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# Kinetic Studies on the Specificity of Chelate-Iron Uptake in *Aspergillus*

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Three strains of the fungus *Aspergillus*, *Aspergillus quadricinctus* (E. Yuill), *A. fumigatus* (Fresenius), and *A. melleus* (Yukawa), each producing different iron-chelating compounds during iron-deficient cultivation, were used for $^{55}Fe^{3+}$ uptake measurements. Iron from chelates of the ferrichrome-type family was taken up by young mycelia of all strains tested, irrespective of the ferrichrome-type compound these strains predominantly produce in low-iron cultures. Ferrichrysin-producing strains, however, seem to favor ferrichrysin iron uptake, whereas ferrichrome, ferricrocin, and even ferrirubin showed similar iron transport properties in all of these strains. Compared to iron uptake from ferrichrome-type compounds ($K_m \sim 5$ $\mu$M) iron uptake from fusigen revealed completely different kinetic values ($K_m \sim 50$ to 80 $\mu$M). Iron from exogenous chelates, e.g., from coprogen produced by *Neurospora crassa* or ferrioxamine B produced by *Streptomyces pilosus*, can obviously not be taken up by *Aspergillus*, confirming the pronounced specificity of chelate-iron transport in fungi.

A common feature of the iron-chelating compounds excreted under iron-deficient conditions by the genus *Aspergillus* is that they belong mainly to the ferrichrome-type family (18). The ferrichrome-type compounds are cyclohexapeptides containing 3 mol of $\delta$-*N*-acyl-$\delta$-*N*-hydroxyornithine and a tripeptide chain consisting of either triglycine (ferrichrome), glycylserylglycine (ferricrocin), or serylserylglycine (ferrichrysin) (13). Although ferrichrome, ferricrocin, and ferrichrysin are the main components of the iron chelates of aspergilli (9, 18), other chelates may occur, e.g., ferrirubin, ferrirhodin, fusigen, and some unknown minor components (4, 8). The composition of excreted compounds may vary to some extent and may depend on the cultivation medium (2). In general, however, the pattern of the produced iron-chelating compounds is highly characteristic and typical for the producing strain.

An interesting question, therefore, is whether diverse chelating compounds synthesized by one organism are all equally well used for iron accumulation or whether there exist pronounced differences in iron transport properties of various compounds with structural similarities. In this paper we provide evidence that, as we have assumed earlier (15), a pronounced specificity of chelate-iron uptake can also be observed among aspergilli.

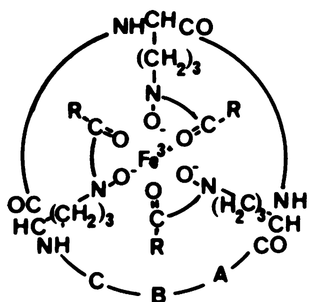
## MATERIALS AND METHODS

**Chemicals.** Ferrichrome was a gift from J. B. Neilands, Berkeley, Calif. Ferricrocin, coprogen, and fusigen were kindly supplied by H. Diekmann, Hannover, West Germany. Ferrirubin was from W. Keller-Schierlein, ETH Zürich, Switzerland. Ferrichrysin and ferrioxamine B were from the stock of the Institut für Biologie, Lehrbereich Mikrobiologie I, Tübingen. All other chemicals were purchased from Merck, Darmstadt. $^{55}FeCl_3$ in 0.1 M HCl (carrier free) was obtained from the Radiochemical Centre, Amersham, England.

**Fungal strains.** *Aspergillus quadricinctus* (E. Yuill), CBS 135.52, was from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. *Aspergillus melleus* Yukawa (Tü 142) and *Aspergillus fumigatus* Fresenius (Tü 149), previously designated *Aspergillus versicolor* (ETH M 3636), were from the stock of the Institute. H. Diekmann has kindly made these strains available to us.

**Growth of cultures.** All strains were grown on agar medium containing 0.4% yeast extract, 1% malt extract, 0.4% glucose at 27 C until extensive conidiation was reached. The conidia were harvested with 0.9% NaCl solution containing 1 drop of Tween 80 per 100 ml and washed repeatedly by centrifugation. Mycelia for kinetic measurements were grown in culture flasks containing a chemically defined medium with the following composition: L(−)-asparagine, 5 g; $K_2HPO_4 \cdot 3H_2O$, 1 g; $MgSO_4 \cdot 7H_2O$, 1 g; $CaCl_2 \cdot 2H_2O$, 0.5 g; and distilled water to 1 liter, pH 6. Glucose (2%) was added after separate sterilization.

**Kinetic measurements.** Chemically defined me-



**Ferrichrome:** R = $CH_3$

ABC = Gly - Gly - Gly

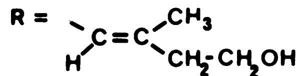
**Ferricrocin:** R = $CH_3$

ABC = Gly - L-Ser - Gly

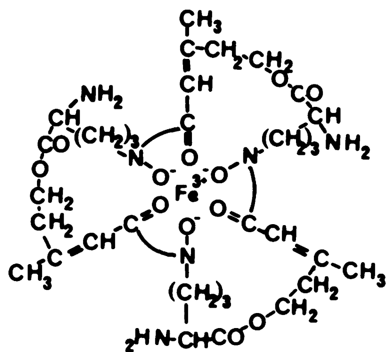
**Ferrichrysin:** R = $CH_3$

ABC = L-Ser - L-Ser - Gly

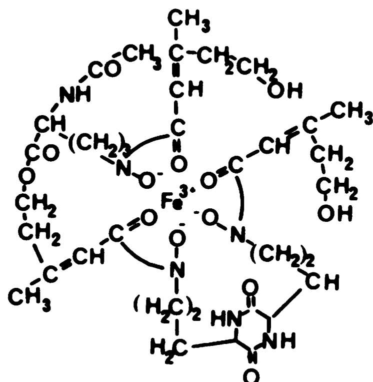
**Ferrirubin:** R =



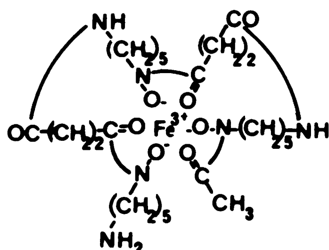
ABC = L-Ser - L-Ser - Gly



**Fusigen**



**Coprogen**



**Ferrioxamine B**

FIG. 1. *Structures of iron chelates (siderochromes) tested for iron uptake in Aspergillus. The ferrichrome-type compounds (ferrichrome, ferricrocin, ferrichrysin, ferrirubin) and fusigen occur in the genus Aspergillus, whereas coprogen is found among the genera Penicillium and Neurospora. Ferrioxamine B is produced by several strains of Streptomyces. All compounds are excreted during iron deficiency as iron-free compounds.*

dium (100 ml) containing no additional iron was inoculated with $10^9$ conidiospores. After 20 h of incubation at 27 C on a rotating shaker at 120 rpm the young mycelia were used for kinetic measurements. The kinetic assay for the saturation kinetics contained increasing amounts of $^{55}$Fe-labeled iron chelates and was started by addition of a suspension of 2 ml of young mycelia to a volume of 2.2 ml. After 10 min of incubation the mycelia were rapidly filtered through paper filters and subsequently washed three times with 10 ml of 0.9% cold NaCl solution. The mycelia on the filter disks were counted with Unisolve I (Koch-Light Lab. Ltd., England) in a liquid scintillation counter (Nuclear-Chicago, Mark I) with external quench correction. Samples were counted after 24 h of equilibration with Unisolve I.

**Colorimetric determination of iron chelates.** The concentration of the iron chelates (called "siderochromes" in the terminology of Neilands [13]) was determined spectrophotometrically using the absorption values given by Neilands (12): ferrichrome $E_{430}^{1\%}$ = 39.2; ferricrocin $E_{430}^{1\%}$ = 33; ferrichrysin $E_{430}^{1\%}$ = 38; ferrirubin $E_{450}^{1\%}$ = 33; coprogen $E_{440}^{1\%}$ = 36; ferrioxamine B $E_{430}^{1\%}$ = 39. For fusigen, $E_{436}^{1\%}$ = 35.2 was used, as described by Diekmann (4). The purity of the siderochromes was examined by thin-layer chromatography on silica gel (Merck, Darmstadt) using chloroform-methanol-water as the solvent system. The radioactive siderochromes were prepared by addition of 4 nmol of $^{55}FeCl_3$ in 0.1 M HCl to a solution of 2 ml of desferrisiderochrome (5 nmol). For concentration-dependent uptake measurements 100 $\mu$l of $^{55}$Fe-labeled siderochrome and increasing amounts of a nonradioactive siderochrome solution (1 $\mu$mol/ml) were mixed in the incubation vials just before use and filled up to an equal volume of 200 $\mu$l.

**Preparation of the desferri compounds.** The desferrisiderochromes were obtained after treatment with 8-hydroxyquinoline. To a solution of 2 ml of siderochrome (1 $\mu$mol/ml) 0.5 ml of methanolic 8-hydroxyquinoline (20 $\mu$mol/ml) was added. After incubating at 70 C for 1 h, the reaction was allowed to complete by standing at room temperature for 24 h. The colorless solution obtained was separated from the black iron-hydroxyquinoline sediment and extracted five times with 2 ml of chloroform to remove excess hydroxyquinoline. The remaining solution was evaporated to dryness. The desferrisiderochromes were dissolved in 2 ml of distilled water and adjusted colorimetrically to the appropriate concentration. To remove any free ionic iron, all siderochrome solutions were passed through a carboxymethyl-cellulose column.

## RESULTS

In the present investigation iron uptake was studied from several siderochromes that were known to occur in aspergilli, e.g., ferrichrome-type compounds and fusigen. As a comparison two exogenous siderochromes, coprogen and ferrioxamine B, were tested. Structures of the siderochromes used are given in Fig. 1. After incubation for approximately 20 h in an iron-free, chemically defined asparagine medium, the conidia usually germinated to a dense mycelial suspension. Atomic absorption measurement revealed that the conidia contained approximately 1 nmol of Fe per mg (dry weight). Thus, an iron deficiency does not seem to be apparent at this stage of growth. Furthermore, there is no excretion of any iron-chelating compounds until day 2 of cultivation in iron-deficient media (2). The iron-chelate uptake system in aspergilli, however, is already present at that time, as shown for iron uptake from $^{55}$Fe-labeled ferrichrysin by *A. melleus* (Fig. 2).

**Chelate-iron uptake by A. quadricinctus.** The main iron chelate produced by *A. quadricinctus* is ferrichrome (E. Krezdorn, personal communication). The concentration-dependent uptake of iron from $^{55}$Fe-labeled ferrichrome revealed a saturation kinetic with $K_m \sim 5$ $\mu$M and $V_{max} \sim 0.10$ nmol of Fe per min per mg (Fig. 3a). Besides ferrichrome, ferricrocin and ferrirubin also gave similar transport kinetic data. The ferrichrysin-mediated iron uptake, however, was significantly slower with maximal rates of about 0.05 nmol of Fe per min per mg. Coprogen, an iron chelate from *Neurospora crassa* (10), and ferrioxamine B from *Streptomyces pilosus* (1) were ineffective as

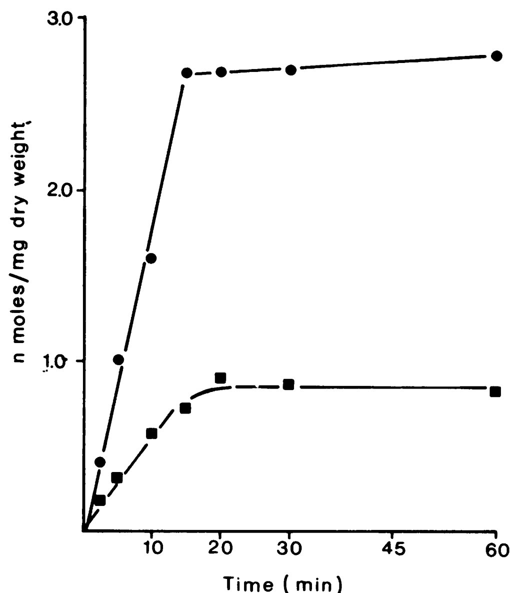



FIG. 2. *Uptake of iron from $^{55}$Fe-labeled ferrichrysin by A. melleus. The labeled chelate was added to a washed iron-deficient culture (20 h old) and incubated for 60 min at 27 C. Samples were taken at intervals, filtered, washed, and counted in a liquid scintillation counter. Symbols: ferrichrysin, 50 $\mu$M (●) and 5 $\mu$M (■).*

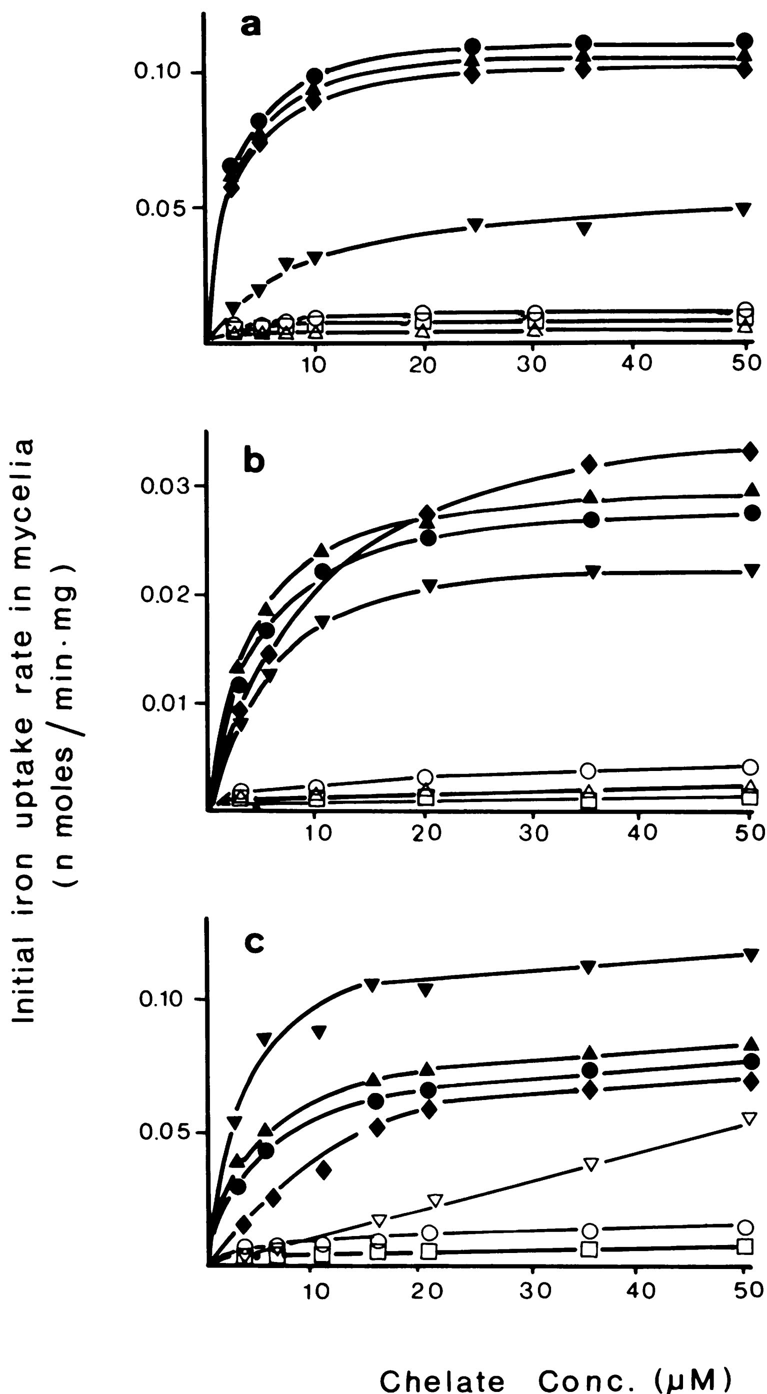



FIG. 3. *Initial iron uptake versus concentration of siderochromes measured with three different Aspergillus strains: (a) A. quadricinctus, (b) A. fumigatus, (c) A. melleus. Washed cells were exposed with fresh medium to different concentrations of $^{55}$Fe-labeled siderochromes for 10 min and were subsequently filtered, washed, and counted after 24 h of equilibration in the counting solvent. Symbols: ferrichrome (●); ferricrocin (▲); ferricrocin with sodium azide, $10^{-3}$ M (△); ferrichrysin (▼); ferrichrysin with sodium azide, $10^{-3}$ M (▽); ferrirubin (◆); coprogen (○); and ferrioxamine B (□).*

transporting agents. The typical saturation kinetics found for ferrichrome-type iron uptake were completely abolished after addition of sodium azide ($10^{-3}$ M) to the incubation medium, as shown for ferricrocin iron uptake by *A. quadricinctus* (Fig. 3a) and *A. fumigatus* (Fig. 3b) and for ferrichrysin-iron uptake by *A. melleus* (Fig. 3c). Fusigen at high concentrations showed iron uptake rates which were several-fold higher than those of the hexapeptides of the ferrichrome-type family. The kinetic data revealed $K_m$ values which were approximately 10 times higher than those found for the ferrichrome-type compounds (Fig. 4).

**Chelate-iron uptake by A. fumigatus.** *A. fumigatus* produces ferricrocin and ferrirhodin. Ferrirhodin is a ferrirubin analogue containing *cis*-anhydromevalonic acid residues instead of *trans*-anhydromevalonic acid. The iron uptake kinetics with various iron chelates showed no principal differences (Fig. 3b). Even from those iron chelates which are obviously not excreted by this strain an iron uptake could be observed. In addition, coprogen and ferrioxamine showed no iron transport properties. Fusigen iron uptake was just as much as in the *A. quadricinctus* strain. Fusigen iron uptake could, however, be depressed by addition of ferricrocin (Fig. 5). On the other hand, the ferricrocin iron uptake was slightly increased when fusigen was present.

**Chelate-iron uptake by A. melleus.** *A. melleus* is a well-known producer of ferrichrysin (2, 18). Several minor components have been found, among which ferrirubin and ferrirhodin could be identified. In contrast to the other aspergilli tested, *A. melleus* revealed the highest iron uptake rates when ferrichrysin iron was supplied. Iron from ferricrocin, ferrichrome, and ferrirubin was taken up at significantly lower rates (Fig. 3c). Fusigen iron uptake exceeded that found with the other two strains (Fig. 4). Coprogen and ferrioxamine B showed no iron donor properties in this strain.

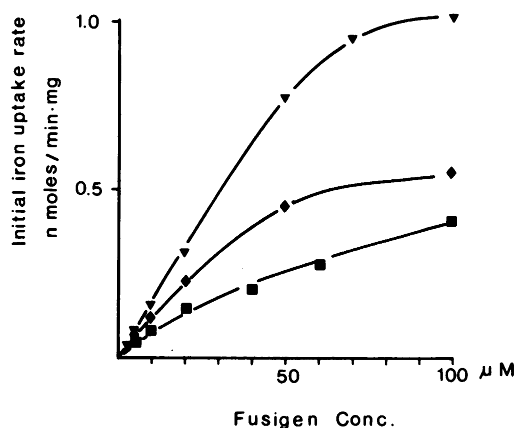



FIG. 4. *Initial iron uptake with [$^{55}$Fe]-labeled fusigen. Conditions were as described in Fig. 3. Symbols: A. quadricinctus* (■); *A. fumigatus* (◆); *A. melleus* (▼).

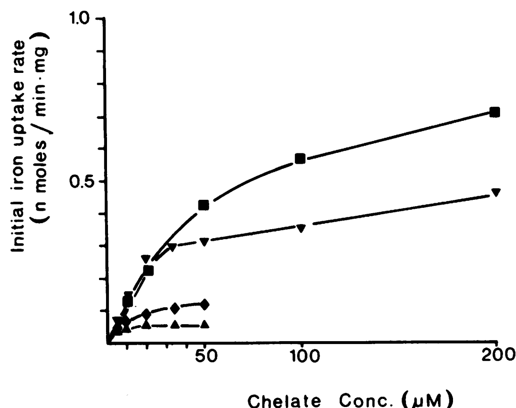



FIG. 5. *Initial iron uptake from $^{55}$Fe-labeled fusigen* (■); *$^{55}$Fe-labeled fusigen with ferricrocin, 25 µM* (▼); *$^{55}$Fe-labeled ferricrocin* (▲); *$^{55}$Fe-labeled ferricrocin with fusigen, 25 µM* (◆). *Strain: A. fumigatus.*

## DISCUSSION

Iron uptake studies on the two fungi *Ustilago spaerogena* (5) and *N. crassa* (17) have proved the existence of special iron uptake systems from siderochrome chelates in which iron is transported against a concentration gradient, showing all characteristics of an active transport, such as saturation kinetics, sensitivity for energy poisons or anaerobiosis, and competitive behavior in the presence of other fungal ferrichrome-type compounds (15). There is good evidence that these transport systems take up chelate iron by recognizing the chelate conformation (5, 6, 11, 16). The existence of siderochrome-binding proteins in membranes of bacteria have been reported (7, 14). From the observed specificity of siderochrome uptake in fungi there is every reason to believe that similar binding proteins are active during chelate iron uptake in fungi also. Previous results had revealed that *N. crassa* takes up iron from its own chelate coprogen and from the two ferrichrome-type compounds ferricrocin and ferrichrysin (15). Several other siderochromes, such as ferrioxamines, fusigen, ferrichrome A, and iron-rhodotorulic acid, were completely excluded. Emery (5) has reported that, contrary to ferrichrome, ferrichrome A is not transported in *U. spaerogena*, although both siderochromes are excreted by this strain. As *Aspergillus* produces a variety of siderochromes with some differences, depending on the type of strain, it

was of interest to know whether there exist differences in transport behavior among those siderochromes produced by the same strain and among strain-specific and exogenous siderochromes.

The three strains of *Aspergillus* studied in the present investigation exhibit fundamental differences in the pattern of the siderochromes produced under iron-deficient conditions in chemically defined medium. *A. quadricinctus* produces mainly ferrichrome, *A. fumigatus* is a ferricrocin producer, and the main siderochrome of *A. melleus* is ferrichrysin. Although the biosynthesis of the ferrichrome-type compounds of these *Aspergillus* strains is definitely prescribed, the strains do not seem to distinguish between closely related iron-chelate structures such as ferrichrome, ferricrocin, ferrichrysin, and even ferrirubin. However, uptake of ferrichrysin iron was somewhat superior in the ferrichrysin-producing strain. At present no decision can be made as to whether only one uptake system is responsible for the observed iron uptake behavior.

The strikingly different uptake kinetics found with fusigen in all strains studied should be emphasized. It may be assumed that because of the higher $K_m$ values for fusigen iron uptake separate uptake mechanisms exist. Iron transfer to endogenous acceptors may possibly be easier with fusigen than with the ferrichrome-type compounds. Diekmann (3) has reported that fusigen appeared early in the culture medium of *A. fumigatus* and some other fungi. After 3 days of fermentation fusigen may represent over 90% of the total siderochromes produced. Fusigen generally disappears in older cultures. Because of the instability of the ester groups, decomposition may occur during prolonged fermentation. Also, surface-bound esterase attack cannot be excluded. The competitive behavior between fusigen- and ferrichrome-type iron uptake is not unequivocal, as the iron of the chelates is not kinetically inert and exchange mechanisms render a conclusive interpretation more difficult. From a physiological point of view the fusigen iron supply may play a role in fast-growing young mycelia, whereas in older cultures the more stable ferrichrome-type compounds may provide the indispensible iron for growing.

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