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# Roles of Divalent Cations and pH in Mechanism of Action of Nitroxoline against *Escherichia coli* Strains

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**The antibacterial activity of nitroxoline (NIT), an antibiotic used in the treatment of acute or recurrent urinary tract infections caused by *Escherichia coli*, is decreased in the presence of $Mg^{2+}$ and $Mn^{2+}$ but not $Ca^{2+}$. In order to elucidate the interaction between this drug and the divalent cations, spectrophotometric studies based on the natural absorption of the nitroxoline moiety were conducted. In the presence of the divalent metal ions, a shift in the NIT $A_{448}$ suggested the formation of drug-ion complexes, for which the stability followed the order $Mn^{2+} > Mg^{2+} > Ca^{2+}$. A clear correlation was found between the chelating property and antibacterial activity of NIT; both were pH dependent. A convenient colorimetric method for the determination of NIT uptake by bacterial cells was also developed. Uptake was energy independent and showed biphasic kinetics: a rapid association with cells and then a slower increase in cell-associated NIT which reached a plateau. NIT uptake was reduced in the presence of magnesium. The implications of metal ion complexation and pH on the clinical efficacy of NIT are discussed.**

Nitroxoline (NIT), or 5-nitro-8-hydroxyquinoline, is an antibiotic which does not belong to any known antimicrobial class. This drug is used in France in the treatment of acute or recurrent urinary tract infections (UTIs) (14, 26) since it shows bacteriostatic activity against *Escherichia coli* strains frequently encountered in UTIs. On the other hand, the pharmacokinetics of NIT in plasma and urine are well established (4). NIT also possesses fungistatic activity (11) and bactericidal properties against *Mycoplasma* spp. (7).

Recent studies have shown an inhibition of adherence of uropathogenic *E. coli* to uroepithelial cells (27) and urinary catheters (8) at sub-MICs of NIT. In order to explain this activity Bourlioux et al. (9) proposed that NIT promotes a disorganization of the bacterial outer membrane resulting from the chelation by NIT of the divalent ions $Mg^{2+}$ and $Ca^{2+}$. The same investigators observed a decrease in the antibacterial activity of NIT on *E. coli* in the presence of some divalent metal ions (9). It is interesting to note that 8-hydroxyquinoline (oxine) and its derivatives have been reported to complex with metal ions (23, 37).

To acquire further information on the mechanism of action of NIT and the behavior of the molecule toward the bacterial envelope, the interaction between NIT and some divalent metal ions was spectrophotometrically examined by using the absorption properties of the molecule in the visible region. In addition, microbiological investigations were carried out to determine the role of these ions and the pH in the antibacterial activity of NIT. The uptake of NIT by *E. coli* was also studied.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** Three strains of *E. coli* were studied. Strains J96 and AL46 were isolated from patients with UTIs. J96 is a standard strain, frequently used in bacterial adherence assays, expressing type 1 and P fimbriae (24); the AL46 strain was already included in our previous studies (38). The third strain is the reference K-12 strain, which was used as a control. These strains were kindly provided by A. Labigne (Institut Pasteur, Paris, France). On the basis of lipopolysaccharide serotyping, *E. coli* AL46 and K-12 were characterized as rough strains and *E. coli* J96 was characterized as a smooth strain ($O_4$).

All strains were subcultured and grown at 37°C for 24 h in Luria-Bertani (LB) liquid medium (31) containing 1% Bacto Tryptone (Difco), 0.5% yeast extract (Difco), and 1% NaCl prepared in distilled water; the pH was adjusted to 7.2 with NaOH.

To study the impacts of acidification and alkalinization of the culture medium on the antibacterial activity of NIT, the pHs of the media were adjusted by the dropwise addition of 1 N hydrochloric acid or sodium hydroxide. To minimize the changes in pH that occur during autoclaving, the media were filter sterilized by passage through 0.2-μm-pore-size filter flask units (Nalgene sterilization filter units with nylon membrane; Poly-Labo, Paris, France).

**Chemicals.** NIT (Nibiol) was provided by the Laboratoires Debat (Garches, France) and was used after dissolution in boiling 0.01 M sodium hydroxide. A stock solution with a final concentration of 10 $g \cdot liter^{-1}$ (pH 11.7) was stored at 4°C until use.

The salts ($CaCl_2 \cdot 2H_2O$ and $MgCl_2 \cdot 6H_2O$ [Prolabo] and $MnCl_2 \cdot 4H_2O$ [Touzart et Matignon]) and the buffer components (sodium acetate [Prolabo], maleate [Fluka], and Tris [Serva]) were obtained commercially. Metal ion solutions (2 M) as well as buffers were prepared in distilled water.

All dilutions were made with the appropriate buffer for spectrophotometric studies.

**Spectrophotometric methods.** The interaction between NIT and divalent cations ($Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$) was studied spectrophotometrically at room temperature (22 ± 2°C) by using the absorption properties of NIT in the visible region ($\lambda_{max}$ = 448 nm in water). The binding of metal ions to NIT was accompanied by changes in NIT absorption easily measured at 448 nm. All absorption measurements were obtained in triplicate. The relative standard deviation of the measured absorbances never exceeded 0.8% throughout the study.

The study was divided into two steps. First, a series of dilutions was prepared with a constant NIT concentration (21 μM) and a constant metal ion concentration (50 mM) at different pHs. The change in NIT absorption in relation to changing pH was then monitored over the pH range of 3.8 to 8.6. For this purpose, two biological buffers were used: a 0.1 M acetate buffer (pH 3.8 to 5.6) and a 0.2 M Tris-maleate buffer (pH 5.2 to 8.6) (13a). As a control, the absorbance of a NIT solution without metal ion was measured in the corresponding buffers.

Second, another series of solutions was prepared in Tris-maleate buffer (pH 8.4) with a constant drug concentration (21 μM) but with increasing metal ion concentrations ranging from 10.5 μM to 50 mM. The modification in NIT $A_{448}$ relative to the changing metal ion concentration was again monitored. The determination of the conditional association constants (β) at pH 8.4 between NIT and $Ca^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ ions was directly calculated from the $A_{448}$ as a function of the metal ion concentration.

**Determination of MICs and MBCs in LB liquid medium.** The MICs of NIT for various *E. coli* strains were determined by the use of serial twofold dilutions of the antibiotic. The inoculum was from an overnight culture in the LB liquid medium. Two milliliters of liquid medium containing about $10^5$ to $10^6$ $CFU \cdot ml^{-1}$ plus NIT at concentrations ranging from 0.25 to 1,024 $\mu g \cdot ml^{-1}$, as

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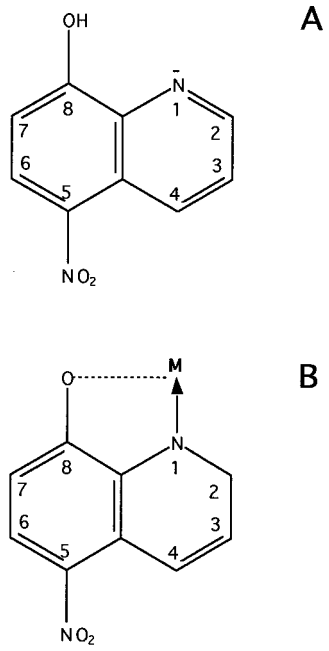


FIG. 1. Chemical structure of NIT in its unchelated form (A) and chelated form (B) with metal ion (M).

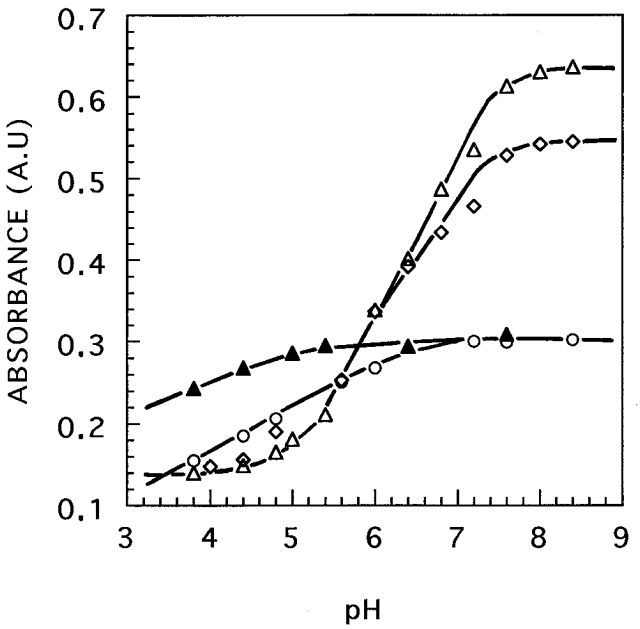


FIG. 2. $A_{448}$ of NIT (4 μg/ml; 21 μM) as a function of pH. Acetate buffer (0.1 M) was used for pHs between 3.8 and 5.6, and Tris-maleate buffer 0.2 M was used for pHs ranging from 5.2 to 8.6. Each datum point represents the mean of three experiments. Symbols: △, NIT alone or in the presence of monovalent cation ($Na^+$, $K^+$); ◇, NIT in the presence of 50 mM $CaCl_2$; ○, NIT in the presence of 50 mM $MgCl_2$; ▲, NIT in the presence of 50 mM $MnCl_2$.

well as controls (incubations without the antibiotic), were incubated for 24 h at 37°C. The lowest concentration that prevented visible growth was assessed as the MIC. The MBC of NIT for bacterial strains was determined by plating a sample (0.01 ml) with a calibrated platinum loop from each tube without visible growth issued from the MIC determination onto an LB medium agar plate and incubating at 37°C overnight. The MBC was defined as the lowest concentration at which the count was reduced to less than 99.9% of that of the bacterial inoculum.

When the influences of monovalent and divalent cations on the antibacterial activity of NIT were investigated, the final concentrations of $Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ in cation-supplemented broth ranged from 0.1 to 50 mM.

**Measurement of NIT uptake.** The uptake of NIT was measured by a modification of the fluorimetric method of Chapman and Georgopapadakou (12, 13). Mid-log-phase bacterial cells were harvested by centrifugation (10,000 × *g* for 15 min), washed once with 50 mM sodium phosphate buffer (pH 7.2), and suspended in the same buffer to $5 \times 10^9$ CFU/ml. Samples of 60 ml were dispensed into 150-ml Erlenmeyer flasks and incubated in a shaking water bath at 37°C for 15 min. NIT was added to final concentrations ranging from 0.25 to 20 μg/ml; at timed intervals 2.0-ml samples were removed and were immediately centrifuged at 15,000 × *g* for 2 min (at 4°C). Samples were washed once with 2.0 ml of buffer and were again pelleted. To each cell pellet 2.0 ml of 20 mM sodium hydroxide (pH 11.9) was then added, and the tubes were vortexed vigorously to completely resuspend the pellet. Incubation at room temperature for 60 min was sufficient to fully extract NIT from *E. coli* by this treatment. Samples were centrifuged at 15,000 × *g* for 15 min; the $A_{448}$ of the supernatant was measured and was compared with the $A_{448}$ of a standard curve of 0 to 100 μg of NIT per ml (0 to 0.53 mM) in 20 mM NaOH to determine the amount of NIT uptake.

In some experiments, before the addition of NIT the cells were treated at 4°C for 15 min to inhibit the proton motive force and therefore the active transport processes. The samples were then processed as described above for NIT uptake. The reason for not using the two frequently employed energy inhibitors, 2,4-dinitrophenol (an uncoupler of oxidative phosphorylation from electron transport) and carbonyl cyanide *m*-chlorophenylhydrazone (an inhibitor of electron transport), was due to the absorptions of these compounds at wavelengths close to that at which NIT is absorbed.

**Instruments.** A Shimadzu UV-visible 2100 recording spectrophotometer equipped with 10-mm glass cuvettes was used to obtain the measurements. All pH measurements were performed with a Hanna 8520N pH meter at 22 ± 2°C.

## RESULTS

**Spectrophotometric assays. (i) Effects of pH on NIT absorption.** NIT exhibits a β-hydroxyquinoline skeleton, with the presence of a nitro (—$NO_2$) group at the 5 position of the quinoline nucleus (Fig. 1A), which allows this molecule to absorb light in the visible region with a maximum at 448 nm. The effect of pH on the absorbance of NIT alone is depicted in Fig. 2. The hydroxyl group acts as a weak acid. As the pH rises, it dissociates to —$O^-$, giving another electronic resonance to the molecule, which causes a change in absorbance. The pH-titration curve obtained showed that half of the —OH groups are dissociated at pH 6.3, corresponding to the $pK_a$; this was in agreement with the value of 5.97 obtained from previous chemical studies with a different solvent (50). The pH-dependent absorption intensity of NIT was maximal at pH 8.4 (Fig. 2). Throughout the study the Beer-Lambert law was followed at concentrations ranging from 0.1 to 100 μg of NIT per ml.

**(ii) Chelating property of NIT.** As observed in Fig. 2, changes in the $A_{448}$ of NIT were obtained in the presence of an excess (50 mM) of different divalent cations ($Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$), which constitute the major cationic species involved in bacterial metabolism (5, 32). However, NIT absorbance was less modified by $Ca^{2+}$ than by $Mg^{2+}$ and $Mn^{2+}$. Such an effect was absent when monovalent cations ($Na^+$, $K^+$) were used. At high pHs, a plateau was reached in each case, when the —OH group of NIT was completely dissociated (and, eventually, entirely complexed with the divalent cations present) (Fig. 2). For manganese ions, this plateau was reached at a lower alkaline pH. Indeed, the lower was the pH when the plateau was reached, the higher was the stability constant (*Ks*), and the stronger was the cation bound. This was clearly the case with $Mn^{2+}$ versus $Mg^{2+}$, although the respective absorption molar coefficients of their complexes were similar, as confirmed by a common plateau (Fig. 2). On the other hand, upon complexation with magnesium and manganese ions, NIT exhibited a new absorption maximum toward shorter wavelengths at around 430 nm (blue shift) (Fig. 3). According to the previous observation concerning $Mn^{2+}$, the largest shift obtained with this ion was interpreted as a result of the stronger ability of NIT to chelate $Mn^{2+}$ versus $Mg^{2+}$ (Fig. 3).

Furthermore, the evolution of the complexation of NIT at

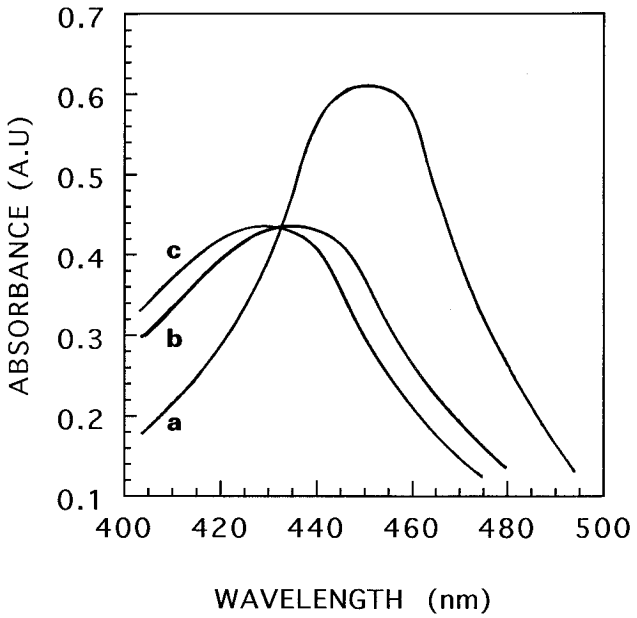


FIG. 3. Absorption spectra of NIT (4 μg/ml; 21 μM) in Tris-maleate buffer (pH 8.4). (a) NIT alone; (b) NIT in the presence of 10 mM $MgCl_2$; (c) NIT in the presence of 10 mM $MnCl_2$.

pH 8.4 as a function of the metal ion concentration was studied (Fig. 4). We attempted to evaluate the stoichiometry of chelation by measuring the change in NIT absorption when increasing amounts of divalent cations were added to NIT solutions. However, magnesium and manganese concentrations 1,700- and 130-fold higher than the drug concentration (21 μM), respectively, were required to obtain a complete complexation of NIT.

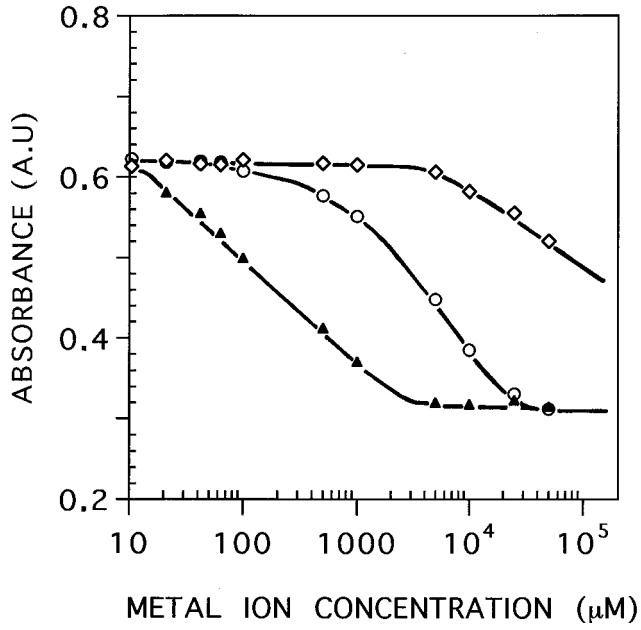


FIG. 4. $A_{448}$ of NIT (4 μg/ml; 21 μM) at 448 nm in 0.2 M Tris-maleate buffer (pH 8.4) in the presence of different concentrations of divalent cations. Each value represents the mean of three separate experiments. Symbols: ◇, $CaCl_2$; ○, $MgCl_2$; ▲, $MnCl_2$.

**Effects of divalent cations on MICs and MBCs of NIT.** The effects of monovalent and divalent cation supplementation on the antimicrobial activity of NIT against three *E. coli* strains in LB broth are listed in Table 1. A substantial reduction in the activity of NIT was obtained in the presence of $Mg^{2+}$ and $Mn^{2+}$, whereas it was constant with $Ca^{2+}$, $Na^+$, and $K^+$.

With increasing concentrations of $Mg^{2+}$ and $Mn^{2+}$, a progressive decrease in the bacteriostatic and bactericidal activities of the drug was evident. It seems that these two properties are interdependent actions of the molecule. Furthermore, the results revealed a correlation of 0.989 and 0.748 between increasing concentrations of divalent cations and the MIC increase (for an average of three strains) in the presence of magnesium and manganese, respectively: the higher the cation concentration, the higher the MIC. The decrease in activity correlated well with the increasing complexation of NIT with ions. Similar results were obtained with MBCs.

Moreover, the MICs of NIT increased fourfold in the presence of $Mg^{2+}$ and $Mn^{2+}$ at 50 mM. In parallel, the MBCs obtained with $Mn^{2+}$ increased only 4- to 8-fold, in contrast to 32-fold or more with $Mg^{2+}$. These data indicate that the better antagonistic effect of magnesium ions could be attributed to a potentially more important implication of these ions in the mechanism of the action of NIT.

**Effects of pH of the culture medium on MICs and MBCs of NIT. (i) Effect of pH alone.** The intrinsic role of the pH of the medium on the bacterial growth was first investigated (Table 2). *E. coli* grew well (doubling time, <1 h) in medium acidified with HCl at levels as low as pH 5.0. At pH 4.5, the generation time of the organism increased, with a longer lag period of 3 h; pH 4.5 was the lowest pH that allowed perceptible bacterial growth (20, 44). At pH 4.0 the population remained viable but could not multiply. Under alkaline conditions up to pH 8.5, differences in the lag times and the generation times were minor. At pH 9.0, the highest pH tested, the growth of the organisms began to be affected. These results agree well with previous data, which showed that *E. coli* is able to adapt to drastic changes in its environment and in particular grows over a wide range of external pHs, from 5.0 to 9.0 (20, 44, 52).

Second, the in vitro susceptibility of *E. coli* to NIT under various pH conditions was evaluated (Table 2). Changes in pH appeared to influence the antibacterial activity of the drug: NIT became progressively more active as the pH fell. The bacteriostatic and bactericidal activities of NIT were likewise affected by alkaline conditions. MICs and MBCs increased with an almost identical ratio of 4 when the pH of the medium increased; they were four- and eightfold higher when the pH increased from 5.0 to 9.0, respectively.

**(ii) Combined effects of pH and divalent cations.** As shown in Table 2, the in vitro efficacy of NIT against *E. coli* was more greatly affected by the combined effects of pH and metal ion supplementation. In the presence of 50 mM magnesium, the bacteriostatic activity of NIT was strongly reduced. MICs increased from 8 μg/ml at pH 5.0 to 128 μg/ml at pH 9.0, i.e., an increase 16-fold higher in comparison with the effects of pH alone. The bactericidal activity of NIT was also progressively reduced at alkaline pHs.

**Uptake of NIT.** The accumulation of NIT by *E. coli* at 37°C over a 75-min time course is shown in Fig. 5. Uptake of the drug demonstrated biphasic kinetics, possibly indicating different target site affinities. After the addition of NIT to the cell suspension, a rapid initial association of the drug occurred during the first 10 min of contact with the cells. This step was followed by a slower increase in cell-associated NIT, which continued for at least 1 h and then reached a plateau.

In all strains tested, the uptake was proportional to the

TABLE 1. MICs and MBCs of NIT for *E. coli* strains in the presence of divalent cations at different concentrations

| *E. coli* strain | MIC[a] (μg/ml) with the following cations at the indicated concn (mM): | | | | | | | | | | | MBC[a] (μg/ml) with: | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control[b] | $Ca^{2+}$ | | | | $Mg^{2+}$ | | | | $Mn^{2+c}$ | | | Control[b] | $Ca^{2+}$ | | | | $Mg^{2+}$ | | | | $Mn^{2+c}$ | | |
| | | 0.1 | 1 | 10 | 50 | 0.1 | 1 | 10 | 50 | 0.1 | 1 | 10 | | 0.1 | 1 | 10 | 50 | 0.1 | 1 | 10 | 50 | 0.1 | 1 | 10 |
| AL46 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 16 | 32 | 16 | 16 | 32 | 128 | 128 | 128 | 128 | 128 | 128 | 128 | 256 | >1,024 | 128 | 128 | 512 |
| J96 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 8 | 16 | 8 | 32 | 32 | 32 | 32 | 64 | 64 | 64 | 32 | 32 | 64 | 1,024 | 64 | 64 | 128 |
| K12 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 16 | 32 | 8 | 32 | 32 | 64 | 64 | 64 | 64 | 64 | 64 | 64 | 256 | >1,024 | 128 | 128 | 512 |

[a] The MIC and MBC of NIT for *E. coli* were determined at 37°C in LB broth (pH 7.2). Each value represents the average of the results of one experiment done in triplicate. Similar results were obtained in MT minimal medium (38) and Mueller-Hinton broth.
[b] The MIC and MBC of NIT for *E. coli* were determined in nonsupplemented LB broth or in the presence of 50 mM monovalent cations ($Na^+$ and $K^+$).
[c] Manganese at 10 mM was the highest concentration used in the study because it was sufficient to entirely chelate NIT (Fig. 4).

extracellular NIT concentrations from 0.1 to 8 μg/ml (0.5 to 42 μM) (Fig. 6). In this range, the concentration of NIT reached within the cell became progressively 40- to about 70-fold higher than that in the medium (data not shown). A saturation of NIT uptake was observed from 12 μg/ml, a concentration for which the NIT intracellular/NIT extracellular ratio (cf. the previous sentence) was stabilized at 70.

Lineweaver-Burk plots of uptake were inconsistent with uptake by simple diffusion. The results were also incompatible with a facilitated (carrier-mediated) diffusion. In fact, NIT appeared to be concentrated in the cell. The energy dependence of NIT uptake was therefore investigated. The identical kinetics of NIT association with cells obtained at 4°C provided evidence that the uptake was not energy dependent.

NIT uptake experiments performed with strains having a rough (strains AL46 and K12) or a smooth (strain J96) lipopolysaccharide showed the same type of kinetics, confirming that the uptake is neither strain specific nor outer membrane composition dependent.

Washing of the cells with NIT-free buffer decreased NIT accumulation by up to 28% ± 3% (washed once) and 59% ± 4% (washed five times) in *E. coli* (data not shown). This extensive loss of cell-associated NIT was probably due to a nonspecific adherence of the drug to the cell wall. This easily removable fraction suggests reversible binding of NIT to an external bacterial structure.

NIT uptake was greatly inhibited in the presence of magnesium (Fig. 5). Increases in the concentrations of $Mg^{2+}$ progressively diminished the steady-state level of drug binding. For example, the prior addition of 50 mM $MgCl_2$ to the cell suspension containing 4 μg of NIT per ml (all of the NIT present was complexed by the excess of magnesium) reduced the amount of cell-associated NIT from $7.8 \times 10^5$ to $3 \times 10^5$ molecules per cell, a loss of 62%.

TABLE 2. Effects of medium pH on *E. coli* growth and the antibacterial activity of NIT against *E. coli*

| Medium pH | Doubling time (min)[a] | Lag time (h)[a] | Antibacterial activity of NIT (μg/ml) | | | |
|---|---|---|---|---|---|---|
| | | | Control[b] | | With 50 mM $Mg^{2+}$ | |
| | | | MIC[c] | MBC[c] | MIC[c] | MBC[c] |
| 4.0 | 495 | ND[d] | ND | 16 | ND | 16 |
| 4.5 | 75 | 3.0 | 1 | 16 | 4 | 32 |
| 5.0 | 67 | 2.5 | 4 | 64 | 8 | 64 |
| 5.5 | 61 | 2.0 | 4 | 64 | 16 | 64 |
| 6.0 | 59 | 2.0 | 8 | 64 | 32 | 128 |
| 6.5 | 58 | 2.0 | 8 | 128 | 32 | 256 |
| 7.0 | 56 | 2.0 | 8 | 128 | 32 | 1,024 |
| 7.5 | 55 | 2.0 | 8 | 128 | 32 | >1,024 |
| 8.0 | 58 | 2.0 | 8 | 256 | 64 | >1,024 |
| 8.5 | 62 | 2.0 | 16 | 512 | 128 | >1,024 |
| 9.0 | 79 | 2.5 | 16 | 512 | 128 | >1,024 |

[a] Similar results were obtained in the presence of 50 mM $Mg^{2+}$.
[b] LB medium alone.
[c] The MIC and MBC of NIT for *E. coli* AL46 were determined at 37°C in LB broth. Each value represents the average of the results of one experiment done in triplicate. Similar results were obtained in MT minimal medium (38) and Mueller-Hinton broth.
[d] ND, not determined.

## DISCUSSION

Inasmuch as there have been many reports in the literature indicating that different metal ions affect the antimicrobial activities (1, 6, 15, 21, 39), bacterial uptake (12), and bioavailabilities (17, 29, 48) of numerous antibiotics, we were interested in studying how some metal ions affect the activity of NIT against *E. coli* strains.

A spectrophotometric methodology was found to be the most convenient analytical method for studying the interaction

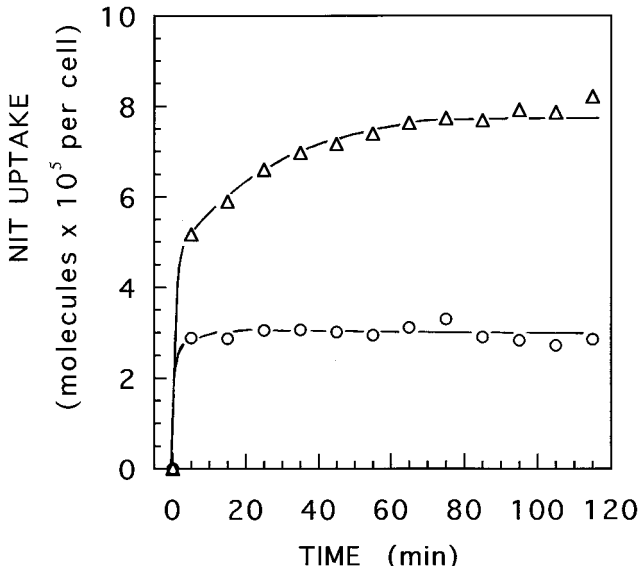


FIG. 5. Uptake of NIT (4 μg/ml; 21 μM) by *E. coli* AL46 as a function of incubation time. The uptake experiment was performed in 50 mM sodium phosphate buffer (pH 7.2) with shaking. Each value of NIT uptake is the mean of six separate experiments. Symbols: △, uptake of NIT alone at 37 or 4°C; ○, reduction in NIT uptake in the presence of 50 mM $MgCl_2$ preliminarily added to the cell suspension.

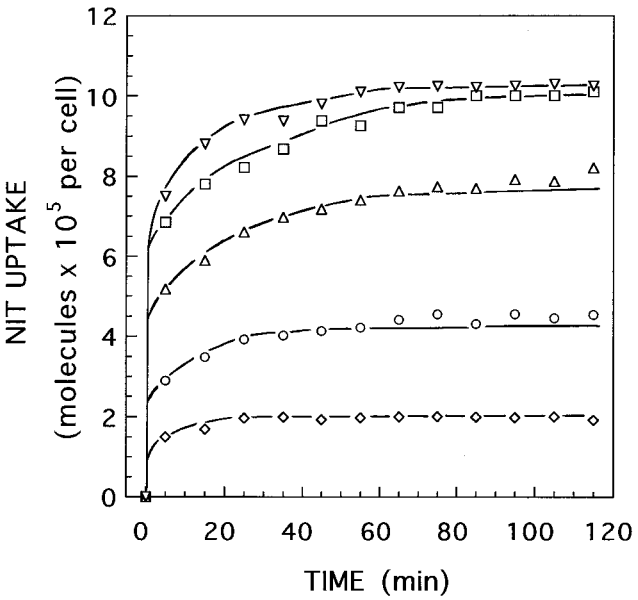


FIG. 6. Effects of external NIT concentration on NIT uptake by *E. coli* AL46. The uptake experiment was performed at 37°C in 50 mM sodium phosphate buffer (pH 7.2) with shaking. Saturation of NIT uptake was observed from 12 μg/ml. Each value of NIT uptake is the mean of six separate experiments. Symbols: ◇, 1 μg/ml; ○, 2 μg/ml; △, 4 μg/ml; □, 8 μg/ml; ▽, 12 μg/ml.

between NIT and metal ions. A substantial modification of NIT absorption in the presence of divalent cations demonstrated the formation of drug-ion complexes, for which the stability followed the order $Mn^{2+} > Mg^{2+} > Ca^{2+}$. As described previously concerning the complexation of 8-hydroxyquinoline (oxine) and its derivatives (23), the reactivity of NIT with divalent cations was due to the formation of cyclic stable five-membered chelates (Fig. 1B) through the replacement of the hydrogen from the acidic phenolic group (at position 8) and coordination to the nitrogen atom (at position 1), because these chemical groups have donor atoms. These observations show the interdependence between the acido-basic and the chelating properties of NIT. Moreover, the stability constant of the formation of chelates is related to the dissociation constant of the hydroxyl group: the chelation became much more pronounced as the pH increased, corresponding to the progressive dissociation of the hydroxyl group. Already, earlier studies with oxine found a linear relationship between the stability constants of complexes and the corresponding protonation constant of the phenoxide donor atom (46, 47). Hence, at a pH of greater than 8.3 [$pK_a$ (—OH) + 2], all of the molecule was considered to be entirely complexed. As a consequence, a pH of 8.4 was chosen for the further physicochemical studies. Higher pH values were not chosen because of the risk of precipitation of the corresponding metal hydroxide (10).

As reported by Fraser and Creanor (19), the stability constant of the NIT-cation chelates is comparatively low, and very high metal ion concentrations were necessary to ensure complete occupation of the available chelating sites (Fig. 4). This is consistent with the suggestion that oxine binds $Mn^{2+}$ more strongly than it does $Mg^{2+}$ (37). A survey of the large body of information in the literature indicated that the complexation of all derivatives of 8-hydroxyquinoline with divalent cations obeys a 2:1 (chelating compound [*L*]:metal ion [*M*]) stoichiometry (23). This allows calculation of a conditional stability constant β, which can be reasonably considered the same as the overall *Ks* stability constant of an $ML_2$ complex, since pH 8.4 yields a completely dissociated form of NIT. The calculated log *Ks*'s for $Ca^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ were 5.68 ± 0.18, 7.45 ± 0.25, and 8.82 ± 0.4, respectively. The order of the stability constants for these different cations is in good agreement with the one reported for 5-nitro-oxine with log *Ks* values of 2.88, 4.09, and 5.75, respectively (46). Even if the stoichiometry of NIT:divalent-cation complexes is 2:1, the difference between several ions may be due to the charge density (42), the ionic charge (46), and the nature of the solvent that is used (47).

We next studied the influence of some metal cations on the bacteriostatic and bactericidal activities of NIT; both were reduced in the presence of magnesium and manganese, thus confirming the interaction of the drug with these cations, whereas sodium potassium, and calcium were ineffective. The results concerning magnesium are in agreement with those of an earlier report (9). The inefficiency of calcium, correlated with the low level of complexation of NIT with this ion, demonstrates the importance of the chelating property of NIT in its antimicrobial activity. The mechanism of this activity has not yet been elucidated. However, it is interesting to observe that an analog of NIT, 8-hydroxyquinoline, inhibits the RNA polymerase in yeasts solely by chelating the $Mg^{2+}$ and $Mn^{2+}$ cations required for enzyme activity (18, 19). The same investigators reported that oxine is also capable of inhibiting isolated *E. coli* RNA polymerase by essentially removing $Mn^{2+}$ and $Mg^{2+}$ in the absence of any direct contact between oxine and the enzyme (19).

In our study, the magnesium ions, in contrast to manganese, were found to play a major role in the reduction of NIT activity. This difference could be attributed to a stronger stabilization of the outer membrane with high external $Mg^{2+}$ concentrations (50 mM) (36, 38, 49), thus inducing limited diffusion of components through the membrane. This effect could also be due to the formation of drug-ion complexes that are less rapidly absorbed by bacteria, but this does not explain why $Mg^{2+}$ is more efficient. It seems, in fact, that magnesium ions bridging lipopolysaccharide molecules favor the accumulation of NIT by *E. coli* (see above), although no correlation between NIT uptake level and the susceptibility of *E. coli* to NIT was noted.

The pH of the medium was shown to affect in an identical manner the bacteriostatic and bactericidal properties of NIT; i.e., they were decreased under alkaline conditions. On the contrary, an increase in the susceptibility of *E. coli* to NIT was noted with lower pH values. However, the acidification of the medium increases the risk of crystalluria of NIT (pH < 4.5) (33). Fortunately, the normal pH in human urine is approximately 5.5 to 6.2 (3, 45). Furthermore, during UTIs, the pH of the urine is close to normal in patients with cystitis, whereas it is higher (6.0 to 9.0) in patients with pyelonephritis (45), mostly as a result of the bacteria expressing the urease enzyme (e.g., *Proteus* spp.); this is not the case with *E. coli*, which is urease negative.

The effects of pH plus cation supplementation were also investigated. When the pH rose, the inefficiency of NIT in the presence of $Mg^{2+}$ became more pronounced. NIT is progressively more ionized under more elevated pHs, increasing its ability to chelate $Mg^{2+}$ in excess and thereby inhibiting its antibacterial activity. These observations provided evidence for the relationship between the chelating property, which is a function of pH, and the antibacterial activity of NIT and are in agreement with our earlier hypothesis suggesting that the efficacy of NIT is likely to be associated with its physicochemical property. Ample examples in the literature of antibiotics with a pH-dependent activity (3, 25, 28, 41) and of the combined antagonistic effects of metal ions and pH (6, 21) are available.

NIT uptake was also examined. We observed a pronounced accumulation of NIT in bacterial cells, in spite of no energy-dependent step. Furthermore, an important reduction in uptake was found in the presence of magnesium. These data indicate a binding of the drug with superficial structures of the bacteria and a probable interaction between NIT and magnesium ions complexed with the polyphosphate groups of the adjacent lipopolysaccharide molecules. Similar observations were reported by many investigators for quinolones (12) and tetracyclines (30, 51), in which an association of drugs to outer membrane-bound $Mg^{2+}$ was suggested to explain the inhibition of antibiotic uptake by adding free $Mg^{2+}$ in excess.

It is possible that the second step in the cell association of NIT may be due to a diffusion of the molecule through the outer and inner membranes. However, our results do not allow us to draw conclusions about the mode of penetration of NIT through the outer membrane, but neither porin nor nonporin (presumably phospholipid bilayers) pathways can be excluded. The physicochemical parameters of the molecule, for example, its small size (molecular weight, 190.15) and its hydrophobic character, do not exclude these two possibilities (12, 22, 34, 35).

Numerous studies have demonstrated that the antimicrobial activities of antibiotics are markedly influenced by the abilities of these drugs to chelate metal ions. The antagonistic effects of divalent cations ($Mg^{2+}$, $Mn^{2+}$) on NIT activity reported here are similar to those seen between some metal ions (e.g., $Mg^{2+}$, $Ca^{2+}$, and $Al^{3+}$) and quinolones (1, 6, 12, 39), tetracyclines (41), aminoglycosides (6, 29), and other agents (28, 41). Because urine contains appreciable quantities of inorganic ions (2, 16), it is possible that the formation of chelates between NIT and metal ions may affect in vivo the efficacy of this drug. In particular, the magnesium is found as the preponderant divalent cation in urine, with concentrations ranging from 0.9 to 9.2 mM (16); these concentrations are well within the range used in the present study and may have dramatic effects on the antimicrobial activity of NIT. These data raise the necessity of taking such antagonizing factors into consideration, especially during standard in vitro susceptibility tests, as noticed recently (40, 43). On the other hand, such a complexation between NIT and metal ions would also limit the absorption of this compound by the human gastrointestinal tract because of the formation of insoluble chelates. Indeed, a reduced bioavailability has especially been shown for quinolones in the presence of magnesium, iron, and aluminum, which are commonly found in antacids (17, 29, 48).

In conclusion, the work presented here presents evidence that the chelating property of NIT plays a role in the mechanism of action of the molecule. In spite of the unfavorable factors (pH, divalent cations) on MICs and MBCs, the concentrations of NIT reached in the urine are nevertheless largely sufficient to achieve clinical and microbiological cure (4). Similar antagonistic factors have also been shown to reduce the potencies of many other antibiotics. Since such factors may have dramatic effects and may limit the therapeutic outcome, it is necessary that an antibiotic should be minimally affected by the physicochemical conditions of the medium (e.g., pH, osmolarity, and metal ion composition). For this purpose, the physicochemical properties of the drug must be taken into account from the beginning of any similar study in order not to lead to an overestimation of the in vivo activity of the antibiotic and compromise the treatment of infection.

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