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Ferricrocin functions as the main intracellular iron-storage compound in mycelia of *Neurospora crassa*

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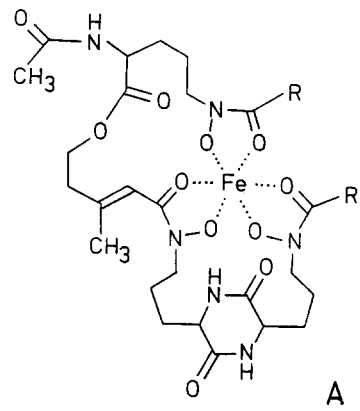
Summary. *Neurospora crassa* produces several structurally distinct siderophores: coprogen, ferricrocin, ferrichrome C and some minor unknown compounds. Under conditions of iron starvation, desferricoprogen is the major extracellular siderophore whereas desferriferricrocin and desferriferrichrome C are predominantly found intracellularly. Mössbauer spectroscopic analyses revealed that coprogen-bound iron is rapidly released after uptake in mycelia of the wild-type *N. crassa* 74A. The major intracellular target of iron distribution is desferriferricrocin. No ferritin-like iron pools could be detected. Ferricrocin functions as the main intracellular iron-storage peptide in mycelia of *N. crassa*. After uptake of ferricrocin in both the wild-type *N. crassa* 74A and the siderophore-free mutant *N. crassa arg-5 ota aga*, surprisingly little metabolization (11%) could be observed. Since ferricrocin is the main iron-storage compound in spores of *N. crassa*, we suggest that ferricrocin is stored in mycelia for inclusion into conidiospores.

Key words: Siderophores — Ferricrocin — Iron storage — Sporulation — In vivo Mössbauer spectroscopy

Introduction

Iron is required for an enormous variety of metabolic processes in virtually all organisms. The low solubility of Fe^{3+} under aerobic conditions at near neutral pH severely restricts its bioavailability. Thus, microorganisms have evolved siderophores (see, e.g., Fig. 1), low-molecular-mass (0.5–1.5 kDa) ferric ion chelators, exhibiting extraordinarily high complex formation constants (Raymound et al. 1984). The major role of siderophores is extracellular solubilization of iron from minerals or organic substrates under conditions of cellular iron deprivation. Siderophore-mediated transport of Fe^{3+} is in general energy-dependent



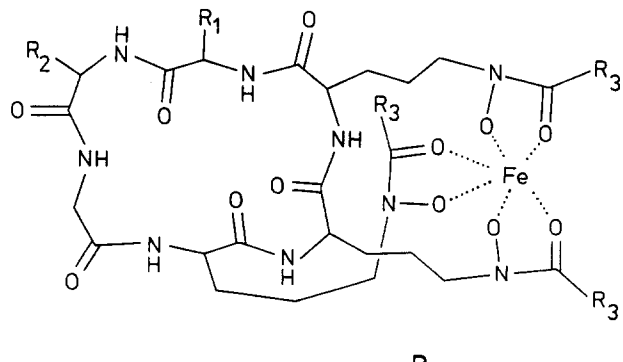


Fig. 1A, B. Structural formulae of **(A)** coprogen [R = $-CH=C(CH_3)-CH_2)_2OH$] and **(B)** ferricrocin ($R_1=H$, $R_2=CH_2OH$, $R_3=CH_3$)

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and involves stereo- and enantio-specific membrane-receptor and transport systems (Chung et al. 1986; Huschka et al. 1985, 1986). Recently we demonstrated that siderophores are not restricted to iron solubilization and transport. In addition, they exhibit iron-storage functions in mycelia and spores of certain fungi (Matzanke et al. 1987a, c).

Under conditions of iron deficiency various siderophores are synthesized by the fungus *Neurospora crassa*. The major component excreted is desferricoprogen (Fig. 1A). Relatively high amounts of a second siderophore, ferricrocin (Fig. 1B), can be extracted from iron-supplied mycelia (Horowitz et al. 1976). Only traces of ferricrocin are detectable in the extracellular space. However, *N. crassa* displays comparable uptake rates for both ferricrocin and coprogen (Huschka et al. 1985). The structures of these two trihydroxamate siderophores are very different. Whereas ferricrocin belongs to the family of ferrichrome-type cyclic hexapeptides, coprogen, containing a diketopiperazine ring, is a noncyclic compound. In fact, there are indications that the transmembrane transport (Huschka et al. 1985, 1986) and the intracellular role of these two siderophores might differ. Ferricrocin is regarded as an essential factor in germination (Horowitz et al. 1976). Recent observations derived from Mössbauer spectra revealed an iron-storage function of ferricrocin in spores of *Neurospora* (Matzanke et al. 1987c). Moreover, ferricrocin is formed by coprogen degradation products in *Neurospora crassa arg-5 ota aga*, a siderophore-free mutant, at a late state of growth (Matzanke et al. 1987a).

In order to clarify the intracellular role of ferricrocin, the application of classical methods like radioactive labelling is insufficient. Thus, we employed in vivo Mössbauer spectroscopy which allows iron uptake and transfer to be monitored and discrimination between different iron environments and redox states. This technique has proved to be powerful in such investigations (Matzanke and Winkelmann 1981; Matzanke et al. 1986a, b; Matzanke 1987; Matzanke et al. 1987a–c). In this study we have followed uptake and metabolization of [^{57}Fe]coprogen and [^{57}Fe]ferricrocin in *N. crassa* 74A. We present evidence for a rapid intracellular iron transfer from coprogen to desferriferricrocin and for a significant metabolic intertness of ferricrocin in mycelia of *N. crassa*.

Materials and methods

Preparation of [^{57}Fe]siderophores. ^{57}Fe (enrichment: 95%) was purchased from Rohstoffeinfuhr, Düsseldorf. The metal was dissolved in a small volume of HNO_3/HCl (1:2, by vol.). The pH of the solution was adjusted to 1.0 with KOH. Isolation, purification, and preparation of desferri-ferricrocin and desferri-coprogen was performed as described previously (Wong et al. 1983). Formation of [^{57}Fe]siderophores was achieved by mixing an equimolar solution of $^{57}Fe^{3+}$ with an aqueous solution of desferrisiderophore. The pH was quickly adjusted to 6 and the mixture was stirred for 4 h to complete the reaction. The red-brown complexes were passed through an XAD-2 column (Serva, Heidelberg, FRG) (Wong et al. 1983) and the purity was checked by thin-layer chromatography (TLC) on silica gel using freshly prepared solvent sytem I (chloroform/methanol/water, 70/24/4, by vol.) and solvent system II (*n*-propanol/acetic acid/water, 80/20/20, by vol.). R_f values were 0.31, 0.21 in solvent system I and 0.10, 0.15 in solvent system II for ferricrocin and coprogen respectively. The volume of the siderophore solutions wasd adjusted with incubation buffer (pH 5.6) to a final complex concentration of 4 mM. Concentrations were determined spectrophotometrically on an HP8450A spectrophotometer (Hewlett-Packard Co., Palo Alto, Calif.) using the molar absorption coefficients reported elsewhere (Wong et al. 1983).

Cultures and maintainance. N. crassa arg-5 ota aga and the corresponding wild-type strain *N. crassa* 74A were a gift from R. H. Davis, Irvine, Calif. Since biosynthesis of ornithine is blocked in *N. crassa arg-5 ota aga*, no desferrisiderophore is produced by this mutant under iron-limited growth conditions in ornithine-free media. The strains were maintained on YMG agar containing 4 g yeast extract (Difco laboratories, Detroit, Mich.), 10 g malt extract (Oxoid Ltd, London, England) and 4 g glucose/l. Conidiospores were harvested after 2 weeks of growth at 27° C with saline (0.9%) containing 0.5% Tween 80. The conidiospores were washed twice with saline by centrifugation. Optimum germination was achieved when freshly harvested spores were kept for 48 h at 4° C. Spores (6×10^8) were used to inoculate 1 l of a chemically defined low-iron medium containing 5 g L-aspargine, 1 g $K_2HPO_4 \cdot 3H_2O$, 1 g $MgSO_4 \cdot 7H_2O$, 0.5 g $CaCl_2 \cdot 2H_2O$, 20 mg $ZnSO_4 \cdot 7H_2O$, 0.01 mg biotin, 50 mg L-arginine, 100 mg putrescin, and 1 l distilled water (pH 6). Glucose (4%) was added after separate sterilization.

Sample preparation. The spores were incubated at 27° C in a rotary shaker at 120 rpm. After 72 h the mycelia were washed twice with 0.9% NaCl solution and were resuspended in fresh minimal medium. Depending on the experiment, [^{57}Fe]ferricrocin or [^{57}Fe]coprogen was added to the culture to give a final metal complex concentration of 20 μM. After 1 h of incubation, cells were washed again to remove residual [^{57}Fe]siderophore and resuspended again for additional growth. Cell material was removed at various stages of additional growth (see Results), cooled to 0° C within 1 min, loaded into a cylindrical Delrin Mössbauer sample holder (volume 1.3 ml), shock-frozen to 78 K, and kept at this temperature until measurement. Sample thickness did not exceed 9 mm. Samples of ferricrocin were prepared as lyophilized powder, frozen aqueous solution diluted with bovine serum albumin (BSA) (200 mg/ml), and lyophilized powder of a ferricrocin/BSA solution (1:200, wt/wt). Spectra of coprogen have been described elsewhere (Matzanke et al. 1987a).

Mössbauer measurements. Measurements were performed at 4.2 K in horizontal transmission geometry. Further conditions have been reported previously (Matzanke et al. 1986b, 1987a). Spectral analysis was executed by subtracting nonphysical fits of the [^{57}Fe]coprogen and [^{57}Fe]ferricrocin spectra from the

measured cell spectra yielding the contribution of the siderophores to the envelope spectra. The resulting metabolite spectra were processed further by stripping and fit procedures.

Extraction of siderophores from mycelia. For extraction and purification of desferriferricrocin from iron-deficient cultures of *N. crassa* 74A, EDTA-washed glassware and double-distilled water were used throughout. The mycelium was washed free of medium, resuspended in methanol and stirred at 60° C for 1 h. Trichloroacetic acid was added to the filtrate and the precipitate was removed by centrifugation and discarded. The supernatant was evaporated three times to dryness, dissolved again in ethanol and allowed to stand at 4° C for 24 h. The precipitate was removed again by centrifugation. Extraction of ferric siderophores from mycelia of *N. crassa* 74A and *N. crassa arg-5 ota aga* was achieved by the same procedure except that no iron-limited conditions were required. The extracts were tested for siderophores by TLC using the solvent systems I and II mentioned above. The brown TLC spots were characterized by the reference siderophores ferricrocin, coprogen and ferrichrome C. Desferrisiderophores were made visible by formation of the brown ferric ion complexes.

Results

In Fig. 2A the Mössbauer spectrum of ferricrocin powder recorded at 4.2 K is shown. As in the related paramagnetic ferrichrome A, strong spin-spin relaxation effects can be observed. However, only slight residual relaxation is observed at 4.2 K if the material is diluted with bovine serum albumin (BSA). BSA simulates the intracellular protein-rich environment and it minimizes spin-spin interactions of the metal centers. The spectrum shown in Fig. 2B represents a frozen aqueous solution of ferricrocin diluted with BSA (200 mg/ml) measured at 4.2 K. Changes of pH in the range 4–7.5 do not alter the Mössbauer spectra of ferricrocin. The spectra of other members of the ferrichrome family like ferrichrome, ferrichrysin, and ferrirubin are very similar to ferricrocin (data not shown). Figure 2C shows the spectrum of a ferricrocin sample, which was diluted with BSA and lyophilized. Again, the spectrum is well resolved, indicating only slight residual relaxation. However, the overall splitting differs from that in the spectrum of Fig. 2B. The spectrum of the frozen aqueous sample spans a range from -7.9 mm s^{-1} to $+9.5$ mm s^{-1}, whereas the absorption pattern of the freeze-dried sample stretches from -7.7 mm s^{-1} to $+9.2$ mm s^{-1}. When the lyophilized sample is dissolved again in H_2O (200 mg BSA/ml), the same Mössbauer spectrum is obtained as shown in Fig. 2B. This difference of the overall splitting was unexpected and needs a detailed analysis in a forthcoming paper. It indicates, however, that ferricrocin spectra are very sensitive to changes of the microenvironment

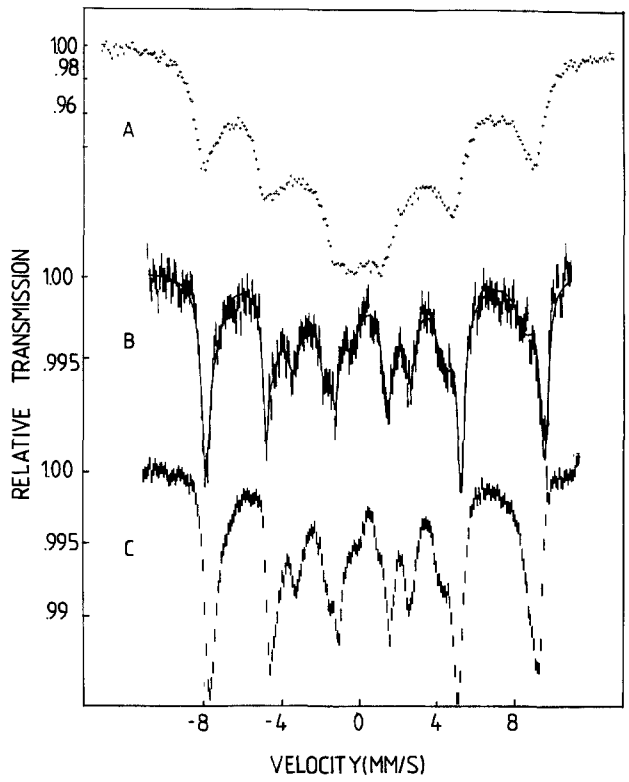

Fig. 2A–C. Mössbauer spectra of [^{57}Fe]labeled ferricrocin at 4.2 K in a magnetic field $H_{app} = 20$ mT perpendicular to the γ-rays. **A** A powder of ferricrocin; **B** a frozen aqueous solution, diluted with BSA; **C** a lyophilized powder of **B**. The solid line in **B** results from a non-physical least-square fit in order to obtain a theoretical envelope spectrum used for stripping ferricrocin contributions from measured cell spectra

which has to be born in mind when ferricrocin subspectra are subtracted from the spectra of whole cells. Fortunately, the absorptions due to ferricrocin observed in cell spectra (Figs. 3, 5, 6) are in most cases very similar to those shown in Fig. 2B, thus enabling an unambigous identification of the ferricrocin contribution to the cell spectra. The spectrum of frozen aqueous coprogen diluted with BSA has been reported elsewhere (Matzanke et al. 1987a). It can be distinguished easily from the various ferricrocin-type and other ferrichrome-type spectra since the outermost resonance absorptions are near -7.7 mm s^{-1} and $+9.0$ mm s^{-1}. Furthermore, the absorptions are considerably broader than those of ferricrocin.

Ferricrocin uptake was monitored by Mössbauer spectroscopy in both *N. crassa* 74A and the siderophore-deficient mutant *N. crassa arg-5 ota aga*. Figure 3 shows spectra of whole cells of *N. crassa arg-5 ota aga*. After growth for 72 h in a low-iron mineral salt medium, the mycelia were

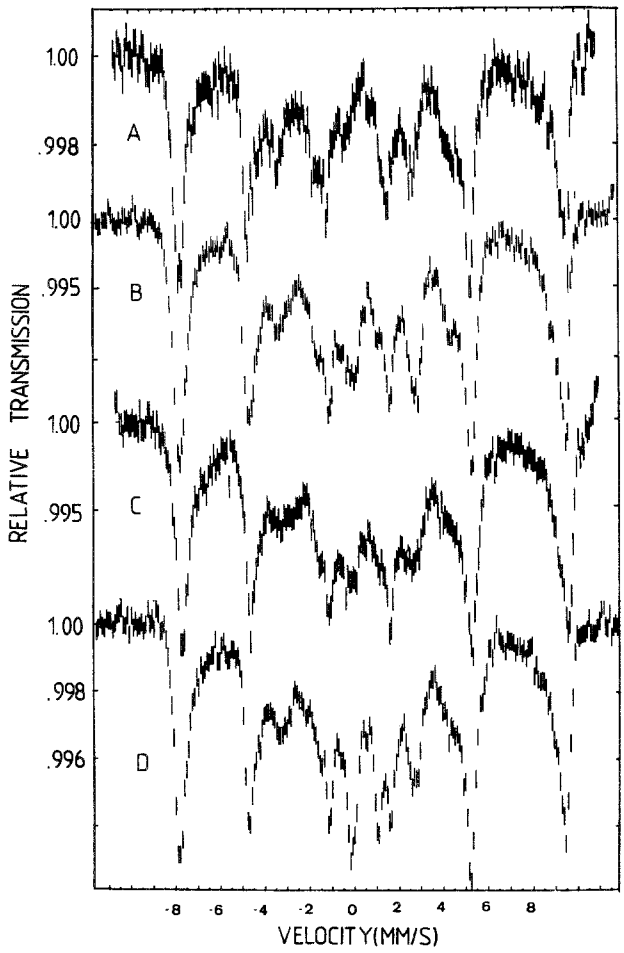

Fig. 3A–D. Mössbauer spectra of frozen *N. crassa arg-5 ota aga* cells recorded at 4.2 K in a perpendicular field of 20 mT **(A–D)**. The mycelia were grown in iron-deficient minimal medium and subsequently incubated for 1 h with [^{57}Fe]ferricrocin. The cells were then washed and grown for an additional period of 0.5 h **(B)**, 4 h **(C)**, and 65 h **(D)** in minimal medium. For comparison, the spectrum of [^{57}Fe]ferricrocin is included **(A)**

incubated for 1 h with [^{57}Fe]ferricrocin. Iron metabolization was stopped by shock-freezing ice-cold filtered mycelia after 0.5, 4.0, and 65 h of additional growth (Fig. 3B–D). For comparison a spectrum of aqueous [^{57}Fe]ferricrocin, magnetically diluted with BSA, is included (Fig. 3A). All cell spectra exhibit magnetically split subspectra which closely resemble ferricrocin. From these spectra the ferricrocin subspectrum was stripped yielding ferricrocin contributions to the envelope spectra of 99%, 89%, and 90%, respectively. Unexpectedly, over the whole time range observed, the proportion of iron metabolites did not exceed 11%. The stripped metabolite spectra are shown in Fig. 4A–C. After 0.5 h of metabolization, a broadened Fe^{2+} high-spin species could be observed ($\delta = 1.2$ mm s^{-1}, $\Delta E_Q = 2.95$ mm s^{-1}) and an additional component, which probably represents Fe^{3+} in a high-spin state ($\delta = 0.45$ mm s^{-1}; $\Delta E_Q = 1.2$ mm s^{-1}). After 4 h of metabolization, the metabolite spectrum was broad and featureless. After 65 h of further growth, the stripped spectrum exhibited an Fe^{3+} species similar to that of Fig. 4A. In addition, a magnetic split species might also be present which was not, however, analyzed further.

Figure 5 displays spectra of [^{57}Fe]ferricrocin uptake and metabolization in wild-type *N. crassa* 74A over a time range of 65 h. Conditions were kept as in the previous experiment with *N. crassa arg-5 ota aga*. Again, stripping ferricrocin from the cell spectra revealed that very little iron was transferred from ferricrocin to iron-containing metabolites, namely, 5%, 10% and 10% after 0.5 h, 4 h and 65 h of growth, respectively (stripped spectra not shown).

In the envelope cell spectra of *N. crassa* 74A after 4 h and of *N. crassa arg-5 ota aga* after 27 h of [^{57}Fe]ferricrocin metabolization (not shown), again, 65% and 85% of the total absorption area, respectively, could be attributed to ferricrocin. However, subsequent to ferricrocin stripping, an

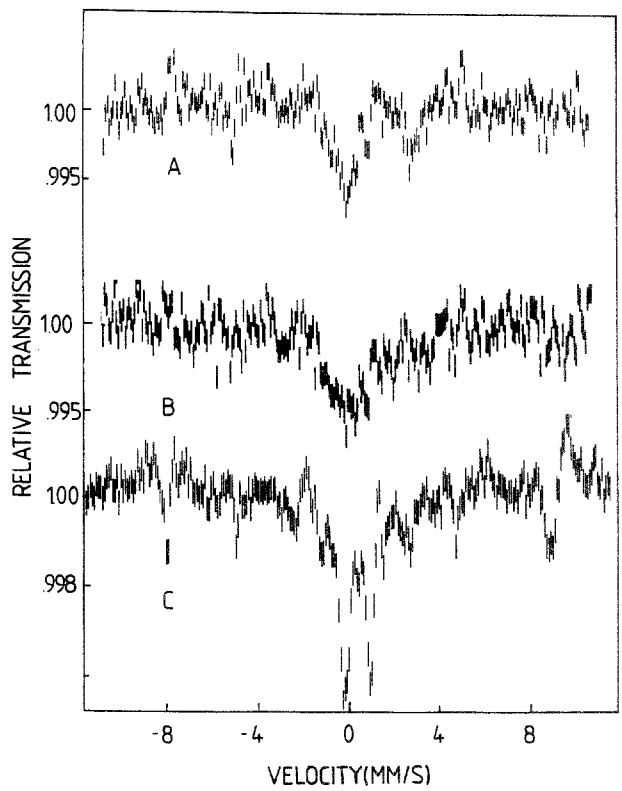

Fig. 4A–C. Mössbauer spectra of frozen *N. crassa arg-5 ota aga* cells corresponding to Fig. 3 B–D after stripping of ferricrocin

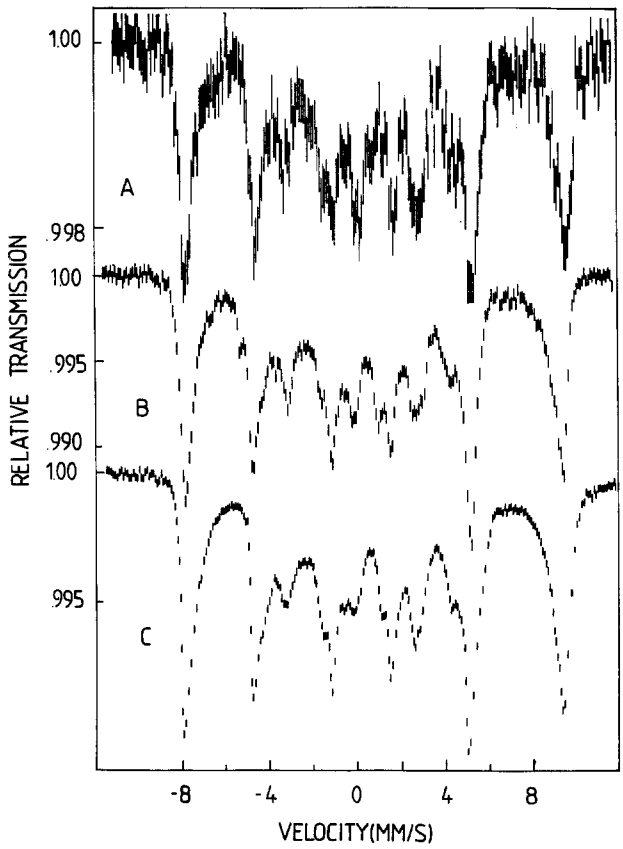

Fig. 5A–C. Mössbauer spectra of *N. crassa* 74A. Growth and uptake conditions were kept as described in Fig. 3. Subsequent to [^{57}Fe]ferricrocin uptake, additional growth was allowed for 0.5 h **(A)**, 4 h **(B)** and 65 h **(C)**

additional magnetic species was detectable in the Mössbauer spectra. This component fits the spectrum in Fig. 2C, indicating a different ferricrocin microenvironment or another iron complex. In order to clarify, siderophores have been isolated from cell extracts of mycelia grown under the same conditions as the Mössbauer samples (see Materials and methods). On TLC major spots were obtained with $R_f = 0.30$, 0.31 in solvent system I and $R_f = 0.11$, 0.10 in solvent system II for for the mutant and wild-type strain, respectively. Moreover, traces of a second component could be identified: $R_f = 0.44$, 0.45 in solvent system I, and $R_f = 0.12$, 0.13 in solvent system II. Ferricrocin, coprogen and ferrichrome C were used as reference compounds yielding R_f values of 0.31, 0.20, and 0.46 in solvent system I, and R_f values of 0.11, 0.16, and 0.12 in solvent system II. Thus, TLC confirms the existence of a second siderophore-like component similar to ferrichrome C, although the R_f values of the cell extracts are slightly different from the reference.

From a previous Mössbauer study of coprogen uptake in *N. crassa arg-5 ota aga*, it is konwn that ferricrocin is synthesized at a late state of growth (Matzanke et al. 1987a). In order to elucidate the intracellular role of ferricrocin, information on its formation in wild-type *N. crassa* was desirable. Figure 6 displays Mössbauer spectra of frozen *N. crassa* 74A cells after uptake of [^{57}Fe]coprogen. Growth and uptake conditions were kept similar to the experiments described above. Metabolization was stopped after 0.5 h (A), 4 h (B), and 27 h (C) of additional growth. From these spectra the coprogen subspectrum was subtracted. Only 65% of the total absorption area could be attributed to coprogen after 0.5 h (Fig. 6A). With additional growth the coprogen contribution decreased further, namely, to 36% and 0% after 4 h and 27 h respectively (Fig. 6B, C).

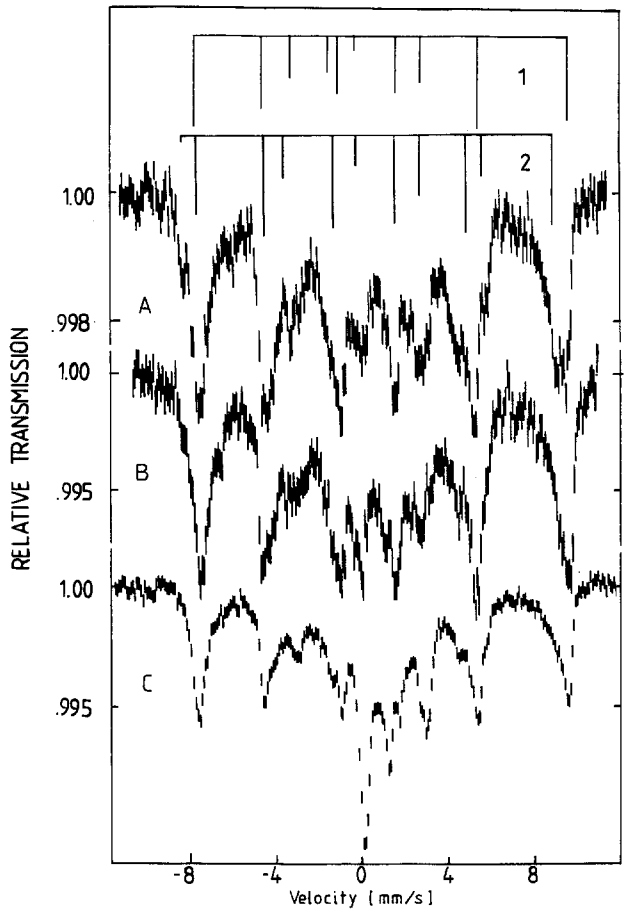

Fig. 6A–C. Mössbauer spectra of *N. crassa* 74A grown 72 h under low-iron stress and incubated for 1 h with [^{57}Fe]coprogen. Metabolization was stopped by freezing samples after 0.5 h **(A)**, 4 h **(B)** and 27 h **(C)**. Spectra were recorded as described in Fig. 3. For comparison the bar diagrams of ferricrocin **(1)** and of coprogen **(2)** are plotted on top of spectra A

The remaining contributions of 35%, 64% and 100% represent various iron metabolites. The major fraction exhibits a six-line pattern, which could be stripped from the residual spectrum employing the spectrum of ferricrocin depicted in Fig. 2B. From this a ferricrocin contribution has been evaluated of 28%, 47% and 44% of the total absorption area after 0.5 h, 4 h and 27 h of additional growth, respectively. In fact, in an earlier paper, isolation of ferric siderophore complexes has been reported from mycelial extracts of *N. crassa* wild-type strain 74A, including three components: ferricrocin (80%), ferrichrome C (10%) and a third compound, probably coprogen (10%) (Horowitz et al. 1976). This culture was grown under iron-sufficient conditions. We have repeated the experiment with mycelia grown for 72 h under low-iron stress without adding coprogen. From 100 g wet mycelium, 5 mg desferri-ferricrocin could be isolated by extraction. The compound was characterized on silica gel TLC by complexation of ferric ion and was quantified spectrophotometrically (see Materials and methods). Traces of two other siderophores were observable on the chromatograms but were not analyzed further.

After subtraction of coprogen and ferricrocin, a third magnetic species appeared in Fig. 6C similar to that found in the spectrum of Fig. 5B. This component, comprising 29% of the total absorption area, was removed from the residual cell spectrum employing the spectrum of lyophilized and BSA-diluted ferricrocin (Fig. 2C). In order to identify this species, siderophores have been isolated again from cell extracts of 50 g wet mycelia grown under the same conditions (see Materials and methods). Two spots could be detected on silica gel. The major component exhibits R_f values of 0.31 and 0.12 in solvent systems I and II, respectively, corresponding to ferricrocin; the values of the second component are 0.45 (I) and 0.14 (II), fitting those reported for ferrichrome C. The ferrichrome-C-like magnetic species disappears again after further growth while ferricrocin still accounts for 44% of the cellular iron after 65 h (spectrum not shown).

In Fig. 7A–C the remaining metabolite spectra of Fig. 6 are depicted, representing 7%, 17%, and 27% of the cellular iron pool after 0.5 h, 4 h and 27 h of additional growth, respectively. Whereas spectra A and B show poor resolution, spectrum C exhibits two species, an Fe^{3+} component and high-spin Fe^{2+}. From least-square fits, using Lorentzians, isomer shift δ, quadrupole splitting ΔE_Q and line width Γ has been derived: $\delta_1 = 0.42$ mm s^{-1}, $\Delta E_Q = 1.05$ mm s^{-1}, $\Gamma_1 = 0.64$ mm s^{-1}, 18.4%;

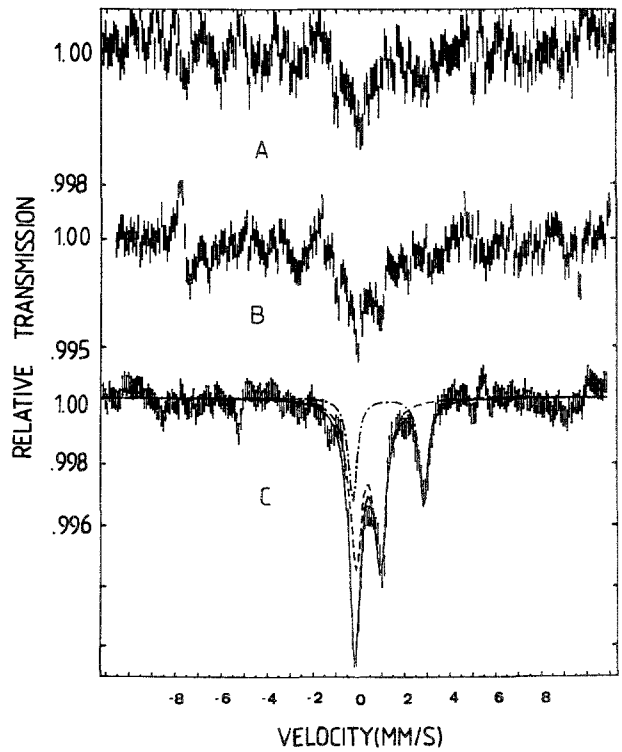

Fig. 7A–C. Metabolite spectra of *N. crassa* 74A after stripping of coprogen and ferricrocin. Experimental conditions as in Fig. 6A–C. The metabolite spectrum of C was fitted with two quadrupole doublets of Lorentzian line shape (see text)

$\delta_2 = 1.33$ mm s^{-1}, $\Delta E_Q = 3.07$ mm s^{-1}, $\Gamma_2 = 0.46$ mm s^{-1}, 8.6%. It is important to note that these metabolites exhibit the same parameters as those observed in *Escherichia coli* K12 after [^{57}Fe]ferricrocin uptake (Matzanke et al. 1987b).

Discussion

It has been reported earlier that ferricrocin represents the main intracellular siderophore in *N. crassa* (Horowitz et al. 1976). In the present study we have shown that coprogen-bound iron in mycelia of *N. crassa* 74A is rapidly transferred to ferricrocin and other iron-requiring compounds. Coprogen is completely decomplexed within 27 h and the ligand is reexcreted (Matzanke et al. 1988). About 45% of the incorporated iron is transferred to ferricrocin and about 55% to other metabolites. Moreover, within a time interval of 4 h subsequent to coprogen uptake, iron is preferentially shunted to desferriferricrocin.

In various microbial systems it could be demonstrated that metal transfer from siderophores to metabolism involves an oxidoreductase (Ernst

and Winkelmann 1977; Matzanke et al. 1988). It has to be stressed, however, that ligand exchange of iron from coprogen to ferricrocin is not necessarily mediated enzymatically. The complex formation constants of both ferricrocin and coprogen are in the same order of mangnitude (Wong et al. 1984). Although metal exchange is kinetically very slow between the complexes, excess of free ligand increases the exchange rate dramatically (Tufano and Raymond 1981). In fact, we could demonstrate that a significant intracellular amount of desferriferricrocin exists prior to coprogen uptake. Our findings provide, for the first time, evidence for an intracellular metal transfer from one siderophore to another. Moreover, this metal transfer might be achieved directly by ligand exchange not requiring metabolic energy.

As in a previous study on coprogen uptake in *N. crassa arg-5 ota aga*, no ferritin-like structures were detectable in *N. crassa* 74A (Matzanke et al. 1987a). However, the Mössbauer spectroscopic data of coprogen uptake and metabolization in *N. crassa* 74A clearly indicate that ferricrocin is the major intracellular pool of iron. The specific importance of intracellular ferricrocin becomes even more apparent when the data of ferricrocin uptake in both the wild-type and mutant strain of *Neurospora* are taken into account. In these experiments ferricrocin comprises not less than 65% of the total intracellular iron pool over a time period of 65 h. The contribution of intracellular non-siderophore iron species does not exceed 11% of the total absorption area, revealing that iron is scarcely shunted from ferricrocin to other metabolic functions. The large amount of metal transfer from coprogen to ferricrocin and the scarcity of metabolization of ferricrocin-bound Fe^{3+} indicates a quasi-inert iron-storage function of this siderophore in *N. crassa*. Moreover, we have shown that ferricrocin acts as an iron-storage compound in spores of *N. crassa*. These observations point to a very important intracellular function of ferricrocin in sporulation. In fact, many nutritional factors contribute to the induction of fungal sporulation and this process is associated with tremendous metabolic activity. Macromolecules must be synthesized to provide sporulation-specific structures. It is known that a number of fungal spores have prepackaged RNA and/or proteins that are required for the preliminary stages of spore germination (Brambl et al. 1978; Van Etten et al. 1981; Dahlberg and Van Etten 1982). In addition, it has been suggested that mycelial vesicles of *N. crassa* which contain basic amino acids serve as a reservoir for precursors required during sporulation (Brody 1981). In this context, the intracellular accumulation of ferricrocin seems to warrant an iron pool sufficiently large for sporulation. Thus, the rationale for the relative inertness of ferricrocin in mycelia of *N. crassa* has to be seen from the viewpoint of propagation.

The Mössbauer spectroscopic analyses of the second intracellular magnetically split species indicate either two different microenvironments of ferricrocin or an additional component. TLC data favour the latter possibility. In fact, ferrichrome C has been identified earlier as a minor fraction of intracellular siderophores in *N. crassa*. Therefore, this second intracellular siderophore very probably represents ferrichrome C. However, the metabolic function of this component remains enigmatic. Perhaps it mediates between the cellular needs for iron and the metabolic inertness of ferricrocin.

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