielig and Bayer (1955a) extended these results to several biological reducing agents including ascorbic acid, reduced glutathione and cysteine. They also found that liver slices incubated under anaerobic conditions released iron from ferritin.

The fact that chemical reduction released iron from ferritin le to the search for a biochemical mechanism in which ferritin was enzymatically reduced. Green and Mazur (1957) and Mazur et al. (1958) observed that xanthine oxidase could reduce ferric iron in ferritin using either xanthine or hypoxanthine as a substrate. Xanthine oxidase is nonspecific, however, and can also use molecular oxygen, methylene blue, and cytochrome c as an electron acceptor. Attempts to demonstrate that this reaction was physiologically important were unconvincing.

More recently Pape et al. (1968b) have shown that iron can be removed from ferritin in vitro with nitrilotriacetate. The process only goes to 50% completion and is very slow. The nature of the biologically active chelator, if it exists, is not known. Thus the mechanism of iron removal from ferritin in vivo and the manner in which the cell controls the rate of removal (as well as addition) are not clear.

Role of Ferritin in Iron Metabolism

In mammals iron is primarily involved in hemoglobin metabolism (70% of mammalian iron is bound in hemoglobin). Hemoglobin is synthesized in maturing reticulocytes in bone marrow and catabolized during the destruction of erythrocytes by reticuloendothelial cells. The iron released by this catabolism is stored in ferritin until picked up by transferrin in the circulatory system and transferred back to bone marrow to repeat the cycle. A measure of the magnitude of iron conservation in humans is that 20-40 mg Fe are released by erythrocyte catabolism daily. Essentially all of this must be reincorporated into hemoglobin since the net daily intake is less than 0.5 mg Fe (Bothwell and Finch, 1962).

Ferritin stores iron only temporarily in reticuloendothelial cells but it also is involved in more permanent storage of iron in liver and spleen. This latter store does not mix with the daily iron cycle associated with hemoglobin breakdown since iron released by hemoglobin breakdown is not incorporated into liver ferritin to any significant extent. Nevertheless, this store can be mobilized when there is insufficient

iron in the erythrocyte-marrow-reticuloendothelial system. Long periods of anemia lead to the complete mobilization of the body's iron reserves.

With different sites of ferritin iron storage, all connected through transferrin iron in the blood and yet strictly regulated into two types of storage, it is clear that a relatively delicate balance must control the sites where transferrin picks up iron and unloads it so that normally pick up is in the reticuloendothelial cells and discharge in the marrow. Jandl, et al. (1959) have obtained evidence that the level of saturation of transferrin with iron is the controlling factor in the pick up and discharge of iron at the correct sites. They have shown that uptake by erythrocytes increases with increasing saturation of transferrin up to 60% saturation when transferrin begins to unload iron into liver ferritin. These are just the bare outlines of the system and stress the need to know more about the uptake and release of iron by ferritin.

Higher plants require iron for normal chloroplast development ("greening"). Growth on iron deficient medium leads to a yellow appearance of the leaves (chlorosis) which can be altered, at least in Xanthium, by addition of ferrous sulfate to the leaves. Such chlorotic leaves contain badly developed chloroplasts (Bogorad et al. 1958). In maize the mutant yellow-stripe 1 has been shown to be deficient in iron metabolism (Bell, et al., 1958).

In view of the iron requirement for normal chloroplast development, the discovery of ferritin in proplastids - the precursor to chloroplasts -

is not surprising Hyde et al. (1963) found ferritin in thin sections of Pisum seeds and cotyledons. It appears in large crystalline sheets in proplastids. Sitte (1961) and Schnepf (1962) have reported similar structures in Pisum root meristems and Passiflora nectary glands. More recently Robards and Humpherson (1967) have described sheets of ferritin in proplastids in Salix cambium and Jacobson et al. (1963) have reported ferritin in proplastids of the monocotyledon Zea mays. Several of these authors report that the large crystalline sheets of ferritin conspicuous in proplastids are not found in mature chloroplasts. Since development of chloroplasts is known to require iron and since several iron-containing proteins have been isolated from chloroplasts, this suggestion of ferritin's role as iron supply for synthesis of the photosynthetic apparatus is reasonable.

Ferritin in Phycomyces

Chapter 2 of this thesis describes the isolation of ferritin from the fungus Phycomyces by a butanol extraction procedure, precipitation at pH 5.0, and equilibrium sedimentation in CsCl. The purity of the ferritin isolated in this way is assessed from its UV absorption spectrum and protein/iron ratio and by disc electrophoresis. In structure and morphology it resembles mammalian ferritin very closely. Unlike the latter, however, it is found associated with lipid droplets in cytoplasm.

Phycomyces ferritin is selectively incorporated into spores where it accounts for at least 40-50% of the total spore iron. This suggests that it functions as an iron storage protein. Evidence in favor of this hypothesis is given in Chapter 3 where the loss of iron from ferritin is demonstrated following germination of ^{59}Fe-labeled spores.

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CHAPTER 2

PURIFICATION AND PROPERTIES OF PHYCOMYCES FERRITIN

INTRODUCTION

Ferritin is a large protein containing up to 30% iron by weight. It was discovered in horse spleen and first purified by Laufberger (1937). Since then ferritin from mammalian tissues has been extensively studied (see Granick, 1946, and Harrison, 1964, for reviews). More recently ferritin has been demonstrated in invertebrates (Roche et al., 1961; Towe, Lowenstam, and Nesson, 1963) and plants (Hyde et al., 1963). This paper presents evidence that it is also present in the fungus Phycomyces blakesleeanus indicating an even wider occurrence for this protein.

Ferritin is unusual because of its high iron content. It consists of a spherical protein shell (apoferritin) of molecular weight 465,000 (Rothen, 1944) and a dense core of ferric hydroxide. In the electron microscope the iron core is sufficiently electron dense to be directly visible in unstained preparations while the apoferritin can be revealed by negative staining as an electron transparent annulus around the core. Due to the iron content of its core, ferritin has a high buoyant density in CsCl (1.8 g/cm^3) and a high sedimentation coefficient (approximately 60S). Moreover, the iron content of individual molecules is not constant but can vary from no iron (apoferritin) to about 4000 atoms in a full core (Fischbach and Anderegg, 1965). Ferritin molecules with different iron contents have correspondingly altered sedimentation coefficients and buoyant densities. In ferritin preparations from mammalian liver and spleen a broad distribution of S-values is observed indicating heterogeneity in iron content. Such preparations also contain up to

25% apoferritin molecules. Although heterogeneous in mass ferritin can be crystallized and moves as a single species in moving boundary electrophoresis.

In Part I of this paper the purification from Phycomyces of a protein having the properties of mammalian ferritin is described. In electron micrographs it is indistinguishable from mammalian ferritin. Its UV absorption spectrum is very similar to that of mammalian ferritin. The Phycomyces protein has a protein/iron ratio (w/w) of 4.7, sediments at about 55S, and has a buoyant density of 1.82 g/cm^3 in CsCl density gradients. Because of these properties the Phycomyces protein has been identified as Phycomyces ferritin.

Phycomyces ferritin is, however, not identical to mammalian ferritin. It does not cross-react with antibodies to horse or human ferritin and cross-reacts only weakly with antibodies to rabbit ferritin. Electrophoretically in acrylamide gels Phycomyces ferritin moves more slowly at pH 9.5 than horse ferritin.

Ferritin in Phycomyces can be isolated from mycelium, sporangiophores (spph), and spores. It is found associated with large lipid droplets in the cytoplasm. Part II of this paper describes in detail the localization of the ferritin in large two dimensional crystalline arrays over the surface of lipid droplets in spph cytoplasm. A method for isolating this ferritin-lipid complex from spph lysates and the lability of the complex to detergent treatment are described. Experiments are also presented showing the distribution of iron and ferritin among various subcellular fractions of spph.

The major portion of ferritin in spph is incorporated into spores. It will be shown that supplemental iron in the growth medium leads to an absolute increase in the amount of ferritin in spph and spores although the relative proportion of spph ferritin incorporated into spores is lower than in spph grown on unsupplemented medium. The implication of these findings for the hypothesized role of ferritin as an iron storage compound in Phycomyces spores is discussed.

MATERIALS AND METHODS

Stocks of Phycomyces blakesleeanus

All experiments were done with mycelium and vegetative spph. The strain used was NRRL 1555(-) which has been used by Delbrück et al. for biophysical experiments.

Mycelium was prepared by germinating spores in aerated liquid culture. After 2-5 days of growth the mycelium was harvested by filtration through a Buchner funnel. To prepare spph, spores were germinated on solid medium in petri dishes. After 3 days, mycelial growth covered the agar surface and spph were initiated. On GA medium about 3000 spph grew on a single petri dish. Spph (4-6 cm high) were harvested in stage IV (Shropshire, 1963) by cutting them off with scissors or plucking them from the mycelium.

Media

A synthetic glucose-asparagine (GA) medium was used for all experiments (Zankel, Burke and Delbrück, 1967). The medium was solidified by the addition of 1% agar.

To induce higher levels of ferritin biosynthesis, 15 μg/ml Fe (as $FeCl_3$) was added to liquid cultures; 500 μg Fe/petri dish was added to solid medium.

Reagents

Horse ferritin was obtained from Sigma Chemical Co. (2X crystallized). Horse apoferritin was prepared from horse ferritin by exhaustive dialysis against $Na_2S_2O_4$ at pH 5 in sodium acetate buffer (Behrens and Taubert, 1952). Rabbit ferritin was a gift of Dr. Joseph Seckbach. Rabbit antisera against horse ferritin and human ferritin, sheep antiserum against rabbit ferritin, and normal rabbit serum were a gift of Dr. Mattioli. Pooled normal sheep serum was a gift of Hyland Laboratories. $^{59}FeCl_3$ in 0.1 M HCl (5.8 mc/mg) was obtained from New England Nuclear Corp. Bovine serum albumin and tripyridyl-s-triazine were obtained from Sigma Chemical Co.

Electron Microscopy

Phycomyces spph were fixed for two hours in 2.5% glutaraldehyde at room temperature followed by osmium tetroxide at $4^{\circ}C$ for one half hour. Specimens were dehydrated with ethanol and propylene oxide prior to embedding in maraglas (Polysciences, Inc.). Thin sections were cut on an LKB microtome and stained with 0.5% lead citrate.

Purified ferritin was prepared for examination by placing a drop of ferritin solution onto a grid covered with a thin film of carbon. After allowing a minute for adsorption, the excess was removed by a thorough washing with distilled water and the sample was allowed to dry. Negative staining was achieved by adding a drop of a saturated aqueous solution of uranyl acetate to the grid before drying. After 15 seconds, it was diluted with an equal volume of water and then drained with filter paper.

Unstained material was examined in a Phillips EM200 operating at 40KV with a 20 μ aperature; stained material was examined at 80KV with a 40 μ aperature.

Electron diffraction was performed by the selected area method in a Siemens Elmiskop I using a 200 μ aperature (Alderson and Halliday, 1965).

Ultracentrifugation

Preparative centrifugations were carried out using SW 39 and SW 50 rotors in a Spinco Model L ultracentrifuge. Analytical runs for sedimentation velocity and buoyant density determinations were performed in a Spinco Model E ultracentrifuge using UV absorption optics. The resulting photographs were traced with a Joyce-Loebl microdensitometer.

Immunodiffusion

A modification (Dr. R. Sanders, personal communication) of the Ouchterlony gel diffusion method was used. Gels (1% agar in 0.01 M veronal

buffer pH 8.6) were made by spreading 0.7 ml over a 4.5 x 2.5 cm area on a microscope slide. After the gel had hardened a lucite form was placed on the agar. It contained a pattern of four wells at the corners of a square with one well in the center. The wells were 1.5 mm in diameter; the center-well to corner-well distance was 2.5 mm. Each well held 20 ul of solution. After filling the wells, the slides were incubated 24 hours at room temperature. This was enough time for the solutions to completely diffuse into the gel. The lucite form was lifted off and the precipitin bands observed with dark field illumination.

Acrylamide Gel Electrophoresis

Reagents for acrylamide gels were obtained from Canalco, Bethesda, Md. The method of Ornstein (1964) was modified to use only one gel at pH 8.9. The electrode buffer was at pH 8.3. Electrophoresis was carried out at 500 V (5 mamp/tube). Following electrophoresis, gels were stained for protein with amidoblack in acetic acid-methanol or for iron with 1% potassium ferrocyanide in 2.5% acetic acid. Gels were destained with 5% acetic acid.

Protein Determinations

The Folin-phenol reagent was used as described by Lowry et al. (1951). Bovine serum albumin and horse apoferritin were used as standards. Optical densities were read at 660 mμ in a Cary 15 spectrophotometer.

Iron Determinations

Iron was determined with the color reagent tripyridyl-s-triazine according to the procedure of Fischer and Price (1964). To assure complete recovery of ferritin iron in the assay, the initial iron extraction procedure of Fischer and Price was modified. Large samples (up to

1 ml) were diluted with 1 ml $Na_2S_2O_4$ in 10% acetic acid and heated 10 minutes at 100°C to release iron from ferritin. Small samples (10 µl) were diluted with 0.15 ml 6 N HCl and heated as above. The latter procedure was used for quantitative work.

Ferrous ammonium sulfate was used as an iron standard. Optical densities of the colored iron complex were measured in a Cary 15 spectrophotometer.

Preparation of ^{59}Fe Labelled Spph Lysates

Heat shocked spores were spread on GA plates with $^{59}FeCl_3$ at a given specific activity. Spph were harvested from the plates after 4-5 days and ground in a mortar with several volumes of cold buffered isotonic sucrose solution (0.44 M sucrose, 0.05 M phosphate buffer pH 6.7). The lysate was filtered through miracloth to remove spph cell walls and large debris prior to fractionation by differential centrifugation as described in the text.

Sucrose Gradients

Linear sucrose gradients (5 ml, 5-20%) were preformed in cellulose nitrate centrifuge tubes. The gradients were buffered with 0.1 M phosphate at pH 6.7 and contained 0.1 M NaCl. Following centrifugation, drops were collected from the bottom of the centrifuge tube using a canula piercing unit (Buchler Instruments). The RNA bacteriophage MS2 (81S) was used as a centrifuge marker. Assay of MS2 followed the procedure of Strauss and Sinsheimer (1963).

Radioactivity Measurement

Aqueous samples (1 ml) were added to 10 ml portions of Bray's scintillation solution (Bray, 1960) in glass or polyethylene vials and their radioactivity measured with a Packard Tricarb Scintillation Counter. Quenching was monitored by the channels ratio method. In practice corrections for quenching were only required when samples contained large quantities of β-carotene.

RESULTS

Part I - Purification and Properties of Ferritin from Phycomyces Mycelium

A 32 liter culture of Phycomyces mycelium, grown 4-5 days at 23°C in liquid GA medium with 15 μg/ml Fe, was harvested by centrifugation. The mycelium (300 g) was squeezed dry and homogenized in cold 0.5 M phosphate buffer (pH 6.0) in a Waring blendor. This suspension was cycled twice through a Gaulin homogenizer which broke the mycelium into pieces 50-100 μ in length and extruded the cellular contents into solution.

Since Phycomyces ferritin is associated with lipids in the cell, the initial fractionation of the crude lysate involved extraction with n-butanol (Morton, 1953). This step was particularly useful since most cellular proteins precipitated at the interface between the butanol phase containing the lipid and the aqueous phase containing the ferritin. To make this separation effective there had to be a high salt concen-

tration (0.5 M) in the aqueous phase and an elevated temperature (35-40°C) to minimize the butanol concentration in the aqueous phase.

To 1500 ml of crude lysate, 600 ml of n-butanol was added with vigorous stirring over a 10 minute period. The resultant emulsion was separated into two phases by centrifugation at 10,000 x g for 10 minutes. The aqueous phase was retained. It was dialyzed to remove salt and butanol and concentrated by lyophilization.

The lyophilized material was resuspended in a 10-fold smaller volume and dialyzed at pH 5.0 in 0.1 M acetate buffer. At this pH Phycomyces ferritin was selectively precipitated. This step also removed a UV-absorbing contaminant from ferritin preparations derived from spph and spores. The UV absorption spectrum of this contaminant is identical to that of gallic acid, a plentiful compound in spph (10 mg/ml in cytoplasm; Dennison, 1959). However, the UV-absorbing contaminant does not show the same spectral changes as a function of pH that samples of known gallic acid show. This contaminant has not been further characterized.

Phycomyces ferritin precipitated at pH 5.0 was more than 50% pure as judged by its protein/iron ratio (Table 1). As a final purification step this material was centrifuged to equilibrium in a CsCl density gradient (Meselson, Stahl, and Vinograd, 1957; Fischbach and Anderegg, 1965) at a density of 1.8 g/cm^3. Ferritin prepared in this way from mycelium was somewhat heterogeneous in density (iron content). However, a main red band was clearly visible in the tube. The material in this band was dialyzed against 0.05 M phosphate buffer (pH 6.0) and used for all subsequent experiments.

Ferritin could be isolated from lysates of spph by a similar n-butanol extraction and pH 5.0 precipitation. Spores were found to be an excellent source of starting material (see Part II) although lysis of the spores was only possible on a small scale in a Nossal cell disintegrator (Nossal, 1953).

Protein/Iron Ratio and UV Absorption Spectrum

Phycomyces ferritin had the characteristically low protein/iron ratio of a mammalian ferritin. The values from several determinations are presented in Table I.

Concentrated solutions (10 mg/ml) of mammalian ferritin are dark red due to the ferric iron in the core of the molecule. The color is not due to a specific absorption peak in the visible spectrum, but to continuously increasing absorption starting in the blue and running into the UV (Drysdale and Munro, 1965a). Solutions of Phycomyces ferritin are also red. The spectrum from 240-450 mu is given in Figure 1. The absorbance is plotted per mg Fe per ml as determined from the iron assay. The contribution of the iron core to the extinction at 280 mu is so great that the characteristic protein absorption peak at this wavelength is obscured.

Disc Electrophoresis

The protein/iron ratio and absorption spectrum indicated a high degree of purity in the ferritin preparation. The purity was assessed independently by disc electrophoresis at pH 9.5. Horse ferritin was

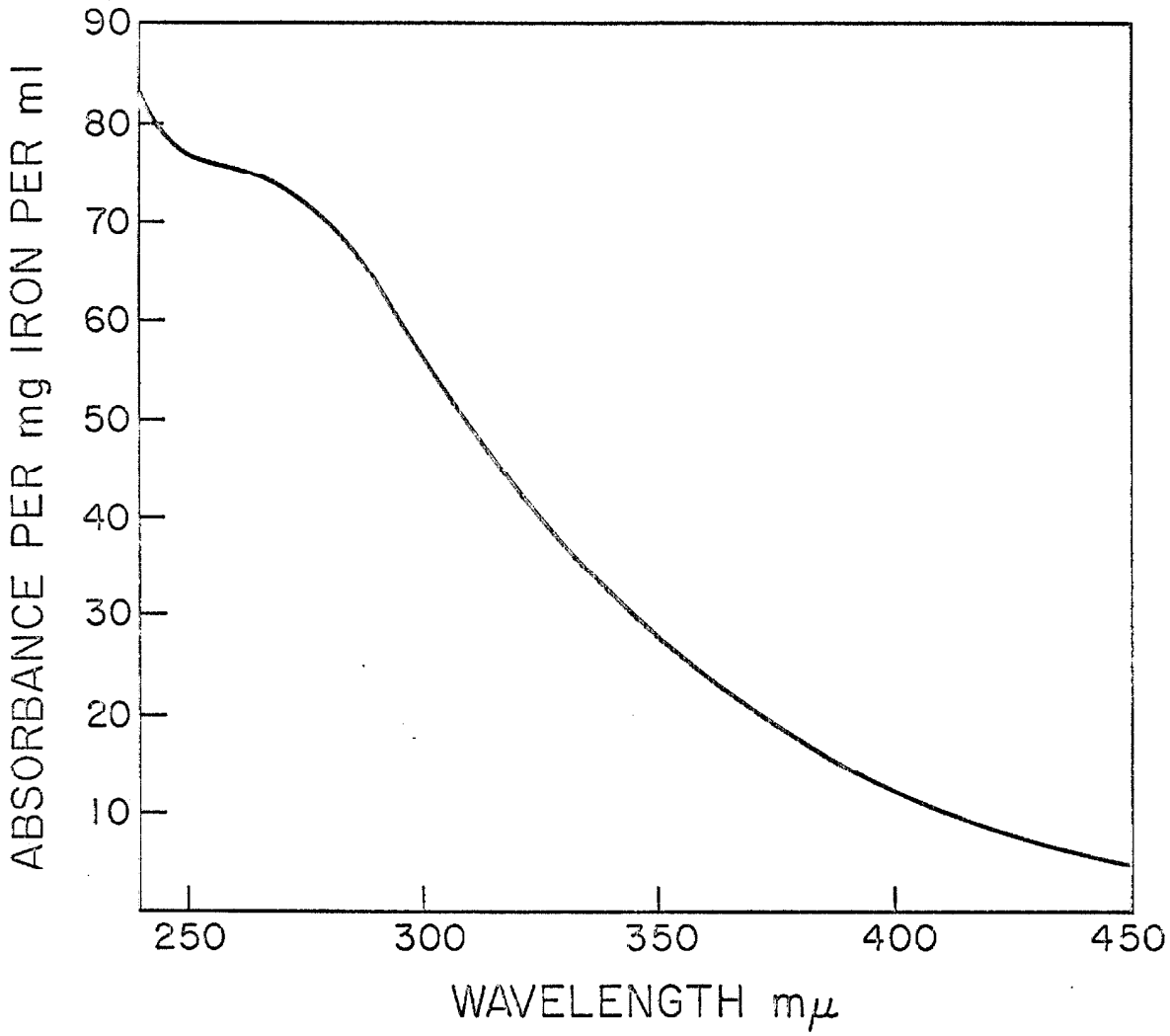
TABLE 1

Iron and Protein Content of Purified Phycomyces Ferritin

Material	Iron mg/ml	Protein mg/ml	Protein/Iron
Phycomyces Ferritin Main band from CsCl Gradient	0.51	2.38	4.7
above main band CsCl Gradient	0.39	1.94	5.0
Phycomyces Ferritin pH 5.0 precipitate	1.49	8.7	5.8
Horse Ferritin	0.43	1.08	2.5

FIGURE 1. UV Absorption Spectrum of Phycomyces Ferritin

Ferritin was purified by n-butanol extraction and pH 5.0 precipitation. The latter step removed the UV-absorbing contaminant. Absorbance is plotted per mg/ml Fe as determined from the iron assay. The absorbance due to protein on this scale would be approximately 4 at 280 mμ.


ABSORBANCE PER mg IRON PER ml
90
80
70
60
50
40
30
20
10
250
300
350
400
450
WAVELENGTH mμ

run simultaneously for comparison. Phycomyces ferritin gave one band containing both iron and protein as determined by staining the gels with either ferrocyanide or amidoblack (Figure 2). Horse ferritin gave two bands both of which contained iron and protein. The leading band is the monomer; the trailing band is the dimer. The dimer does not have a lower net charge but is retarded more than the monomer by the gel (Harrison and Gregory, 1965; Suran and Tarver, 1965). Phycomyces ferritin electrophoresed at a position corresponding closely to that of the horse ferritin dimer. It may, therefore, be a dimer at pH 9.5.

Electron Microscopy

The morphology of the Phycomyces ferritin was examined in the electron microscope in the unstained condition (Figure 3a) and after negative staining (Figure 3b). Its appearance was essentially the same as that of horse ferritin: an electron dense core 50-60 Å in diameter which could be seen, in the negatively stained preparation, to be surrounded by an electron transparent annulus with an overall diameter of 120 Å.

Crystallization

Phycomyces ferritin, purified by precipitation at pH 5.0, was crystallized from 0.0025 M Tris - 0.02 M glycine buffer (pH 8.3). Dark red crystals as large as 0.1 mm were grown although the process was slow and not convenient for purification. No attempt was made to use cadmium sulfate for crystallization as with mammalian ferritins.

FIGURE 2. Disc Electrophoresis at pH 9.5

Horse and Phycomyces ferritin (approximately 100 μg per tube) were electrophoresed for two hours (15 mA/cm^2). Gels stained with amidoblack (A) and potassium ferrocyanide (B). H Horse ferritin; P Phycomyces ferritin.

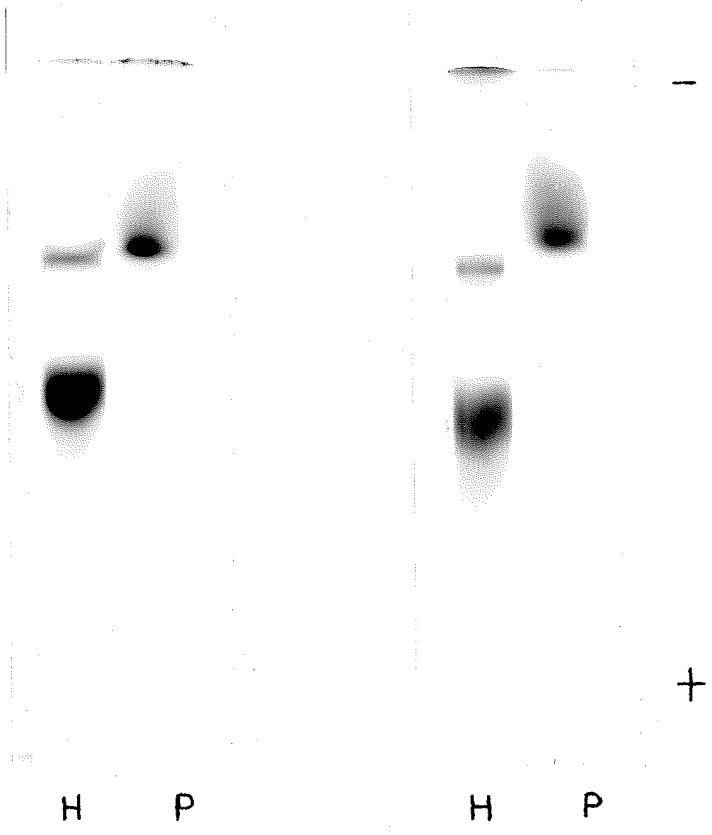
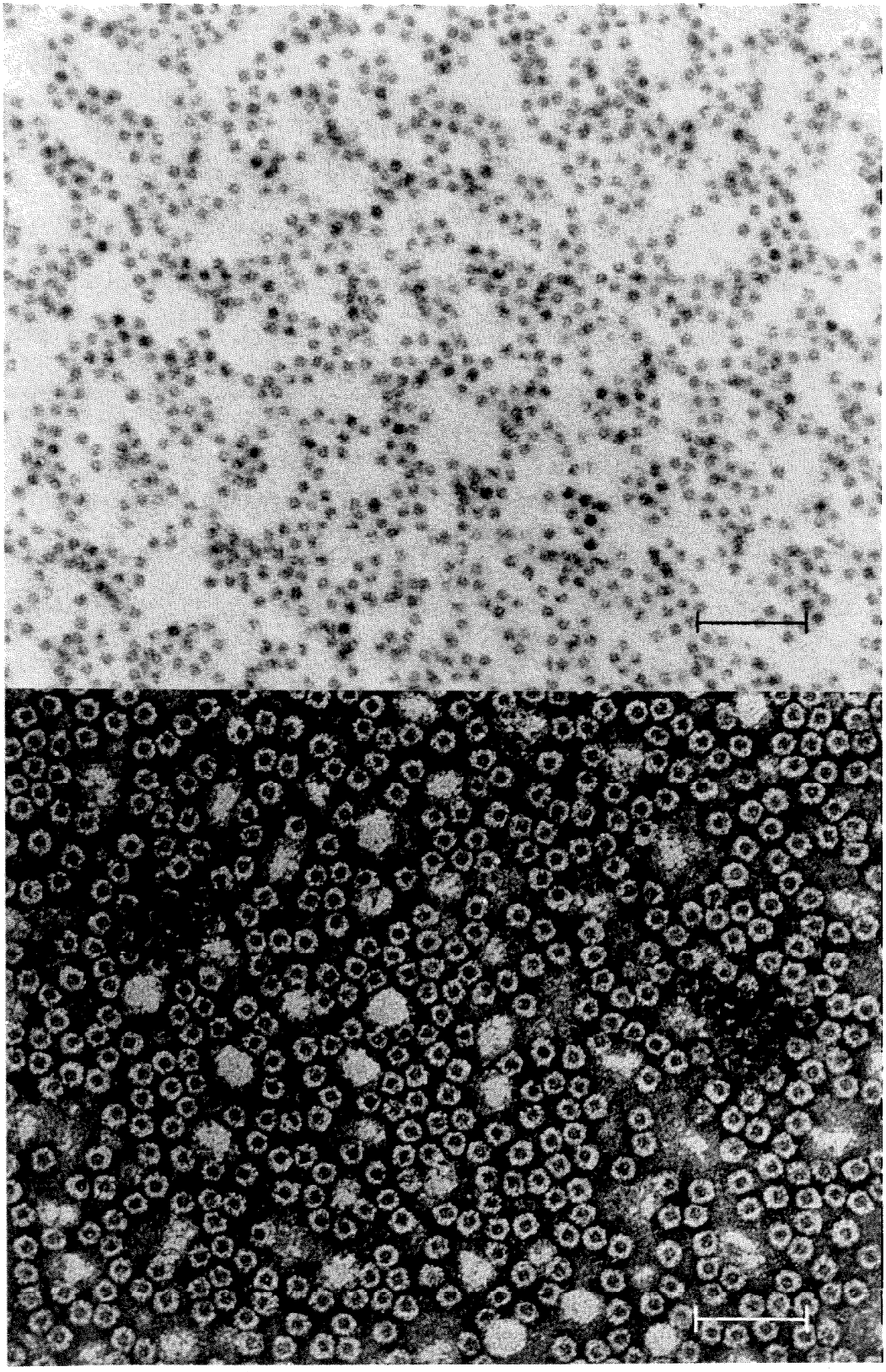

H
P
A
H
P
B
−
+

FIGURE 3. Purified Preparation of Phycomyces Ferritin

Top: unstained

Bottom: negatively stained with uranyl acetate

Scale: 500 Å



Thin sections (300-400Å) of glutaraldehyde-fixed crystals have been examined in the electron microscops (Figure 4a). In the absence of a heavy metal stain only the iron core of the ferritin is visible. The resolution of individual molecules in such a thin section, several ferritin diameters thick, indicated that the section was almost perpendicular to a crystal axis. The ferritin molecules in this projection formed a hexagonal pattern with the axes making angles of 55^{o}, 53^{o} and 72^{o}.

The question arises whether there exists a projection of the face-centered cubic lattice of crystalline ferritin (Harrison, 1959) which is compatible with the projection in the electron micrograph. The projection down the face diagonal of a face-centered cubic lattice is given in Figure 4b. It forms a hexagonal pattern with angles and center-center spacings similar to those in the electron micrograph. Slight rotation of this lattice also gives rise to the "blurring" along one axis which is conspicuous in the electron micrograph. Although an exhaustive search has not been made, at least one other crystal lattice gives a projection similar to the one in the electron micrograph (i.e. the projection down the A axis of the unit cell of a hexagonal close packed lattice).

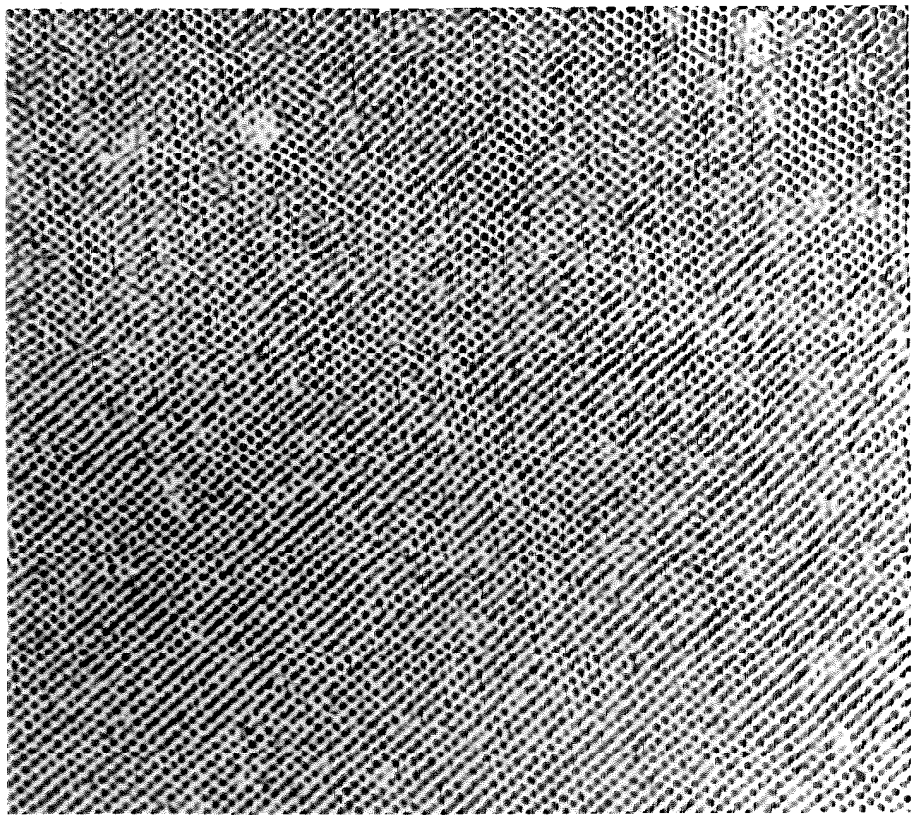
Determination of Sedimentation Coefficient and Buoyant Density in CsCl

Phycomyces ferritin was sedimented in a boundary velocity experiment in an analytical ultracentrifuge. Figure 5 shows a densitometer trace of the boundary in a representative run indicating the homogeneity of Phycomyces ferritin. The boundary positions were measured and the

FIGURE 4.

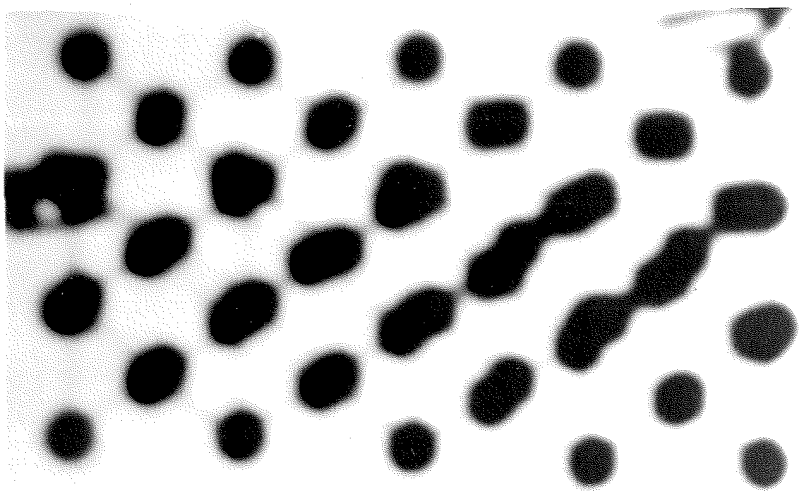
(A) This section of a gluteraldehyde-fixed crystal of Phycomyces ferritin. The electron dense cores of individual ferritin molecules form a hexagonal pattern in this projection.

(B) Projection down the face diagonal of a face-centered cubic lattice showing the hexagonal arrangement of projected lattice points. The lattice has been tipped slightly to match the blurring along one axis seen in the crystal thin section.



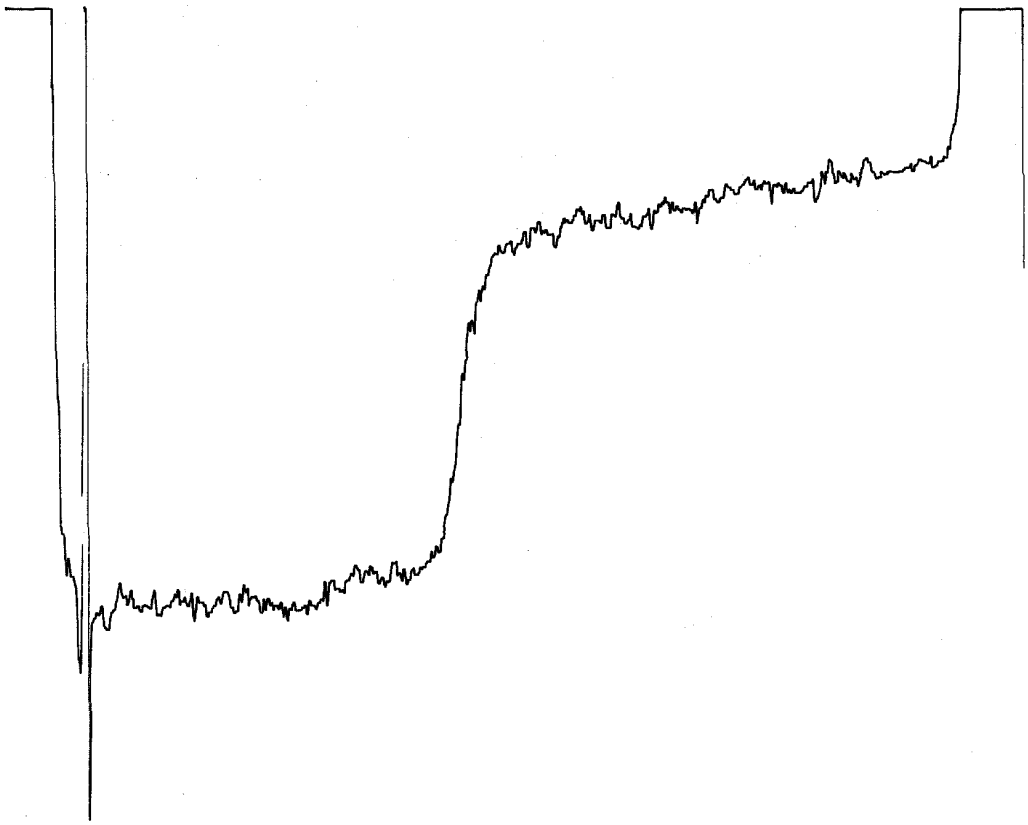
A

1000 Å



B

FIGURE 5. Densitometer trace of boundary sedimentation experiment with purified Phycomyces ferritin 40 minutes after reaching speed of 24,630 rpm (20°C). Ferritin concentration is 10 μg/ml Fe. Sedimentation is from left to right.



sedimentation coefficients calculated using the relationship

$$S = \frac{1}{\omega^2 r} \frac{dr}{dt}$$

where S is the sedimentation coefficient; r, the radius; ω the angular velocity; and t, the time. The experimental S-values were taken as $S_{20,w}$ since the sedimentation runs were done at 20°C in dilute aqueous solvent.

The results for three ferritin concentrations are given in Table 2. Because of the high absorption of ferritin in the UV low concentrations could be used in the sedimentation runs making extrapolation of the S-values to infinite dilution unnecessary.

The buoyant density of ferritin in CsCl was determined in the analytical ultracentrifuge (Meselson, Stahl, and Vinograd, 1957; Vinograd and Hearst, 1962). Solutions of ferritin and CsCl (ρ = 1.81 g/cm^3) were centrifuged for 36 hours at 44,770 rpm. At equilibrium photographs were taken and microdensitometer traces made. After correcting for a rising baseline, the film density (equivalent to ferritin concentration) was plotted against the radius (to center of rotation) for two Phycomyces ferritin preparations (Figure 6). Both preparations -- one from mycelium, the other from spores -- were obtained by n-butanol extraction and pH 5.0 precipitation. The mycelial preparation was further purified by taking the main band after centrifuging the material in a preparative CsCl density gradient.

The buoyant density of the ferritin bands was determined from the calculated composition density gradient as described by Vinograd and

TABLE 2

Sedimentation Coefficient of Purified Phycomyces Ferritin

Ferritin Concentration µg/ml Fe	$S_{observed}$
17.6	55.2
8.1	54.8
4.5	54.7

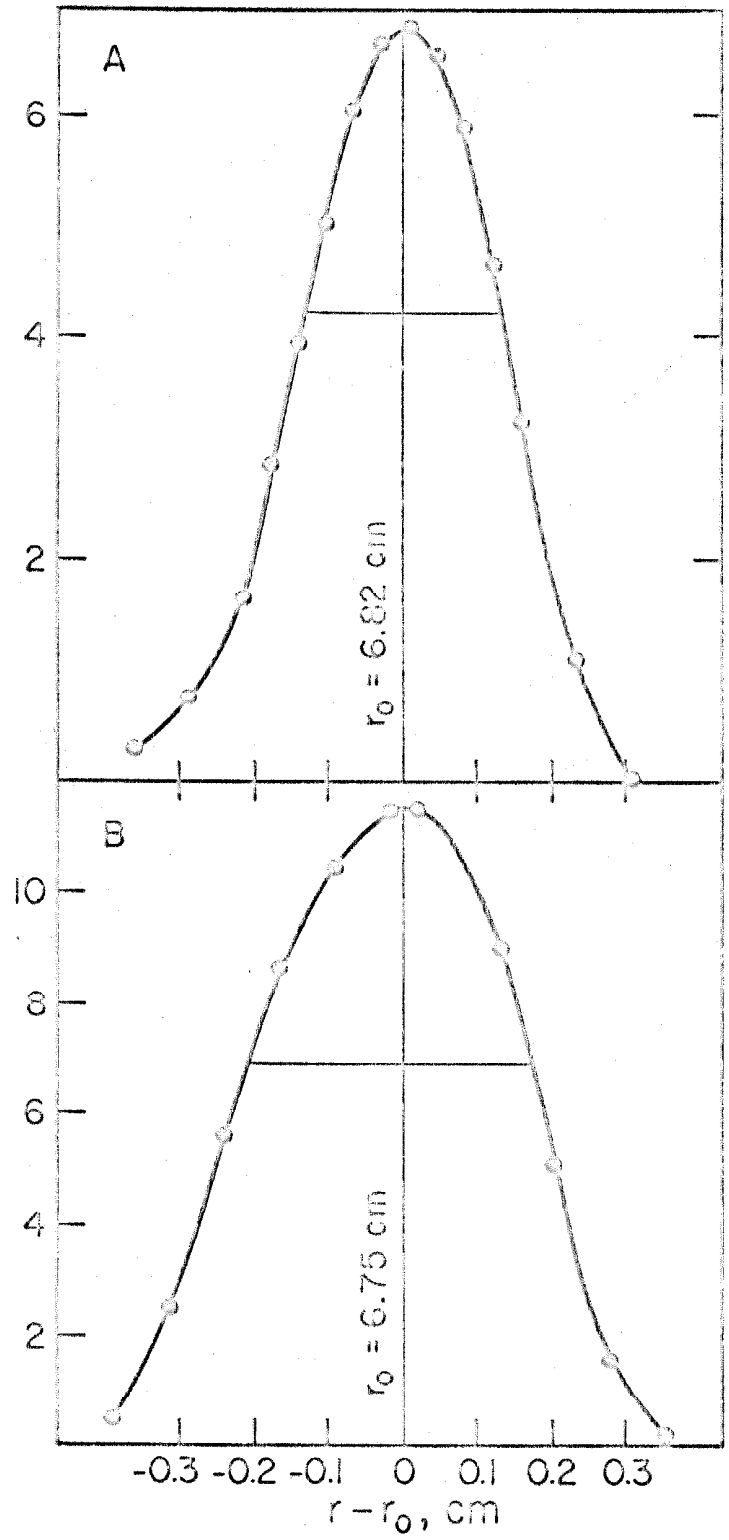
Phycomyces ferritin was purified through the CsCl banding step as described in the text. The main band was collected and dialyzed against 0.05 M phosphate buffer at pH 6.0. Boundary sedimentation was performed with this material at the concentrations given above. Centrifugation was performed at 20°C at 24,630 rpm. Pictures were taken at 4 minute intervals using the UV optical system.

FIGURE 6. Distribution of Phycomyces Ferritin in CsCl Density Gradient at Equilibrium. Ferritin concentration: 20 μg/ml Fe; 44,770 rpm; 25° C. The density at band center is 1.82 g/cm^3. The original densitometer trace has been replotted to correct for a rising baseline. The ordinate is an arbitrary concentration scale related to film density. On the abscissa, r is the distance from the center of rotation; r_o is band center. The band width (2σ) is indicated by the line across each peak.

(A) spore ferritin

(B) mycelial ferritin (main band from preparatative CsCl gradient)

Both preparations are from iron supplemented medium.


A
6
4
2
r_o = 6.82 cm
B
10
8
6
4
2
r_o = 6.75 cm
-0.3 -0.2 -0.1 0 0.1 0.2 0.3
$r - r_o$, cm

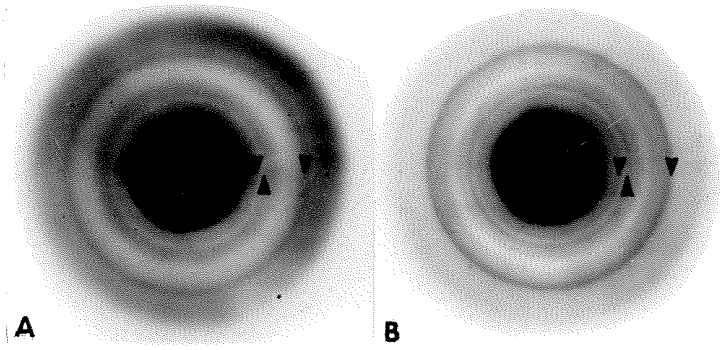
Hearst (1962). Mycelial and spore ferritin, both from Phycomyces grown on iron supplemented medium, have a buoyant density of 1.82 g/cm^3. Both preparations formed relatively narrow bands in CsCl density gradients indicating rather less density heterogeneity than in similar mammalian preparations (Fischbach and Anderegg, 1965). Their respective band widths are 0.38 and 0.27 cm which are equivalent to 0.046 and 0.033 g/cm^3 for the density gradient present in these runs.

Electron Diffraction

To compare the structure of the iron cores of Phycomyces ferritin and horse ferritin, unstained films of ferritin were subjected to selected area (200 μ) diffraction in the electron microscope. The ring patterns observed were calibrated against MgO films and are shown (after rephotographing) in Figure 7. Considerable detail is lost in printing and the relative intensities of the rings are altered. It is, however, possible to compare the stronger lines in each pattern. These lines are identical in position and lie at 2.52Å, 2.23Å, and 1.48Å. They are indicated by arrows in the figure. In horse ferritin (Figure 7b) rings are also visible at 1.94Å and 1.72Å. Qualitatively the lines in Phycomyces ferritin are broader than in horse ferritin indicating that the size of the crystalline regions within the core is probably smaller than in horse ferritin. This line broadening, combined with the diffraction pattern of the supporting carbon film (broad zones from 1.0Å to 1.25Å and above 1.6Å) may make weaker lines from Phycomyces ferritin undetectable.

FIGURE 7. Electron Diffraction Pattern of Phycomyces Ferritin (A) and Horse Ferritin (B).

The arrows identify the principal rings in both patterns. They are identical and lie at 2.52 Å, 2.23 Å, and 1.48 Å.


A
B

Immunological Specificity

To determine if there was any antigenic similarity between Phycomyces ferritin and the well characterized mammalian ferritins, the former was tested with antisera against horse, human, and rabbit ferritin. A weak precipitin band was detected when Phycomyces ferritin was tested with sheep antiserum to rabbit ferritin (Figure 8). Pooled normal sheep serum gave no precipitin band with Phycomyces ferritin. Rabbit ferritin at 100X lower concentration than Phycomyces ferritin still gave a strong precipitin band with the sheep antiserum.

Part II - Intracellular Ferritin: Localization and Properties

Phycomyces ferritin is found tightly bound to large lipid droplets in the cytoplasm of spph, mycelium and spores. This ferritin-lipid complex forms an unusual subcellular structure which is clearly visible in electron micrographs of spph in thin section (Figure 9). It was this observation which prompted the isolation of ferritin described in Part I. In osmium tetroxide fixed tissue the lipid droplets in the spph cytoplasm are large round uniformly staining particles 0.5-2.0 μ in diameter. At high magnification, dense granules are visible around the edge or over part of the surface of each lipid droplet, depending on whether the section through it is equatorial or tangential. These granules are arranged in a partially crystalline two dimensional pattern over the surface of the lipid particle. They have the same dimensions as Phycomyces ferritin described in Part I and are naturally electron

FIGURE 8.

Ouchterlony double diffusion plate showing the weak cross-reaction between Phycomyces ferritin and antiserum to rabbit ferritin. Center well contains sheep antiserum to rabbit ferritin. Side wells A and B contain Phycomyces ferritin at 90 and 45 μg/ml Fe. Side wells C and D contain rabbit ferritin at 2 and 1 μg/ml Fe.

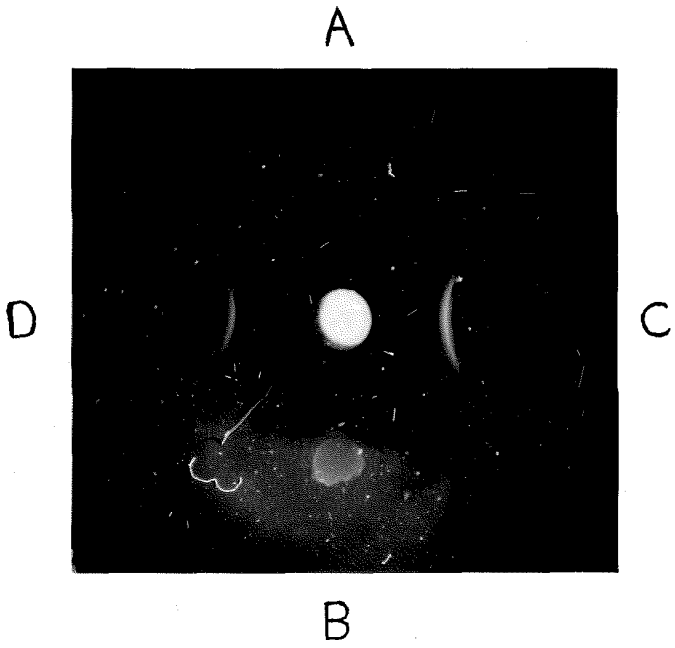
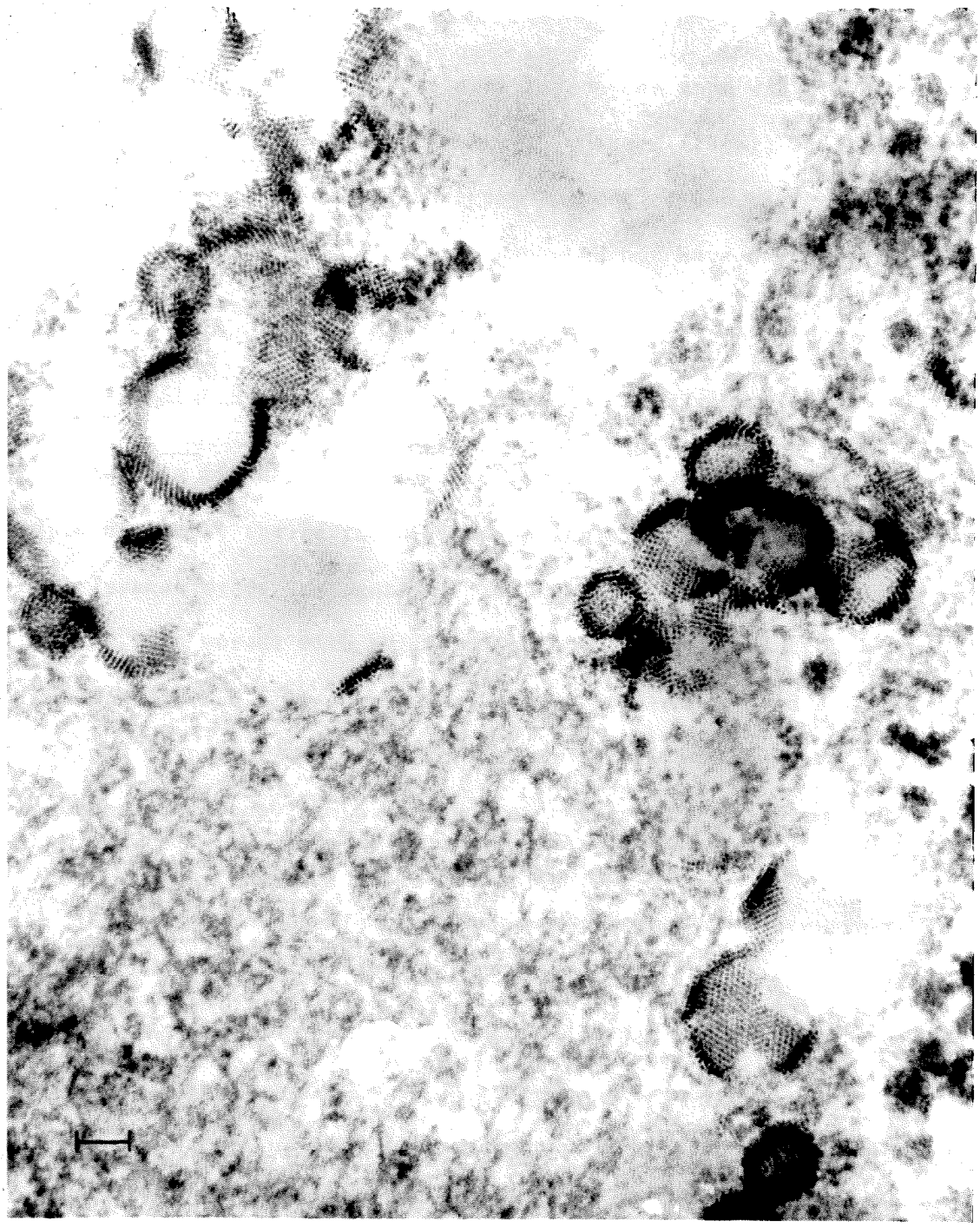

A
D
C
B

FIGURE 9.

Thin section near the top of a glutaraldehyde-osmium tetroxide fixed stage 3 spph showing lipid droplets in cytoplasm covered with crystalline arrays of ferritin. Some of the lipid droplets are cut tangentially showing extensive crystalline regions; others are cut more equatorially showing only a ring of ferritin (the medium was not supplemented with iron). Scale: 1000 Å.



dense (i.e. visible in tissue prepared for electron microscopy without heavy metal fixative or stain).

The ferritin-lipid particle in Phycomyces spph was first noted by Hyde (1964, unpublished experiments). Sassen (1965), Thornton (1966), and Peat and Banbury (1967) have published electron micrographs showing the ferritin-lipid particles in Phycomyces but have not identified the dense granules as ferritin molecules.

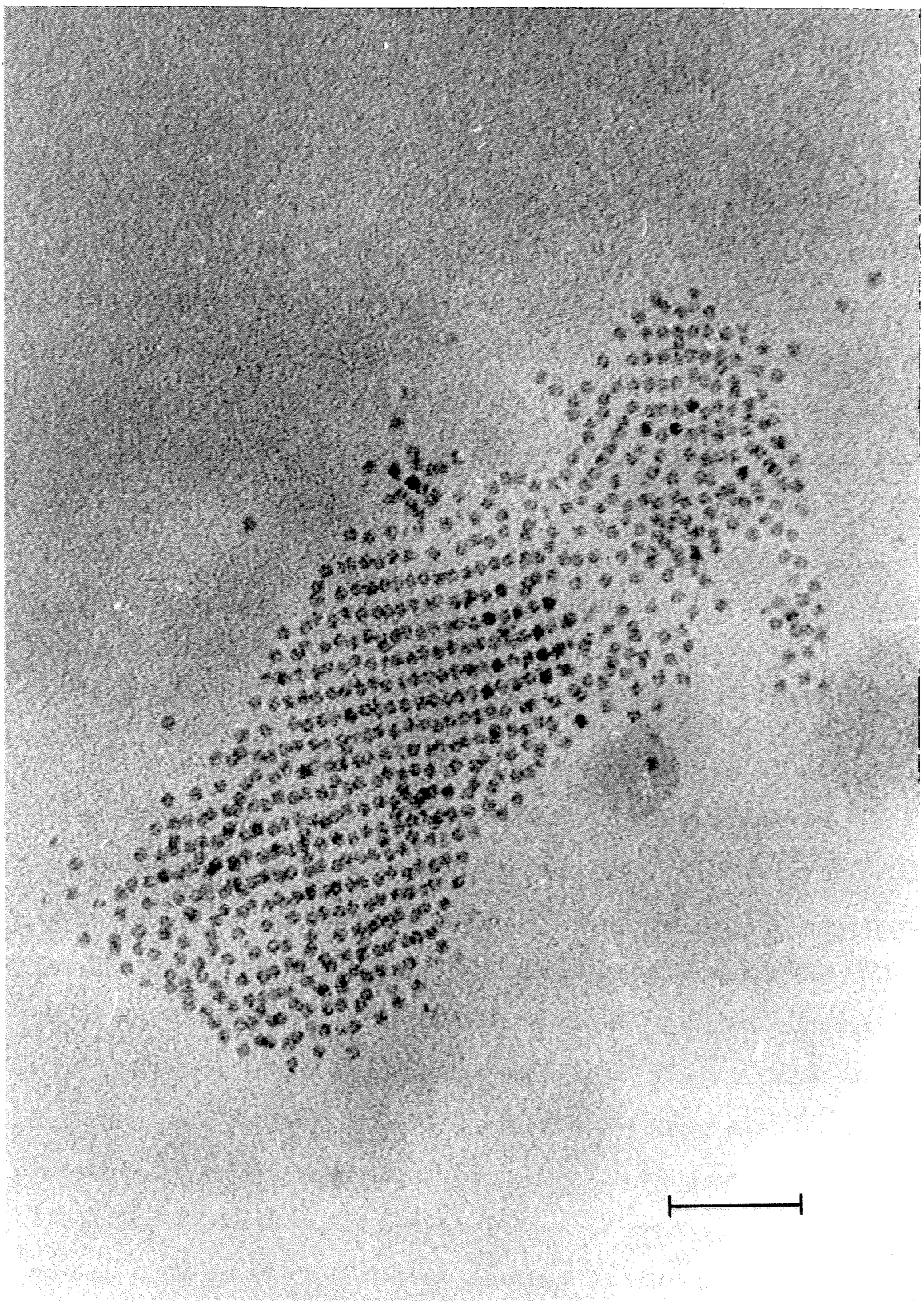
Isolation of Ferritin-Lipid Particles From Cell Lysates

Because of the large size and low density (< 1.0 g/cm^3) of the lipid droplets, the ferritin-lipid particle is easilly isolatable from cell lysates by centrifugation. To prepare lysates spph were ground briefly in a mortar with buffered isotonic sucrose solution. Centrifugation (10,000 x g; 10 minutes) of the lysate produced a thick scum of lipid on top of the solution. After fixation and embedding for electron microscopy, thin sections of this scum were observed to consist mainly of the ferritin-lipid particles.

A simpler, more effective method to examine the ferritin in these complexes was to dilute a spph lysate to the point where centrifugation produced a monolayer of lipid particles rather than a thick scum. Such monolayers could be picked up on electron microscope grids, washed with several drops of water to remove most of the lipid, and examined directly in the electron microscope (Figure 10). In such preparations the crystalline array of ferritin molecules from the surface of the lipid droplets stuck to the grid. At high magnification the fine structure typical of ferritin was easily resolved.

FIGURE 10.

Crystalline monolayer of Phycomyces ferritin from the surface of a lipid droplet. The preparation was obtained by diluting a spph lysate (from iron supplemented medium) to the point where centrifugation produced a monolayer of lipid on the surface of the solution. A specimen grid was touched to the surface of the solution, washed with a drop of water, dried, and examined without staining in the electron microscope. The electron dense cores of ferritin are visible. Scale: 500 Å



Disruption of the Ferritin-Lipid Complex

Phycomyces spph were grown on GA medium to which $^{59}FeCl_3$ (sp. Act. 0.5 μc/mg Fe) was added. Spph lysates were prepared in isotonic sucrose and the lipid layer, heavily labeled with ^{59}Fe, was collected following centrifugation. It was dispersed in a small volume of buffer containing 0.5% Brij and an aliquot centrifuged through a 5-20% sucrose gradient. The result is shown in Figure 11. The ^{59}Fe, which originally moved centripetally in association with large lipid droplets, now sedimented centrifugally as a single peak between 50 and 60S. This suggests that treatment with 0.5% Brij dissociates the ferritin from the lipid droplets which remain at the top of the gradient.

Distribution of Ferritin Among Subcellular Fractions of Phycomyces Spph

To determine the distribution of iron and ferritin in spph, Phycomyces was grown on ^{59}Fe-supplemented medium, spph were harvested and the resulting lysate fractionated by differential centrifugation. Low speed centrifugation (500 x g) pelleted the spores, nuclei, and aggregated debris. Centrifugation of the low speed supernatant for 15 minutes at 10,000 x g pelleted the mitochondria and glycogen and collected the lipid particles on top of the solution (scum). Table 3 gives the distribution of ^{59}Fe among these fractions. The bulk of the ^{59}Fe was incorporated into the low speed spore pellet. Subsequent purification of the spores from the nuclei and debris in this fraction (by centrifugation through 1.9M sucrose and repeated washings in fresh buffer) caused no loss of ^{59}Fe indicating that the label was located in the spores.

FIGURE 11.

Sucrose gradient sedimentation of ^{59}Fe-labeled scum treated with 0.5% Brij which releases the ferritin from the lipid droplets. The material was centrifuged at 37,000 rpm for 2 hours at 4°C.

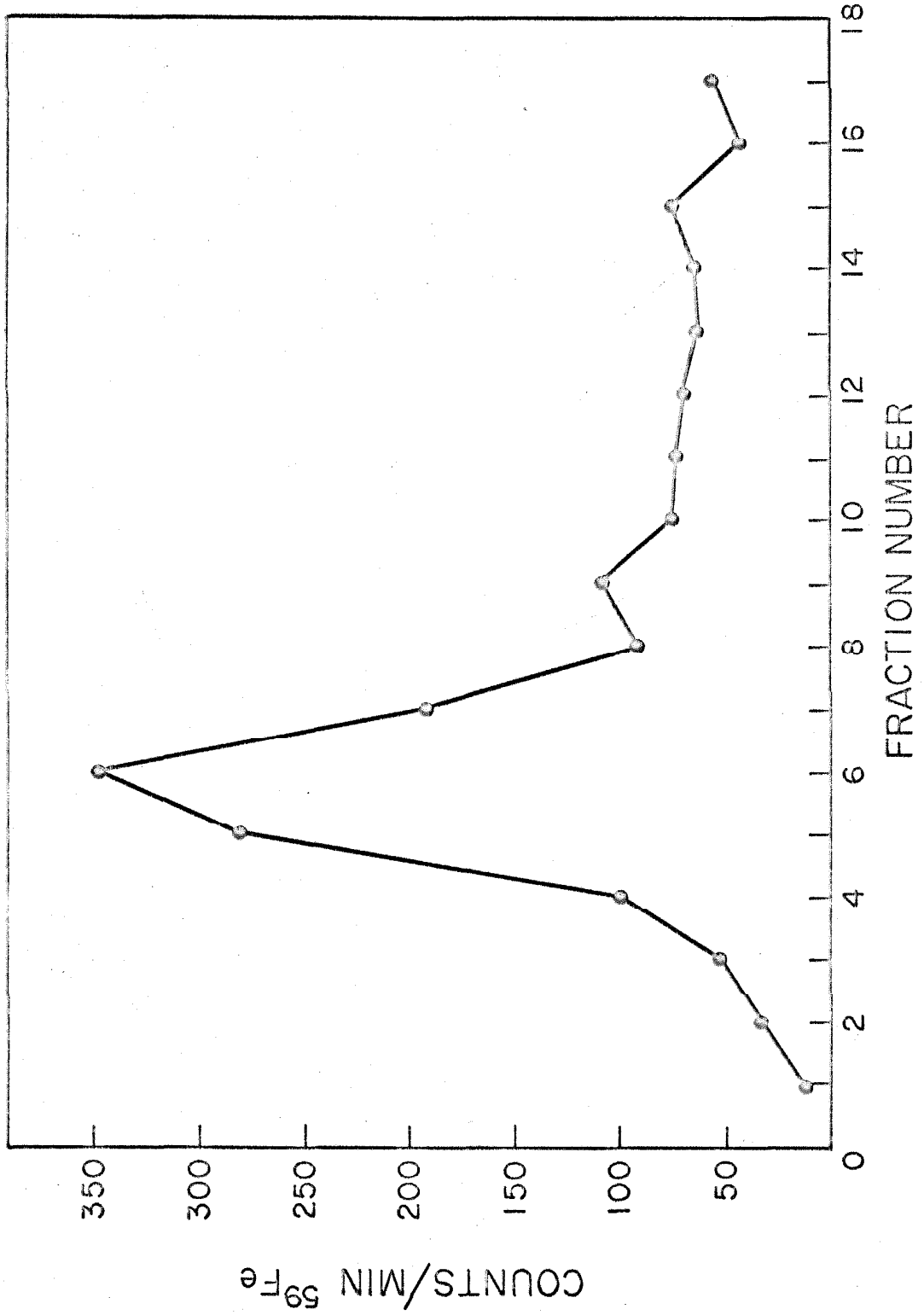

COUNTS/MIN ^{59}Fe
350
300
250
200
150
100
50
0
2
4
6
8
10
12
14
16
18
FRACTION NUMBER

TABLE 3

Distribution of ^{59}Fe and Ferritin in Spph Lysate

Fraction	Total cpm $\times 10^{-5}$	%	Fraction of ^{59}Fe in Ferritin (as determined by sedimentation in 60S region of sucrose gradient)
Lysate	4.7	100	---
500 x g pellet (spores)	3.5	72	100% of extractable ^{59}Fe (but only ~50% of total cpm are extractable)
10,000 x g Scum (lipid droplets)	0.4	8	100%
10,000 x g Supernatant	0.8	16	variable but < 50%
10,000 x g Pellet (mitochondria, glycogen)	0.2	4	(not investigated)

Phycomyces was grown on GA medium supplemented with 500 µg Fe per petri dish. $^{59}FeCl_3$ was added to a specific activity 4.2 x 10^5 cpm/mg Fe. Spph were harvested and ground in buffered isotonic sucrose in a mortar.

The amount of ^{59}Fe in ferritin in each subcellular fraction was determined by sedimenting samples in sucrose gradients. Prior to sedimentation ferritin was freed from any associated lipid in the fractions by treatment with n-butanol (see Part I) or 0.5% Brij. To analyze the radioactivity in the spores it was necessary to disrupt them in a Nossal cell disintegrator before proceeding with the n-butanol extraction. The results are given in Table 3.

The lipid fraction contained only ^{59}Fe which sedimented in the 60S region of the gradient and was therefore identified as ferritin (Figure 11). In a spore lysate all the extractable radioactivity sedimented about 60S in a sucrose gradient, similar to the result obtained with the lipid fraction (Figure 12a,c). It should be noted, however, that only 40-50% of the ^{59}Fe in a spore lysate could be recovered in the aqueous phase after butanol extraction. Re-extraction of the precipitated material at the interface, containing the remaining radioactivity, yielded some more ^{59}Fe which sedimented as ferritin although it was never possible to recover all the ^{59}Fe from spore lysates. No radioactivity was recovered which sedimented differently than ferritin.

The nature of the ^{59}Fe in the 10,000 x g supernatant varied from experiment to experiment. At least 50% of the radioactivity was always dialyzable; of the remaining ^{59}Fe some always sedimented as ferritin.

Ferritin Content of Spph and Spores as a Function of Iron Content of the Growth Medium

Much of the work already discussed in this paper has depended on the fact that supplemental iron in the growth medium markedly enhances the ferritin content of Phycomyces. This effect has been examined quantitatively. Two levels of iron in the growth medium were used: low iron (no iron added to GA medium; iron level in the medium due to impurities is ~ 0.1 ug/ml) and high iron (GA medium supplemented with 15 µg/ml Fe). Phycomyces was grown at these two iron levels in the presence of $^{59}FeCl_3$. Spph lysates (from 30 petri dishes) were prepared and separated by low speed centrifugation into a spore fraction and a cytoplasmic fraction. (The cytoplasmic fraction contained the mitochondria, glycogen, lipid, and supernatant fractions which were examined independently in the previous section.) The distribution of ^{59}Fe between these two fractions is given in Table 4. The absolute amounts of iron calculated from the specific activities are also given in the table. It is evident that the iron content of both spph cytoplasm and spores increases sharply when the growth medium is supplemented with iron.

The cytoplasmic and spore fractions were extracted for ferritin using the n-butanol procedure. The cytoplasmic fraction was treated directly with n-butanol. The spores were first disrupted in a Nossal cell disintegrator and then extracted with n-butanol. The distribution of ^{59}Fe between the aqueous phase and the interface is shown in Table 5.

TABLE 4

^{59}Fe Content of Spph Cytoplasm and Spores from Low Iron and High Iron Growth Media

Fraction	High Iron			Low Iron		
	cpm x 10^{-6}	µg Fe	%	cpm x 10^{-6}	µg Fe	%
Lysate	14	950	100	52.7	24	100
500 x g Supernatant (cytoplasm)	4.7	320	35	10.2	4.6	22
500 x g Pellet (spores)	10	680	65	41.4	19	78

Specific Activity:

High Iron medium 1.47×10^{7} cpm/mg

Low Iron medium 2.2×10^{9} cpm/mg

TABLE 5

Distribution of ^{59}Fe Between Aqueous Phase and Interface in n-Butanol Extracts of Cytoplasm and Spore Fractions

Fraction	High Iron cpm x 10^{-5}	Low Iron cpm x 10^{-5}
Cytoplasm		
Aqueous phase	22	25.6
Interface	10	44.0
Spore		
Aqueous phase	1.6	4.1
Interface	1.1	4.1

Portions of the cytoplasm and spore fractions of Table 4 were extracted with n-butanol as described in Part I. Following centrifugation to separate the phases, the aqueous phase and the interface (containing precipitated material) were assayed for ^{59}Fe. These two phases contained all the radioactivity.

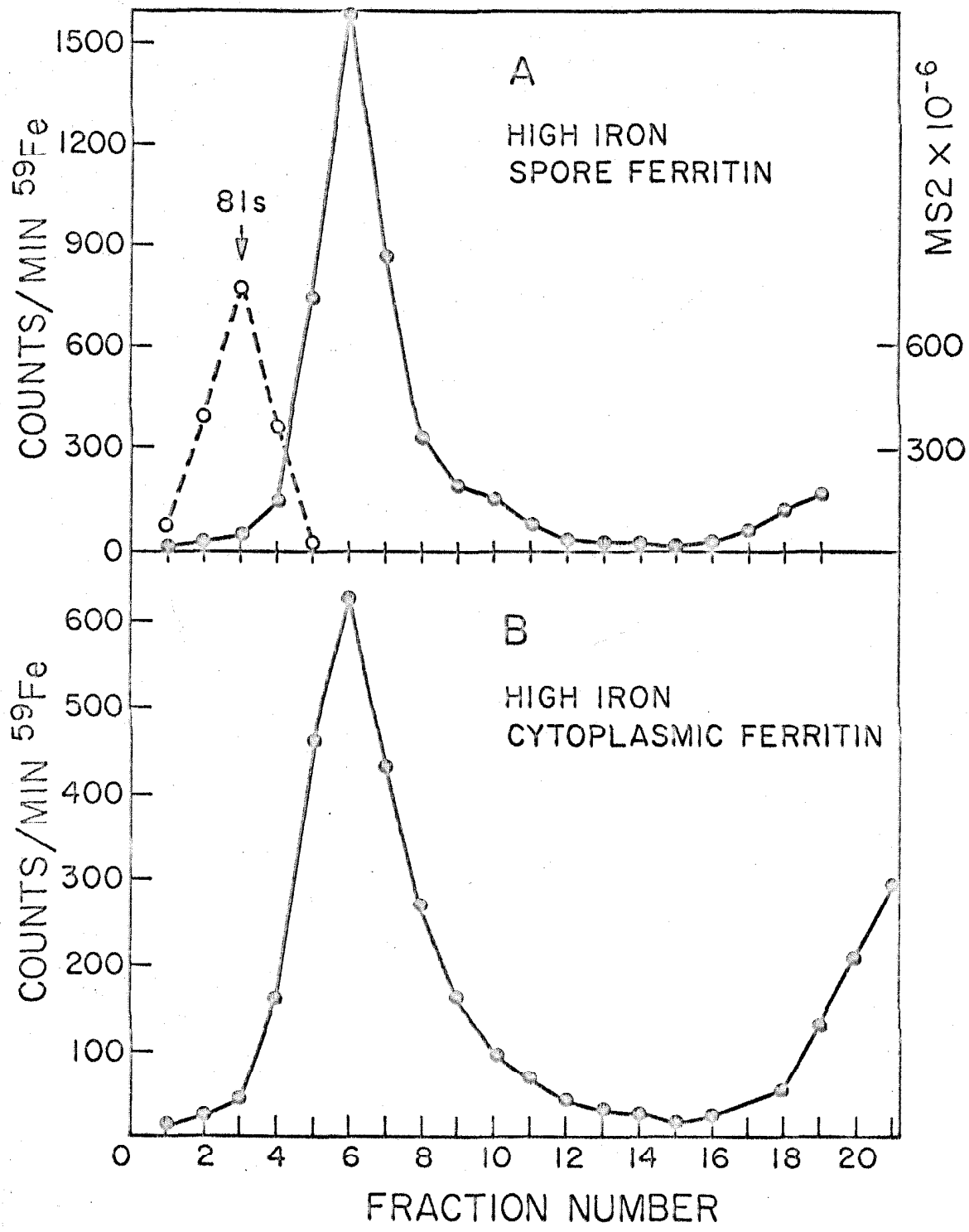
The aqueous phases from these extractions were analyzed for ferritin by centrifuging samples in sucrose gradients. The results are given in Figure 12. All extractable radioactivity from both high iron and low iron spores sedimented in the 60S region of the gradients (Figure 12a,c). In the two cytoplasmic samples (Figure 12b, d) 1/2 to 2/3 of the ^{59}Fe also sedimented as ferritin. The conclusion from this experiment is that the additional iron in high iron spores and spph cytoplasm is contained largely in ferritin.

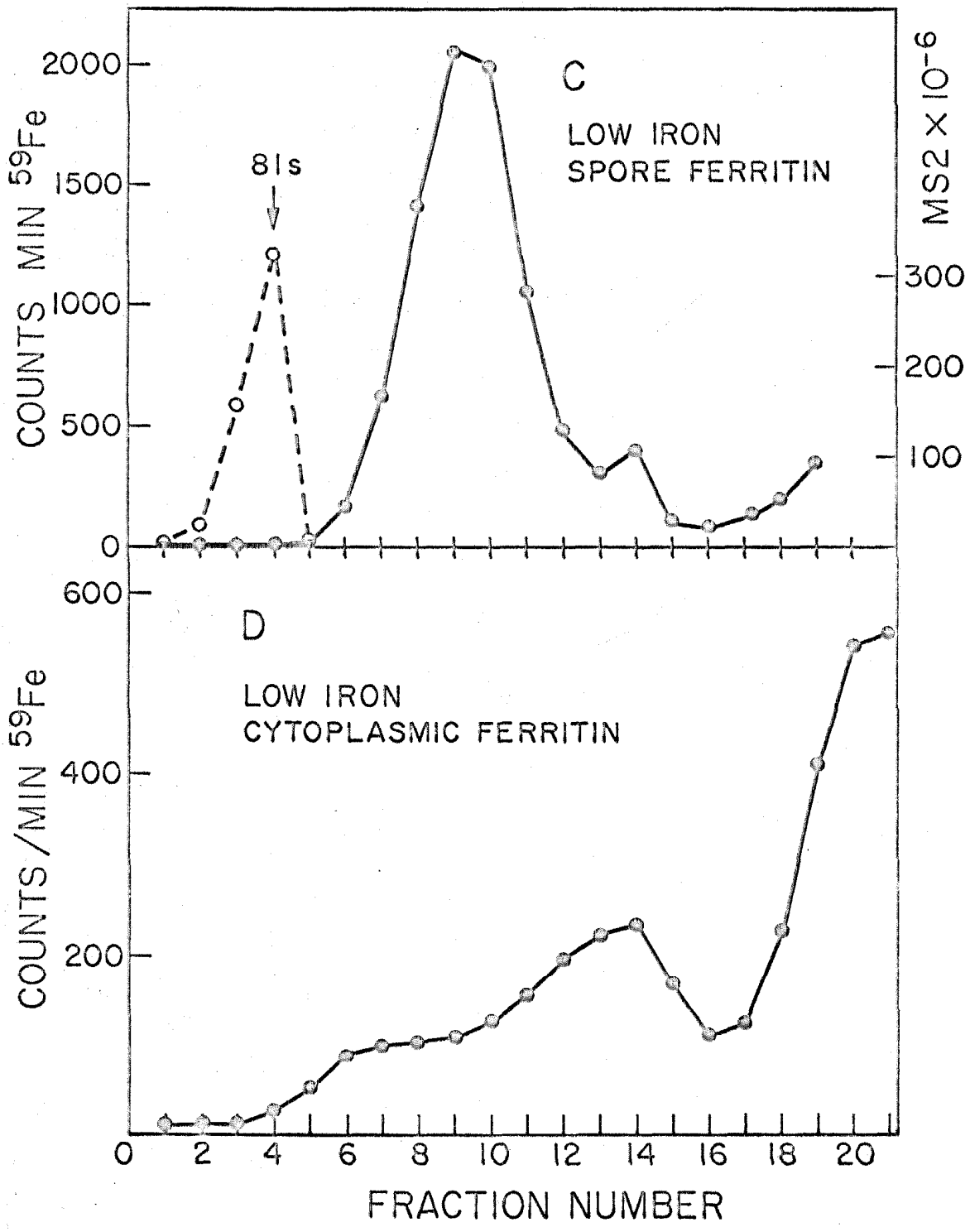
Sedimentation Properties of Cytoplasmic and Spore Ferritin from High and Low Iron Growth Medium

Supplementing the growth medium with iron not only induces the formation of additional ferritin in both cytoplasm and spores but also alters the sedimentation properties (i.e. iron content) of the average ferritin molecule. This is shown in the four sucrose gradients in Figure 12. When the medium was supplemented with iron, the ferritin isolated from spores and spph cytoplasm was indistinguishable by sedimentation (Figure 12a, b). It had an S-value of 67. By comparison, on low iron medium, the ferritin from spores and spph cytoplasm did not have the same sedimentation characteristics (Figure 12c,d). The former was relatively homogeneous and sedimented at 54S; the latter was very heterogeneous and sedimented over a broad range of S-values from 54S to 30S. Clearly, under low iron growth conditions, two populations of ferritin exist in Phycomyces spph: one in spph cytoplasm and one in spores.

FIGURE 12. Sedimentation Properties of Spph Ferritin.

Lysates of spph grown on high iron and low iron media were separated into spore and cytoplasmic fractions. Samples from the aqueous phase of n-butanol extracted fractions were layered on sucrose gradients and centrifuged for 2 hours at 37,000 rpm, $4^{\circ}C$. Twenty drop fractions were collected for ^{59}Fe assay. One drop between each fraction was collected into broth for assay of bacteriophage MS2 (centrifuge marker, 81S). A and B are spore and cytoplasmic fractions from spph grown on high iron GA medium; C and D are spore and cytoplasmic fractions from spph grown on low iron GA medium.


A
HIGH IRON
SPORE FERRITIN
81s
COUNTS/MIN 59Fe
MS2 X 10-6
1500
1200
900
600
300
0
600
300
B
HIGH IRON
CYTOPLASMIC FERRITIN
COUNTS/MIN 59Fe
600
500
400
300
200
100
0
2
4
6
8
10
12
14
16
18
20
FRACTION NUMBER


C
LOW IRON
SPORE FERRITIN
81s
COUNTS MIN 59Fe
MS2 X 10-6
2000
1500
1000
500
0
300
200
100
D
LOW IRON
CYTOPLASMIC FERRITIN
COUNTS /MIN 59Fe
600
400
200
0
2
4
6
8
10
12
14
16
18
20
FRACTION NUMBER

The sedimentation coefficient (67S) found for ferritin following n-butanol extraction in these experiments is higher than the value (55S) determined by analytical ultracentrifugation in Part I for ferritin purified by n-butanol extraction and pH 5.0 precipitation. In the latter step a significant amount of "UV-absorbing contaminant" was dissociated from Phycomyces ferritin (Part I). The difference in S-value may be due to this contaminant.

DISCUSSION

Part I of this paper presents an isolation procedure for a large iron-containing protein from Phycomyces. This protein exhibits properties very similar to those of ferritin from mammals and higher plants. On the basis of these similarities we have identified it as Phycomyces ferritin.

Phycomyces ferritin is a very stable protein. It survives butanol extraction, which denatures most cellular proteins, and, after purification, can be maintained in solution in the cold for many months without obvious degradation. In electron micrographs of unstained or negatively stained preparations Phycomyces ferritin is indistinguishable from mammalian ferritins. The structure of the iron hydroxide micelle, as judged by its electron diffraction pattern, appears to be the same in both Phycomyces and horse ferritin. Since the structure of the ferritin micelle is different from any of the naturally occurring oxyhydroxides of iron (Haggis, 1967; Harrison, et al, 1967; Towe and

Bradley, 1967) its presence in both Phycomyces and horse ferritin is strong evidence for the similarity of these two proteins.

Like other ferritins the Phycomyces protein has a low protein/iron ratio. Although the actual ratio found for Phycomyces ferritin is not as low as that reported for horse ferritin (Table 1), this may be due to the experimental method rather than a real difference between the two ferritins. The Folin reagent used for protein determinations gives different extinction coefficients with different proteins (Lowry, 1951). Since horse apoferritin was used as the standard for the protein assay, a two fold error in the protein determination for Phycomyces ferritin is conceivable. Thus the observed two fold difference in the protein/iron ratio between Phycomyces and horse ferritin may not be significant. An accurate determination of protein nitrogen by the Kjeldahl procedure would be desirable.

Sedimentation to equilibrium in CsCl density gradients provides an independent check of the comparative protein/iron ratio of horse and Phycomyces ferritin. The buoyant density of ferritin in CsCl is a function of its iron content (Fischbach and Anderegg, 1965). The protein moiety alone has a density typical of proteins (1.28 g/cm^3). Addition of iron to apoferritin increases its density to about 1.8 g/cm^3 in "full" ferritin having a protein/iron ratio of about 2.0. Phycomyces ferritin has a density of 1.82 g/cm^3, which is close to that of similarly "full" horse ferritin molecules. Since Phycomyces and horse ferritin appear to have very similar sizes and iron cores of similar composition, it would require approximately the same amount of protein to "float" the cores of both molecules at a density of 1.8 g/cm^3 in CsCl.

One remarkable feature of Phycomyces ferritin compared to horse ferritin is its homogeneity. Horse ferritin preparations, which contain molecules of widely differing iron content, exhibit inhomogeneity in sedimentation velocity (Rothen, 1944) and in buoyant density in CsCl (Fischbach and Anderegg, 1965). By comparison, the homogeneity of Phycomyces ferritin in sedimentation (Figure 5) and buoyant density (Figure 6) is striking. Nevertheless, Phycomyces ferritin preparations are not entirely homogeneous. An approximation of the density heterogeneity can be made from the band widths in Figure 6. The density gradient in these experiments was 0.122 g/cm^4. For band widths of 0.38 cm and 0.27 cm the density changes across the bands are 0.046 g/cm^3 and 0.033 g/cm^3. Such density variations reflect differences of 7-9% in the iron content of the core which are equivalent to a 4% variation in the total molecular weight.

Despite the structural and morphological similarity of Phycomyces ferritin to other ferritins, there are some marked differences. The most obvious is the lipophilic character of Phycomyces ferritin. In cytoplasm Phycomyces ferritin is bound to large lipid droplets where it forms a crystalline monolayer over the lipid surface. Such crystalline monolayers are also characteristic of plant ferritin as it occurs in proplastids although there is no evidence that lipid is involved (Hyde et al, 1963; Robards and Humpherson, 1967). Mammalian ferritin does not usually form crystalline structures in cytoplasm, nor is it associated with lipid. Characteristically, it is scattered throughout the cytoplasm as individual molecules although, under some conditions of excess iron

metabolism, it is observed in large disordered cytoplasmic aggregates (Kuff and Dalton, 1957; Kerr and Muir, 1960; Bessis and Breton-Gorius, 1957,1959).

In two other properties Phycomyces ferritin is distinguishable from mammalian ferritins: antigenic specificity and electrophoretic mobility. Phycomyces ferritin fails to cross-react with antibodies against two mammalian ferritins (human and horse) although it does cross-react weakly with antibodies to rabbit ferritin. In view of similar results with cytochrome c, the lack of antigenic homology between mammalian ferritins and Phycomyces ferritin probably does not reflect differences in the total structure of the protein. Indeed, Margoliash has found that, despite strong structural and functional similarity between cytochrome c's from widely divergent sources, there may be almost no antigenic homology (e.g. cytochrome c from yeast and horse) (Margoliash, et al., 1967).

Phycomyces and horse ferritin differ in electrophoretic mobility in acrylamide gels. The Phycomyces protein migrates, at pH 9.5, behind the main monomeric band of horse ferritin at a position corresponding closely to that of the horse ferritin dimer. Although the electrophoresis data suggest that Phycomyces ferritin is a dimer at pH 9.5, the sedimentation data obtained at pH 6.0 appear incompatible with a dimer of molecules the size and density of ferritin. It is possible, however, that Phycomyces ferritin reversibly dimerizes at high pH. Unfortunately sedimentation data have not been obtained with Phycomyces ferritin at high pH.

This paper has demonstrated a net increase in the level of ferritin in Phycomyces when the fungus is grown on iron supplemented medium. This increase, demonstrated for both spph cytoplasm and spores, is approximately 30-50 fold. A similar increase has also been shown for mycelium in liquid culture (David, unpublished experiments). Increases in the level of ferritin following iron administration have been well documented in mammalian systems, particularly rat liver.(Fineberg and Greenberg, 1955; Loftfield and Eigner, 1958; Saddi and von der Decken, 1964, 1965; and Drysdale and Munro, 1965b, 1966). These authors have also shown that the increase in ferritin represents an increase in the net synthesis of the protein moiety of ferritin. Evidence for or against accelerated apoferritin synthesis in Phycomyces following iron addition to the growth medium has not been obtained. However the increase in the number of ferritin molecules (at least 20 fold in spores) is such that de novo protein synthesis seems necessary if the cell is not to maintain large excesses of apoferritin.

Before considering the function of ferritin in Phycomyces, its function in other organisms will be briefly mentioned. Because of its structure ferritin has been implicated as an iron storage molecule -- either storage for future biosynthetic purposes or storage for otherwise toxic iron concentrations in cytoplasm. Evidence for the latter function comes from the large accumulations of ferritin in the cells of animals and humans suffering from hemolytic anemias and pathological iron uptake syndromes. In such cases ferritin appears to sequester the excess iron which mammals are unable to excrete from the body. Ferritin

is also present in normal animals in liver, spleen, and bone marrow where it plays a badly defined role as an intermediate in the "iron cycle" between hemoglobin breakdown and resynthesis.

Although it is normally an iron storage protein, there are two examples of ferritin functioning in iron transport. In mammals Bessis and Breton-Gorius (1957) described ferritin transport between macrophages and erythroblasts by pinocytosis. In chitons, where ferritin is present in epithelial cells associated with radular tooth synthesis (Towe and Lowenstam, 1967), it has also been found in the blood stream and may function as the primary iron transport agent there (Nesson, personal communication).

In higher plants, both monocotyledons and dicotyledons, there is evidence which suggests that ferritin may function as an iron storage protein for iron required in the biosynthesis of the photosynthetic apparatus. In electron micrographs of meristematic and cambial tissue crystalline masses of ferritin are conspicuous in proplastids and absent in mature chloroplasts (Hyde, et al, 1963; Robards and Humpherson, 1967). However, there is not yet any biochemical evidence to substantiate a precursor-product relationship between plant ferritin and iron proteins in chloroplast grana.

Byanalogy with its role in higher plants and in mammals, ferritin in Phycomyces is believed to function as an iron storage compound. Several facts support this idea. (1) Compared to the distribution of dry weight between spph and spores, a disproportionately large amount of iron and ferritin is contained in spores. Between 65 and 80% of spph iron can be

recovered in spores. By comparison, spores contain only 30% of the spph dry weight. (2) The disproportionate concentration of iron and ferritin in spores is greater when iron is less available in the growth medium (Table 4). (3) The partition of iron between ferritin and the soluble iron pool in spph is about even (Figure 12) whereas in the mycelium soluble iron is present in 5-10 fold excess over ferritin (David, unpublished experiments).

These facts strongly suggest that ferritin is selectively incorporated first into spph and then into spores where it can function as an iron storage compound for biosynthetic steps requiring iron during the early stages of spore germination. It should be noted that the selective incorporation of ferritin into spores, particularly under conditions of low iron availability, is not the result expected if ferritin acted simply as a detoxifying agent for Fe^{+++}. Presumably Nature would not deposit garbage in the organ through which the species is being propagated.

In conclusion, mention should be made of the advantages of Phycomyces for studying the role of ferritin in iron metabolism. Unlike the mammalian systems, in which several organs are involved, Phycomyces has ferritin located inside a comparatively simple cell. Furthermore, Phycomyces ferritin is accumulated in spores where it accounts for at least one half of all the iron present and where its level can be regulated by the iron concentration in the growth medium. Starting with spores, the kinetics of iron mobilization from ferritin during germination and growth can be studied and correlated with the synthesis of iron-

containing products. Such studies have been undertaken and are reported in the second part of the thesis.

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CHAPTER 3

METABOLISM OF FERRITIN IRON IN GERMINATING SPORES

INTRODUCTION

In Chapter 2 it was postulated that ferritin in Phycomyces functions as a source of iron for biosynthesis during the early stages of mycelial growth following spore germination. This seemed to be a logical explanation for the observed localization in spores of a disproportionately large amount of spph iron and ferritin. The present chapter describes exploratory experiments performed to test this hypothesis indirectly, by investigating the fate of spore ferritin following germination.

Phycomyces spores represent a potentially very favorable system for such a study because: (1) spores can be prepared with differing ferritin contents over at least a 40 fold range; (2) at least 50% of spore iron is in ferritin, and, prior to germination, there is no soluble iron pool immediately available for biosynthesis; (3) the iron content of the germination medium can be varied; and (4) in a two step procedure Phycomyces ferritin can be sufficiently purified for isotope analysis. Thus, iron mobilization from ferritin can be studied as a function both of the intracellular ferritin content and of the extracellular iron concentration.

The role of ferritin in cellular iron metabolism has been previously studied in three systems: mammals, higher plants and the invertebrate chiton. Mammalian ferritin is located in liver, spleen, and bone marrow where it stores iron. Addition of iron to the mammalian circulatory system leads to its rapid incorporation into liver ferritin. Here iron is sequestered until it is transferred to bone marrow for hemoglobin

biosynthesis (Hahn, et al.,1943; Moore and Dubach, 1962). Mobilization of iron from liver ferritin is thought to require an oxidation-reduction system (Green and Mazur, 1957), however the mechanism of iron release is not clear.

In plants and chitons little is known about the role of ferritin in iron metabolism beyond electron microscopic observations of its intracellular location and its disappearance in conjunction with the synthesis of cellular components known to require iron (Hyde et al., 1963; Towe and Lowenstam, 1967). The situation in Phycomyces spores may be more analogous to higher plants and chitons than to mammalian liver where iron appears to cycle constantly into and out of ferritin.

The work described below demonstrates iron mobilization from spore ferritin and points the way toward a more detailed investigation into the control mechanisms involved. A two-step procedure is described for separation of ferritin from a soluble iron fraction which begins to appear in spores following germination. Using this procedure to follow ^{59}Fe in labeled spores containing two different levels of ferritin and iron, it is shown that in ferritin-poor spores the ferritin iron is almost completely mobilized in 24 hours whereas the ferritin-rich spores show no significant loss of ferritin iron even after 48 hours. Coincident with the loss of ferritin iron upon germination, soluble iron begins to appear inside the cell. These processes are not inhibited by high concentrations of iron in the germination medium.

MATERIALS AND METHODS

All materials and methods used in the following experiments were described in Chapter 2 except those listed below.

Preparation of ^{59}Fe-labeled spores

^{59}Fe-labeled spores were prepared at two different specific activities, 1.0 and 0.007 μc/ug. Heat shocked spores were diluted into sterile water (50 spores/ml) containing 1.67 μc/ml $^{59}FeCl_3$ at a specific activity 8.7 μc/μg. This spore suspension was distributed at 2 ml per plate to 30 petri dishes each containing about 30 ml solid GA medium and approximately 3.0 μg Fe as impurity. The specific activity was thus reduced to about 1.0 μc/μg. To obtain the lower specific activity 500 μg Fe was added with 2 ml of the spore suspension to each of 30 more plates. Thus, the spores in the first set were grown on minimal iron medium, the spores in the second set on high iron medium.

After 4.5 days the spph were harvested and ground in a mortar with dilute phosphate buffer. Spores were recovered from the lysate by low speed centrifugation followed by several washings in fresh buffer. The yield from 30 plates was 4-6 x 10^9 spores.

For germination studies spore preparations were sterilized by filtration on a 5 μ millipore filter with sterile phosphate buffer. This procedure, followed by a long heat shock (1-2 hours at 50°C), sterilized spore samples so that, upon dilution into GA medium, no bacterial growth occurred for at least 48 hours.

Germination of ^{59}Fe-labeled Spores

In germination experiments 2-4 x 10^8 spores (5-20 x 10^5 cpm) were diluted into 200 ml liquid GA medium in a 1 liter Erlenmeyer flask. The cultures were shaken on a reciprocating shaker at 22°C for 12, 24 and 48 hours. Using a heat shock, spore germination was quite synchronous and over 90% complete.

Preparation of Lysates from Spores and Germinated Spores

Germinated spores were harvested by centrifugation at 5000 rpm for 10 minutes (12 hour sample) or filtration in a Buchner funnel (24 and 48 hour samples). The harvested material was resuspended in 10-20 ml of 0.5 M phosphate buffer pH 6.0 for lysis. Ungerminated spores were also suspended in 0.5 M phosphate buffer for lysis.

Ungerminated spores and 12 hour samples were disrupted in a Nossal cell disintegrator (Nossal, 1953). Spore suspensions, mixed with 2 mm glass beads (7 ml spore solution per 15 g glass beads), were shaken in a stainless steel capsule on a reciprocating arm at 100 cps. Two minutes of such treatment (with intermittent cooling of the capsule in an ice bath) broke 70-90% of ungerminated spores and essentially all spores in the 12 hour sample. A French press was used to lyse 24 and 48 hour samples.

Purification of Ferritin - Butanol Extraction and Gel Filtration Chromatography

Lysates prepared in 0.5 M phosphate buffer were directly extracted

with butanol at 40°C (Chapter 2). Following centrifugation to separate the phases, the aqueous phase was recovered. This solution contained ^{59}Fe-ferritin and low molecular weight ^{59}Fe-labeled material. To determine the fraction of ^{59}Fe in ferritin, the two components were separated by gel filtration chromatography.

Bio-Gel A 1.5 M (Agarose) was obtained from BioRad Corp.. Columns were prepared by slurrying the hydrated agarose beads in elution buffer (0.05 M phosphate buffer pH 6.0, 0.15 M NaCl) and gradually filling a column at a slow rate of flow. Columns 1.2 cm x 50 cm were prepared in the cold. Samples of 1.0 ml or less were loaded in the top of the column and washed in with eluting buffer. Elution was carried out at flow rates of 10-20 ml/hr. Generally fifty 2.5 ml fractions were collected automatically with a Gilson Fraction Collector. Aliquots (1.0 ml) of each fraction were assayed for ^{59}Fe by scintillation counting in Bray's solution. Fractions near the void volume were diluted and assayed for MS2 when this bacteriophage was present as an excluded marker. (The ^{59}Fe assay and the bacteriophage assay were described in Chapter 2.) Under these conditions the excluded marker was found in fraction 14, the ferritin peak in fractions 16-18, and the included "soluble iron" peak in fraction 29-30. Recovery from the column was quantitative and the column could be used repeatedly.

RESULTS

Identification of Ferritin and Soluble Iron on Agarose Gel Filtration Column

In n-butanol extracted lysates of ^{59}Fe-labeled spores, ferritin is the only ^{59}Fe-labeled component in the aqueous phase (Chapter 2). Gel filtration of such extracts on Agarose 1.5 M gave the elution pattern shown in Figure 1a and 2a. Phycomyces ferritin eluted as a broad peak of ^{59}Fe-labeled material partially included by the gel. Figure 1b and 2b show the position of a void column marker (bacteriophage MS2) which was excluded by the gel and eluted as a very sharp peak just ahead of ferritin.

Following germination of ^{59}Fe-labeled spores, a second radioactive component appeared in the aqueous phase. It was entirely included on the Agarose 1.5 M column (Figure 1b,c). This material was tentatively identified as soluble iron because: (1) it was slowly dialyzable; (2) it was not precipitated by n-butanol extraction or 5% trichloroacetic acid; and (3) it did not sediment from the meniscus in sucrose gradients centrifuged for two hours at 37,000 rpm. It can not be the Fe^{+++} ion, since that iron is very insoluble at neutral pH, but it could be Fe^{+++} bound in a low molecular weight chelate.

Ferritin Content of Iron-Rich and Iron-Poor Spores; Yield of Ferritin from Butanol Extraction

Table I gives the iron content, calculated from the specific activity,

TABLE I

Iron Content of Iron-rich and Iron-poor Spores for Germination Experiment

	Specific Activity μc/μg	counts/min per spore	μg Fe/spore	Fe atoms/spore
Iron-rich spores	0.007	2.3×10^{-3}	160×10^{-9}	170×10^{7}
Iron-poor spores	1.0	7.7×10^{-3}	3.5×10^{-9}	3.8×10^{7}

Spores were prepared from Phycomyces grown on GA medium supplemented with ^{59}Fe at the specific activities given. Spores were counted in a Petroff-Hauser bacteria counter and assayed for ^{59}Fe.

of the iron-rich and iron-poor spores used in this experiment. They differ 46 fold in iron content.

In Chapter 2 it was shown that 40-50% of the ^{59}Fe could be extracted from lysates of iron-rich or iron-poor spores, and that all of this extractable iron was in the form of ferritin. Table II confirms this result. Thus, at least part of the additional iron in iron-rich spores is in ferritin. A short calculation shows that this extra ferritin iron is in <u>additional</u> molecules. The S-values of ferritin from iron-rich and iron-poor spores are 67S and 54S indicating about 25% difference in iron content (Chapter 2). Since iron-rich spores contain about 60X more ferritin iron than iron-poor spores, they must contain 45X more ferritin molecules.

It is necessary to consider carefully the yield of ferritin in the n-butanol extraction procedure since the fraction of spore iron in ferritin is critical to the conclusions to be drawn from this experiment. In control experiments the yield of purified ^{59}Fe-ferritin added to unlabeled lysates before n-butanol extraction indicated that the procedure was about 90% efficient in extracting ferritin. However, this was not a reliable measure of the efficiency of extraction since the added ferritin was probably not bound to lipid particles in the same way as it is normally in lysates.

Attempts to deduce yields by comparing the amount of extractable ferritin in ferritin-rich and ferritin-poor spores depend on assumptions about the proportions of ferritin in the unextractable iron in such samples. The experimental data did, however, provide an approximate

TABLE II

Distribution of ^{59}Fe in Iron-rich and Iron-poor Spores Following Germination

I-R iron-rich spores

I-P iron-poor spores

The counts/min in the lysates of ungerminated spores have been corrected for unbroken spores which pellet following centrifugation of the n-butanol extraction mixture.

The sum of the counts/min in the Aqueous Phase and Interface are about 80% of the counts/min in the Lysate. This is a systematic error which is probably attributable to the low counting efficiency of the precipitated material in the Interface.

Counts/min in Ferritin (B) are the sum of the counts/min in Fractions 14-25 of the agarose column divided by the total counts/min eluted from the column. Thus, ungerminated spores contain only 90% ferritin since 10% of the ferritin counts/min tail into the soluble iron region of the column (Figure 1a,2a).

TABLE II

Distribution of ^{59}Fe in Iron-rich and Iron-poor Spores Following Germination

	Ungerminated Spores		12 hr. Germination		24 hr. Germination		48 hr. Germination	
	I-R	I-P	I-R	I-P	I-R	I-P	I-R	I-P
Counts/min x 10^{-5} Lysate	3.4	11.1	2.9	2.7	3.3	5.9	3.1	8.2
Counts/min x 10^{-5} Aqueous Phase	1.6	4.1	1.5	1.3	2.3	4.0	1.9	4.9
Counts/min x 10^{-5} Interface	1.1	4.1	1.1	0.8	0.3	0.7	0.5	1.9
(A) Counts/min in Aqueous Phase (as percent of Lysate)	47	37	50	49	69	68	63	60
(B) Counts/min in Ferritin Peak on Agarose Column (as percent of Aqueous Phase)	90	87	90	75	80	17	82	25
Extractable Ferritin (as Percent of Lysate Equal to A x B)	42	32	45	37	55	12	52	15

answer. In Table II the recovery of ^{59}Fe in the aqueous phase following n-butanol extraction rises with samples taken at longer times after germination. This rise is independent of the nature of the ^{59}Fe component in the aqueous phase since in iron-rich samples the rise is due to increased extraction of ferritin and in iron-poor samples it is due to increased extraction of soluble iron. Thus, it can be concluded that extraction of spores does not yield all the ferritin present in spores. However, it is not possible to determine exactly what fraction of spore iron is ferritin and what fraction is another iron-containing component (e.g. iron bound to spore walls).

Fate of Ferritin Iron in Germinating Spores

Using the n-butanol extraction procedure followed by gel filtration, the fate of ^{59}Fe-ferritin has been studied after germination of ferritin-rich and ferritin-poor spores. The results are shown in Figures 1 and 2. Qualitatively there was a marked difference in ferritin iron metabolism between the two kinds of spores. In ferritin-poor spores the extractable ferritin was almost entirely gone by 24 hours. By comparison, ferritin-rich spores contained at 24 hours more extractable ferritin than ungerminated spores. The gel filtration results at 48 hours were essentially identical to the 24 hour samples.

Coincident with the loss of ^{59}Fe from the ferritin peak was the appearance of ^{59}Fe at the position of soluble iron on the column. This material appeared to arise by transfer of ^{59}Fe from ferritin. However, because of the enhanced recovery of radioactivity in the aqueous phase at

FIGURE 1. Distribution of ^{59}Fe Between Ferritin and Soluble Iron in Ungerminated and Germinating Iron-Poor Spores.

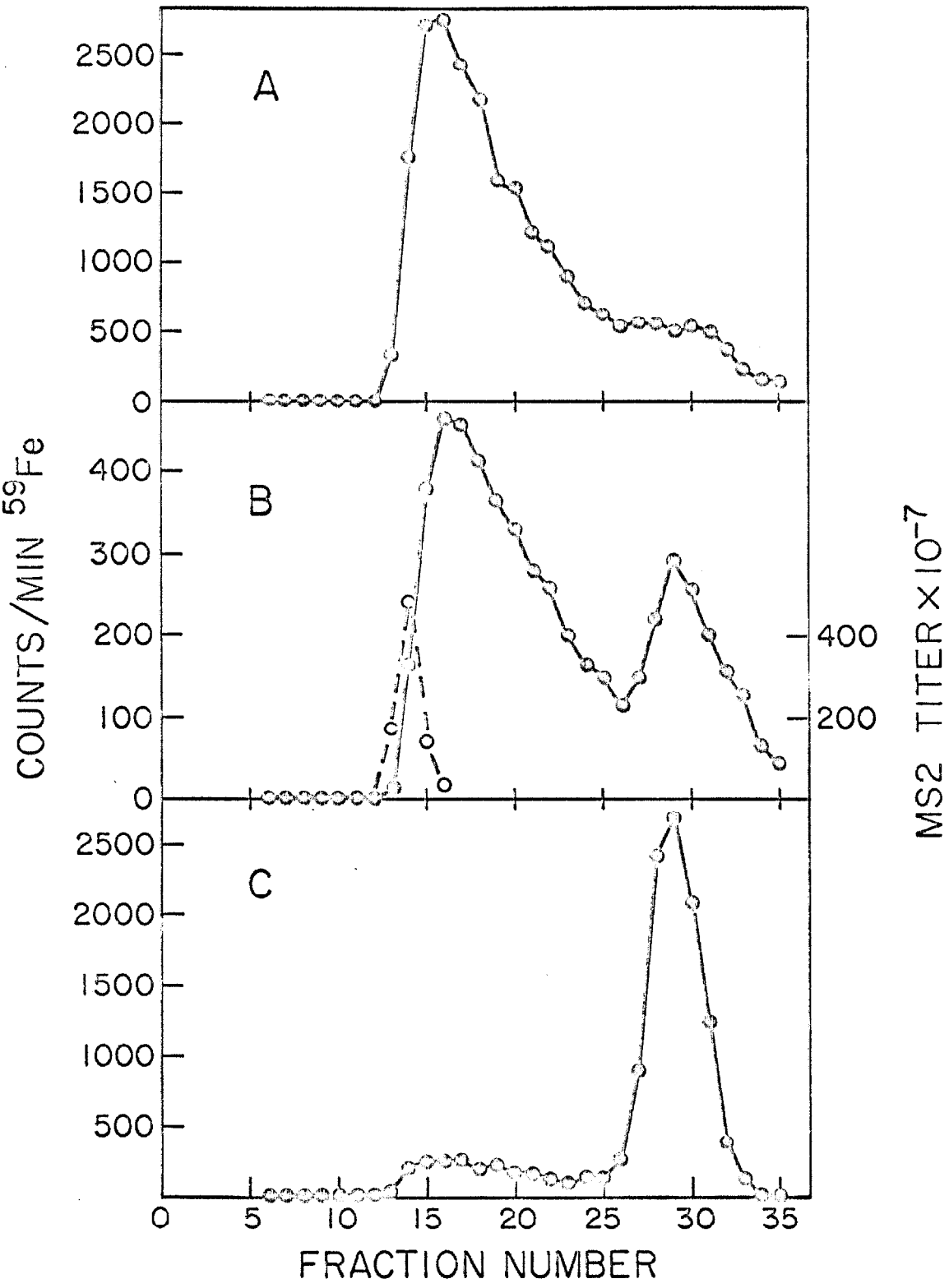
After germination for 12 and 24 hours, lysates were prepared and extracted with n-butanol. The aqueous phase was fractionated by gel filtration on an Agarose 1.5 M column (1.2 x 50 cm). Elution was carried out with 0.05 M phosphate buffer (pH 6.0) containing 0.1 M NaCl. Fractions (2.5 ml) were collected and assayed for ^{59}Fe.

Ungerminated spores were similarly extracted and analyzed by gel filtration.

(A) Ungerminated spores

(B) 12 hour germination

(C) 24 hour germination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COUNTS/MIN ^{59}Fe
MS2 TITER X 10^{-7}
FRACTION NUMBER

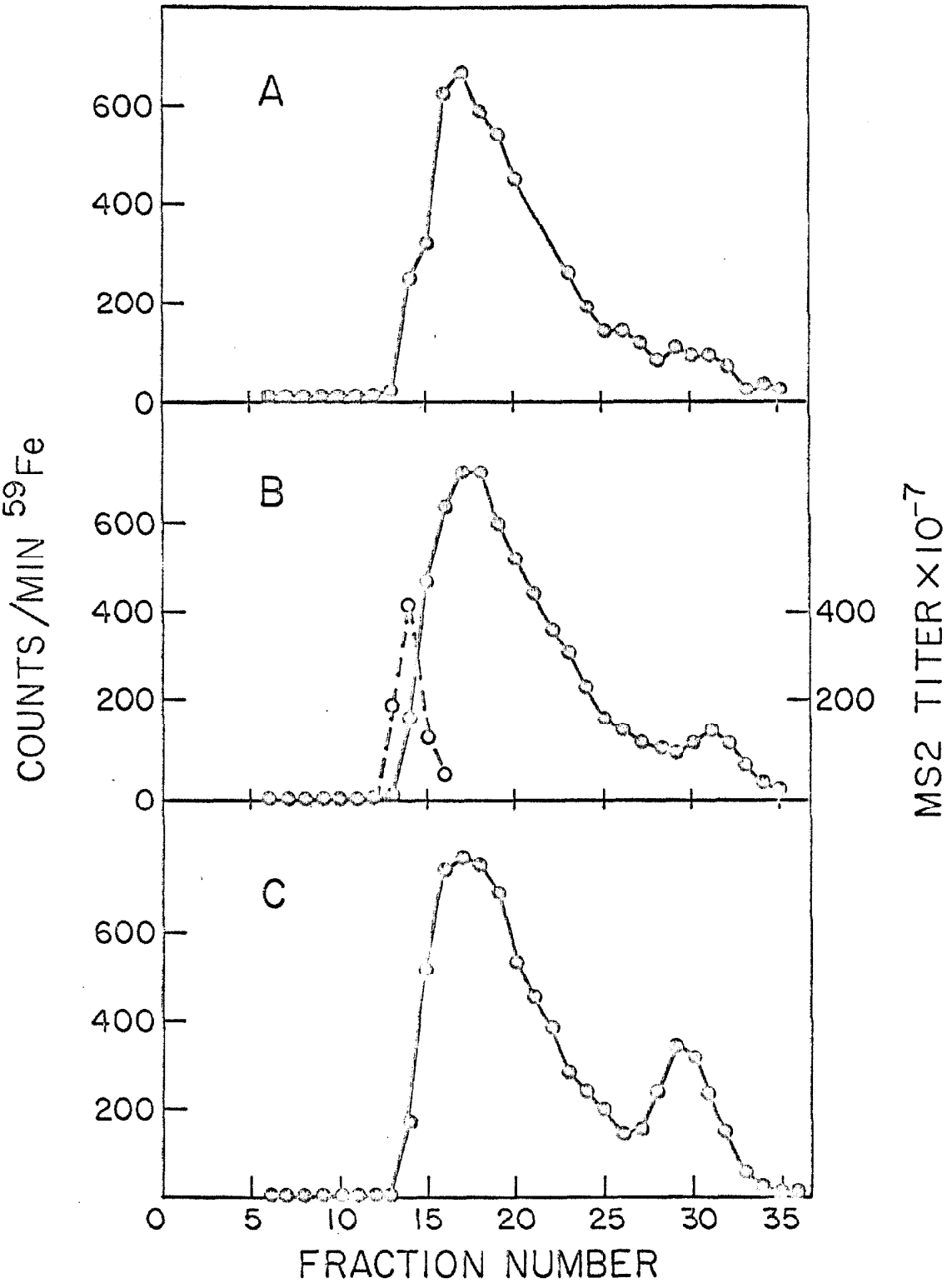
FIGURE 2. Distribution of ^{59}Fe Between Ferritin and Soluble Iron in Ungerminated and Germinating Iron-Rich Spores

For methods, see Figure 1.

(A) Ungerminated spores

(B) 12 hour germination

(C) 24 hour germination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longer times after germination, it was not possible to determine quantitatively whether ferritin iron was the precursor to soluble iron.

The quantitative data for the germination experiments are presented in Table II. In order to compare different experiments, the results have been expressed as percentages. They are the same as in Figures 1 and 2 but indicate more precisely what percentage of the total ^{59}Fe is accounted for. There is a significant increase in the extractable ^{59}Fe in the 24 and 48 hour samples. In the iron-rich spores this has the effect of increasing the absolute amount of ferritin iron extracted although the proportion of ferritin iron in the aqueous phase is decreased. In the case of iron-poor spores the effect of improved extractability is to reduce the apparent loss of ^{59}Fe from ferritin.

Effect of Extracellular Iron on Ferritin Iron Metabolism

The effect of external iron on ferritin iron mobilization was tested by germinating iron-poor spores in GA medium containing 5 μg/ml Fe (as $FeCl_3$). The germination was carried out for 12 hours at which point the aqueous phase contained about a 3:1 ratio of ferritin to soluble iron (Table II). No difference could be observed in the amounts of label in the ferritin and soluble iron components of the aqueous phases between spores germinated in the iron-supplemented GA medium and spores germinated in unsupplemented GA medium.

To be certain that the supplemental iron in the medium was being taken up, unlabeled spores were germinated in GA medium containing ^{59}Fe (approximate specific activity 1 μc/μg). Over 95% of the ^{59}Fe was bound to spores in 24 hours (L. Brown, unpublished experiments, 1966).

DISCUSSION

The experiments in this paper strongly suggest, but do not prove, that iron is mobilized from ferritin in germinating spores of Phycomyces and that the initial product is probably soluble iron. Rigorous demonstration of a precursor-product relationship between spore ferritin and soluble iron has not been achieved because: (1) not all the iron present in spores was extracted and accounted for, and (2) an increasing amount of spore iron became extractable by our procedure at longer times after germination. Thus, it is formally possible to argue that ferritin is becoming unextractable in germinating iron-poor spores and that the soluble iron is being derived from the unextractable iron present in spores. However, reference to the control experiment with iron-rich spores makes this an unlikely possibility since in this case ferritin continues to be extractable after germination. Thus, the simplest explanation — that ferritin iron is becoming soluble iron — is the most satisfactory. Soluble iron derived from ferritin is presumably the cytoplasmic pool from which iron is drawn for biosynthesis of iron proteins. Unextractable iron may be iron bound to protein as heme or non-heme iron; it may also include some ferritin.

The germination experiments show that the extent of iron mobilization is not proportional to the ferritin content of spores. In spores containing "limiting" amounts of ferritin, the mobilization of ferritin iron is rapid and continues almost to completion in 24 hours. By comparison, in spores containing large amounts of ferritin, there is

even an increase in the amount of extractable ferritin during 48 hours of growth. We believe that this increase does not represent synthesis of new ferritin but increased extractability.

The amount of iron in the soluble iron pool increases sharply with the extent to which the spores were loaded with iron. The iron-rich and iron-poor samples at 24 (and at 48) hours after germination in iron-poor medium contain approximately the same amount of tissue wet weight. From the counts per minute in ferritin (Table 2) and the specific activities of the labeled spores (Table 1) it is possible to calculate the absolute amount of soluble iron derived from iron of the spores. The iron-rich sample contains 10-20X more soluble iron that the iron-poor sample. The origin of this soluble iron derived from spore iron is uncertain.

The appearance of soluble iron following germination of iron-poor spores was not blocked by addition of iron to the germination medium. This finding may indicate that mobilization of a certain fraction of spore iron is intimately associated with the germination process and is independent of mycelial iron metabolism.

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