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Accumulation of Enoxacin by *Escherichia coli* and *Bacillus subtilis*

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Several methods were used to determine enoxacin uptake in *Escherichia coli* strains because washing of cells removed all or most cell-associated enoxacin whereas no washing was associated with large amounts of cell-bound enoxacin. Washing after up to 40 to 45 min of exposure to enoxacin followed by suspension in drug-free medium prevented a significant effect of enoxacin on cell growth. Cell uptakes obtained with different methods showed no difference in the shape of the timed uptake curves but did show significant quantitative differences. These results are consistent with cell-associated enoxacin comprising a freely exchangeable pool of drug. Lineweaver-Burk plots of uptake were consistent with uptake of enoxacin by simple diffusion. No saturability and no competition with ciprofloxacin were observed. Low temperature (4°C) was associated with decreased uptake. Arsenate, carbonyl cyanide *m*-chlorophenylhydrazone, sodium fluoride, sodium azide, and 2,4-dinitrophenol had no effect on uptake. We conclude that the mechanism of transport of enoxacin into cells is by simple diffusion. Mutants of *E. coli* with deficiency of outer membrane proteins F and C and an enoxacin-resistant mutant selected by serial passage with increasing enoxacin concentrations demonstrated that F porins play a significant role in enoxacin uptake and influence susceptibility to enoxacin. Uptake was shown to be similar in a strain of *Bacillus subtilis*.

Fluoroquinolone (6-fluoro-7-piperazino-4-quinolones) antimicrobial agents are a group of compounds which differ from older quinolone antibiotics in their much greater activity and wider spectrum of bacterial inhibition. As a group they show a marked increase in hydrophilicity compared to nalidixic acid and most have amphoteric properties (6). Like nalidixic acid, they apparently share the A subunit of DNA gyrase as their primary site of action (2, 11), although DNA has been reported as the principal binding site of quinolones (10). It has been reported that the major increase in activity seen with fluoroquinolones does not fully correlate with a corresponding increase in capability to inhibit isolated DNA gyrase (12).

Reduced susceptibility to fluoroquinolones may be brought about by mutations affecting DNA gyrase, outer membrane permeability, and other unrecognized cellular changes. Several low-level resistance mutations have reduced accumulation of norfloxacin in some cases associated with an apparent loss of the F-porin protein in strains of *Escherichia coli* (6, 7).

We have undertaken a study of the mechanism of uptake of enoxacin in view of the probability that uptake is a major contributing factor to the improved activity of this agent. This view is supported, because of the association of diminished uptake and reduced susceptibility, by the lack of clear correlation between activity and inhibition of isolated DNA gyrase for several fluoroquinolones and the major increase in hydrophilicity of enoxacin compared to nalidixic acid. Our studies support the conclusion that uptake is by simple diffusion in *E. coli* and *Bacillus subtilis*.

MATERIALS AND METHODS

Bacterial strains. The source and characteristics of each strain used are described in Table 1.

Media. Nutrient broth (BBL Microbiology Systems, Cockeysville, Md.) was used for overnight growth, MIC studies, and enoxacin uptake measurement. Tryptic soy agar (Difco Laboratories, Detroit, Mich.) was used for colony counts and nutrient agar (BBL) was used for disk susceptibility studies. MICs were determined in nutrient broth (NB), using a final inoculum of 10^6 to 10^7 CFU/ml. Incubation was done for 18 h at 35°C. Disk susceptibility testing was performed by standard diffusion methodology (1). Enoxacin was obtained from Parke, Davis & Co., Toronto, Ontario, Canada, ciprofloxacin was from Miles Pharmaceuticals, Toronto, and nalidixic acid from Sigma Chemical Co., St. Louis, Mo.

Uptake studies of enoxacin. Radiolabeled enoxacin (5.8 Ci/mmol; Warner-Lambert Co.) was generously provided by Parke, Davis & Co., Toronto. Radioactive enoxacin was shown to be bioactive by comparing zones of inhibition produced by 6-mm paper disks containing 2 or 10 μg of either a standard enoxacin preparation or [^{14}C]enoxacin. Zones of inhibition were identical. In uptake studies a constant ratio of 0.2 mg of [^{14}C]enoxacin was mixed with 4 mg of unlabeled enoxacin. The standard uptake procedure was performed by growing cells overnight in NB, inoculating this culture to fresh broth to give an optical density at 600 nm (OD_{600}) reading of about 0.1, and allowing cells to reach log phase of growth by incubation with shaking at 35°C. Uptake studies were initiated by adding the mixture of unlabeled and ^{14}C-labeled enoxacin to cells at 35°C in a shaking water bath with an OD_{600} reading of approximately 0.4. Samples of 1 ml were removed and centrifuged for 3 min at room temperature at 10,000 × *g*, and the entire supernatant was removed. The pellet was suspended into 1 ml of nutrient broth and immediately filtered through a 0.5-μm GV filter (Millipore Corp., Bedford, Mass.) prewashed with 1 ml of unlabeled enoxacin at a concentration of 1 mg/ml. Filters were counted in Omnifluor (4 g of toluene per liter; New England Nuclear Corp., Boston, Mass.). Washing of filters following the filtration of cells with 2 ml of NB, 3% NaCl in water, 0.1% sodium dodecyl sulfate in water, or unlabeled enoxacin (2 mg/ml) in NB all produced approximately a 20% reduction in cell counts. None of the wash steps was performed as part of

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TABLE 1. *E. coli* K-12 strains

Strain	Source	Characteristics
J5-3 derivatives		
J5-3	B. J. Bachmann, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.	F^+, *pro-22 metF63*
SA1306	K. E. Sanderson, University of Calgary, Calgary, Alberta, Canada	*pro-22 metF63 gyrA* Rif^r
JB-5R[a]	Our laboratory	*pro-22 metF63 gyrA* $Enox^r$
JF derivatives		
JF-568	R. K. Poole, The University of British Columbia, Vancouver, Canada	*proC24 aroA357 his53 purE41 ilv277 met65 lacY29 xyl114 rpsL97 cycA1 cycB2 tsx63* (parent)
JF-694	B. J. Bachmann	F^- *proC24 ompF254 his53 ompC263 purE41 nmpA1 ilv227 met65 lacY29 xyl-14 rpsL97 cycA1 cycB2 tsx63* ($OmpC^-$ $OmpF^-$ $PhoE^+$)
JF-701	R. K. Poole	Isogenic to JF-568 but *ompC264* ($OmpC^-$)
JF-703	R. K. Poole	Isogenic to JF-568 but *ompF254* ($OmpF^-$)

[a] Obtained by serial passage in the presence of increasing concentrations of enoxacin (Enox).

the standard uptake protocol. Examination of a variety of other uptake procedures demonstrated that the amount of cell-associated enoxacin was highly dependent on the wash procedure used. However, for each method the shape of time-dependent uptake and the zero intercept of Lineweaver-Burk plots was the same, although the total quantity of enoxacin bound varied. In general, the greater the volume or the number of wash steps, the less the quantity of cell-associated enoxacin. Control samples were used which contained an identical quantity of labeled and unlabeled enoxacin but no bacterial cells. Values obtained with this control sample were used to correct for background binding in the absence of bacterial cells. Cell-associated enoxacin is used to mean the quantity of enoxacin determined from ^{14}C counts following subtraction of background filter counts. Heat-killed cells suspended to an OD_{600} reading of about 0.4 were used for uptake determinations by the standard protocol. Time-dependent uptake with these heat-killed cells showed the same pattern as viable cells but accumulated 20 to 30% less enoxacin.

Bioassay method. Cells were grown to log phase in NB at 37°C with shaking, enoxacin was added at different concentrations, and the preparation was incubated at 37°C with continued shaking. At various times thereafter, 10 ml of bacterial suspension was removed and centrifuged at 10,000 × *g* for 3 min, the supernatant was discarded, and cells were washed once with 2 ml of saline. This preparation was centrifuged again, cells were suspended in 1 ml of saline, and the bacterial suspension was immersed in boiling water for 7 min to elute enoxacin. The boiled suspension was centrifuged at 10,000 × *g* for 3 min, and the activity of enoxacin in the supernatant (1 ml) was determined. A cork bore was used to produce 10-mm-diameter holes in nutrient agar inoculated with sufficient *E. coli* J5-3 to produce just confluent growth. A volume of 100 μl of the supernatant was placed in the wells, and the zones of inhibition were compared with those produced by standards of different concentrations of enoxacin. The bioactivity of heated enoxacin was determined to be identical to unheated drug by comparing standards of enoxacin boiled for 7 min with unheated enoxacin.

To study energy inhibitors, each inhibitor was added 2 min before the addition of enoxacin at the following final concentrations: carbonyl cyanide *m*-chlorophenylhydrazone (Sigma), 2×10^{-5} M; sodium azide (Fisher Scientific Co., Fairlawn, N.J.) and sodium arsenate (J. T. Baker Chemical Co., Phillipsburg, N.J.), 10^{-3} M; 2,4-dinitrophenol (DNP) (Sigma), 2×10^{-3} M; sodium fluoride (Fisher Scientific), 10^{-2} M. Anaerobic culture and manipulations including incubation were carried out in an anaerobic chamber (Forma Scientific, Marietta, Ohio).

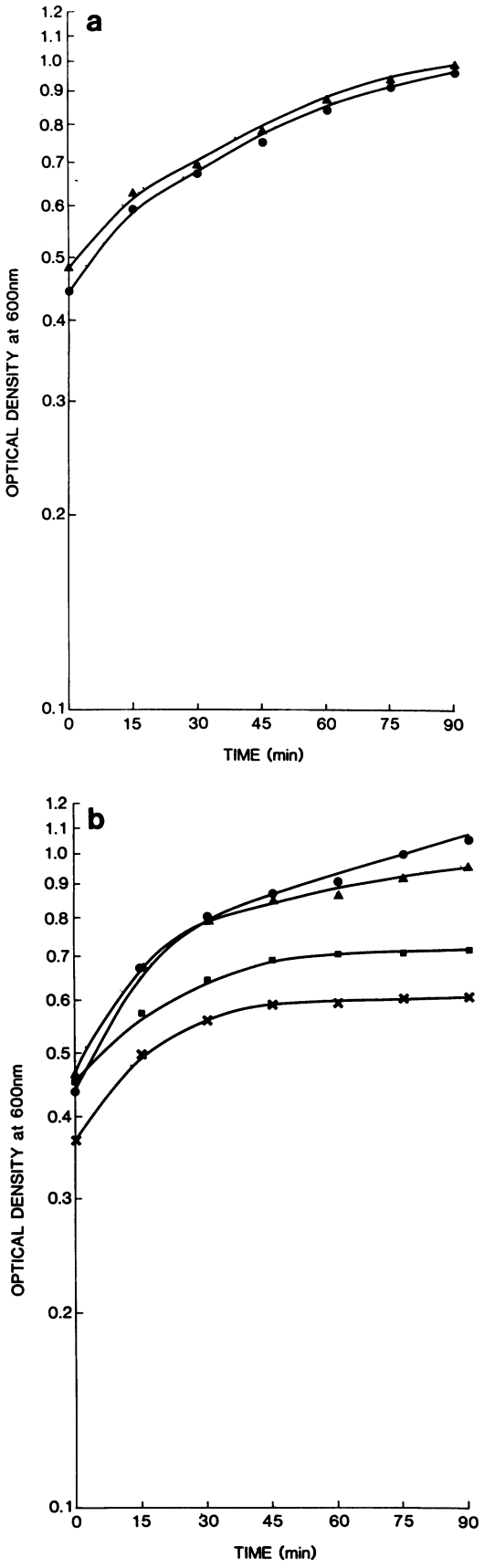
For purposes of preparing Lineweaver-Burk plots, cells were exposed for 1 min to a range of enoxacin concentrations from 0.5 (sub-MIC) to 150 μg/ml. The uptake procedure was identical to that of the standard protocol except that after centrifugation the pellet was suspended to 300 μl of NB and the entire volume was counted in PCS solubilizer (Amersham Corp., Arlington Heights, Ill.).

Growth studies. Studies carried out to determine the effects of washing conditions on the capability to reverse the action of enoxacin were performed as follows. Cells were grown in NB at 37°C with shaking to an OD_{600} reading of about 0.4, and enoxacin was added at 1, 2, 5, 10, or 20 μg/ml. After 2 min of exposure to the enoxacin at 37°C, 20-ml samples were removed and centrifuged at 10,000 × *g* and 25°C for 2 min, and the supernatant was discarded. The cells were washed with 20 ml of nutrient suspended in the same volume of prewarmed (37°C) medium and reincubated, with OD_{600} determined every 15 min over the next 90 min. Additional studies were performed by identical methodology for enoxacin concentrations of 20, 40, 60, and 80 μg/ml. However, time of removal of samples was at 10-min intervals from 0 to 120 min. Preparations were washed with prewarmed medium and the OD_{600} was determined as described above. Studies were also performed at 1, 5, and 10 μg of enoxacin per ml, allowing a 60-minute exposure to the drug before washing and suspension as above.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis studies. Outer membrane preparations were prepared by a method previously described (4), and electrophoresis was performed as previously described, using 12.5% polyacrylamide gels (8).

RESULTS

Uptake and growth studies. Preliminary studies with the [^{14}C]enoxacin suggested that the uptake process was one of simple diffusion with rapid entry of enoxacin. To determine if the rapidly accumulated enoxacin inhibited cells, growth was monitored after 2 min of exposure of *E. coli* SA1306 to 1, 5, 10, or 20 μg of enoxacin per ml. No effect on growth rate was detected (data for 20 μg/ml; Fig. 1a).



We further exposed *E. coli* SA1306 to higher enoxacin concentrations of 20, 40, 60, and 80 μg/ml for times ranging from 10 to 120 min before washing cells free of enoxacin at 10-min intervals within this time range. A period of 40 to 50 min of treatment with the drug before washing and suspension was needed to produce a major (a one-third or greater reduction in doubling time) impairment of growth (data not shown). Exposure to lower concentrations of 1, 5, and 10 μg/ml for 60 min before washing and suspension caused a significant decrease of growth rate at 5 and 10 μg/ml and a questionable decrease at 1 μg/ml (Fig. 1b).

These studies demonstrated that the effect of enoxacin could be reversed by washing cells for up to 40 to 45 min even at a concentration as high as 80 μg/ml. Therefore, enoxacin was not bound firmly to its target(s). The effect after 40 to 45 min likely indicated that this period of prolonged inhibition of the target was needed to inhibit cell growth.

Time-dependent uptake of [^{14}C]enoxacin, using the standard protocol and a bioassay method, are shown in Fig. 2a and b. Both methods produced similarly shaped time-dependent uptake plots, although small quantitative differences existed between these methods. Failure to reproduce washing procedures stringently produced quantitative differences between repetitions of uptake studies at identical enoxacin concentrations.

When the standard protocol was modified such that the filtered cells were washed with a larger volume (10 ml) of NB or two repetitions of 1 ml of NB, cell-associated counts dropped significantly to values of 20 to 80% of those obtained with the standard protocol.

Our results were consistent with the conclusion that the cell-associated enoxacin consists of a pool of freely exchangeable enoxacin. Cell localization studies did not allow us to determine whether this drug was bound on the cell surface or located intracellularly due to rapid relocalization of label when cells were broken and subject to differential centrifugation. We also conclude that a variety of washing methods removes an extensive component of the cell-associated enoxacin, including that bound to those critical targets needed to inhibit cell growth.

Characteristics of cell uptake. In view of the flat uptake pattern seen with time, 1-min samples were chosen for purposes of preparing Lineweaver-Burk plots (Fig. 3a and b). These uptakes were performed over a concentration range of 0.5 to 150 μg/ml, which included uptake at about the MIC (i.e., 0.62 to 1.25 μg/ml). The plots showed an intercept of zero, indicating a lack of saturability of the transport system. Plots prepared with different wash methods all showed a zero intercept. The rate of uptake was clearly decreased at 4°C. Both of these findings were consistent with a process of simple diffusion. Increasing uptake with time was only seen with very high concentrations of 80 μg/ml or greater (Fig. 2a and b).

FIG. 1. Effect of enoxacin on growth. (a) Cells were treated with 20 μg of enoxacin per ml for 2 min at 35°C with shaking. A 20-ml aliquot of cells was removed and centrifuged at 10,000 × *g* for 3 min at 22°C, the supernatant was discarded, and cells were washed once with 20 ml of prewarmed NB and suspended finally in the same volume of NB at 35°C. Cell growth was followed by reading the OD_{600} every 15 min for 90 min. Symbols: ●, no enoxacin, ▲, 20 μg of enoxacin per ml. (b) Cells were exposed for 1 h with 1, 5, or 10 μg of enoxacin per ml at 35°C with shaking. Thereafter cells were treated to remove enoxacin and suspended as given for (a). Symbols: ●, no enoxacin; ▲, 1 μg/ml; ■, 5 μg/ml; ×, 10 μg/ml.

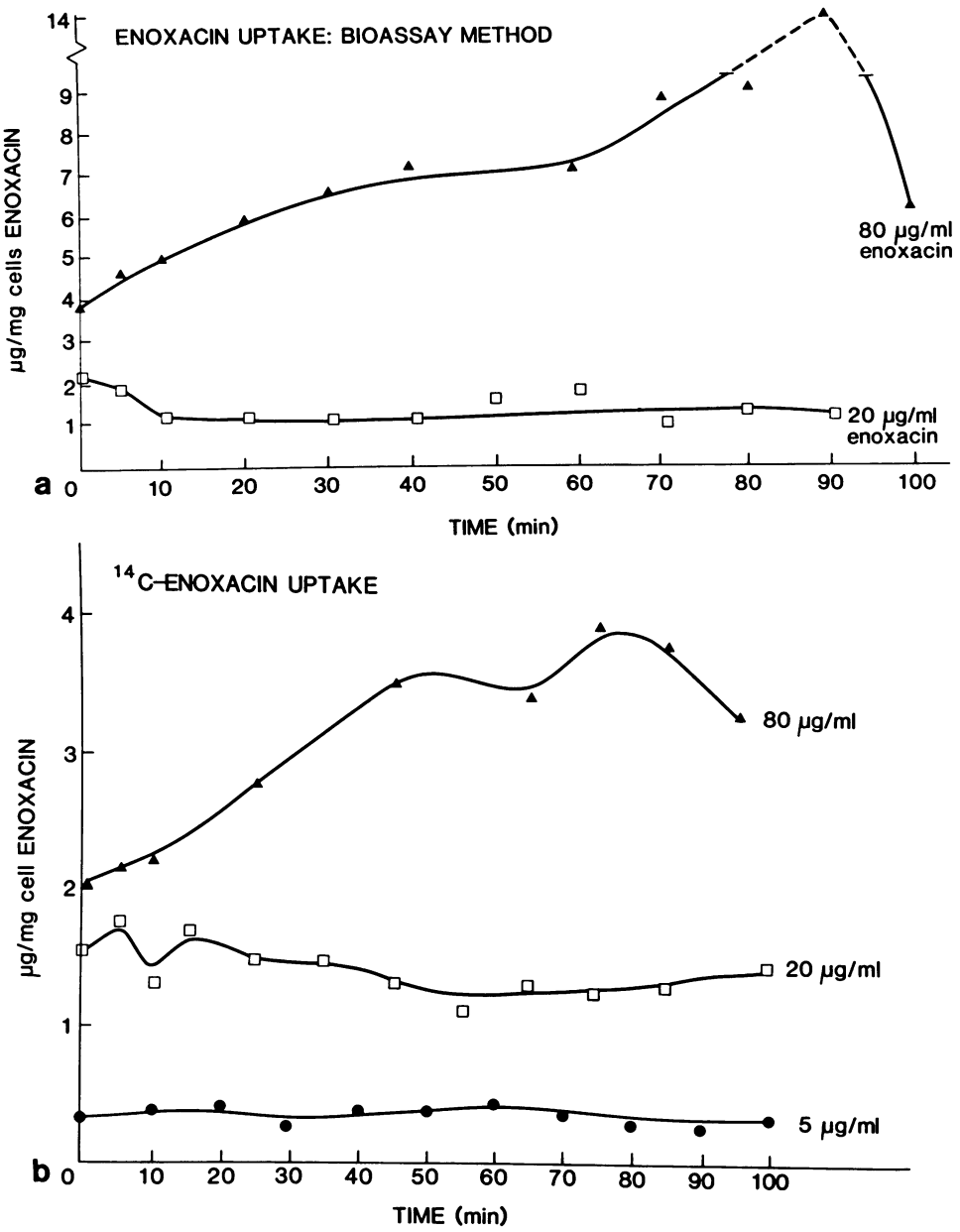


FIG. 2. Time-dependent uptake by *E. coli* SA1306 of enoxacin at 37°C in NB with shaking. (a) Samples of 10 ml were removed at the times given, and enoxacin uptake was determined by the bioassay method. (b) Samples of 1 ml were removed at the times given, and enoxacin uptake was determined by the standard protocol of centrifugation and filtration.

Treatment of cells with sodium azide, carbonyl cyanide *m*-chlorophenylhydrazone, sodium arsenate, or sodium fluoride did not inhibit uptake (Table 2). Thus, uptake occurs independently of electron transport systems, proton motive force, ATP hydrolysis, or glycolytically derived energy. DNP has been reported to reduce uptake of ciprofloxacin, pefloxacin, amifloxacin, and norfloxacin with a negligible effect on enoxacin (J. M. Diver, L. J. V. Piddock, and R. Wise, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 946, 1986). DNP had no effect on enoxacin uptake in our studies (Table 2). Uptake measured by bioassay, while not examined as extensively, showed the same insensitivity to energy inhibitors as uptake measured with radiolabeled enoxacin.

Unlabeled ciprofloxacin used at concentrations ratios of ciprofloxacin/enoxacin of 5:5, 5:3.5, 5:2, 5:1, 5:0.67, 1:5, 2:5, and 3.5:5 μg/ml did not modify enoxacin uptake, supporting the unsaturable nature of uptake.

Enoxacin uptake in resistant mutants. Mutation affecting the A subunit of DNA gyrase caused a 2- to 4-fold decrease in susceptibility to enoxacin but about a 40-to 80-fold decrease in susceptibility to nalidixic acid (Table 3). This mutation had no effect on cell uptake (Fig. 3b). However, mutation causing a loss of F porin did cause a significant decrease in uptake and a twofold decrease in enoxacin susceptibility (Table 3), but loss of the C porin did not (Table 3; Fig. 3c). The *E. coli* mutant JB-5R derived in our laboratory and selected by multistep passage in several

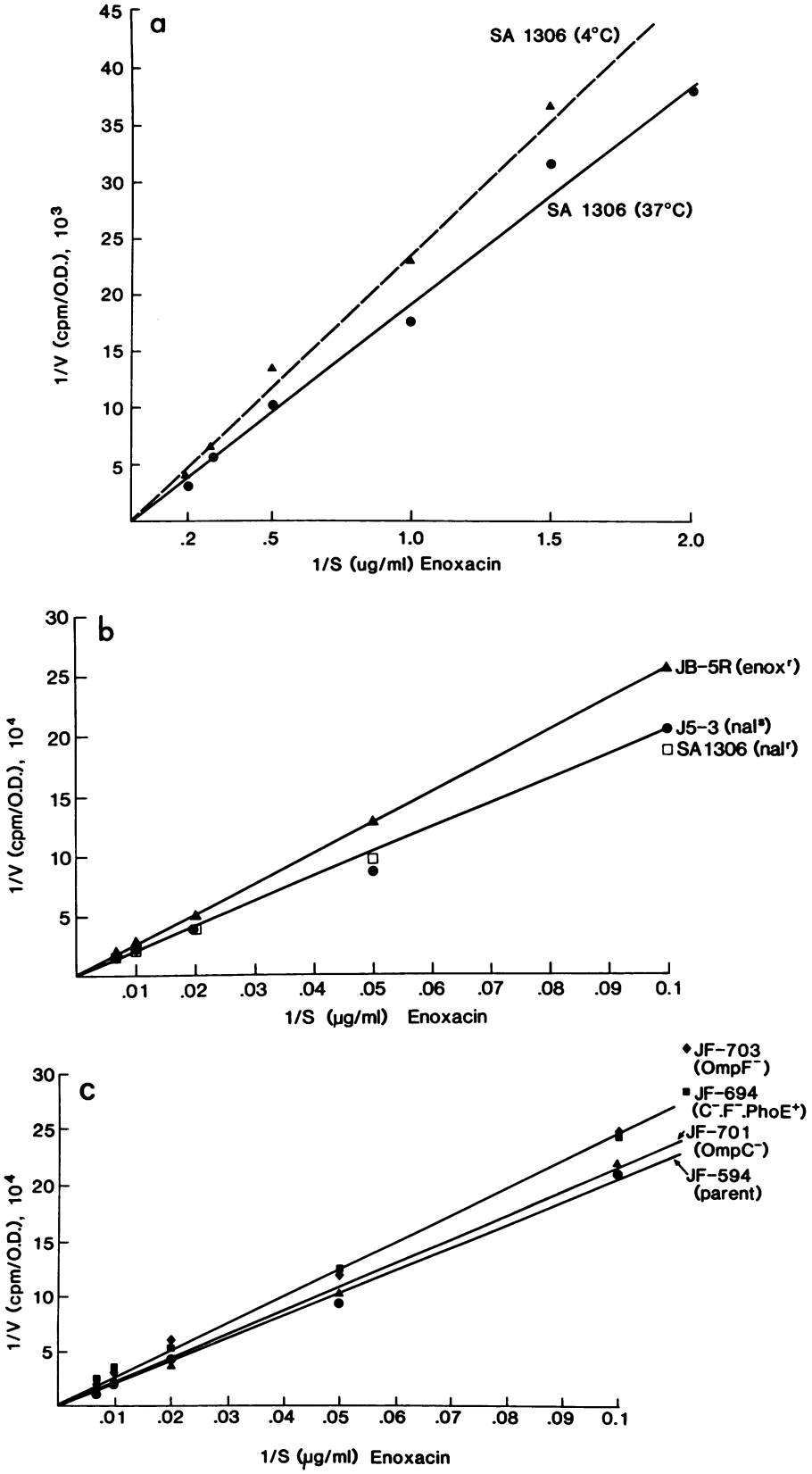


FIG. 3. Lineweaver-Burk plots for *E. coli* strains at (a) 4 and 37°C or (b, c) 37°C after 1-min enoxacin exposure over a concentration range of (a) 0.5 to 5.0 or (b, c) 10 to 150 μg/ml. (a) *E. coli* SA1306. Uptake was determined by centrifugation of a 1-ml sample for 3 min at 10,000 × *g* and 22°C, removal of all supernatant, and suspension into 300 μl of NB. The entire sample was counted in PCS solubilizer. (b) *E. coli* J5-3, JB-5R, and SA1306. The uptake method was the same as that for (a). (c) *E. coli* JF strains. Uptake was measured as noted in (a).

TABLE 2. Effect of cell inhibitors on enoxacin uptake[a]

Condition	Concn (M)	Enoxacin uptake (cpm/OD unit) at given time (min)			
		0	5	10	20
No inhibitor		114	97	109	111
NaF	1×10^{-2}	94	92	106	118
NaN_3	1×10^{-3}	124	87	101	125
$NaHAsO_4$	1×10^{-3}	99	124	125	134
2,4-DNP	2×10^{-3}	124	105	114	128
CCCP	2×10^{-5}	133	98	138	144

[a] The method used to measure the uptake was the standard centrifugation filtration protocol. The inhibitors were added to the culture cells 2 min before the addition of enoxacin at a final concentration of 20 μg/ml. Each time value is the average of two determinations. CCCP, Carbonyl cyanide *m*-chlorophenylhydrazone.

concentrations of enoxacin also showed a loss of F porin (Fig. 4) and a decrease in uptake (Fig. 3b) and susceptibility (Table 3).

Enoxacin uptake in *B. subtilis*. Enoxacin uptake was measured by the standard uptake protocol with centrifugation and filtration or bioassay uptake methods at a concentration range of 5 to 80 μg/ml (data not shown). The shapes of curves were highly similar to those seen with *E. coli*, with flat uptake at 20 μg/ml and lower and increasing uptake with time at very high concentrations (80 μg/ml) of enoxacin. The amount of uptake was about twice that of *E. coli* for *B. subtilis* at equivalent concentrations. Kinetic analysis demonstrated that the uptake pattern was again unsaturable. Thus, the nature of the uptake process in *B. subtilis* is highly similar or identical to that seen in *E. coli*.

DISCUSSION

Our investigations are consistent with the conclusion that enoxacin enters bacteria by means of simple diffusion. We examined a variety of uptake conditions which we feel allow us to conclude that the cell-associated enoxacin is a freely exchangeable pool which could include drug bound to the envelope as well as intracellularly. Our conclusions were the same with respect to simple diffusion whichever uptake procedure was utilized, although the quantity of drug bound varied among methods. Uptake was very rapid, with plateau levels being reached by the time the first sample could be taken.

Simple diffusion was supported by the lack of saturability, independence of energy, lack of competition by ciprofloxacin, and reduced uptake at 4°C. A similar conclusion has been reached for norfloxacin by Hooper et al. (D. C. Hooper, J. S. Wolfson, K. S. Souza, and M. N. Schwartz, 26th ICAAC, abstr. no. 944, 1986). However, Diver et al. (26th ICAAC) have found the characteristics of uptake for ciprofloxacin, pefloxacin, amifloxacin, and norfloxacin consistent with a lack of saturability but a reduction of uptake with DNP. They also examined enoxacin and found near agreement with our conclusions in that the effect of DNP on enoxacin uptake was minimal.

Our studies showed that a deficiency of F porin in an isogenic set of mutants and a laboratory-derived mutant caused reduced uptake and susceptibility for enoxacin. C-porin deficiency had no apparent effect. These findings are in agreement with results reported for norfloxacin by Hirai et al. (6) and Hooper et al. (7). Thus, it appears that the outer membrane of *E. coli* represents a modest permeability barrier to fluoroquinolone uptake in gram-negative bacteria.

TABLE 3. MICs of enoxacin and nalidixic acid for *E. coli* strains

Strain	MIC, μg/ml (fold increase)	
	Enoxacin	Nalidixic acid
E. coli J5-3 derivatives[a]		
J5-3	0.16–0.32	1.25–2.50
SA-1306	0.62–1.25 (2–4)	50–100 (40–80)
JB-5R	5.0–7.5 (16–24)	50–100 (40–80)
E. coli JF568 derivatives[b]		
JF-568 (parent)	0.15	ND[c]
JF-694 (OmpF$^-$ OmpC$^-$ PhoE$^+$)	0.32 (2)	ND
JF-701 (OmpC$^-$)	0.16	ND
JF-703 (OmpF$^-$)	0.31–0.62 (2–4)	ND

[a] Inoculum 5×10^6 to 1×10^7 CFU/ml.
[b] Inoculum, 10^6 CFU/ml.
[c] ND, Not determined.

Hirai et al. (6) have also shown that rough lipopolysaccharide does not markedly increase susceptibility to fluoroquinolones, unlike the case for nalidixic acid which is more active on rough lipopolysaccharide mutants than smooth isogenic strains. The effect of the F-porin deficiency and lack of effect of lipopolysaccharide roughness are consistent with the hydrophilic nature of most fluoroquinolones. The modest effect of F-porin deficiency suggests that these compounds can traverse the outer membrane by alternative uptake routes. In the case of *E. coli*, C porin does not seem to play a role based on our results and those of Hirai et al. (6). The alternative route is not clear at this time as neither hydrophobic (9) nor membrane disorganization (5) pathways would seem applicable to fluoroquinolones in view of their noncationic character and relative hydrophilicity.

The modest nature of the outer membrane permeability barrier is consistent with similar activities of fluoroquinolones on many gram-positive and gram-negative bacteria (11). We also have shown that the pattern and characteristics

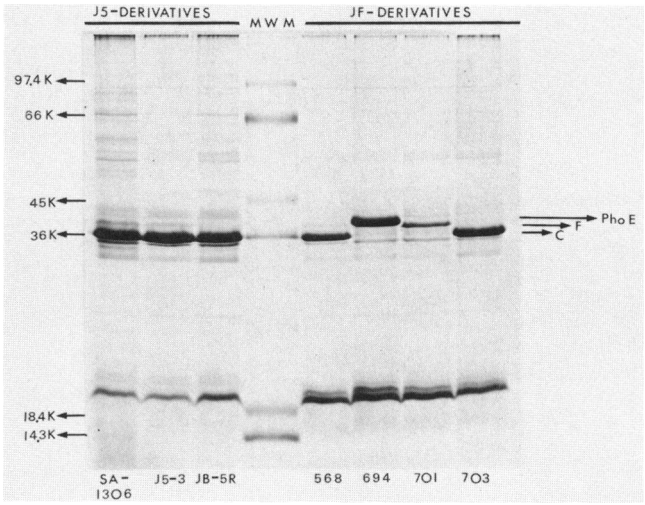


FIG. 4. Sodium dodecyl sulfate (12.5%)-polyacrylamide gel electrophoresis (SDS-PAGE) of outer membrane proteins for *E. coli* J5 (SA1306, J5-3, and JB-5R) and JF (568, 694, 701, and 703) strains. Arrows on the left-hand side indicate the position of molecular weight markers (MWM). Arrows on the right side indicate the positions of F, C, and Pho-E porins. A 200-μg portion of protein was loaded for each lane of gel.

of enoxacin uptake for a gram-positive bacillus, *B. subtilis*, are very similar to those of *E. coli* although the quantity taken up is greater for *B. subtilis*. We feel this further exemplifies the role of the outer membrane as well as the remarkable capability of this fluoroquinolone to rapidly and effectively transverse a range of bacterial envelopes.

Our studies have also shown that a DNA gyrase mutation does not significantly affect uptake. This could be because fluoroquinolones have an additional target(s) such as DNA (10). Alternatively, the binding to DNA gyrase may be very small relative to total cell binding, and our uptake procedures are unable to discriminate changes in gyrase binding.

At very high concentrations we repeatedly noted a time-dependent increase in uptake after 60 min or more. This was associated with some evidence of cell lysis in that the OD consistently declined by 10 to 20% in concert with the increased uptake. This finding is consistent with that reported by Dougherty and Saukkonen (3) with nalidixic acid. That study showed a modification of permeability on treatment with nalidixic acid and proposed that such could play a role in cell death. It is possible that a similar effect may occur with enoxacin.

Even though enoxacin enter cells rapidly, growth inhibition was significantly delayed in our studies. We believe this is explained by the fact that inhibition results from inhibition of DNA gyrase (2, 11), a process independent of drug uptake. Higher concentrations of enoxacin produce greater uptake as expected with diffusion but do not markedly decrease the time to growth inhibition (up to 80 μg/ml). Apparently, the targets critical to growth inhibition are saturated at low concentrations.

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