

Iron Transport in *Escherichia coli*: Uptake and Modification of Ferrichrome

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During the transport of iron as ferrichrome complex into cells of *Escherichia coli* K-12, the ligand was modified and excreted into the medium. The rate of the formation of the modified product corresponded with the rate of iron transport. The modified product showed a decreased affinity for ferric iron and did not serve as an effective iron ionophore. After all of the ferrichrome had been converted, the modified product was taken up into the cell in an iron-free form. The uptake of ferrichrome and of the modified product depended on the transport system specified by the *tonA* and *tonB* genes. The modified product could be converted back into ferrichrome by mild acid or alkaline hydrolysis. One mole of acetate was released per mole of ferrichrome. It is proposed that one *N*-hydroxyl group of ferrichrome is acetylated to explain the low affinity for iron as the *N*-hydroxyl groups form the ligands for iron (III). A weak ester linkage by which the acetyl group is covalently bonded would account for the easy hydrolysis. The iron-free form of ferrichrome, deferrichrome, was also rapidly converted when incubated with cells with a functional transport system. It is therefore likely that iron is released from ferrichrome by reduction before modification takes place. The conversion of the ligand could be a mechanism by which cells rid themselves of a potentially deleterious ligand for iron in the cytoplasm. A possible role in ferrichrome transport is discussed.

The iron supply of cells under aerobic conditions requires ligands to complex the extremely insoluble iron. The equilibrium concentration of ferric iron at pH 7 is about 10^{-18} M (8, 27, 32) which is orders of magnitude below the concentration needed to support cellular growth and function. Iron is actively transported into cells of *Escherichia coli* as ferric citrate (11), ferric enterochelin (3, 5, 8, 12, 15, 18, 22, 25, 27, 30-33), and as ferrichrome (3-5, 9, 17, 19, 20, 23, 24, 27, 33, 34) complex. There is also a low affinity system for the accumulation of iron for which no ligand is known. With regard to ferrichrome, it is of interest that it is not produced by *E. coli* but by certain fungi (27). Nevertheless, ferrichrome is taken up by cells of *E. coli* via a highly specific transport system. An outer membrane receptor protein, specified by the *tonA* gene, and a function in the cytoplasmic membrane, controlled by the *tonB* gene, are required for the transport of ferrichrome (1, 3-7, 9, 10, 13, 15-20, 23-25, 27-34). Additional mutants have been isolated, but they were not sufficiently characterized biochemically to reveal the details of their functional defects (4).

Ferrichrome is a cyclic hexapeptide composed of three residues of glycine and three residues of

δ -*N*-acetyl-L- δ -hydroxyornithine. It forms with iron a ferritrihydroxamate with a stability constant of 10^{29} (32). Previously it was thought that the iron was released intracellularly from the complex by reduction, that the ligand was excreted unchanged, and that it could be recycled again for iron transport (4, 5, 23). In this paper we show that the ligand is modified during transport into a form which does not serve as an effective iron ionophore. This finding leads to a revised view of iron transport via the ferrichrome complex and aligns the mechanism of ferrichrome transport more closely to that of ferric enterochelin.

MATERIALS AND METHODS

Strains and culture conditions. The strains of *E. coli* K-12 used include AB2847 *aroB tsx thi malA*, its *tonA* derivative P8, and its *tonB* derivative BR158, which were described previously (20). Growth was usually started from single colonies, kept on tryptone-yeast agar (19, 20) in M9 minimal medium supplemented with the required substrates and with 2 μ M iron (19, 20). After growth overnight, cells were spun down, washed twice with iron-extracted M9 salts (medium without carbon source, supplemented with 1 mM $MgSO_4$), and diluted into iron-extracted M9 medium to a density of 5×10^7 cells per ml. Ferric chloride was added to a final concentration of 0.15 μ M. Cells were grown at 37°C in a gyratory water bath shaker at 150

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rpm to a density of 5×10^8 cells per ml which corresponded to 0.19 mg (dry weight) per ml. The growth medium with 0.4% acetate (without glucose) was supplemented with 0.1% Casamino Acids to improve the growth rate. For growth of the *tonB* mutant, citrate (1 mM) was replaced by dihydroxybenzoate (20 μ M).

Transport assay. Exponentially growing cells in M9 minimal medium with 0.15 μ M ferric ions were harvested and washed twice at 4°C with iron-extracted M9 salts. They were then suspended in M9 salts to a density of 5×10^8 cells per ml at 4°C. The transport assay was initiated by the addition of the labeled compounds after the cell suspension had been shaken for 5 min at 37°C in the presence of 0.1% glucose. After appropriate time intervals, samples of 0.5 ml were filtered through Selectron filters (type BA 85, 0.45 μ m), washed twice with 4 ml of 0.1 M LiCl, dried, and counted in a liquid scintillation counter. The uptake rates were related to the cell dry weight at the beginning of the experiment. All experiments were reproduced at least three times.

Adsorption of ferrichrome and its derivative to Servachrome XAD-2 (100 to 200 μ m). The resin was purchased from Serva, Heidelberg. Before use, the resin was washed once with methanol and then thoroughly washed with double-distilled water. An aqueous suspension of the resin (15 mg/100 μ l) was added to 1 ml of the transport assay medium after centrifugation of the cells or to 0.25 ml of concentrated cell extract. After careful mixing, the resin sedimented. The supernatant was removed, and the resin was washed three times with 1 ml of water. The adsorbed material was extracted with 0.5 ml of methanol-water (1:1, vol/vol) and dried in the vacuum.

Assay of iron binding. The material to be tested was dissolved in 0.5 ml of HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-ethanesulfonic acid) buffer (100 mM, pH 6) and incubated at room temperature with a 5 times molar excess of ferric chloride in 0.1 M HCl. After 3 h, the material was extracted with Servachrome XAD-2 as described.

Chromatography on thin-layer plates. The material was applied in 20 μ l of methanol on silica gel thin-layer plates (number 5553, Merck, Darmstadt). The chromatography was performed for 3 h at room temperature with the solvent chloroform-methanol-water (65:25:4, vol/vol). The distribution of radioactivity was determined with a scanner equipped with an integrative scanning device with which the relative amounts of the products were quantitatively determined.

Ion-exchange chromatography. The cation-exchange resin AG50 W-X2 (100 to 200 mesh, Bio-Rad Laboratories, Richmond, Calif.) was prepared as suggested by the supplier. The modified compound (20 μ l, 1.6×10^6 cpm) was applied together with ferrichrome (0.1 mg/100 μ l) and 60 μ l of 6 N HCl to a column (65 by 0.8 cm) previously equilibrated with the starting buffer containing 0.2 M pyridine acetate (pH 2.6) at 40°C. The anion-exchange resin Dowex 1-X8 (100 to 200 mesh; Bio-Rad) was prepared by the protocol of the supplier and poured at 40°C into a column (90 by 0.8 cm). It was equilibrated with 0.5 M pyridine acetate (pH 9). A mixture of [3 H]ferrichrome and the tritium-labeled modified product was applied

to the column at pH 10. After 100 ml had been eluted, the buffer was replaced by 0.5 M acetic acid. Samples of 0.5 ml were counted, and a portion was analyzed by silica gel thin-layer chromatography.

Electrophoresis on Cellogel strips. The tritium-labeled products were applied to strips of Cellogel (17 by 2.5 cm, Serva, Heidelberg), and the electrophoreses were run for 2 to 3 h with 0.2 M triethanolamine adjusted with HCl to pH 8.5 and 7.0, or with a 0.2 M pyridine acetate buffer (pH 4.5), or with formic acid (2.5 ml of 88% formic acid in 97.5 ml of water [pH 2.1]). Ferrichrome (5 μ g) was used as the visible internal standard. The radioactive compounds were identified by scanning the wet gels.

Labeling with dimethylamino naphthalene-5-sulfonyl chloride (dansyl chloride). The method of Gray was employed for labeling with dansyl chloride (14). [3 H]ferrichrome and the tritium-labeled modified product (10 μ l, 8×10^4 cpm of each) were dried together with 10 μ l of 0.1 M NaHCO₃ solution. The material was dissolved in 20 μ l of water, and 20 μ l of [3 H]dansyl chloride in acetone (112 nmol, 10^6 cpm) was added. After 30 min at room temperature the material was dried, dissolved in 20 μ l of acetic acid-acetone (2:3, vol/vol), and chromatographed on silica gel thin-layer plates with the solvent chloroform-methanol-water (65:25:4, vol/vol) for 3 h. The chromatograms were scanned to identify the radioactive spots.

Preparation of cell homogenates. Cells (5×10^8) from the transport experiments were spun down, washed with 5 ml of Tris-hydrochloride (10 mM, pH 7.8), and resuspended in 1 ml of the same buffer. They were then treated with lysozyme (50 μ l, 2 mg/ml) for 2 min at 4°C, 2 ml of 1.5 mM EDTA (pH 8.0) was added, and the suspension was then sonicated twice with ultrasound for 10 s in an ice bath. The supernatant was recovered after centrifugation for 1 h at 200,000 $\times g$.

Quantitative determination of acetate. The enzymatic assay involved the conversion of acetate plus ATP to acetyl phosphate and ADP, catalyzed by acetate kinase. ADP and phosphoenol pyruvate were converted with pyruvate kinase to pyruvate and ATP. Pyruvate was reduced in the presence of NADH with lactate dehydrogenase to lactate and NAD⁺. The decrease in the absorbance at 366 nm was followed to measure the consumption of NADH which was proportional to the amount of acetate present. The experiment followed exactly the described method (2).

Other methods. The following procedures were described previously (20) and include the labeling of ferrichrome with tritium and radioactive iron, the extraction of the growth and uptake medium with Chelex 100 to reduce the iron content (final concentration about 0.05 μ M), gel filtration on Bio-Gel P-2, high-pressure liquid chromatography of ferrichrome, and the preparation of [3 H]deferriferri-ferrichrome from [3 H]ferrichrome by the extraction of iron with 8-hydroxyquinoline (20, 23).

RESULTS

Uptake of ferrichrome. Ferrichrome uptake was studied using radioactive $^{55}\text{Fe}^{3+}$ and with the tritiated ligand which was chemically syn-

thesized by substitution of the acetyl residues by ^3H -labeled acetyl groups. To avoid interference by the other iron transport systems, mutants (*aroB*) were used which were unable to synthesize enterochelin. The low-affinity iron transport system was suppressed by the addition of nitrilotriacetate (NTA) which is a strong iron ligand and which is not taken up by cells of *E. coli* (11). For all transport studies the iron supply of the cells was limited by growth in iron-extracted minimal medium in which the iron concentration was adjusted to $0.15\ \mu\text{M}$.

During the rapid uptake of iron via ferrichrome, only very little of the ligand was found associated with the cells (Fig. 1). After all iron of the medium had been taken up, the ligand was then accumulated in the cells. These observations have been reported previously by various investigators (20, 23). The uptake of iron and of the ligand required the functions specified by the *tonA* and the *tonB* genes.

To understand the delayed uptake of the ligand, we began a study of ferrichrome uptake in

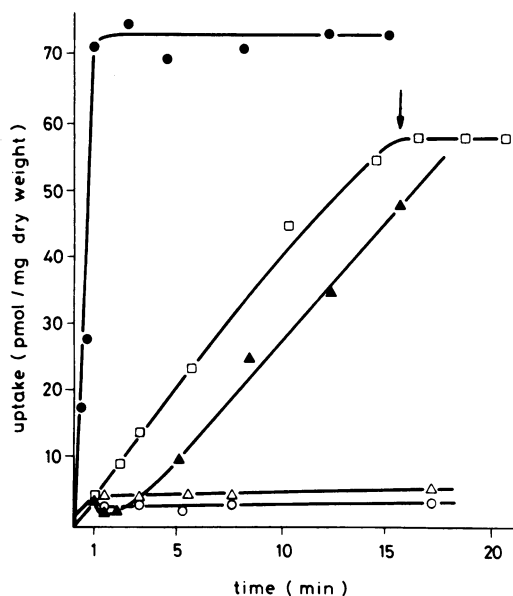


FIG. 1. Uptake of $[^{56}\text{Fe}(\text{III})]\text{ferrichrome}$ (●), of added $[^3\text{H}]\text{ferrichrome}$ (▲), and of added $[^3\text{H}]\text{deferriferrichrome derivative}$ (□) into cells of *E. coli* AB2847. Uptake of $[^{56}\text{Fe}(\text{III})]\text{ferrichrome}$ into the *tonA* mutant P8 (○) and into the *tonB* mutant BR158 (△). The uptake curves of the tritium-labeled compounds into the mutants were identical to the uptake of iron shown. The arrow indicates the addition of $10\ \mu\text{M}$ nonradioactive ferrichrome. Uptake was measured using a concentration of $0.03\ \mu\text{M}$ for each substrate. The cells were suspended in iron-extracted minimal growth medium to which $100\ \mu\text{M}$ NTA was added.

greater detail. Addition of the iron-free ligand, $[^3\text{H}]\text{deferriferrichrome}$, resulted in an immediate uptake of the ligand (Fig. 1). No uptake occurred in *tonA* and *tonB* mutants (the curves were identical with the ones shown for iron uptake in Fig. 1). We concluded that the delay in the uptake of the ligand when supplied as $[^3\text{H}]\text{ferrichrome}$ was due to the time it took to transport iron into the cells, to release it from the ferrichrome complex, and to excrete the unloaded ligand. Furthermore, the uptake of the free ligand was inhibited as long as ferrichrome was present in the medium. Addition of ferrichrome after 15 min of transport (Fig. 1) immediately inhibited the uptake of the ligand. When the supernatant of a cell culture, which had been incubated for 6 min with $0.1\ \mu\text{M}$ $[^3\text{H}]\text{ferrichrome}$ to take up all iron, was inoculated with fresh cells, it was found that the tritium label was immediately taken up at a rate as shown in Fig. 1. However, added $^{56}\text{Fe}^{3+}$ was only very poorly taken up (data not shown). To corroborate this finding, the following experiment was performed. A surplus of iron ($0.5\ \mu\text{M}$ $^{56}\text{Fe}^{3+}$) over deferriferrichrome ($0.1\ \mu\text{M}$) was incubated for 11 min with cells. After this time at least 20% of the iron had been taken up by the cells but more than 90% of the ligand remained in the medium. The cells were centrifuged, and the supernatant was divided into equal halves. The same amount of fresh cells was added to both supernatants. One culture was supplied with deferriferrichrome ($0.1\ \mu\text{M}$). Only the latter culture accumulated iron at the same fast rate characteristic of ferrichrome (Fig. 2). This experiment again showed that the excreted ligand was unable to support the transport of iron effectively.

To measure iron transport at very low ligand concentrations, it was important that the experiments documented in Fig. 2 were carried out with only $1\ \mu\text{M}$ NTA in the uptake assay since higher concentrations of NTA competed with deferriferrichrome for the iron. This is demonstrated in Fig. 3 where three concentrations of NTA ($1\ \mu\text{M}$, $10\ \mu\text{M}$, $100\ \mu\text{M}$) were incubated with a mixture of $0.5\ \mu\text{M}$ $^{56}\text{Fe}^{3+}$ and $0.1\ \mu\text{M}$ deferriferrichrome for 4 h before transport was started. It was evident that $1\ \mu\text{M}$ NTA but not $100\ \mu\text{M}$ NTA allowed ferrichrome transport to be measured under the conditions employed. NTA alone did not support iron transport.

The question arose as to whether the ligand was only used once for the transport of iron. We studied uptake with a fixed concentration of iron ($0.5\ \mu\text{M}$) in the medium mediated by various concentrations of added deferriferrichrome. The values obtained after 20 min (Table 1) were compared with the expected values calculated

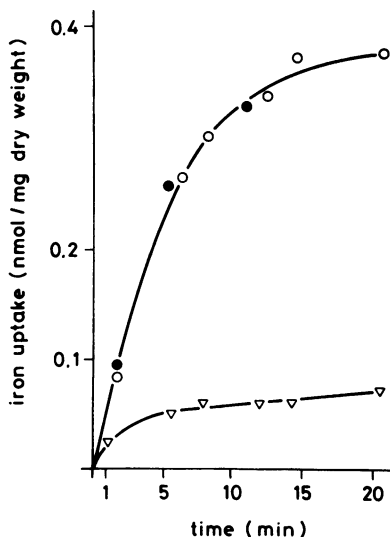


FIG. 2. Uptake of iron into cells of *E. coli* K-12 AB2847 suspended to a concentration of 5×10^8 cells per ml in iron-extracted M9 salts supplemented with 0.1% glucose, $1 \mu\text{M}$ NTA, $0.5 \mu\text{M}$ ^{55}Fe (III), and $0.1 \mu\text{M}$ deferri-ferrichrome (●). After 11 min of iron transport, two samples of 3 ml of culture were withdrawn, the cells were spun down, and the supernatants were inoculated with fresh cells (5×10^8 /ml). Iron uptake was measured in the cultures with the addition of $0.1 \mu\text{M}$ deferri-ferrichrome (○) and without the addition of ligand (▽).

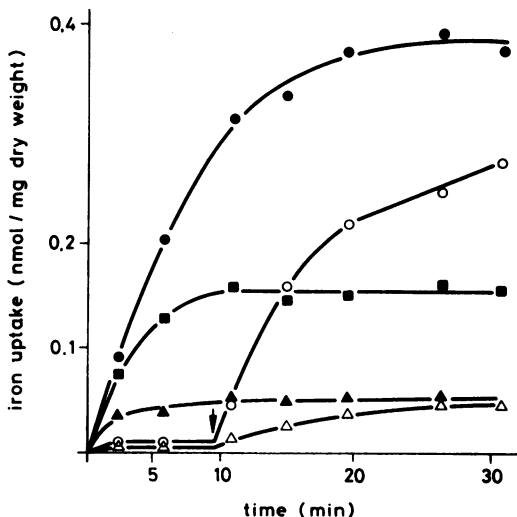


FIG. 3. Effect of NTA on $[^{55}\text{Fe}$ (III)]ferrichrome transport. Cells of *E. coli* K-12 AB2847 were incubated in M9 salts with 0.1% glucose, $0.5 \mu\text{M}$ ^{55}Fe (III), and $0.1 \mu\text{M}$ deferri-ferrichrome in the presence of $100 \mu\text{M}$ (▲), $10 \mu\text{M}$ (■), and $1 \mu\text{M}$ (●) NTA. In addition, cells were incubated with $0.5 \mu\text{M}$ ^{55}Fe (III), $100 \mu\text{M}$ NTA (Δ), and $1 \mu\text{M}$ NTA (○) for 9 min, and then (indicated by the arrow) $0.1 \mu\text{M}$ deferri-ferrichrome was added. The iron was always incubated with the

TABLE 1. Uptake of iron into cells

Deferri-ferrichrome (μM)	Fe^{3+} uptake (pmol/mg [dry wt])		
	Expected	Measured after 20 min ^a	
		$1 \mu\text{M}$ NTA	$100 \mu\text{M}$ NTA
0.01	24	50	19
0.03	73	115	57
0.05	122	185	100
0.10	244	265	160

^a Binding of iron to cells in the absence of ferrichrome with $1 \mu\text{M}$ NTA or $100 \mu\text{M}$ NTA in the assay mixture was subtracted from the measured values.

on the basis that one ligand molecule transported only one iron ion into the cell. As can be seen, the measured values in the presence of $1 \mu\text{M}$ NTA exceeded the calculated values at most by a factor of two, so that at low ligand concentrations not more than two iron ions were transported by one ligand molecule. At the lowest ligand concentration applied, 50 times more iron was supplied in the medium. When the ratio of iron to the ligand in the medium reached the value of 5, the amount of transported iron per ligand approached the value of 1. With $100 \mu\text{M}$ NTA, it was always below 1.

Modification of ferrichrome. The above results suggested that, after transport of ferrichrome, a modified product was excreted which could be reaccumulated but showed little ability to transport iron. We therefore searched for indications that there was some modification of the ligand. The modified product was in very small amounts in the supernatant of the uptake assay together with other compounds released by the cells. It was therefore important to find a procedure that extracted it selectively. Tritium-labeled ferrichrome, deferri-ferrichrome, and the modified product could be reproducibly adsorbed by a batch procedure to Servachrome XAD-2 with a yield of 95% and desorbed again with a yield of 65 to 70%. Charged compounds adsorbed only very little to XAD-2. The product obtained was sufficiently pure for chromatographic analysis. When it was chromatographed on a column of Bio-Gel P-2, it was eluted at a position which was different from that of ferrichrome, albomycin (an antibiotic, part of its structure is similar to ferrichrome), or deferri-ferrichrome (Fig. 4). The modified tritiated product was chromatographed together with ferrichrome as the internal standard, to determine exactly its elution position. As will be shown later, it was unlikely that any iron exchange had

ligands for 4 h at 22°C before the experiment was started, except in the last two experiments shown above.

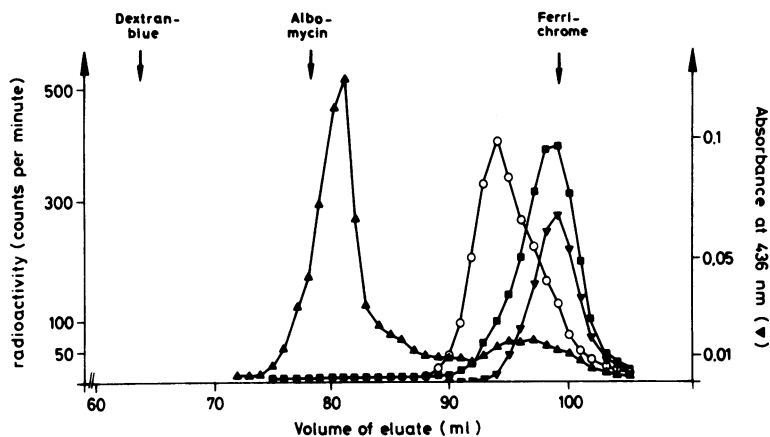


FIG. 4. Chromatography of 134 nmol of nonradioactive ferrichrome (▼), 147 nmol of [^3H]deferri-ferrichrome (▲), 30 pmol of [^3H]ferrichrome derivative with 134 nmol of ferrichrome (○), and 30 pmol of [^3H]deferri-ferrichrome with 134 nmol of ferrichrome (■) on columns of Bio-Gel P-2 (90 by 0.6 cm) (20). The elution rate was 12 ml/h. The adsorbance at 436 nm and the radioactivity were measured in the eluted fractions.

occurred between ferrichrome and the modified deferri-ferrichrome which may have changed the chromatographic behavior of the modified product.

For routine identification of the modified ferrichrome in the medium or in cells extracts, the XAD-2 adsorption method was applied with subsequent chromatography on silica gel thin-layer plates. Ferrichrome had an R_f value of 0.49; deferri-ferrichrome and the modified ferrichrome had an R_f value of 0.41 (Fig. 5). To distinguish the modified product from deferri-ferrichrome, a surplus (usually five times more) of Fe^{3+} over the tritium-labeled product was added to all samples and the ferrichrome products were extracted a second time before chromatography. Under these conditions, the total amount of deferri-ferrichrome was converted to ferrichrome. In contrast, the R_f value of the modified product remained unchanged after iron supplementation. It required a 100 times higher concentration of iron to convert about 60% of the modified product into a form which chromatographed like ferrichrome. The kinetics of the formation of the modified deferri-ferrichrome is also shown in Fig. 5. The radioactivity of the peaks was quantitatively determined and compared with the iron uptake rate in the same assay culture (Fig. 6). The modified deferri-ferrichrome appeared in the medium with the same rate as iron was taken up into the cells. This result was corroborated by uptake experiments with 1 μM ferrichrome. After 15 min, as much modified [^3H]deferri-ferrichrome was detected in the medium as ^{56}Fe was taken up into the cells. As a control, no modified ferrichrome was formed when [^3H]ferrichrome was incubated with a *tonA* mutant.

When deferri-ferrichrome was incubated with wild-type cells, the rate of formation of the modified compound was much faster (Fig. 6). This can be explained by the lack of inhibition of the deferri-ferrichrome uptake by ferrichrome (see Fig. 1). In fact, the affinity of the transport system for the various substrates was different. It required at least a 100-fold excess of deferri-ferrichrome over ferrichrome to reduce the iron transport rate by 50%. An equimolar concentration of deferri-ferrichrome inhibited the uptake of the modified form very little, whereas an equimolar concentration of ferrichrome reduced the uptake of the modified form by 60% (data not shown).

The chromatographic analysis of the ^3H -labeled ligand which was taken up into the cells whether added as [^3H]ferrichrome or [^3H]deferri-ferrichrome (see Fig. 1) revealed only the modified product. At high concentrations of ferrichrome (1 μM), when the iron uptake was not complete, the small amount of the cell-associated ^3H label was ferrichrome, which agrees with the competition experiments.

Characterization of the modified deferri-ferrichrome. Unlike ferrichrome, the modified product (57 nmol) showed no adsorption in the region of 425 nm. Since the modified product transported iron very poorly, and large amounts of iron were required to form a complex similar to ferrichrome, it was assumed that it was modified in such a way that iron binding was affected. This could have been caused by cleavage of the hexapeptide ring or by a modification at the three hydroxamate residues. Cleavage of peptide bonds would have resulted in a charged molecule and in free amino and carboxylate groups. However, in electrophoreses at pH values between

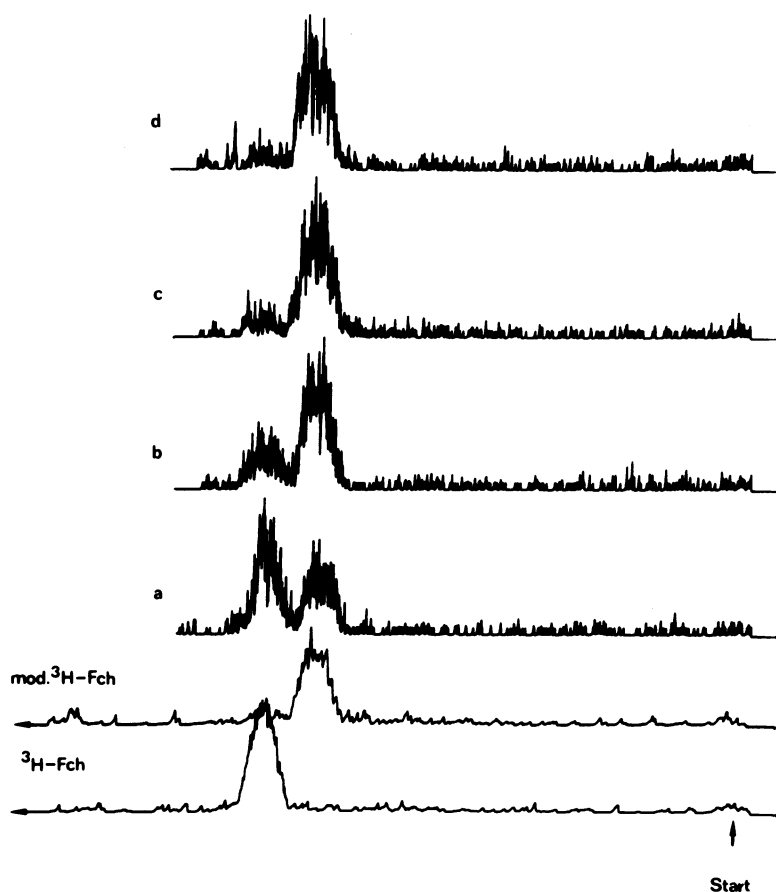


FIG. 5. Chromatography on a silica gel thin-layer plate of [^3H]ferrichrome and its modified product. The details of the uptake assay are described in Fig. 6. Samples (2 ml) were taken from the transport assay after 3 (a), 6 (b), 9 (c), and 15 min (d). Cells were removed by centrifugation. Ferrichrome and its derivatives were extracted with Servachrome XAD-2 and eluted again, and a surplus (5 \times) of ferric iron was added. They were subjected to an additional extraction cycle and then chromatographed. [^3H]ferrichrome ($^3\text{H-Fch}$) and the modified product (mod. $^3\text{H-Fch}$) were applied as reference substances.

2.1 and 8.5, the modified product showed a mobility identical to that of ferrichrome. On cation- and anion-exchange columns, no difference between ferrichrome and the modified product was noticed. Incubation with dansyl chloride revealed no free amino group. It was therefore unlikely that the peptide ring was cleaved. Moreover, the tritium label, introduced into ferrichrome by the exchange of unlabeled and labeled acetyl groups, could be quantitatively adsorbed (more than 95%) to Servachrome XAD-2 from the medium of a transport experiment (0.03 μM [^3H]ferrichrome, 5 min). When the lyophilized medium of a similar transport assay was chromatographed on a column of Bio-Gel P-2, the [^3H]acetyl label employed was quantitatively recovered at the elution position of the cyclic hexapeptide. This experiment was performed with a column which clearly separated

acetate from ferrichrome but did not resolve ferrichrome from ferrichrome derivatives (Fig. 4). Both experiments show that no acetyl groups were released from ferrichrome and metabolized in the cell.

The possibility remained that something was added to the molecule. Weak acid hydrolysis (0.1 M HCl, 1 h, 22 $^\circ\text{C}$) converted the modified product with a yield between 70 and 100% to a compound, which, when supplemented with an equimolar amount of Fe^{3+} , showed the same chromatographic behavior as ferrichrome when subjected to silica gel thin-layer chromatography and high-pressure liquid chromatography. In addition, weak alkaline hydrolysis (in methanol, saturated with ammonia, for 1 h at 50 $^\circ\text{C}$) converted the modified product back to ferrichrome with a yield of 35%. Of still greater importance was the restoration of the transport

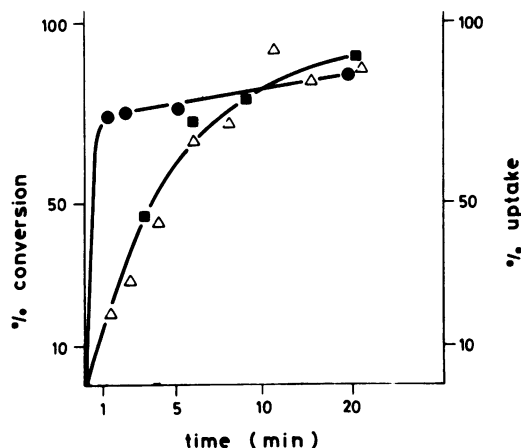


FIG. 6. Comparison of the rate of iron transport with the rate of the modification of ferrichrome. The data of the amount of the ferrichrome derivative (Δ) and of the uptake of $[^{56}\text{Fe}]$ ferrichrome (\blacksquare) in the same experiment were taken from Fig. 5. Parallel cultures of *E. coli* K-12 AB2847, assayed in M9 salts with 0.1% glucose and 100 μM NTA, were incubated with 0.2 μM $[^{56}\text{Fe}]$ - and $[^3\text{H}]$ ferrichrome, respectively. Samples were filtered to measure uptake of radioactive iron into the cells. In addition, samples were withdrawn from the $[^3\text{H}]$ ferrichrome-containing culture and treated further as described in Fig. 5. In a second experiment, 0.2 μM $[^3\text{H}]$ deferriferrichrome was added to an iron-free (not more than 0.05 μM iron) culture of cells, and the formation of the modified product (\bullet) was measured as described for ferrichrome. The uptake was referred to the amount of radioactive iron added to the assay.

activity (Fig. 7). The iron transport rate was much faster after hydrolysis than before. A high concentration of NTA (100 μM) was employed in the transport assay to demonstrate the strong affinity of the restored compound for Fe^{3+} . We found no evidence against the conclusion that the restored compound was ferrichrome. Larger amounts of pure substance will have to be prepared to prove this assumption chemically.

To test the assumption that the modification reaction was an acetylation which was reversed by weak acid hydrolysis, cells were grown in a medium with 0.4% acetate and incubated with 350 μM $[^{14}\text{C}]$ acetate for 5 min before the addition of 0.1 μM ferrichrome. In parallel cultures, transport of iron via ferrichrome and the uptake of $[^3\text{H}]$ ferrichrome were determined to make sure that iron uptake and the conversion of the ligand took place under the conditions employed. Servachrome XAD-2 was added to the supernatant of the ^{14}C -labeled culture after 35 min. The adsorbed products were recovered and separated on a silica gel thin-layer plate. Ferrichrome and tritium-labeled modified product were chromat-

ographed on the same plate. ^{14}C label was found with an R_f value identical to the modified deferriferrichrome but none at the position of ferrichrome (Fig. 8). The identity of the compounds in the other two peaks is unknown. It was not unexpected that additional radioactive compounds were excreted into the medium which adsorbed to the resin.

To determine whether acetate or a metabolic product had been transferred to the ligand, tritium-labeled modified product was isolated by preparative thin-layer chromatography and by subsequent column chromatography on Bio-Gel P-2. The product was hydrolyzed with 0.1 N HCl for 1 h at room temperature. The ferrichrome products were extracted with Servachrome XAD-2, desorbed again, supplemented with iron, and quantitatively determined after silica gel thin-layer chromatography. In the remaining supernatant, acetate was determined enzymatically with acetate kinase. The results are listed in Table 2. In three independent experiments, 1

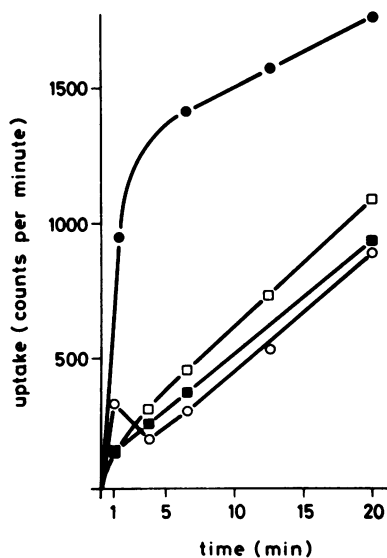


FIG. 7. Uptake of the tritium-labeled modified product of ferrichrome without iron before (\square) and after acid hydrolysis (\circ). Uptake of radioactive iron mediated by the modified product before (\blacksquare) and after acid hydrolysis (\bullet). The isolated modified product (0.4 nmol) was incubated with 0.1 M HCl for 1 h at room temperature and extracted with Servachrome XAD-2. One half was supplemented with five times the amount of $^{56}\text{Fe}^{3+}$ in HEPES buffer, incubated for 3 h, and extracted again. Unhydrolyzed modified product was treated accordingly. Uptake for all four preparations was measured with cells of *E. coli* K-12 AB2847 in M9 buffer supplemented with 0.1% glucose and 100 μM NTA. The final concentration of the ligand was 0.06 μM in the four samples.

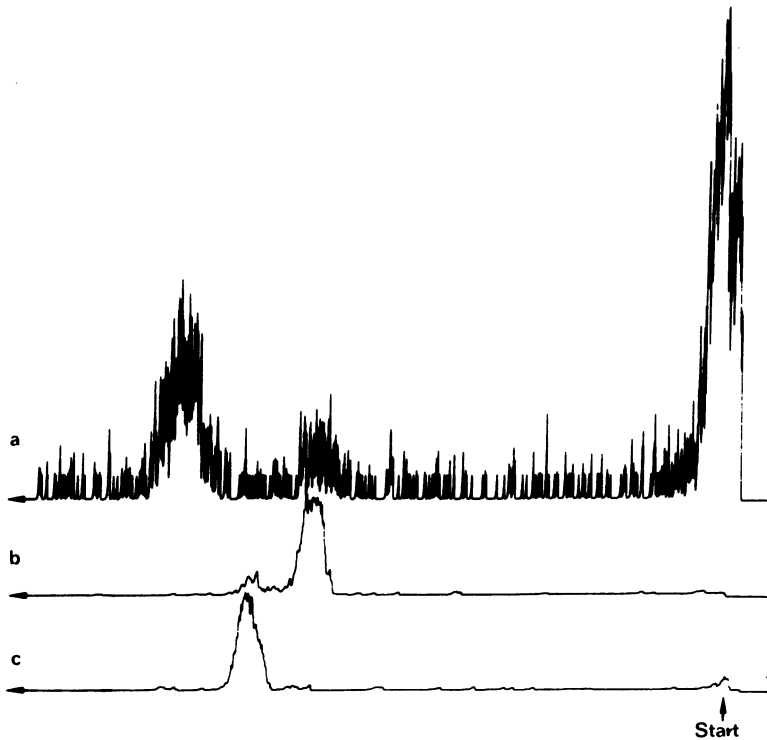


FIG. 8. Chromatography of the labeled modified product (a) obtained by incubating cells of *E. coli* K-12 AB2847 with $0.1 \mu\text{M}$ ferrichrome in the presence of $350 \mu\text{M}$ of [^{14}C]acetate ($100 \mu\text{Ci}$). After 35 min, cells were spun down, and the labeled products were extracted with Servachrome XAD-2 and chromatographed on a silica gel thin-layer plate. The tritium-labeled modified product (b) was prepared by incubating $0.2 \mu\text{M}$ [^3H]ferrichrome with cells in M9-glucose medium for 10 min and co-chromatographed with the ^{14}C -labeled compounds and with [^3H]ferrichrome (c) as reference substance.

TABLE 2. Hydrolysis products of [^3H]ferrichrome derivative^a

Substrate	Hydrolysis products (nmol)	
	Ferrichrome	Acetate
Ferrichrome		0
Ferrichrome derivative	17	19
Ferrichrome derivative	13.5	13
Ferrichrome derivative	13	13

^a Results of three experiments.

mol of acetate was obtained per mol of recovered ferrichrome. We therefore conclude that, during transport, ferrichrome was modified by acetylation or by a group containing an acetyl residue.

DISCUSSION

Up to now it was thought that deferriferri-chrome could be repeatedly used to transport Fe^{3+} into cells of *E. coli* (4, 23). According to this model the ferric complex was transported into the cell, Fe^{3+} was released by reduction to Fe^{2+} ,

and the ligand was excreted for another cycle of iron uptake. Raymond and Carrano (32) called the recycling "the European approach" to microbial iron transport in contrast to "the American approach" to iron transport via enterochelin which is hydrolyzed and thus wasted for further iron transport (33). As this paper shows, to our regret, there is no European exemplar of how energy and material are conserved at least in iron transport. The ligand was modified at the same rate as iron was transported (Fig. 6), and the modified form had little affinity to iron and would thus be a very inefficient iron transport vehicle. The observation that deferriferri-chrome was rapidly converted into the modified form (Fig. 6) showed that the ligand rather than ferrichrome was probably the substrate for the modification reaction. The redox potential of ferrichrome at pH 7 was estimated to be at about -450 mV which is in the range of physiological reductants (8). We therefore assume that ferric iron is reduced and released from the complex before modification occurs.

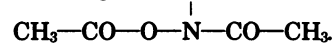
What is the purpose of the modification of the

ligand? Modification would prevent the reformation of the complex with reoxidized iron. The accumulation of deferriferrichrome into the cytoplasm during the secondary uptake (Fig. 1) could poison the cell since the high affinity of the ligand for iron could shorten the iron supply for the synthesis and function of the many iron-containing proteins. In fact, only the modified product with its low iron affinity was found in the cells, so that secondary uptake was confined solely to the modified product. It was taken up in the iron-free form since, under the conditions used, all iron had already been accumulated from the medium via ferrichrome before the secondary uptake had begun. The delay in the uptake of the modified form was mainly due to the competition of ferrichrome for the uptake system (Fig. 1). The uptake of the modified form also depended on the functions specified by the *tonA* and *tonB* genes. Additional mutations outside the *tonA* and *tonB* genes (5, 20), which conferred resistance to albomycin and led to an impaired ferrichrome uptake, also lacked the secondary uptake (20). Therefore, ferrichrome, deferriferrichrome, and the modified deferriferrichrome were all taken up by the same transport system. This transport system also tolerated the introduction of a negative charge by reaction of ferricrocin with succinic anhydride (9). The affinity of the transport system for the various substrates was very different. Competition experiments showed the highest affinity of ferrichrome for the transport system, lower affinity for the modified form, and the lowest affinity for deferriferrichrome.

Ferrichrome was recovered from the modified ligand by mild acid hydrolysis. This excluded any cellular conversion by hydrolytic reactions. Leong and Neilands came to the conclusion that the ligand was not altered during transport since it could be extracted into benzyl alcohol with the same yield as deferriferrichrome (23). This observation agrees with our finding that no charged molecule was created. A molecule with extra charges would have been separated from ferrichrome in the ion-exchange chromatographies and the electrophoreses performed at a pH range from 2.1 to 8.5. The ligand was labeled with radioactive acetate, and 1 mol of acetate was determined per mol of ligand after acid hydrolysis of the purified product. The assay with acetate kinase does not prove that acetate had been transferred to the ligand since the enzyme also phosphorylates propionate (2). However, we are studying at the moment a purified system obtained from *E. coli* cells in which the conversion of deferriferrichrome is stimulated by the addition of acetyl phosphate

which makes it highly probable that the modification reaction is an acetylation. In deferriferrichrome there exists only one reactive site for acetylation, the *N*-hydroxyl residues.

Acetylation of one hydroxyl group explains the easy hydrolysis under mild conditions which is known from the total chemical synthesis of enantio-ferrichrome (26). It also accounts for the low affinity for iron since the three *N*-hydroxyl groups together with the carboxyl groups of the acetyl residues bind Fe^{3+} in an octahedral array (27). Replacement of the hydrogen atom of one *N*-hydroxyl group by an acetyl residue impedes the formation of the iron chelate. The following structure is tentatively proposed:



From the data described in this paper emerges the following model for iron uptake as ferrichrome complex. Ferrichrome is transported as efficiently as ferric enterochelin, the ligand synthesized by *E. coli*. The iron is released possibly by reduction. Whether the acetylation facilitates the release of iron remains to be determined in the *in vitro* system. The acetylated deferriferrichrome is released into the medium and not taken up as long as ferrichrome is present in the medium. Under conditions where all of the ligand is used up in the iron transport, the acetylated product is taken up by the same transport system. If an excess of iron is in the medium, the acetylated ligand may also carry iron into the cell. This can account for the observation (Table 1) that, at a high ratio of iron to ligand, two iron ions are transported by one ligand molecule. This uptake has a low affinity for iron since higher concentrations of NTA inhibit the uptake. Deferriferrichrome is also taken up by the same transport system, converted to the acetylated derivative, and left in the cell. One also has to consider the possibility that ferrichrome transport is accelerated by the excretion of the ligand and that acetyl phosphate serves as an energy source for ferrichrome transport, as was recently suggested for a number of substrates which are taken up by transport systems in which binding proteins participate (21). Appropriate mutants will have to be studied to decide whether modification of ferrichrome is obligatory for iron transport or whether it is a secondary event.

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