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Effect of Magnesium Complexation by Fluoroquinolones on Their Antibacterial Properties

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By using infrared and ^{19}F nuclear magnetic resonance spectroscopies, we localized the binding site and measured the affinity of magnesium for six fluoroquinolones. It was proven that magnesium is situated between the ketone and the carboxylate groups. We determined the binding constants for the 1:1 Mg^{2+}-drug complex in solution. Sparfloxacin and pefloxacin, with affinity constants (K_a) of (10.1 ± 0.6) × 10^2 M^{-1} and (21 ± 1) × 10^2 M^{-1}, respectively, were the least and the most bound, respectively. The trend of the affinities of the assayed fluoroquinolones for magnesium was correlated with their antimicrobial activities against four bacteria and with their accumulation by these bacteria. The reference strain, *Escherichia coli* KL16, and two resistant mutants, NalA (gyrase mutation) and NalB (uptake defect), plus *Staphylococcus aureus* 209P were used. It appeared that, in every case, an impairment of accumulation is responsible for the increase in the MICs observed upon the addition of magnesium.

The advantages of fluoroquinolone antibiotics include their excellent activity against various bacteria, a low frequency of adverse effects, and good absorption on oral administration. However, different studies (5, 11, 14, 22) have shown that some cations, such as magnesium and aluminum, cause malabsorption of most fluoroquinolones, which may result in therapeutic failure. Moreover, the MIC of an antibacterial agent is the result of two steps: the entry of the molecule into the bacterial cell and its interaction with its target within the cell. The rate of penetration of fluoroquinolones across the bacterial cell envelope depends on various physical properties of the drug (6, 12, 18, 20), with its hydrophobicity (3) and the presence of magnesium (7, 13) being major factors. While conflicting reports have been accumulating in the literature on the way in which quinolone antibacterial agents inhibit DNA gyrase-catalyzed supercoiling (17, 24, 25, 32, 34), magnesium is also implicated in the binding of the drug to DNA (17, 21, 33). To better understand the role of magnesium in these various features of quinolone activity, we aimed to determine the site of the binding and to measure the affinity of this ion for some fluoroquinolones. Our approach includes infrared (IR) and ^{19}F nuclear magnetic resonance (NMR) spectroscopies, which have never been used for this purpose, and a comparative study of the effect of magnesium concentration on the in vitro activities of the assayed fluoroquinolones and on their uptake by four bacterial strains.

(This work was presented in part at the 32nd Interscience Conference on Antimicrobial Agents and Chemotherapy, Anaheim, Calif., 11 to 14 October 1992 [16a].)

MATERIALS AND METHODS

Chemicals and strains. The fluoroquinolones were kindly provided by their manufacturers; ciprofloxacin was a gift from Bayer France (Puteaux, France), ofloxacin was from Diamant (Puteaux, France), norfloxacin, pefloxacin, and sparfloxacin were from Rhone-Poulenc Rorer (Vitry sur Seine), and BMY 40062 (7[(1*R*,4*R*)-2,5-diazobicyclo[2.2.1]heptan-2-yl]-1-(1,1-dimethyl-ethyl)-1,4-dihydro-6-fluoro-4-oxo-1.8 naphthyridine-3-carboxylic acid) (4) was from Bristol-Myers Squibb France (Marne la Vallée, France). Nalidixic acid was from Sigma. The structures of pefloxacin and sparfloxacin are given in Fig. 1.

Three *Escherichia coli* strains were provided by J. T. Smith (London, United Kingdom): KL16 (*thy*$^+$ *his*$^+$ *trp*$^+$ *str*^{s}, prototroph, fluoroquinolone susceptible) and two resistant mutants, NalA and NalB, which have a gyrA mutation and a defect in permeability, respectively (15, 26). *Staphylococcus aureus* 209P was from our laboratory collection.

Silicone oils DC 550 and DC 556 were from Touzart et Matignon, Vitry sur Seine, France. Oil with a density of 1.043 was made by mixing three parts of DC 550 and seven parts of DC 556.

IR spectra. The solutions of pefloxacin (1 mM) and sparfloxacin (5 mM) were prepared from 10 mM stock solutions either in 100% D_2O or in buffered D_2O [20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 20 mM KCl) at pD 7.4. Because the spectra were differential spectra (the buffer was subtracted), their qualities may vary with the compound, and in order to ensure better-quality spectra, the concentration of sparfloxacin had to be increased to 5 mM. The pH meter reading was adjusted by DCl or KOD at 0.4 unit less than the required value in order to take into account the change in the glass electrode potential because of D_2O (13). The $MgCl_2$ concentration was 30 mM. IR spectra were recorded on a Perkin-Elmer FT 1720X instrument. They were obtained with CaF_2 cells (50 μm) for the solutions and on suspension in fluorolube between CaF_2 plates for the solids.

NMR spectra. The solutions of fluoroquinolones (0.1 mM) were prepared in the same buffer used for the solutions for the IR spectra, with 80% (vol/vol) H_2O and 20% (vol/vol) D_2O used for the field frequency lock. The concentration of $MgCl_2$ varied from 0 to 15 mM, which doubled the ionic strength; a control on pefloxacin showed that it did not affect the results. Proton-decoupled ^{19}F NMR spectra were recorded at 282.4 MHz on a Bruker AM-300 spectrometer under the following conditions: flip angle, ca. 52°; recycle time, 1.2 s (to prevent any saturation of the signal); digital resolution, 1.22 Hz per point.

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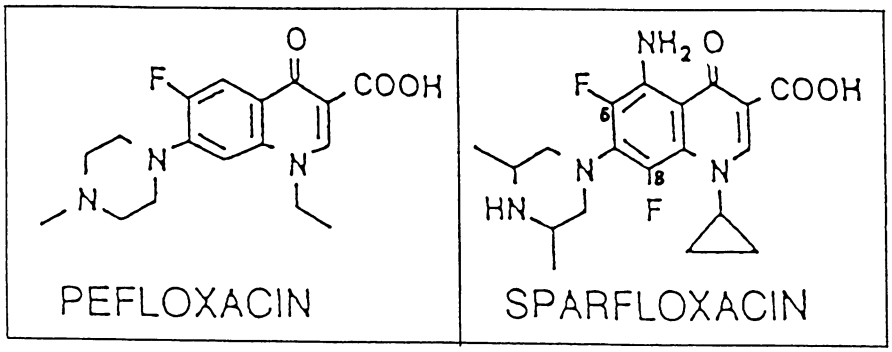

FIG. 1. Structures of pefloxacin and sparfloxacin.

The number of scans was up to 5×10^4, depending on the concentrations of the free and bound species. The temperature was lowered to 283 K in order to slow down the exchange between these species. Theoretical line shapes were calculated on a VAX 750 computer by using a program written by Stephenson and Binsch (29).

MIC determinations. The MICs of the fluoroquinolones for the four strains were determined by a microdilution method in 96-well microdilution plates, with Mueller-Hinton broth, after 18 h of incubation at 37°C. Final inocula of 10^6 CFU/ml were used, and the maximum concentration of the fluoroquinolones was 20 μg/ml. Several series of microdilutions were carried out, each with a different magnesium diacetate [$Mg(OAc)_2$]/fluoroquinolone ratio, from 0 to 2×10^4 (wt/wt). This ratio was kept constant for a specific series. The values are the means of three determinations. Controls with magnesium alone were carried out and showed that bacterial growth was normal, whatever the magnesium concentration.

Intracellular accumulation of quinolones. The method described by Bazile et al. (3) was used, with minor modifications, to measure the intracellular accumulation of the quinolones. Cells were grown in Mueller-Hinton broth to 180 Klett units (blue filter), washed with phosphate-buffered saline (PBS; 80 mM KH_2PO_4 and 77 mM NaCl [pH 7.2]), and resuspended in the same medium to an A_{530} of 25. Accumulation was assayed at 37°C in PBS upon the addition of 10 μg of the drug per ml. At 15 min, a 500-μl sample was placed onto the surface of 500 μl of ice-cold silicone oil (density, 1.043) in a 1.5-ml conical plastic tube, and the mixture was centrifuged at $10,000 \times g$ for 2 min by using a 5415C Eppendorf centrifuge. The tubes were frozen and cut in the middle of the oil layer and were then inverted to eliminate excess oil from the pellet. The pellet was suspended in 1 ml of PBS, boiled for 7 min, and centrifuged at $10,000 \times g$ for 5 min. Norfloxacin (λ_{ex} = 273 nm, λ_{em} = 415 nm) and ciprofloxacin (λ_{ex} = 273 nm, λ_{em} = 418 nm) levels in the supernatant were estimated by fluorimetry. For pefloxacin and sparfloxacin, a ^{14}C-labeled product (5 μCi/mg) was used, and the quinolone concentration was estimated by liquid scintillation counting. Series of uptake experiments (three measures for each point) were carried out for each compound and each bacterium at four $Mg(OAc)_2$/fluoroquinolone ratios: 0, 3,000, 5,000, and 10,000 (wt/wt).

TABLE 1. IR frequencies of some characteristic bands of quinolones in the solid state

Compound	IR frequency (cm^{-1})		
	νCO ketone	νCO carboxylic acid	νOH
Nalidixic acid	1618	1714	3200
Pefloxacin	1630	1712	3340
Sparfloxacin	1642	1716	3462

RESULTS AND DISCUSSION

Structure of COOH group and site of drug-magnesium interaction. Nakano et al. (19) and Timmers and Sternglanz (31) had suggested, on the basis of UV data, that the site of interaction between quinolones and magnesium involves the 4-keto oxygen and the ionized 3-carboxylic acid group. It was important to confirm this localization by a technique that was able to probe these groups directly: IR spectroscopy.

The frequencies and assignments of the stretching vibrations of the carbonyl groups are summarized in Table 1 for nalidixic acid, pefloxacin, and sparfloxacin in the solid state and in Table 2 for the solutions of pefloxacin and sparfloxacin without and with magnesium. Some spectra are given in Fig. 2.

An X-ray study of nalidixic acid (9) showed that there is a

TABLE 2. Stretching frequencies of the carbonyl groups of pefloxacin and sparfloxacin in D_2O[a]

Compound and group	IR frequency (cm^{-1}) at the following pD and the indicated Mg^{2+} concn (M)[b]:					
	4		7.4		12	
	0	3×10^{-2}	0	3×10^{-2}	0	3×10^{-2}
Pefloxacin						
COOH species[c]						
νCO ketone	1631	1632	1632			
νCO carboxylic acid	1698	1700	1698			
COO^- species[d]						
νCO ketone			1622	1632	1622	1632
$\nu_a COO^-$			1580	1590	1580	1590
$\nu_s COO^-$			1400	1409	1400	1410
Sparfloxacin, COO^- species[d]						
νCO ketone	NS[e]	NS	1632	1638	1631	1642
$\nu_a COO^-$	NS	NS	1583	1583	1582	1582
$\nu_s COO^-$	NS	NS	1424	1438	1424	1439

[a] Pefloxacin was used at 1×10^{-3} M, and sparfloxacin was used at 5×10^{-3} M.
[b] Blank spaces indicate that there are no species of that group.
[c] The COOH species includes the cationic and neutral forms.
[d] The COO^- species includes the zwitterionic and anionic forms.
[e] NS, not soluble.

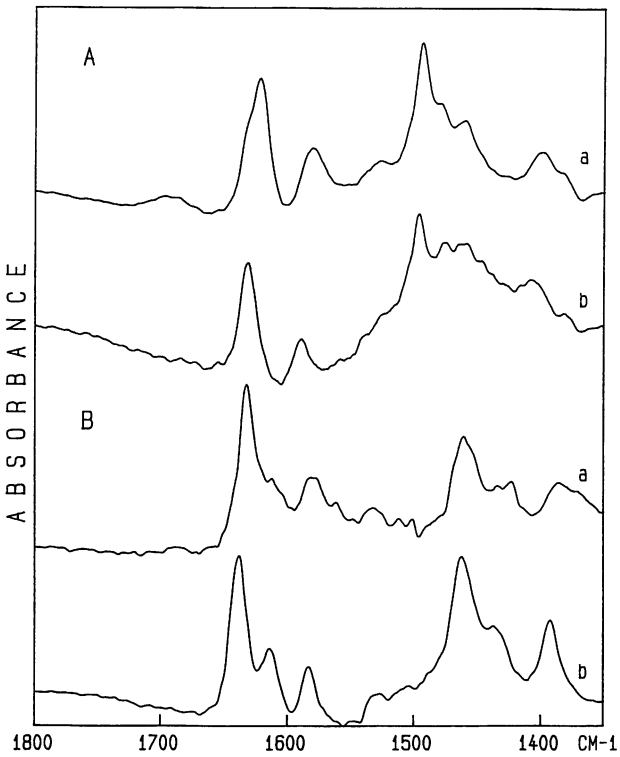

FIG. 2. IR spectra of pefloxacin (A) (1 mM) and sparfloxacin (B) (5 mM) in buffered D_2O (pD 7.4): curves a, no magnesium; curves b, with 30 mM magnesium.

strong intramolecular hydrogen bond between the CO_{ketone} and the OH of the COOH group. The same structural feature holds for pefloxacin and sparfloxacin since the data in Table 1 show that, for the three compounds, the $\nu CO_{carboxylic}$ frequencies were similar and the νOH frequencies were low. The frequencies for νCO and νOH for a free carboxylic acid are 1,740 and 3,600 cm^{-1}, respectively. However, νOH for pefloxacin, higher than for nalidixic acid, implies a weaker hydrogen bond. This bond is even weaker for sparfloxacin, as indicated by the still higher νOH, which is in line with the involvement of the CO_{ketone} group in a double intramolecular interaction with both the OH and the NH_2 groups, as has been seen by X ray for 5-aminooxolinic acid (16). For pefloxacin in acid solution, a 10-fold dilution (from 10 to 1 mM) did not change either the intensities or the frequencies of the carbonyl bands (data not shown). Therefore, there is no intermolecular hydrogen bonding in acid solution and the internal chelation observed in the solid state remains in acid solution. As a matter of fact, the νCO_{ketone} frequencies were the same for the solid state and the acid solution, and the -14 cm^{-1} shift of the $\nu CO_{carboxylic}$ from solid to solution could be explained by the solvation by water molecules.

At pD 4, the addition of magnesium to pefloxacin did not affect its carbonyl vibrations. At pD 12, the carboxylic functions of pefloxacin and sparfloxacin were deprotonated, and as expected, the carboxylate vibrations (antisymmetric $\nu_a COO^-$ and symmetric $\nu_s COO^-$) were observed. Upon the addition of magnesium to pefloxacin solution, a similar increase of 10 cm^{-1} was observed for the νCO_{ketone}, ν_a, and $\nu_s COO^-$ modes (Table 2). For sparfloxacin, the addition of Mg^{2+} induced the same shift on the νCO_{ketone} mode, but $\nu_a COO^-$ remained unchanged, while $\nu_s COO^-$ was increased by 15 cm^{-1}. At pD 7.4, pefloxacin and sparfloxacin spectra showed features different from each other. The pefloxacin spectrum indicated the presence of both carboxylic and carboxylate groups. A band analysis allowed us to evaluate the percentages of these two groups; these were found to be ca. 20% for the COOH species and 80% for the COO^- species, which was in very good agreement with the results obtained by potentiometry (30), i.e., 56% zwitterionic and 26% anionic forms (COO^- species) and 15% neutral and 3% cationic forms (COOH species). Upon the addition of magnesium, the bands corresponding to the COOH species disappeared and the carbonyl bands of the COO^- species underwent the same shift (10 cm^{-1}) as they did at pD 12. The sparfloxacin spectrum at pD 7.4 displayed only the bands of the COO^- species. Upon the addition of magnesium, the carboxylate shifts were similar to those observed at pD 12, but the shift of νCO_{ketone} was smaller (Table 2).

The shift of -10 cm^{-1} for the νCO_{ketone} of both pefloxacin and sparfloxacin, from acid to basic solutions (Tables 1 and 2), reflects the disappearance of the intramolecular hydrogen bond because of the deprotonation of the carboxylic function.

Upon the addition of magnesium to the pefloxacin solutions at pD 7.4 and 12, the frequency increase in all the carbonyl vibrations in the COO^- species indicates that both the CO_{ketone} and COO^- groups interact with Mg^{2+}. This is in agreement with the bathochromic shifts (4 and 6 nm) of the two lower-energy transitions that we observed by UV analysis (data not shown), which implies that complexation by Mg^{2+} increases the electronic density on both the ketone and the carboxylate groups. The fact that the bands corresponding to the COOH species at pD 7.4 disappeared upon the addition of magnesium reflects the shift of the equilibrium COOH $\Leftrightarrow$ COO^- toward COO^- because of the complexation of the COO^- species with magnesium. No spectral change occurs at pD 4, which indicates no Mg^{2+} complexation with the COOH species.

For sparfloxacin, the addition of magnesium in a basic solution induced on the νCO_{ketone} mode an effect similar to that observed for pefloxacin. However, the different shifts of the $\nu_a COO^-$ and $\nu_s COO^-$ modes indicate that the COO^- group is involved in the complex with a different geometry. At pD 7.4, the smaller shift of the νCO_{ketone} vibration is probably due to a specific change in the electronic density of the ketone group, which is induced by the nearby amino group positively charged at this pD (Fig. 1).

It is noteworthy that, for both fluoroquinolones, the intramolecular hydrogen bond and the interaction with magnesium induce similar perturbations of the ketone group. This similarity had already been observed for nalidixic and oxolinic acids (19, 31).

Affinity constant. The affinity constants (K_a) of quinolones for magnesium were determined by NMR. The fluorine nucleus was chosen for its large range of chemical shifts, which makes it very sensitive to weak interactions. Furthermore, its large gyromagnetic ratio and 100% natural abundance allowed us to study dilute solutions (0.1 mM). The ^{19}F chemical shifts, with respect to trifluoroacetic acid, were -49.30, -49.10, -48.71, -48.63, and -46.62 ppm for ciprofloxacin, norfloxacin, pefloxacin, BMY 40062, and ofloxacin, respectively. The two fluorine resonances F6 and F8 of sparfloxacin were assigned, by proton-selective decoupling, at -73.01 and -67.40 ppm, respectively. Some spectra for pefloxacin and sparfloxacin are given in Fig. 3.

They show that, at the frequency and temperature used, the F6 signals for the free and bound species were in the slow exchange limit on the NMR time scale. One can observe the peak for the free drug at 0 mM Mg^{2+}, the peak for the bound

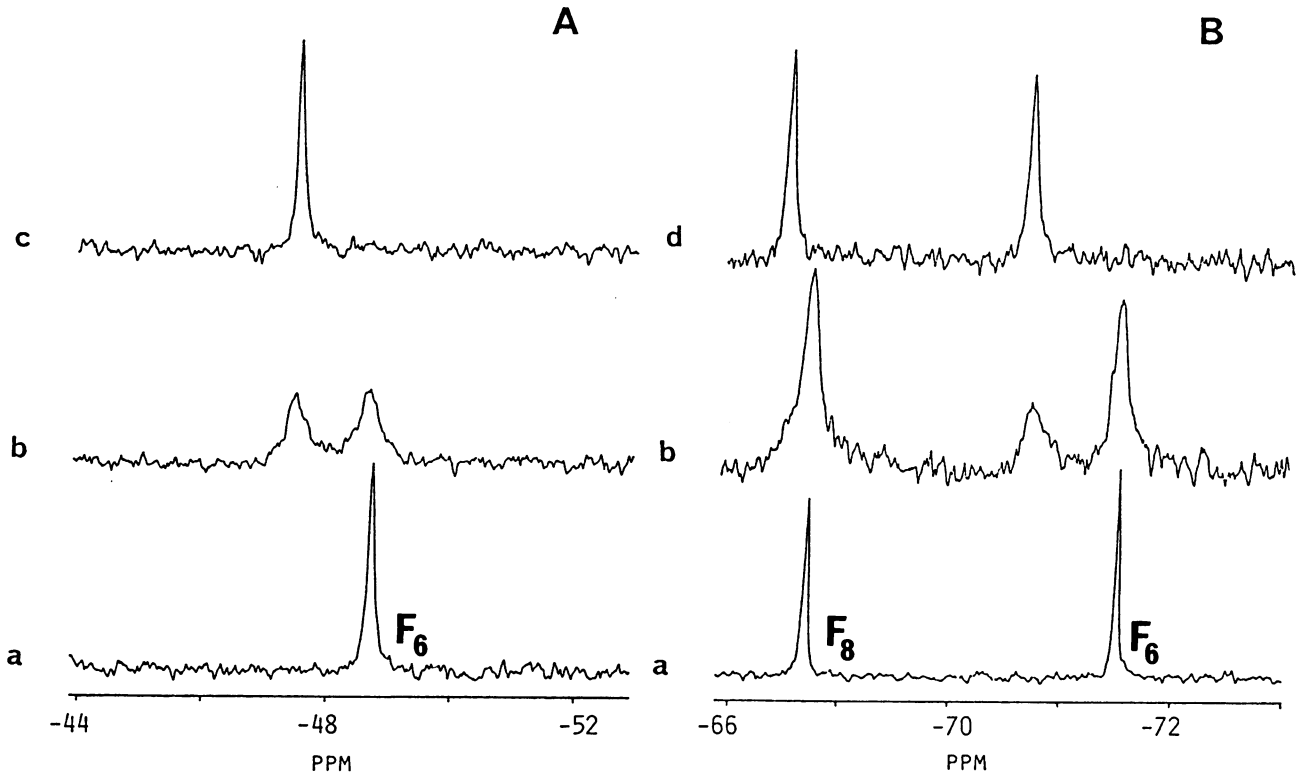

FIG. 3. ^{19}F NMR spectra of pefloxacin (A) and sparfloxacin (B) (0.1 mM) with different concentrations of magnesium: curves a, no magnesium; curves b, 0.5 mM magnesium; curve c, 10 mM magnesium; curve d, 15 mM magnesium.

drug at 10 mM Mg^{2+} for pefloxacin and 15 mM for sparfloxacin, and both peaks in the intermediate situation (0.5 mM). Therefore, the populations of these two species were evaluated by integration and refined by a band shape analysis in order to calculate the affinity constants. Data obtained for different ratios of [$MgCl_2$]/[drug], from 0 to 150, were fitted with two stoichiometries (1:1 and 2:1) for the drug-ion complex. Only the 1:1 stoichiometry yielded coherent values for K_a. The affinity constants were similar for sparfloxacin and ofloxacin, $(10.1 \pm 0.6) \times 10^2\ M^{-1}$, and then increased for ciprofloxacin [$(13.0 \pm 0.5) \times 10^2\ M^{-1}$], norfloxacin [$(13 \pm 1) \times 10^2\ M^{-1}$], BMY 40062 [$(17.0 \pm 0.8) \times 10^2\ M^{-1}$], and pefloxacin [$(21 \pm 1) \times 10^2\ M^{-1}$].

There are only a few reports in the literature on the K_as of quinolones for magnesium. Timmers and Sternglanz (31) fitted their UV data for nalidixic acid on the basis of a 1:1 complex. Cole et al. (8) and Bailey et al. (1) assumed both 1:1 and 2:1 stoichiometries to explain their UV data for nalidixic acid and their potentiometric titration data for methoxyquinolones. The 2:1 stoichiometry was the only one considered by Ross and Riley (23) in their study of very concentrated solutions of lomefloxacin with a ratio of [drug]/[Mg^{2+}] equal to 4. The K_a values (1:1) determined in the studies described above (from 400 to 1,000 M^{-1}) are similar to those that we calculated for the fluoroquinolones. Very recently, Palu et al. (21) have determined, from fluorescence measurements, a K_a of 990 ± 36 M^{-1} for a 1:1 norfloxacin-magnesium complex.

Influence on antimicrobial activity. The K_a values indicated that the binding of magnesium increases from sparfloxacin to ciprofloxacin, norfloxacin, and pefloxacin, with pefloxacin being bound twice as much as sparfloxacin. Besides, the hydrophobicities of these compounds, measured as the distribution

TABLE 3. Some physicochemical and antibacterial properties of four fluoroquinolones

Drug[a]	K_a (M^{-1}, 10^2)[b]	Hydrophobicity (log D)[c]	MED gyrase (mg/liter)[d]	KL16		NalA		NalB		209P	
				MIC (mg/liter)[e]	Uptake (mg/liter)	MIC (mg/liter)	Uptake (mg/liter)	MIC (mg/liter)	Uptake (mg/liter)	MIC (mg/liter)	Uptake (mg/liter)
Spfx	10.1 ± 0.6	0.08	0.2	0.032	70	0.064	55	0.032	35	0.075	180
Cflx	13.0 ± 0.5	−0.70	0.1	0.016	80	0.128	75	0.016	70	0.15	130
Nflx	13 ± 1	−0.60	1.4	0.04	80	0.313	65	0.04	75	0.62	125
Pflx	21 ± 1	0.25	1.0	0.08	55	0.62	55	0.08	45	0.31	150

[a] Spfx, sparfloxacin; Cflx, ciprofloxacin; Nflx, norfloxacin; Pflx, pefloxacin.
[b] Affinity constant for Mg^{2+}.
[c] From reference 3.
[d] MED *E. coli* gyrase for the susceptible strain KL16 taken from reference 3.
[e] The values are the means of three determinations and varied by no more than 10%.

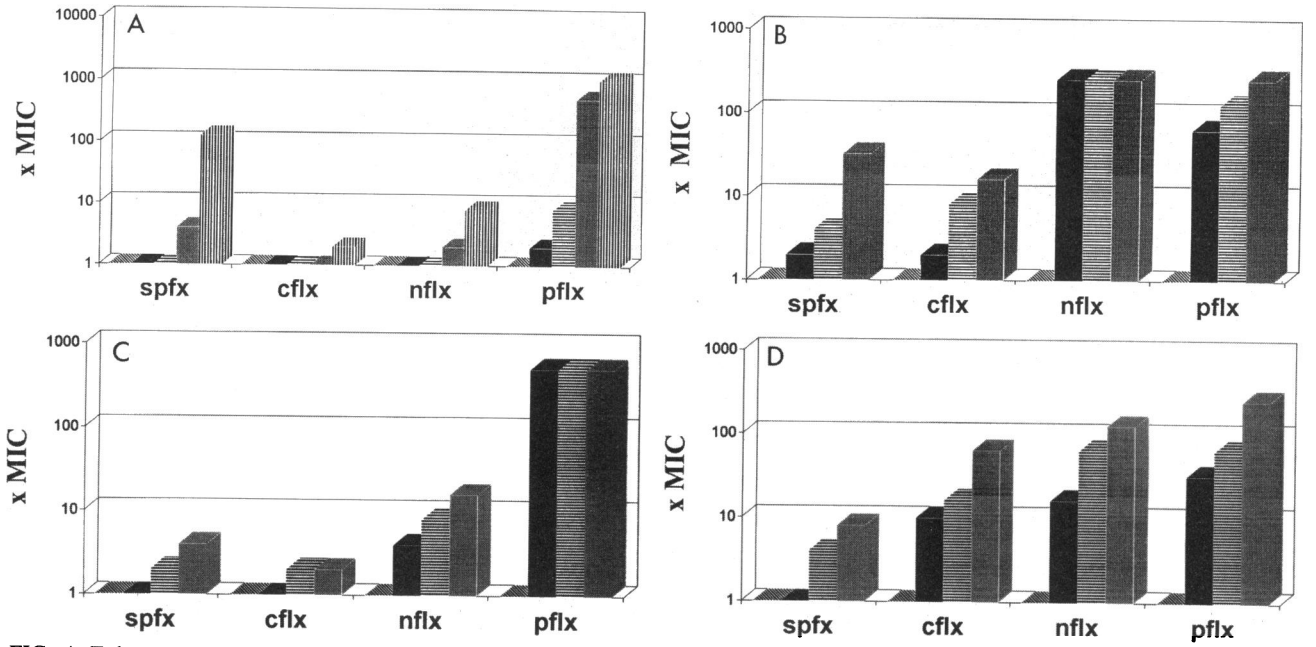

FIG. 4. Enhancement factors of the MICs for bacterial strains KL16 (A), NalA (B), NalB (C), and 209P (D) versus the magnesium/drug ratio (0, 3,000, 5,000, 10,000, and 20,000 [wt/wt], indicated by the bars from left to right, respectively). The Mg^{2+}/drug ratio of 20,000 was used only for strain KL16. *, no greater MIC was observed because of limitations in drug concentrations (20 μg/ml). spfx, sparfloxacin; cflx, ciprofloxacin; nflx, norfloxacin; pflx, pefloxacin.

coefficient (*D*) between *n*-octanol and phosphate buffer (pH 7.2) (3), increased from ciprofloxacin to norfloxacin, sparfloxacin, and pefloxacin (Table 3). Finally, it is important to note that, under the experimental conditions used, the magnesium concentration within the cell does not vary (10, 28), and therefore cannot affect the affinities of quinolones for internal targets.

The MICs of sparfloxacin, ciprofloxacin, norfloxacin, and pefloxacin for the four assayed strains are given in Table 3 together with their levels of accumulation. The activity of the drug on gyrase is expressed as the minimal effective dose (MED; the minimum amount of drug required to cause *E. coli* gyrase-catalyzed DNA supercoiling inhibition) (3).

Upon the addition of magnesium, the variations in the MICs for each strain are shown in Fig. 4, and the variations in uptake are shown in Fig. 5. We chose to use a fixed Mg^{2+}/drug ratio rather than fixed Mg^{2+} concentrations, as did Smith and Ratcliffe (27), in order to compare all the quinolones at the same Mg^{2+}/drug ratio, irrespective of the MICs of the quinolones.

The variations in the MICs observed upon the addition of Mg^{2+} prompted us to examine the uptake of the assayed quinolones by bacterial cells. It is now established that for gram-negative organisms, the more hydrophilic a drug, the more it enters the cell via the porin pathway (3). The hydrophobic quinolones also use the so-called self-promoted pathway in which they need to chelate outer membrane-bound magnesium to diffuse across the exposed lipid domains of the outer membrane (7). The observed effect of Mg^{2+} on the uptake of quinolone by *E. coli* strains confirms this fact; pefloxacin, which is both most bound and most hydrophobic, accumulates least in the presence of Mg^{2+} (10% of the accumulation without Mg^{2+} when a 3,000 Mg^{2+}/drug ratio was used). The relatively high degree of hydrophobicity of sparfloxacin can explain why it surpasses ciprofloxacin and norfloxacin in its susceptibility to magnesium (Fig. 5).

The MICs should reflect both the uptake in the cell and the activities of quinolones on the DNA gyrase. As expected, the addition of magnesium increased the MICs, depending on the strain and the drug. Because sparfloxacin is more active than pefloxacin against DNA gyrase (Table 3), impairment of its accumulation was not very deleterious to its antimicrobial activity, whereas it dramatically affected the activity of pefloxacin. For KL16, the degree of susceptibility of sparfloxacin to Mg^{2+} was higher than those of ciprofloxacin and norfloxacin, although its K_a was the lowest. Thus, at a Mg^{2+}/drug ratio of 10,000, the MICs of sparfloxacin, ciprofloxacin, norfloxacin, and pefloxacin were multiplied by 4, 1, 2, and 512, respectively, whereas at a ratio of 20,000, the multiplying factors were 128, 4, 8, and >1,024, respectively (Fig. 4). For the ratio of >1,024, the higher MIC of sparfloxacin that was obtained, in comparison with those of ciprofloxacin and norfloxacin, may be correlated to sparfloxacin's lower level of uptake because of its higher degree of hydrophobicity.

For both mutant *E. coli* strains and for the Mg^{2+}/drug ratios studied (≤10,000), the increase in the MIC upon the addition of magnesium (Fig. 4) was correlated with the affinities of the drugs for this ion, as calculated by NMR, and the decrease in uptake (Table 3 and Fig. 5). For the NalA strain, the effect of added magnesium on the MICs, although impairing the uptake, was more obvious. This large influence may be explained by the resistance produced by the *gyrA* mutation, which requires a higher concentration of fluoroquinolone within the cell in order to lead to bacterial death. The addition of magnesium increased the MICs more for the NalB mutant than for the susceptible KL16 strain, in spite of the same MICs in the absence of magnesium. This could have been due to the enhancement, by the drug-Mg^{2+} interaction, of the defect in uptake inherent to this mutant.

For *S. aureus*, the effect of magnesium on the MICs was correlated with the affinity of this ion for the drug. Figure 5 shows that this effect is due to a defect in accumulation. In

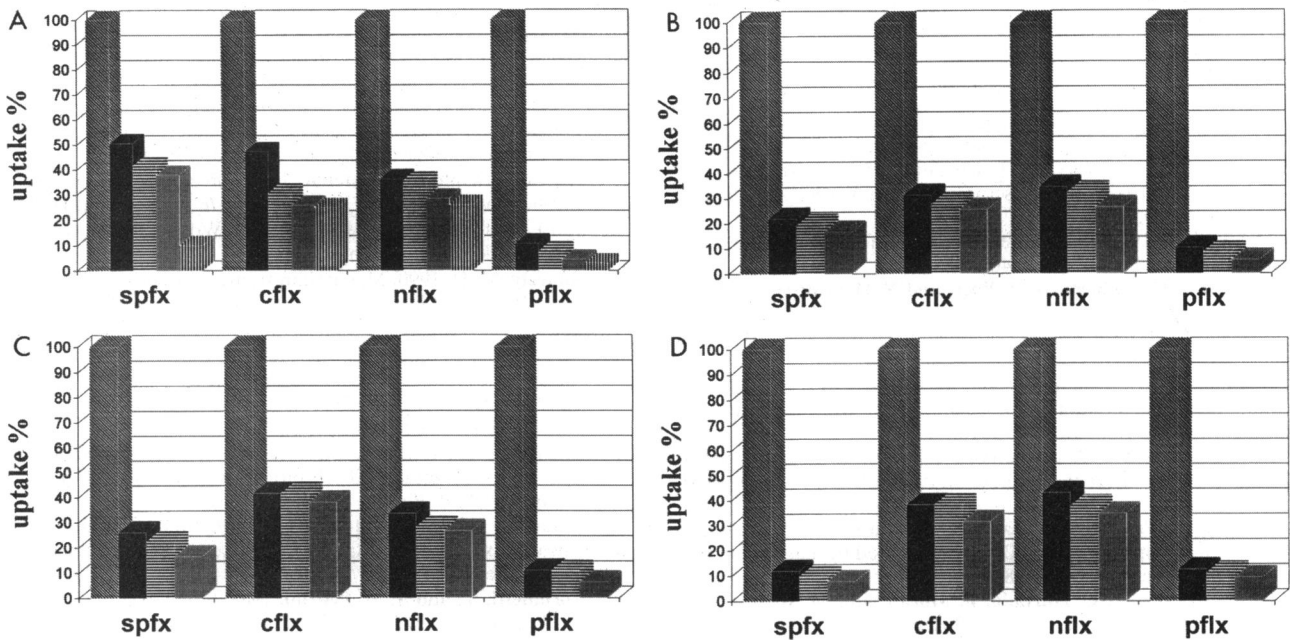

FIG. 5. Variations in uptake by bacterial strains KL16 (A), NalA (B), NalB (C), and 209P (D) versus the magnesium/drug ratio (0, 3,000, 5,000, 10,000, and 20,000 [wt/wt], indicated by the bars from left to right, respectively). The Mg^{2+}/drug ratio of 20,000 was used only for KL16. spfx, sparfloxacin; cflx, ciprofloxacin; nflx, norfloxacin; pflx, pefloxacin.

order to explain this phenomenon, we suggest that teichoic acids may play, for the chelation of Mg^{2+}, a role similar to that of lipopolysaccharides in gram-negative strains.

In conclusion, the interaction of magnesium with the CO_{ketone} and the COO^- groups of fluoroquinolones has been proved without ambiguity, and K_as were determined. The affinities of the quinolones for magnesium are very consistent with the impairment of uptake of the drugs by the cell and with the resulting antimicrobial properties. The role of hydrophobicity is very important for the susceptible *E. coli* strain, since hydrophobic drugs (pefloxacin, sparfloxacin) are more susceptible to the effects of magnesium. This supports very well the existence of a self-promoted transport for hydrophobic drugs (7).

The intracellular concentration of Mg^{2+} in *E. coli* was recently found to be about 100 mM (28). With an intracellular quinolone concentration of about 0.1 mM (evaluated from the data in Table 3) and taking into account the K_as that we measured, we can assume that in the cell, the quinolones studied are totally associated with Mg^{2+}. Therefore, they interact as the complex quinolone-Mg^{2+} with their target: the DNA-gyrase complex. This is in good agreement with the results of Willmott and Maxwell (33), Palu et al. (21), and Bazile and Moreau (2), which suggest an interaction of such a quinolone-Mg^{2+} complex with DNA and gyrase and not a direct interaction of free quinolones with DNA (25).

REFERENCES

1. **Bailey, A. J. G., A. Cole, J. Goodfield, P. M. May, M. E. Dreyfuss, J. M. Midgley, and D. R. Williams.** 1984. The complexation of transition series metal ions by nalidixic acid and related methoxyquinolones: its influence on partition coefficients with reference to antibacterial activity. Int. J. Pharm. **22**:283–290.
2. **Bazile, S., and N. J. Moreau.** 1994. Interactions between fluoroquinolones, Mg^{2+}, DNA and DNA gyrase, studied by phase partitioning in an aqueous two-phase system and by affinity chromatography. J. Chromatogr. A **668**:241–247.
3. **Bazile, S., N. J. Moreau, D. Bouzard, and M. Essiz.** 1992. Relationships among antibacterial activity, inhibition of DNA gyrase, and intracellular accumulation of 11 fluoroquinolones. Antimicrob. Agents Chemother. **36**:2622–2627.
4. **Bouzard, D., P. Di Cesare, M. Essiz, J. P. Jacquet, J. R. Kiechel, P. Remuzon, A. Weber, T. Oki, M. Masuyoshi, R. E. Kessler, J. P. Fung-Tomc, and J. V. Desiderio.** 1990. Fluoro-naphthyridines and quinolones as antibacterial agents. 2. Synthesis and structure-activity relationships of new 1-*tert*-butyl 7 substituted derivatives. J. Med. Chem. **33**:1344–1352.
5. **Brouwers, J. R. B. J., H. J. Van Der Kam, J. Sijtsma, and J. H. Proost.** 1990. Important reduction of ciprofloxacin absorption by sucralfate and magnesium citrate solution. Drug Invest. **2**(3):197–199.
6. **Celesk, R. A., and N. J. Robillard.** 1989. Factors influencing the accumulation of ciprofloxacin in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **33**:1921–1926.
7. **Chapman, J. S., and N. H. Georgopapadakou.** 1988. Routes of quinolone permeation in *Escherichia coli*. Antimicrob. Agents Chemother. **32**:438–442.
8. **Cole, A., J. Goodfield, D. R. Williams, and J. M. Midgley.** 1984. The complexation of transition series metal ions by nalidixic acid. Inorg. Chem. Acta **92**:91–97.
9. **Czugler, M., G. Argay, J. Frank, Z. Meszaras, L. Kutschabsky, and G. Reck.** 1976. 1-Ethyl-1,4-dihydro-4-oxo-5-amino-6,7-methylenedioxy-3-quinoline-carboxylic acid. Acta Cryst. Ser. B **32**: 3124–3126.
10. **Flatman, P. W.** 1984. Magnesium transport across cell membranes. J. Membr. Biol. **80**:1–14.
11. **Flor, S., D. Guay, J. Opsal, K. Tack, and G. Matzke.** 1990. Effect of magnesium-aluminum hydroxide and calcium carbonate antacids on bioavailability of ofloxacin. Antimicrob. Agents Chemother. **34**:2436–2438.
12. **Furet, Y. X., J. Deshusses, and J. C. Pechere.** 1992. Transport of pefloxacin across the bacterial cytoplasmic membrane in quinolone-susceptible *Staphylococcus aureus*. Antimicrob. Agents Chemother. **36**:2506–2511.
13. **Glasoe, P. K., and F. A. Long.** 1960. Use of glass electrodes to measure acidities in deuterium oxide. J. Phys. Chem. **64**:188–190.
14. **Gurdal, H., F. Tulunay, and G. Altay.** 1990. Post antibiotic effect of

ofloxacin and the activity of Mg^{2+}. J. Antimicrob. Chemother. **26:** 291–292.
15. **Hane, M. W., and T. H. Wood.** 1969. *Escherichia coli* K-12 resistant to nalidixic acid; genetic mapping and dominance studies. J. Bacteriol. **99:**238–241.
16. **Huber, C. P., D. S. S. Gowda, and R. Acharya.** 1980. Refinement of structure of nalidixic acid. Acta Cryst. Ser. B **36:**497–499.
16a. **Lecomte, S. C., Coupry, M. T. Chenon, and N. J. Moreau.** 1992. Program Abstr. 32nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. 785.
17. **Maxwell, A.** 1992. The molecular basis of quinolone action. J. Antimicrob. Chemother. **30:**409–416.
18. **McCaffrey, C., A. Bertasso, J. Pace, and N. H. Georgopapadakou.** 1992. Quinolone accumulation in *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Antimicrob. Agents Chemother. **36:**1601–1605.
19. **Nakano, M., M. Yamamoto, and T. Arita.** 1978. Interactions of aluminum, magnesium, and calcium ions with nalidixic acid. Chem. Pharm. Bull. **26:**1505–1510.
20. **Nikaido, H.** 1989. Outer membrane barrier as a mechanism of antibacterial resistance. Antimicrob. Agents Chemother. **33:**1831–1836.
21. **Palu, G., S. Valisena, G. Ciarrochi, B. Gatto, and M. Palumbo.** 1992. Quinolone binding to DNA is mediated by magnesium ions. Proc. Natl. Acad. Sci. USA **89:**9671–9675.
22. **Perez-Giraldo, C., C. Hurtado, F. Moran, and M. Blanco.** 1990. The influence of magnesium on ofloxacin activity against different growth phases of *Escherichia coli*. J. Antimicrob. Chemother. **25:**1021–1026.
23. **Ross, D. L., and C. M. Riley.** 1992. Physicochemical properties of the fluoroquinolone antimicrobials. III. Complexation of lomefloxacin with various metal ions and the effect of metal ion complexation on aqueous solubility. Int. J. Pharm. **87:**203–213.
24. **Shen, L. L., L. A. Mitscher, P. N. Sharma, T. J. O'Donnell, D. W. T. Chu, C. S. Cooper, T. Rosen, and A. G. Pernet.** 1989. Mechanism of inhibition of DNA gyrase by quinolone antibacterials: a cooperative drug-DNA binding model. Biochemistry **28:** 3886–3894.
25. **Shen, L. L., and A. G. Pernet.** 1985. Mechanism of inhibition of DNA gyrase by analogues of nalidixic acid: the target of the drugs is DNA. Proc. Natl. Acad. Sci. USA **82:**307–311.
26. **Smith, J. T.** 1984. Awakening the slumbering potential of the 4-quinolone antibacterials. Pharm. J. **233:**299–305.
27. **Smith, J. T., and N. T. Ratcliffe.** 1985. Effect of pH and magnesium on the *in vitro* activity of ciprofloxacin. *In* H. Neu, and H. Weuta (ed.), First International Ciprofloxacin Workshop. Excerpta Medica, Leverkusen, Germany.
28. **Snavely, M. D.** 1990. Magnesium transport in prokaryotic cells, p. 155–175. *In* H. Siegel (ed.), Metal ions in biological systems, vol. 26. Marcel Dekker, New York.
29. **Stephenson, D. S., and E. Binsch.** 1978. Iterative dynamic nuclear magnetic resonance program for unsaturated exchange-broadened band shapes. Program 365, Quantum chemistry program exchange. Indiana University, Indianapolis.
30. **Takacs-Novak, K., B. Noszal, I. Hermecz, G. Keresztur, B. Podanyi, and G. Szasz.** 1990. Protonation equilibria of quinolone antibacterials. J. Pharm. Sci. **79:**1023–1028.
31. **Timmers, K., and R. Sternglanz.** 1978. Ionization and divalent cation dissociation constants of nalidixic and oxolinic acids. Bioinorg. Chem. **9:**145–155.
32. **Tornaletti, S., and A. M. Pedrini.** 1988. Studies on the interaction of 4-quinolones with DNA by DNA unwinding experiments. Biochim. Biophys. Acta **949:**279–287.
33. **Willmott, C. J. R., and A. Maxwell.** 1993. A single point mutation in the DNA gyrase a protein greatly reduces binding of fluoroquinolones to the gyrase-DNA complex. Antimicrob. Agents Chemother. **37:**126–127.
34. **Yoshida, H., M. Nakamura, M. Bogaki, H. Ito, T. Kojima, H. Hattori, and S. Nakamura.** 1993. Mechanism of action of quinolones against *Escherichia coli* DNA gyrase. Antimicrob. Agents Chemother. **37:**839–845.