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132.* Uptake of Iron by *Neurospora crassa*
III. Iron Transport Studies with Ferrichrome-Type Compounds

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Abstract. The transport of $^{55}Fe^{3+}$, mediated by various fungal sideramines, was tested using an ornithine-deficient mutant of *Neurospora crassa* (arg-5, ota, aga), which can be cultivated completely free of its own sideramines. We have found that *Neurospora crassa* is able to accumulate iron by its own chelate coprogen ($K_m \sim 20\ \mu M$, $V_{max} \sim 1.74$ nmol/mg min) and also by the two exogenous sideramines ferricrocin ($K_m \sim 5\ \mu M$, $V_{max} \sim 0.22$ nmol/mg min) and ferrichrysin ($K_m \sim 20\ \mu M$, $V_{max} \sim 0.4$ nmol/mg min) produced by members of the genus *Aspergillus*. Other sideramines like ferrichrome, ferrichrome A, and ferrirubin were relatively ineffective as iron transport molecules for this organism. Competitive inhibition of coprogen uptake was observed with all ferrichrome-group compounds: ferrichrysin ($K_i \sim 140\ \mu M$), ferrichrome and ferricrocin ($K_i \sim 5\ \mu M$). Ferrirubin ($K_i \sim 0.5\ \mu M$) was the strongest inhibitor of coprogen uptake in *Neurospora crassa*. Inhibition experiments indicate that ferrirubin may possibly block the uptake of coprogen and ferrichrome-type compounds by occupying the receptor sites without being preferably transported. For further characterisation of the coprogen uptake system in *Neurospora crassa* comparative uptake experiments were performed with the parent wild-type strain 74 A, and with a ferricrocin-producing *Aspergillus fumigatus* strain.

Key words: Iron Transport — Sideramines — *Neurospora* — *Aspergillus.*

A variety of iron-chelating compounds, collectively termed siderochromes have been isolated from culture fluids of fungi and bacteria. Although several siderochromes exhibit antibiotic activity, the majority of these compounds are growth promoting substances known as sideramines. It is widely accepted that siderochromes can dissolve ferric iron and facilitate its transport into microbial cells. There is, however, some uncertainty about the specificity of the iron transport systems in microorganisms. In bacteria a variety of sideramines are able to antagonize growth inhibition by sideromycins (Zähner *et al.*, 1960; Alexanian *et al.*, 1972). Recently, Luckey *et al.* (1972) reported the ability of an entero-

* 131.: Widmer, J., Keller-Schierlein, W.: Synthese des δ-N-Hydroxyarginins. Helv. chim. Acta (in preparation).

bactin-less mutant of *Salmonella typhimurium* to utilize, in addition to enterobactin, different types of siderochromes produced by other microorganisms. Our previous studies on *Neurospora crassa* revealed that ferrioxamine-type siderochromes are unsuitable iron transport molecules for the coprogen transport system (Winkelmann and Zähner, 1973). Since we have also found that *Neurospora crassa* accumulated considerable amounts of $^{55}Fe^{3+}$ by ferricrocin, we decided to investigate the uptake behaviour of iron by other ferrichrome-type compounds[1]. For further characterisation of the coprogen transport system, we studied the influence of different sideramines on coprogen uptake.

Materials and Methods

Chemicals. Ferricrocin, ferrirubin and ferrichrome A were gifts from Prof. W. Keller-Schierlein. Coprogen, rhodotorulic acid, fusigen and ferrichrome were generously supplied by Prof. H. Diekmann. Ferrioxamin B · HCl was provided by Prof. H. Zähner. $^{55}FeCl_3$ in 0.1 M HCl was purchased from Radiochemical-Centre, Amersham, England. The desferri-compounds were prepared with 8-hydroxyquinoline in 20% methanol. Salts and other chemicals, if not otherwise stated, were purchased from Merck, Darmstadt.

Fungal Strains. The strain used for the main part in these investigations was the *Neurospora crassa* triple mutant (arg-5, ota, aga), which could be cultured under sideramine-free conditions. This strain and the corresponding parent wild-type strain 74 A were gifts from Prof. R. H. Davis. *Aspergillus fumigatus* Fresenius (Tü 149) was from the stock of the Institut für Biologie, Lehrbereich Mikrobiologie.

Growth of Cultures. For all strains, a chemically defined medium was used, containing 5 g L(−)-asparagine, 1 g $K_2HPO_4 \cdot 3H_2O$, 1 g $MgSO_4 \cdot 7H_2O$, 0.5 g $CaCl_2 \cdot 2H_2O$, 100 mg L( )-arginine, 100 mg putrescine, 20 mg $ZnSO_4 \cdot 7H_2O$, 10 mg biotin and destilled H_2O to 1 l, pH 6. Glucose (2%), separately sterilized, was added to the culture flasks. After inoculation with approximately 10^9 conidia, the culture flasks containing 100 ml medium were incubated on a rotating shaker with 120 RPM at 27° C approximately 12 h. The final amount of young mycelia (dry weight) contained in the culture was determined separately before each kinetic measurement by filtration.

Kinetic Measurements. The incubation mixture for the saturation kinetic measurements contained increasing amounts of sideramines (10—100 μM) and 100 μl of a ^{55}Fe-sideramine solution (10^6 dpm/0.15 nmol) in a final volume of 2 ml. The reaction was initiated by the addition of 1.8 ml cell suspension. Before use, the cell suspension was filtered through a gauze to remove the largest mycelial pieces. The incubation mixture was shaken on a New Brunswick Rotary Shaker Model G-2 at room temperature. After 10 min of incubation, the mycelial mass was filtered and washed 3 times with 10 ml of cold 0.9% NaCl-solution. The radioactivity was then measured using 10 ml Unisolve-1 (Koch-Light Laboratories) in a Liquid Scintillation counter (Nuclear-Chicago, Mark II). Lineweaver-Burk-diagrams were plotted on a Wang 700-Computer using a n^{th} order-regression analysis. K_m and V_{max} were calculated from the printed coefficients.

1 In the present context, the following hexapeptides are designated as ferrichrome-type compounds: ferrichrome, ferrichrome A, ferricrocin, ferrichrysin, ferrirubin and ferrirhodin.

Results

Uptake of ^{55}Fe-Coprogen in *Neurospora crassa* arg-5, ota, aga and in Its Corresponding Wild-Type Strain 74A

The coprogen transport system in *Neurospora crassa* is found to be present immediately after germination of the conidia. Although some variations in the K_m values between the two strains could be observed, the most striking differences were found in the calculated V_{max} values. There is much evidence to suggest that a correlation between sideramine-deficiency and the rate of uptake exists. We have found, that the mutant, arg-5, ota, aga, which is unable to synthesize desferricoprogen under ornithine-free cultivation (Wais, 1971), exhibited a markedly higher coprogen uptake than did its corresponding wild-type strain (Fig. 1).

Concentration-Dependent Uptake of Some Exogenous ^{55}Fe-Sideramines

A more detailed experimental approach concerning the specificity of the coprogen transport system in *Neurospora crassa* arg-5, ota, aga revealed, that coprogen is not the only sideramine possessing iron transport properties in this organism. As is demonstrated in Fig. 2, ferricrocin and ferrichrysin are also relatively good iron-transport molecules. From the observation, that the simplest compound of the ferrichrome group,

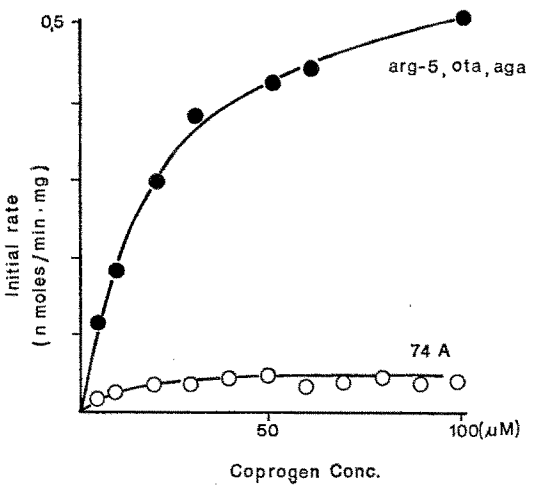

Fig. 1. Plot of initial uptake rate vs. coprogen concentration for the *Neurospora crassa* mutant arg-5, ota, aga and for the parent wild-type strain 74A. The initial rate was determined after 10 min incubation of young mycelia in the cultivation medium containing increasing amounts of ^{55}Fe-coprogen. The mycelia were filtered, washed with 3×10 ml cold 0.9% NaCl-solution and counted with 10 ml Unisolve-1

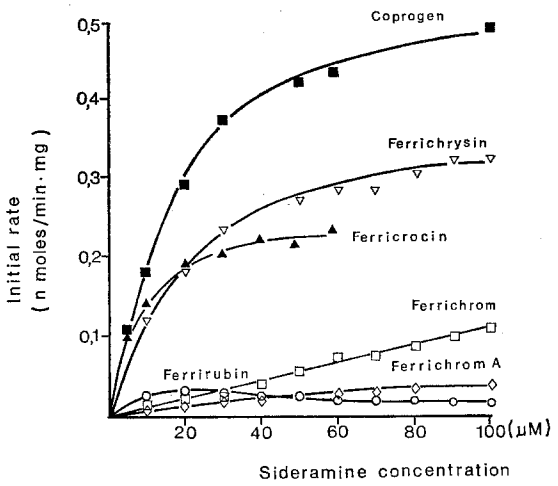

Fig. 2. Initial rate of uptake vs. concentration relationship for various ^{55}Fe-siderамines of the ferrichrome-type group by *Neurospora crassa* (arg-5, ota, aga). The conditions are as described in Fig. 1

ferrichrome, showed very different uptake kinetics, it may be assumed that OH-groups may be involved in uptake mechanism. Although coprogen exhibited the highest rate of uptake (~ 2 nmol/min · mg dry weight), ferricrocin seems to be the compound possessing the higher affinity for the uptake system. The mean K_m values for coprogen and ferrichrysin approximated 20 μM, whereas for ferricrocin K_m values of 5 μM were measured. The other sideramines, ferrichrome, ferrichrome A and ferrirubin exhibited significantly lower transport properties.

Inhibition of ^{55}Fe-Coprogen Uptake by Other Sideramines

The fact that sideramines other than coprogen could be accumulated by *Neurospora crassa* suggests that a competition during uptake may be involved. Indeed, such molecules, that were taken up in similar amounts proved to be good competitive inhibitors of coprogen transport (Fig. 3). Even ferrichrome, which seemed to be taken up in a non-saturable manner, revealed good competitive properties. The most surprising finding, however, was that ferrirubin, which is obviously not accumulated in significant amounts, could act as a powerful inhibitor of coprogen uptake. The degree of inhibition can be estimated from the K_i value, which describes the affinity of the cells for the inhibitor: ferrirubin ($K_i \sim 0.5$ μM), ferricrocin ($K_i \sim 5$ μM), ferrichrome ($K_i \sim 5$ μM) and ferrichrysin ($K_i \sim 140$ μM).

Some other sideramines such as ferrioxamine B, fusigen, rhodotorulic acid, ferrichrome A and ferrirhodin were also tested. They inhibited

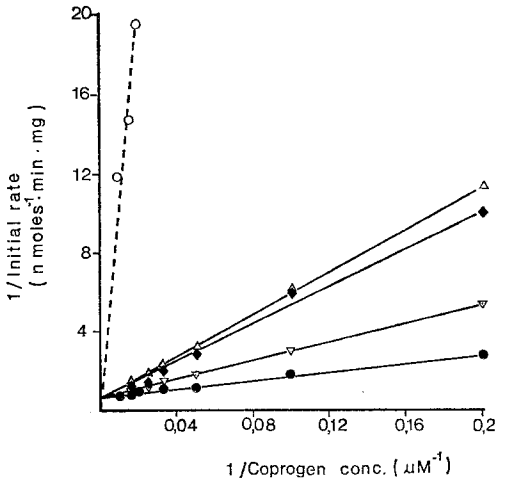

Fig. 3. Plot of the reciprocal initial rates of uptake against the reciprocal concentrations of ^{55}Fe-coprogen (Lineweaver-Burk-Plot). ^{55}Fe-coprogen without inhibitor • and in the presence of 50 μM ferrichrysin ▽, 50 μM ferrichrome ◆, 50 μM ferricrocin △ and 50 μM ferrirubin ○. Organism: *Neurospora crassa* (arg-5, ota, aga). Conditions as described in Fig. 1

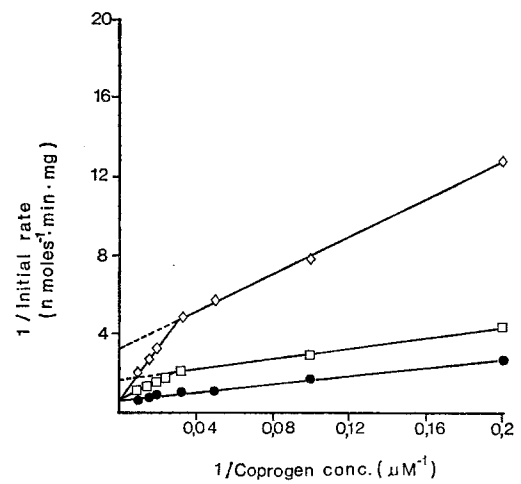

Fig. 4. Double reciprocal plot (Lineweaver-Burk-Plot) for ^{55}Fe-coprogen uptake without inhibitor • and in the presence of 50 μM ferrichrome A ◇ and 50 μM rhodotorulic acid □. Organism: *Neurospora crassa* (arg-5, ota, aga). Conditions as described in Fig. 1

coprogen uptake only to a small extent. The more complex kinetic data for the inhibition of coprogen uptake by ferrichrome A and rhodotorulic acid are given in Fig. 4. These two compounds revealed competitive behaviour only at high concentrations, whereas at low concentrations

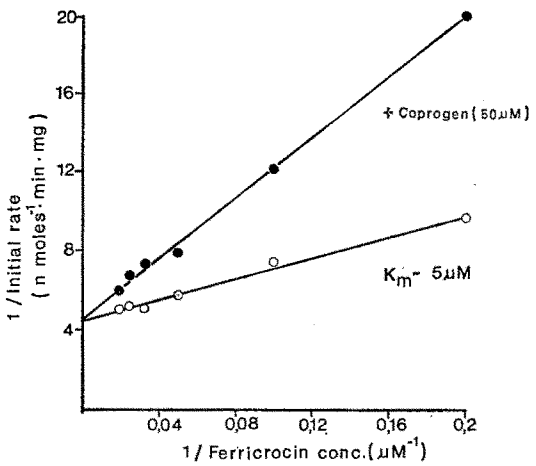

Fig. 5. Double reciprocal plot (Lineweaver-Burk-Plot) for ^{55}Fe-ferricrocin uptake ○ and in the presence of 50 µM coprogen ●. Organism: *Neurospora crassa* (arg-5, ota, aga). Conditions as described in Fig. 1

they showed a pronounced non-competitive behaviour, which might be attributed to their negative charge. The affinity constant for ferrichrome A ($K_i \sim 6.5$ µM) thus lies in the same range as those of the other ferrichrome group compounds. Ferrichrome A is related to the ferrirubin molecule, but differs from it in that it possesses 3 β-methylglutaconic acid moieties instead of 3 trans-5-hydroxy-3-methyl-pentenoic-(2)-acid moieties (Keller-Schierlein, 1963). In order to conclude that two compounds of related structure are transported by the same transport system, it has been proposed that several conditions should be met (Sanford and Smyth, 1972): The corresponding K_m and K_i values should be the same for each compound, and a third compound should possess the same K_i value when acting as an inhibitor of each compound. Our results indicate that the above-mentioned conditions are fulfilled.

The apparent Michaelis constant for coprogen uptake was 20 µM. When acting as an inhibitor of the ferricrocin uptake the K_i value also approximated 20 µM. The corresponding values for ferricrocin uptake and for ferricrocin acting as an inhibitor of coprogen uptake were 5 µM (Fig. 5). Moreover, ferrirubin exhibited a high inhibitory effect directed towards both uptake processes ($K_i \sim 0.5$ µM). These results indicate that several ferrichrome-group iron chelates are more or less suitable for the coprogen transport system in *Neurospora crassa*.

The Inhibitory Effect of Ferrirubin

From Fig. 2 it was clear, that ferrirubin itself was not accumulated in higher amounts, as demonstrated in the low rate of uptake. The strong

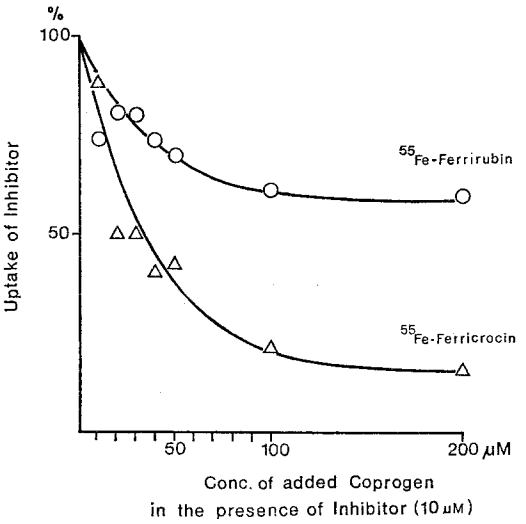

Fig. 6. Uptake of $^{55}Fe^{3+}$ mediated by ferrirubin ○ and ferricrocin △ in the presence of increasing amounts of coprogen. Organism: *Neurospora crassa* (arg-5, ota, aga). Incubation time: 10 min

inhibitory effect of ferrirubin on coprogen and ferricrocin uptake therefore cannot be due to a competition of the translocation process. It seems more probable, that the specific binding, which is responsible for the observed saturation kinetics and which is the reason for the high specificity of the iron transport molecules, is blocked. Some experiments should prove this hypothesis. If ^{55}Fe-ferrirubin and, for comparison, ^{55}Fe-ferricrocin is added to the incubation medium together with increasing amounts of coprogen, uptake of each inhibitor will decrease (Fig. 6). The results clearly demonstrate the competitive character of a compound which is not transported in greater amounts. Preincubation with ferrirubin revealed that the receptors for coprogen uptake are occupied within a few minutes. The uptake of ^{55}Fe-ferricrocin is also far more inhibited by ferrirubin than by other sideramines (Table 1).

If a tight connection between recognition and translocation of $^{55}Fe^{3+}$ or the whole chelate during uptake exists, a displacement of coprogen from the receptor sites is difficult to demonstrate. We would expect that only minimal amounts of ^{55}Fe-coprogen can be displaced after a short time of incubation; the greater part of radioactivity will be located inside the cell. Nevertheless, Table 2 shows that the displacement of ^{55}Fe-coprogen is demonstrable for several sideramines, but it is most effective for ferrirubin. These data correspond roughly with the observed K_i values. But they also demonstrate that the strong inhibitory effect of

Table 1. Inhibition of ferricrocin uptake by ferrirubin, coprogen and ferrichrysin[a]

Inhibitor added	% Inhibition
0.1 μM ferrirubin	63
10.0 μM ferrirubin	94
50.0 μM ferrirubin	98.2
50.0 μM coprogen	64
50.0 μM ferrichrysin	45

[a] The incubation mixture contained 10 μM ^{55}Fe-ferricrocin and the above specified inhibitors with young mycel in culture medium in a volume of 2 ml. After 10 min incubation on a rotating shaker at room temperature the mycelial mass was filtered, washed and counted.

Table 2. Displacement of previously taken up coprogen by various sideramines[a]

Sideramine added	% Coprogen displaced
Ferrichrysin	2.6
Ferricrocin	12.0
Ferrichrome	12.0
Coprogen	12.0
Ferrirubin	16.2

[a] Young mycelia were preincubated for 10 min with 10 μM ^{55}Fe-coprogen in culture medium. The control was filtered, washed and counted, to the remaining incubation mixtures 100 μM of the above specified sideramines were added. After 10 min of further incubation the uptake values were compared with the control.

ferrirubin may be attributed rather to the inability to be transported than to the higher affinity.

Based on these findings we are able to estimate the numbers of transport sites, assuming that each site will be blocked by one molecule of ferrirubin. After saturation with ferrirubin we found 0.2 nmol ferrirubin bound to 1 mg dry weight of young mycelia of *Neurospora crassa*, which corresponds to 12×10^{13} coprogen transport sites per mg dry weight.

Uptake Studies on *Aspergillus fumigatus*

The data of sideramine uptake in *Neurospora crassa* might suggest that coprogen may possibly be also a suitable iron transport molecule for those organisms which produce ferricrocin or ferrichrysin. *Aspergillus fumigatus*, under iron deficient conditions will take up desferri-fusigen as well as desferri-ferricrocin. Measuring the uptake of ^{55}Fe-ferricrocin and ^{55}Fe-coprogen (Fig. 7) showed, that the initial velocity of coprogen uptake is considerably slower than that of ferricrocin uptake, with K_m values of 111 μM and 4.7 μM, respectively. Further, the uptake of ^{55}Fe-coprogen is obviously unaltered by the addition of ferrirubin, whereas inhibition of ^{55}Fe-coprogen uptake could be observed after addition of

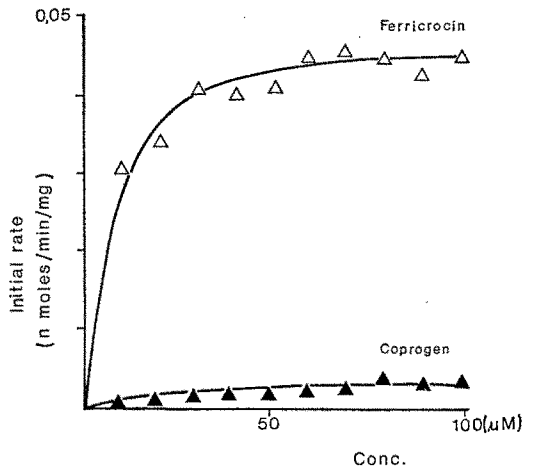

Fig. 7. Concentration-dependent uptake of ^{55}Fe-ferricrocin △ and ^{55}Fe-coprogen ▲ by *Aspergillus fumigatus*. The initial uptake rates were determined after 10 min incubation of young mycelia (24 h cultivation), in the same medium as used for *Neurospora crassa*. Conditions as described in Fig. 1

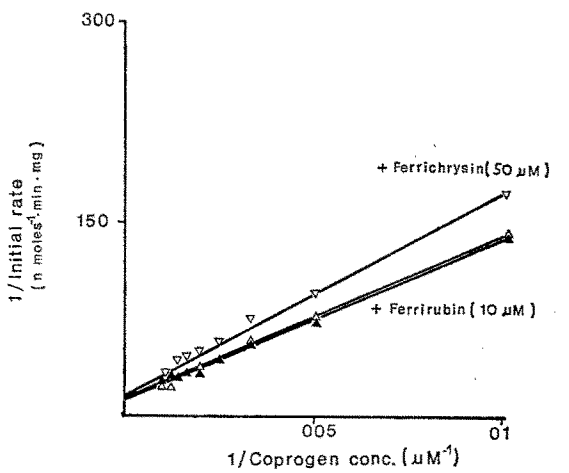

Fig. 8. Double reciprocal plot (Lineweaver-Burk-Plot) showing the inhibitory influence of 10 μM ferrirubin △ and 50 μM ferrichrysin ▽ on ^{55}Fe-coprogen uptake in *Aspergillus fumigatus*. ^{55}Fe-coprogen without inhibitor ▲. Conditions as described in Fig. 7

ferrichrysin (Fig. 8). Hence, the transport system in *Aspergillus fumigatus* does appear to favour ferrichrome group compounds and might afford other properties of the iron transport molecules. This finding emphasizes

the tight relation between specific coprogen uptake and ferrirubin action. The fact, that ferricrocin uptake is inhibited by ferrirubin in coprogen producing organisms but not in ferricrocin producing organisms additionally support the hypothesis, that uptake of various sideramines of the ferrichrome group in *Neurospora crassa* proceeds by only one sideramine uptake system with a relatively broad specificity.

Discussion

Although, in the present investigation, only the fate of the radioactivity of iron was followed, other observations justify the assumption that the whole molecule is taken up. Previous experiments with ^{14}C-labelled coprogen (Winkelmann and Zähner, 1973; Winkelmann *et al.*, 1973) have shown that in the sideramine-deficient *Neurospora crassa* mutant (arg-5, ota, aga) coprogen is taken up as a whole. Moreover, uptake of ^{14}C-labelled ferrichrysin in a wild-type strain of *Neurospora crassa* Shear et Dodge (Tü 154) has been reported (Hartmann, 1973). It is, however, necessary to differentiate between the process of uptake and the process of translocation within the cell. Although the whole iron chelate may be taken up, only the iron may be translocated through the membrane. At present, however, nothing is known about details of the coprogen transport. As our experiments were performed with an ornithine-free organism, any iron transfer among those sideramines supplied and other endogenous ornithine-containing sideramines, which may possibly occur in wild-type strains, can be excluded.

The present kinetic studies with different sideramines represent further evidence for the existence of specific iron uptake mechanisms in microorganisms. In *Neurospora crassa* none of the exogenous sideramines tested exhibited a higher uptake-rate than coprogen. Although it can be inferred from the observed values for K_m and K_i that at least at higher concentrations ferricrocin and ferrichrysin possibly use the coprogen transport system, other uptake system cannot be entirely excluded (Christensen, 1969). At low concentrations the interaction of rhodotorulic acid and ferrichrome A with coprogen uptake is obviously not competitive suggesting two different uptake ways. It must be pointed out, however, that in some cases, a larger part of non-mediated transport may possibly be measured. This means that some compounds, like ferrichrome, possess considerable diffusion properties. Because of the relatively high inhibition of coprogen uptake by these compounds, it could be assumed that although migration inside the cell is possible, their affinity to the receptor sites is reduced. The other extreme is that a compound may have such a strong interrelation with the receptor sites that a further transport inside the cell is impossible. Such a mechanism may possibly exist for ferrirubin in *Neurospora crassa*. Ferrirubin seems to be the first sideramine exhibiting specific inhibition of sideramine uptake. The mode of action may be

similar to the sideramine antibiotics such as albomycin and ferrimycin (Zähner *et al.*, 1960), concerning their specific affinity to sideramine transport systems. The antibiotic effect of the ferrimycins, however, is believed to develop inside the cell (Knüsel *et al.*, 1969) and is not a result of iron deprivation. At present, however, nothing is known about the structural requirements of the coprogen molecule necessary for specific uptake. Previous results have revealed that the central ion plays an important role in the accumulation of coprogen (Winkelmann *et al.*, 1973). Data obtained in this investigation may possibly suggest that OH-groups are required for recognition by the receptor sites in the *Neurospora* coprogen transport system. From the possible significance of OH-groups for mediated uptake of sideramines, it could be assumed that these sideramines are transported by a system analogous to that described for sugar transport (Cirillo, 1972). In *Neurospora*, a variety of sugar transport systems exists (Scarborough, 1973). Although such a system may be responsible for coprogen uptake, it seems more probable that special iron-chelate transport systems have evolved in microorganisms. As reported for the inhibitory action of diphenolic compounds, on sugar transport, ferrirubin may similarly cause a deformation of the orientation of the coprogen receptor sites or a generalized non-specific obstruction by hydrophobic attachment to adjacent regions. Specific interactions, however, seem to be more probable. Coprogen and ferrirubin both contain trans-5-hydroxy-3-methylpentenoic-(2)-acid moieties, which may be the reason for the strong competitive character of ferrirubin during uptake. As ferricrocin and ferrichrysin lack such structural similarities, trans-5-hydroxy-3-methyl-pentenoic-(2)-acid does not seem to be required for uptake. Ferrichrysin and ferricrocin only possess serine-OH groups. The fact that the uptake of these compounds is also inhibited by ferrirubin, may account for the conception of only one uptake system in *Neurospora crassa*. On the other hand, the trans-5-methyl-3-pentenoic-(2)-acid moieties may be directly involved in a transport regulating mechanism of the coprogen transport system. Because of the peptide nature of the iron chelates, a peptide transport system would be an attractive model. Payne and Gilvarg (1971) have summarized the properties of amino-acid transport systems and peptide transport systems. Because of the essentiality of free α-amino groups for both transport systems, identification of the coprogen transport system with one of these systems may be excluded. From the results obtained with *Aspergillus fumigatus* it can be assumed that in this organism coprogen uptake is an unspecific uptake. This has been further substantiated by demonstrating the ineffectiveness of ferrirubin action. The inhibition of coprogen-mediated iron uptake by ferrichrysin, however, seems to provide evidence that in this case coprogen or iron from coprogen can be slowly transported by a possibly existing ferrichrome-type uptake system of *Aspergillus* strains.

These findings may be analogous to the observed sideramine-mediates iron uptake by the coprogen transport system in *Neurospora crassa*.

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