MICROBIAL IRON METABOLISM

A COMPREHENSIVE TREATISE

Edited by J. B. Neilands

DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA

ACADEMIC PRESS New York and London 1974
A Subsidiary of Harcourt Brace Jovanovich, Publishers

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Chapter 7

FERRITIN AND IRON METABOLISM IN *PHYCOMYCES*

CHARLES N. DAVID

I. INTRODUCTION

The iron protein ferritin has recently been isolated from the fungus *Phycomyces blakesleeanus* (David, 1968; David and Easterbrook, 1971). Ferritin is a large molecule (MW ~ 900,000) consisting of a shell of protein subunits surrounding a massive core of iron. The iron content of individual molecules can vary from no iron (apoferritin) to about 4000 atoms in a full core. The sedimentation coefficient varies correspondingly from 18 to 67 S (Fischbach and Anderegg, 1965). Although it has long been characterized in mammalian tissues (Granick, 1946; Harrison, 1964; Crichton, 1973a) and more recently in plant chloroplasts (Hyde *et al.*, 1963), its occurrence in microorganisms was previously unknown. The present contribution summarizes briefly the characteristics of ferritin from *Phycomyces* and presents in some detail a series of unpublished experiments on the synthesis and degradation of ferritin and its role in iron metabolism.

II. PURIFICATION AND PROPERTIES OF *PHYCOMYCES* FERRITIN

Ferritin in *Phycomyces* was initially identified in electron micrographs of thin sections of sporangiophores (Fig. 1; Sassen, 1965; Peat and Banbury, 1968; David and Easterbrook, 1971). The molecules are arranged in a two-dimensional crystalline pattern on the surface of lipid droplets in cytoplasm. The lipid droplets bearing ferritin monolayers can be isolated in the lipid pellicle following centrifugation of cell homogenates. Subsequent treatment of the lipid with detergent or *n*-butanol releases ferritin.

David and Easterbook (1971) purified and characterized ferritin from *Phycomyces*. Extraction of cell homogenates with *n*-butanol effectively solubilizes ferritin from lipid and precipitates most nonferritin cellular protein. *Phycomyces* ferritin can be purified from the aqueous phase following butanol extraction by isoelectric precipitation at pH 5.0 and isopycnic banding in CsCl density gradients. Purified ferritin from *Phycomcyes* grown on iron-supplemented medium sediments as a single molecular species at 55 S and yields one band in gel electrophoresis. Removal of iron by reduction with $Na_2S_2O_4$ yields apoferritin which sediments at 18 S. Disruption of ferritin with sodium dodecyl sulfate yields protein subunits of molecular weight 18,500 (R.R. Crichton, personal communication).

The characteristic structure of mammalian and plant ferritin revealed in the electron microscope by negative staining is also shown by *Phycomyces* ferritin

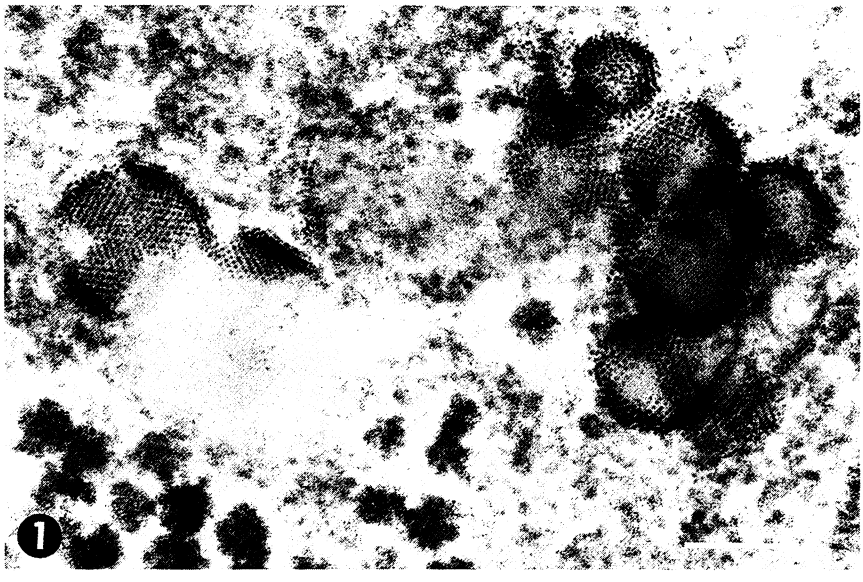

Fig. 1 Thin section of *Phycomyces* sporangiophore showing lipid droplets covered with crystalline arrays of ferritin molecules. Sporangiophore fixed with gluteraldehyde and osmium tetroxide. × 83,000. Bar = 0.2 μm.

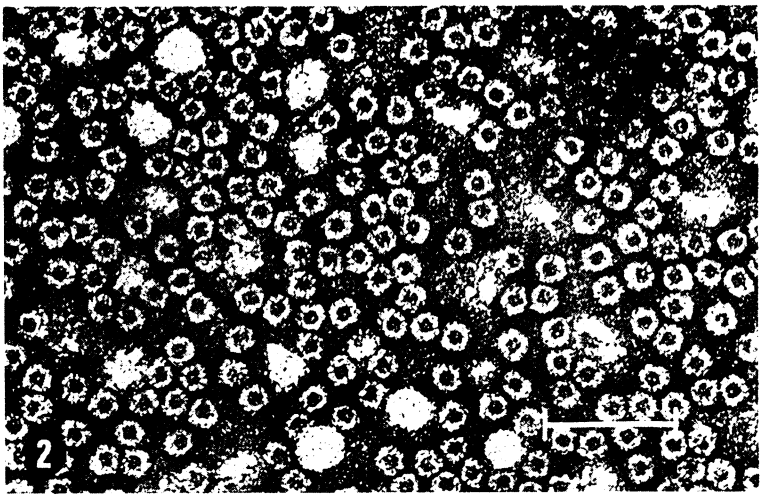

Fig. 2 Purified *Phycomyces* ferritin negatively stained with uranyl acetate. The electron-opaque iron core is surrounded by an electron-lucent protein shell. × 340,000. Bar = 500 Å.

(Fig. 2). The electron-opaque iron core (50–60 Å in diameter) is surrounded by an electron-lucent annulus of protein (105 Å diameter). Although similar in structure to plant and animal ferritins, *Phycomyces* ferritin contains maximally about one-half as much iron per unit protein and sediments, at 55 S, more slowly than horse ferritin.

III. INDUCTION OF FERRITIN SYNTHESIS BY IRON

Growth of *Phycomyces* on medium supplemented with iron causes a marked increase in the level of iron in mycelium, sporangiophores, and spores. Figure 3 shows the iron content of spores with increasing iron in the growth medium. The additional iron is present in ferritin molecules (Fig. 6). The fiftyfold increase in ferritin iron is probably too great to be accounted for simply by addition of iron to unfilled ferritin molecules. Thus, the results suggest that *de novo* synthesis of apoferritin has occurred similar to the induction of apoferritin which has been shown in animal tissues in response to iron administration (Fineberg and Greenberg, 1955; Loftfield and Eigner, 1958; Saddi and von der Decken, 1965; Drysdale and Munro, 1966).

Ferritin isolated from iron-supplemented growth medium (15 μg/ml iron) has a uniformly high S value (see above) and contains a full complement of iron. Ferritin isolated from *Phycomyces* grown on iron-poor medium (0.1 μg/ml iron) is more heterogeneous and has a somewhat lower S value (40–50 S) indicating less iron per molecule.

The ferritin content of *Phycomyces* grown on limiting amounts of iron has not been investigated. The level of 0.1 μg/ml iron present as impurities in growth medium is somewhat more than the minimal requirement since normal mycelial growth and sporangiophore development occur. Media from which iron has been

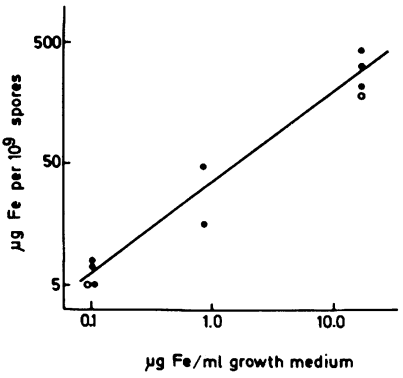

Fig. 3 Iron content of *Phycomyces* spores in cultures supplemented with iron. Phycomyces was grown on glucose–asparagine medium containing defined amounts of iron and ^{59}Fe. Sporangiophores were harvested and spores isolated. The iron content per spore was calculated from the radioactivity per spore and the specific activity of ^{59}Fe in the medium. Each point represents an independent experiment. The two open circles show the results of direct chemical determination of iron spore (see David and Easterbrook, 1971, for details of iron determinations).

At least 50% of the spore iron in all samples is in ferritin molecules (see Fig. 6). Similar increases in iron and ferritin content occur in mycelium and sporangiophores when the growth medium is supplemented with iron.

completely removed support almost no mycelial growth and do not permit sporangiophore development (Odegard, 1952). Under such conditions spores containing more ferritin iron grow better than spores with less ferritin iron but the level of growth on iron supplemented medium is not attained (C. N. David, unpublished).

IV. FERRITIN SYNTHESIS AND LOCALIZATION IN SPORES

Ferritin can be isolated from all developmental stages of *Phycomyces*—mycelium, sporangiophores, and spores. In all cases it appears to be associated with lipid droplets. During the course of spore formation, sporangiophore ferritin is selectively incorporated into spores. In cultures grown on iron-poor medium, 80% of the total sporangiophore ferritin is found in spores and 20% remains in sporangiophore cytoplasm after spore formation. Dry weight is partitioned approximately equally between spores and sporangiophores. On iron-supplemented medium, where the level of ferritin is about fiftyfold higher, the partitioning of ferritin between spores and sporangiophore cytoplasm more nearly approaches the partitioning of dry weight.

Sporangiophore development is dependent on mycelial growth. Many of the components of sporangiophore cytoplasm and spores, e.g., nuclei, polyphosphates, and lipid droplets are taken up directly from the mycelium and are not synthesized in the elongating sporangiophore (Bergman *et al.*, 1969; Galle, 1964). Some of these components are in fact synthesized in the mycelium prior to the initiation of sporangiophore outgrowth. Ferritin is synthesized in the mycelium and per-

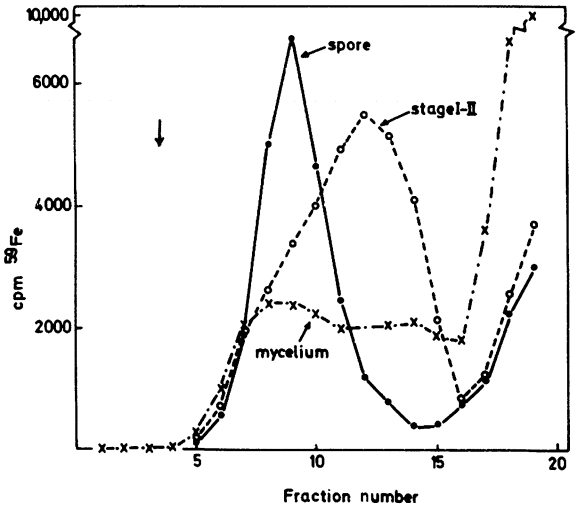

Fig. 4 Sucrose gradient sedimentation of ^{59}Fe-labeled ferritin from mycelium, Stage I–II sporangiophores, and spores. *Phycomyces* was cultured on glucose–asparagine medium (0.1 μg/ml iron) with ^{59}Fe. Mycelium was harvested immediately prior to initiation of sporangiophores; stage I–II sporangiophores were harvested from a parallel culture 8 hours later; spores were prepared from a third parallel culture 1 day later. All three samples were homogenized with a Nossal disintegrator and extracted with *n*-butanol. Aliquots of the aqueous phase were sedimented in 5 to 20% sucrose gradients (37,000 rpm, 2.5 hours, 6°C). Fractions were collected and assayed for ^{59}Fe. Sedimentation is from right to left. The arrow indicates the position of a sedimentation marker at 81 S. The results of all three gradients have been superimposed for comparison.

haps during the early stages of sporangiophore outgrowth. This ferritin has a broad spectrum of S values (iron contents) in cultures grown on 0.1 μg/ml iron (Fig. 4). During sporangiophore initiation a large population of ferritin molecules of intermediate iron content is concentrated in the cytoplasm of young (Stage I) sporangiophores. Eighty percent of this ferritin is destined to be incorporated into spores. In conjunction with spore formation at the end of Stage I, ferritin undergoes a maturation process. Ferritin molecules of intermediate S value become more homogeneous and acquire a higher S value (Fig. 4). A similar addition of iron to mammalian ferritin has been shown *in vivo* (Drysdale and Munro, 1966) and *in vitro* (Macara *et al.* 1972).

V. FERRITIN AND IRON METABOLISM IN GERMINATING SPORES

The selective incorporation of iron and ferritin into spores of *Phycomyces* grown on iron-poor medium suggests that ferritin iron is a storage form of iron destined to be used for biosynthesis following spore germination. In the following it will be demonstrated that ferritin in germinating spores releases iron to a soluble pool and that the release of iron from ferritin is controlled by the cell. The results suggest further that the soluble iron pool is involved in biosynthesis.

A. The Soluble Iron Pool

Phycomyces mycelium, sporangiophores, and germinating spores have a pool of soluble iron. Since ferric iron is quite insoluble at cellular pH, this "soluble" iron is probably complexed with a low-molecular weight substance. Nothing is known of the nature of the chelator involved. Chelators of the sideramine type (Neilands, 1957) are not excreted by *Phycomyces* (Müller, 1968). However, no attempt has been made to identify sideramines in cellular homogenates containing soluble iron.

The soluble iron pool in *Phycomyces* has the following characteristics: (1) it is slowly dialyzable; (2) it is not precipitated by 5% trichloroacetic acid or by *n*-butanol extraction of cell homogenates; and (3) it does not sediment during ultracentrifugation at 100,000 *g* for 3 hours. Soluble iron is present in biosynthetically active parts of *Phycomyces,* namely, in growing mycelium, sporangiophores, and germinating spores. It is not present in dormant spores although the spores contain ferritin and other elements of sporangiophore cytoplasm. This result suggests that soluble iron may be an intermediate or donor of iron for macromolecular biosynthesis.

B. Release of Ferritin Iron During Germination

The availability of spores with varying ferritin contents and containing no soluble iron pool provides an experimental basis for studying the release of iron from ferritin associated with the initiation of biosynthetic activities following spore germination. *Phycomyces* spores labeled with ^{59}Fe and containing two different levels of iron—6×10^{-9} and 3×10^{-7} μg/ spore—were obtained (Fig. 3). At least 50% of this iron was extractable as ferritin in both cases (Fig. 6). The fate of ferritin iron during germination and early mycelial growth was investigated. Butanol extraction of cell homogenates precipitates most cellular proteins leaving ferritin and soluble iron as the only major ^{59}Fe-containing components in the aqueous phase. Gel filtration of this material clearly separates ferritin and soluble iron.

Heat-shocked *Phycomyces* spores germinate rapidly and synchronously at 20°C in glucose-asparagine medium. The spores first swell and then build a large central vacuole. By 12 hours all spores are vacuolated and by 16 hours most have developed young germ tubes which grow out to become mycelium.

Ungerminated spores contain only iron extractable as ferritin. Following germination of ferritin-poor spores, extractable ferritin decreases and disappears almost entirely by 24 hours (Fig. 5). By comparison ferritin-rich spores show no detectable loss of ferritin. Figure 6 shows the quantitative results from several independent experiments. There is a rapid loss of ferritin between 12 and 20 hours after germination in ferritin-poor spores. Under the same conditions ferritin-rich spores show a slight increase in ferritin due to more efficient extraction of growing mycelium compared to spores. In the case of ferritin-poor spores the increased efficiency of extraction probably hides the initial stages of ferritin degradation since at 12 hours ^{59}Fe has already started to appear in the soluble pool.

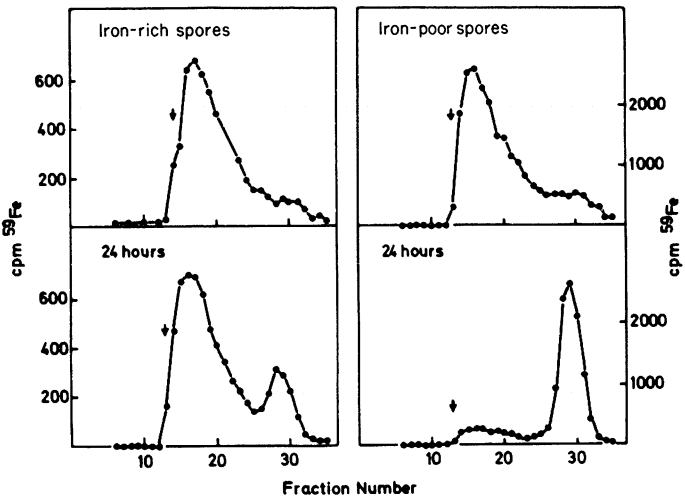

Fig. 5 Fate of ferritin iron during germination of ^{59}Fe-labeled spores. Spores containing two different levels of ferritin iron and ^{59}Fe were prepared from iron-poor (0.1 μg/ml) and iron-rich (15 μg/ml) cultures (see Fig. 3 for details). The labeled spores were heat-shocked and germinated in glucose–asparagine medium (10^6 spores/ml) containing about 0.1 μg/ml iron. Samples were taken at 0 and 24 hours, homogenized in a Nossal disintegrator, and extracted with *n*-butanol. The aqueous phase was analyzed by gel filtration on an agarose column (Bio Gel A 1.5 *M*; eluted with 0.05 *M* phosphate buffer, pH 6.0, 0.15 *M* NaCl). The excluded volume is indicated by the arrow. Ferritin is partially included and elutes about fraction 16. Low-molecular weight soluble iron is completely included and elutes about fraction 29.

Coincident with the loss of ^{59}Fe from ferritin in germinating ferritin-poor spores, is the appearance of ^{59}Fe at the position of soluble iron on the gel filtration column. Soluble ^{59}Fe begins to appear at 12 hours and increases rapidly up to 24 hours when most of ^{59}Fe-ferritin has been degraded (Fig. 5). Following germination of iron-rich spores, ^{59}Fe appears in the soluble pool by 24 hours. A coincident decrease in the level of ferritin can not be demonstrated because of the increasing efficiency of extraction of germinated tissue compared to spores. Calculating from the specific activity of ^{59}Fe in the germinating spores, the pools of soluble iron at 24 hours were: 0.04 μg/100 mg wet weight for germinated iron-poor spores and 0.3 μg/100 mg wet weight for germinated iron-rich spores. The levels of ferritin iron remaining in the same tissue were 0.005 and 1.2 μg/100 mg, respectively, for ferritin-poor and ferritin-rich tissue.

The results show that (1) release of iron from ferritin occurs upon spore germination and (2) the extent of this release is controlled by the cytoplasm of the germinating spores. When the cytoplasm is saturated with iron, further release of ferritin iron is blocked. Emery (1971) and Winkelmann and Zähner (1973) have shown a similar saturation of cytoplasmic sites by sideramine iron taken into cells of *Neurospora* and *Ustilago* from the external medium. Above a defined intracellular concentration, the uptake of sideramine iron is blocked. Both results strongly suggest that control mechanisms regulate the level of cytoplasmic iron acquired either from an intracellular iron-storage molecule or from an extracellular source.

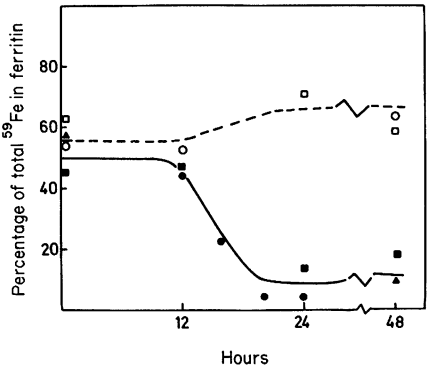

Fig. 6 Quantitative changes in ferritin iron during germination of ferritin-rich and ferritin-poor spores. The figure summarizes the results from three independent experiments of the type shown in Fig. 5. The ^{59}Fe extractable as ferritin is expressed as the percentage of total ^{59}Fe in the sample (% ^{59}Fe in ferritin peak after gel filtration of the aqueous phase × % of total ^{59}Fe in the aqueous phase; see David, 1968). Filled symbols, ferritin-poor spores; open symbols, ferritin-rich spores.

C. Mechanism of Iron Release from Ferritin

The release of iron from ferritin during germination of ferritin-poor spores provides an opportunity to study the mechanism involved. In particular, by measuring the S value (iron content) of the ferritin as a function of the amount of iron released, it is possible to determine if all molecules in the population are losing iron equally or if release is an "all-or-none" reaction in individual molecules. Figure 7 shows the sedimentation properties of the ferritin remaining after various degrees of iron release during the germination of ferritin-poor spores. During the rapid loss of ferritin iron no ^{59}Fe-labeled ferritin molecules appear which sediment more slowly than spore ferritin. Thus, the release of iron from ferritin appears to occur in an all-or-none reaction.

This result eliminates simple models of iron release based on chelation (Pape *et al.*, 1968) or reduction (Bielig and Bayer, 1955) in which all ferritin molecules are attacked statistically. These mechanisms would only yield the all-or-none result if the process in individual molecules were highly cooperative such that nucleation was the rate-limiting step or if the cytoplasm were compartmentalized into local regions where ferritin was being degraded and other regions where ferritin was protected. In either case, the release of iron from individual molecules must be a rapid process.

A more tempting explanation, in view of the all-or-none result, is the enzymatic degradation of ferritin—ferritin molecules being the substrate for an enzyme which releases iron from the core. Recently, evidence for such an enzymatic activity has been presented (Osaki and Sirivech, 1971) and Crichton (1973b) has suggested a mechanism, involving loss of protein subunits from the shell, by which the degrading enzyme could gain access to the iron core. It remains to be determined, however, if this enzymatic activity, in fact, causes all-or-none release of iron. Furthermore, the enzyme must be subject to feedback inhibition by cytoplasmic iron since its activity is clearly limited under conditions prevailing in germinating ferritin-rich spores.

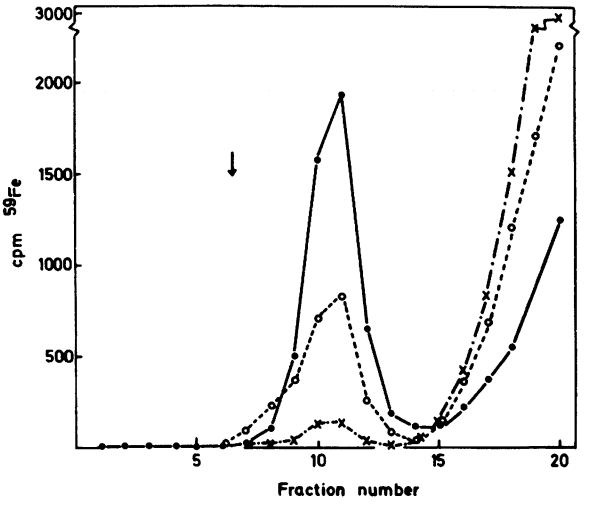

Fig. 7 Sucrose gradient sedimentation of ^{59}Fe-labeled ferritin from germinating ferritin-poor spores. Samples of the aqueous phase from 12 (●—●)-, 16 (○—○)-, and 24 (×—×)-hour germinated ferritin-poor spores (Fig. 6) were sedimented in 5 to 20% sucrose gradients (37,000 rpm, 2 hours, 6°C). Fractions were collected and assayed for ^{59}Fe. Sedimentation is from right to left. The arrow indicates the position of a sedimentation marker at 81 S. The results from the three gradients have been superimposed to facilitate comparison.

If the all-or-none result is indicative of the general mechanism of iron release from *Phycomyces* ferritin—not just a special mechanism associated with spore germination—then it must be concluded that ferritin molecules having intermediate S values are the product of iron accumulation rather than the result of random release and addition of iron. Drysdale and Munro (1966) have demonstrated in rat liver that the synthesis of ferritin molecules occurs by slow accumulation of iron. Molecules of intermediate iron content are precursors to molecules of higher iron content. The changes in sedimentation properties of ferritin during spore formation in *Phycomyces* (Fig. 4) also suggest that molecules of intermediate iron content are precursors which take up more iron to form the final product—ferritin in spores.

VI. SUMMARY

The iron-storage protein ferritin has been isolated from the fungus *Phycomyces* thus extending the range of occurrence of ferritin to microorganisms. *Phycomyces* ferritin is closely similar in structure to plant and animal ferritins and its synthesis is also stimulated by supplemental iron in the growth medium.

Ferritin iron is concentrated in *Phycomyces* spores. During spore germination the ferritin iron is released to a soluble iron pool which appears to be involved in biosynthetic processes utilizing iron. The amount of ferritin iron released is limited by the cell even in the presence of excess ferritin. The nature of this feedback control is not known.

In *Phycomyces* the uptake and release of iron by ferritin appear to occur by different mechanisms. The release of iron during spore germination is an all-or-none process. By comparison, synthesis of full ferritin which occurs during spore formation involves slow accumulation of iron thus giving rise to molecules of intermediate S value and iron content.

ACKNOWLEDGMENTS

I wish to thank Dr. E. W. Goodell and Dr. G. Winkelmann for their useful discussions during the preparation of this manuscript. The research was supported by National Science Foundation Grant GB-4642 and U.S. Public Health Service Grant GM-00086.

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