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Antibiotic Action of *N*-Methylthioformohydroxamate Metal Complexes

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The antibiotic activity of metal complexes of *N*-methylthioformohydroxamic acid against gram-negative *Escherichia coli* NIHJ and gram-positive *Staphylococcus aureus* 209P was investigated. The kinetically labile, square-planar, divalent (Cu, Ni, and Pd) and octahedral, trivalent (Fe, Co, and Cr) complexes displayed activity, whereas the more inert platinum(II) or rhodium(III) complex displayed no activity, or activity only at elevated concentrations. The free ligand did not suppress the growth of the above organisms, and the sulfur atom of the ligand in its metal complexes appears crucial for activity. Uptake studies of radioactively labeled *N*-methylthioformohydroxamic acid, its nickel(II), platinum(II), iron(III), and rhodium(III) complexes were conducted in the *Escherichia coli* K-12 RW193 mutant, which is defective in the production of its native iron(III) transport agent, enterobactin. Uptake of ^{55}Fe or ^{63}Ni label from their metal complexes appeared to occur as free inorganic metal ions. The antibiotic activity of the iron(III) and nickel(II) complexes was not due merely to an enhanced accumulation of metal ions by the cell. Metal complexes of [^{35}S]*N*-methylthioformohydroxamic acid enhanced the accumulation of ^{35}S label compared to that of the free ligand. The antibiotic activity and uptake data are discussed in terms of possible modes of action.

Bis(*N*-methylthioformohydroxamato)copper (II) (also known as fluopsin C, antibiotic YC 73, or antibiotic B_1) and tris(*N*-methylthioformohydroxamato)iron(III) (fluopsin F) display broad-spectrum antibiotic activity against both gram-positive and -negative bacteria and fungi (5). These naturally occurring antibiotics have been isolated from the culture supernatant fluids of *Pseudomonas* (1, 10, 16) and *Streptomyces* species (S. Miyamura, Japan Patent 87087, 1973). Ferric complexes of chemically synthesized *N*-substituted thioformohydroxamic acids, which are analogs of thioformin (*N*-methylthioformohydroxamic acid), also exhibit antibiotic activity against bacteria such as *Staphylococcus aureus* 209P and *Escherichia coli* NIHJ, and fungi such as *Candida albicans* Eiken (2, 4, 12). Ferric complexes of the corresponding *N*-substituted oxyhydroxamic acids presumably display no antibiotic activity since these ligands, which are known to have large formation constants for ferric ion, exhibited no antibiotic activity when tested in natural media rich in iron and other metals (12). However, the ferric complex of the oxygen analog of thioformin had not been tested for antibiotic action.

The mode of antibiotic action of these ferric or cupric complexes currently remains obscure, except for the following report. Miyagishima quantitatively correlated the antibiotic activity of ferric complexes of *N*-substituted thioformohydroxamic acids with the hydrophobic character of the molecule for both gram-positive and -negative bacteria (11). He proposed that the antibiotic activity of these agents is dependent upon their ability to penetrate the cell (8, 11). The more hydrophobic the *N*-substituent, the less effective that agent is against gram-negative bacteria. Miyagishima suggested that hydrophobic agents might be retained more strongly than hydrophilic ones by the relatively lipid-rich outer membrane of gram-negative bacteria, hence this would account for their diminished antibiotic activity (11).

In a previous study, we prepared square-planar, divalent bis(*N*-methylthioformohydroxamate)-metal complexes and octahedral, trivalent tris(*N*-methylthioformohydroxamate)-metal complexes, $M(th)_2$ (M = Cu, Ni, Pd, and Pt) and $M(th)_3$ (M = Fe, Co, Cr, and Rh), respectively. The platinum(II) and rhodium(III) complexes were substitutionally inert on a time scale of 6 to 8 h (6). We report here the antibiotic action of the above metal ion-substituted complexes against gram-positive *S. aureus* 209P and gram-negative *E. coli* NIHJ and *E. coli* K-12 RW193. We also describe uptake studies of radioactively labeled *N*-methylthioformohydrox-

amic acid and its kinetically labile and inert complexes in the latter organism in an effort to elucidate their mode of action.

MATERIALS AND METHODS

Bacterial strains. The strains *E. coli* NIHJ (ATCC 26) and *S. aureus* 209P (ATCC 6938P) were obtained from the American Type Culture Collection. The *E. coli* K-12 strain RW193, which is defective in the production of its native iron transport agent (enterobactin), was obtained from R. W. Wayne.

Media. Bacteria were maintained at 37°C either in nutrient broth (Difco Laboratories) cultures or on nutrient broth plates (7). Minimal medium A (9, 15) was used for susceptibility tests of *E. coli* NIHJ. The minimal medium for antibiotic tests with *S. aureus* consisted of 2.0 g of KH_2PO_4, 5.7 g of Na_2HPO_4, and 1.0 g of $(NH_4)_2SO_4$ per liter, and it was made 0.01% (wt/vol) in $MgSO_4 \cdot 7H_2O$, 0.1% (wt/vol) in Casamino Acids (Difco certified), and 0.5% (wt/vol) in glucose. A chemically defined medium for growth of *E. coli* K-12 RW193 has been described previously (7).

Specially treated metal ion-deficient minimal media were used for antibiotic testing of the free ligand thioformin (*N*-methylthioformohydroxamic acid) against *S. aureus* and *E. coli* K-12 RW193 as well as for uptake experiments. For *S. aureus*, an aqueous solution of phosphates, ammonium sulfate, and Casamino Acids was extracted with 1% (wt/vol) 8-hydroxyquinoline in chloroform solution. For *E. coli* K-12 RW193, an aqueous solution of phosphates, sodium chloride, and ammonium chloride was extracted with 8-hydroxyquinoline. Each extracted solution was then supplemented with the appropriate amounts of other nutrients. Glassware for these experiments was presoaked overnight with 0.5% ethylenediaminetetraacetic acid (EDTA) solution and thoroughly rinsed with doubly distilled water.

Antibiotics and chemicals. Thioformin was prepared by procedures described previously (2, 6, 17). Metal complexes of thioformin were prepared as described previously (6); *O*-methylthioformin (*N,O*-dimethylthioformohydroxamate) was prepared from *O*-ethylthioformate and *N,O*-dimethylhydroxylamine by a previously described procedure (19), as was *N*-methylformohydroxamic acid (18). Tris(*N*-methylformohydroxamato)iron(III) was prepared from *N*-methylformohydroxamic acid and excess $FeCl_3 \cdot 6H_2O$ in aqueous solution and purified by benzyl alcohol extraction (13). The reddish brown iron(III) complex was recrystallized from chloroform-hexane solution. Results of analysis for $Fe(C_2H_4NO_2)_3$ were as follows: C, 25.93; H, 4.35; N, 15.12; found: C, 26.17; H, 4.49; N, 14.91. Chemical analyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley.

Antibiotic stock solutions. Inorganic metal salts, thioformin, *O*-methylthioformin, copper(II) and nickel(II) complexes of thioformin, and tris(*N*-methylformohydroxamato)iron(III) were dissolved in distilled water. The palladium(II) and platinum(II) complexes of thioformin were dissolved in a minimal volume of acetone and then diluted with distilled water. Acetone in these solutions was removed in vacuo. The concentrations of platinum(II) geometrical isomers were confirmed spectrophotometrically (6). All trivalent metal complexes of thioformin were dissolved in a minimal volume of chloroform. After the addition of distilled water, chloroform was removed in vacuo from the mixture. The aqueous solution was filtered, and the concentration of the metal complex was determined spectrophotometrically (6). All stock solutions were prepared immediately before testing.

Susceptibility testing. The following protocol was identical for all organisms except for variation in the minimal medium used. Single colonies of bacteria were inoculated from nutrient broth plates into 2 ml of nutrient broth medium, and the culture was incubated with shaking at 37°C for 10 h. The cells were washed aseptically twice with 2 ml each of the appropriate minimal medium and then resuspended in 2 ml of the same medium. For *E. coli* K-12 RW193, sodium citrate was omitted from its minimal medium. Approximately 4 ml of resuspended cells was transferred to 100 ml of minimal medium. The culture was incubated at 37°C until its optical density was between 0.06 and 0.08 at 620 nm. Appropriate volumes (less than 0.5 ml) of antibiotic stock solutions were placed in culture tubes and then diluted to 0.5 ml with a combination of distilled water and a tenfold concentrated minimal medium to make the final solution of unit strength. After 2 ml of bacterial culture was added to each tube, the cultures were incubated at 37°C. The contents of each tube were suspended before their optical density was measured at 620 nm using a Spectronic 20 (Bausch and Lomb). Measurements were made at 2-h intervals for 8 h and then overnight. The minimal inhibitory concentration (MIC) was arbitrarily chosen to be that concentration of antibiotic at which there was no growth overnight.

Radioactively labeled antibiotics. Solid $Na_2{}^{35}S \cdot 5H_2O$ (specific activity, 70 mCi/mmol) and $^{63}NiCl_2$ in 0.5 N HCl (specific activity, 630 mCi/mmol) were purchased from New England Nuclear Corp., Boston, Mass. The $^{55}FeCl_3$ in 0.5 N HCl (specific activity, 1,500 mCi/mmol) was obtained from ICN Chemical and Radioisotope Division, Irvine, California.

[^{35}S]*N*-methylthioformohydroxamic acid was prepared by a modification of an earlier procedure (6). The starting material, sodium dithioformate, was prepared as follows. Approximately 15-μl amounts of $CHCl_3$ were added at three 15-min intervals, followed by two additions of 75 μl of $CHCl_3$ at 20-min intervals, to a slurry of approximately 50 mg of $Na_2{}^{35}S \cdot 5H_2O$ (20 mCi; specific activity, ca. 70 mCi/mmol) in 0.2 ml of 0.1 M NaOH in methanol, maintained at 45°C. The reaction mixture was incubated further for 20 min at 45°C after the last addition of chloroform. After 50 mg of *N*-methylhydroxylamine hydrochloride (6) was added to the above sodium dithioformate solution, the solution was adjusted to pH 7 with 0.1 M HCl and was allowed to stand for 2 h at room temperature. The free ligand was purified by extraction into $CHCl_3$ (4 × 1 ml) after the pH of the solution had been adjusted to 1 with 1.0 M HCl. The combined $CHCl_3$ extracts were extracted with a saturated $NaHCO_3$ solution (2 × 1 ml). After the pH of the combined aqueous extracts was adjusted to 1 with 1.0 M HCl, the resultant solution was extracted with $CHCl_3$ (4 × 1 ml). Yields

of ligand ranged from 10 to 24%, based upon $Na_2{}^{35}S \cdot 5H_2O$. The ligand was stored in $CHCl_3$ solution at −70°C.

Tris([^{35}S]*N*-methylthioformohydroxamato)iron(III) and tris(*N*-methylthioformohydroxamato)[^{55}Fe]iron(III) were prepared as described previously (17). Both radioactively labeled metal complexes were prepared immediately before uptake experiments. Bis([^{35}S]*N*-methylthioformohydroxamato)nickel(II), bis(*N*-methylthioformohydroxamato)[^{63}Ni]nickel(II), bis([^{35}S]*N*-methylthioformohydroxamato) platinum(II), and tris([^{35}S]*N*-methylthioformohydroxamato)-rhodium(III) were prepared as described previously (6). The specific activity of ^{35}S label in each compound was 10 mCi/mmol, whereas the specific activity of the [^{55}Fe]- and [^{63}Ni]-labeled complexes was 500 mCi/mmol. All metal complexes except those of iron(III) were stored in chloroform solution at −20°C. All radioactively labeled compounds were determined to be radiochemically pure by scanning their silica gel thin-layer chromatograms (6) with a Packard 7201 radiochromatogram strip scanner. The purity of each compound was checked again immediately before uptake experiments. Stock solutions of labeled compounds were prepared as follows. After the chloroform solution of each compound was concentrated to dryness in vacuo, the compound was dissolved in doubly distilled water. The concentration of all metal complexes was determined spectrophotometrically (6), whereas the concentration of [^{35}S]*N*-methylthioformohydroxamic acid was determined from its specific activity.

Uptake measurements. Single colonies of *E. coli* K-12 RW193 were inoculated from nutrient broth plates into 3 ml of minimal medium supplemented at 1 mM citrate, and the culture was incubated with shaking at 37°C for 10 h. After the cells were inoculated into 75 ml of fresh medium, the culture was incubated at 37°C until its optical density was 0.4 at 650 nm. The cells were harvested by centrifugation at 8,000 × *g* for 10 min at room temperature and were washed twice at room temperature with equal volumes of culture medium not containing citrate. The cell pellet was suspended in a sufficient volume of minimal medium without citrate to give an optical density of 0.4, and 6- to 8-ml portions were dispensed into 25-ml Erlenmeyer flasks. The flasks were incubated at 37°C with shaking on a water bath for 15 min before the addition of labeled compounds. After appropriate volumes of stock solutions were added to the cells at 37°C, 0.50-ml portions of cell suspensions were removed at regular intervals and were added to 2.3 ml of minimal medium supplemented at 1 mM in trisodium nitrilotriacetate maintained at 0°C. The samples were centrifuged at 10,000 × *g* for 5 min at 4°C, and the cell pellets were washed twice with 2.3 ml each of minimal medium supplemented with trisodium nitrilotriacetate. The cell pellets were resuspended in 2.3 ml of the same medium, and the suspensions were rapidly filtered through 0.45-μm membrane filters (Millipore Corp., Bedford, Mass.). The filters were washed with minimal medium supplemented with trisodium nitrilotriacetate (2 × 2.3 ml) and were allowed to dry overnight at room temperature. Radioactivity in the filters was determined in 5 ml of Aquasol scintillation fluid (New England Nuclear), using a Beckman LS-250 liquid scintillation counter. The amount of labeled compound taken up per 0.50 ml of cell suspension was calculated by comparison with a control containing the compound but no cells.

RESULTS

Susceptibility. The requirement that thioformin (*N*-methylthioformohydroxamic acid) form a metal complex for antibiotic action was ascertained by testing the free ligand and an analog (*O*-methylthioformin) which would not be expected to complex metal ions efficiently. Neither compound at elevated concentrations inhibited the growth of *E. coli* NIHJ or *S. aureus* 209P in minimal media (Table 1). *E. coli* K-12 RW193 was susceptible to the free ligand at a substantially higher concentration. *O*-methylthioformin was inactive against *S. aureus* even in a minimal medium which was not extracted to remove trace metal ions. Thioformin, however, was active at 5 μM under the same conditions.

The requirement of the sulfur atom in metal complexes of thioformin for antibiotic activity was ascertained by testing the oxygen-substituted metal complex, tris(*N*-methylformohydroxamato)iron(III). This analog was not inhibitory at elevated concentrations against *E. coli* NIHJ or K-12 RW193 or *S. aureus* 209P, whereas tris(*N*-methylthioformohydroxamato)-

TABLE 1. *MICs (μM)*[a]

Compound	*S. aureus* 209P	*E. coli* NIHJ	*E. coli* K-12 RW193
Th	NE at 1,000	NE at 400	1,500
O-Me(th)	NE at 400	NE at 490	—
$Fe(h)_3$	NE at 300	NE at 200	NE at 1,100
$Fe(th)_3$	0.5	70	25
$Co(th)_3$	5	75	—
$Cr(th)_3$	25	75	—
cis-$Rh(th)_3$	150	NE at 250	NE at 260[b]
trans-$Rh(th)_3$	150	NE at 240	
$FeCl_3 \cdot 6H_2O$	NE at 200	NE at 240	NE at 1,000
$CoCl_2 \cdot 6H_2O$	75	50	—
$CrCl_3 \cdot 6H_2O$	NE at 300	NE at 300	—
$RhCl_3 \cdot 3H_2O$	NE at 420	320	NE at 450
$Cu(th)_2$	0.5	5	—
$Ni(th)_2$	0.5	5	5
$Pd(th)_2$	0.5	5	—
cis-$Pt(th)_2$	NE at 150	NE at 150	NE at 140[b]
trans-$Pt(th)_2$	NE at 150	NE at 150	
$CuSO_4 \cdot 5H_2O$	NE at 300	50	—
$NiCl_2 \cdot 6H_2O$	NE at 300	25	50
$PdCl_2$	—	15	—
Na_2PdCl_4	200	—	—
K_2PtCl_4	300	50	25

[a] Th, Thioformin (*N*-methylthioformohydroxamic acid); *O*-Me(th), *O*-methylthioformin (*O,N*-dimethylthioformohydroxamate); h, oxygen derivative of thioformin (*N*-methylformohydroxamic acid); NE, no effect; —, not determined.

[b] Geometrical isomers were not resolved for the rhodium(III) or platinum(II) complex.

iron(III) was inhibitory at much lower concentrations (Table 1).

Labilization of the ligand in metal complexes of thioformin as a requirement for antibiotic action was determined by testing a series of square-planar, divalent bis(*N*-methylthioformohydroxamate)-metal complexes and a series of octahedral, trivalent tris(*N*-methylthioformohydroxamate)-metal complexes. Both series exhibit a range in substitutional lability (6). About the same antibiotic activity against *E. coli* NIHJ (Table 1) was observed for the iron(III), cobalt(III), and chromium(III) complexes of thioformin; all three complexes were previously shown to exhibit kinetic lability within a time scale of 6 to 8 h (6). The iron(III), cobalt(III), and chromium(III) complexes were more potent against *S. aureus* 209P than against *E. coli* NIHJ, and they exhibited progressively decreasing activity, Fe > Co > Cr, against *S. aureus*. Neither the *cis* nor the *trans* geometrical isomer of the rhodium(III) complex exhibited antibiotic action against *E. coli* NIHJ at elevated concentrations, but they exhibited antibiotic action against *S. aureus* 209P at much higher concentrations than those of the other trivalent complexes (Table 1). The extent of the lability of the isomers of the rhodium(III) complex was determined from a competition experiment as follows. Methanolic solutions of each isomer and an excess of cupric sulfate were mixed together and incubated at 37°C. Thin-layer chromatography (using silica gel plates with methyl ethyl ketone as the solvent system and iodine vapor as the visualizing agent) indicated within 6 to 8 h a very faint new band with an R_f value identical to that of authentic bis(*N*-methylthioformohydroxamato)copper(II) in addition to the original band for each rhodium(III) isomer. Thus, the rhodium(III) isomers displayed a small yet finite lability. The iron(III) complex inhibited the growth of *E. coli* K-12 RW193, whereas the unresolved rhodium(III) complexes had no effect (Table 1).

The copper(II), nickel(II), and palladium(II) complexes of thioformin were about equally potent against *E. coli* NIHJ, with MICs of 5 μM; the same complexes were also equally effective against *S. aureus* 209P, with MICs of 0.5 μM (Table 1). These complexes have been demonstrated previously to exhibit substitutional lability within a time scale of 6 to 8 h (6). Neither the *cis* nor *trans* geometrical isomer of the platinum(II) complex, which has been shown previously to be substitutionally inert on a time scale of 8 h (6), exhibited antibiotic action against *E. coli* NIHJ or *S. aureus* 209P at elevated concentrations (Table 1). *E. coli* K-12 RW193 was susceptible to the nickel(II) complex, but not to the unresolved platinum(II) complexes (Table 1).

Gram-positive *S. aureus* was more susceptible than gram-negative *E. coli* to metal complexes of thioformin (Table 1). The trivalent metal complexes are generally less potent antibiotics against *E. coli* NIHJ or K-12 RW193 than are the divalent complexes. Simple inorganic metal salts were generally found to be less potent than the corresponding metal complex of thioformin against *E. coli* or *S. aureus* (Table 1).

Uptake. Uptake studies of [^{35}S]*N*-methylthioformohydroxamic acid, ^{35}S- and ^{55}Fe-labeled iron(III) complexes, ^{35}S-labeled rhodium(III) complex, ^{35}S- and ^{63}Ni-labeled nickel(II) complexes, and ^{35}S-labeled platinum(II) complex in *E. coli* K-12 RW193 were undertaken to determine whether their antibiotic activity could be correlated with their uptake. Since thiohydroxamic acids exhibit much less affinity for iron(III) than a cell's iron(III) transport agent (6, 7), uptake studies of tris(*N*-methylthioformohydroxamato)iron(III) must be performed in the absence of the cell's iron transport agent. *E. coli* K-12 RW193 was selected for study because it does not produce its native iron(III) transport agent, enterobactin. Although the nickel(II) complex is not naturally occurring, to the best of our knowledge, uptake of this metal complex was studied because its radioisotope (^{63}Ni) is easier to handle than the corresponding radioisotope (^{64}Cu) of the naturally occurring copper(II) complex. Since both complexes exhibit similar lability, the nickel(II) complex was studied.

Experiments were performed to determine whether the ligand, *N*-methylthioformohydroxamic acid, increases the rate of uptake of the metal ion in metal complexes compared to that of the corresponding inorganic metal salt. The rates of uptake of the ^{55}Fe label of tris(*N*-methylthioformohydroxamato)[^{55}Fe]iron(III) and of $^{55}FeCl_3$, both at 1.0 μM, were identical (Fig. 1). The rates of uptake of the ^{63}Ni label of bis(*N*-methylthioformohydroxamato)[^{63}Ni]nickel(II) and of $^{63}NiCl_2$, both at 1.0 μM, were likewise identical (Fig. 2). The uptake rates of the ^{55}Fe- and ^{63}Ni-labeled complexes were found to be linear to at least 4 μM. Although the iron(III) complex was a less potent antibiotic than the nickel(II) complex (Table 1), the extent of uptake of the ^{55}Fe label of the iron(III) complex was ten times that of the ^{63}Ni label of the nickel(II) complex at the end of 2 h (Fig. 1 and 2).

Experiments were performed to determine whether metal ions increase the rate of uptake of the ligand in metal complexes compared to

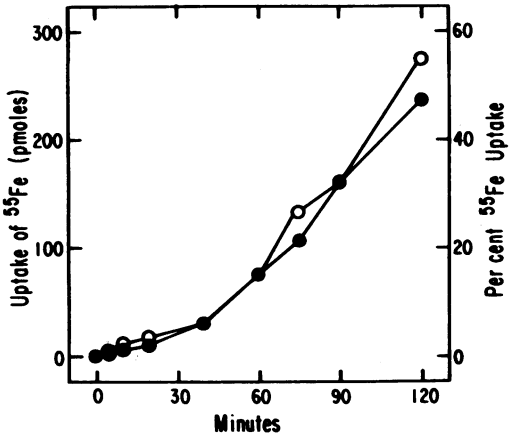

FIG. 1. *Uptake of radioactive label of $^{55}FeCl_3$ (●) and tris(N-methylthioformohydroxamato)[^{55}Fe] iron(III) (○). E. coli K-12 RW193 cultures were incubated at 37°C with final metal concentrations of 1.0 μM. Uptake is expressed as percent and as picomoles per 0.50 ml of cell suspension.*

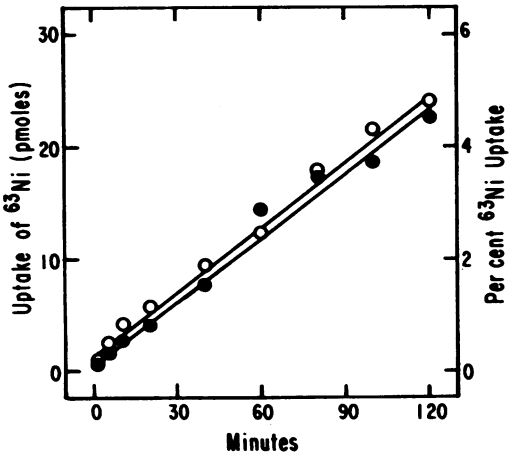

FIG. 2. *Uptake of radioactive label of $^{63}NiCl_2$ (●) and bis(N-methylthioformohydroxamato)[^{63}Ni] nickel(II) (○). E. coli K-12 RW193 cultures were incubated at 37°C with final metal concentrations of 1.0 μM.*

that of the free ligand. The extent of uptake of the ^{35}S label of tris([^{35}S]*N*-methylthioformohydroxamato)iron(III) (10 pmol of ^{35}S label per 0.50-ml cell suspension) at 1.0 μM was nearly three times that of [^{35}S]*N*-methylthioformohydroxamic acid (4 pmol) at 3.0 μM at the end of 2 h (Fig. 3). However, the MIC of the latter was nearly 60 times that of the former (Table 1). The extent of uptake of the ^{35}S label of substitutionally inert, nonantibiotic tris([^{35}S]*N*-methylthioformohydroxamato)rhodium(III) (70 pmol of ^{35}S label per 0.50 ml of cell suspension) was about seven times that of the kinetically labile antibiotic [^{35}S]iron(III) complex (10 pmol) at the end of 2 h (Fig. 3). The effective concentration of ^{35}S label was 3.0 μM for each experiment in Fig. 3. The uptake rates of the ^{35}S label of the ligand, its iron(III) complex, and its rhodium(III) complex were found to be linear to at least 15, 6, and 115 μM, respectively.

The extent of uptake of the ^{35}S label of bis([^{35}S]*N*-methylthioformohydroxamato)nickel(II) (8 pmol of ^{35}S label per 0.50 ml of cell suspension) at 1.5 μM was twice that of the ^{35}S-free ligand (4 pmol) at 3.0 μM at the end of 2 h (Fig. 3). However, the MIC of the latter was about 300 times that of the former (Table 1). The extent of uptake of the ^{35}S label of kinetically inert, nonantibiotic bis([^{35}S]*N*-methylthioformohydroxamato)platinum(II) (13 pmol of ^{35}S label per 0.50 ml of cell suspension) was almost twice that of the kinetically labile, antibiotic [^{35}S]nickel(II) complex (8 pmol) at the end of 2 h (Fig. 3). The effective concentration of ^{35}S

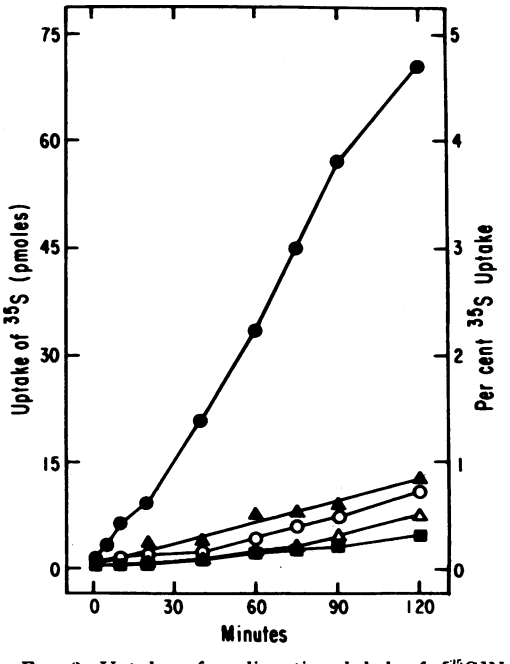

FIG. 3. *Uptake of radioactive label of [^{35}S]N-methylthioformohydroxamic acid (■); tris([^{35}S]N-methylthioformohydroxamato)iron(III) (○); tris([^{35}S]N-methylthioformohydroxamato)rhodium(III) (●); bis([^{35}S]N-methylthioformohydroxamato)nickel(II) (△); and bis([^{35}S]N-methylthioformohydroxamato)platinum(II) (▲). E. coli K-12 RW193 cultures were incubated at 37°C with final trivalent metal concentrations of 1.0 μM, with final divalent metal concentrations of 1.5 μM, and with a final free ligand concentration of 3.0 μM.*

label was 3.0 μM for each experiment in Fig. 3. The uptake rates of the ^{35}S label of the nickel(II) and platinum(II) complexes were found to be linear to at least 2 μM.

The uptake experiments in each figure were performed in duplicate simultaneously and repeated at least twice. Although the cells did grow in the uptake medium, the cell density at the end of 2 h was identical for each uptake experiment in a given figure.

DISCUSSION

Susceptibility. Since neither thioformin nor *O*-methylthioformin inhibited the growth of *E. coli* NIHJ or *S. aureus* 209P, and since thioformin only inhibited *E. coli* K-12 RW193 at very high concentrations, a metal complex of thioformin appears essential for antibiotic action. However, Egawa et al. (2) reported that the sodium salt of thioformin suppressed the growth of *S. aureus* 209P in natural culture media. Since such media probably contained trace amounts of iron(III) and other metal ions, free thioformin would have complexed any substitutionally labile metal ions present (6). Hence, the actual antibiotic agent was very likely a labile metal complex of thioformin. Egawa et al. (2), also reported that *O*-acyl (acetyl, *p*-nitrobenzoate, etc.) derivatives of thioformin, which would not be expected to complex metal ions efficiently, exhibited potent antibiotic activity against *S. aureus* 209P in natural culture media. However, it is quite conceivable that the labile ester function underwent chemical or enzymatic hydrolysis in vivo to yield free thioformin, which then would have complexed adventitious metal ions in the medium. Once again, the actual antibiotic agent was probably a labile metal complex of thioformin. The *O*-methyl derivative of thioformin would not be expected to be antibiotic against bacteria in media rich in metal ions because its ether function should not be very susceptible to hydrolysis to afford free thioformin.

Kinetically labile divalent (Cu, Ni, and Pd) and trivalent (Fe, Co, and Cr) complexes of thioformin displayed antibiotic activity against *E. coli* and *S. aureus*, whereas the more kinetically inert platinum(II) or rhodium(III) complex displayed no antibiotic activity, or activity only at elevated concentrations (Table 1). Thus, labilization of the ligand in metal complexes appears to be a requirement for antibiotic activity. The observed order of potency of the trivalent complexes Fe > Co > Cr > Rh against *S. aureus* 209P is in accord with the observed order of kinetic lability (6).

In accordance with Miyagishima's correlation of the hydrophobic nature of an antibiotic agent with its activity (11), kinetically labile trivalent metal complexes of thioformin may be less potent against *E. coli* than labile divalent metal complexes because the former complexes are more soluble in chloroform (6). The more hydrophobic trivalent complexes may be more effectively retained by the outer membrane, thus negating their potency. Miyagishima implied that gram-positive *S. aureus* may be more susceptible than gram-negative *E. coli* (Table 1) because the former organism contains less lipid in its cell envelope and, hence, might retain an antibiotic agent less effectively (11). However, it is clear that solubility criteria alone cannot account for the lack of antibiotic action of substitutionally inert metal complexes which have essentially the same solubility as their labile, antibiotic counterparts (6).

The sulfur atom in labile metal complexes of thioformin appears to be required for antibiotic activity, since the oxygen-substituted metal complex, tris(*N*-methylformohydroxamato)-iron(III), was not inhibitory, whereas the parent sulfur derivative was (Table 1). Miyagishima demonstrated earlier that *N*-substituted formohydroxamate complexes are not inhibitory, whereas *N*-substituted thioformohydroxamate complexes of iron(III) are (4, 12). The antibiotic activity of hydroxamate complexes might be precluded, since this functional group is responsible for the coordination of iron(III) in a number of iron(III) transport agents manufactured by microorganisms under iron-deficient conditions (14).

Uptake. The uptake results in Fig. 1 and 2 are consistent with the operation of at least the following mechanism. Rapid and complete dissociation of the substitutionally labile iron(III) or nickel(II) complex occurs, followed by accumulation of the ^{55}Fe or ^{63}Ni label as free inorganic ions by the cell. The accumulation of inorganic iron(III) presumably occurs by the low-affinity iron transport system for which no complexing agent has been described (3). Since this mechanism presumably involves donation of the iron to the cell membrane without penetration of the complex or ligand, the enhanced accumulation of the ^{35}S label of the above complexes compared to that of the free ligand cannot be accounted for by the above mechanism. Since the kinetically inert rhodium(III) or platinum(II) complex cannot be readily dissociated under physiological conditions, the accumulation of the ^{35}S label in the inert complexes apparently occurred as the intact metal complex. A comparison of the percent uptake of ^{55}Fe label of the iron(III) complex (Fig. 1) with that of ^{35}S label of the rhodium(III) complex (Fig. 3) indi-

cates that, at most, 10% of the accumulated ^{55}Fe label occurred as the intact metal complex. Similarly, a comparison of the percent uptake of ^{63}Ni label of the nickel(II) complex (Fig. 2) with that of ^{35}S label of the platinum(II) complex (Fig. 3) indicates that, at most, 20% of the accumulated ^{63}Ni label occurred as the intact complex. If the accumulation of ^{35}S label of the labile metal complexes did not occur as intact complex, the mechanism of its accumulation remains obscure and needs further investigation. Since neither the rhodium(III) nor platinum(II) complex was resolved for uptake experiments, no information can be gleaned as to which geometrical isomer was accumulated by the cell. The uptake of the ^{35}S label of the free ligand apparently proceeded in the absence of metal ions. However, the possibility that the free ligand complexed adventitious metal ions in the uptake medium to form a metal complex, which in turn stimulated the uptake of the ^{35}S label of the free ligand cannot be ruled out, although the medium was treated with 8-hydroxyquinoline to remove trace metal ions.

The antibiotic activity of the iron(III) and nickel(II) complexes is not due merely to an enhanced accumulation of metal ions by the cell, since the uptake rates of the metal label as inorganic ions or as metal complexes are identical, and the metal complexes have lower MICs than their corresponding inorganic salts. The antibiotic activity is also not a result of accumulation of a nondissociable metal complex. Additionally, since the ligand did not enhance the uptake rate of the ^{55}Fe label of the iron(III) complex compared to that of inorganic iron(III), the ligand probably does not behave as an iron(III) transport agent.

Although uptake of the ^{35}S label of [^{35}S]*N*-methylthioformohydroxamic acid was linear to approximately 15 μM, accumulation of its ^{35}S label was measured up to 500 μM, where it was found that approximately 150 pmol (compared to 600 pmol if linearity were obeyed) of the ^{35}S label was taken up per 0.50 ml of cell suspension in 2 h. Therefore, at least 150 pmol of the ^{35}S label would have been accumulated in 2 h at the MIC of the ligand against *E. coli* K-12 RW193. If the uptake rates of the ^{35}S label of the nickel(II) and iron(III) complexes are assumed to be approximately linear to their respective MICs against *E. coli* K-12 RW193, then the extent of accumulation of the ^{35}S label of the free ligand at 2 h would be at least six times greater than that of the nickel(II) complex (25 pmol of ^{35}S label) and would be at least comparable to that of the iron(III) complex (250 pmol). A substantially smaller accumulation of the ^{35}S label of the nickel(II) complex than that of the free ligand is apparently required for antibiotic activity. This difference might be explained by one of the following: (i) the labile nickel(II) complex is more reactive than the free ligand at the target site; (ii) the free ligand does not have access as facile to the target as the nickel(II) complex; or (iii) the labile nickel(II) complex reacts at a different target than that of the free ligand.

Experiments are in progress to locate the intracellular site(s) of accumulation of the ^{35}S label of the ligand and of its nickel(II) and iron(III) complexes in an effort to elucidate further the mode of action of these compounds.

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