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Pyoverdine-mediated iron transport

Fate of iron and ligand in *Pseudomonas aeruginosa*

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Summary. Incubated in the presence of [^{55}Fe]ferri[^{14}C]pyoverdine, iron-poor *Pseudomonas aeruginosa* accumulated more ^{55}Fe than ^{14}C over a 60-min period. Distribution studies showed (a) more ^{14}C than ^{55}Fe in the soluble fraction during the first 20 min, (b) approximately 60% of the ^{55}Fe associated with the membranes at 60 min, and (c) approximately 85% of the ^{14}C in the soluble fraction at 60 min. Cells osmotically shocked after incubating with [^{55}Fe]ferri[^{14}C]pyoverdine for 60 min released ^{55}Fe but not ^{14}C, suggesting separation of metal and ligand in the periplasmic space. Whereas the mechanism of dissociation of iron and ligand is not known, the decrease in transport observed in the presence of dipyridyl suggests involvement of reduction in this process. Transport of iron was energized by the proton motive force instead of by intracellular levels of ATP. The hydrogen ion gradient was the major driving force of transport. Cyanide-poisoned cells accumulated more ^{14}C than ^{55}Fe over 60 min. Here, iron accumulated in the soluble fraction instead of on the membranes.

Key words: Siderophore - Iron transport - Pyoverdine - *Pseudomonas aeruginosa*

Introduction

When grown under iron-poor conditions, many bacteria synthesize and secrete into the environment iron chelators termed siderophores (Neilands 1981). By solubilizing the ferric ion of insoluble complexes that form under aerobic conditions at physiological pH, siderophores make iron available for use by the cell. In the host it is expected that siderophores sequester iron from iron-containing molecules such as transferrin and lactoferrin, and subsequently deliver iron to the microbial cell.

The first step in transport of siderophore-bound iron into the Gram-negative bacterial cell is recognition of the iron-containing siderophore, i.e. the ferrisiderophore, by a specific outer membrane receptor. Following binding of the ferrisiderophore to the receptor, the iron must pass through the outer membrane, the periplasmic space and the cytoplasmic membrane before entering the cytoplasm of the cell. Release of iron from a siderophore could occur by one of the following mechanisms: (a) upon reduction of the ferric ion; (b) following hydrolysis of the chelator; or (c) via exchange with another chelator. Following dissociation of the iron from its chelator within the cell envelope or the cytoplasm, iron metabolism or storage occurs.

The major exogenous siderophore of the fluorescent pseudomonads is pyoverdine, a water-soluble yellow-green fluorescent pigment characteristically produced by iron-starved cells (Meyer et al. 1987). It has been demonstrated that pyoverdine stimulates growth of *Pseudomonas aeruginosa*, presumably by delivering iron to the cell (Cox and Adams 1985). Studies have revealed that pyoverdine isolated from other fluroescent *Pseudomonas* strains does serve as an iron transporter in these cells (Hohnadel and Meyer 1988). One of the high-molecular-mass proteins apparent on the outer membrane of iron-starved *P. aeruginosa* may serve as a receptor for ferripyoverdine (Meyer et al. 1979).

This study examined the steps of pyoverdine-mediated iron transport into *P. aeruginosa* and the subsequent fate of intracellular iron. The results of experiments performed using single-labeled ferripyoverdine ([^{55}Fe]ferripyoverdine) and the double-labeled ferrisiderophore ([^{55}Fe]ferri[^{14}C]pyoverdine) are presented here. These findings are correlated with results of Mossbauer spectroscopy studies using [^{57}Fe]ferripyoverdine presented in another paper (Mielczarek et al. 1990). Here, the distribution of ^{55}Fe and ^{14}C in the cells following incubation with the dual-labeled ferrisiderophore is presented, as is the effect of energy inhibitors on iron transport and accumulation. It is seen that energy-rich cells accumulate more ^{55}Fe than ^{14}C. Energy-poor cells, however, accumulate more ^{14}C than ^{55}Fe, and the distribution pattern of the two isotopes differs in these cells as compared with that in energy-rich cells.

Evidence also suggests that dissociation of iron from the ferrisiderophore occurs in the periplasmic space.

Materials and methods

Bacteria and culture conditions

Pseudomonas aeruginosa ATCC 15692 was used in all experiments. Cells were routinely grown in 0.25% casamino acids containing 0.2 mM $MgCl_2$, pH 7.2. These cells are referred to as iron-poor cells. Iron-fed cells are those grown in the same medium containing 64 μM $FeCl_3$. Cultures were grown shaking in a New Brunswick rotatory shaker at 150 rpm at 30° C. Mid-to-late logarithmic-phase cells were used in all experiments.

Preparation of the ferrisiderophore and the deferrisiderophore

A modification of the method of Meyer and Abdallah (1978) was used to prepare [^{55}Fe]ferripyoverdine. Here, cells were grown in casamino acid medium instead of succinate synthetic medium which contains phosphate. This allowed for the direct addition of radiolabeled iron to spent medium without formation of [^{55}Fe]ferric phosphate. Specifically, 8 μCi $^{55}FeCl_3$ (New England Nuclear) was added to each 100 ml spent media. After stirring for 1 h at room temperature, 100 μmol $FeCl_3$ was added and the mixture stirred for 1 h more. The ferripyoverdine was then extracted with 0.5 vol. chloroform/phenol, and driven into water with ethyl ether. Following lyophilization, the sample was reconstituted in water and applied to a Sephadex CM-25 column equilibrated with 0.5 M pyridine/acetic acid pH 5.0 and eluted with the same buffer. This chromatography resulted in the appearance of two bands, the top one of which was collected, washed twice in distilled water, and used for transport studies. The specific activity of these preparations ranged over 25–51 Ci/mol.

To make dual-labeled ferripyoverdine, cells were grown in 125 ml casamino acid medium to mid-logarithmic phase. Then 10 μCi mixed ^{14}C-labeled amino acids (New England Nuclear) was added, and the culture shaken for another 15 h. After centrifugation, radiolabeled and unlabeled iron was added to the spent medium as above, and [^{55}Fe]ferri[^{14}C]pyoverdine was purified as stated. The ratio of radioactivity (dpm) of ^{55}Fe to that ^{14}C in these preparations was approximately 30/1. The specific activity of iron in the preparations was 28–30 Ci/mol and that of ^{14}C, 0.92–1 Ci/mol. On graphs showing data using these preparations, the ranges used on the axes reflect the approximate 30/1 difference.

[^{14}C]Pyoverdine was prepared as above, adding only unlabeled iron to the spent medium of cells grown in the presence of ^{14}C-labeled amino acids. Following purification of ferri[^{14}C]pyoverdine, deferration was accomplished using 8-hydroxyquinoline, and the deferrated siderophore was recovered on a Sephadex CM-25 column using distilled water as described by Meyer and Abdallah (1978). The specific activity of [^{14}C]pyoverdine was 1 Ci/mol.

Transport assays

Mid-logarithmic cells were centrifuged and washed twice with 60 mM 4-morpholinepropanesulfonic acid, pH 7.2 (Mops) at room temperature. Cells were resuspended in Mops at a total absorbance of 3.0 at 600 nm. Inhibitors at concentrations indicated in the text, 2 mM succinate, or 6 mM dipyridyl was added, and the suspension kept at 30° C for 20 min at which time [^{55}Fe]ferri[^{14}C]pyoverdine or [^{55}Fe]ferripyoverdine (containing approximately 1 μCi ^{55}Fe) was added. The total volume of cell suspension with additions was 5.0 ml. Aliquots (0.5 ml) were withdrawn at designated times and added to 5 ml cold Mops on a Metricel GA-6 filter, porosity 0.45 μm. Following filtration, the cells on the filter were washed twice with 3 ml cold Mops, and the filter transferred to a liquid scintillation vial. Optifluor (Packard) was added and the sample counted in a Packard 300 CD liquid scintillation counter. Adsorption of radioactivity to the filters was determined by filtering radiolabeled substrate diluted as above in the absence of cells. The filters were washed as above and counted. Results are expressed as nmol $^{55}Fe \times 30/g$ dry mass of cells and nmol $^{14}C/g$ dry mass of cells. At this time it is not known if the cell-associated ^{14}C is intact pyoverdine or hydrolyzed pyoverdine.

To determine if soluble ^{55}Fe or ^{14}C is cytoplasmic or periplasmic, this transport assay was carried out using twice the volume of cells. At select time intervals, 1-ml aliquots were filtered and washed. After 60 min, the cells of 1 ml were osmotically shocked (Hoshino 1979) by resuspending in 20 ml 20 mM Tris/HCl pH 8.4, containing 0.2 M $MgCl_2$, and kept at 30° C for 5 min followed by a 15-min incubation in an ice bath. After one more temperature shift, the suspension was filtered and the cells washed as above. A second 1-ml aliquot of cells was resuspended in 20 ml Mops buffer and treated as above. This experiment was repeated four times.

Distribution studies

The cells of 1 or 2-l culture media were washed as above and resuspended in 100 ml Mops containing 2 mM succinate or 0.1 mM KCN. After 20 min at 30° C, [^{55}Fe]ferri[^{14}C]pyoverdine containing 2 μCi ^{55}Fe was added. At the designated times, 20-ml aliquots were withdrawn, centrifuged, washed twice in cold Mops, and broken in a Carver pressure cell as previously described (Royt 1988). Total membranes and soluble fraction were collected upon centrifuging at $147500 \times g$ for 30 min and aliquots of each were counted. To isolate inner and outer membranes, the membrane suspension was instead applied to a sucrose density gradient and centrifuged, as previously described (Royt 1988), and total inner membranes and outer membranes were collected. In all cases, membranes were washed twice before being counted. Total radioactivity in the soluble fractions or bound to membranes was calculated, and these results expressed as above.

Results

The effect of energy inhibitors on transport of [^{55}Fe]ferripyoverdine

As in *Pseudomonas fluorescens* (Meyer and Hornsperger 1978), the rate of transport via pyoverdine of radiolabeled iron into iron-stressed *P. aeruginosa* was stimulated by the presence of a utilizeable substrate, succinate, and decreased by the presence of 2 mM sodium azide (Fig. 1). To determine the major energy source for this transport, the rate of transport was examined in the presence of other energy inhibitors (Table 1). It is seen that at 1 mM, potassium cyanide, another inhibitor of electron flow in the electron transport chain, inhibited uptake of iron without affecting viability: 80% inhibition of [^{55}Fe]ferripyoverdine uptake occurred in the presence of 0.1 mM cyanide. Sodium arsenate, a phosphate analogue that inhibits ATP formation, was less effective in preventing iron uptake via pyoverdine than were both sodium azide and potassium cyanide. The uncoupler of oxidative phosphoryla-

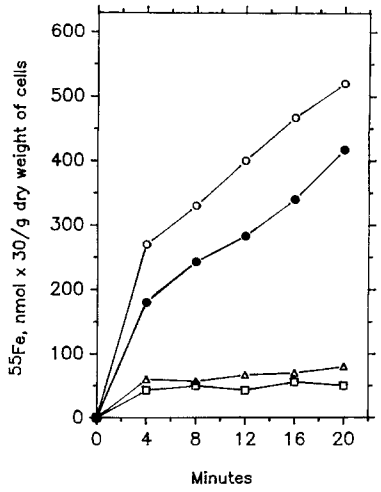

Fig. 1. Transport of ^{55}Fe via [^{55}Fe]ferripyoverdine into cells grown in casamino acid medium. Transport into iron-poor cells in the presence of (○) 4 mM succinate, (△) 2 mM sodium azide, and (●) no additions. (□) Transport into cells grown in iron-supplemented casamino acid medium

tion, dinitrophenol, at 5 mM, inhibited iron uptake, whereas the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was effective in inhibiting uptake at a lower concentration, i.e. 1 mM. Together, these results indicate that the proton motive force drives the transport of iron. To determine if the membrane electrical potential or the transmembrane hydrogen ion gradient is the major driving force, transport was measured in the presence of the ionophores nigericin, which reduces the proton gradient, and valinomycin, a potassium ionophore which reduces the electrical potential of the membrane (Table 1). Incubation in the presence of valinomycin for 10 min resulted in minimal inhibition of uptake. At the same concentrations, nigericin more effectively inhibited transport than did valinomycin. Hence, the trans-membrane hydrogen ion gradient is of more importance in transport of iron via pyoverdine than is the membrane electrical gradient.

Table 1. Effects of energy inhibitors on viability and [^{55}Fe]ferripyoverdine uptake by iron-poor cells

Inhibitor	Final concn (mM)	Time of measurement (min)	Inhibition of uptake (%)	Viability (%)
KCN	0.1	20	80	96
	1.0		100	100
Sodium azide	0.1	20	0	100
	1.0		13	100
	2.0		100	94
Sodium arsenate	5.0	20	0	95
	10.0		37	97
Dinitrophenol	0.1	20	0	102
	0.5		15	95
	2.0		32	103
	5.0		100	89
Valinomycin	0.1	10	8	100
	0.5		8	98
Nigericin	0.1	10	47	95
	0.5		48	96
CCCP	0.1	10	87	82
	1.0		95	85

Transport of [^{55}Fe]ferri[^{14}C]pyoverdine into iron-deficient cells and intracellular distribution of radioisotopes

Upon incubating cells grown in casamino acid medium with the dual-labeled ferrisiderophore for 1 h, it is seen that the initial rates of accumulation of ^{55}Fe and of ^{14}C are similar (Fig. 2). After 10 min, however, only ^{55}Fe continues to accumulate in the cells. Upon disrupting cells incubated with [^{55}Fe]ferri[^{14}C]pyoverdine and determining the location of the isotopes, it was found that ^{14}C accumulates in the soluble fraction; only trace amounts of ^{14}C were found on the total membranes of these cells (Fig. 3A). Radiolabeled iron, however, was found in both the soluble fraction and on the membranes. Greater amounts of ^{14}C than of ^{55}Fe were consistently found in the soluble fraction at 20 min. This indicates dissociation of ligand and metal within this time period. Separation of the membranes revealed that the iron accumulated on the inner membrane, with residual amounts being found on the outer membrane (Fig. 3B). Similarly, more ^{14}C was found on the inner membrane than on the outer membrane.

Cells exposed to [^{55}Fe]ferri[^{14}C]pyoverdine for 1 h were osmotically shocked to ascertain if any soluble ^{55}Fe and/or ^{14}C was in the periplasmic space. It is seen in Fig. 4 that osmotically shocked cells contained less ^{55}Fe than did the cells at 1 h. Control cells, i.e. cells which had been transferred instead to Mops buffer and subjected to two temperature shifts, did not show a de-

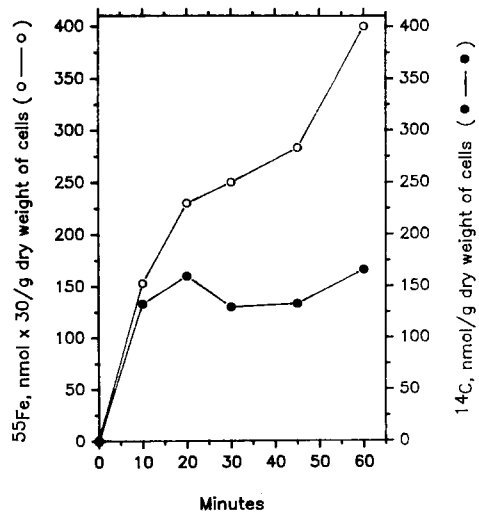

Fig. 2. Transport of [^{55}Fe]ferri[^{14}C]pyoverdine into iron-poor cells. Total accumulation of (○) ^{55}Fe and (●) ^{14}C by iron-poor cells as determined by the transport assay

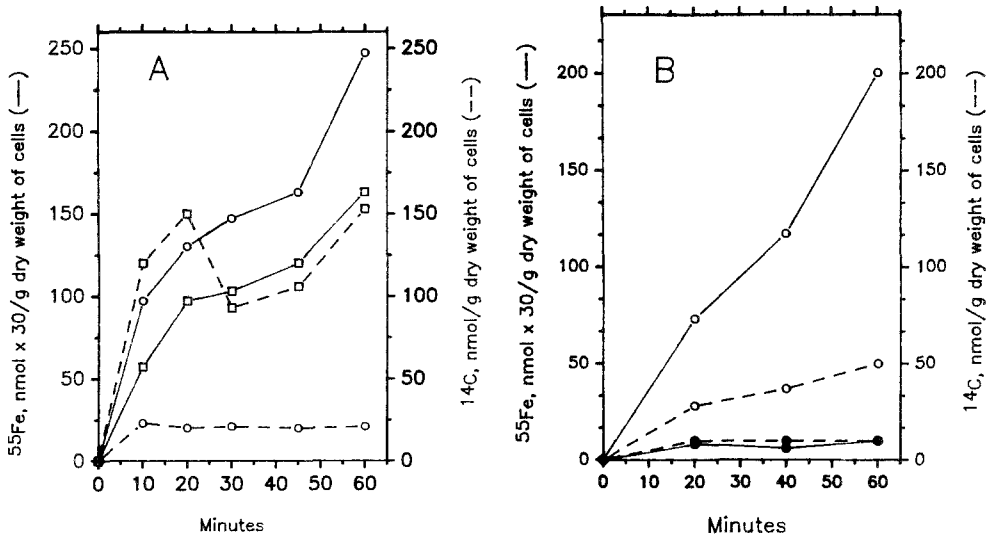

Fig. 3. The distribution of ^{55}Fe and ^{14}C in iron-poor cells incubated with [^{55}Fe]ferri[^{14}C]-pyoverdine. (A) The distribution of (—) ^{55}Fe and (--) ^{14}C on (○) the membranes (outer and inner), and in (□) the soluble fraction. (B) The amount of (—) ^{55}Fe and of (--) ^{14}C on the (○) inner membrane and (●) outer membanes

crease in total ^{55}Fe. The total cellular amount of ^{14}C did not decrease upon osmotic shocking. These results were consistently found in four experiments, with the decrease in ^{55}Fe radioactivity of osmotically shocked cells ranging between 16%–21% total ^{55}Fe. Hence, some ^{55}Fe is released from the periplasmic space while ^{14}C is not released.

In another set of experiments, the effect of dipyridyl, a chelator of the ferrous but not the ferric ion, on the total accumulation of label was investigated. In the presence of 6 mM dipyridyl, the total accumulation of both ^{55}Fe and ^{14}C was less than that found in untreated cells, but the relative amounts of the two isotopes in the cells were the same, i.e. more ^{55}Fe accumulated in the cells after 20 min than did ^{14}C (Fig. 5). It is also seen in this figure that deferri[^{14}C]pyoverdine did not enter the cells.

It is noted from Fig. 6 that in the presence of 0.1 mM cyanide, the total uptake of both ^{55}Fe and of ^{14}C was less than that of unpoisoned cells. At 1 h, a residual amount of ^{55}Fe entered cyanide-poisoned cells, whereas approximately 65% of the ^{14}C that entered the control cells entered the poisoned cells. Contrary to the findings with nonpoisoned cells, cyanide-poisoned cells consistently transported greater amounts of ^{14}C than of ^{55}Fe over a 1-h period. The relative amounts of membrane-associated and soluble ^{55}Fe in cyanide-poisoned cells were the transposition of those found in unpoisoned cells, for in the presence of the inhibitor, more ^{55}Fe was found in the soluble fraction than on the membranes (Fig. 7). However, the distribution of the ^{14}C in the poisoned cells was the same as in the control cells, in that more ^{14}C was found in the soluble fraction than on the membranes (Fig. 7).

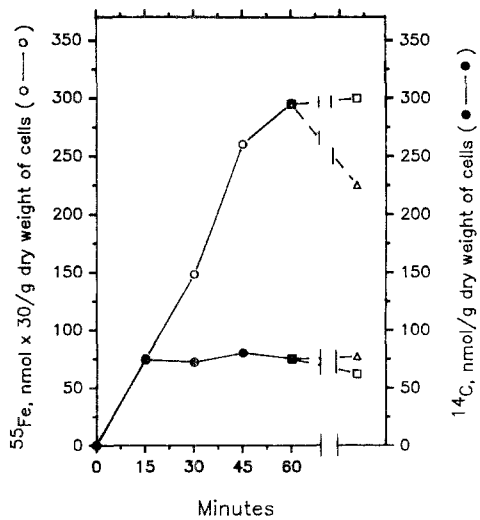

Fig. 4. The effect of osmotic lysis of cells incubated with [^{55}Fe]ferri[^{14}C]pyoverdine. The total accumulation of (○) ^{55}Fe and (●) ^{14}C by iron-poor cells was determined using the transport assay over a 60-min period, at which time aliquots of cells were resuspended in (△) osmotic lysing buffer or (□) Mops and subjected to two temperature shifts

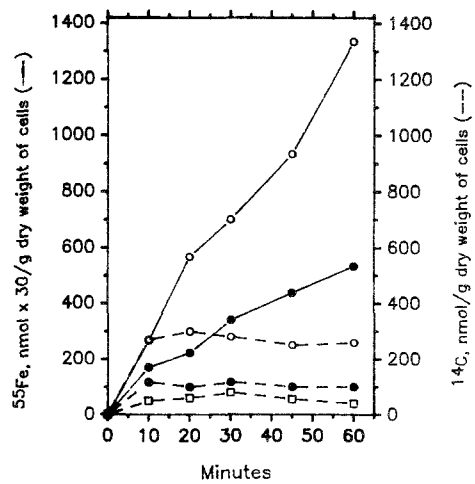

Fig. 5. Transport of [^{55}Fe]ferri[^{14}C]pyoverdine into iron-poor cells. (○) Control cells. Cells incubated with (●) 6 mM dipyridyl, or with (□) [^{14}C]pyoverdine. (—) ^{55}Fe; (--) ^{14}C

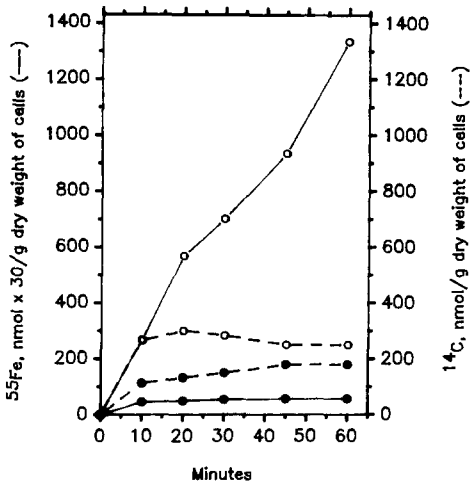

Fig. 6. Transport of [^{55}Fe]ferri[^{14}C]pyoverdine into iron-poor cells. (O) Control cells. Cells incubated in the presence of (●) 0.1 mM KCN. (—) ^{55}Fe; (--) ^{14}C

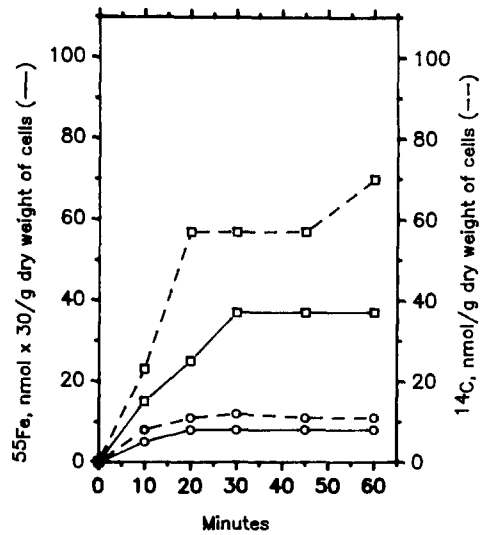

Fig. 7. Distribution of ^{55}Fe and ^{14}C in cyanide-poisoned cells incubated with [^{55}Fe]ferri[^{14}C]pyoverdine. The distribution of (—) ^{55}Fe and (--) ^{14}C in the (□) soluble fraction and on the (O) membranes (outer and inner)

Discussion

Incubation of iron-poor *P. aeruginosa* with [^{55}Fe]ferri[^{14}C]pyoverdine results in the uptake of both isotopes by the cells. Initially, the rates of uptake of the two isotopes are similar. However, after 10 min of incubation, ^{55}Fe continues to enter the cell, but further accumulation of ^{14}C by the cells ceases. At 1 h, approximately 60% of the iron is associated with the inner membranes. Whether this iron is bound to the membrane-associated iron chelator found in these cells (Royt 1988) is not known at this time. The remaining iron taken up by the cells is in the soluble fraction. Upon separating the periplasmic fluid from the cytoplasm, approximately half of the total soluble ^{55}Fe is found in the periplasmic fluid. ^{14}C accumulated primarily in the soluble fraction, with only 15% of total ^{14}C found on the membranes after a 60-min incubation with [^{55}Fe]ferri[^{14}C]pyoverdine. No radiolabeled carbon was released from the cells during plasmolysis.

The initial rapid influx of ^{14}C into the cytoplasm during the first 20 min of incubation was consistently found (Fig. 3A). Since radiolabeled iron accumulates in the soluble fraction more slowly during this time period, it is suggested that iron dissociates from the siderophore during this period. Release of iron, but not of ^{14}C, from osmotically shocked cells suggests that dissociation of the iron and chelator occurs within the periplasm. This dissociation is probably followed by separate transport of iron and pyoverdine (or a degradation product of it if release occurs via ligand hydrolysis) across the inner membrane of the cells, for carbon initially accumulates in the cytoplasm of the cells, whereas iron is distributed between the inner membrane and the cytoplasm. Experiments using the metabolic inhibitor cyanide confirm the notion of separate transport systems for iron and pyoverdine. While cyanide reduced the rate of transport of both iron and carbon, more significantly it transposed the pattern of iron and carbon accumulation, i.e. more ^{14}C accumulated in these cells than did ^{55}Fe. Also, lack of significant accumulation of iron on the membranes of poisoned cells shows a requirement of energy, or more specifically, of electron flow for this process. Whether transport of the pyoverdine through the inner membrane is energy-driven, or if instead it is a passive process driven by the iron–siderophore dissociation, is not known at this time. Knowledge of the excretion mechanism of pyoverdine from the cell may give insight into this question. In any event it appears that, following iron-ligand dissociation, the transport of pyoverdine or a degradation product of it through the cytoplasmic membrane is a more efficient process than is the transport of iron through this membrane.

Iron may be released from pyoverdine upon reduction. Cell-free extracts of the fluorescent pseudomonad *P. fluorescens* exhibit ferripyoverdine reductase activity (Halle and Meyer 1989) but it is not known if the enzyme is located on the inner membrane, or if it exists in the cytoplasm, periplasm, or both of these compartments of the cell. Because of the lack of specificity of this enzyme, it is not known if the enzyme serves to release iron from pyoverdine in vivo. While the exact mechanism of release of iron from ferripyoverdine in *P. aeruginosa* is not known, a reductive mechanism is suggested by the findings using dipyridyl, an Fe^{2+} chelator. It was shown that, after 10 min, transport of iron into cells incubated with the dual-labeled ferrisiderophore and dipyridyl stopped (Fig. 5), indicating a role of reduction in the accumulation process (Ecker and Emery 1983). Also, the lower pH of the periplasmic space of Gram-negative bacteria (Stock 1975) promotes iron reduction in this compartment (Lee et al. 1985). In *P. aeruginosa*, iron may instead be released by an ex-

change mechanism or following hydrolysis of the chelator. In any event, the experiments presented here indicate that separation of iron from pyoverdine takes place in the periplasmic space. This conclusion implies that the ferrated siderophore does not accumulate in the cell. Mossbauer spectroscopy studies presented in another paper confirm this presumption (Mielczarek et al. 1990).

The mechanism of iron transport via pyoverdine suggested by these studies is one postulated by Leong and Neilands (1976) in that dissociation of iron and ligand occurs at the level of the cytoplasmic membrane surface, followed by separate uptake of iron and siderophore. Accumulation of ^{14}C in cells indicates that a taxi cab mechanism (Carrano and Raymond 1978), i.e. one in which the ligand shuttles iron to the cell, but itself does not enter the cell, is not occurring. Also, the possibility that the intact ferrisiderophore instead enters the cytoplasm followed by intracellular dissociation of iron is not likely due to the presence of pyoverdine-free iron in the periplasmic space.

The decreased rate of pyoverdine-mediated uptake of iron in the presence of metabolic inhibitors that interfere with the proton motive force shows a role of an energized membrane in the transport process. The more pronounced inhibition of uptake in the presence of nigericin, as compared with that produced by valinomycin, shows a greater role of the pH gradient than of the electrical gradient in the transport process. This differs from the energy requirement of iron transport via the siderophore pseudobactin into *Pseudomonas putida* WCS358 (de Weger et al. 1988). There, the electrical gradient was more influential in iron uptake than was the pH gradient. Iron transport via enterochelin into *Escherichia coli* is also dependent on an energized membrane (Pugsley and Reeves 1977), as is siderophore-mediated iron transport by other Gram-negative species (Knosp et al. 1984; Chart and Trust 1983).

It is of interest now to determine if the iron released during the osmotic shock procedure is bound to a periplasmic protein, and hence to determine if pyoverdine-mediated iron transport occurs by a periplasmic transport system (Ames 1986), as is involved in iron transport via enterochelin into *E. coli* (Pierce and Earhart 1986). Also, as we continue to monitor the fate of iron in *P. aeruginosa*, we will investigate a possible role of the membrane-associated iron chelator (Royt 1988) in iron storage or metabolism.

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