# SOME ASPECTS OF MICROBIAL IRON METABOLISM

J. B. NEILANDS

*Department of Biochemistry, University of California, Berkeley, California*

## INTRODUCTION

In recent years it has become evident that the total iron content of the animal organism is greater than the sum of the iron found in all of the known iron compounds (1). The nature of this remaining iron is unknown although in part it may be accounted for by the "non-heme iron" which Green and Beinert (2) have discovered as components of various respiratory enzymes. It is apparent that, eventually, all of the iron compounds of living cells will have to be identified before a total picture of iron metabolism and function can be established. Since all living cells are faced with more or less similar metabolic problems it should be possible to use almost any species as a subject for the study of unknown iron compounds. On the other hand, as Kluyver and van Niel have pointed out in their elegant little book *The Microbe's Contribution to Biology* (3), there are certain advantages in the selection of microorganisms as subjects for the examination of metabolic processes. Thus it is possible that an organism could be found with an exaggerated iron metabolism. After studying this species it might further be possible to apply the results to the general problem of iron metabolism in plants and animals.

## EXPERIMENTS WITH *USTILAGO SPHAEROGENA*

*Ustilago cytochrome c.* The smut fungus *Ustilago sphaerogena* is an organism with an exaggerated iron metabolism. Weisel and Allen (4) showed that when grown on a medium containing yeast extract, this fungus formed large amounts of a substance which by direct spectrophotometric examination of the cells appeared to be cytochrome *c*. In subsequent work they found that the addition of zinc to a simple synthetic medium was an important factor in the realization of high yields of the hemoprotein (5).

In order to be certain that the substance observed by Weisel and Allen was indeed cytochrome *c*, the pigment was extracted from the cells and obtained in the pure state (6) by use of an ion exchange chromatography procedure previously worked out for beef heart cytochrome *c* (7). A comparison of some of the properties of the fungal and beef heart cytochrome *c* follows:

| Source | $M_{s,D}$ | pI | Hemochrome Absorption Maxima (mμ) | |
|---|---|---|---|---|
| | | | α | β |
| *U. sphaerogena*.... | 18–20 × $10^3$ | 7.0 | 550 | 520 |
| Beef heart..... | 12 × $10^3$ | 10.5 | 550 | 520 |

On a molar basis the beef heart and fungal cytochromes were found to be of about equal activity in the rat liver succinoxidase system.

*Ferrichrome.* Extracts of *U. sphaerogena* from which all cytochrome components had been removed by ammonium sulfate precipitation still contained an appreciable amount of both color and iron. These extracts were accordingly fractionated by solvent extraction, and a crystalline iron containing substance named ferrichrome was obtained (8). Crystals of this substance are shown in figure 1.

In the production of ferrichrome, *U. sphaerogena* was grown in 200-liter batches using vigorous aeration and a medium containing yeast extract and glucose. The yield of pigment was 0.4 to 0.5 g per kg of dried cells. The isolation of ferrichrome from the culture fluid has not been achieved. Ferrichrome is also formed by *U. sphaerogena* when the fungus is grown on a simple salts-glucose medium (9). With such a medium the ferrichrome concentration within the cells has been increased to about 1.5 g per kg of dried cells in the absence of added zinc (9).

*Chemical properties of ferrichrome.* The purity of thrice recrystallized ferrichrome can be conveniently checked by paper chromatography, using ultraviolet inspection. The $R_f$ values are 0.40 and 0.68 with a butanol-acetic acid-water and with a 50 per cent methanol-0.1 M phosphate buffer solvent system, respectively. Thus the substance is extremely soluble in water and sparingly soluble in all organic solvents with the exception of hot methanol.

The approximate empirical formula is $C_{27\text{-}29}H_{42\text{-}46}O_{12}N_9Fe$. The mol wt of ferrichrome has

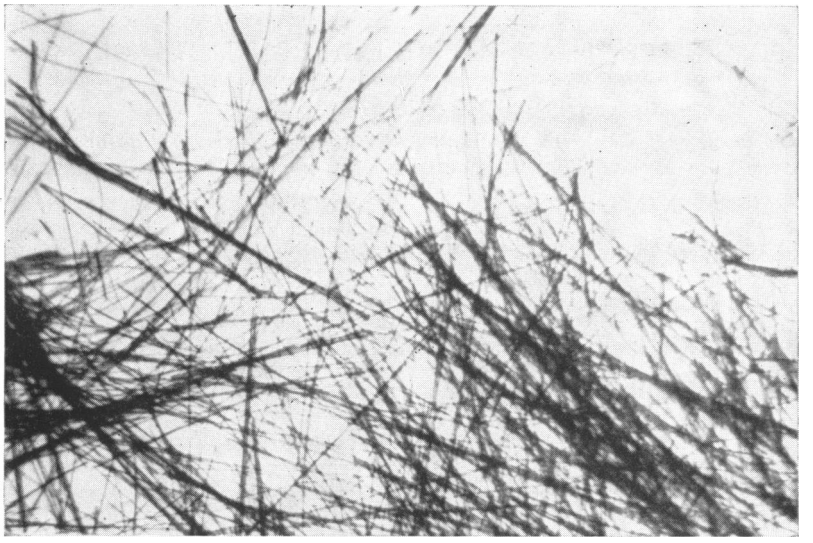


*Figure 1.* Crystalline ferrichrome from methanol

been established by three independent criteria to be in the region of 725. This is the minimum figure obtained by assuming the 11.0 per cent ash to be pure $Fe_2O_3$. That the ash is essentially pure ferric oxide is indicated by the fact that direct analyses for iron give values of 7.35 per cent. Diffusion experiments with the sintered glass disc of Northrop and Anson (10) yield a mol wt of 700 to 800. Finally, a mol wt of $7 \times 10^2$ has been obtained by sedimentation analysis in the synthetic boundary cell (11) coupled with measurements of the diffusion coefficient and partial specific volume. Values of $0.4 \times 10^{-13}$ sec, $4 \times 10^{-6}$ cm$^2$ sec$^{-1}$ and 0.65 ml g$^{-1}$ were obtained for the sedimentation coefficient, diffusion constant and partial specific volume, respectively.

The iron atom of ferrichrome is bound with great strength to the organic part of the molecule. Aqueous solutions are stable indefinitely up to pH 11 or 12 but in more alkaline solutions the iron is lost as the hydroxide. On removal of iron with dilute sodium hydroxide a colorless substance is obtained, the nitrogen content of which has increased to 18.2 per cent. This increase in nitrogen content corresponds to the loss of only the iron atom. The addition of inorganic iron salts to this preparation results in resynthesis of ferrichrome.

Thenoyltrifluoroacetone, a reagent which chelates ferric iron, is capable of removing this metal from ferrichrome provided very long extraction times and high concentrations of the reagent are used. Unsaturated conalbumin is incapable of extracting iron from ferrichrome under optimum conditions for iron binding by this egg white protein (Neilands, J. B., unpublished experiments). On the other hand, iron-free ferrichrome cannot remove iron from saturated conalbumin. This seemingly paradoxical situation can be resolved by assuming that (a) the dissociation of iron-conalbumin is a slow reaction and (b) ferrichrome binds iron with much higher avidity than does conalbumin. Radioactive inorganic iron rapidly exchanges with ferrichrome iron, just as it does with ethylenediaminetetraacetic acid (EDTA). The latter fact enables the equilibration of ferrichrome with EDTA according to the equations:

$$\text{Ferrichrome} \rightleftharpoons \text{iron-free ferrichrome} + Fe^{+++}$$

$$\text{EDTA} + Fe^{+++} \rightleftharpoons Fe^{III}\ \text{EDTA}$$

Since ferrichrome has an absorption band in the visible while $Fe^{III}$ EDTA is almost colorless, the composition of the reaction mixture can be analyzed spectrophotometrically. The results show that ferrichrome binds iron with a stability constant about 10 times that of EDTA.

The high stability constant for the iron in ferrichrome is an index of the metal ion specificity of the compound. The spectrum of ferrichrome is not altered by the addition of other metal ions such as those of copper or zinc. It is therefore concluded that ferrichrome is a relatively specific iron binding agent.

Treatment of dilute aqueous solutions of ferrichrome with hydrosulfite causes the characteristic amber color to vanish. Shaking in air causes the visible spectrum to reappear. These data plus the fact that the iron and color are removed with either thenoyltrifluoroacetone or 0.1 N NaOH indicate that the visible light absorption by ferrichrome is a consequence of the presence of ferric iron in the molecule. If the reduction is carried out in the presence of cyanide the spectrum of the oxidized compound fails to reappear on subsequent aeration (12). The addition of orthophenanthroline to a solution of reduced ferrichrome results in the appearance of the red color of the expected ferrous chelate. Finally, an aerated solution of reduced ferrichrome regains its full color rather slowly, especially at neutral or slightly alkaline pH. All of these data support the conclusion that ferrous ion is bound only weakly, if at all, to the organic part of ferrichrome.

The final proof that ferrichrome contains ferric iron was established by Ehrenberg (13). He found both oxidized and reduced ferrichrome to be strongly paramagnetic. The decrease in paramagnetic susceptibility accompanying reduction, from 5.68 to 4.93 Bohr magnetons, is typical of a change from fully paramagnetic trivalent iron to fully paramagnetic divalent iron. Ferrichrome iron is therefore held mainly through ionic forces.

Since ferrichrome shows only end absorption in the ultraviolet, the presence of aromatic rings can be ruled out. In contrast to intact ferrichrome the iron free compound exhibits light absorption only in the very deep ultraviolet. In alkaline solutions the ultraviolet absorption is moved to longer wavelengths. This transformation can be reversed by the addition of acid and exhibits a $pK_a$ of 9.0. Such behavior is characteristic of enolic groupings. Electrometric titration of iron free ferrichrome reveals a broad buffer zone with a $pK_a$ of 9.0, corresponding to three equivalents per mole. Since no ionization constant can be located in ferrichrome over the range pH 2.5 to 9.5, one or more enolic groupings probably participate in the binding of the ferric ion.

The infra-red spectrum reveals the presence of amide or substituted amide linkages (8). Unfortunately the crystals were too small for mounting for x-ray analysis.

Iron free ferrichrome gives a strongly positive biuret reaction. Hydrolysis for 16 hours with 6 N HCl at 105 C liberates at least two moles of ammonia and two amino acids, glycine and ornithine. The amino acids have been identified by paper chromatography, both in the free state and as their dinitrophenyl derivatives, and by chromatography on ion exchange resins. The ornithine has been determined by tests with *Escherichia coli* mutant no. G 160-37 to be the L(+) form. There are three or four moles of glycine but only one of L(+) ornithine per mole. Hydrolysis in 2 N NaOH liberates, in addition to the amino acids, several moles of acetic acid. The latter product was identified by steam distillation and titration ($pK_{a'}$ = 4.75), by paper chromatography and by crystallization of sodium uranyl acetate.

In summary, then, ferrichrome can be regarded as a heteromeric peptide, in the sense of Bricas and Fromageot (14), since it contains constituents other than amino acids. Approximately half of the molecule is accounted for as the iron plus the amino acids. Part of the remaining structure gives rise to acetic acid and ammonia on vigorous hydrolysis. The substance is an extremely powerful, and relatively selective, chelating agent for ferric iron. The iron is held by mainly ionic bonds some of which are enolic.

*Biological properties of ferrichrome.* At about the same time that the isolation of ferrichrome was described, two reports appearing in the literature seemed of special interest. The first of these, by Page (15), was concerned with the nutrition of the *Pilobolus* fungus. He found that hemin served as a growth factor for this organism. The second report was from the Lederle Laboratories (16) and described the isolation of an iron containing compound from an *Actinomyces* fermentation. The new product, named coprogen, was a thousand times as active as hemin in supporting the growth of the coprophylic *Pilobolus* species.

Because of its possible relation to coprogen, ferrichrome was tested as a growth factor for *Pilobolus kleinii*, using the Page (15) medium. The results shown in figure 2 show the compound to be highly active for this test organism.

More recently, Lochhead and coworkers (17) in Canada have described a factor of microbial origin which was required for the growth of *Arthrobacter terregens*. The substance was named "Terregens factor." Subsequently both coprogen

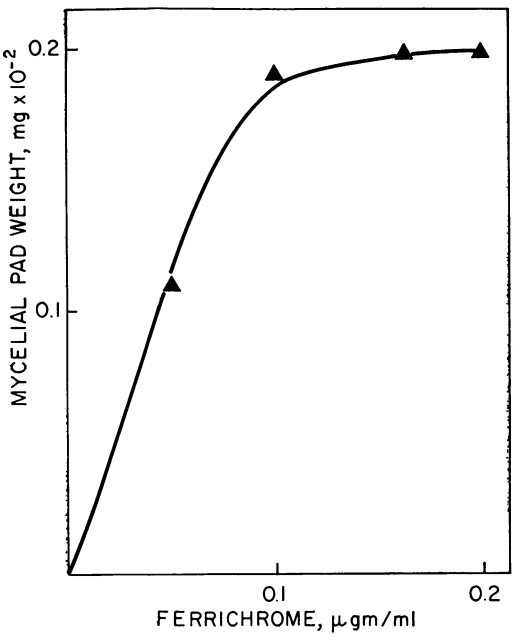



*Figure 2.* Growth factor activity of ferrichrome for *Pilobolus kleinii.*

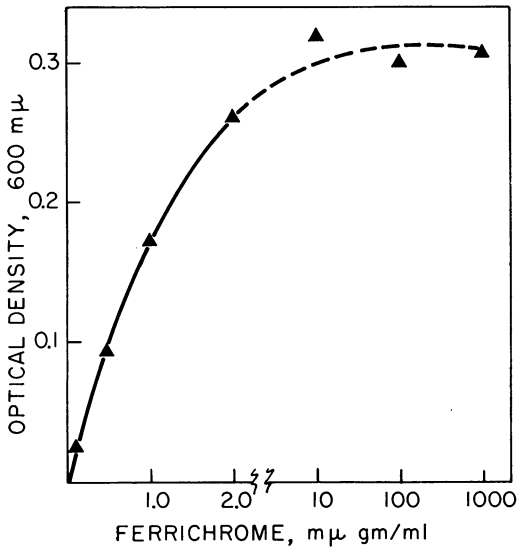



*Figure 3.* Growth factor activity of ferrichrome for *Arthrobacter terregens.*

and ferrichrome were found to be potent growth factors for *A. terregens* (18) (figure 3). Also, hemin and aspergillic acid showed some activity. Although ferrichrome, coprogen and terregens factor all exhibit similar biological activity, the three products differ in several of their chemical and physical properties. Ferrichrome contains more iron and nitrogen than does coprogen. The $R_f$ of coprogen with 80 per cent methanol-phosphate buffer is 0.80 while that of ferrichrome is 0.68. Terregens factor (18) contains a large number of different amino acids and appears to be quite distinct from either ferrichrome or coprogen as a chemical entity.

The fact that ferrichrome is autoxidizable might indicate that the compound acts as an electron transfer catalyst. However, all attempts to reduce ferrichrome by either extracts or broken cell preparations of *Ustilago sphaerogena* or *A. terregens* have been unsuccessful. In addition, a number of plant and animal tissues have been found incapable of reducing ferrichrome.

Iron free ferrichrome is active for *A. terregens* grown on the medium of Burton and Lochhead (17). However, since this medium contains appreciable quantities of iron (even without added iron) and since iron free ferrichrome readily combines with iron, it cannot be concluded that the presence of this element in ferrichrome is not required for biological activity. It has also been observed that the extent of growth of *A. terregens* on the Burton and Lochhead medium is independent of added inorganic iron, even at concentrations of ferrichrome as low as 1.0 mμg/ml. From one such experiment using medium without added inorganic iron, the cell yield in dry weight was found to be 95 mg per L. Since this growth was realized from a total of 1.0 μg of ferrichrome, the iron content of the cells would have to have been vanishingly small, in fact less than $8 \times 10^{-5}$ per cent, if only ferrichrome iron were utilized. Hence it seems probable that, given ferrichrome, the organism is able to utilize inorganic iron.

When *U. sphaerogena* is grown on a simple medium containing ammonia, acetate, sugar and salts, copious quantities of cytochrome *c* are produced (5). Without added iron, the growth on this medium is less vigorous, the cells are white and contain much less cytochrome *c*. When such cells are aseptically centrifuged, washed and replaced in fresh medium containing ferrichrome, the disappearance of the latter compound from the medium parallels the synthesis of cytochrome within the cells. Further, Grimm and Allen (5) showed that with this medium high levels of zinc, up to 2.0 mg per L, are required for optimum synthesis of cytochrome *c*. The amount of

ferrichrome extractable from the cells is a function of the prior zinc nutrition of the organism (9). Without added zinc the cells contain little cytochrome *c* and yield relatively large amounts of ferrichrome. When zinc is added to a growing culture of *U. sphaerogena* there is, concomitant with cytochrome *c* synthesis, an immediate drop in the extractable ferrichrome. The loss in ferrichrome is greater than can be accounted for simply by dilution of the existing levels of ferrichrome by the increased growth occasioned by the presence of zinc. Thus there is evidence that (a) externally supplied ferrichrome can penetrate the cells of *U. sphaerogena* and (b) cytochrome *c* is synthesized at the expense of ferrichrome.

Ferrichrome is without antibiotic activity against either *Micrococcus pyogenes* var. *aureus* or *Bacillus subtilis* (8). It is toxic when injected into rats, exhibiting a pronounced hemodiluting effect.

*Ferrichrome A.* It was noted above that the growth of *U. sphaerogena* without added iron, *i.e.*, suboptimal amounts, results in the formation of white cells containing low levels of cytochrome *c*. At the same time, as Garibaldi found (19), several iron binding compounds appear in the cell free medium. The iron addition compounds of these materials are quite highly pigmented and are therefore readily detected by paper chromatography. The major component found by paper chromatography has been crystallized from water in the form of regular plates. Since this substance contains iron, ornithine and glycine, it is obviously related to ferrichrome and has therefore been named "ferrichrome A." Crystals of ferrichrome A are shown in figure 4.

The "threshold" level of added iron needed to prevent the extracellular appearance of ferrichrome A (iron free form) is about 1 mg per L. When the iron level of the medium is too low neither growth nor ferrichrome A formation occurs. Intermediate levels of iron permit both vigorous growth and the appearance of up to 1 g of crystalline ferrichrome A per L.

Ferrichrome A differs from ferrichrome in the following respects (20): It contains serine in addition to those amino acids found in ferrichrome; it has less nitrogen and iron; is very soluble in methanol and is sparingly soluble in water. The $R_f$ values for ferrichrome A in butanol-acetic acid-water and in 80 per cent methanol-phosphate buffer are 0.51 and 0.52, respectively.

The iron atom is bonded to the organic part of ferrichrome A with even greater strength than to ferrichrome. The element has been shown by both Garibaldi (20) and Ehrenberg (13) to be present in the ferric state. The sedimentation coefficient, diffusion coefficient and partial specific volume were found to be $0.6 \times 10^{-13}$ seconds, $3 \times 10^{-6}$ cm$^2$ sec$^{-1}$ and 0.69 ml g$^{-1}$, respectively. These data yield a mol wt of $1.5 \times 10^3$. From the iron content, 5.3 per cent, a minimum mol wt of 1100 is obtained. This value is supported by x-ray crystallographic measurements. From the dimen-

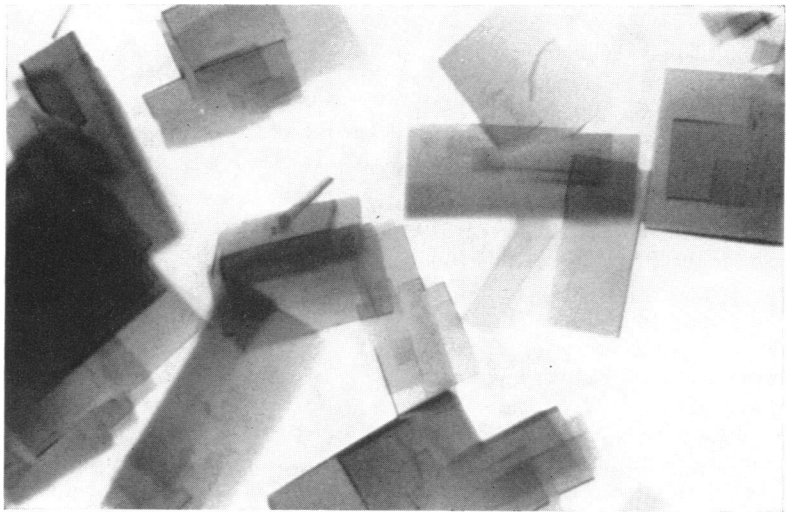


*Figure 4.* Crystalline ferrichrome A from water

sions, $a = 11.31$ A, $b = 13.18$ A and $c = 17.32$ A, a volume of $25 \times 10^2$ cu A is obtained for the unit cell. Since the density is 1.45 g $ml^{-1}$, the mol wt, assuming 2 molecules per unit cell, is $(25 \times 10^2\ 1.45 \times 6.02 \times 10^{23})/(2 \times 10^{24})$ or $11 \times 10^2$.

Little is known of the structural features of ferrichrome A. The isolated compound is an acid. Three equivalents of strong base are consumed in the region pH 3 to 6. As contrasted to ferrichrome, the three characteristic properties are the presence of serine, the acidity (which affects the solubility) and the stronger binding of the iron.

Ferrichrome A, like ferrichrome, can be utilized by *U. sphaerogena* for the synthesis of cytochrome *c* (20). The substance is a hundred times less active as a growth factor for *A. terregens* than ferrichrome.

### EXPERIMENTS WITH AEROBIC BACILLI AND *ASPERGILLUS NIGER*

*Formation of iron binding compounds.* It was stated previously that the addition to the modified Grimm-Allen (5) medium of as little as 1 mg of iron per L prevented the formation of iron free ferrichrome. Since the excretion of powerful iron binding compounds under conditions of iron deprivation would be an evolutionally useful metabolic act, a study was made of the tendency of various microbial species to form such compounds under conditions similar to those used for the production of ferrichrome A. The results demonstrated that at least three species (*Bacillus subtilis* and *B. megaterium*, and *A. niger*) could be induced to form such compounds (21). Several strains of *B. subtilis* and *B. megaterium* were examined and found to give similar results.

The formation of the iron binding compounds was detected by the addition of 0.25 mg of ferric ion per ml of the cell free medium. The optical density was then read at the wavelength of maximum absorption of the principal colored iron complex present. These wavelengths were 510, 440, and 440 mμ for the *B. subtilis*, *A. niger* and *B. megaterium* cultures, respectively.

In a series of experiments designed to show that only intermediate levels of iron are effective, the basal medium was treated with 8-hydroxyquinoline and extracted with chloroform prior to sterilization and inoculation. The formation of the ferric ion binding agent by *B. subtilis* as a function of time and initial iron concentration is shown in figure 5.

The measured product of this fermentation, the ferric derivative of which exhibits a deep purple color at neutral pH, has been extensively purified by Ito at the University of California (unpublished experiments) and appears to have the general properties of a polyhydric phenol. The chemical nature of the compounds formed by *A. niger* and *B. megaterium* is unknown, although judging from their spectra they may be more closely related to ferrichrome or ferrichrome A. The ferric chelate of citric acid, although yellow in color, does not have an absorption maximum in the higher wavelength region employed in the present work.

*Escherichia coli* and a *Pseudomonas* species, when grown in the unpurified medium, gave no visual evidence of the formation of iron binding agents. It is conceivable that some of these compounds might provide colorless derivatives with ferric ion; in that event they would be more difficult to detect. Success in finding the substances with the three species described above may be connected with the more vigorous growth and higher iron requirement of the bacilli and fungi. It should also be mentioned that *Ustilago zeae*, when grown in the medium without added iron, formed a substance with the chromatographic properties of ferrichrome A.

*B. subtilis*, but not *B. megaterium*, *Ustilago* species or *A. niger*, excretes large amounts of

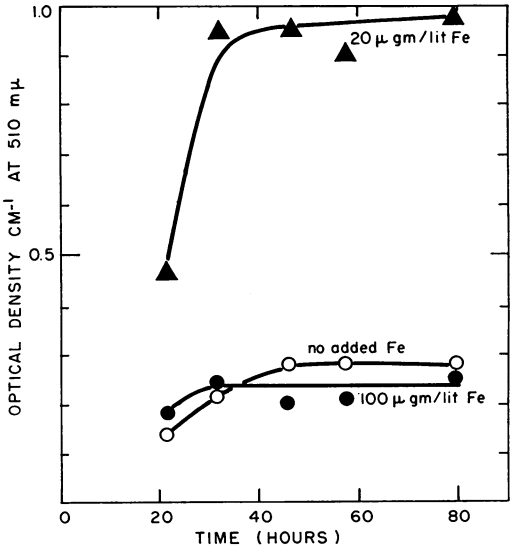



*Figure 5.* Formation of iron-binding substances by *Bacillus subtilis* as a function of time and initial iron concentration (21).

porphyrin in addition to the ferric ion binding agents described above. The porphyrin excreted by *B. subtilis* has been shown by Townsley (22) to be mainly coproporphyrin III.

### EXPERIMENTS WITH *MICROCOCCUS LYSODEIKTICUS*

*Formation of coproporphyrin III.* Any attempt to elucidate the complete pathway of iron in metabolism must eventually cope with the problem of the origin of the iron atom of heme. It has been known for a very long time that neither ferrous nor ferric ion is spontaneously bound by porphyrins; *i.e.*, drastic chemical treatment is needed for insertion of iron into the tetrapyrrole (23). Radioactive iron does not exchange with hematin iron (24). The two obvious possibilities are (a) an enzyme exists which is capable of combination with both porphyrin and an iron donor chelate, the activation energy of the reaction is lowered, and iron is transferred to the porphyrin; or (b) the reaction is nonenzymatic, the iron entering a porphyrin precursor which is capable of spontaneous binding of the element. Investigation of this problem has been greatly facilitated by our current extensive knowledge of the mechanism of porphyrin formation (25).

The excretion of porphyrin by microbial cultures growing under controlled iron supply has been studied by several investigators. The list of organisms examined includes *Corynebacterium diphtheriae* (26), *Rhodopseudomonas sphaeroides* (27), *Bacillus cereus* (28) and *B. subtilis* (20). Since the organism *M. lysodeikticus* excretes porphyrin, this species offers several advantages as an experimental subject for study of the mechanism of heme synthesis. In the first place it is known to form large quantities of heme (as catalase). Secondly, since the organism is very susceptible to lysozyme, the work with whole cells can be readily extended to soluble preparations. Finally, such preparations are relatively colorless, as contrasted to lysed erythrocyte preparations, and it is possible to carry out *in situ* spectrophotometric analysis of the reaction mixture.

Since details of the experimental work with *M. lysodeikticus* have been published elsewhere (29), only the main conclusions need to be recorded here.

Cultures of *M. lysodeikticus* growing at low levels of iron were found to excrete large amounts of porphyrin. This porphyrin, which was characterized through the crystalline methylester, was found to be almost exclusively coproporphyrin III (29). Starting with δ-aminolevulinic acid, lysed cell preparations were found to synthesize compounds exhibiting the properties of the porphyrinogens, *i.e.*, substances which on aeration, or exposure to light, were rapidly converted to porphyrins. The types and amounts of porphyrins formed by the lysed cells were a function of the initial iron level of the medium on which the organism had been cultivated. Thus a "low iron" lysate (iron level low enough to cause porphyrin excretion) gave more porphobilinogen, uroporphyrin and coproporphyrin than an "adequate iron" lysate (iron levels high enough to prevent porphyrin excretion).

There was no evidence for the formation of large amounts of ferric ion binding agents by *M. lysodeikticus* under the above growth conditions.

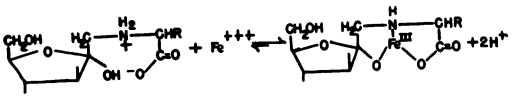
*In vitro synthesis of coproheme.* When coproporphyrinogen III was added to a "low iron" lysate, the theoretical yield of coproporphyrin III could be recovered. The addition of ferrous sulfate to this system greatly reduced the recovery of coproporphyrin. Finally, it was found that the disappearance of coproporphyrin III in the presence of iron could be correlated with the synthesis of coproheme.

The system devised for the *in vitro* synthesis of coproheme contained 300 mg dry weight of cell lysate, 1.3 μmole of coproporphyrinogen III and 5 μmole ferrous sulfate. The system was stable to mild heat but was partially destroyed by drastic heat treatment. There was no detectable binding of ferrous or ferric ion by coproporphyrinogen III in the absence of the cell lysate. The lysed cell preparation was found incapable of converting coproheme itself to protoheme. Hence, the role of coproheme as a direct precursor of protoheme is questionable. In spite of this the *in vitro* system for coproheme synthesis will serve as a useful model for the iron incorporation reaction.

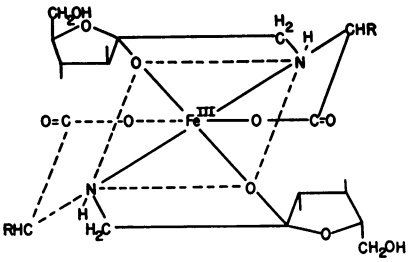
*Sugar amino acids as iron binding compounds.* The *M. lysodeikticus* culture used in this work was a strain which Wolin (30) had "trained" to grow on a simple medium containing glucose, sodium glutamate, salts and biotin. It was found that when the ingredients of the medium were sterilized separately no growth was obtained. However, after much experimentation it was dis-

covered that the addition of various compounds or preparations to such a medium would permit growth. Thus trace amounts of citric acid, 8-hydroxyquinoline, ferrichrome or small amounts of an autoclaved solution of glucose and sodium glutamate were all active. Since sugar-amino acids (ketose-amino acids) are well known products of such a browning reaction, [N-(1′-carboxy) - phenylethyl - 1 - amino - 1 - deoxy-D-fructose] or "fructose phenylalanine" prepared by the method of Gottschalk (31) was tested and found to be highly active as a growth stimulant for *M. lysodeikticus* on the medium described above. Ethylenediamine tetraacetic acid was inactive.

Since most of the active compounds were iron binding substances, fructose-phenylalanine was tested for its capacity to chelate ferric ion, using the difunctional pH recorder (32). Ferric ion was found to eliminate two protons from the *zwitterion* (33) probably by the following reaction:



The stability constant of the reaction, $Fe^{3+} + A^{2-} \rightleftharpoons Fe^{1+}$ was $10^{16}$. At higher ratios of anion:metal ion, a 2:1 complex was formed. This compound was an extremely stable ferric chelate with the probable structure:



The ring structure is shown in the sugar moiety, since (a) the ionization at carbon 2 is without influence on the ultraviolet absorption spectrum and (b) the ferric chelates are not highly colored. The structural similarity to the well known ferric EDTA chelate will be noted.

## DISCUSSION

One possible conclusion from the work with the ferrichrome compounds is that these substances act as "coenzymes" for the transfer of iron in microbial metabolism. The main observations in support of this idea are as follows: (a) the substances active as growth factors for *Pilobolus* species and *A. terregens* are strong iron binding agents. Aspergillic acid, which shows some activity for *A. terregens*, forms a stable ferric chelate compound. (b) The binding constant for iron is so high that it can be concluded that the iron of the metabolic pool must exist in this form. (c) Ferrichrome and ferrichrome A are taken into the cells of *U. sphaerogena* and the iron is eventually used for heme synthesis. (d) The formation of iron free ferrichrome compounds is stimulated during iron deficiency. This does not imply that the microbe is possessed of a central nervous system. From the practical point of view it does mean, however, that organisms which can produce such compounds have become equipped with an important survival mechanism. Such a mechanism could easily have been developed during the relentless processes of evolution. (e) The ferrichrome compounds appear relatively specific for ferric iron. Ferrous ion is bound only loosely if at all. Thus a mechanism is provided for release of the iron without degradation of the organic part of the molecule. This is an important point in view of the very high potency of ferrichrome as a growth factor. (f) There is no evidence that the ferrichrome compounds act as electron transfer catalysts. Such a catalyst would have to bind strongly both valency states of iron, a condition which is met by the porphyrins but not by the ferrichromes. An autoxidizable compound like ferrichrome would in fact starve *U. sphaerogena* should there exist an enzyme which rapidly reduced the substance without concomitant phosphorylation. (g) The requirement of certain higher plants for chelated iron is well known (34). Thus ethylenediaminetetraacetic acid is widely used for treatment of iron deficiency chlorosis in the field. On the other hand, it has been pointed out (35) that lichens and other plants capable of growth on rocks must be able to excrete organic compounds which dissolve the required mineral elements. By analogy, one might expect to find microbial species of two types, those capable and those incapable of secreting iron binding agents. Living organisms with the former capability might be termed *autosequesteric* whereas those unable to synthesize their own sequestering agent might be called *anautosequesteric*. *U. sphaerogena* and the aerobic bacilli mentioned in this report would be examples

of autosequesteric organisms, while the strain of *M. lysodeikticus* described as well as various green plants would be classed as anautosequesteric. Indeed, Orlando at this university (unpublished experiments) has found that the ferrichrome compounds are effective in supplying iron to tomato plants growing in hydroponic solution. (h) The net effect of a "coenzyme" for the transfer of iron would have a two-fold advantage in the nutrition of the cell. In the first place, the iron would be protected from combination with the myriad of organic structures within the cell. Rather, it would be concentrated in one or two compounds which are prepared to donate it to the apoproteins of the iron enzymes. Secondly, the iron would be protected from precipitation as ferric hydroxide, a reaction which free ferric ion would readily undergo under conditions prevailing within living cells.

Although the theory is attractive from several points of view, the definite identification of the ferrichrome compounds as specific iron transfer catalysts must await further research. It needs to be demonstrated, for instance, that only specific iron compounds are absorbed by *Pilobolus* species and *A. terregens* and utilized for heme synthesis in these organisms. It will be recalled that fructose-phenylalanine, ferrichrome, 8-hydroxyquinoline and citric acid, but not ethylenediaminetetraacetic acid, can promote growth of *M. lysodeikticus* on a simple medium with the components sterilized separately. Thus some degree of specificity exists for the type of chelating agent acceptable for this organism. The observation that ethylenediaminetetraacetic acid is inactive in this system also rules out the possibility that the compounds act by detoxifying some poisonous metal ion.

An important role of ferrichrome and related compounds in microbial metabolism is indicated by their widespread distribution. Thus the *Pilobolus* fungus only exists in nature by virtue of the compounds synthesized by the intestinal flora. Lyr (36) has isolated from horse dung three microbial species which actively synthesize such factors. A survey of 32 common microbial species showed that 10 of these produced coprogen-like substances (37). There is no evidence that these products occur in animal tissues.

It is remarkable that in each and every case of porphyrin production by organisms growing at low levels of iron, the isolated porphyrin is coproporphyrin III rather than protoporphyrin IX. This fact suggests that the enzymes which synthesize protoporphyrin are able to proceed only to a four-carboxyl porphyrin in the absence of iron. The synthesis of coproheme from coproporphyrinogen and iron in the presence of lysed cell preparations of *M. lysodeikticus* appears not to be an enzymatic process. Since Orlando (unpublished experiments) has found that the reaction can be carried out with lyophilized preparations, it should now be possible to elucidate the mechanism of this model reaction for iron incorporation within the macrocyclic porphyrin ring. It has been emphasized (29) that the lack of planarity of the porphyrinogens might be an important aspect of their iron-accepting capacity. It has so far not been possible to transfer ferrichrome iron to coproporphyrinogen by an *in vitro* synthesis.

Apart from the work reported in this review, several additional facts point to the role of the ketose-amino acids in iron metabolism. Borsook *et al.* (38) found them to stimulate incorporation of amino acids into the proteins of reticulocytes only in the presence of iron. Rogers *et al.* (39) found this type of compound to stimulate the initial growth of *Lactobacillus gayonii* and recently Rogers (40) has reported the isolation of several *E. coli* mutants requiring N-glucosylglycine or its Amadori rearrangement product (fructose-glycine). The ketose-amino acid is a novel iron binding site that could readily be introduced into proteins. Fructose-lysine, fructose-aspartic acid or fructose-glutamic acid could be combined in peptide linkage through the omega functional group of the amino acid moiety to an appropriate functional group in proteins.

ACKNOWLEDGMENTS

The author is indebted to J. A. Garibaldi, T. Ito, J. Orlando, and P. M. Townsley for much of the experimental work reported in this review. The crystallographic measurements were made by L. Pauling and co-workers. E. E. Snell kindly read the manuscript. Financial support has been provided by the Office of Naval Research under Contract Number *222(39)*.

REFERENCES

1. Theorell, H., Beznak, M., Bonnichsen, R., Paul, K. G., and Åkeson, Å. 1951 On the distribution of injected radioactive iron in guinea pigs and its rate of appearance

in some hemoproteins and ferritins. Acta Chem. Scand., **5,** 445–475.
2. GREEN, D. E., AND BEINERT, H. 1955 Biological oxidations. Ann. Rev. Biochem., **24,** 1–44.
3. KLUYVER, A. J., AND VAN NIEL, C. B. 1956 *The microbe's contribution to biology.* Harvard University Press, Cambridge, Mass.
4. WEISEL, P., AND ALLEN, P. J. 1951 Abstr. Inst. Biol. Sci. Meeting, Minneapolis, September.
5. GRIMM, P. W., AND ALLEN, P. J. 1954 Promotion by zinc of the formation of cytochromes in *Ustilago sphaerogena.* Plant Physiol., **29,** 369–377.
6. NEILANDS, J. B. 1952 The isolation and properties of cytochrome *c* from different sources. J. Biol. Chem., **197,** 701–708.
7. PALEUS, S., AND NEILANDS, J. B. 1950 The preparation of cytochrome *c* with the aid of ion exchange resin. Acta Chem. Scand., **4,** 1024–1032.
8. NEILANDS, J. B. 1952 A crystalline organo iron compound from the fungus *Ustilago sphaerogena.* J. Am. Chem. Soc., **74,** 4846–4847.
9. NEILANDS, J. B. 1953 Factors affecting the microbial production of ferrichrome. J. Biol. Chem., **205,** 647–650.
10. NORTHRUP, J. H., AND ANSON, M. L. 1929 A method for the determination of diffusion constants and the calculation of the radius and weight of the hemoglobin molecule. J. Gen. Physiol., **12,** 543–554.
11. PICKELS, E. G., HARRINGTON, W. F., AND SCHACHMAN, H. K. 1952 An ultracentrifuge cell for producing boundaries synthetically by a layering technique. Proc. Natl. Acad. Sci. U. S., **38,** 943–948.
12. NEILANDS, J. B. 1953 An assay method for ferrichrome. J. Biol. Chem., **205,** 643–646.
13. EHRENBERG, A. 1956 Magnetic properties of ferrichrome and ferroverdin. Nature, **178,** 379–380.
14. BRICAS, E., AND FROMAGEOT, C. 1953 Naturally occurring peptides. Advances in Protein Chem., **8,** 1–125.
15. PAGE, R. M. 1952 The effect of nutrition on growth and sporulation of *Pilobolus.* Am. J. Botany, **39,** 731–739.
16. HESSELTINE, C. W., PIDACKS, C., WHITEHILL, A. R., BOHONOS, N., HUTCHINGS, B. L., AND WILLIAMS, J. H. 1952. Coprogen, a new growth factor for coprophylic fungi. J. Am. Chem. Soc., **74,** 1362–1363.
17. LOCHHEAD, A. G., BURTON, M. O., AND THEXTON, R. H. 1952 A bacterial growth-factor synthesized by a soil bacterium. Nature, **170,** 282–283.
18. BURTON, M. O., SOWDEN, F. J., AND LOCHHEAD, A. G. 1954 Studies on the isolation and nature of the "terregens factor." Can. J. Biochem. Physiol., **32,** 400–406.
19. GARIBALDI, J. A., AND NEILANDS, J. B. 1955 Isolation and properties of ferrichrome A. J. Am. Chem. Soc., **77,** 2429–2430.
20. GARIBALDI, J. A. 1957 Iron metabolism in microorganisms. Doctoral dissertation. University of California, Berkeley.
21. GARIBALDI, J. A., AND NEILANDS, J. B. 1956 Formation of iron-binding compounds by microorganisms. Nature, **177,** 526–527.
22. TOWNSLEY, P. M. 1956 The iron and porphyrin metabolism of *Micrococcus lysodeikticus.* Doctoral dissertation. University of California, Berkeley.
23. LEMBERG, R. AND LEGGE, J. W. 1949 *Hematin compounds and bile pigments.* Interscience Publishing Co., New York.
24. RUBEN, S., KAMEN, M. D., ALLEN, M. B., AND NABINSKY, P. 1942 Some exchange reactions with radioactive tracers. J. Am. Chem. Soc., **64,** 2297–2298.
25. SHEMIN, D. 1956 In *Essays in biochemistry,* Graff, S., ed. J. Wiley and Sons, New York, 241–258.
26. PAPPENHEIMER, A. M., JR. 1947 Diphtheria toxin. III. A reinvestigation of the effect of iron on toxin and porphyrin production. J. Biol. Chem., **167,** 251–259.
27. LASCELLES, J. 1956 The synthesis of porphyrins and bacterio-chlorophyll by *Rhodopseudomonas sphaeroides.* Biochem. J., **62,** 78–93.
28. SCHAEFFER, P. 1952 Recherches sur le métabolisme bactérien des cytochromes et des porphyrines. Biochem. et Biophys. Acta, **9,** 362–368.
29. TOWNSLEY, P. M., AND NEILANDS, J. B. 1956 The iron and porphyrin metabolism of *Micrococcus lysodeikticus.* J. Biol. Chem., **224,** 695.
30. WOLIN, H. L., AND NAYLOR, H. B. 1955 Basic nutritional requirements of *Micrococcus lysodeikticus.* Bacteriol. Proc., 55th General Meeting, p. 47.
31. GOTTSCHALK, A. 1952 Some biochemically relevant properties of *N*-substituted fructosamines derived from amino-acids and *N*-arylglucosylamines. Biochem. J., **52,** 455–460.
32. NEILANDS, J. B., AND CANNON, M. I. 1955 Automatic recording pH instrumentation. Anal. Chem., **27,** 29–33.

33. NEILANDS, J. B. AND TOWNSLEY, P. M. 1956 Iron binding activity of sugar-amino acids. Federation Proc., **15,** 320.
34. WEINSTEIN, L. H., ROBBINS, W. R., AND PERKINS, H. F. 1954 Chelating agents and plant nutrition. Science, **120,** 41–43.
35. HUTNER, S. H., PROVASOLI, L., SCHATZ, H., AND HASKINS, C. P. 1950 Some approaches to the study of the role of metals in the metabolism of microorganisms. Proc. Am. Phil. Soc., **94,** 152–170.
36. LYR, H. 1953 Zur Kenntnis der Ernährungsphysiologie der Gattung Pilobolus. Arch. mikrobiol., **19,** 402–434.
37. HESSELTINE, C. W., WHITEHILL, A. R., PIDACKS, C., TEN HAGEN, M., BOHONOS, N., HUTCHINGS, B. L., AND WILLIAMS, J. H. 1953 Coprogen, a new growth factor present in dung, required by *Pilobolus* species. Mycologia, **45,** 7–16.
38. BORSOOK, H., ABRAMS, A., AND LOWY, P. H. 1955 Fructose-amino acids in liver: stimuli of amino acid incorporation *in vitro*. J. Biol. Chem., **215,** 111–124.
39. ROGERS, D., KING, T. E., AND CHELDELIN, V. H. 1953 Growth stimulation of *Lactobacillus gayonii* by N-D-glucosylglycine. Proc. Soc. Exptl. Biol. Med., **82,** 140–144.
40. ROGERS, D. 1956 N-glucosylglycine-requiring mutants of *Escherichia coli*. Federation Proc., **15,** 339.