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RELEVANCE OF IONIC EFFECTS ON NORFLOXACIN UPTAKE BY *ESCHERICHIA COLI*

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Abstract—The uptake of the quinolone drug norfloxacin by *Escherichia coli* was investigated at initial rate kinetics at different pH and monovalent/divalent metal ion concentration. The results support a simple diffusion mechanism for quinolone incorporation into cells. The uptake process decreases under acidic conditions. The presence of Na^+ or K^+ ions does not affect the results to an appreciable extent, whereas divalent ions cause a dramatic decrease in drug incorporation. The antibacterial activity, evaluated under identical experimental conditions, shows a direct relationship with the uptake data. As a general explanation for the above results it is suggested that the ability of the drug to penetrate into cells is a function of its net charge. The molecule in the zwitterionic form exhibits maximum permeation properties, whereas the uptake is remarkably reduced when the drug bears a net charge as a result of ionization or complex formation with bivalent ions. These results allow further insight into the mechanism of quinolone access to the intracellular compartment.

The mechanism of quinolone access to the target site has important implications in the antibacterial activity of this class of compounds. Contradictory reports have been presented on the molecular nature of the quinolone acceptor(s) [1–6] and it has not been properly dissected, in addition to their precise mechanisms of action [7–13], why the majority of quinolones are less potent against Gram positive bacteria and anaerobes [14]. In this regard, the quinolone resistance observed in some strains of *Escherichia coli* has been connected with the absence of OmpF and OmpC porins in the outer membrane [15–17]. However, the differences in quinolone MICs between the wild type and the porin deficient mutants were generally less than four-fold and correlated with the hydrophobicity of the quinolone. Thus a porin mediated permeation does not fully account for the total uptake of these drugs into bacterial cells as seems to be the case for hydrophilic cephalosporins [18–20]. Both an energy independent diffusion and an active transport mechanism have been claimed to account for quinolone uptake in *E. coli* [21–24]. Somewhat conflicting results appeared in recent literature which warrant further investigations in this matter. Moreover, data from *in vitro* sensitivity testing showed that remarkable effects on antibacterial activity of quinolone occurred as a function of the presence of varying concentrations of hydrogen ions or bivalent metal ions (Ca^{2+} and Mg^{2+}) in the growth medium [22, 25–27]. These effects could be reasonably related to an interference in the mechanisms of drug uptake. In an attempt to shed further light into the problem of quinolone activity and cell permeation, we envisaged studying the assumption of the model drug norfloxacin by *E. coli* cells at initial rate kinetics. The experiments were performed at different pH, ionic strength, divalent cation concentrations and state of the energized cell.

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MATERIALS AND METHODS

Drugs and chemicals. Norfloxacin was kindly provided by Merck Sharp and Dohme (Rahway, NJ) and its purity checked by means of HPLC. [^{14}C]Norfloxacin (sp. act. 45.6 μCi/mg) was also from the same supplier. Tritiated water (1 Ci/mL) and inulin [^{14}C]carboxilic acid (sp. act. 2–10 μCi/mol) were purchased from Amersham (Amity-PG, Milan, Italy). Other antibiotics were from the Sigma Chemical Co. (St Louis, MO). All reagents used in this study were analaR grade.

Drug susceptibility testing. The *E. coli* strains used in the present work were the reference strain Kp05124, kindly provided by Glaxo (Verona, Italy) and a clinical isolate (wt 05) obtained from a patient with cystitis. The two strains exhibited a virtually identical pattern of sensitivity to norfloxacin and to those antibiotics that are currently used to treat urinary tract infections [28]. A conventional broth dilution method was employed to assay minimal inhibitory concentrations (MICs) [29] using an inoculum of 2×10^6 bacteria. The broth was a Mueller–Hinton medium whose relative Mg^{2+} and Ca^{2+} ion concentration, as calculated by atomic absorption measurements, was generally less than 10 μM.

Uptake studies. E. coli cells, harvested from exponentially growing cultures, were washed and resuspended in a buffer containing 10 mM Tris pH 7.0 and 150 mM NaCl, to which 50 mM glucose was occasionally added. The parameters of pH, ionic strength and divalent cation concentration were varied in the same buffer according to the different experimental conditions. Experiments of norfloxacin uptake were carried out by mixing simultaneously equal volumes (100 μL) of norfloxacin and cell suspension (2×10^9 *E. coli* cells/mL) with a dual syringe device [30]. The mixture was then dispensed in a series of 500 μL microhaemocytometer (microfuge) tubes placed in an Eppenodorf centrifuge 5414. These pretreated microfuge tubes contained 50 μL

Table 1. Cellular uptake (15 sec) and MIC values* for norfloxacin in *E. coli* Kp05214 strain as a function of Ca^{2+} and Mg^{2+} concentrations

	MIC (μg/mL)	Uptake (pmol/10^8 cells)
Ca^{2+} (mM)		
0	0.09	20.0
1	0.08	20.0
10	0.42	12.1
30	0.64	9.1
60	1.28	4.5
100	2.56	1.8
Mg^{2+} (mM)		
0	0.08	20.0
1	0.14	19.2
3	0.41	18.1
5	0.70	11.0
10	1.40	5.6
100	6.14	1.2

* The means of five experiments are shown.

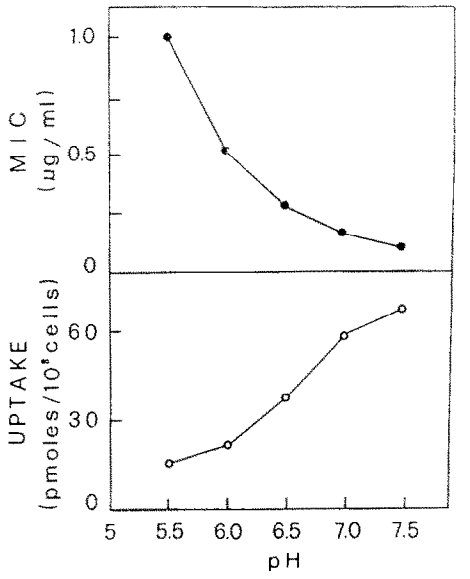

Fig. 1. pH effects on the MIC values (top) and uptake at 15 sec (bottom) of norfloxacin by *E. coli* Kp05214 cells. Quite comparable results were obtained with wt 05 strain.

of a (20% w/v) solution of trichloroacetic acid (TCA) overlaid with 100 μL of a dense mixture of silicon oil (Dow Corning 550 fluid, Nye Inc, New Bedford, MA) and liquid paraffin (Carlo Erba, Milan) in a proportion of 84 and 16 parts, respectively, to give a final density of 1.13 g/mL. The [^{14}C]norfloxacin–*E. coli* cells mixture was allowed to lay on top of the oil-paraffin for varying periods of time (from 0 to 180 sec) before the cell suspensions were sedimented by centrifugation (12,000 *g* for 30 sec) through the oil-paraffin and into TCA. The study on the mechanism of the uptake process and on the interference upon it by varying monovalent and divalent cation concentrations was performed at initial velocity, as calculated by kinetics data. In this case, after being dispensed, the cells were allowed to stay in contact with the drug for 15 sec, before centrifugation. The microfuge tubes were frozen in dry ice and ethanol, and the tube bottom containing the cell pellet in TCA was cut away. The intracellular incorporation of norfloxacin for each of the tubes was measured using a liquid scintillation spectrometer (LKB 1214 Rackbeta). When unlabelled compound was employed, as for the experiments on the dependence of norfloxacin incorporation by increasing extracellular drug molarities, the cell pellet was extracted with 10 volumes of methanol. The drug methanolic extract was submitted to fluorometric determinations in a Perkin–Elmer MPF 66 apparatus equipped with a computerized data station. The excitation wavelength was set at 330 nm and the emission at 420 nm. Calculation of the intracellular and extracellular space was accomplished by measuring incorporation of tritiated water and [^{14}C]inulin as described previously [31].

RESULTS

Antibacterial activity of norfloxacin

The antibacterial activity of norfloxacin was assayed on a clinical isolate of *E. coli* and on the reference Kp05124 strain. As already described [25–27], growth inhibition caused by norfloxacin was largely dependent on the relative concentrations of Mg^{2+} and Ca^{2+} in the medium. Ca^{2+} increases the MICs beginning from 10 mM onwards by about one order of magnitude. Mg^{2+} is substantially more effective in the same test as the MIC values can be increased virtually by 100-fold (Table 1). The effect starts already at the concentration of 1 mM which is close to the physiological value of this ion in the extracellular fluid. pH effects are also prominent. In fact lowering pH causes a substantial decrease in sensitivity. The MIC values obtained in the pH range 5.5 to 7.5 decrease dramatically from 1.0 to 0.12 μg/mL (Fig. 1).

Norfloxacin uptake by E. coli *cells*

This study was performed by a phase partition technique that allows a precise definition of the uptake process even at periods as early as 10–12 sec and that is more accurate than filtration methods used so far [21–22]. This procedure enables evaluation of the uptake at initial velocity which is crucial to assess the transport mechanism. As shown in Fig. 2, the uptake is linearly related to time up to 40–50 sec after which a plateau value is reached. This corresponds to about 65 pmol/10^8 cells. The nature of the uptake phenomenon was further investigated by evaluating the amount of drug incorporated at initial rate velocity as a function of external concentration (Fig. 3). This experiment could be performed by fluorescence measurements due to the very high emission response of quinolone drugs, including norfloxacin. The maximum concentration used in this study was 600 μM due to solubility limits of norfloxacin in aqueous media. However, the examined range was wide enough and representative of pharmacological conditions, to allow safe characterization of the process. This is clearly a non-saturable one, as it is classically the case for passive diffusion.

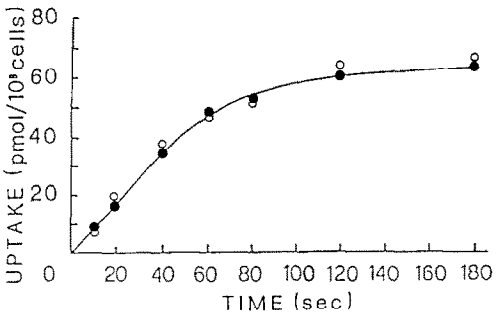

Fig. 2. Kinetics of norfloxacin uptake by the examined *E. coli* strains. Reaction was performed at 21°, using a [^{14}C]norfloxacin concentration of 100 μM. Full circles refer to the uptake of wt 05 cells, open circles to that of Kp05214. See Materials and Methods for a description of the method.

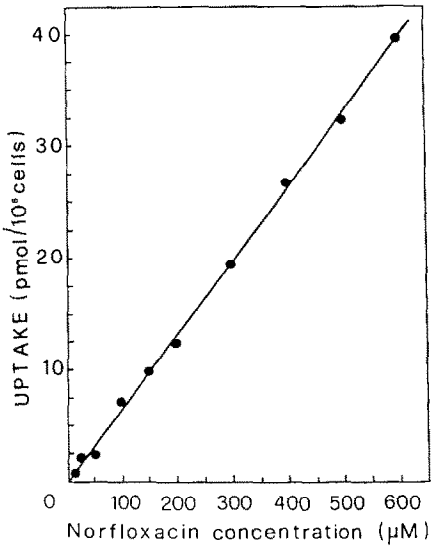

Fig. 3. Fluorescence response of norfloxacin extracted from *E. coli* cells, exposed to the drug for 15 sec, as a function of extracellular quinolone concentration. Results did not differ between the two strains.

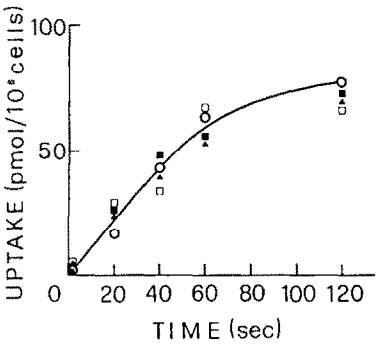

Fig. 4. Effect of 20 min preincubation of *E. coli* wt 05 cells with 10 mM cyanate (filled squares), 10 mM arsenate (filled triangles), and combined cyanate and arsenate (open squares) on the kinetics of norfloxacin uptake. The open circles refer to the uptake data in the absence of the above compounds. Experimental conditions as in Fig. 1. No appreciable difference was found between the two strains.

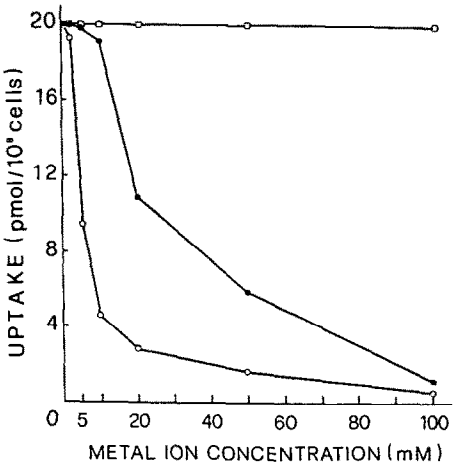

Fig. 5. Effects of metal ion concentration on the uptake of norfloxacin by *E. coli* wt 05 cells. Uptake was estimated at 15 sec. The same behaviour was observed with strain Kp05214. Open squares refer to Na^+ or K^+, filled circles to Ca^{2+}, and open circles to Mg^{2+}. The monovalent ions did not exhibit any effect up to 1 M concentration (not shown).

Influence of the state of energized cell on the uptake process

In order to confirm the above findings, measurements of the quinolone uptake were performed after treatment of the cells with concentrations of arsenate and cyanate that normally depress cell respiratory functions [21, 24]. The kinetics are presented in Fig. 4. It is immediately evident that the treatment does not affect norfloxacin uptake, as it should be expected if an active transport were to occur.

Effects of pH *on the uptake process*

In addition to modifying the MICs, the hydrogen ion concentration plays a clear role in the uptake process (Fig. 1). Indeed, on lowering the pH value to acidic conditions, a lower amount of the drug is incorporated into cells. The decrease can be as high as four-fold on going from pH 7.5 to pH 5.5.

Effect of monovalent and divalent cations on the uptake process

The uptake of norfloxacin by *E. coli* was studied at different monovalent (Na^+/K^+) and divalent (Ca^{2+}/Mg^{2+}) metal ion concentrations. While the monovalent alkaline cations did not affect the drug influx process at all, even at 1 M salt, dramatic effects occurred upon addition of Ca^{2+} and Mg^{2+}, the latter being considerably more effective (see Fig. 5). Mg^{2+}, in fact, inhibited the quinolone uptake which dropped to about 20% of the control value at biologically relevant concentrations (5–10 mM). At higher molarities (100 mM) there was practically no difference between Ca^{2+} and Mg^{2+}.

DISCUSSION

The mechanisms of quinolone uptake by susceptible cells have been the matter for recent controversy in the literature. A number of conflicting reports have in fact appeared, suggesting that this class of compounds are taken up by either a passive diffusion process or by a mechanism of active transport. Very recently Cohen *et al.* [33] have suggested that the net uptake of norfloxacin in *E. coli* was governed by an endogenous active efflux, which was not occurring through the conventional OmpF porin route. However, no precise indication has been thus far presented on the kinetics of drug influx. In addition, all the uptake experiments were based on either filtration or centrifugation methods, followed normally by extensive washing of bacterial cells that were exposed to the drug. By so doing it is quite hard to obtain measurements at initial rate kinetics, and, in addition, redistribution effects can take place following cell washing. These can ultimately influence the kinetic parameters and the appreciation of what gets really incorporated inside cells. The technique reported in this paper, adopted from studies with eukaryotic cells, enabled us to follow drug uptake at early time periods and does not suffer either from pitfalls due to redistribution phenomena or from non-specific binding to materials other than cell components. It is evident that the uptake is linear up to 40 sec. At the stage when a plateau is reached, the amount of drug which is cell-associated is much superior to the level that could be attained when equilibrium is present between intracellular and extracellular compartments (the former accounting for 0.5 $\mu L/10^8$ cells including periplasmic and intercellular space). Hence, this phenomenon of drug accumulation could depend on an active process of concentration or on the quinolone partition within cell constituents (most likely membranes) and binding to them. As the drug which is incorporated at initial velocity shows a linear dependence from extracellular concentration, it is straightforward to conclude that the uptake proceeds through simple diffusion. This evidence is further strengthened by the observation that arsenate and cyanate, which should influence the state of the energized cell by inhibiting ATP synthesis in Emden–Myerhoff pathway (KCN) and cytochrome oxidase (arsenate), had no effect on uptake itself. This result agrees with previous data on enoxacin by Bedard *et al.* [21] but is in contrast with the report of Kotera *et al.* [24], based on UV titration assays, that norfloxacin is taken up by active transport. It is noteworthy that we failed to observe any change in drug uptake by either addition to the medium of 50 mM glucose or by starving the cells in a solution with no carbon source added. Thus, also from these data, an energy dependent influx of norfloxacin seems rather unlikely to be activated in *E. coli*. The pH effects on drug uptake are most probably related to acid–base equilibria involving the quinolone. In fact norfloxacin is an amphiphilic (amino acidic) compound, which can undergo the following protonation–deprotonation steps.

$$NorH_2^+ \underset{+H^+}{\overset{-H^+}{\longleftrightarrow}} NorH \underset{+H^+}{\overset{-H^+}{\longleftrightarrow}} Nor^-$$

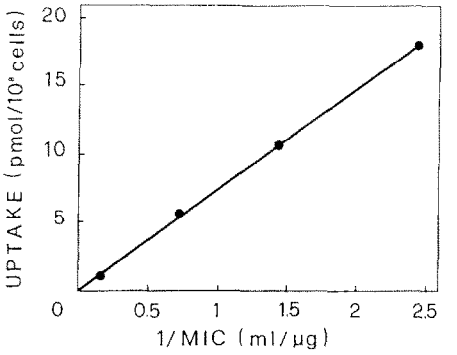

Fig. 6. Relationship between the cellular uptake at 15 sec of norfloxacin and the reciprocal of the MIC at corresponding Mg^{2+} concentrations.

where $NorH_2^+$ is the fully protonated species (both at the carboxyl and aliphatic amino group), NorH is the zwitterionic form and Nor^- is the fully deprotonated species. These equilibria do not take into account the presence of the aromatic amino function, which exhibits very low basicity and is always in the deprotonated form in the pH range we examined. The first equilibrium is relevant under acidic conditions. Titration experiments (not shown) indicate that while at pH 5.5 about 20% of the drug is in the zwitterionic form, more than 90% NorH is present at pH 7.5. Interestingly, the change in Nor uptake and activity parallels the ionization state of the drug. In fact both the penetration and antibacterial activity drop dramatically going from pH 7.5 to pH 5.5. These data suggest that the charged species are mainly prevented from crossing the membrane, which on turn points to the relevance of the lipophilic uptake of the quinolone. Thus, our pH results are well in agreement with those of Smith and Ratcliffe [25] who had originally proposed that pH affected either drug uptake or the target site. The present work is now providing evidence supporting the former, rather than the latter hypothesis.

Although some authors [15] suggested that Nor, being an hydrophilic quinolone should efficiently enter the cells through OmpF porins, it has to be pointed out that in their experiments Nor was accumulating in the OmpF deficient strain to an extent of almost 50% that of the sensitive wild type strain. Moreover, no appreciable difference in drug uptake appeared to build up during the first 5 min. The results obtained in the presence of bivalent metal ions show that both Mg^{2+} and Ca^{2+} impair drug penetration and activity. A plot of relative uptake as a function of the reciprocal of the MIC at different metal ion concentrations (Figs 6 and 7) shows a linear relationship between these two parameters. Thus, earth-alkaline ions act primarily by inhibiting quinolone penetration into cells. It is suggested in the literature that fleroxacin binds to magnesium ions [22]. Preliminary data obtained from potentiometric titrations show that indeed norfloxacin is able also to form complexes with calcium and magnesium ions, the latter being more stable than the former. Our results strengthen the suggestion that quinolone

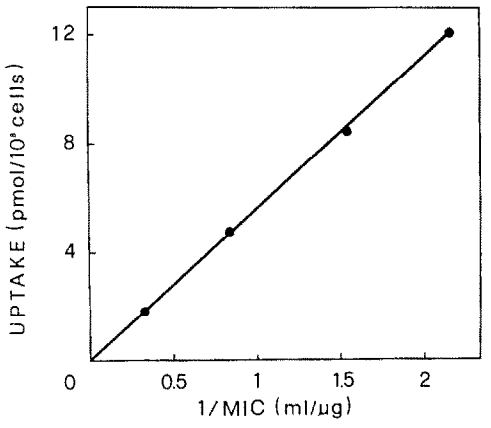

Fig. 7. Relationship between the cellular uptake at 15 sec of norfloxacin and the reciprocal of the MIC at corresponding Ca^{2+} concentrations.

binding to the above ions plays a general role in the transport mechanism. It is worth considering that different complex species can be present in solution. In particular, the stoichiometry of the complexes and the concentration of the various species change as a function of drug, ion and pH. At low drug concentrations it is reasonable to assume that $(Me\text{-}NorH)^{2+}$, i.e. the 1:1 complex with the zwitterionic form, is the predominant complex species. This positively charged compound does not appear to be able to cross the membrane, as indicated by the clear relationship between decrease of penetration and lowering of the MIC. If porine transport were the prevalent mechanism of drug uptake, it should be even facilitated by an increase in the polarity of the drug, as it is in the case of complex formation with bivalent metal ions. Two possible explanations could account for the experimental findings: either the cations impair the mechanism through which the drug gets to porins from the bulk solution, or lipophilic transport is important predominantly under our conditions, so that an increase in drug polarity prevents the drug from crossing the hydrophobic membrane. In this connection, it was suggested that the presence of Mg^{2+} in solution could compete for the binding of fleroxacin to magnesium ions already bound to glycolipid components of the outer membrane, thus limiting the diffusion process to porins. If we take into an account the above discussed pH effects on drug penetration and activity, which are clearly related to changes in the net charge of the drug (and cannot of course be explained in terms of metal ion complexation phenomena), we feel confident in extending the same concepts to the case of the metal complexes of quinolones. Thus, we propose that the reduced quinolone uptake in the presence of Mg^{2+} and Ca^{2+} should be primarily related to a decrease in lipophilic transport of the charged species formed when the metal binds to the drug, rather than to an impairment of porine-mediated uptake. This provides a simple, unifying explanation for the various phenomena we observed, which can obviously be modulated by the characteristics of the single quinolone drugs as far as the relative importance of the different mechanisms of drug uptake are concerned. It is noteworthy that the Ca^{2+} and Mg^{2+} results presented herein agree with those of other workers [32]. Moreover, for the first time, they provide proof for the hypothesis that the effects of metal ions are operating on drug uptake as suggested previously by the same authors [32]. The proposed active efflux of norfloxacin deserves some further comments. If such a process were to take place, as suggested by Cohen *et al.* [33], one should expect to see drug accumulation in the deenergized cells, This has not been the case in our experiments, but it cannot be excluded that other kinds of inhibitors could increase the quinolone intracellular concentration by more directly affecting cell structures involved in the formation and maintenance of the proton motive force. The active endogenous efflux of norfloxacin that has been described in *E. coli* was based on experiments with vescicles formed by everted membranes derived from susceptible strains. Although this situation is reminiscent of the one described for tetracycline in *E. coli* cells harbouring resistance *tet* determinants [34], no clear indication was given on the nature of the norfloxacin transporter neither on how this process could affect the sensitivity to quinolones. Therefore, such an efflux phenomenon deserves to be further confirmed under more physiological conditions and its function to be more clearly established. Studies on this topic and on quinolone uptake in Gram positive bacteria are now in progress and will be the subject of future reports.

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