JOURNAL OF BACTERIOLOGY, May 1976, p. 823-830
Vol. 126, No. 2
Copyright © 1976 American Society for Microbiology
Printed in U.S.A.

Mechanisms of Siderophore Iron Transport in Enteric Bacteria

JOHN LEONG AND J. B. NEILANDS*

Department of Biochemistry, University of California, Berkeley, California 94720

Received for publication 10 December 1975

Uptake of ^{55}Fe- and ^{3}H-labeled siderophores and their chromic analogues have been studied in *Salmonella typhimurium* LT-2 and *Escherichia coli* K-12. In *S. typhimurium* LT-2, at least two different mechanisms for siderophore iron transport may be operative. Uptake of ^{55}Fe- and ^{3}H-labeled ferrichrome and kinetically inert Λ-*cis*-chromic [^{3}H]deferriferrichrome by the *S. typhimurium* LT-2 *enb7* mutant, which is defective in the production of its native siderophore, enterobactin, appears to occur by two concurrent mechanisms. The first mechanism is postulated to involve either rapid uptake of iron released from the ferric complex by cellular reduction without penetration of the complex or ligand or dissociation of the complex and simultaneous uptake of both ligand and iron coupled with simultaneous expulsion of the ligand. The second mechanism appears to consist of slower uptake of the intact ferric complex. Uptake of ferrichrome by the *E. coli* K-12 RW193 mutant, which is also defective in the production of enterobactin, appears to occur by at least one mechanism, which is postulated to consist of transport of the intact ferric complex with simultaneous expulsion of the free ligand by the cell. Cellular release of iron from the complex is presumed to involve reduction. Transport of intact chromic deferriferrichrome in both organisms demonstrates that the Λ-*cis* coordination isomer can be accepted by the uptake system and suggests that uptake of the labile ferric complex cannot rely on rapid isomerization or dissociation. In *S. typhimurium enb7*, at least three possible mechanisms of transport are consistent with the uptake data for ferrioxamine B. Neither the *cis* nor *trans* geometrical coordination isomers of chromic [^{3}H]deferriferrioxamine B are taken up. Uptake of the above siderophores with mutants resistant to the antibiotic albomycin was also studied. These findings demonstrate the existence of multiple mechanisms for assimilation of siderophore iron and implicate reduction of the metal ion as a prominent feature of the uptake process.

Siderophores (also called siderochromes) are high-affinity microbial ferric ion transport agents. The ligands of these compounds display diverse structures, although most may be classified as hydroxamates or catechols (14).

Bacterial uptake of siderophores has been followed usually by monitoring either the labeled ligand or metal, seldom together. Workers in the field generally have assumed that label accumulation in such experiments represents uptake of the intact ferric complex, which may be a very tenuous assumption. Relatively few bacterial uptake studies have been performed with doubly labeled siderophores. Uptake of ligand and metal of ferric schizokinen in *Bacillus megaterium* SK11 (1), ferric aerobactin in *Aerobacter aerogenes* 62-1 (1), and ferric enterobactin (enterochelin) in *Escherichia coli* K-12 (7) has been followed and appears to be synchronous in each case. In the first two systems the labeled free ligand appears to be released by the cell after uptake of the intact ferric complex. However, there may exist other bacterial transport systems in which the function of the siderophore is to solubilize iron by complexation, followed by delivery of the metal ion to the cell membrane.

In this paper we examine transport of ^{55}Fe- and ^{3}H-labeled siderophores in the enteric bacteria to elucidate details of the mechanism of high-affinity iron uptake. We have prepared metal ion-substituted siderophores to compare their uptake behavior to that of the native ferric siderophores and to examine the stereospecificity of transport. The high-spin d^5 ferric octahedral siderophore complexes have no crystal-field stabilization energy, and such complexes are consequently kinetically labile (2). However, replacement of ferric ion by chromic ion, which has large crystal-field stabilization en-

ergy, induces kinetic inertness (2). Therefore, the individual coordination isomers of these chromic complexes can be isolated, and the role that these specific coordination isomers might play in bacterial iron transport can be studied (10–12).

Although *E. coli* and *S. typhimurium* do not produce ferrichrome or ferrioxamine B, they nevertheless have high-affinity uptake systems for such exogenous siderophores derived from fungal and *Streptomyces* species.

We describe in this communication uptake of ^{55}Fe- and ^{3}H-labeled ferrichrome and kinetically inert Λ-*cis*-chromic deferriferrichrome (11) in *E. coli* K-12 RW193 and in *S. typhimurium* LT-2 strains *enb7* and *sidC33*. In addition, transport of ^{55}Fe- and ^{3}H-labeled ferrioxamine B and of the *cis* and *trans* geometrical coordination isomers of chromic deferriferrioxamine B (12) has been examined in *S. typhimurium* LT-2 *enb7* and *sidK62*.

MATERIALS AND METHODS

Chemicals. Chemicals used were of the highest purity obtainable and, unless otherwise specified, were not purified further. Ferrichrome (11), chromic deferriferrichrome (11), ferrioxamine B (12), *cis*-chromic deferriferrioxamine B, and *trans*-chromic deferriferrioxamine B (12) were obtained as described previously.

Radioactively labeled siderophores were prepared as follows. The $^{55}FeSO_4$ (usual specific activity, 5 to 10 Ci/g) in 0.05 M H_2SO_4 was purchased from New England Nuclear Corp., Boston. The [^{55}Fe]-ferrichrome (1.9×10^{10} counts/min per mmol) was prepared from $^{55}FeSO_4$, diluted threefold with $FeCl_3 \cdot 6H_2O$ and excess deferriferrichrome, and purified using the procedure for chromic deferriferrichrome (11). The [^{55}Fe]ferrioxamine B (1.6×10^{10} counts/min per mmol) was prepared from $^{55}FeSO_4$, diluted fourfold with $FeCl_3 \cdot 6H_2O$ and excess Desferal, and purified as described previously (12). Desferal is the trade name of the mesylate salt of deferriferrioxamine B.

Ferrichrome, labeled by microwave discharge activation of tritium gas at the Lawrence Berkeley Laboratory, was partially purified using the procedure for chromic deferriferrichrome (11). Final purification was achieved by silica gel column chromatography with 30% H_2O-CH_3OH as the solvent system (11). The [^{3}H]ferrichrome had a specific activity of 10^{11} counts/min per mmol. Chromic [^{3}H]deferriferrichrome was prepared as follows. The [^{3}H]-deferriferrichrome, obtained by treatment of [^{3}H]-ferrichrome with 8-hydroxyquinoline (14), was diluted 125-fold with cold deferriferrichrome and reacted with $CrCl_3 \cdot 6H_2O$ as described previously to afford chromic [^{3}H]deferriferrichrome (11). Final purification was achieved by silica gel column chromatography with 30% H_2O-CH_3OH as the solvent system (11). Chromic [^{3}H]deferriferrichrome had a specific activity of 7.2×10^{8} counts/min per mmol.

Desferal was also labeled by microwave discharge activation of tritium gas at the Lawrence Berkeley Laboratory. The crude [^{3}H]Desferal was diluted 15-fold with cold Desferal and purified by preparation of the ferric complex with excess $FeCl_3 \cdot 6H_2O$ (12). The [^{3}H]ferrioxamine B had a specific activity of 1.2×10^{10} counts/min per mmol. Chromic [^{3}H]deferriferrioxamine B was prepared as follows. Pure [^{3}H]Desferal, obtained by treatment of [^{3}H]ferrioxamine B with 8-hydroxyquinoline (14), was diluted 10-fold with cold Desferal and reacted with anhydrous $CrCl_3 \cdot 3THF$ as described earlier to afford chromic [^{3}H]deferriferrioxamine B (12). *Cis*- and *trans*-chromic [^{3}H]deferriferrioxamine B (1.7×10^{9} counts/min per mmol) were separated by cation-exchange column chromatography (12).

All radioactively labeled siderophores were determined to be radiochemically pure by scanning their thin-layer chromatograms with a Packard 7201 radiochromatogram strip scanner. Thin-layer chromatography of ferrichrome compounds was performed on silica gel plates with 30% H_2O-CH_3OH as the solvent system, whereas cellulose powder plates with *n*-butyl alcohol–*n*-propyl alcohol–water (9:6:5) as the solvent system were used for ferrioxamine B compounds.

Stock solutions (0.1 mM) of labeled siderophores were used for bacterial transport experiments.

Bacterial strains. All strains used in this study were defective in the production of enterobactin, their native siderophore. *E. coli* K-12 strain AN193 (F^-, *thi*$^-$, *proC*$^-$, *leu*$^-$, *trp*$^-$, *entA403*, *tsx*$^-$, *tonA*$^-$) was obtained from I. G. Young. Strain RW193, a *tonA*$^+$ strain derived from AN193, was obtained from R. Wayne. *S. typhimurium* LT-2 strain *enb7* (class II) and *enb sid* mutants *sidC33* (strain TA2732) and *sidK62* (strain TA2761) derived from the *enb7* parent were obtained from M. Luckey. All *Salmonella sid* strains are resistant to the antibiotic albomycin. In addition, *sidC33*, displays good growth response to ferrichrome, and *sidK62* displays a similar response to ferrioxamine B.

Media. Precautions were taken in the preparation of the media used both for iron uptake and for growth of cells in liquid culture to minimize their iron content. Major media components, such as phosphates, ammonium sulfate, and glucose, with the lowest iron content available were chosen, and the water was deionized and twice quartz-distilled. In addition, for ferrioxamine B uptake the glassware was cleaned and then autoclaved with 0.5% (wt/vol) ethylenediaminetetraacetate, followed by thorough rinsing with double-distilled water, and finally rinsed twice with boiling double-distilled water.

All bacteria used in this study were grown in nutrient broth (Difco) at 37 C for overnight cultures and storage. Nutrient broth plates contained 8 g of nutrient broth (Difco), 5 g of NaCl, and 13 g of agar per liter.

Medium A (14, 15) was used for growth of *enb7* for ferrichrome uptake. A specially treated iron-deficient medium A was used for growth of *sidC33* for ferrichrome uptake and for growth of *enb7* and *sidK62* for ferrioxamine B uptake. A 10-ml solution

consisting of 4.0 g of 85% (wt/vol) H_3PO_4, 0.1 g of KCl, and 1.0 g of $(NH_4)_2SO_4$ was passed through a column (3 by 20 cm) of carefully washed Chelex-100 (sodium form; Bio-Rad Laboratories, Richmond, Calif.). Approximately 100 ml of eluate was collected and diluted to 500 ml with double-distilled water, the pH was adjusted to 6.8 with concentrated H_2SO_4, and 0.1 g of $MgSO_4 \cdot 7H_2O$ was added. Iron-deficient glucose solution was prepared as follows. Approximately 35 ml of a 60% glucose solution was passed through a column of Chelex-100 to afford approximately 150 ml of eluate, which was adjusted to pH 4.8 with concentrated H_2SO_4. This 13% glucose solution was autoclaved separately and added to the autoclaved medium to give 0.5% glucose.

M9 minimal medium (8), supplemented with 60 mg each of L-proline, L-leucine, and L-tryptophan and 1 mg of thiamine hydrochloride per liter and made 1.0 mM in both citrate and $MgSO_4$ and 0.2% in glucose, was used for growth of *E. coli* RW193 for ferrichrome uptake.

Bacterial preparations. Several single colonies of strains to be tested were inoculated directly from nutrient broth plates into the appropriate growth medium described above and incubated with shaking at 37 C for 10 h. Growth was measured turbidimetrically at 650 nm with a Gilford-Beckman DU spectrophotometer. After growth into early stationary phase, a 20% inoculum was made into fresh growth medium, and the culture was incubated as before. The cells were harvested at an optical density between 0.6 and 0.8 by centrifugation at room temperature and were washed twice at room temperature with equal volumes of culture medium supplemented with either 1 mM citrate for *Salmonella* strains or 100 μM nitrilotriacetate (NTA) for *E. coli* strains to scavenge adventitious iron from the medium and make it unavailable to cells (15). Citrate was omitted from the *E. coli* uptake medium. The cell pellet was suspended in a sufficient volume of the above-described medium to give an optical density of 1.2 and then divided into 10- or 20-ml portions in 125-ml Erlenmeyer flasks. The flasks were incubated at 37 C with shaking on a water bath for 5 min before addition of labeled siderophores and iron supplements.

Siderophore uptake measurements. Cells were routinely assayed for uptake of labeled siderophores under the following conditions. Stock solutions of the labeled siderophores (0.1 mM) were added with or without iron supplements to the cells and incubated at 37 C. Iron supplements included ferric citrate (prepared from $FeCl_3 \cdot 6H_2O$ and a 50-fold molar excess of citrate) and ferric NTA (prepared from $FeCl_3 \cdot 6H_2O$ and a 20-fold molar excess of NTA). The iron supplements were prepared with excess ligand to prevent formation of highly polymerized species, which would not be readily available to siderophores (16, 17). Approximately 0.5-ml aliquots of ferric cell suspensions and 1.0-ml aliquots of chromic cell suspensions were removed at regular intervals and treated as described earlier (5, 10). The radioactivity in the supernatant fluid was determined in Bray scintillation fluid with a Nuclear-Chicago Uni-Lux II counter (5, 10).

Fate of [^{3}H]deferriferrichrome. A crystal of $FeSO_4 \cdot 7H_2O$ (to reform ferric complex) was added to the supernatant fluids of transport samples at a time corresponding to quantitative uptake of the ^{55}Fe label of [^{55}Fe]ferrichrome (0.5/μM) with little or no uptake of the ^{3}H label of [^{3}H]ferrichrome (0.5 μM) in *enb7* and RW193. The supernatant fluid was then extracted with an equal volume of benzyl alcohol. The radioactivity in the aqueous and organic phases was determined in Bray scintillation fluid. Controls were performed without cells, with [^{3}H]ferrichrome (0.5 μM).

Chromic deferriferrichrome growth tests. The ability of chromic deferriferrichrome to support growth of *S. typhimurium* LT-2 *enb7* either in the presence or absence of 20 μM ferric citrate was examined by placing filter paper disks containing 2, 20, or 200 pmol of the siderophore on medium E plates (14). The top agar (2 ml) in each case contained 1 mM citrate. The radius of growth around the disk was measured after 20 h of incubation at 37 C.

RESULTS

Siderophore uptake by *S. typhimurium*. Uptake of [^{55}Fe]ferrichrome, [^{3}H]ferrichrome, and kinetically inert Λ-*cis*-chromic [^{3}H]deferriferrichrome, all at 0.5 μM, was first investigated in separate experiments in the *S. typhimurium* mutant *enb7* in the presence of 1 mM citrate (Fig. 1). Uptake of the ^{55}Fe label of [^{55}Fe]ferrichrome was found to be linear and quantitative within 2 min. In contrast, uptake of the ^{3}H label of Λ-*cis*-chromic [^{3}H]deferriferrichrome was found to be linear and quantitative within 10 min. The rate of uptake of the ^{55}Fe label of ferrichrome was about three times faster than that of the ^{3}H label of chromic deferriferrichrome. Uptake of the ^{3}H label of [^{3}H]ferrichrome was virtually identical to that of the chromic complex for the first 2 min, followed by a lag of approximately 5 min. It concluded with a slower linear rate of uptake, which was complete, although not quantitative, after 30 min. If an available ferric ion source such as ferric citrate at 5 μM was also added to *enb7*, the uptake rate of the ^{3}H label of [^{3}H]ferrichrome was once again identical to that of the chromic complex and reached saturation at 55% within 4 min. The initial lag period and slower secondary rate of uptake of the ^{3}H label of the ferric complex were thereby eliminated.

Since uptake of ferrichrome iron might involve hydrolysis of its ligand, the fate of the ^{3}H label of [^{3}H]ferrichrome at that point in time corresponding to quantitative removal of the ^{55}Fe label of [^{55}Fe]ferrichrome with little or no uptake of the ^{3}H label of [^{3}H]ferrichrome in *enb7* was of interest. Since the products of the

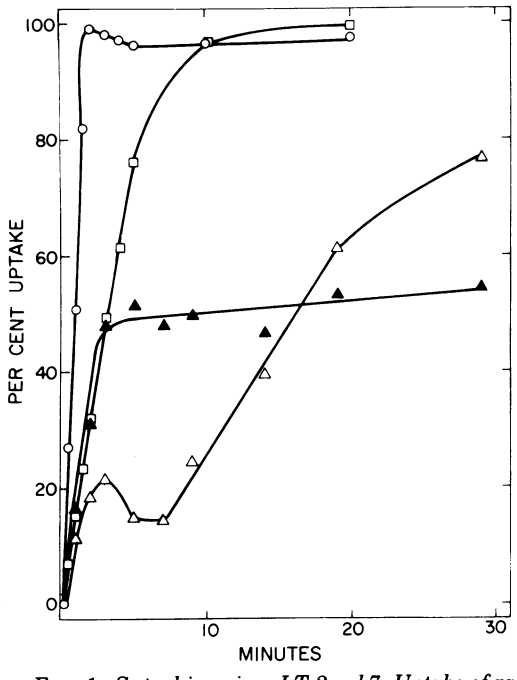

FIG. 1. *S. typhimurium LT-2 enb7. Uptake of radioactive label of [^{55}Fe]ferrichrome (○), Λ-cis-chromic [^{3}H]deferriferrichrome (□), [^{3}H]ferrichrome (△), and [^{3}H]ferrichrome with 5 μM ferric citrate (▲). Cultures were incubated at 37 C with final metal complex concentrations of 0.5 μM in the presence of 1 mM citrate.*

hydrolysis of ferrichrome would be zwitterionic, they would not be extractable into benzyl alcohol. Therefore, after ferrichrome uptake had been allowed to proceed for 2 min, the [^{3}H]ferrichrome supernatant fluid was subjected to benzyl alcohol extraction, and the radioactivity of both the aqueous and organic phases was determined. Seventy-nine percent of the radioactivity of the [^{3}H]ferrichrome in the supernatant fluid was extractable into benzyl alcohol, compared to 84% of the radioactivity in a control without cells. These results suggest that the ligand is not structurally altered metabolically by the cell.

Since resistance to the antibiotic albomycin might arise as a result of a specific defect in the uptake of the ligand of ferrichrome, we investigated ferrichrome uptake in *sidC33*, which is resistant to albomycin but is able to utilize ferrichrome for growth (14). Uptake of the ^{55}Fe label of [^{55}Fe]ferrichrome (0.5 μM) by *sidC33* was linear and 75% complete within 90 min, considerably slower than that of the parent *enb7*. In a separate experiment, no uptake of the ^{3}H label of [^{3}H]ferrichrome (0.5 μM) was observed. However, uptake of the ^{3}H label of kinetically inert Λ-*cis*-chromic [^{3}H]deferriferrichrome (0.5 μM) was linear and reached saturation at about 25% within 30 min.

Uptake of [^{55}Fe]ferrioxamine B, [^{3}H]ferrioxamine B, and the kinetically inert *cis* and *trans* geometrical coordination isomers of chromic [^{3}H]deferriferrioxamine B, all at 0.1 μM, was examined in separate experiments in *enb7* (Fig. 2). The role that specific coordination isomers might play in bacterial iron transport was of particular interest. Uptake of the ^{55}Fe label of [^{55}Fe]ferrioxamine B was found to be linear and complete within 20 min. In contrast, uptake of the ^{3}H label of [^{3}H]ferrioxamine B was found to lag for about 10 min, before reaching 80% completion within 2 h. If ferric citrate at 1.0 μM was also added to *enb7*, the lag period of the ^{3}H label of [^{3}H]ferrioxamine B was extended to approximately 20 min, followed by a slightly slower rate of uptake reaching 50% completion within 2 h. Finally, no uptake of ^{3}H label of either *cis*- or *trans*-chromic [^{3}H]-deferriferrioxamine B was observed.

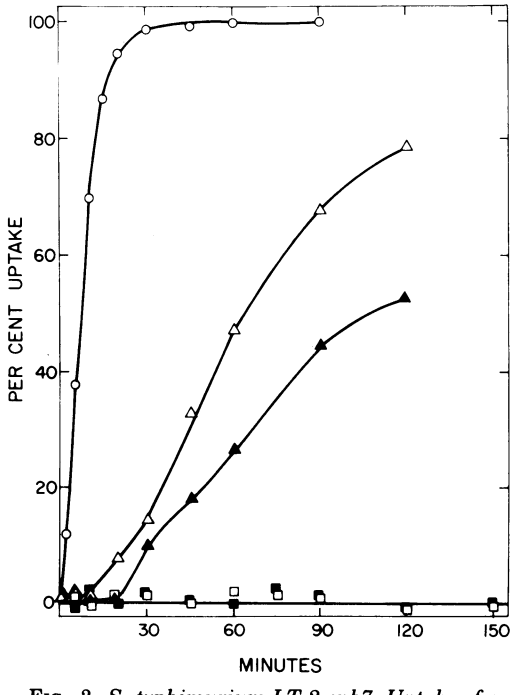

FIG. 2. *S. typhimurium LT-2 enb7. Uptake of radioactive label of [^{55}Fe]ferrioxamine B (○), cis-chromic [^{3}H]deferriferrioxamine B (□), trans-chromic [^{3}H]deferriferrioxamine B (■), [^{3}H]ferrioxamine B (△), and [^{3}H]ferrioxamine B with 1 μM ferric citrate (▲). Cultures were incubated at 37 C with final metal complex concentrations of 0.1 μM in the presence of 1 mM citrate.*

Uptake of the same radioactively labeled siderophores, all at 0.1 μM, was also examined in another *S. typhimurium enb sid* mutant, *sidK62*. Since the *enb sid* mutants exhibit a variety of defects in siderophore uptake, we wanted to know whether *sidK62* might exhibit reduced uptake of the ligand of ferrioxamine B. Uptake of the ^{55}Fe label of [^{55}Fe]ferrioxamine B was linear and complete within 30 min. No uptake of the ^{3}H label of either *cis-* or *trans*-chromic [^{3}H]deferriferrioxamine B or [^{3}H]ferrioxamine B, in particular, was observed. No binding of either [^{55}Fe]ferrioxamine B (0.1 μM), [^{3}H]ferrioxamine B (0.1 μM), or *cis-* or *trans*-chromic [^{3}H]deferriferrioxamine B (0.1 μM) was observed to cells of *sidK62* within 2 min of mixing at 37 C when measured by membrane filtration (Millipore Corp.) (8, 9).

Siderophore uptake by *E. coli*. Cell suspensions of the enterobactin-negative *E. coli* mutant RW193, grown under iron-deficient conditions, were tested for their ability to take up [^{55}Fe]ferrichrome, [^{3}H]ferrichrome, and Λ-*cis*-chromic [^{3}H]deferriferrichrome, all at 0.5 μM, in separate experiments in the presence of 100 μM NTA, to scavenge adventitious iron in the medium (Fig. 3). The uptake rates of the ^{55}Fe label of [^{55}Fe]ferrichrome and the ^{3}H label of chromic [^{3}H]deferriferrichrome were virtually identical, both being linear and quantitative within 20 min. Uptake of the ^{3}H label of [^{3}H]ferrichrome was found to lag for about 10 min, before reaching 90% completion within 1 h. If an available ferric ion source such as ferric NTA at 5 μM was also added to RW193, the uptake behavior of the ^{3}H label of [^{3}H]ferrichrome was virtually unaltered for the first 30 min and then displayed a lag for about 30 min before reaching 40% completion after 90 min.

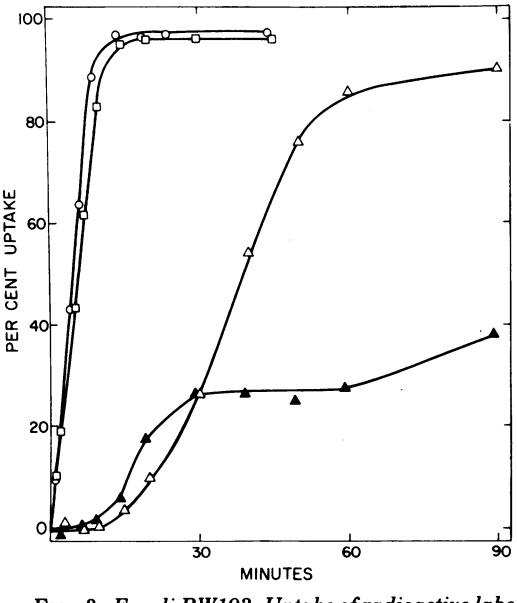

FIG. 3. *E. coli RW193. Uptake of radioactive label of [^{55}Fe]ferrichrome (○), Λ-cis-chromic [^{3}H]deferriferrichrome (□), [^{3}H]ferrichrome (△), and [^{3}H]-ferrichrome with 5 μM ferric NTA (▲). Cultures were incubated at 37 C with final metal complex concentrations of 0.5 μM in the presence of 100 μM NTA.*

Seventy-six percent of the radioactivity of the [^{3}H]ferrichrome supernatant fluid at that point in time corresponding to quantitative uptake of the ^{55}Fe label of [^{55}Fe]ferrichrome, with little or no uptake of the ^{3}H label of [^{3}H]ferrichrome, was extractable into benzyl alcohol. The radioactivity of a control without cells was 87% extractable into benzyl alcohol. Thus no appreciable metabolic structural alterations of the ligand by the cell occurred.

Effect of *S. typhimurium* LT-2 on kinetic lability of chromic deferriferrichrome. We wanted to determine whether kinetically inert chromic deferriferrichrome (11) might be labilized by bacterial action. If such were the case, then addition of a ferric ion source such as ferric citrate would result in the rapid formation of ferrichrome, which would support the growth of *S. typhimurium* LT-2 *enb7* on medium E plates. No growth was observed with 2 or 20 pmol of chromic deferriferrichrome, and only a constant radius of growth of 3 mm was observed with 200 pmol of the chromic complex, either in the presence or absence of 20 μM ferric citrate. A 3-mm radius of growth corresponds to approximately 2 pmol of ferrichrome. Thus, no significant labilization of chromic deferriferrichrome occurred with *enb7*.

DISCUSSION

Several possible mechanisms can be imagined for the cellular acquisition of iron from exogenous siderophores. We offer three mechanisms, which are not meant to be inclusive. Mechanism 1 features donation of the iron to the cell membrane without penetration of the complex or ligand. Mechanism 2 involves uptake of the intact metal complex followed by internal release of the iron. Mechanism 3 features dissociation of the complex with simultaneous uptake of both ligand and iron. The use of ^{55}Fe- and ^{3}H-labeled and chromic ion-substituted siderophores helps to resolve these pathways.

In mechanism 1, iron would be accumulated by cells, but the ligand of the labile ferric siderophore complex would remain in the medium,

as would the intact chromic siderophore complex, which cannot be readily dissociated or reduced under physiological conditions. Both the ligand and metal ion of Λ-*cis*-chromic deferriferrichrome are taken up together as the intact complex by *Ustilago sphaerogena* (10), suggesting that this microorganism cannot dissociate the kinetically inert chromic complex. Therefore, siderophore-mediated uptake of the ^{55}Fe label would be observed, but accumulation of the ^{3}H label of the ligand of either the ferric or chromic complex would not occur.

In mechanism 2, both the chromic complex and the ligand of the ferric complex could potentially accumulate in the cell along with the iron, since dissociation of the ferric complex would occur after its penetration into the cell. Therefore, uptake of the ^{3}H label of the chromic siderophore at a rate similar to that of the ^{55}Fe label would distinguish transport by mechanism 2 from transport by mechanism 1 or 3. However, if the ^{3}H label of the chromic complex is not accumulated, mechanism 2 would not be ruled out, since on the one hand the mixture of chromic coordination isomers obtained by metal ion substitution may not contain the specific isomer recognized by the transport system, if such an isomer exists, and on the other hand the transport system might require isomerization of the intact complex during uptake.

In mechanism 3, the non-dissociable chromic siderophore complex would not be accumulated, but the ligand of the labile ferric complex would enter the cell along with the iron. The rate of exit of the free ligand would govern the extent of its accumulation within the cell. Therefore, an accumulation of the ^{3}H label of the ferric siderophore complex at a rate equal to or less than the rate of uptake of the ^{55}Fe label, combined with a lack of uptake of the ^{3}H label of the chromic complex, would characterize transport by mechanism 3.

Siderophore uptake by *S. typhimurium*. The results of uptake experiments with ^{55}Fe- and ^{3}H-labeled ferrichrome and the ^{3}H-labeled chromic analogue in *enb7* (Fig. 1) are consistent with the operation of two concurrent mechanisms, mechanism 2 and mechanism 1 or 3. Either rapid uptake of iron from the ferric complex by the cell without penetration of the complex or ligand (mechanism 1) or dissociation of the complex with simultaneous uptake of both ligand and iron (mechanism 3), coupled with simultaneous explusion of ligand, occurs. A slower rate of uptake of the intact ferric complex (mechanism 2) also occurs. The accumulation of the Λ-*cis* coordination isomer of the chromic analogue demonstrates that this specific isomer can be accepted by the uptake system and suggests that uptake of the labile ferric complex cannot rely on rapid isomerization or dissociation. Either mechanism 1 or 3 would likely require reduction of the ferric complex at the cell membrane to allow dissociation of the complex, since the half-life of iron exchange in ferrichrome (pH 6.3, 37 C) is approximately 10 min (13), yet the initial uptake of the ^{55}Fe label of [^{55}Fe]ferrichrome is complete within 2 min. The lag in the accumulation of the ^{3}H label of [^{3}H]ferrichrome is consistent with uptake of the intact ferric complex (mechanism 2), most of which has been reformed from the ligand and adventitious ferric ion in the medium. Supplementation with ferric citrate appears to result in a more rapid reformation of the ferric complex, which is then transported intact at a rate identical to that of the chromic analogue, eliminating the lag period in the ^{3}H uptake of [^{3}H]ferrichrome.

In the case of the *enb sid* mutant *sidC33*, no uptake of ^{3}H label from [^{3}H]ferrichrome was observed; however, uptake of the ^{55}Fe label of [^{55}Fe]ferrichrome was much slower than in *enb7* and there was a residual uptake of the chromic analogue. Since both uptake systems seen in the parent strain are operative but function at reduced levels, a possible regulatory defect is suggested.

The uptake results with ^{55}Fe- and ^{3}H-labeled ferrioxamine B and the *cis* and *trans* isomers of chromic [^{3}H]deferriferrioxamine B in *enb7* (Fig. 2) do not allow us to distinguish among mechanisms 1, 2, or 3. No uptake of the chromic coordination isomers was observed, as predicted by either mechanism 1 or 3. However, this result would also be expected for mechanism 2 if the mixture of chromic isomers utilized does not contain the specific isomer recognized by the transport system or if isomerization of the intact ferric complex is required for transport. Similarly, the lack of initial accumulation of the ^{3}H label of [^{3}H]ferrioxamine B is consistent either with dissociation of the complex without penetration of the complex or ligand (mechanism 1) or with immediate expulsion of the ligand after its penetration through the membrane (mechanism 2 or 3). The mechanism of accumulation of the ^{3}H label of [^{3}H]ferrioxamine B that occurs several minutes later needs further investigation. Since supplementation with ferric citrate did not measurably affect the rate of accumulation of the ^{3}H label of [^{3}H]ferrioxamine B, its uptake is apparently not limited by the rate of reformation of the intact ferric complex. Therefore, this ^{3}H label accumulation may indicate that uptake over the entire time course occurs by mechanism 2 or 3, with rapid expulsion of the ligand

from the cell initially but with slower expulsion later to allow net influx of ligand. The uptake results with the same radioactively labeled siderophores in the *enb sid* mutant *sidK62* also do not allow us to distinguish among mechanisms 1, 2, and 3. Unlike the parent *enb7, sidK62* did not accumulate the ^{3}H label of [^{3}H]ferrioxamine B.

Siderophore uptake by *E. coli*. The uptake results with ^{55}Fe- and ^{3}H-labeled ferrichrome and ^{3}H-labeled chromic analogue by RW193 (Fig. 3) show important differences from those in the *S. typhimurium* LT-2 mutant, *enb7*. Since the uptake rates of the ^{55}Fe label of [^{55}Fe]ferrichrome and its chromic analogue are virtually identical, the uptake mechanism appears to involve transport of the intact ferric complex (mechanism 2) with simultaneous expulsion of the ligand, since no counts of the latter are accumulated initially. Reduction of the ferric complex to allow its dissociation seems likely in view of the rapid uptake of the ^{55}Fe label. As in *enb7*, accumulation of the Λ-*cis* coordination isomer of the chromic analogue demonstrates that this specific isomer can be accepted by the uptake system and suggests that uptake of the labile ferric complex cannot rely on rapid isomerization or dissociation. Since supplementation with ferric NTA affected only the extent of the ^{3}H label of [^{3}H]ferrichrome incorporated, uptake is apparently not limited by the rate of reformation of the ferric complex. The mechanism of the slower accumulation of the ^{3}H label remains obscure, although retardation of the expulsion of free ligand, originally transported as the intact ferric complex, is possible.

Synchronous uptake of both the ligand and iron of the siderophore (mechanism 2) has been demonstrated with ferric schizokinen in *B. megaterium* SK11 (1), ferric aerobactin in *A. aerogenes* 62-1 (1), and ferric enterobactin in *E. coli* K-12 (7). In none of the above systems has the mechanism of release of iron from the siderophore been conclusively established, but genetic and biochemical evidence has been adduced for participation of an esterase in rendering available the iron of ferric enterobactin (9). Recently, Flatmark and Romslo demonstrated that energy-dependent accumulation of iron from ferric sucrose in rat liver mitochondria requires reducing equivalents supplied by the respiratory chain, suggesting that iron crosses the inner membrane only in the ferrous form (6). Our uptake results with ferrichrome in *E. coli* K-12 RW193 and *S. typhimurium* LT-2 *enb7* also suggests that release of iron from ferrichrome requires reduction.

We have found that at least two different mechanisms for siderophore iron uptake may be operative in enteric bacteria. Furthermore, *E. coli* K-12 and *S. typhimurium* LT-2, after growth in comparable media, show important differences in ferrichrome uptake. Thus, in *S. typhimurium* LT-2, both a synchronous mechanism involving uptake of the intact ferric complex (mechanism 2) and a second process take place, the latter involving either donation of the iron to the cell membrane without penetration of the complex or ligand (mechanism 1) or dissociation of the complex and simultaneous uptake of both ligand and iron (mechanism 3), coupled with simultaneous expulsion of ligand. In *E. coli* K-12, a synchronous process characterized by uptake of the intact ferric complex (mechanism 2) and simultaneous expulsion of the free ligand takes place.

Mechanisms 1 and 3 would confer upon the cell certain biological advantages. Assuming that the process involves reduction, it would preclude a siderophore-mediated transport of non-essential metal ions such as aluminum. In addition, it would prevent the unnecessary accumulation of the free ligand in the cytoplasm, a circumstance that might be deleterious to the cell. On the other hand, uptake of the intact complex, as in mechanism 2, would provide the cell with a specific small molecule, which could be utilized as a regulator of iron-related metabolism or as an indicator of the ecological composition of the extracellular environment.

The differences we have observed between *E. coli* K-12 and *S. typhimurium* LT-2 in ferrichrome uptake are not the only discrepancies in metabolism of this siderophore known in the two organisms. The ferrichrome receptor in *E. coli* K-12 binds the T1, T5, and Φ80 groups of phages, whereas the corresponding receptor in *S. typhimurium* LT-2 binds phage ES18 (M. Luckey and J. B. Neilands, Pacific Slope Biochemistry Conference, Abstracts, p. 53, 1975).

ACKNOWLEDGMENTS

We thank Rush Wayne and Mary Luckey for their assistance and many helpful discussions and K. N. Raymond for use of his equipment and helpful discussion.

This research was supported by Public Health Service grants AI 04156 from the National Institute of Allergy and Infectious Diseases and AM 17146 from the National Institute of Arthritis, Metabolism, and Digestive Diseases.

LITERATURE CITED

1. **Arceneaux, J. E. L., W. B. Davis, D. N. Downer, A. H. Haydon, and B. R. Byers.** 1973. Fate of labeled hydroxamates during iron transport from hydroxamate-iron chelates. J. Bacteriol. 115:919-927.
2. **Basolo, F., and R. G. Pearson.** 1967. Mechanisms of inorganic reactions, 2nd ed. John Wiley & Sons, New York.
3. **Davis, W. B., and B. R. Byers.** 1971. Active transport of

iron in *Bacillus megaterium:* role of secondary hydroxamic acids. J. Bacteriol. **107**:491–498.

4. **Downer, D. N., W. B. Davis, and B. R. Byers.** 1970. Repression of phenolic acid-synthesizing enzymes and its relation to iron uptake in *Bacillus subtilis*. J. Bacteriol. **101**:181–187.
5. **Emery, T.** 1971. Role of ferrichrome as a ferric ionophore in *Ustilago sphaerogena*. Biochemistry **10**:1483–1488.
6. **Flatmark, T., and I. Romslo.** 1975. Energy-dependent accumulation of iron by isolated rat liver mitochondria. J. Biol. Chem. **250**:6433–6438.
7. **Frost, G. E., and H. Rosenberg.** 1973. The inducible citrate-dependent iron transport system in *Escherichia coli* K-12. Biochim. Biophys. Acta **330**:90–101.
8. **Guterman, S. K.** 1973. Colicin B: mode of action and inhibition by enterochelin. J. Bacteriol. **114**:1217–1224.
9. **Langman, L., I. G. Young, G. E. Frost, H. Rosenberg, and F. Gibson.** 1972. Enterochelin system of iron transport in *Escherichia coli:* mutations affecting ferric-enterochelin esterase. J. Bacteriol. **112**:1142–1149.
10. **Leong, J., J. B. Neilands, and K. N. Raymond.** 1974. Coordination isomers of biological iron transport compounds. III. Transport of Λ-*cis*-chromic desferriferrichrome by *Ustilago sphaerogena*. Biochem. Biophys. Res. Commun. **60**:1066–1071.
11. **Leong, J., and K. N. Raymond.** 1974. Coordination isomers of biological iron transport compounds. II. The optical isomers of chromic desferriferrichrome and desferriferrichrysin. J. Am. Chem. Soc. **96**:6628–6630.
12. **Leong, J., and K. N. Raymond.** 1975. Coordination isomers of biological iron transport compounds. IV. Geometrical isomers of chromic desferriferrioxamine B. J. Am. Chem. Soc. **97**:293–296.
13. **Lovenberg, W., B. B. Buchanan, and J. C. Rabinowitz.** 1963. Studies on the chemical nature of clostridial ferredoxin. J. Biol. Chem. **238**:3899–3913.
14. **Luckey, M., J. R. Pollack, R. Wayne, B. N. Ames, and J. B. Neilands.** 1972. Iron uptake in *Salmonella typhimurium:* utilization of exogenous siderochromes as iron carriers. J. Bacteriol. **111**:731–738.
15. **Pollack, J. R., B. N. Ames, and J. B. Neilands.** 1970. Iron transport in *Salmonella typhimurium:* mutants blocked in the biosynthesis of enterobactin. J. Bacteriol. **104**:635–639.
16. **Spiro, T. G., G. Bates, and P. Saltman.** 1967. The hydrolytic polymerization of ferric citrate. II. The influence of excess citrate. J. Am. Chem. Soc. **89**:5559–5562.
17. **Spiro, T. G., L. Pape, and P. Saltman.** 1967. The hydrolytic polymerization of ferric citrate. I. The chemistry of the polymer. J. Am. Chem. Soc. **89**:5555–5559.