THE UNIVERSITY OF CALGARY

FACTORS DETERMINING BACTERIAL SUSCEPTIBILITY TO FLUOROQUINOLONES

BY

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ABSTRACT

Uptake mechanisms of two fluoroquinolones, enoxacin and ciprofloxacin, were investigated in bacterial cells. The drug accumulation was found to be a very rapid process, reaching an equilibrium within a minute to two and one-half minutes. We determined that cell-associated drug was reversible, non-saturable and occurred independently of cellular energy requirements. Moreover, no competition for uptake was observed with structural analogs; drug-cell permeation was found to be decreased at low temperature (4^{o}C) and with increased magnesium concentration. Lineweaver-Burk plots of uptake were consistent with uptake of fluoroquinolones by simple diffusion. Furthermore, using different washing regimens, we established that enoxacin was not firmly bound to its target(s). Studies with *E. coli* isogenic mutants provided evidence that the F porin, as opposed to the protein F in *Pseudomonas aeruginosa*, plays a major role contributing to the overall uptake process. Studies of ciprofloxacin uptake and SDS-induced cell lysis in the presence of various quinolones were performed on isogenic *P. aeruginosa* strains exhibiting rough and smooth lipopolysaccharide phenotypes. The conclusion from these studies was consistent with the LPS core region being a binding site for fluoroquinolones. This conclusion was also supported by a differential effect of washing on

ciprofloxacin accumulation in *P. aeruginosa* as compared to *E. coli* indicating the drug was more firmly bound at an extracytoplasmic site. As an initial step in the transmembrane diffusion process, ciprofloxacin and enoxacin were found to interact with liposomes in a pH-dependent fashion maximal at acidic and neutral pHs, suggesting that both ionic and hydrophobic forces are involved in the binding process. High quinolone concentrations showed an effect on liposome integrity which was greatest at the drug's maximimal hydrophobicity, since at these pHs greater loss of [^{14}C]ATP from liposomes was observed. The relative contribution of ciprofloxacin accumulation in intact cells and ability to inhibit DNA synthesis were investigated among *E. coli*, *P. aeruginosa*, and *Alcaligenes faecalis* in order to establish a correlation between bacterial cell susceptibility to ciprofloxacin and magnitude of uptake and cell-target sensitivity. We concluded that susceptibility to inhibition of DNA synthesis was the major determinant of ciprofloxacin susceptibility in *E. coli*, whereas for *A. faecalis*, low cell permeability was the limiting factor. In *P. aeruginosa*, a combination of low permeability and reduced susceptibility of DNA synthesis to inhibition were contributing factors to the lower activity of ciprofloxacin in this organism.

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TABLE OF CONTENTS

TABLE OF CONTENTS, Continued:

TABLE OF CONTENTS, Continued:

TABLE OF CONTENTS, Continued:

TABLE OF CONTENTS, Continued:

TABLE OF CONTENTS, Continued:

TABLE OF CONTENTS, Continued:

TABLE OF CONTENTS, Continued:

TABLE OF CONTENTS, Continued:

TABLE OF CONTENTS, Continued:

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES PAGE

LIST OF FIGURES PAGE

LIST OF FIGURES

LIST OF FIGURES PAGE

LIST OF FIGURES PAGE

LIST OF FIGURES

LIST OF FIGURES PAGE

LIST OF FIGURES PAGE

LIST OF ABBREVIATIONS

ATP	adenosine-5'-triphosphate
β	beta
BHIB	brain heart infusion broth
BSA	Bovine Serum Albumin
^{o}C	celcius
CCCP	carbonylcyanide m-chlorophenyl-hydrazone
CFU	colony forming unit
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
2,4-DNP	2,4-dinitrophenol
EDTA	ethylenediaminetetraacetic acid
h	hour
ID_{50}	drug concentration required to obtain 50% inhibition of DNA synthesis
Kd	kilodalton
L	liter
LB	Luria broth
LPS	lipopolysaccharide
mA	milliamperes
MBC	minimal bactericidal concentration
MIC	minimal inhibitory concentration
μg	microgram

LIST OF ABBREVIATIONS, Continued:

μM	micromolar
μm	micrometer
mM	millimolar
min	minute
N	normality
NaF	sodium fluoride
$NaHAsO_4$	sodium arsenate
NaN_3	sodium azide
NB	nutrient broth
OD_{600}	optical density at 600 nm
omp	outer membrane protein
PAGE	polyacrylamide gel electrophoresis
pmsf	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulfate
TSA	Tryptic Soy Agar
UV	ultraviolet
VB	Vogel-Bonner
VBGAM	Vogel-Bonner medium supplemented with 0.4% glucose, 0.01% arginine, and 1 mM methionine

LIST OF COMMERCIAL SUPPLIERS

- American Type Culture Collection (ATCC), Rockville, MD
- Amersham Corporation, Arlington Heights, IL
- BBL Microbiology Systems, Cockeysville, MD
- Beckman Instruments Canada Inc., Mississauga, Ontario
- Boehringer Mannheim, Dorval, Quebec
- Bio-Rad Laboratories, Richmond, CA
- Calbiochem, Behring Diagnostics, La Jolla, CA
- Difco Laboraties, Detroit, MI
- *Escherichia coli* Genetic Stock Center, New Haven, CT
- Fisher Scientific Co., Fairlawn, NJ
- Gelman Sciences, Ann Arbor, MI
- International Biotechnologies, Inc.
- J.T. Baker Chemical Co., Phillipsburg, NJ
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- Sigma Chemical Co., St. Louis, MO
- Terochem Laboratories, Edmonton, Alberta
- Whatman Inc., Clifton, NJ
- Whatman International Ltd., Maidstone, England

INTRODUCTION

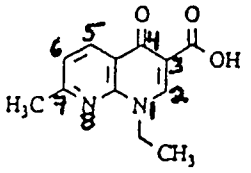
1.0 INTRODUCTION

1.1 Clinical Aspects

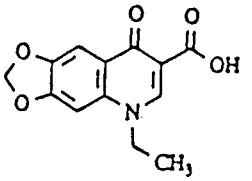
A new family of antimicrobial agents associated with high potential for clinical use has been introduced recently in the medical area. These compounds, fluoroquinolones, can be divided into two classes: naphthyridines and quinolones, which are derived from nalidixic acid and oxolinic acid precursors, respectively (Figure 1) (Chu and Fernandez, 1989). Chemical modifications of these two prototypes have led to new fluoroquinolone derivatives displaying an increased drug potency, broad spectrum of activity, relatively low toxicity and excellent pharmakokinetic properties (Hooper and Wolfson, 1985, Wolfson and Hooper, 1985).

Fluoroquinolones have been the object of intensive clinical studies, for example in a relatively recent trial, ciprofloxacin was administered to 2,018 patients suffering from various infectious diseases. Ciprofloxacin was found to be a drug of great therapeutic value in the treatment of osteomyelitis, gonorrhea, and infections of the urinary and gastrointestinal tracts, respiratory tree, and skin and soft tissues (Sanders, 1988). In that study, ciprofloxacin was found to be as safe as other conventional drugs used in the treatment of a particular type of infection. Since ciprofloxacin can be administered as an oral agent, lower costs are

FIG. 1. Chemical structure of quinolone and naphthyridine-derived antimicrobial agents (Shen *et al.*, 1989c).



NALIDIXIC ACID



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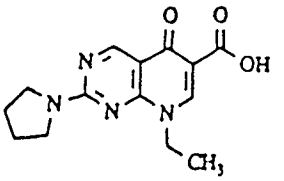
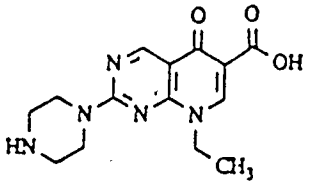


PIROMIDIC ACID



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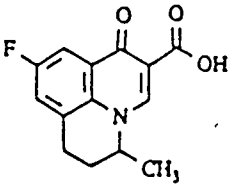

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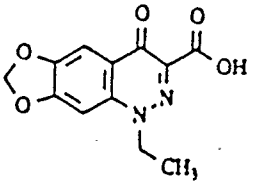
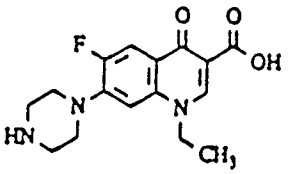
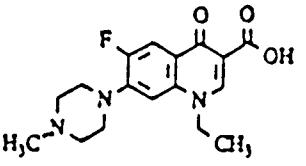


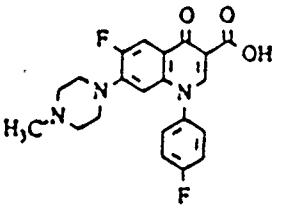
CINOXACIN



NORFLOXACIN



PEFLOXACIN



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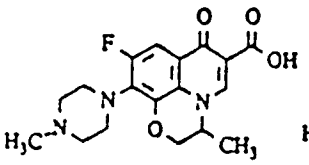
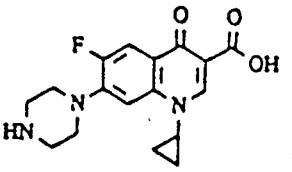
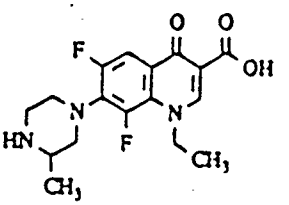


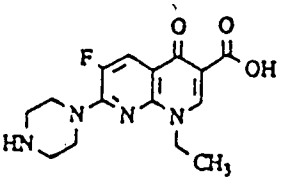
OFLOXACIN



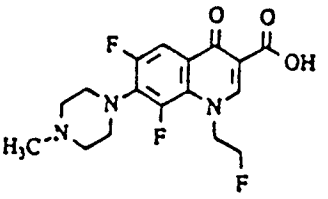
CIPROFLOXACIN



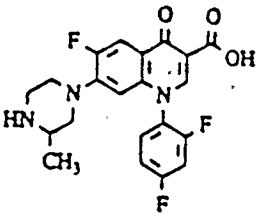
LOMEFLOXACIN



ENOXACIN



FLEROXACIN



TEMAFLOXACIN

associated with this antimicrobial agent (Sanders, 1988). Adverse reactions were found infrequently and were generally mild, most often occurring in the gastrointestinal tract. Some central nervous system symptoms have been also reported during drug therapy. Some studies have shown that fluoroquinolones are associated with some side effects on host defense mechanisms. Human lymphocyte function has been studied in the presence of 4-quinolones. For instance, increased ^{3}H-thymidine incorporation as a measurement of DNA synthesis was observed in phytohemagglutinin-stimulated lymphocytes pre-exposed to ciprofloxacin (Forsgren *et al.*, 1986). However, paradoxically, lymphocyte growth and progression through the cell cycle was inhibited as well as immunoglobulin secretion (Forsgren *et al.*, 1987). It has been shown that the phagocytic and bactericidal activities of human polymorphonuclear leucocytes were unaffected after exposure to ciprofloxacin (Forsgren and Bergkvist, 1985). Interestingly, a *Staphylococcus aureus* strain pre-exposed to ciprofloxacin for 60 minutes was found to be more susceptible to phagocytosis and killing by neutrophils (Forsgren and Bergkvist, 1985). As mentioned above, fluoroquinolones have been found to possess very good pharmacokinetic features characterized by rapid oral absorption, achievement of urine, serum and tissue concentrations well above the MIC for most gram-

negative and many positive organisms and relatively long half-lives in serum (Hooper and Wolfson, 1985; Hoiby, 1986).

1.2 Structure-Activity Relationships

Computer-automated structure-evaluation program analysis has revealed that the nature of the C-7 substituent was very important in determining cell permeability to the drug, an N-substituted piperazine leading to greater antimicrobial activity (Klopman *et al.*, 1987). The presence of a carboxylic acid and oxo groups at position 3 and 4 on the molecule is directly related to its antimicrobial activity. Moreover, a C-6 fluorine substitution resulted in an enhancement of antibacterial potency including gram-positive bacteria and obligate anaerobes (Crumplin, 1988). Fluoroquinolones are regarded as amphoteric molecules. For example, for norfloxacin the pK values for the carboxyl group and piperazine 4'-nitrogen are 6.2 to 6.4 and 8.7 to 8.9, respectively (Hooper *et al.*, 1989). They are relatively hydrophilic compounds compared to older quinolones such as nalidixic and oxolinic acid (Hirai *et al*, 1986a; Ashby *et al*, 1985). In fact, their hydrophobicity, given as the partition coefficient in n-octanol-0.1 M phosphate buffer (pH 7.2) is generally below the value of 1, varying from 0.007 for enoxacin to 0.33 for ofloxacin compared to a value of 2.23 and 3.92

for oxolinic and nalidixic acid, respectively. Biological activity as a result of effects of DNA gyrase activity is also greatly affected by the nature of the N-1 substitution, the optimal substituent to date being the cyclopropyl group since ciprofloxacin is the most potent fluoroquinolone against *Enterobacteriaceae* and *Pseudomonas aeruginosa* (Chu and Fernandez, 1989; Hooper and Wolfson, 1988).

1.3 Mode of Action

1.3.1 Mechanistic studies

1.3.1.1 Inhibition of DNA gyrase activity: DNA gyrase is postulated to be the primary drug target.

There is evidence from genetic and biochemical studies that DNA gyrase is the drug target (Hooper and Wolfson, 1988). This enzyme, a class II topoisomerase, is able to catalyze the conversion of relaxed DNA into a negatively supercoiled form through the breakage and rejoining of double-stranded DNA changing the DNA linking number in steps of two (Drlica, 1984; Gellert, 1981; Wang, 1985, 1987).

In *Escherichia coli*, DNA gyrase holoenzyme is a tetramer containing two protein subunits, A and B (A_2B_2) with molecular weights ranging from 100 - 105 kd and 90 - 95 kd, respectively (Gellert, 1981). These subunits are

encoded by the *gyr*A (48 min, chromosomal map) and *gyr*B genes (82 min), respectively (Bachmann, 1990). The A subunit is postulated to be involved in transient double-stranded breakage-reunion, whereas the B subunit is responsible for the ATPase activity of the enzyme (Cozzarelli, 1980). The enzyme acts by cutting the double-stranded DNA at four base pair staggered positions. The *E. coli gyr*A gene has been recently cloned and sequenced (Swanberg and Wang, 1987). Studies of the amino acid sequence derived from the nucleotide sequence showed about 50% homology compared with the *B. subtilis* GyrA protein (Swanberg and Wang, 1987). Moreover, the *E. coli* gyrase A active site tyrosine has been shown to possess sequence similarities with other proteins associated with topoisomerase activities suggesting that the active site is a conserved region (Swanberg and Wang, 1987). Topoisomerase sequence similarities also strongly suggest that several domains in the prokaryotic class II enzymes have their counterparts in the yeast DNA topoisomerase II enzyme (Wang, 1987; Lynn, 1986).

Recently, studies on topoisomerase-catalyzed reactions have demonstrated that tyrosine at position 122 of the A subunit of *E. coli* DNA gyrase is the amino acid involved in the formation of an O^4-phosphotyrosine linkage with the 5'phosphoryl group of the DNA molecule

leading to a covalent protein-DNA linkage (Horowitz and Wang, 1987). The A subunit was previously found to be the target of the older quinolones, nalidixic acid and oxolinic acid, whereas novobiocin and coumermycin A were found to inhibit the B subunit activity by competitive inhibition at the ATP binding site (Cozzarelli, 1980).

Fluoroquinolones act by blocking the DNA rejoining step mediated by the enzyme, which results in the formation of a stable DNA-DNA gyrase complex. Recently, a gyrase-mediated DNA strand breakage-reunion assay has been improved allowing a study of the strand religation event separate from the cleavage reaction by using calcium instead of magnesium in the reaction mixture. Quinolones were found to significantly alter the rate of the gyrase-mediated religation event consistent with stabilization of the enzyme-DNA intermediate (Sutcliffe, 1990). This assay is very interesting and will certainly be useful in comparing the rate of ligation of wild-type and resistant DNA gyrases, and in establishing the results obtained with specific mutations.

1.3.1.2 Inhibition of DNA gyrase activity: DNA is postulated to be the primary drug target.

Recent studies have demonstrated that the primary target of fluoroquinolone antimicrobial agents is the DNA molecule itself (Tornaletti and Pedrini, 1988; Shen and

Pernet, 1985). Evidence of DNA unwinding activity associated with 4-quinolones has been provided by Tornaletti and Pedrini (1988). In their experiments, different ratios of supercoiled DNA and norfloxacin molecules were mixed together, then DNA topoisomerase I was added to allow complete DNA relaxation. Interestingly, a new distribution of topoisomers on chloroquine-agarose gel was observed with DNA previously treated with norfloxacin compared to the DNA control. The average linking numbers of the topoisomer population were compared and the amount of drug bound to the DNA was estimated. Thus, each norfloxacin molecule bound to DNA would unwind the double helix by about 7.2^{o}. DNA unwinding was observed only in the presence of magnesium and varied among different DNA molecules suggesting a sequence preference for norfloxacin binding to DNA. Moreover, DNA unwinding was not found to be mediated through an intercalative process. Binding studies of ^{3}H-labelled quinolone norfloxacin with DNA gyrase and various DNA molecules have been performed in order to bring some clarification regarding the molecular mechanism of quinolone inhibition of DNA gyrase (Shen and Pernet, 1985; Shen *et al.*, 1989a, b). They provided evidence supporting the hypothesis that (i) quinolones do not bind to DNA gyrase at their inhibitory concentration; (ii) quinolones bind poorly to relaxed double-stranded

DNA; (iii) quinolones bind preferentially to single-stranded DNA, but in a nonspecific and noncooperative manner; (iv) quinolones bind specifically to a saturable site on supercoiled DNA in a highly cooperative manner; (v) DNA gyrase enhances drug binding to relaxed forms of DNA. A cooperative drug-DNA binding model has been proposed from these studies (Shen *et al.*, 1989c).

The model proposes (i) from crystallography studies of a quinolone, nalidixic acid, that strong intermolecular drug-drug interactions do occur via stacking of the naphthyridine rings and tail-to-tail hydrophobic interactions between the ethyl groups located at the N1 position. This phenomenon would explain the cooperativity and high binding affinity observed with DNA molecule; (ii) the DNA binding site of the drug molecule which is induced by the bound DNA gyrase in an ATP-dependent process is located in a single-stranded DNA pocket, more precisely at the protruding four bases of cleaved DNA strands.

Binding of the drug molecules would basically lead the enzyme-DNA complex locked permanently at the intermediate gate-opening step of the DNA supercoiling process (Shen *et al.*, 1989c).

1.3.2 Biological features associated with fluoroquinolone antibacterial and bactericidal activities.

1.3.2.1 SOS response induction.

The exact mechanisms of cell killing by the type II topoisomerase-targeting drugs remains unclear. In bacteria, DNA gyrase has been shown to be involved in the regulation of DNA supercoiling level, DNA replication, transcription and repair (Drlica, 1984; Gellert, 1981; Wang, 1985). Therefore, inhibition of the gyrase enzymatic activity would alter these biological processes. It has been suggested that cell-killing is mediated through the DNA SOS repair response induced by the quinolones. The SOS response is a cascade of biological events induced by DNA damage and characterized by an increased cell mutability, enhanced resistance to killing effects of genotoxic agents, prophage induction and cellular filamentation due to inhibition of gene product activities involved in septum formation (Van Houten, 1990). The initial event leading to quinolone-induced cell death by triggering the SOS-related DNA repair process has been postulated to be the early inhibition of DNA synthesis *in vivo* (Chow *et al.*, 1988) which is followed by a recovery of DNA synthesis dependent on the presence of wild-type *rec*A and *lex*A genes (Engle *et al*, 1982). A correlation was established between the drug-induced DNA damage phenomenon and the drug potencies (MIC, MBC) (Chow *et al.*, 1988). A dose-response curve in *E. coli* has been observed between the

extent of early inhibition of DNA synthesis induced by a quinolone and the drug concentration (Snyder and Drlica, 1979; Chow *et al.*, 1988) and in *P. aeruginosa* (Bedard *et al.*, 1989b). This quinolone-induced inhibition of DNA synthesis was previously shown to be a supercoiling-independent, gyrase-mediated phenomenon, occurring at drug concentrations below the level needed to inhibit DNA supercoiling *in vivo* and was found not to be prevented by chloramphenicol and rifampin (Drlica *et al.*, 1980). The correlation established by Chow *et al.* is an important finding since it would explain, in part, previous data showing discrepancies between quinolone potency and their ability to inhibit DNA gyrase supercoiling activity *in vitro* of *Micrococcus luteus* (Zweerink and Edison, 1986), of *E. coli* (Sato *et al.*, 1986), of *Citrobacter freundii* (Aoyama *et al.*, 1988b), and *P. aeruginosa* (Inoue *et al.*, 1988). However, other studies have demonstrated reasonable correlation between fluoroquinolone MICs and the concentration needed to inhibit DNA gyrase activity (Hooper *et al.*, 1987; Takahata and Nishino, 1988). Interestingly, bactericidal activity of nalidixic acid (Deitz, 1986) and ciprofloxacin (Smith, 1984) have been shown to be reduced at high drug concentrations which inhibit not only DNA synthesis but RNA and protein synthesis, suggesting that active protein synthesis is a requirement for lethal drug activity.

Prolonged inhibition of the septum formation event would lead to cell lysis due perhaps to drastic changes in bacterial membrane permeability. In fact, it has been found that a significant leakage of intracellular material occurred upon treatment of *E. coli* cells with nalidixic acid (Dougherty and Saukkonen, 1985). However, in a similar study with ciprofloxacin, no release of cytoplasmic contents was observed after two hours exposure of *E. coli* cells at 1 μg/ml drug concentration (Diver and Wise, 1986). On the other hand, ciprofloxacin has been shown to have an influence on cell membrane composition since *E. coli* cells showed a reduction in the lipid content of cell envelope following exposure to this antimicrobial agent (Suerbaum *et al.*, 1987). Cohen and McConnell (1986) also suggested that ciprofloxacin might have a direct effect on the cell envelope since a significant increase of endotoxin released from *E. coli* cells was observed after thirty-minute cell exposure to ciprofloxacin.

There is also evidence of an alteration of bacterial DNA structure associated with the presence of fluroquinolone within the cell. In fact, a change in the average DNA mobility was observed with SDS-treated lysates from cells previously incubated with enoxacin (Courtright *et al.*, 1988).

DNA gyrase inhibitors might lead to bacterial cell death by selectively interfering with the expression of genes (Dalhoff and Doring, 1987). It is already known that 4-quinolones selectively interfere with the transcription of some genes, for instance, *P. aeruginosa* isolates exposed to a subinhibitory concentration of ciprofloxacin exhibited a significant reduction of elastase and exotoxin A production (Dalhoff and Doring, 1987; Grimwood *et al.*, 1989). On the other hand, regarding the expression and function of P fimbriae in uropathogenic *E. coli*, no effect was detected following cell exposure to fluoroquinolones (Kovarik *et al.*, 1989). The fact that specific genes are stimulated or inhibited by quinolones has been suggested as a mechanism of drug-induced cell death since deregulation of normal growth cycle was observed (Benbrook and Miller, 1986). This observed phenomenon might result from a decreased supercoiling density of chromosomal DNA topological domains due to inhibition of gyrase activity.

Alteration of DNA structure in the presence of 4-quinolone would induce the cell SOS response, most probably in a permanent fashion, because of the inability of DNA repair mechanisms to overcome the damages induced by quinolones. Upon removal of quinolones, the SOS repair activity would readjust to the normal basic level of its activity (Van Houten, 1990). Another possibility

resides in the induction of the SOS repair system in a more specific manner by fluoroquinolones. Indeed, repetitive extragenic palindromic DNA sequences have been found at the 3' region of the SOS repair gene, *uvr*B. These DNA elements were postulated to be involved in regulation of intraoperonic expression; furthermore, when present in the 3' region of the transcription start site, the mRNA transcribed showed higher stability (Van Houten, 1990). Interestingly, these DNA sequences were shown to be a binding site of the DNA gyrase (Yang and Ames, 1988), which also suggests that DNA supercoiling level might be very important for the expression of such genes.

1.3.2.2 Bactericidal mechanisms.

It seems that fluoroquinolones possess several mechanisms of action. Bactericidal activity of these antimicrobial agents has been extensively studied among bacterial species. One of their remarkable features resides in the fact that they kill bacteria rapidly. Generally, after a one to two hour drug exposure at one to four times the MIC, a thousand-fold decrease in cell viability can be observed (Wolfson and Hooper, 1985). Three bactericidal mechanisms, A, B, and C have been described for 4-quinolones. The A and C mechanisms have been found to be abolished by inhibitors of RNA and protein synthesis (rifampicin and chloramphenicol, respectively). Therefore, active RNA and protein

synthesis are prerequisites for the bactericidal action. It was also established that mechanisms B and C, unlike A, were effective against non-dividing bacteria (Crumplin *et al.*, 1984). Bactericidal activities of norfloxacin, ciprofloxacin and ofloxacin have been studied in *E. coli* for the former and *Staphylococcus aureus* and *E. coli* for the two latter drugs. It has been suggested that the lethal activity of norfloxacin in *E. coli* is an active process since competent RNA and protein synthesis was a requirement. Norfloxacin was found to be associated with a selective activity since, at a concentration of 1 μg/ml, dramatic inhibition of DNA replication was achieved without inhibition of RNA and protein synthesis (Crumplin *et al.*, 1984). For ciprofloxacin, mechanism B was found to be absent in *Staphylococcus aureus*, while both A and B bactericidal mechanisms were effective in *E. coli*. Ofloxacin was found to possess A and B mechanisms in both *Staphylococci* and *E. coli* (Lewin and Smith, 1988). The authors suggested that the observed phenomenon would explain the drug potency difference observed for the strains. Interestingly, the cell lytic activity of DNA topoisomerase poisons seem to be mediated through an energy-dependent process involving the proton motive force (Liu, 1989). An energy inhibitor such as dinitrophenol has been shown to prevent death of L1210 cells treated with eukaryotic topoisomerase II poisons

(Liu, 1989). The same effect was observed in *E. coli* with carbonylcyanide m-chlorophenylhydrazone on a fluoroquinolone ciprofloxacin (Bedard *et al.*, 1989b).

1.3.3 Similarities in the mode of action of eukaryotic topoisomerse II poisons and DNA gyrase inhibitors.

It is interesting to note that the mechanisms of action of quinolones on DNA gyrase and antitumor drugs such as epipodophyllotoxins on eukaryotic DNA topoisomerase II, which are not DNA intercalators, have striking similarities. A similar mode of interaction with the type II DNA topoisomerase-DNA complexes has been suggested for both quinolones and epipodophyllotoxins (Nelson *et al.*, 1989). In fact, a recent study has demonstrated a similar pattern of DNA cleavage produced by ciprofloxacin and etoposide (an epipodophyllotoxin) for an archaebacterial plasmid called pGRB-1 from a *Halobacterium* GRB recipient strain (Sioud and Forterre, 1989). It is well known that high concentrations of quinolone derivatives can interfere with the action of the eukaryotic enzymes, and the sensitivity of eukaryotic type II topoisomerase to high concentrations of nalidixic acid might explain the side effects associated with this compound (Rose, 1988).

1.4 Mechanisms of Quinolone Permeation in Bacterial Cells

1.4.1 Quinolone-cell permeation in *in vitro*-derived bacterial strains and clinical isolates.

A correlation between antibacterial activity and the hydrophobicity of quinolones, measured as the partition coefficient in n-octanol - 0.1 M phosphate buffer (pH 7.2), and the integrity of the lipopolysaccharides of the outer membrane was first established by Hirai *et al.*, 1986a. These investigators demonstrated that LPS-deficient *E. coli* and *Salmonella* mutants were more susceptible to relatively hydrophobic quinolones such as nalidixic acid. Mutants lacking OmpF were less susceptible to all quinolone tested, suggesting a very effective penetration of drug molecules through OmpF porin pores. The observations also suggested that passage of quinolones through the outer membrane was not restricted to porins, but also involved a hydrophobic route through phospholipid bilayers. These data were also supported by a further study of fleroxacin uptake by *E. coli* (Chapman and Georgopapadakou, 1988). Cells exposed to fleroxacin showed an increased cell-surface hydrophobicity due to a release of lipopolysaccharides. They also exhibited an increased susceptibility to lysis by detergents and a higher outer membrane permeability to

β-lactam antibiotics. All these phenomena were found to be antagonized by the presence of magnesium. Chelation activity via adjacent carbonyls at C-2 and C-3 position on the quinolone nucleus was attributed to quinolones, since presence of divalent cations such as magnesium and calcium increased their fluorescence. Moreover, uptake of fleroxacin was found to be reduced and its MIC increased in the presence of magnesium, the latter also being supported by a previous study with ciprofloxacin and enoxacin (Blaser *et al.*, 1986). Fleroxacin accumulation in *E. coli* was found to be nonsaturable up to 100 μl/ml, energy independent, characterized by a rapid cell-associated drug which was postulated to result in part from the chelation ability of quinolones allowing their interaction with magnesium cross-linking adjacent lipopolysaccharide molecules at the outer membrane surface. This initial uptake process was followed by a slight decrease of the amount of drug associated with *E. coli* cells after 15 - 20 min and then followed by a linear increase in cell-associated fleroxacin (Chapman and Georgopapadakou, 1988. From these studies the authors suggested a model derived from the "self-promoted uptake" proposed for polycationic antibiotics (Hancock, 1984). Basically, data from fleroxacin accumulation were consistent with simple diffusion in *E. coli* as shown by results of studies performed for this thesis (Bedard *et*

al., 1987, 1989b). Permeation occurs through the outer membrane via both porin and nonporin pathways and through the inner membrane via the hydrophobic route. The nonporin pathway would consist in a progressive removal of the magnesium cross-bridging the LPS of the outer membrane, resulting from quinolone-magnesium complex formation. This phenomenon is different from the self-promoted uptake for polycationic antibiotics (Hancock *et al.*, 1984) in which magnesium is displaced from the LPS rather than being chelated. Eventually, hydrophobic patches would be exposed at the cell surface and therefore be available for drug partition through the lipid bilayer. The relative contribution of the porin and nonporin pathways into the overall uptake was suggested to be influenced by the hydrophobicity of the quinolone.

Increasing extracellular magnesium concentration (1 to 14 mM) as well as decreasing pH (7.7 to 6.0) was also found to cause a significant decrease in the antibacterial potency of norfloxacin as well as its accumulation in *E. coli* cells (Hooper *et al.*, 1989). Uptake was characteristically rapid, reaching a plateau within 5 min after drug addition. Also of interest, norfloxacin appeared to be concentrated within intact bacterial cells with a level of 4.5-fold greater than the concentration found extracellularly. A mechanism of cell

trapping of quinolones was suggested. Upon cell treatment with CCCP, greater accumulation of norfloxacin was observed, consistent with previously published data from Cohen *et al.*, 1988a, which provided evidence for an endogenous, saturable, energy-dependent efflux system located at the bacterial inner membrane. In this latter study, the authors, using everted membrane vesicles, demonstrated a carrier-mediated active efflux system for norfloxacin generated by a proton motive force. Competitive inhibition with hydrophilic quinolones, such as ciprofloxacin, enoxacin and ofloxacin was observed, whereas nalidixic acid and oxolonic acid analogs had no effect.

Data already published from this thesis which were published, preceded or confirmed several of the above studies (Bedard *et al.*, 1987, 1989a,b. More recently, uptake of ciprofloxacin, enoxacin and pefloxacin has been investigated in *E. coli* (Diver *et al.*, 1990). The conclusions were quite different with regard to the drug accumulation characteristics, the results being consistent with a nonsaturable active uptake process generated by a proton motive force.

Very few studies have shown fluoroquinolone accumulation profiles in *P. aeruginosa*. In a study of ciprofloxacin uptake measured by a bioassay method, the amount of drug associated with cells was shown to reach a

steady state during the first minute of drug exposure with no saturability over a range of 5 to 80 μg/ml (Celesk and Robillard, 1989). This rapid phenomenon suggested that a diffusion process is also involved in *P. aeruginosa*. Ciprofloxacin accumulation was also shown to be increased in the presence of the energy inhibitor CCCP suggesting a drug efflux mechanism (Celesk and Robillard, 1989). Studies published by Bedard *et al.*, 1989b preceded, confirmed and extended these findings in *P. aeruginosa*.

Thus, one can conclude that the uptake of fluoroquinolones in bacterial cells is complex since several pathways seem to be involved in permation.

1.4.2 Mechanisms of quinolone permeation in human polymorphonuclear leukocytes.

Ciprofloxacin uptake as well as norfloxacin and ofloxacin have been investigated in human polymorphonuclear leukocytes using three different methods: bioassay, fluorometric and radiolabelling assays, respectively (Zweerink and Edison, 1988; Easmon and Crane, 1985; Pascual *et al.*, 1989). Similar results were obtained by all three methods; drugs were found to be taken up very rapidly by the cells, concentrated intracellularly, and were not firmly bound within the cell in the case of ciprofloxacin and norfloxacin.

However, results from cell viability and energy inhibitor effects on drug accumulation showed differences among fluoroquinolone agents investigated above. No conclusion regarding the nature of the drug uptake process was proposed by Zweerink and Edison, whereas Easmon and Crane proposed a simple diffusion system for ciprofloxacin, and an energy-dependent uptake via an amino acid transport system was proposed for ofloxacin by Pascual *et al.*

1.5 Mechanism of bacterial cell resistance to fluoroquinolones

1.5.1 Characteristic features of drug resistance.

Enzymatic inactivation of fluoroquinolones encoded by a plasmid, transposon or chromosomal-borne gene has not been described in bacteria. Generally, bacterial resistance to quinolones seems to be transmitted predominantly on chromosome (Burman, 1977). Only one case of transfer of quinolone resistance mediated through a plasmid has been reported for nalidixic acid in *Shigella dysenteriae* (Munshi *et al.*, 1987). However, no confirmation of this finding has been forthcoming.

Recently however, the authenticity of the transconjugant bacterial strain obtained with regard to the nalidixic acid resistance phenotype mediated

presumably through a plasmid has been object of a controversy (Courvalin, 1990). Instead, *E. coli* strains harboring diverse plasmids such as F' *lac* have been found to be cured following quinolone exposure at one-half to one-quarter the MIC (Weisser and Wiedeman, 1985). However, in that study plasmid elimination was found to be selective. Resistance to fluoroquinolone mediated through mutations affecting the DNA gyrase activity or decreased drug accumulation has been extensively described for *in vitro*-derived bacterial strains as well as clinical isolates. In general, these mutants were characterized (i) using *in vitro* biochemical assays to evaluate the activity of purified DNA gyrase by measuring the extent of DNA supercoiling inhibition induced by fluoroquinolone or as an indicator of DNA gyrase activity, by measuring the inhibition of replicative DNA biosynthesis *in vivo*; (ii) by genetic analysis: gene mapping (interrupted matings, co-transduction with selected markers), gene cloning, DNA sequencing, and complementation studies. As a measurement of cell permeability alterations to an antimicrobial agents, SDS-PAGE profile of outer membrane proteins and lipopolysaccharide patterns were used as tools for indication of cell membrane component modifications, but a definitive answer was obtained by performing drug uptake assays.

1.5.2 Mechanism of resistance in *E. coli* and in *S. aureus*.

Laboratory-derived *E. coli* strains, mutations conferring nalidixic acid resistance through the *gyr*A (Yoshida *et al.*, 1988) and *gyr*B genes (Yamagishi *et al.*, 1986) have been reported and the type of mutation characterized at the molecular level. Interestingly, regarding the *gyr*B mutants, the site of mutations confering low-level nalidixic acid resistance were found to be located in the carboxy-terminal end of the gyrase B protein, a part of the *gyr*B gene coding for the postulated domain responsible for binding to the gyrase A subunit (Yamigishi *et al.*, 1986). This finding also was consistent with the slight effect obtained on the sensitivity to novobiocin with these *gyr*B mutants. It is well known that novobiocin exerts its antibacterial activity by binding to the ATP-binding site domain of the gyrase B subunit. In agreement with this fact, the mutation sites were not found to be located within this domain. Mutations of *gyr*A were found to be located close to one another and situated in the vicinity of the active site (tyrosine at amino acid 122) of the gyrase enzyme (Yoshida *et al.*, 1988). Genetic analysis of spontaneous norfloxacin-resistant mutants of *E. coli* by Hooper *et al.* have identified two types of mutations conferring drug resistance. One called *nfx*A was found to map around 48

min on the *E. coli* genetic map and be an allele of the *gyr*A gene. Cross-resistance with unrelated antibiotics such as cefoxitin, chloramphenicol and tetracycline was observed with the second mutation, *nfx*B, which mapped at about 19 min, therefore being genetically distinct from *omp*F (21 min). However, this mutant was associated with a decreased OmpF production (Hooper *et al.*, 1986). Another locus conferring ciprofloxacin resistance, *cfx*B, has also been characterized by the same group of researchers (Hooper *et al.*, 1987). The *cfx*B mutation was shown to be very closely linked to *mar*A locus (34 min) known to confer multiple antibiotic resistance phenotype (George and Levy, 1983a, b). Studies have revealed that the *mar*A locus was associated with a decreased amount of OmpF expressed resulting from an enhancement of *mic*F expression (Cohen *et al.*, 1988b). Mar mutants of *E. coli* were also found to manifest cross-resistance to fluoroquinolones linked to decreased cell permeability for which the OmpF reduction accounted for only a part (Cohen *et al.*, 1989). Interestingly, as noticed for *nfx*B, *cfx*B was associated with a decreased OmpF production and pleiotropic resistance to quinolones, chloramphenicol and tetracycline. Moreover, both *cfx*B and *nfx*B mutation were found to downregulate the *omp*F expression at the posttranscriptional level (Hooper *et al.*, 1989). Mechanisms of *E. coli* resistance to

norfloxacin have also been extensively studied by Hirai *et al.*, 1986b. The spontaneous mutants which exhibited low-level resistance to norfloxacin were classified into three phenotypes: *nor*A, which consisted of *gyr*A mutants (48 min); *nor*B (34 min) and *nor*C (8 min) which were associated with reduced OmpF proteins, and in addition for the latter, an alteration in the lipopolysaccharide structure. It was also demonstrated in this study that *nor*C mutant was hypersusceptible to nalidixic acid and rosoxacin, suggesting that lipopolysaccharides might form a hydrophilic permeability barrier to hydrophobic quinolones (Hirai *et al.*, 1986a, 1986b). It is interesting to notice that *nor*B mapped also at the *mar*A locus. Spontaneous fleroxacin-resistant mutants of *E. coli* K12 have also been isolated (Chapman *et al.*, 1989). None of these bacteria were found to have changes solely in porin proteins, and all strains were also found to be associated with decreased susceptibility to inhibition of replicative DNA biosynthesis by fluoroquinolones. It was suggested that reduced outer membrane permeability via porins was not sufficient to produce resistance to fleroxacin (Chapman *et al.*, 1989) consistent with the proposed model suggesting that passage of hydrophobic quinolones through the outer membrane is not limited to porins, but might involve a self-promoted uptake and/or a hydrophobic route (Chapman and Georgopapadakou, 1988).

Several studies have described in detail the mechanisms of fluoroquinolone resistance in *E. coli* clinical isolates which were also found to be associated with altered drug target and reduction in drug uptake (Aoyama *et al.*, 1987; Sato *et al.*, 1986; Cullen *et al.*, 1989; Fisher *et al.*, 1989; Nakamura *et al.*, 1989). The same phenomena have also been reported for clinical isolates of *Citrobacter freundii* resistant to quinolones (Aoyama, 1988a; 1988b). Interestingly, a uropathogenic *E. coli* strain isolated during enoxacin therapy exhibited mutations at three different positions on the gyrase A gene compared with the wild-type *E. coli* K-12 (Cullen *et al.*, 1989). These investigators constructed chimeric *gyr*A genes in an *E. coli* KNK453 *gyr*A (ts) recipient strain. Genetic studies demonstrated that only one mutation, Ser 83 with Trp substitution was solely responsible for resistance. In this study, quinolone-resistant *gyr*A genes on a plasmid were found to be dominant over the susceptible *gyr*A 43 (ts) allele. Though the more common observation is that the susceptible allele is dominant. Two rapid and simple screening methods have been recently developed in order to detect and evaluate the frequency of *gyr*A mutations among *E. coli* clinical isolates. One of these methods involves transformation of quinolone-resistant *E. coli* with a plasmid carrying the wild-type *gyr*A gene. Since *gyr*A (wt) is postulated

to be dominant over the quinolone-resistant *gyr*A allele (Hane and Wood, 1969; Yamagishi *et al.*, 1986; Yoshida *et al.*, 1988), a significant increase in quinolone susceptibility of the transformed cell compared to the nontransformants was observed (Nakamura *et al.*, 1989). The other method was based on restriction fragment length polymorphism, since mutations at codon 83 in the *gyr*A gene were found not only to be frequently associated with resistance to 4-quinolone, but also to cause removal of a Hinf 1 site overlapping the codons 82 and 83 of the gyrase A gene (Fisher *et al.*, 1989).

Very few reports have focussed on the mechanism of resistance to fluoroquinolones among gram-positive bacterial species. Hybridization studies of a mutation (*nor*A) on the *Staphylococcus aureus* chromosome (Ubukata *et al.*, 1989) suggested that the cloned *nor*A gene is an allele of one of the gyrase genes, but gene expression data did not show the gyrase wild-type gene to be dominant over the quinolone-resistant gyrase alleles. Recently, Hopewell *et al.* (1990) have isolated the *gyr*A and *gyr*B from a *Staphylococcus aureus* clinical isolate susceptible to methicillin and ciprofloxacin. Interestingly, these two genes were found to map contiguously as previously observed in *Bacillus subtilis* (Lampe and Bott, 1985). Homology studies demonstrated that amino acid residues whose mutation in *E. coli*

confers resistance to quinolones were also conserved in *S. aureus*. The authors suggested that quinolone resistance in *E. coli* and *S. aureus* mediated through DNA gyrase might follow similar mutational pathways.

1.5.3 Mechanism of resistance in *P. aeruginosa*.

A substantial amount of information has been provided regarding the resistance mechanisms to norfloxacin (Hirai *et al.*, 1987) and to ciprofloxacin (Robillard and Scarpa, 1988) in *in vitro* derived mutants of *P. aeruginosa*. With respect to spontaneous norfloxacin-resistant mutants of PAO, mutations *nfx*A and *nfx*B were identified. Cotransductional analysis showed the former to be a *nal*A allele (39 min), whereas the *nfx*B mutation was associated with hypersusceptibility to beta-lactam and aminoglycoside antibiotics with decreased outer membrane permeability to norfloxacin. Studies of outer membrane protein profiles on SDS-PAGE showed a new uncharacterized 54 Kd protein associated with the *nfx*B mutation. In the case of spontaneous ciprofloxacin-resistant mutants of PAO, a mutation *cfx*A was also found to reside at the *nal*A locus (39 min) which codes for the DNA gyrase A subunit, whereas another type of mutation called *cfx*B was mapped within the *nal*B gene (20 min) on the genetic map. Interestingly, as noticed previously with *nal*B mutations (Rella and Haas, 1982; Hane and Wood, 1969), *cfx*B mutation appeared to alter cell permeability

(Robillard and Scarpa, 1988). This type of mutation conferring pleiotropic drug resistance was further investigated in more detail with respect to the accumulation profile of ciprofloxacin. The presence of the energy inhibitor CCCP was found to promote up to four-fold greater ciprofloxacin accumulation in the wild-type parent compared to *cfx*B mutants; however, with no addition of energy inhibitor, similar amounts of drug were associated with all strains (Celesk and Robilland, 1989). Legakis *et al.* (1989) has also reported an increased 54 Kd outer membrane protein among spontaneous ciprofloxacin-resistant *P. aeruginosa*, although several outer membrane protein changes and defects in the LPS ladder pattern (in some cases) were also observed. This suggested that expression of ciprofloxacin resistance might involve a modification of the LPS structure. A broad host-range *E. coli* gyrase A gene probe has been recently constructed in order to be used as a tool to discriminate between quinolone-resistant *gyr*A mutations and permeability alterations in *P. aeruginosa* (Robillard, 1990). The *E. coli* gyrase A protein was found to be functional in *P. aeruginosa* and able to complement the *P. aeruginosa* gyrase with regard to the quinolone susceptibility.

Another study has suggested that changes of a particular outer membrane protein with a molecular weight of 30 - 31 Kd correlated with specific quinolone resistance since no pleiotropic effects were observed with other antibiotics (Daikos *et al.*, 1988). It should be mentioned that the change of the outer membrane protein cited above was described for a *P. aeruginosa* ciprofloxacin-resistant strain isolated during therapy. However, studies of *in vitro*-isolated quinolone-resistant mutants after Tn*5*-insertional mutagenesis have also demonstrated that alterations of cell permeability to fluoroquinolones could be associated with cross-resistance to β-lactams, tetracycline and chloramphenicol. This observed phenomenon correlated with a reduction of a 25.5 Kd outer membrane protein named protein G and a 40-Kd protein, shown by a monoclonal antibody to be antigenically related (Chamberland *et al.*, 1989).

Acquisition of resistance to fluoroquinolones *in vivo* for *P. aeruginosa* has also been analyzed by characterization of clinical isolates obtained during ciprofloxacin therapy (Masecar *et al.*, 1990) and isolates selected during pefloxacin therapy of experimental endocarditis (Chamberland *et al.*, 1989). In both cases, resistance was attributed primarily to mutation through

the *gyr*A gene, although changes were also observed in the lipopolysaccharide structure of the cell envelope.

It has become obvious that mechanisms of fluoroquinolone resistance mediated through decreased cell permeability represent a more complex situation in *P. aeruginosa* compared to the mutational events observed in *E. coli* strains. There is no doubt that one of the major outer membranes, OmpF in *E. coli*, is a very important cell component determining cell susceptibility and resistance to fluoroquinolones. However, with respect to *P. aeruginosa* OprF, which is also a major outer membrane protein, very few reports have suggested its potential role in quinolone susceptibility. There is one case reported of a *P. aeruginosa* strain isolated after enoxacin therapy which showed an absence of OmpF; the mutation reported was presumably in the *nal*B locus (Piddock *et al.*, 1987). More recently, Chamberland *et al.* (1990) have shown that isolates from a cystic fibrosis patient treated with ciprofloxacin showed ciprofloxacin resistance and loss of protein F. However, the strain had no change in ciprofloxacin uptake compared to susceptible pre- and post-therapy isolates. A ciprofloxacin-susceptible revertant retained the loss of F protein. As well, the resistant isolate was shown to require an increased quantity of ciprofloxacin to inhibit DNA synthesis even after EDTA treatment. These findings

were consistent with the F protein deficiency not being the cause of resistance. Despite the fact that OprF is known to be associated with *in vitro* pore-forming activity (Hancock *et al.*, 1979), there is also evidence of that protein F also plays a role in maintaining structural integrity of the *P. aeruginosa* outer membrane (Gotoh *et al.*, 1989). The contribution of the latter role might be more important *in vivo* and might explain the lack of data consistent with OprF involvement in cell susceptibility to fluoroquinolones in *P. aeruginosa*.

RATIONALE

These studies were developed to investigate the factors contributing to fluoroquinolone susceptibility among bacterial species with different MICs to the agents. Since no mechanism of drug inactivation in a prokaryotic system has yet been described, it was postulated that the drug-target affinity and cell permeability to these compounds would be the major determinants of antibacterial activity. Moreover, such studies should provide a better understanding of the mechanisms of resistance mediated through mutations modifying these two determinants. The first objective of our studies was to characterize the permeation processes of fluoroquinolones in bacteria and elucidate by using isogenic mutants, the relative contribution of the specific cell components porins, LPS, and DNA gyrase to the overall accumulation process of these antimicrobial agents.

The data provided from these studies were consistent with use of simple diffusion as an uptake mechanism. Therefore, the possibility of fluoroquinolone interaction with liposomes was determined as an *in vitro* model which could represent the first step of diffusion across the lipid bilayers of the bacterial membrane. Since such interactions were found to exist, the nature of forces involved in the drug-liposomes binding process was

defined as being both ionic and hydrophobic. The second objective consisted of determining (i) the level of susceptibility of inhibition of DNA synthesis to a fluoroquinolone, ciprofloxacin, among selected bacterial species with various MICs; (ii) and the magnitude of drug uptake in order to establish a correlation between these two determinants and the cell susceptibility to ciprofloxacin. Overall, these studies enabled the provision of a model of fluoroquinolone accumulation process in bacteria, and confirmation that the extent of quinolone accumulation controlled susceptibility in certain bacteria. Other published results from various investigators contributed to the elaboration of this model.

MATERIALS AND METHODS

2.0 MATERIALS AND METHODS

2.1 Bacterial Strains

Bacterial strains in this study are given in Table 1.

2.2 Media, Antibiotics, MIC and Disk Susceptibility Studies

Media. Media used in this study were Brain Heart Infusion broth (BHIB) from Difco Laboratories, Detroit, MI, which was used for MICs, cell viability, and uptake studies. Nutrient broth (NB) from BBC Microbiology Systems, Cockeysville, MD was used for MICs, [^{14}C] enoxacin displacement studies, uptake, SDS-induced cell lysis in the presence of quinolones and growth studies. Vogel-Bonner (VB) medium (Vogel and Bonner, 1956) which consists of 0.2 g/L $MgSO_4.7\ H_2O$, 2 g/L citric acid.H_2O, 10 g/L anhydrous K_2HPO_4, 3.5 g/L Na $(NH_4)HPO_4.4\ H_2O$ was stored at room temperature over 10 - 20 ml chloroform. Glucose at a final concentration of 1.37 mM was added before utilization. In DNA inhibition studies and MIC determinations for *P. aeruginosa* PAO 503 and its derivatives which are auxotrophs derived from *P. aeruginosa* PAO1, VB was supplemented with 0.4% glucose, 0.01% arginine;, and 1 mM methionine and the medium (VBGAM) was sterilized by filtration using 0.2 μm diameter pore size filters (Gelman Sciences, Ann Arbor,

Michigan). Luria Broth (LB) from Difco Laboratories, Detroit, MI was used for MICs and DNA inhibition studies. Basic medium (Rella and Haas, 1982) composed of 40 mM tris-hydrochloride (pH 7.4), 80 mM potassium chloride, 7 mM magnesium acetate, 2 mM ethyleneglycol-bis-β-amino-ethyl ether N,N'-tetra-acetic acid, 0.4 mM spermidine trihydrochloride, and 0.5 M sucrose was used for DNA synthesis inhibition studies. Tryptic soy agar (TSA) from Difco Laboratories, Detroit, MI was used for colony counts and disk susceptibility studies.

Antimicrobial Agents. Radiolabelled [^{14}C] enoxacin (4.8 Ci/mol) and [^{14}C] ciprofloxacin (14.8 Ci/mol) were obtained by Parke, Davis and Company, Toronto, Ontario, and A. Dalhoff, Bayer AG, Pharma Research Center, Wuppertal, Federal Republic of Germany, respectively. Ciprofloxacin hydrochloride, from Miles Pharmaceuticals, Toronto; norfloxacin, Merck and Company, Inc., Rahway, NJ; ofloxacin, Ortho Pharmaceutical, Ltd., Don Mills, Ontario; fleroxacin, Hoffmann-LaRoche, Etobicoke, Ontario; nalidixic acid, chloramphenicol, and tetracycline from Sigma Chemical Co., St. Louis, MO

MICs. MICs were performed in media mentioned above by a broth dilution method (Finegold and Martin, 1982) at a final inoculum of 10^6 to 10^7 CFU/ml in 2 ml final

volume of Mueller Hinton broth for an incubation period of 18 h at 35°C.

Disk susceptibility. Disk susceptibility testing was performed by standard diffusion methodology (Bauer *et al.*, 1956). The amount of drug used for fluoroquinolones was, 5 μg/disk; nalidixic acid, 20 μg/disk; tetracycline, 20 μg/disk; and chloramphenicol, 10 μg/disk. In studies of the effect of pH on the susceptibility of *E. coli* JF-derivatives to quinolones, the pH of TSA plates was adjusted to 4.5 using 1 N HCl or 10.0 using 1 N NaOH before autoclaving and checked thereafter.

2.3 Growth Studies and Cell Viability

2.3.1. Growth studies.

In order to study the effects of washing conditions on growth of cells pretreated with enoxacin, *E. coli* SA 1306 was grown in NB at 37°C with shaking to an $O.D._{600}$ of 0.4. Cells were then exposed to enoxacin at 1, 2.5, 10 or 20 μg/ml for a period of 2 min at 37°C. An aliquot of 20 ml was taken and centrifuged at 10,000 x g at 25°C for 2 min. The cell pellet was washed with 20 ml of NB and resuspended with the same volume of medium at 37°C. Growth of this cell suspension was monitored every 15 min for a period up to 90 min. Additional studies using the same methodology as described above were performed using

higher enoxacin concentrations of 20, 40, 60 and 80 μg/ml, however, cell growth was monitored every 10 min instead of 15 min for a period up to 120 min. Studies were also performed at 1, 5 and 10 μg/ml enoxacin for a 60-min cell exposure to the drug before washing and suspension as above.

2.3.2 Cell viability.

An aliquot of an overnight culture of *E. coli* J5-3 in BHIB was used to subculture 100 ml of BHIB to an $O.D._{600}$ of 0.1. Cell growth was monitored until an early log phase ($O.D._{600}$ of 0.4) was achieved. Aliquots of this cell suspension were then taken in order to study the effects of 0.05, and 0.1 μg/ml ciprofloxacin in the presence or absence of 1 mM sodium azide or 20 μM CCCP (final concentration) on cell viability. Cells were incubated at these various conditions for 0.5, 1, 1.5 and 2 hours (and 4 hours in some experiments) at 35°C with shaking. At these time points, an aliquot of 1 ml of cell suspension was removed, centrifuged at 15,000 x g for 4 min, resuspended in the same volume of 0.89% NaCl and various dilutions were made. Viable cells were counted after incubation for 16 h at 35°C on TSA. All counts were in triplicate, and each experiment is the average of two separate determinations.

2.4 Studies of Characteristics of Quinolone-*E. coli* Cell Association

Studies of displacement of enoxacin associated with *E. coli* SA 1306 cells were performed using [^{14}C]enoxacin mixed with unlabelled enoxacin in ratio of 1:20 at a final concentration of 2 μg/ml. An aliquot of 5 ml from an overnight cell culture was used to inoculate 200 ml of fresh NB. Cell growth was monitored until an O.D.$_{600}$ of 0.5. The protonophore CCCP (Sigma) was added at a final concentration of 20 μM 2 min before the addition of the drug mixture.

Studies of the effect of structural analogs on the amount of [^{14}C] enoxacin associated with *E. coli* SA 1306 was performed by the addition of a mixture of 2 μg/ml of [^{14}C]enoxacin with either unlabelled enoxacin, ciprofloxacin, norfloxacin, or nalidixic acid at final concentrations of 1, 20, 50 or 100 μg/ml to the *E. coli* cell culture. As quickly as possible, 10 ml of cells were removed and centrifuged at 10,000 x g for 2 min. The supernatant was discarded, cells washed once with 2 ml of 0.89% NaCl, centrifuged again and resuspended in 1 ml of 0.89% NaCl. A volume of 5 ml of PCS solubilizer (Amersham Corp., Arlington Heights, IL) was added and radioactivity quantitated using a Beckman LS6800 scintillation counter. Studies were also performed with chlor-

amphenicol at concentrations mentioned above and with calcium chloride and piperazine at 1, 50 or 100 mM and 1, 10 or 100 μg/ml, respectively.

2.5 Uptake Studies

2.5.1 Bioassay method for enoxacin.

Growth of an *E. coli* SA 1306 cell suspension previously inoculated from an overnight culture was monitored at 35°c in NB until log phase growth was reached. Enoxacin was added at different concentrations and 10-ml aliquots were removed at 0, 5, 10 min followed by 10-min intervals for a period of up to 100 min. These bacterial suspensions were immediately centrifuged at 10,000 x g for 3 min, the supernatant was removed and cells washed with 2 ml of saline followed by another centrifugation. Cells were resuspended in 1 ml 0.89% NaCl sterile and the bacterial suspension immersed in boiling water for 7 min. This treatment did not affect the bioactivity of enoxacin. The boiled cell suspension was centrifuged at 10,000 x g for 3 min. The activity of enoxacin in the supernatant was determined by measuring the growth inhibition of *E. coli* J5-3 after an incubation of 16 h at 35°C at the periphery of 10-mm diameter holes, produced in nutrient agar medium, which contained 100 μl of the supernate mentioned above.

The zones of inhibition obtained were compared with those produced by standards of different concentrations of enoxacin in a volume of 100 µl.

Studies of the accumulation profile of chloramphenicol at a concentration of 500 µg/ml in *E. coli* SA 1306 and JB-5R strains were performed using the methodology as described above.

2.5.2 Uptake protocols for [^{14}C]enoxacin.

Uptake of enoxacin in *Bacillus subtilis*, and in *E. coli* J5-3 and JF derivatives was performed using a constant ratio of 0.2 mg of [^{14}C]enoxacin (4.8 Ci/mol) with 4 mg of unlabelled enoxacin. Time-dependent uptake and studies of effects of cell inhibitors on enoxacin uptake were performed following this centrifugation-filtration protocol: Cells were grown overnight in NB and an aliquot of this culture was used to inoculate fresh NB to give an O.D.$_{600}$ of about 0.1. Cell growth was monitored at 35^{o}C with shaking until a log phase of growth (O.D.$_{600}$ of about 0.4). A mixture of [^{14}C] labelled-unlabelled enoxacin in ratio 1:20 was added at various concentrations: 5, 20 and 80 µg/ml for time-dependent uptake; and 20 µg/ml for the studies of the effect of energy inhibitors on cell uptake. An aliquot of 1 ml was removed at various times and centrifuged for 3 min at room temperature at 10,000 x g and the supernatant removed. The pellet was resuspended into 1

ml of NB and filtered through a 0.5 μm GV filter (Millipore Corp., Bedford, MA) prewashed with 1 ml of unlabelled enoxacin at 1 mg/ml in order to decrease nonspecific binding. Control samples were used containing identical enoxacin mixtures as mentioned above, but without bacterial cells. Values from these control samples were subtracted from the values found for [^{14}C]enoxacin associated with cells in order to correct for background binding to the filters. Regarding the effect of energy inhibitors on enoxacin uptake: carbonylcyanide m-chlorophenylhydrazone (CCCP), 2,4-dinitrophenol (2,4-DNP), $NaHAsO_4$, NaN_3, or NaF were added 2 min before the drug addition at final concentrations of 2×10^{-5}, 2×10^{-3}, 1×10^{-3}, 1×10^{-3}, and 1×10^{-2} M, respectively. For Lineweaver-Burk plotting studies, *E. coli* SA 1306 was incubated for 1 min with enoxacin at concentrations of 0.5 to 150 μg/ml followed by centrifugation of a 1-ml sample for 3 min at 10,000 x g at 22^{o}C. The supernatant was discarded, cell pellet resuspended into 300 μl of NB and the entire sample was counted in a PCS solubilizer.

2.5.3 Uptake protocol for [^{14}C]ciprofloxacin.

Ciprofloxacin uptake using NB and BHIB was determined in the presence or absence of 20 μM CCCP with and without 10 mM $MgCl_2$. Cells were grown overnight in the appropriate medium at 35^{o}C with shaking. An aliquot of 5 (NB) or 1 (BHIB) ml of the overnight culture was used to

inoculate 50 ml of fresh medium. Cell growth was monitored until an $O.D._{600}$ of 0.5 was reached. Cells were centrifuged and resuspended in the same medium to an $O.D._{600}$ adjusted to 30 to 40. Studies of the effect of CCCP on the uptake process were performed by adding the CCCP at a final concentration of 20 μM 2 min before the addition of ciprofloxacin, which was used at its original specific activity at a concentration of 0.154 μg/ml unless otherwise stated. Uptakes were performed at 35°C in a block heater without agitation. At various times, an aliquot of 80 μl was taken, and uptake was measured using various washing conditions with the same medium used for uptake. For minimal wash, the 80-μl sample was filtered directly through a 2.5-cm Whatman GF/F microfiber glass filter (used for all washing protocols), followed by a washing step with 4 ml of broth. For the 2-ml wash, the 80-μl sample was diluted in 2 ml of broth at room temperature and filtered, followed by a washing step with 2 ml of broth. For the 10-ml wash, the 80-μl sample was diluted in 2 ml of broth at room temperature and filtered, followed by a washing step with 10 ml of broth. Filters were dried and radioactivity counts quantitated in a Beckman Ready Safe liquid scintillation cocktail in a Beckman LS6800 scintillation counter. Uptake is expressed as nanograms of ciprofloxacin per milligram (dry weight) of cells.

2.5.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis studies.

In order to confirm the phenotype of outer membrane proteins of *E. coli* JF-derivative strains used with regard to their porin pattern, outer membranes were purified from these strains and the protein profile studied on 12.5% SDS-PAGE. The same procedure was used for *E. coli* JB-5R since it was assumed that the reduced rate of enoxacin diffusion observed in this strain would correlate with changes of protein commposition in the outer membrane. Outer membranes were isolated by the method of Hancock and Nikaido, 1978, subsequently modified by Godfrey *et al.*, 1984. Cells were grown in 1.5 L of BHIB for 16 h at 35^{o}C with shaking. The cell suspension was centrifuged at 10,000 x g for 10 min and washed once with 0.03 M tris-hydrochloride at pH 8.0, followed by a second centrifugation. Cell pellets were resuspended in 20 ml of 20% sucrose-50 mM tris-hydrochloride at pH 7.9 supplemented with 0.2 mM dithiothreitol (Sigma), final concentration. Thereafter, cells were passed twice through a french press (18,000 lb/in^2) (American Instrument Company, Silver Spring, MA). The protease inhibitor phenylmethylsulfonylfluoride (Sigma) was added to a final concentration of 1 mM. Lysozyme, DNAse, and RNase were sequentially added at a final concentration of 0.1 mg/ml, 0.025 mg/ml, and 0.025 mg/ml,

respectively. Disrupted cells were incubated for 10 min on ice and were then sonicated three times for 20 sec each with 60-sec intervals. Debris was removed by a centrifugation at 1,000 x g for 10 min at 4°C. The supernatant was layered onto a sucrose gradient of 52%, 58%, 64% and 70% sucrose in 50 mM tris-hydrochloride supplemented with 0.2 mM dithiothreitol to prevent oxidation of cell material, and then centrifuged for 16 h at 45,000 rpm (Rotor Ti50, Beckman L5-50B ultracentrifuge). Outer membranes were located at the 58% - 64% sucrose interphase and were collected, washed five times with 50 mM tris-hydrochloride, pH 7.9 - 0.2 mM dithiothreitol, and finally resuspended in the same buffer. The protein content was quantitated using the method of Lowry *et al.*, 1951.

A good resolution of outer membrane proteins was obtained by loading 200 μg of protein per well on a 12.5% SDS-PAGE using the method of Laemmli and Favre (1973). Stacking gel was composed of 5% acrylamide (Boehringer Mannheim Canada), 0.13% N,N'-methylene-bis-acrylamide (SERVA Feinbiochemica, New York, NY), and 0.1% SDS in 0.125 M tris-hydrochloride at pH 6.8. Separating gel consisted of 12.5% acrylamide, 0.33% N,N'-methylene-bis-acrylamide, and 0.1% SDS in 0.375 M tris-hydrochloride at pH 8.8. Gel polymerization was achieved by adding 0.1% ammonium persulfate (Biorad) and 0.1% N,N,N',N-

tetramethylethylene diamine (International Biotechnologies, Inc.). The gel running buffer consisted of 0.6% tris-base, 0.1% SDS, and 2.88% of glycine (Terochem Laboratories, Edmonton, AB) in H_2O at pH 8.3. Outer membranes containing 200 μg of protein were dissolved in a 50-μl solution of sample buffer (2% SDS, 4% 2-mercaptoethanol, 10% glycerol in 1 M tris-hydrochloride, pH 6.8, and 0.002% bromophenol blue), and immersed in boiling water for 3 min. Proteins were electrophoresed at 20 mA for a period of about 4 h. Gel size was 23 x 20 cm and 0.2 cm thick. The gels were stained for 3 h in a solution of 0.1% w/v Coomassie Brilliant Blue R (Sigma) in 50% methanol and 10% acetic acid. Destaining was performed by soaking the gel in a solution of 50% methanol and 10% acetic acid for 16 h at room temperature with gentle shaking.

2.6 Studies of SDS-Induced Cell Lysis by Various Quinolones.

P. aeruginosa strains were grown overnight in NB at 35^{o}C with shaking. An aliquot of this culture (1 ml) was used to inoculate 50 ml of fresh NB and growth monitored until an $O.D._{600}$ of about 0.350 was achieved. Cell suspensions were divided in four parts: (i) control without addition of SDS or fluoroquinolones; (ii) cells incubated with 0.01% or 0.05% SDS; (iii) cells incubated with 10 μg/ml of enoxacin, ciprofloxacin, norfloxacin,

ofloxacin, or fleroxacin; (iv) cells incubated with 0.01% or 0.05% SDS and 10 μg/ml of fluoroquinolone. Cell growth was monitored at intervals of 15 or 20 min for a period of up to 100 min by measuring the $O.D._{600}$ of a 1-ml aliquot removed at appropriate times.

2.7 DNA Synthesis Inhibition Studies

In all cases, the rate of DNA synthesis was measured as the percentage rate compared to the control at 0 min, without addition of ciprofloxacin. The $I.D._{50}$ was defined as the amount of ciprofloxacin required to obtain 50% inhibition of DNA synthesis *in vivo*. The early inibition of DNA synthesis was found to be proportional to the ciprofloxacin concentration in *E. coli*, *P. aeruginosa*, and *A. faecalis*.

2.7.1 Method of Benbrook and Miller, 1986.

This method was modified for use with *P. aeruginosa* strains. Bacteria were subcultued to 20 ml of Vogel-Bonner medium supplemented with 0.4% glucose, 0.01% arginine, and 1 mM methionine for 18 h at 37°C with shaking. Cell suspension was diluted into fresh VBGAM to an $O.D._{600}$ of 0.15 to 0.20, and cell growth monitored at 35°C until an $O.D._{600}$ of 0.4 was obtained. EDTA, when required to permeabilize bacterial cells, was added at a final concentration of 2.5 mM 2 min before the addition of ciprofloxacin. Control rates of DNA synthesis were

determined by sampling cells at 0 and 5 min of DNA synthesis before the addition of the drug. After the 5-min sample was taken, ciprofloxacin was added at various concentrations in order to produce a wide range of inhibition of DNA synthesis by 15 min. To evaluate the extent of inhibition of DNA synthesis, samples were taken at 5-min intervals up to 30 min. The maximal point of inhibition of DNA synthesis was determined at 20 and 25 min from time zero. At the various times mentioned above, an aliquot of 800 μl of cells at $O.D._{600}$ of 0.4 was taken and added to a 3.2 ml of VBGAM containing [^{3}H]adenine (32.3 Ci/mmol, New England Nuclear) and mixed quickly. The experiments for each time sample were performed in duplicate. Labeling was performed for a period of 7 min, then 1-ml aliquot of this reaction mixture was transferred to 400 μl of 0.3 N NaOH-0.1% EDTA in order to stop the reaction and RNA was hydrolyzed by incubating the samples at 37^{o}C overnight. Thereafter, sterile BSA at 40 μg/ml final concentration was added, DNA was precipitated with trichloroacetic acid at a final concentration of 5%. DNA was collected onto cellulose acetate filters and washed with 1 ml 5% TCA; filters were dried, 5 ml Beckman "ready protein" scintillation fluid was added and counts determined using a Beckman LS6800 scintillation counter.

2.7.2 Method of Chow *et al.*, 1988.

This method was used for *E. coli* strains with minor modifications. Cells were grown overnight in LB at 37°C with shaking. Cells were thereafter diluted 20-fold and growth was monitored until an $O.D._{600}$ of 0.6. An aliquot of 200 µl of cells was added to 1800 µl of LB containing ciprofloxacin at various concentrations. Aliquots of 200 µl were taken at various time (0 to 30 min time range at 5-min intervals) and added to 1 µCi [^{3}H]thymidine (2.0 Ci/mmol) (New England Nuclear) in 10 µl LB. Cells were pulse-labelled for 2 min at 37°C, the reaction stopped with trichloroacetic acid at a final concentration of 5%. Cells were filtered onto GF/C filters (Whatman, Inc., Clifton, NJ) which were washed with 5% trichloroacetic acid and 95% ethanol. Filters were then dried. Radioactivity, as a measurement of the rate of DNA synthesis, was determined in a Beckman LS6800 scintillation counter using 10 ml toluene-PPO-POPOP (4 g/L of PPO [2.5 diphenyl-oxazole] and 0.1 g/L dimethyl POPOP [1,4-bis-(5-phenyloxazolyl)benzene].

2.7.3 Method of Rella and Haas, 1982.

This method was used for *P. aeruginosa* and *A. faecalis* bacterial strains with minor modifications (Chamberland *et al.*, 1989). Since these bacterial strains do not incorporate extracellular [^{3}H]dTTP into DNA, ether was used as a permeabilization agent. This

agent was also previously used with success to measure DNA synthesis in *E. coli* cells (Vosberg and Hoffman-Berling, 1971). Cells were grown overnight at 35°C in BHIB with shaking. This overnight culture was diluted 20-fold into 100 ml of BHIB and cell growth was monitored until they reached the log phase. An aliquot of 50 ml was transferred into 20 ml of cold (4°C) basic medium, followed by centrifugation at 10,000 x g for 10 min. Collected cells were washed with a ratio of 5:2 cold BHIB and cold basic medium, and centrifuged. Cells were resuspended in a volume of 1.5 ml of basic medium followed by the addition of an equal volume of cold ether; thereafter, cells were mixed by rotation of a magnetic bar for exactly 45 sec. Then 2 ml of basic medium with 0.8 M sucrose was added to the bottom of the tube. Ether-treated cells were separated from the upper ether-containing phase, and cell pellet obtained by low-speed centrifugation at 1,100 x g for 10 min. Cells were suspended gently in 0.6 ml of basic medium at a final concentration of 2×10^{10} cells/ml and kept on ice. DNA synthesis inhibition assay was performed within 45 min following cell permeabilization. Cell concentrations were adjusted to 2×10^{9} cells/ml by addition of an appropriate volume of prewarmed basic medium which contained various concentrations of ciprofloxacin. At various times within 5-min intervals, an aliquot of 0.1

ml was taken and added to an equal volume of prewarmed (37°C) basic medium supplemented with 80 μM each of dATP, dCTP, dGTP (Boehringer Mannheim, Dorval, Quebec) and [^{3}H] dTTP (104.7 Ci/mmol) (New England Nuclear), 0.4 mM NAD, and 4 mM ATP. Cells were pulse-labelled at 37°C for 2 min. A volume of 3 ml of cold 10% trichloroacetic acid containing 0.05% thymidine and 0.1 M potassium pyrophosphate was added to stop the reaction. Cells were filtered onto GF/C filters (Whatman, Inc., Clifton, NJ) followed by three washes with 10% trichloroacetic acid, followed by 0.1 M hydrochloric acid and 95% ethanol washes, respectively. Filters were dried and 5 ml of Beckman "ready protein" scintillation fluid was added and radioactive counts determined using a Beckman LS6800 scintillation counter.

2.8 Studies of Fluoroquinolone Interactions with Liposomes

2.8.1 Liposome preparation.

The principle of the liposome preparation protocol was taken from the method of Nakae and Nakae (1982). Liposomes were prepared in a solution of chloroform from a liposome kit (Sigma) containing 6.2 x 10^{-6} mole of phosphatidylcholine and dicetylphosphate and cholesterol in a ratio of 100:28.6:14.3, respectively. The lipids were dried in a conical tube previously acid-washed under

a stream of nitrogen in order to evaporate the chloroform. This preparation was further dried for at least one hour in a dessicator under vacuum at room temperature. A fine glass rod was used to scrape the lipid film from the wall of the tube. In our experiments, we arbitrarily referred to phosphatidylcholine as the lipid concentration which was 70% of the lipid content of liposomes.

2.8.2 Drug solubility measurement.

For this study, solutions of enoxacin, ciprofloxacin-HCl, norfloxacin, and nalidixic acid were prepared at a concentration of 10 mg/ml in water. With regard to enoxacin and norfloxacin, complete solubilization was achieved by titration with sodium hydroxide before bringing the solution to the final volume with water. A volume of 100 μl of these solutions was diluted in 800 μl of 0.1 M Mes-NaOH, the pH was adjusted to pHs 3, 4, 5, 6, 7, 8, 9, and 10 using 1 N HCl or NaOH and the volumes adjusted to 1 ml. After an incubation at room temperature for at least an hour, these solutions were centrifuged at 15,000 x g for 5 min. The supernatant was removed and diluted 1:200 with the same buffer previously used and the O.D. measured at the wavelength of maximum absorption for each quinolone, which was 267, 270, 273, and 260 nm for enoxacin, ciprofloxacin, norfloxacin, and nalidixic acid, respectively. The concentration of

quinolone in the supernatant was determined with a standard curve-relating concentration to O.D. established for each of them.

2.8.3 Liposome-drug assays.

2.8.3.1 Effects of quinolones on liposome-liposome interactions and liposome integrity. A volume of 0.5 ml of 5 mM Mes-NaOH, pH 7.0, was added to the dried lipid in the conical tube and a preparation of vesicle membranes was obtained after 30-sec agitation using a vortex mixer (Scientific Industries, Inc., Bohemia, NY). An aliquot of 50 μl was diluted into 1 ml of enoxacin, or ciprofloxacin at a concentration range of 20 mM to 0.16 mM in a 5 mM Mes-NaOH buffer at pH 4.5, 7.0, and 10.0. The O.D. was determined at 450 mn with a blank containing no liposomes used for all drug concentrations tested. The O.D. for each pH was also compared to the O.D. obtained with the liposome preparation without drug addition.

The same protocol as described above for obtaining vesicle membranes was used to study the drug effect on liposome integrity with this following exception. At the vesicle formation step, 2.08×10^{-10} mole of [^{14}C]ATP (0.5 Ci/mmol) (New England Nuclear, Boston, MA) used at its original specificity was incorporated at the same time as the buffer. An aliquot of 50 μl of this liposome

suspension was added to 1 ml of 5 mM Mes-NaOH supplemented with enoxacin, ciprofloxacin, norfloxacin, ofloxacin, and nalidixic acid at final concentrations of 0.02 M at pH 4.5, 7.0 and 10.0. This suspension of liposomes was left in the presence of quinolones for a period of 10 min at room temperature, and then filtered through a 0.22 μm GVWP Durapore filter (Millipore Corp., Bedford, MA) previously washed with 1 ml of unlabelled ATP (Sigma) at a concentration of 1 mg/ml. The [^{14}C]ATP nonspecific binding on paper filters was corrected by using a control with no liposomes. The collected liposomes on paper filter were washed once with 1 ml of 5 mM Mes-NaOH at the appropriate pH and filters counted in 5 ml of omnifluor at a concentration of 4 g/L of toluene (New England Nuclear Corp., Boston, MA). The percentage of [^{14}C]ATP associated with liposomes at physiological pH after washing was estimated at a value of 1.5% of the original amount used. Also, controls which consisted of liposomes suspended in Mes-NaOH buffer without antibiotic or liposomes suspended in 75% ethanol were also used in this study. Liposomes were also suspended in a solution of glucose-Mes-NaOH at the same osmolarity as the antibiotic assay in order to study the osmotic effect which was found to be negligeable on the amount of [^{14}C]ATP released from liposomes.

2.8.3.2 Studies of pH, drug and liposome concentration on liposome-fluoroquinolone interactions. Liposome suspensions were prepared as described above, vesicle membranes were obtained by the addition of 100 μl of 0.1 M Mes-NaOH, pH 7.0 upon agitation on a vortex mixer. An aliquot of 10 μl was diluted in 200 μl of 0.1 M glycine buffer , pH 4.0; 0.1 M Mes-NaOH, pH 6.0 and 7.0, and 0.1 M Hepes buffer, pH 8.0, 9.0, and 10.0, which contained [^{14}C]enoxacin or [^{14}C]ciprofloxacin at their original specific activity at a final concentration of 2 and 1.23 μg/ml, respectively. This preparation was centrifuged in 5 x 20 mm tubes at 100,000 x g for 5 min in a Beckman airfuge. The supernatant was discarded, and the liposome pellet washed with the same volume of appropriate buffer not supplemented with fluoroquinolones. The resuspended liposomes were then centrifuged again under the same conditions as mentioned above. The pellet was then resuspended in the same volume of buffer used for the washing step and the liposome suspension was transferred to 5 ml of PCS solubilizer to determine the radioactivity. Assays were performed at pH 7.0 with regard to the studies of the drug and lipid concentration effects on the amount of drug associated with liposomes. Concentrations used for drug effect studies were for [^{14}C]enoxacin, [^{14}C]ciprofloxacin, and phosphatidylcholine: 1 to 5 μg/

ml, 0.62 to 3.10 μg/ml, and 3.1 x 10^{-6} mole/ml, respectively. Concentrations used for the lipid effect studies were for [^{14}C]enoxacin, [^{14}C]cipro-floxacin, and phosphatidylcholine: 2 μg/ml, 1.23 μg/ml, and 3.1 to 24.8 x 10^{-6} mole/ml, respectively.

RESULTS

3.0 RESULTS

With regard to the experimental data presented, all studies were performed at least three times, and typical results are presented. The graphs presented in this result section were created using regression analysis on the Macintosh computer program, Cricket Graph.

3.1 Bacterial Cell Susceptibilities to Various Quinolones.

Bacterial strain characteristics are given in Table 1. Values for bacterial cell susceptibility of *E. coli*, *P. aeruginosa*, and *A. faecalis* strains used in this study are given in Table 2.

3.2 Study of Factors Determining Bacterial Cell Susceptibility to Fluoroquinolones: Accumulation Process of Fluoroquinolones in Bacteria.

3.2.1 Mechanisms of enoxacin and ciprofloxacin permeation in *E. coli* and *B. subtilis* strains.

3.2.1.1 *E. coli* growth studies.

In order to establish a correlation between the inhibition of cell growth and the cell uptake process of quinolones, we studied the effect of various enoxacin concentrations on *E. coli* growth over different times of exposure. A rapid accumulation of enoxacin in *E. coli* cells was observed (see Section 3.2.1.4) with an

TABLE 1. BACTERIAL STRAINS USED IN THIS STUDY [Page 1 of 4]

Strain	Bacterial Strain Souce	Characteristic
E. coli K-12 Strains		
M1174	K.E. Sanderson University of Calgary Calgary, AB	gal, lacX74, lys(del) Str^r thi trp (cryptic lambda).
SAB3005	K.E. Sanderson	gal, lacX74, lys(del) recA, Str^r, thi, trp (cryptic lambda).
J5-3 derivatives		
J5-3	B.J. Bachmann E. coli Genetic Stock Ctr. Yale University New Haven, CT	F^-, pro-22 met F63
SA 1306	K.E. Sanderson	pro-22 metF63 gyrA nal^r
JB-5R	Our laboratory	pro-22 metF63 gyrA $enox^r$; derived from E. coli SA1306 obtained by serial passage in the presence of increasing concentrations of enoxacin up to 5 µg/ml.

Table 1, Bacterial strains used in this study, continued [Page 2 of 4]

Strain	Bacterial Strain Souce	Characteristic
E. coli K-12 Strains		
JF derivatives		
JF-568	R.K. Poole University of British Columbia, Vancouver, BC	F⁻aroA357 cycA1 cycB2 his53 ilv277 lacY29 met65 proC24 purE41 rpsL97 tsx63 xyl14 (parent)
JF-694	B.J. Bachmann	F⁻cycA1 cycB2 his53 ilv227 lacY29 met65 nmpA ompC263 ompF254 proC24 purE41 rpsL97 tsx63 xyl14 (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⁻)
Pseudomonas aeruginosa strains		
PAO 236	B.W. Holloway Monash University Clayton, Victoria Australia	PAO 1 derivative, gyrA hisIV ilvB/C lys-12 met28 proA trpCD

Table 1, Bacterial strains used in this study, continued [Page 3 of 4]

Strain	Bacterial Strain Souce	Characteristic
Pseudomonas aeruginosa strains		
PAO 503	B.W. Holloway	PAO 1 derivative, met-9011
503-18	L.E. Bryan University of Calgary Calgary, AB	PAO 503 derivative, transductant selected for aminoglycoside resistance, deep rough lipopolysaccharide (Bryan et al., 1984)
PCC 23	A.J. Godfrey University of Calgary Calgary, AB	PAO 503 derivative, mutagenized with ethane methane sulfonate, ticarcillin-resistant, outer membrane protein F mutant (qualitative modification) (Godfrey and Bryan, 1987)
PCC 1989	A.J. Godfrey	PAO 503 derivative, gyrA, F116 transductant

Table 1, Bacterial strains used in this study, continued [Page 4 of 4]

Strain	Bacterial Strain Souce	Characteristic
Pseudomonas aeruginosa strains		
Y 4449	Dr. H.R. Rabin University of Calgary Calgary, AB	Clinical bacterial strain from cystic fibrosis patient isolated before ciprofloxacin therapy
Y 4492	Dr. H.R. Rabin	Clinical bacterial strain from cystic fibrosis patient isolated during ciprofloxacin therapy
Y 4516	Dr. H.R. Rabin	Clinical bacterial strain from cystic fibrosis patient isolated after ciprofloxacin therapy
Alcaligenes faecalis ATCC19018	American Type Culture Collection, Rockville, MD	Porin diameter reported smaller than that of E. coli porins (Ishii and Nakae, 1988)
Bacillus subtilis	Dr. L.E. Bryan	Wild-type

TABLE 2A. SUSCEPTIBILITY OF BACTERIAL STRAINS TO QUINOLONES

Susceptibility of *E. coli*, *P. aeruginosa*, and *A. faecalis* to ciprofloxacin.

Bacterial Strain	MIC (μg/ml) Ciprofloxacin					
	NB		BHIB		VB	LB
E. coli J5-3 derivatives	$-Mg^{++}$	$+Mg^{++}$	$-Mg^{++}$	$+Mg^{++}$		
J5-3	0.006	0.25	0.025	0.15	ND	0.031
SA-1306	0.078	ND	0.31	ND	ND	0.125
JB-5R	ND	ND	ND	ND	ND	1.0
P. aeruginosa						
in vitro-derived strain						
PAO236	ND	ND	ND	ND	ND	4.0
PAO503	0.15	3.0	0.1	1.5	0.125	ND
PCC1989	ND	ND	ND	ND	ND	1.0
in vivo-derived strain						
Y4449	ND	ND	ND	ND	ND	2.0
Y4492	ND	ND	ND	ND	ND	16.0
Y4516	ND	ND	ND	ND	ND	1.0
A. faecalis						
ATCC19018	0.95	3.75	0.25	0.95	ND	ND

TABLE 2B. SUSCEPTIBILITY OF BACTERIAL STRAINS TO QUINOLONES

Susceptibility of *E. coli* strain to enoxacin and nalidixic acid.

Bacterial Strain	MIC (mg/ml) enoxacin	MIC (mg/ml) nalidixic acid
E. coli J5-3 derivatives		
J5-3	0.16 - 0.32	1.25 - 2.50
SA-1306	0.62 - 1.25	50 - 100
JB-5R	5.0 - 7.5	50 - 100
E. coli JF-568 derivatives		
JF-568	0.15	ND
JF-694	0.32	ND
JF-701	0.16	ND
JF-703	0.31 - 0.62	ND

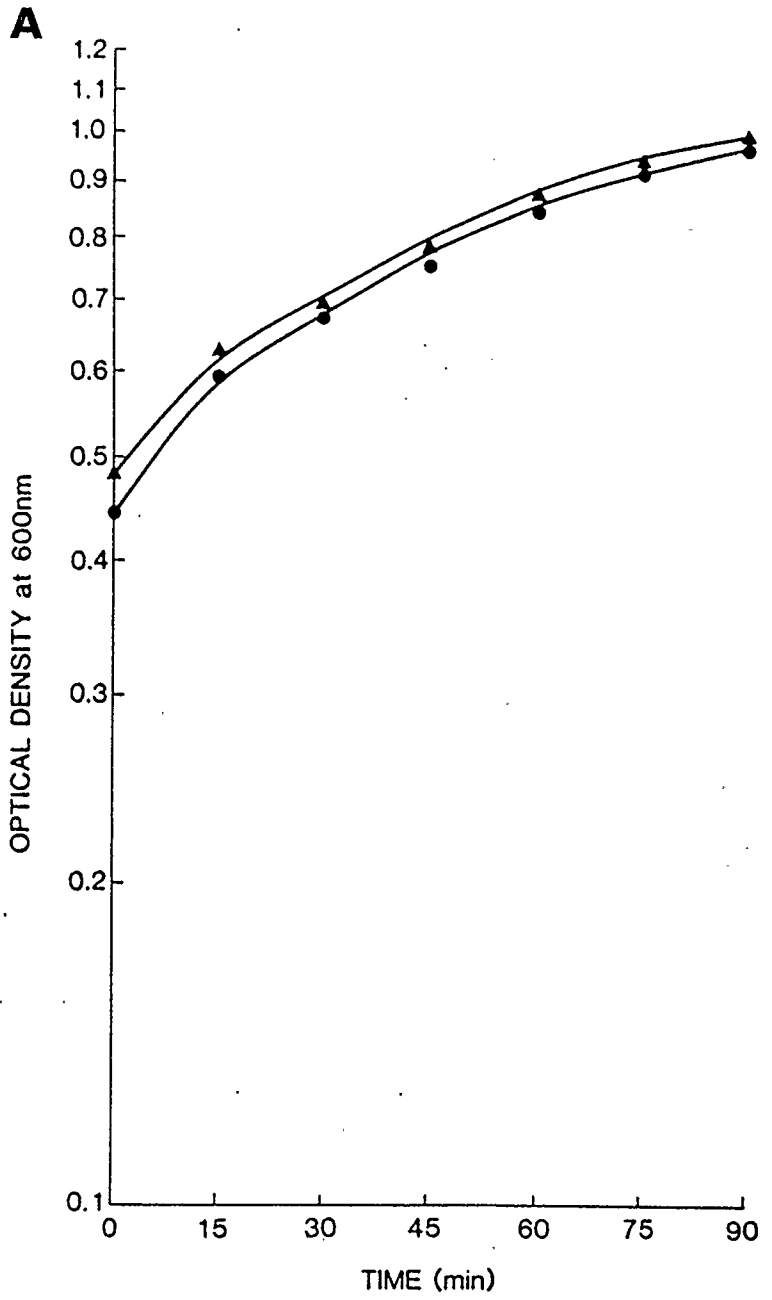
ND = not determined

equilibrium established within a minute. Despite this, no apparent effect on *E. coli* SA 1306 growth rate for a period up to 90 min was seen upon treatment of cells with 1, 5, 10 or 20 μg/ml enoxacin for 2 min, followed by a wash and cell resuspension in an equal amount of medium (Fig. 2a). Cells were exposed to higher concentrations of 20, 40, 60 and 80 μg/ml for a period of time ranging from 10 to 120 min before washing cells free of enoxacin at 10-min intervals (data not shown). This study showed that a period of 40 to 50 min of treatment with the drug before washing and cell resuspension was needed to produce a significant effect (a one-third or greater reduction in doubling time) on cell growth. At lower enoxacin concentrations such as 1, 5 and 10 μg/ml, a significant decrease in cell growth was observed at 5 and 10 μg/ml after a 60-min drug-cell exposure before washing and resuspension (Fig. 2b). These studies show that enoxacin association with *E. coli* SA 1306 is not an irreversible process, drug molecules are not firmly bound to the target(s), and that the growth rate of enoxacin-treated *E. coli* cells can be recovered providing that drug-cell exposure does not exceed 40 to 45 min.

3.2.1.2 *E. coli* cell viability studies.

Cell viability studies were performed in order to define the potential role of the drug efflux system mechanism (see Section 3.2.1.5) in the killing activity

FIG. 2A. Effect of enoxacin pretreatment for a period of 2 min at a concentration of 20 μg/ml on *E. coli* SA 1306 growth in Nutrient Broth at 35°C for a period of 90 min with shaking. Bacterial cells have been washed once and resuspended in equal volumes of the original volume of NB used for cell incubation. Symbols: ●, no enoxacin, ▲, 20 μg/ml enoxacin.


A
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
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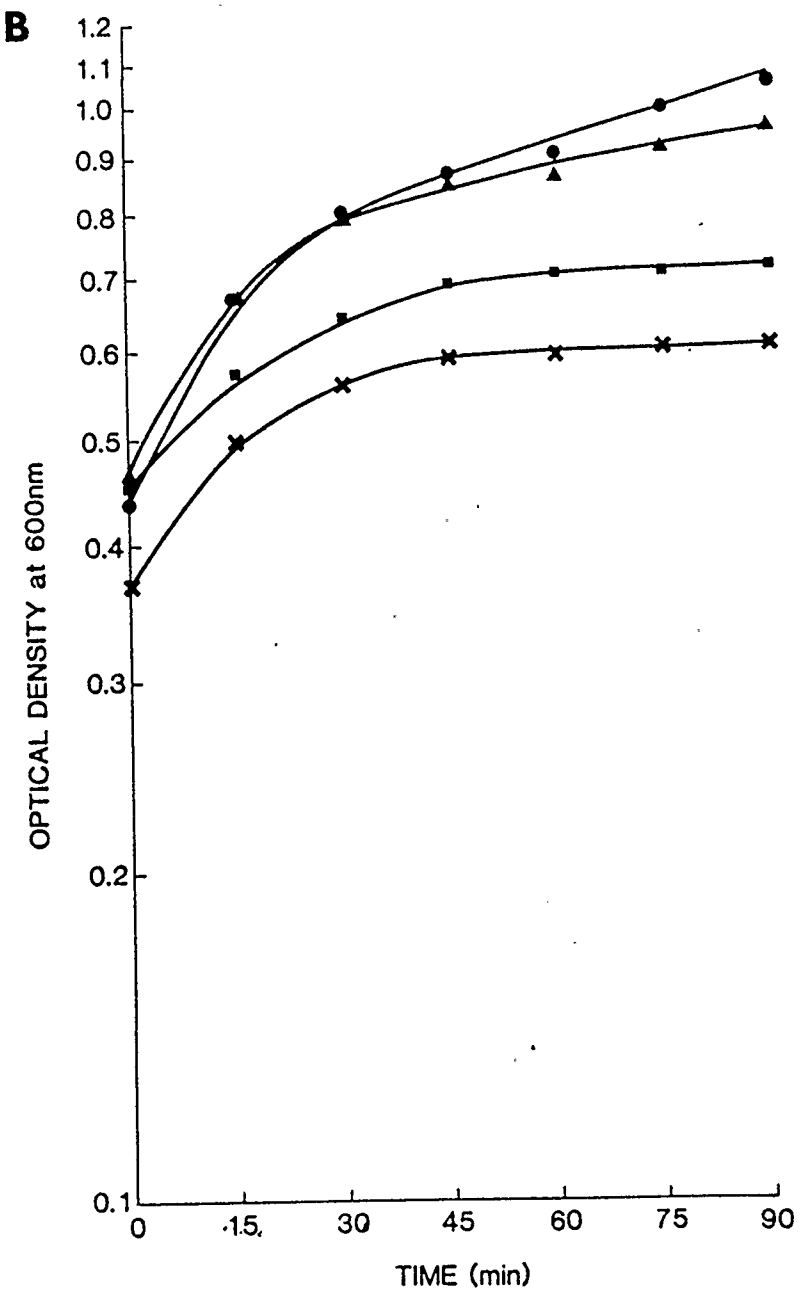
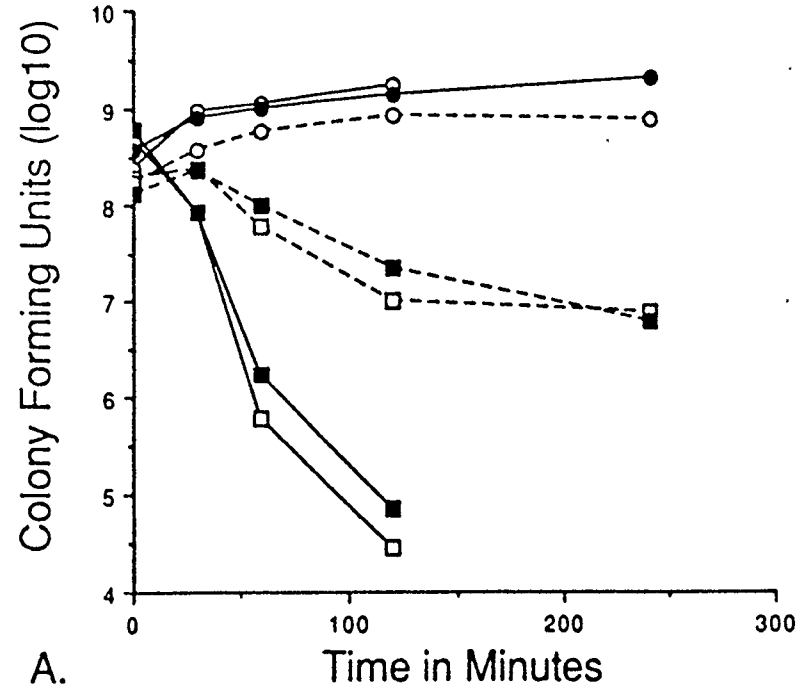
OPTICAL DENSITY at 600nm
0
15
30
45
60
75
90
TIME (min)

FIG. 2B. Effect of enoxacin pretreatment for a period of 60 min at concentrations of 1, 5, or 10 μg/ml on *E. coli* SA 1306 growth in Nutrient Broth at 35°C for a period of 90 min with shaking. Bacterial cells have been washed once and resuspended in equal volumes of the original volume of NB used for cell incubation. Symbols: ●, no enoxacin, ▲, 1 μg/ml; ■ 5 μg/ml; x, 10 μg/ml.

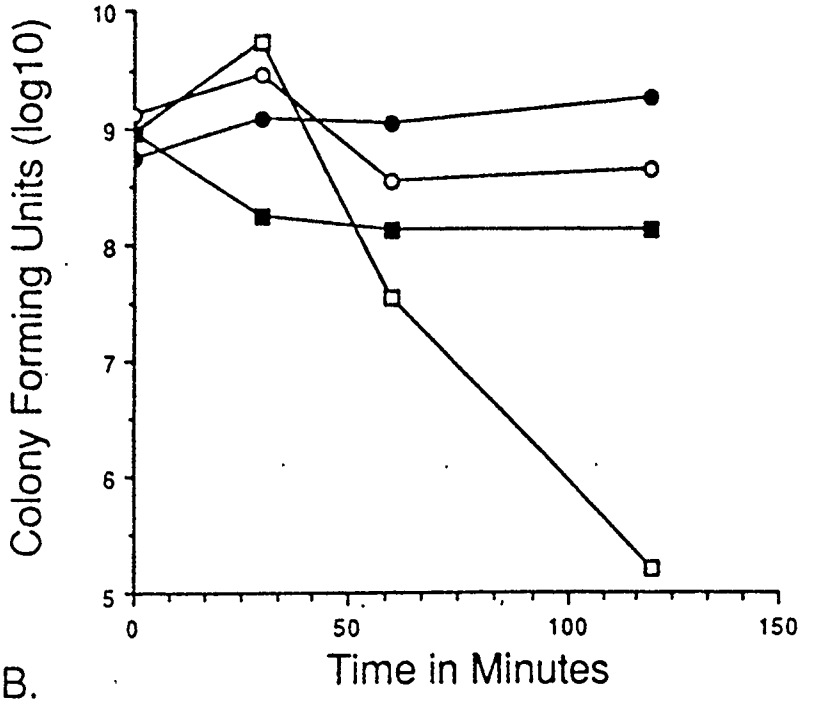

B
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
OPTICAL DENSITY at 600nm
0
15
30
45
60
75
90
TIME (min)

of ciprofloxacin and to investigate the possibility of energy requirement for this process. The capability of ciprofloxacin to exert its bactericidal activity was studied by exposing *E. coli* J5-3 at drug concentrations of 0.05, and 0.1 μg/ml with and without sodium azide (1 mM), an electron transport inhibitor and in the presence or absence of CCCP (20 μM), an inhibitor of the proton motive force. No effect on cell viability was observed at the concentrations used for the energy inhibitors alone. The presence of sodium azide had no effect (Fig. 3a) on killing. However, CCCP reduced the killing capability of ciprofloxacin by greater than 2 log units (Fig. 3b). This study demonstrates that energy derived from a proton motive force system is part of the biological processes involved in the bactericidal activity of ciprofloxacin in *E. coli* J5-3 strain. A similar result was obtained with 2,-4 DNP on the bactericidal activity of nalidixic acid in *E. coli* cells (Cook *et al.*, 1966). Most probably, the drug efflux system (Bryan *et al.*, 1989; Cohen *et al.*, 1988) is not a detrimental factor to the killing activity of ciprofloxacin, since despite an increased drug accumulation observed when this mechanism is blocked (see Section 3.2.1.5), the extent of cell killing is reduced. However, there are likely several factors in determining cell killing activity of ciprofloxacin other than drug accumulation which are affected by CCCP; for instance,

FIG. 3. Cell viability studies of *E. coli* J5-3 strain in BHIB. Cells were pretreated with 0.05 and 0.1 μg/ml ciprofloxacin in the presence or absence of 20 μM CCCP or 1 mM NaN_3 for incubation periods of 0, 30, 60, 90, 120, and 240 min. Thereafter bacteria were centrifuged, resuspended in saline and viable counts performed as given in the Methods. **(A)** Symbols: 0---0 control, no additions; 0——0 , and ●——●, NaN_3 only added; □---□ , ciprofloxacin at 0.05 μg/ml; ■---■ , ciprofloxacin at 0.05 μg/ml with NaN_3; □——□ , ciprofloxacin at 0.1 μg/ml; ■——■ , ciprofloxacin at 0.1 μg/ml with NaN_3. **(B)** Symbols: 0, control, no additions; ●, CCCP only added; □, ciprofloxacin at 0.1 μg/ml; ■ , ciprofloxacin at 0.1 μg/ml with CCCP.


Colony Forming Units (log10)
10
9
8
7
6
5
4
0
100
200
300
Time in Minutes

A.


Colony Forming Units (log10)
10
9
8
7
6
5
0
50
100
150
Time in Minutes

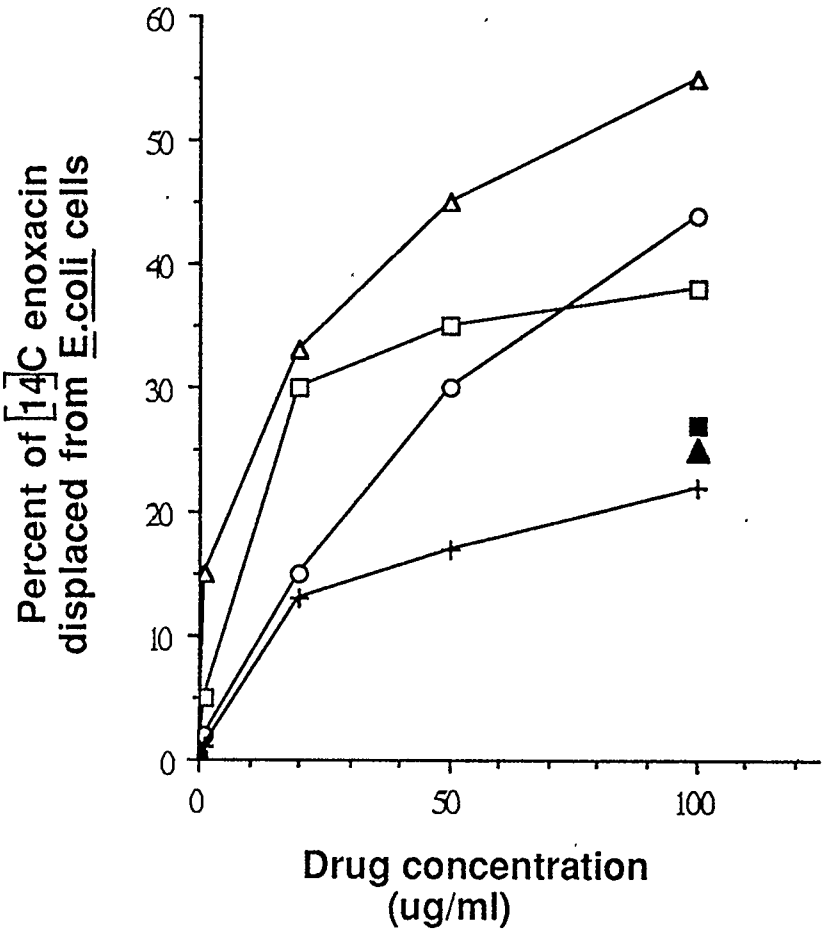
B.

the cell SOS DNA repair system also is most likely to be a candidate. Sodium azide did not either reduce or increase cell killing, but has been shown to increase ciprofloxacin accumulation by blocking quinolone efflux. Thus, it is unlikely that the process of efflux significantly reduces killing under the conditions tested here.

3.2.1.3 Characteristics of association of quinolone with *E. coli* cells.

These investigations were performed by studying the profile of [^{14}C] enoxacin displacement, and the effect of pH on the amount of [^{14}C] enoxacin associated with *E. coli* SA 1306. From uptake studies it was concluded that a relatively large amount of drug is loosely bound to various components of the bacterial cell (see Section 3.2.1.4). We were interested in characterizing the nature of such interactions between *E. coli* SA 1306 and radiolabeled [^{14}C] enoxacin. Cells were pretreated with CCCP before the addition of the drug in order to avoid any interference caused by an active fluoroquinolone efflux system (Bryan *et al.*, 1989; Cohen *et al.*, 1988a). At 2 μg/ml of [^{14}C] enoxacin, drug-cell association was found to be partly reversed by the addition of increasing concentrations of cold enoxacin, ciprofloxacin, or norfloxacin (Figure 4). At 100 μg/ml, 45 to 65 percent of [^{14}C] enoxacin as compared with the control sample was found to be associated with *E. coli* SA 1306.

FIG. 4. Effect of various concentrations of unlabeled structural analogs, enoxacin, ciprofloxacin, norfloxacin, and nalidixic acid on the amount of [^{14}C] enoxacin (2 μg/ml) associated with *E. coli* SA 1306 at 35°C. Also shown, the effect of calcium and piperazine at concentrations of 100 μg/ml. The assays were performed in Nutrient Broth at pH 7.2, cells were pretreated with 20 μM CCCP for 2 min before drug addition. Symbols: □ , enoxacin; o, ciprofloxacin; △ , norfloxacin; +, nalidixic acid; ▲ , piperazine; ■ , calcium.


Percent of [14]C enoxacin displaced from E.coli cells
60
50
40
30
20
10
0
0
50
100
Drug concentration (ug/ml)

Displacement to a lesser but similar extent was also observed with nalidixic acid, piperazine and calcium. Nalidixic acid is capable of chelating divalent cations and piperazine is a component of each of the fluoroquinolones tested and has pka values of 5.55 and 9.81. A much smaller effect was obtained with chloramphenicol, which is not a structural analog (data not shown). At 50 or 100 μg/ml fluoroquinolone concentrations, displacement of [^{14}C] enoxacin was proportionally less than lower concentrations, although partial drug displacement was still observed. This phenomenon might be explained by the fact that the concentration used for [^{14}C] enoxacin and cold quinolones are far below the amount of drug required to saturate all cell binding sites.

Scatchard plots also demonstrated that the nature of such drug-cell interactions was not specific (data not shown). Consequently, even at high drug concentrations, [^{14}C] enoxacin would not be easily displaced from a bacterial cell.

Studies of the amount of [^{14}C] enoxacin associated with *E. coli* intact cells at a concentration range of 1 to 50 μg/ml at pH 4.5 (drug positively charged) and pH 10.0 (drug negatively charged) demonstrated an increase of 18% and a decrease of 25%, respectively, compared to values obtained at pH 7.2. Altogether, these results, indicate that the chelation ability and the positive

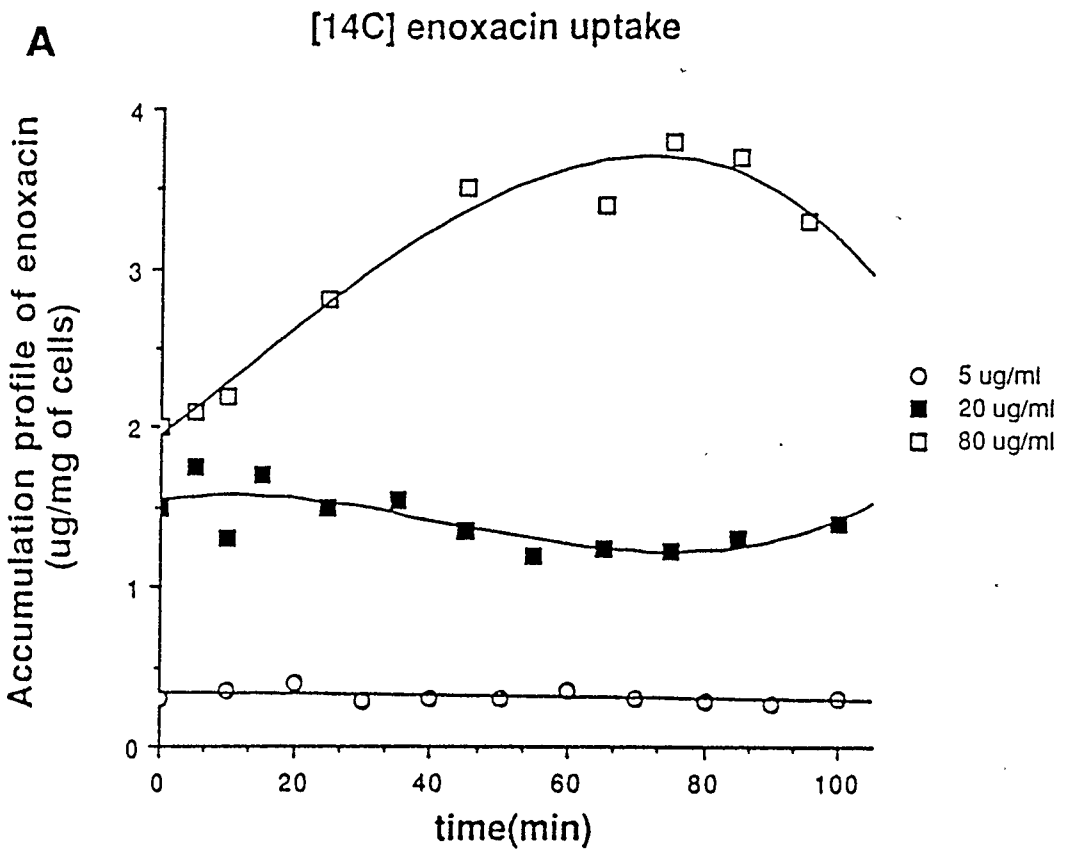
charge of the piperazine substitution of fluoroquinolones are important components involved in the process of drug-cell interactions.

3.2.1.4 Accumulation profile studies: enoxacin.

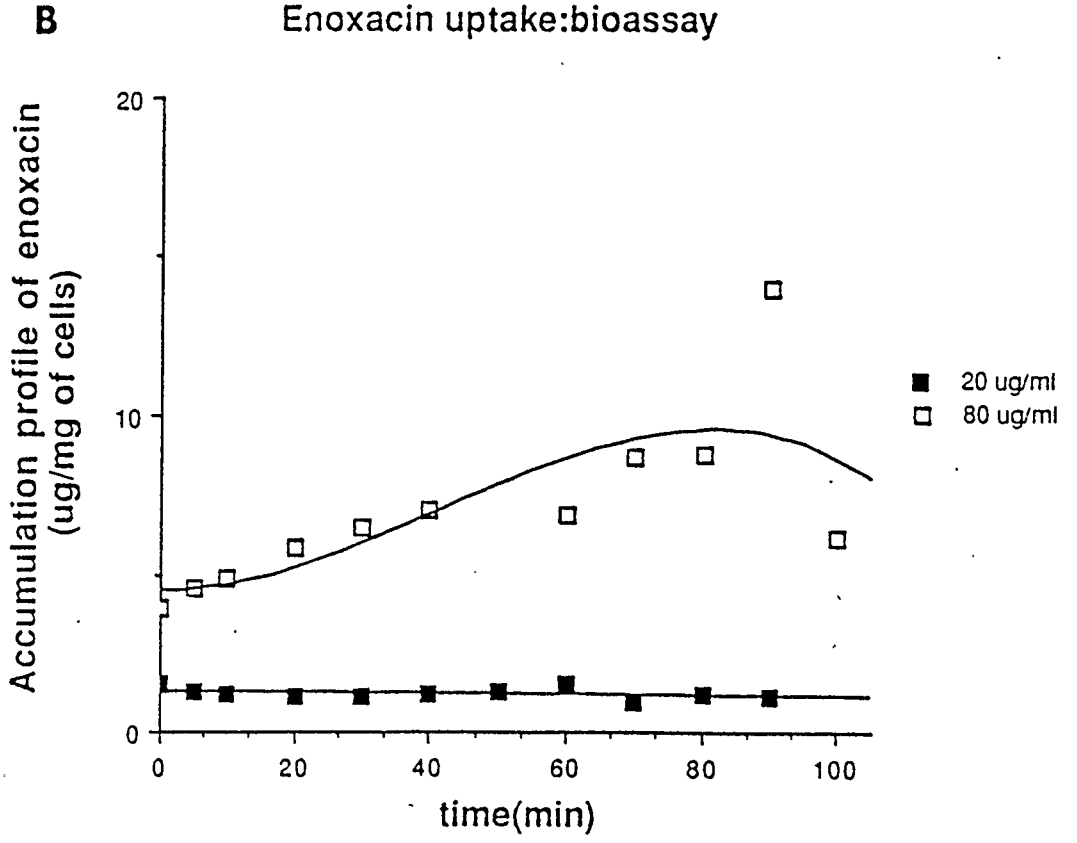
Time-dependent uptake of enoxacin by *E. coli* SA 1306 cells. These studies showed a similar pattern of drug accumulation using two different methods: [^{14}C] enoxacin and the bioassay methods (Figure 5a,b). The amount of enoxacin associated with cells was shown to be influenced by the volume and the number of wash steps used in the uptake protocol. For instance, the amount of cell-associated enoxacin was found to be reduced to a value of 20 to 80% with 10 ml or two repetitions of 1 ml NB wash fluid as compared with the washing regimen used in the standard protocol. Enoxacin uptake performed at drug concentrations up to 20 μg/ml showed the same characteristic of rapid initial uptake followed by a flat uptake pattern seen over a period of time of 90 min. At higher drug concentrations such as 80 μg/ml, time-dependent increased uptake was observed which was associated with a 10 to 20% decrease in optical density, indicating a cell lysis phenomenon.

FIG. 5: Profile of enoxacin accumulation in *E. coli* SA 1306 in NB at 35°C with shaking using **(A)** the [^{14}C] enoxacin uptake protocol and **(B)** enoxacin Bioassay method. **(A)** Symbols: o, 5 μg/ml enoxacin; ■ , 20 μg/ml; □ , 80 μg/ml. **(B)** Symbols: ■ , 20 μg/ml enoxacin; □ , 80 μg/ml.

A


[14C] enoxacin uptake
Accumulation profile of enoxacin (ug/mg of cells)
0
1
2
3
4
0
20
40
60
80
100
time(min)
5 ug/ml
20 ug/ml
80 ug/ml

B


Enoxacin uptake:bioassay
Accumulation profile of enoxacin (ug/mg of cells)
0
10
20
0
20
40
60
80
100
time(min)
20 ug/ml
80 ug/ml

Effect of temperature, energy inhibitors, and a structural analog on enoxacin uptake. Cell uptake was characterized using Lineweaver-Burk plots based on 1-min uptake samples. At concentration ranges of 0.5 to 5 μg/ml (Figure 6) or 10 to 150 μg/ ml (Figures 7 - 8), the plots showed an intercept of zero, indicating a lack of saturability of the transport system. Decreased uptake was observed at 4°C compared to 37°C with *E. coli* SA 1306 cells (Figure 6). The presence of sodium azide, carbonylcyanide m-chlorophenylhydrazone, sodium arsenate, or sodium fluoride did not inhibit the enoxacin uptake, but instead, a slight increase was generally observed comparing no inhibitor to an inhibitor present, especially the protonophores 2,4-DNP and CCCP (Table 3). Moreover, competition studies with unlabeled ciprofloxacin demonstrated no effect on the amount of enoxacin associated with cells using concentration 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, supporting the fact that the uptake process was not a carrier-mediated phenomenon. Time-dependent uptake of enoxacin observed (data not shown) with heat-killed *E. coli* (cells were treated at 70°C for 20 min) showed the same pattern as viable cells, but with a 20 to 30% decrease of drug uptake observed which might be attributed to partial

FIG. 6: Lineweaver-Burk plots of enoxacin uptake in NB at 4 and 37°C after 1 min of drug exposure over a concentration range of 0.5 to 5 μg/ml by *E. coli* SA 1306 strain. Initial rates were based on one-minute values because uptake was so rapid that earlier readings could not be determined.

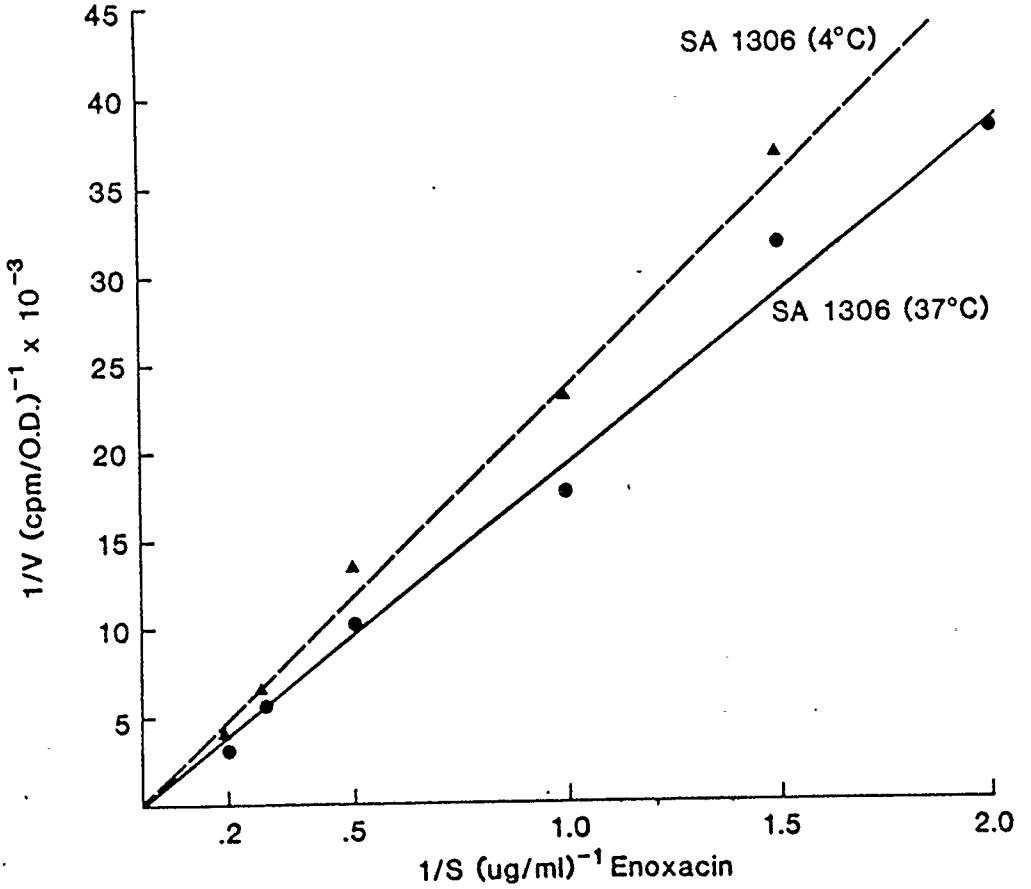

45
40
35
30
25
20
15
10
5
1/V (cpm/O.D.)$^{-1}$ x 10^{-3}
SA 1306 (4°C)
SA 1306 (37°C)
.2
.5
1.0
1.5
2.0
1/S (ug/ml)$^{-1}$ Enoxacin

FIG. 7: Lineweaver-Burk plots of enoxacin uptake in Nutrient Broth at 37°C after 1 min drug exposure over a concentration range of 10 to 150 μg/ml by *E. coli* J5-3, JB-5R and SA 1306 strains. Initial rates were based on one-minute values because uptake was so rapid that earlier readings could not be determined.

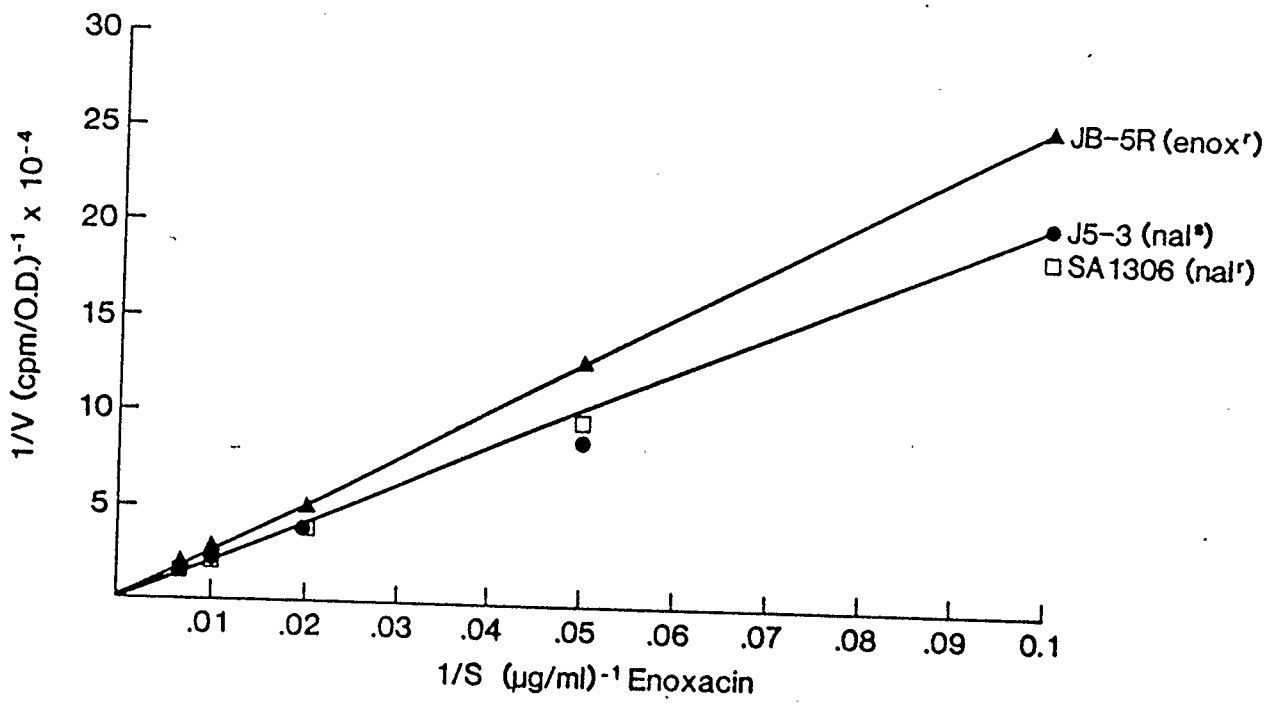

30
25
20
15
10
5
1/V (cpm/O.D.)$^{-1}$ x 10^{-4}
.01
.02
.03
.04
.05
.06
.07
.08
.09
0.1
1/S (µg/ml)$^{-1}$ Enoxacin
JB-5R (enox^r)
J5-3 (nal^s)
SA1306 (nal^r)

FIG. 8: Lineweaver-Burk plots of enoxacin uptake in Nutrient Broth at 37°C after 1 min drug exposure over a concentration range of 10 to 150 μg/ml by *E. coli* JF-strain derivatives. Initial rates were based on one-minute values because uptake was so rapid that earlier readings could not be determined.

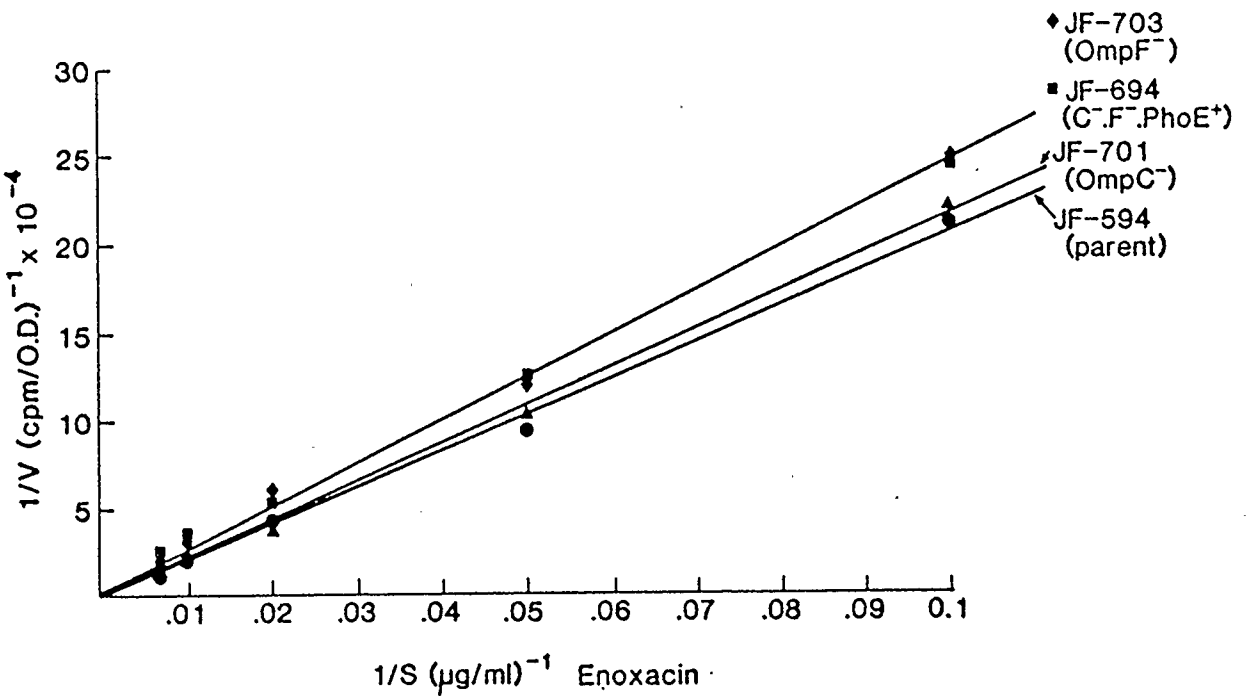

JF-703
(OmpF⁻)
JF-694
(C⁻.F⁻.PhoE⁺)
JF-701
(OmpC⁻)
JF-594
(parent)
30
25
20
15
10
5
1/V (cpm/O.D.)⁻¹ x 10⁻⁴
.01
.02
.03
.04
.05
.06
.07
.08
.09
0.1
1/S (μg/ml)⁻¹ Enoxacin

TABLE 3. EFFECT OF CELL ENERGY INHIBITORS ON ENOXACIN UPTAKE IN *E. COLI* SA 1306.

Energy inhibitor used	Concentration (Molar)	Enoxacin uptake[a] (cpm/O.D. unit) at given time (minute)			
		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]Concentration used: 20 μg/ml

denaturation of porins at this high temperature. This phenomenon has been shown to occur in presence of SDS (Nikaido and Vaara, 1985).

Role of porin and DNA gyrase in enoxacin uptake. Uptake studies with an *E. coli* enoxacin-resistant mutant JB-5R (Tables 1 and 2) selected by multistep passage of the parental strain *E. coli* SA 1306 in several concentrations of enoxacin (Figure 7), as well as studies with isogenic *E. coli* JF derivatives exhibiting various phenotypes with regard to porins (Figure 8) demonstrated that the F porin plays an important role in *E. coli* for susceptibility and resistance to fluoroquinolones. In fact, lower uptake was observed for JB-5R (OmpF decreased) (Figure 7) and for strains JF-694 (Omp C^-, Omp F^-) and JF-703 ($OmpF^-$) (Figure 8). The porin profile of J5-3 and JF derivatives was confirmed by studying the outer membrane protein pattern on SDS-PAGE (Figure 9). The functional effect of the diminished amount of F porin in *E. coli* JB-5R was shown by decreased chloramphenicol uptake and susceptibility and decreased tetracycline susceptibility observed in this strain compared to the parent SA 1306 (Figure 10, Table 4a). It has been well documented that the F porin plays a role with respect to *E. coli* susceptibility to chloramphenicol and tetracycline (Chopra and Eccles, 1978). *E. coli* J5-3 and its DNA gyrase mutant SA 1306 showed no difference with regard to

FIG. 9: Outer membrane protein profile of *E. coli* J5-3 derivatives (J5-3, SA 1306 and JB-5R) and *E. coli* JF-derivatives (568, 694 [$OmpC^-$, $OmpF^-$], 701 [$OmpC^-$] and 703 [$OmpF^-$]) on 12.5% SDS-PAGE.

12.5 % SDS-PAGE OUTER MEMBRANE PROTEINS

E. coli

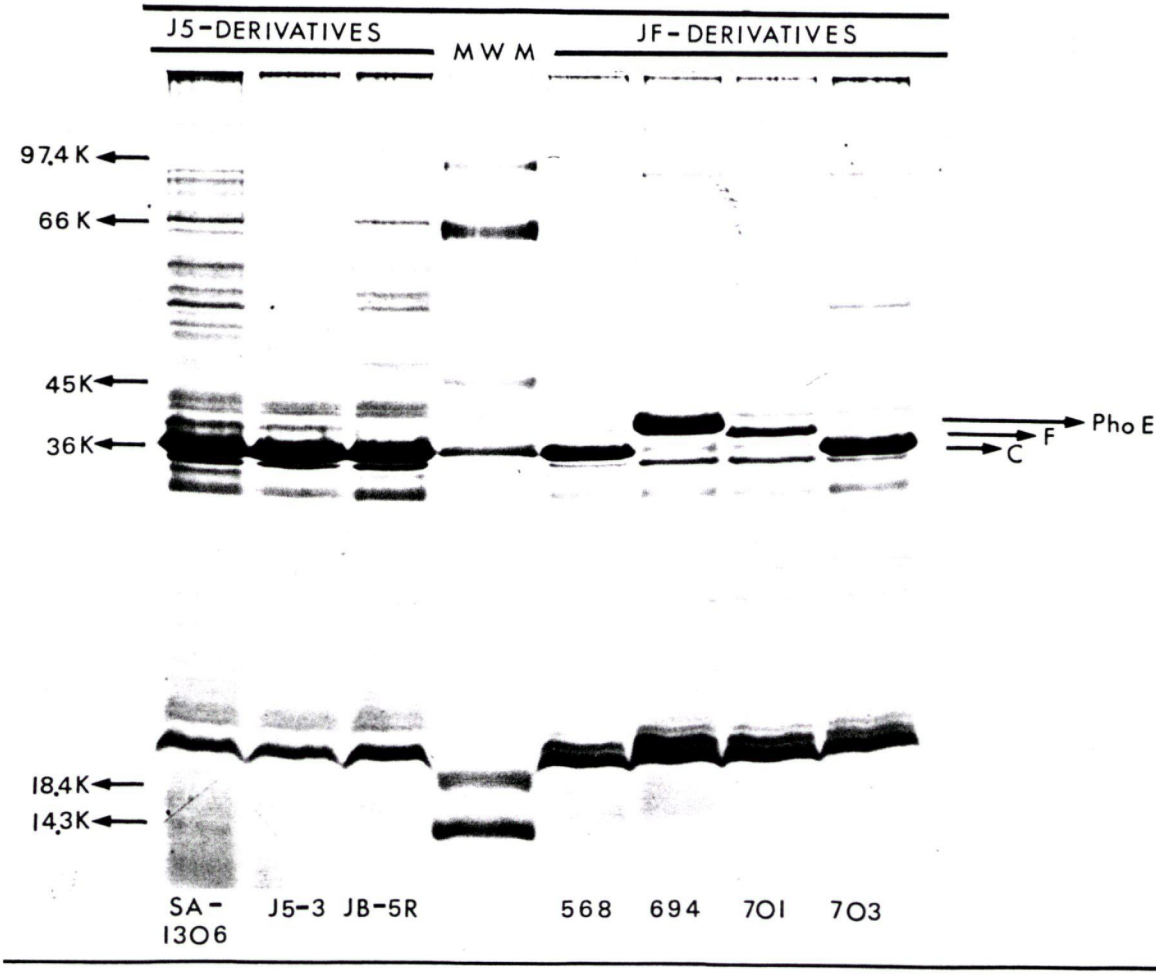


FIG. 10: Accumulation profile of chloramphenicol at a concentration of 500 μg/ml for a period of 60 min at 35°C in Nutrient Broth with shaking by *E. coli* SA 1306 and JB-5R strains. Symbols: ■ , *E. coli* SA 1306; △ , *E. coli* JB-5R.

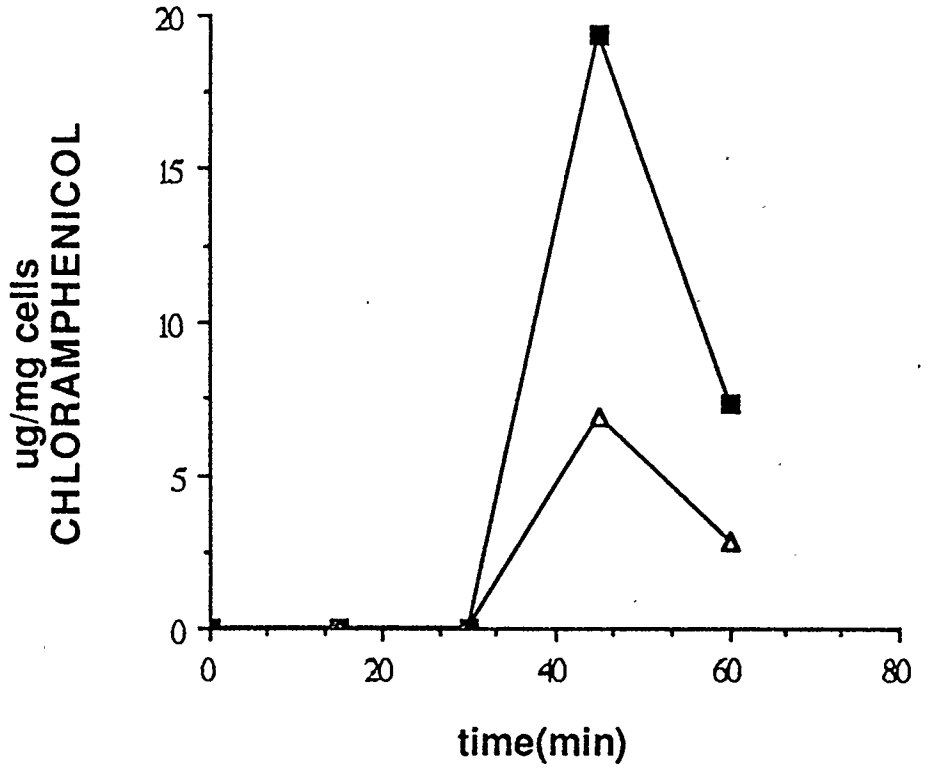

ug/mg cells
CHLORAMPHENICOL
20
15
10
5
0
0
20
40
60
80
time(min)

TABLE 4A. PORINS IN E. COLI: DISC SUSCEPTIBILITY STUDIES

Inhibition zone size (mm) to tetracycline and chloramphenicol for E. coli SA 1306 and JB-5R on tryptic soy agar.

Drug	E. coli Strains	
	SA 1306	JB-5R
tetracycline[a]	15.5	10.5
chloramphenicol[b]	19.0	0

[a]amount of drug per disk: 20 µg

[b]amount of drug per disk: 10 µg

enoxacin uptake, showing that the drug's target DNA gyrase is not an important cell component determining the magnitude of drug uptake. It is interesting to note that the *gyr*A mutation in *E. coli* SA 1306 produced a greater effect on nalidixic acid MIC with 40- to 80-fold increase compared to *E. coli* J5-3, whereas enoxacin MIC was only increased by 2- to 4-fold (Table 2b).

Effect of pH on susceptibility of *E. coli* JF-derivative strains to quinolones. The amount of F porin also correlated with the susceptibility of *E. coli* JF-derivatives to other quinolones at physiological pH, since *E. coli* JF 701 and JF 703 consistently exhibited higher and lesser susceptibility to quinolones, respectively, compared to the JF 568 parent strain (Table 4b) at pH 7.0. This was consistent with the outer membrane profile on SDS-PAGE of JF 701 and JF 703, which showed an increased and reduced amount of F porin, respectively, compared to JF 568 (Figure 9). Despite the 18% increase of the amount of [^{14}C] enoxacin associated with *E. coli* cells at pH 4.7 (Section 3.2.1.3), the antimicrobial activity of enoxacin and other quinolones was found to be decreased to various extents at lower pH (4.7) compared to physiological pH (7.0) for *E. coli* JF derivatives (Table 4b). This suggests that the binding component, but not the drug influx, is increased at acidic pHs. At basic pH (9.2), the bioactivity of the most hydrophilic

TABLE 4B. PORINS IN E. COLI: DISC SUSCEPTIBILITY STUDIES

Inhibitory zone size (mm) to quinolones for E. coli JF strain 568 and derivatives 701 and 703 in tryptic soy agar, pH 4.7, 7.0, and 9.2

Quinolone Tested	pHs:	E. coli JF Strains								
		568 (parent)			701 ($OmpF^+C^-$)			703 ($OmpF^-C^+$)		
		4.7	7.0	9.2	4.7	7.0	9.2	4.7	7.0	9.2
enoxacin[a]		0	22.0	24.4	0	23.0	24.4	0	20.5	24.8
ciprofloxacin[a]		15.0	26.5	31.5	16.1	28.0	31.5	14.4	24.5	33.2
norfloxacin[a]		9.6	23.0	28.0	1.1	26.5	28.0	0	21.0	30.0
fleroxacin[a]		20.1	26.5	20.5	22.6	27.5	20.5	18.8	23.5	18.8
ofloxacin[a]		13.0	24.5	23.8	12.3	27.5	23.8	14.0	23.0	21.5
difloxacin[a]		21.3	21.5	9.0	21.7	23.5	9.0	19.6	21.5	9.0
A56-620[a]		13.4	23.5	25.0	15.0	26.5	24.6	13.3	23.0	22.6
nalidixic acid[b]		14.5	16.5	16.3	15.0	16.5	17.1	14.5	15.0	15.3

[a]amount of drug per disk: 5 µg

[b]amount of drug per disk: 20 µg

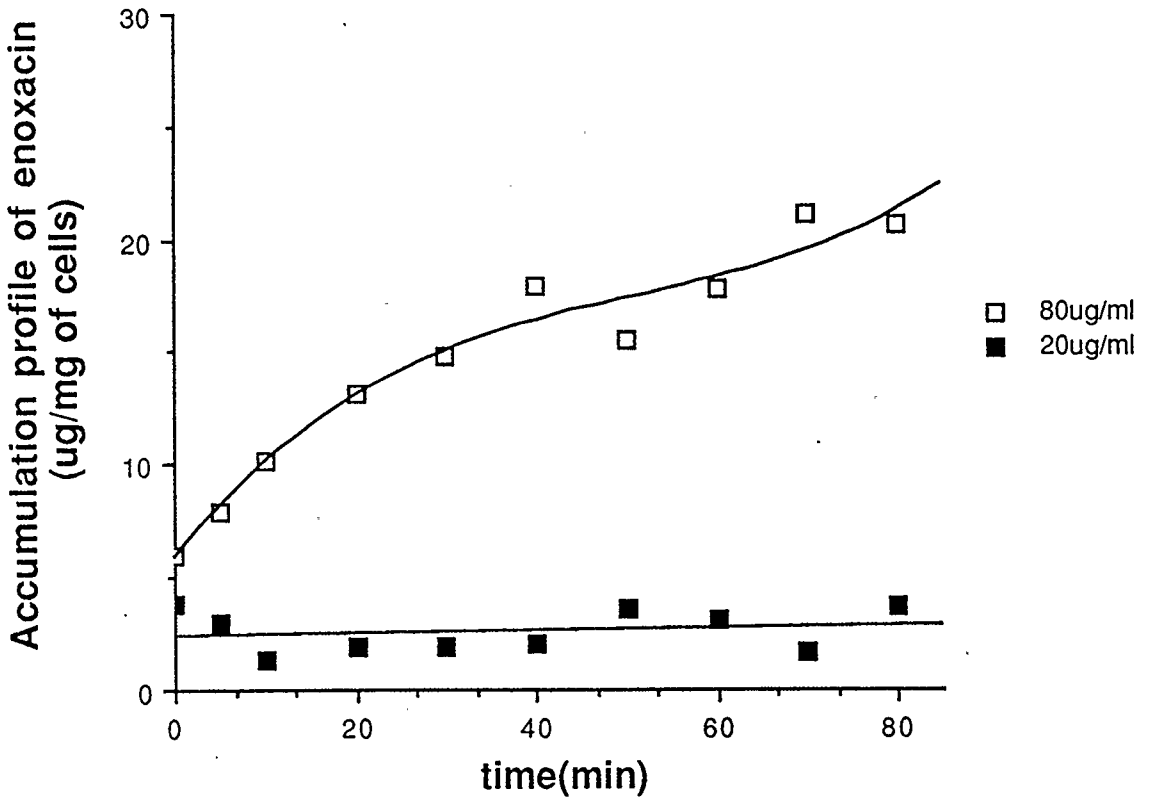
quinolones (enoxacin, ciprofloxacin, norfloxacin) seemed to be enhanced. However, reduced cell growth rate observed at this pH made interpretation difficult.

Enoxacin accumulation profile in *B. subtilis*. The nature of the enoxacin uptake process in *B. subtilis* was found to be very similar to the uptake characteristics found in *E. coli*. Time-dependent uptake at 20 μg/ml (Figure 11) showed the same pattern of rapid initial uptake followed by no further uptake at 20 μg/ml or lower concentrations. At a higher concentration of 80 μg/ml, increased uptake was seen with time after the initial rapid uptake phase (Figure 11). The uptake process was also not saturable up to 80 μg/ml enoxacin (data not shown). However, quantitative differences were noticed, the amount of enoxacin associated with *B. subtilis* was about twice the amount observed for *E. coli* at equivalent drug concentrations.

3.2.1.5 Accumulation profile studies: ciprofloxacin.

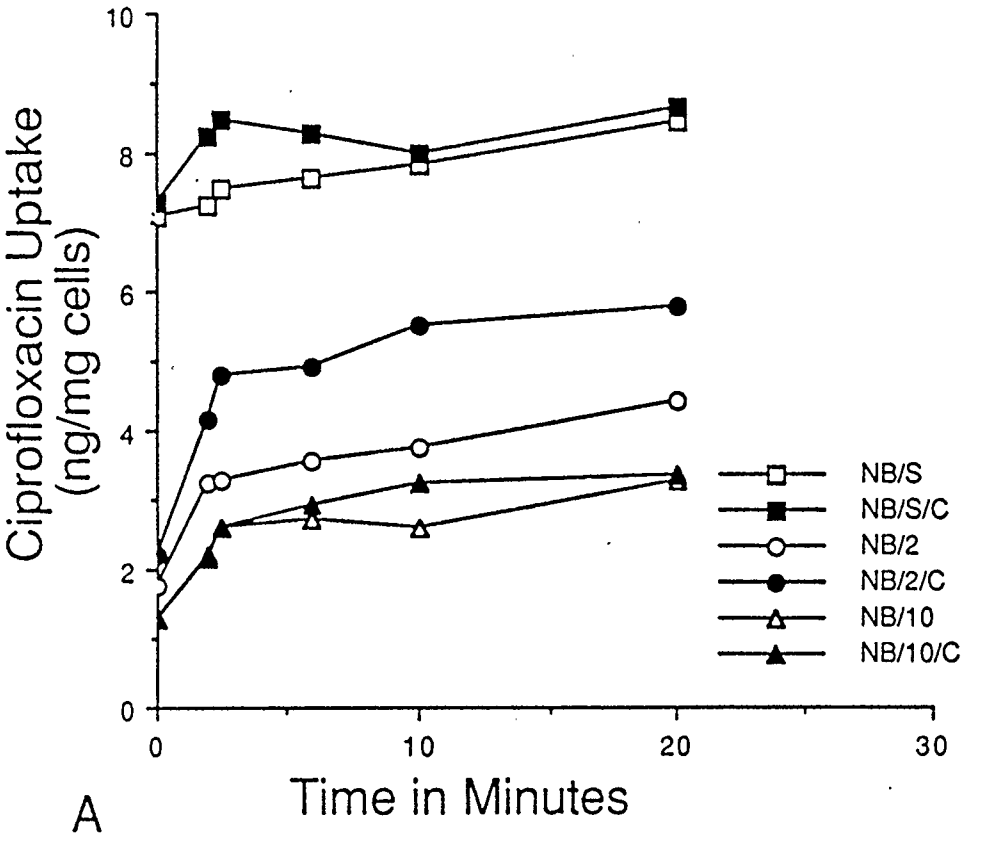
The process of ciprofloxacin permeation in *E. coli* was found to be very similar to enoxacin uptake despite the fact that a higher density of cells was used in the ciprofloxacin uptake protocol. The amount of ciprofloxacin associated with cells was greatly influenced by the washing regimens, for higher cell-associated counts were observed with minimal washing. When filtered cells were

FIG. 11: Accumulation profile of enoxacin by *Bacillus subtilis* in Nutrient Broth at 35°C with shaking over a period of 80 min. Symbols: ■ , 20 μg/ml enoxacin; □ , 80 μg/ml.

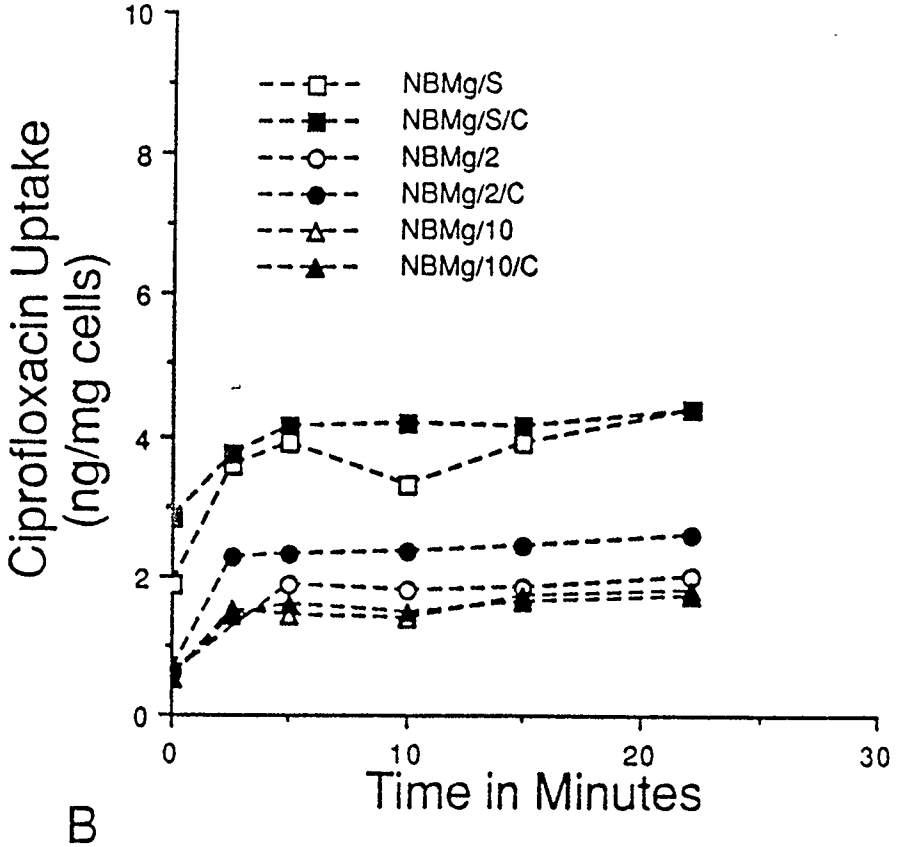

Accumulation profile of enoxacin
(ug/mg of cells)
30
20
10
0
0
20
40
60
80
time(min)
80ug/ml
20ug/ml

washed with a larger volume of medium or when magnesium was added to the medium, time-dependent drug influx could be observed between 0 and 150 seconds since lower cell counts were obtained at zero time (Figures 12a, b). Zero time counts represent the binding phase. Ciprofloxacin, at 0.154 μg/ml was rapidly associated with *E. coli* cells with an equilibrium established at least within 150 seconds. The slight increase observed between 0 and 150 seconds was dependent on the washing regimen and also on the cell density used for the uptake measurements. Cells pretreated with the protonophore CCCP at 20 μM exhibited a slight, but consistent increase of ciprofloxacin uptake in nutrient broth. However, a greater differential amount of drug accumulation was observed in BHIB (Figure 13a). The CCCP effect occurred independently of the addition of magnesium (Figures 12, 13). The ciprofloxacin accumulation was also found to be markedly reduced in the presence of 10 mM $MgCl_2$ in nutrient broth (compare Figure 12a to Figure 12b) and to a lesser extent in brain heart infusion broth (Figure 13a, 13b) independent of the washing regimen. Uptake studies in BHIB showed the same pattern of ciprofloxacin accumulation in *E. coli* cells as observed in NB, but the amount of drug was signigicantly reduced, presumably due to a higher magnesium content found in BHIB with a value determined using fluorophotometry of 0.17 mM compared to 0.06 mM found in NB (Figure 12, 13). The accumulation of ciprofloxacin in BHIB was

FIG. 12: Accumulation profile of ciprofloxacin at a concentration of 0.154 μg/ml with time at 35^{o}C in **(A)** NB and in **(B)** NB with 20 mM $MgCl_2$ by *E. coli* J5-3. Effect of the addition of 20 μM CCCP on the uptake process is also shown. Legend: NB, Nutrient Broth; S, minimal washing; 2, 2-ml wash; 10, 10-ml wash; C, 20 μM CCCP added; Mg, 10 mM $MgCl_2$ added.

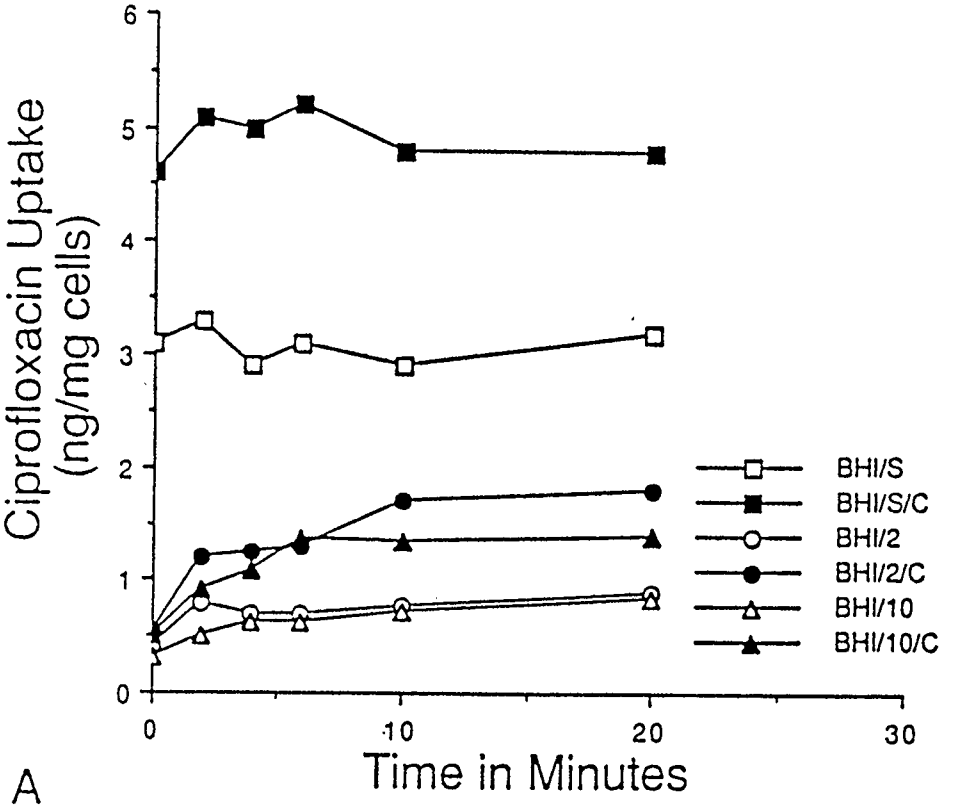

Ciprofloxacin Uptake
(ng/mg cells)
10
8
6
4
2
0
0
10
20
30
Time in Minutes
NB/S
NB/S/C
NB/2
NB/2/C
NB/10
NB/10/C

A

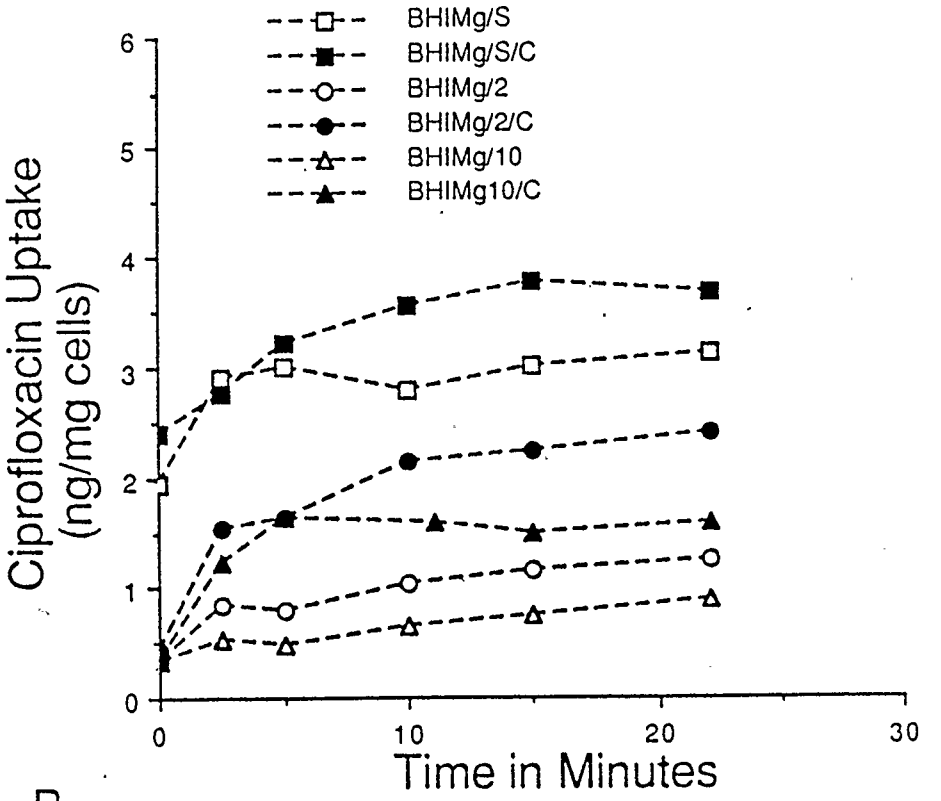

Ciprofloxacin Uptake
(ng/mg cells)
10
8
6
4
2
0
0
10
20
30
Time in Minutes
NBMg/S
NBMg/S/C
NBMg/2
NBMg/2/C
NBMg/10
NBMg/10/C

B

FIG. 13: Accumulation profile of ciprofloxacin at a concentration of 0.154 μg/ml with time at 35^{o}C in **(A)** BHIB and in **(B)** BHIB with 10 mM $MgCl_2$ by *E. coli* J5-3 under minimal, 2-ml and 10-ml washing conditions. Effect of the addition of 20 μM CCCP on the uptake process is also shown. Legend: BHI, Brain Heart Infusion; S, minimal washing; 2, 2-ml wash; 10, 10-ml wash, c, 20 μM CCCP added; Mg, 10 mM $MgCl_2$ added.


Ciprofloxacin Uptake
(ng/mg cells)
6
5
4
3
2
1
0
0
10
20
30
Time in Minutes
BHI/S
BHI/S/C
BHI/2
BHI/2/C
BHI/10
BHI/10/C

A

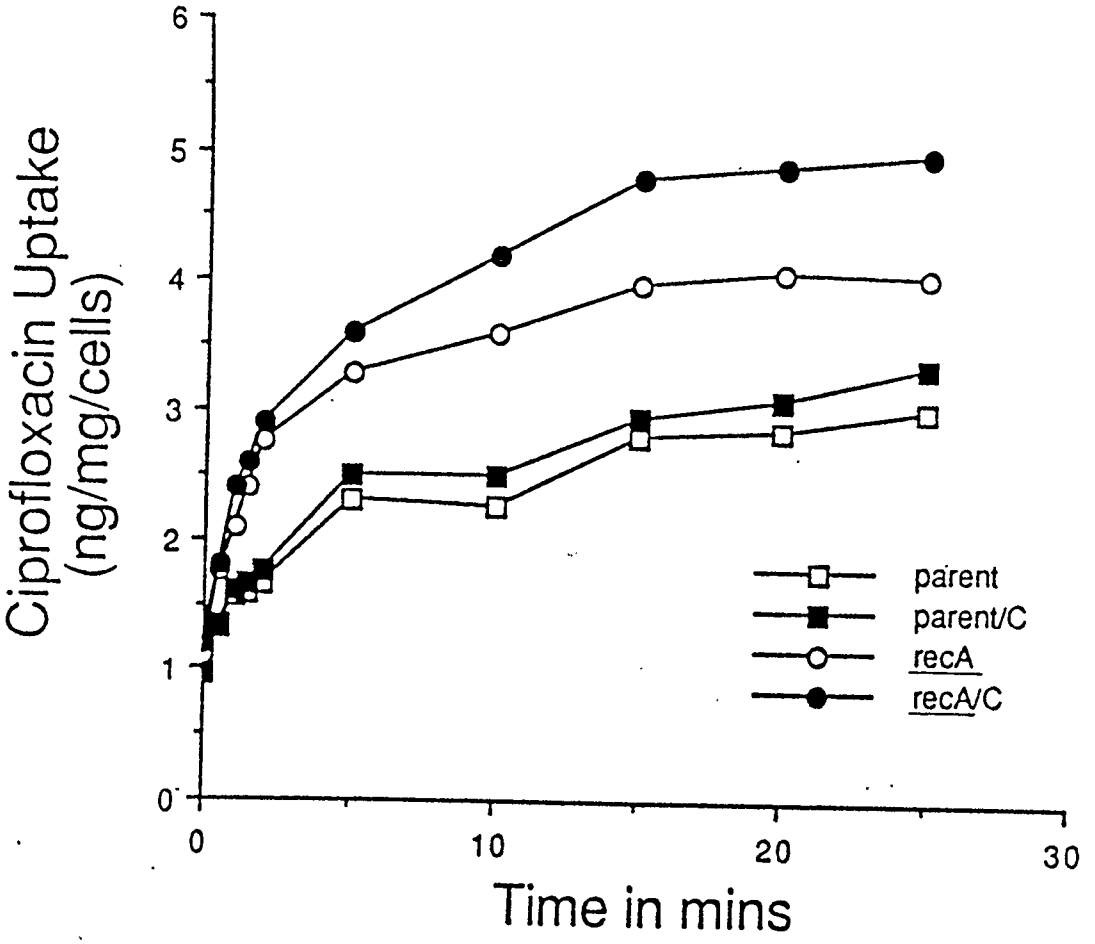

BHIMg/S
BHIMg/S/C
BHIMg/2
BHIMg/2/C
BHIMg/10
BHIMg10/C
Ciprofloxacin Uptake
(ng/mg cells)
0
1
2
3
4
5
6
0
10
20
30
Time in Minutes

B

also slightly increased with *E. coli* cells pretreated with CCCP (Figure 13b). Studies of the effect of *rec*A mutation on ciprofloxacin accumulation in *E. coli* were also performed since increased nalidixic acid susceptibility was observed with *rec*A mutants (McDaniel, *et al.*, 1978), and also such mutations have been found to lead to changes in the outer membrane protein profile (Garvey *et al.*, 1985). The latter may be the explanation for increased uptake of ciprofloxacin observed in an *E. coli* K-12 SAB3005 (a *rec*A mutant) as compared with the parent strain (Figure 14).

From these studies, it can be stated that the uptake mechanism used for enoxacin and ciprofloxacin in *E. coli* cells is a simple diffusion system. This conclusion is supported by the fact that an equilibrium is very rapidly established (Figure 5, 12, 13). The Lineweaver-Burk plots intercept at zero, demonstrating the nonsaturable nature of this process (Figures 6, 7, 8, 18); uptake is reversible, influenced by temperature (Figure 6), slightly reduced in heat-killed *E. coli* cells; and not reduced by various energy inhibitors (Table 3, Figures 12, 13). In *E. coli*, the F porin plays an important role in the uptake process of fluoroquinolones (Figures 7, 8) which contributes to the level of cell susceptibility and resistance to these antimicrobial agents. Interestingly, despite the fact that the results obtained are consistent with a mechanism of diffusion being involved in *E. coli*

FIG. 14: Comparative study of the accumulation profile of ciprofloxacin at a concentration of 0.154 μg/ml with time at 35°C with shaking under 2-ml washing conditions by *E. coli* M1174 (*rec*A+) and SAB 3005 (*rec*A) strains in Nutrient Broth with and without 20 μM CCCP. Legends: parent, *E. coli* M1174; *rec*A, *E. coli* SAB 3005; c, 20 μM CCCP added.


Ciprofloxacin Uptake
(ng/mg/cells)
6
5
4
3
2
1
0
0
10
20
30
Time in mins
parent
parent/C
recA
recA/C

for the ciprofloxacin permeation process, the amount of drug retained with the cells after a 10-ml wash was about 1 μg/ml of cells at a concentration used for uptake of 0.154 μg/ml. Therefore, concentration of ciprofloxacin within the cell would be about 0.6 μg/ml, approximately four times the extracellular concentration. The same phenomenon was observed in the case of norfloxacin in *E. coli* cells (Hooper *et al.*, 1989), and in human polymorphonuclear leucocytes for norfloxacin (Zweerink and Edison, 1988), ciprofloxacin (Easmon and Crane, 1985), and ofloxacin (Pascual *et al.*, 1989). These results suggest that a portion of fluoroquinolone molecules is trapped or remains firmly bound to various or specific cell components; however, an important fraction of drug is loosely bound as proven by the relatively large amount of drug removed upon more stringent washing conditions.

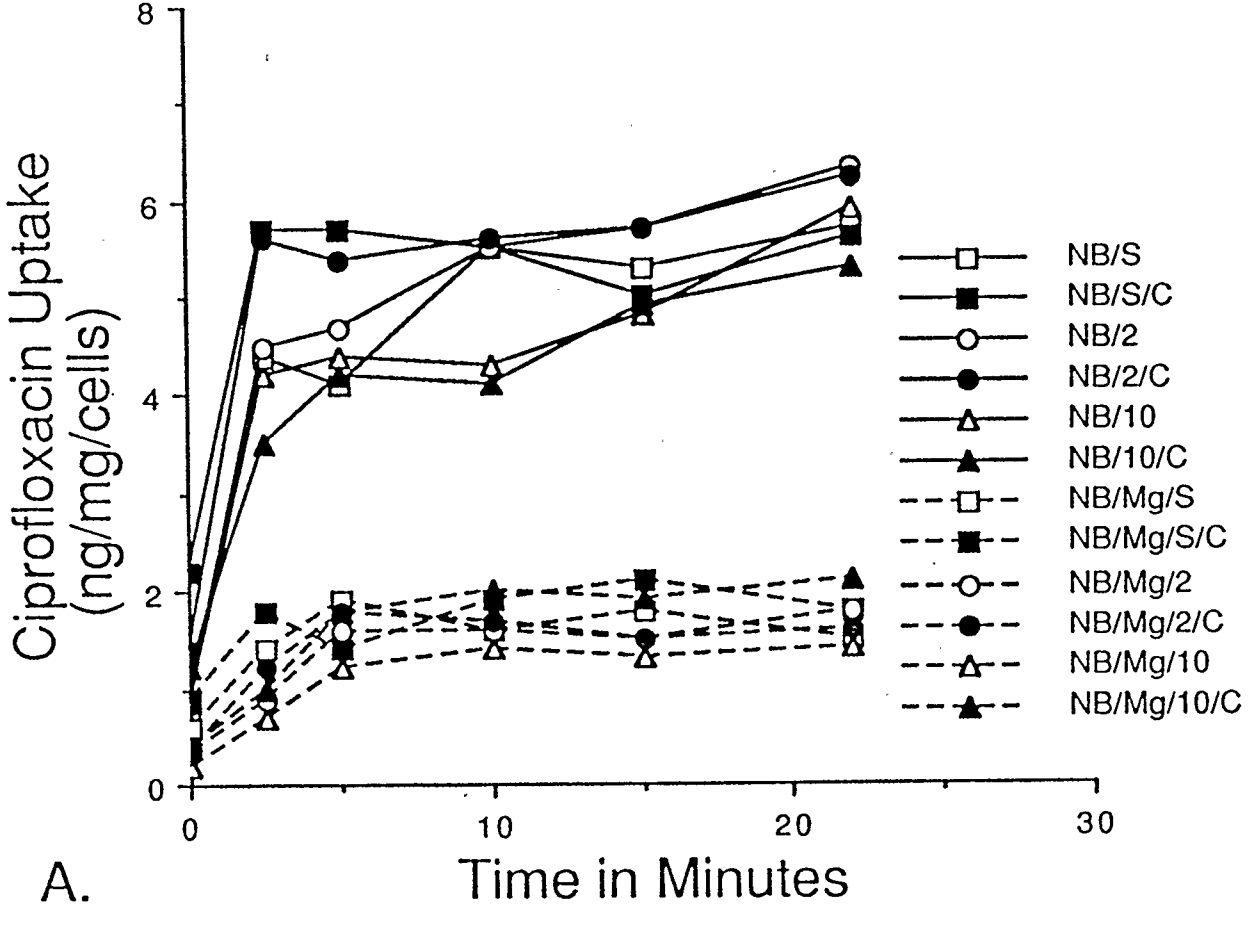
3.2.2 Mechanism of ciprofloxacin permeation in *P. aeruginosa* and *A. faecalis* strains.

3.2.2.1 Accumulation profile of ciprofloxacin in *P. aeruginosa* 503.

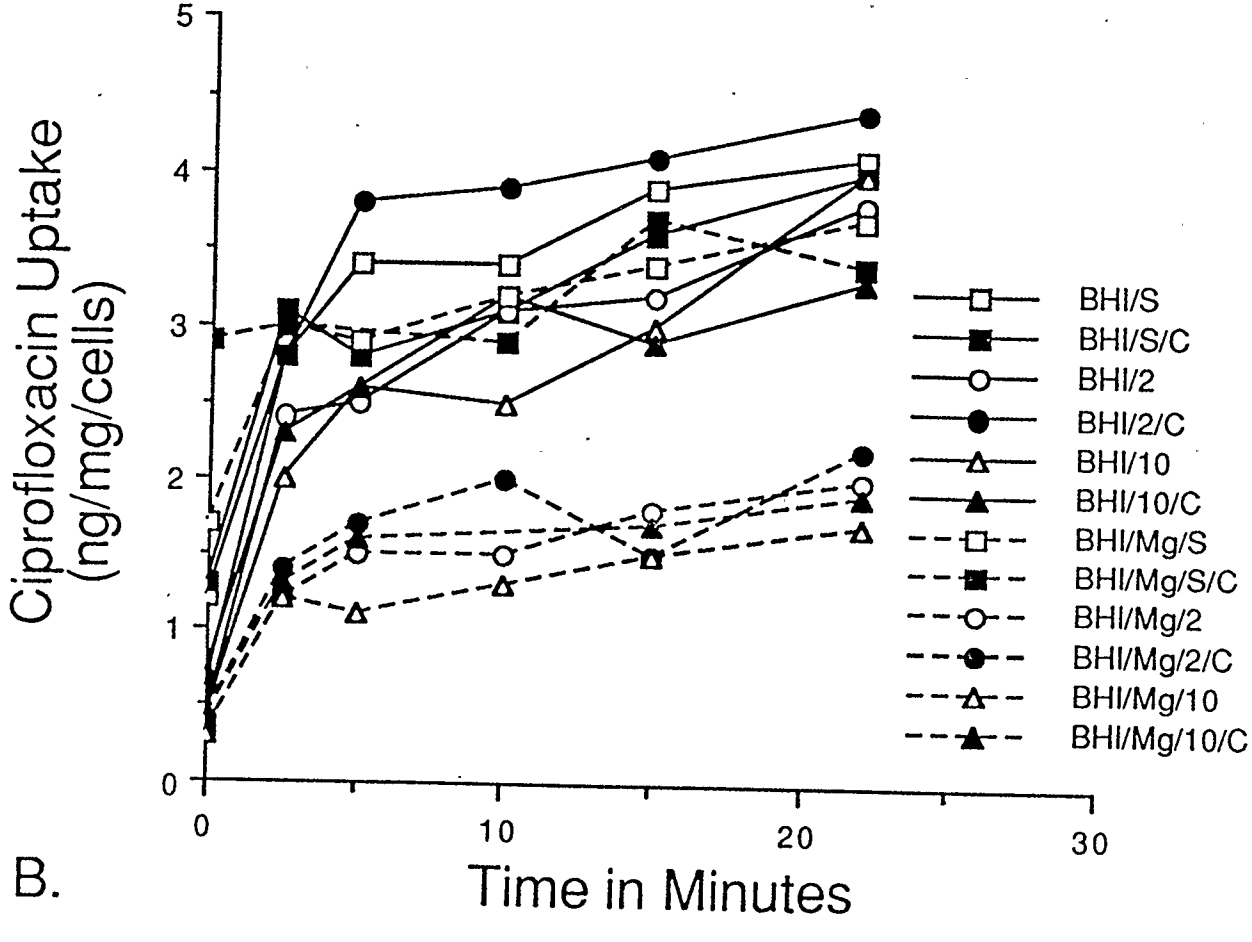
This profile was characterized by a rapid equilibrium established within 150 seconds followed as opposed to *E. coli*, by an obvious influx of drug independent of the washing regimens used (Figure 15). The drug influx rate was greatly reduced upon addition of 10 mM $MgCl_2$ in nutrient broth (Figure 15a), generally, a

lesser effect was observed in BHIB with regard to the uptake phase seen between 0 and 150 seconds (Figure 15b). As previously seen with *E. coli*, the addition of 10 mM magnesium markedly reduced the level of equilibrium achieved for uptakes in NB as well as in BHIB. Moreover, the amount of ciprofloxacin accumulation at equilibrium was reduced in BHIB compared to NB independent of the washing regimen used. Cells pretreated with CCCP were generally associated with a slightly higher uptake in both NB and BHIB (Figure 15). Interestingly, a much smaller effect was obtained with various washing regimens on the amount of drug associated with *P. aeruginosa* in both NB and BHIB (Figure 16) compared to *E. coli* cells. Ciprofloxacin uptake performed with minimal washing at a concentration of 0.154 μg/ ml showed a lower amount of drug associated with *P. aeruginosa* compared with *E. coli*. However, when more extensive washing (2 ml and 10 ml) was used, equal or greater amount of drug was accumulated by *P. aeruginosa* compared to *E. coli* (Figure 16, 17) even at a concentration below MIC (Figure 18). This is consistent with the drug being more firmly bound to an extracytoplasmic site in *P. aeruginosa* than in *E. coli*. The accumulation process of ciprofloxacin presented characteristics of a simple diffusion system process being unsaturable with Lineweaver-Burk plots intercepting zero (Figure 18). As mentioned above, CCCP did not inhibit ciprofloxacin uptake in either *E. coli* or *P.*

FIG. 15: Accumulation profile of ciprofloxacin at a concentration of 0.154 μg/ml with time at 35^{o}C in **(A)** Nutrient Broth and in **(B)** Brain Heart Infusion Broth by *P. aeruginosa* PAO 503 in the presence or absence of 10 mM $MgCl_2$ under minimal, 2-ml, and 10-ml washing conditions. Also shown, the effect of 20 μM CCCP on the uptake process. Legend: NB, Nutrient Broth; BHI, Brain Heart Infusion broth; S, minimal washing; 2, 2-ml wash; 10, 10-ml wash; C, 20 μM CCCP added; Mg, 10 mM $MgCl_2$ added.


Ciprofloxacin Uptake
(ng/mg/cells)
8
6
4
2
0
0
10
20
30
Time in Minutes
NB/S
NB/S/C
NB/2
NB/2/C
NB/10
NB/10/C
NB/Mg/S
NB/Mg/S/C
NB/Mg/2
NB/Mg/2/C
NB/Mg/10
NB/Mg/10/C

A.


Ciprofloxacin Uptake
(ng/mg/cells)
5
4
3
2
1
0
0
10
20
30
Time in Minutes
BHI/S
BHI/S/C
BHI/2
BHI/2/C
BHI/10
BHI/10/C
BHI/Mg/S
BHI/Mg/S/C
BHI/Mg/2
BHI/Mg/2/C
BHI/Mg/10
BHI/Mg/10/C

B.

FIG. 16: Comparative study of the accumulation profile of ciprofloxacin at 0.154 μg/ml by *P. aeruginosa* PAO 503 and *E. coli* J5-3 with time at 35°C in Nutrient Broth under various washing conditions. Symbols: ——, *E. coli*; ---, *P. aeruginosa*; □ , minimal wash; o, 2-ml wash; △ , 10-ml wash.

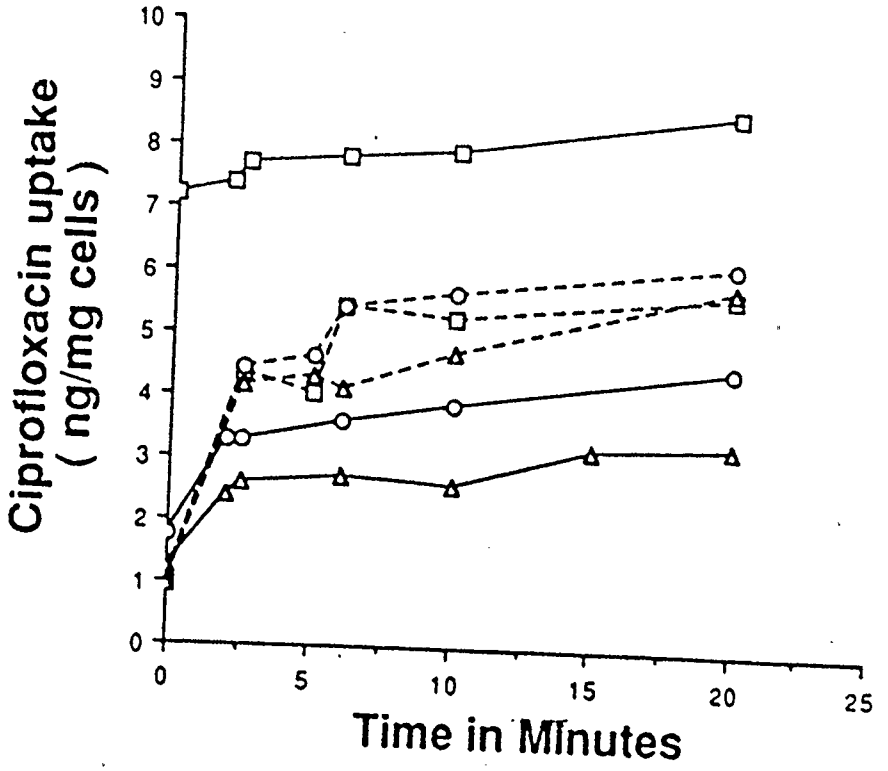

Ciprofloxacin uptake
(ng/mg cells)
10
9
8
7
6
5
4
3
2
1
0
0
5
10
15
20
25
Time in Minutes

FIG. 17: Comparative study of the accumulation profile of ciprofloxacin at 0.154 μg/ml by *E. coli* J5-3 and *P. aeruginosa* PAO 503 with time at 35°C in Nutrient and Brain Heart Infusion Broth under 10-ml washing conditions in the presence or absence of 20 μM CCCP. Symbols:——, *E. coli*; ---, *P. aeruginosa*; △ , NB without CCCP; ▲ , NB with 20 μM CCCP; o, BHIB without CCCP; ●, BHIB with 20 μM CCCP.

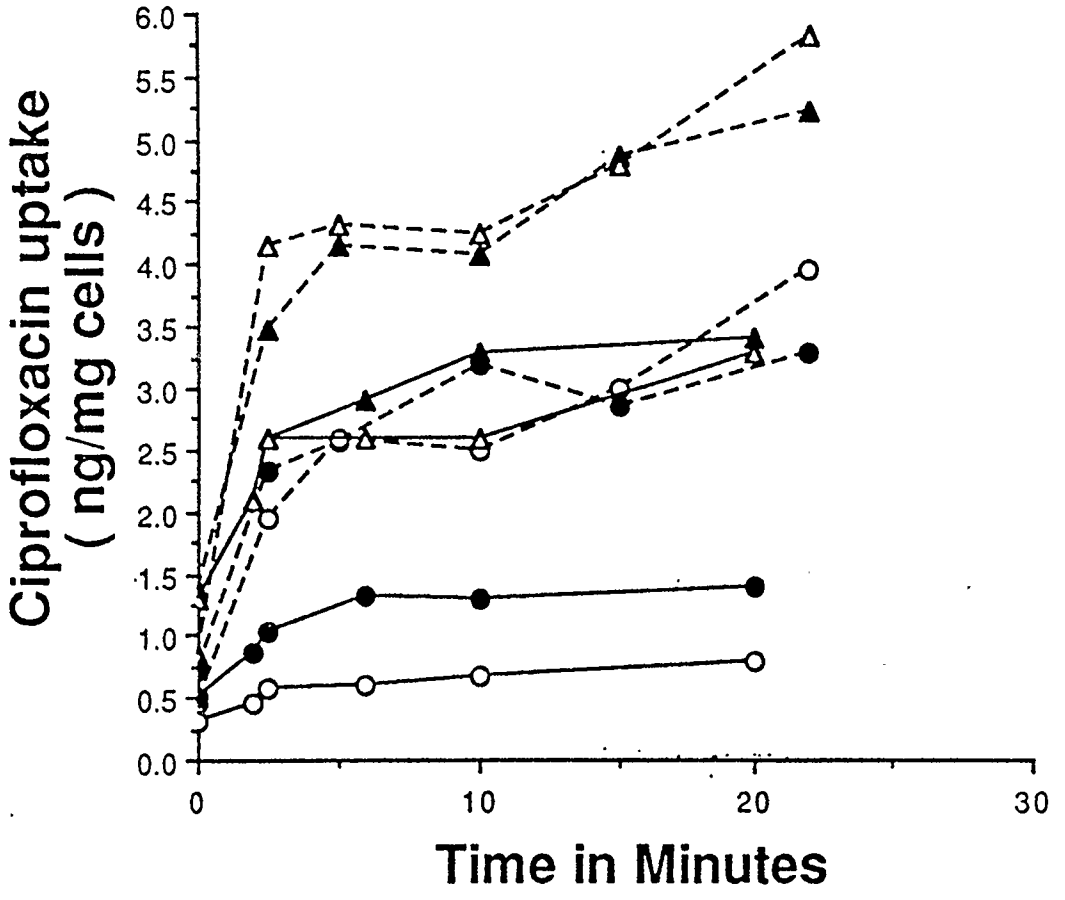
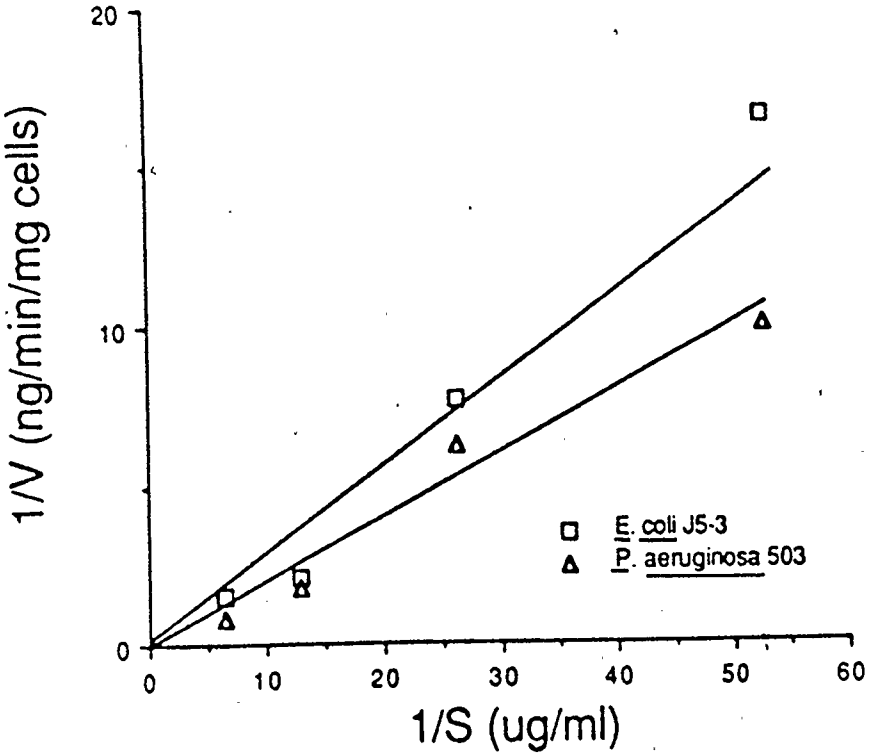

Ciprofloxacin uptake
(ng/mg cells)
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
Time in Minutes

FIG. 18: Lineweaver-Burk plot of ciprofloxacin uptake in Nutrient Broth at 35°C after 2 min drug exposure over a concentration range of 0.019 to 0.154 μg/ml by *E. coli* J5-3 and *P. aeruginosa* PAO 503 strains under 2-ml washing conditions.


20
10
0
0
10
20
30
40
50
60
1/V (ng/min/mg cells)
1/S (ug/ml)
E. coli J5-3
P. aeruginosa 503

aeruginosa. Consistent with results obtained for ciprofloxacin uptake, the presence of 10 mM magnesium also decreased the susceptibility to ciprofloxacin for *E. coli* J5-3, *P. aeruginosa* PAO 503, and *A. faecalis* ATCC 19018 in both NB and BHIB (Table 2a). MICs were usually reduced in BHIB compared to values obtained in NB except for *E. coli* J5-3 which exhibited higher MIC in BHIB without addition of magnesium compared to the MIC obtained in NB under the same conditions.

3.2.2.2 Role of the *P. aeruginosa* outer membrane protein F in ciprofloxacin accumulation.

The role of the outer membrane protein F in *P. aeruginosa* in uptake of ciprofloxacin was investigated using two sets of experiments. Firstly, uptake studies were performed with *P. aeruginosa* PAO 503, and its derivative PCC 23 which is known to possess qualitative and functional changes in protein F. Such a conclusion is supported by several observations including increased resistance to heat denaturation of the mutant F protein as shown by reaction with a monoclonal antibody (MCA 48H3) preferentially recognizing the denatured form. Also, PCC 23 showed reduced susceptibility to many non-quinolone drugs (Godfrey *et al.*, 1986). *P. aeruginosa* PCC 23 showed no significant difference in the total accumulation of drug (Figure 19), despite proven increased susceptibility to quinolones (Table 4c).

FIG. 19: Comparative study of the accumulation of ciprofloxacin at a concentration of 0.154 μg/ml by *P. aeruginosa* PAO 503 and PCC 23 (outer membrane protein F modified) with time at 35°C in Nutrient Broth under 2-ml washing conditions. Cells were pretreated with 20 μM CCCP 2 min before drug addition. Symbols: ■ , *P. aeruginosa* PAO 503; □ , PCC 23.

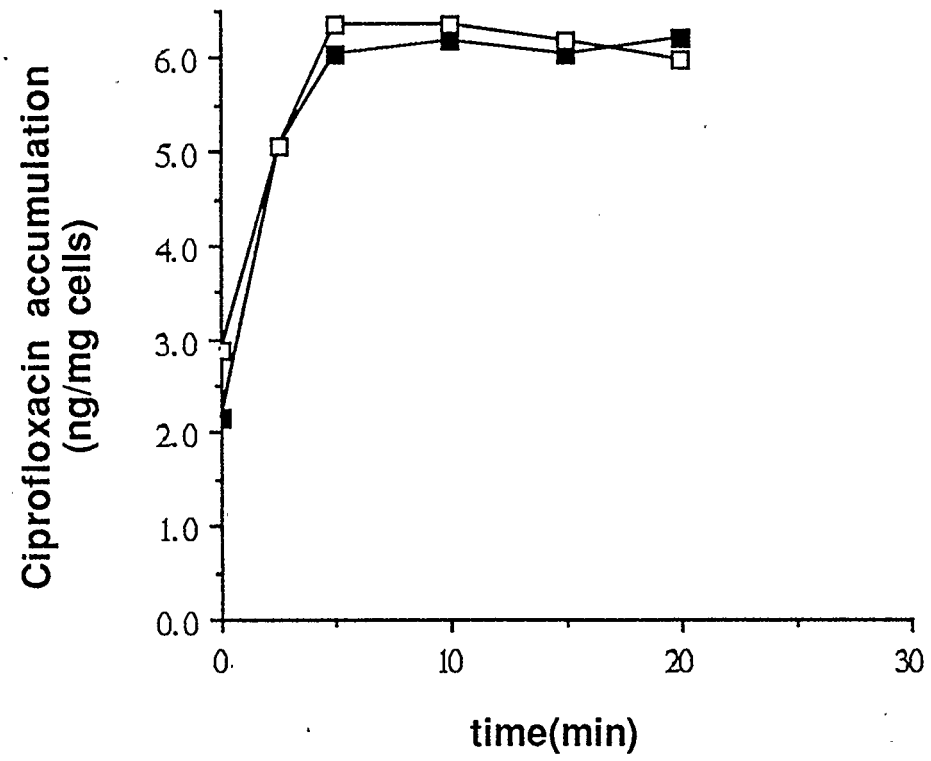

Ciprofloxacin accumulation
(ng/mg cells)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
10
20
30
time(min)

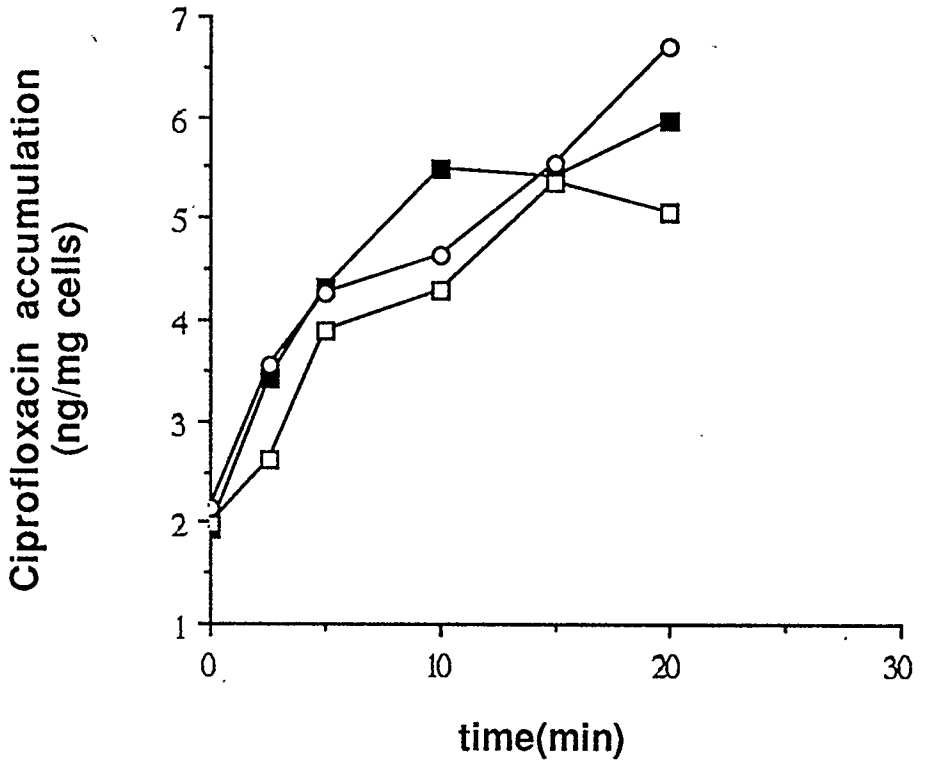
TABLE 4C. Inhibitory zone size (mm) to fluoroquinolones for P. aeruginosa PAO 503 and PCC 23 in tryptic soy agar, pH 7.0

Quinolone Tested	Pseudomonas aeruginosa Strains	
	PAO 503	PCC 23
enoxacin[a]	16.5	19.0
ciprofloxacin[a]	24.0	26.0
norfloxacin[a]	18.0	23.0
fleroxacin[a]	12.5	16.5
ofloxacin[a]	16.0	20.0
difloxaxin[a]	8.5	12.5
A56-620[a]	19.0	22.0

[a]amount of drug per disk: 5 µg

Secondly, no major difference in ciprofloxacin uptake was observed in a set of *P. aeruginosa* clinical strains (Y 4449, Y 4492, Y 4516) isolated from a cystic fibrosis patient before, during and post-therapy with ciprofloxacin (Figure 20). It should be mentioned that the strain isolated during therapy, *P. aeruginosa* Y 4492, exhibited a F^- phenotype, whereas the parent and the revertant (post-therapy) strains showed similar amounts of protein F on SDS-PAGE. Genotyping using the *Pst*I-*Nru*-derived upstream region of exotoxin A as a probe demonstrated that these strains were the same genotype (Ogle *et al.*, 1988). In the uptake studies mentioned above, CCCP was added 2 min prior to drug addition, in order to measure the net amount of drug accumulated at the equilibrium without interference caused by the drug efflux system (Bryan *et al.*, 1989; Cohen *et al.*, 1988a). From these studies it can be concluded that the outer membrane protein F in *P. aeruginosa* does not play a role identical to that of porin F of *E. coli*. Ciprofloxacin upake was not changed in PCC 23 despite enhanced susceptibility, was unchanged in the F^- clinical isolate Y 4492 and in another F^- strain from our laboratory (Chamberland *et al.*, 1990). A structural role for the F protein in the *P. aeruginosa* in the outer membrane has been proposed (Gotoh *et al.*, 1989) and the data in this thesis are more consistent with this view

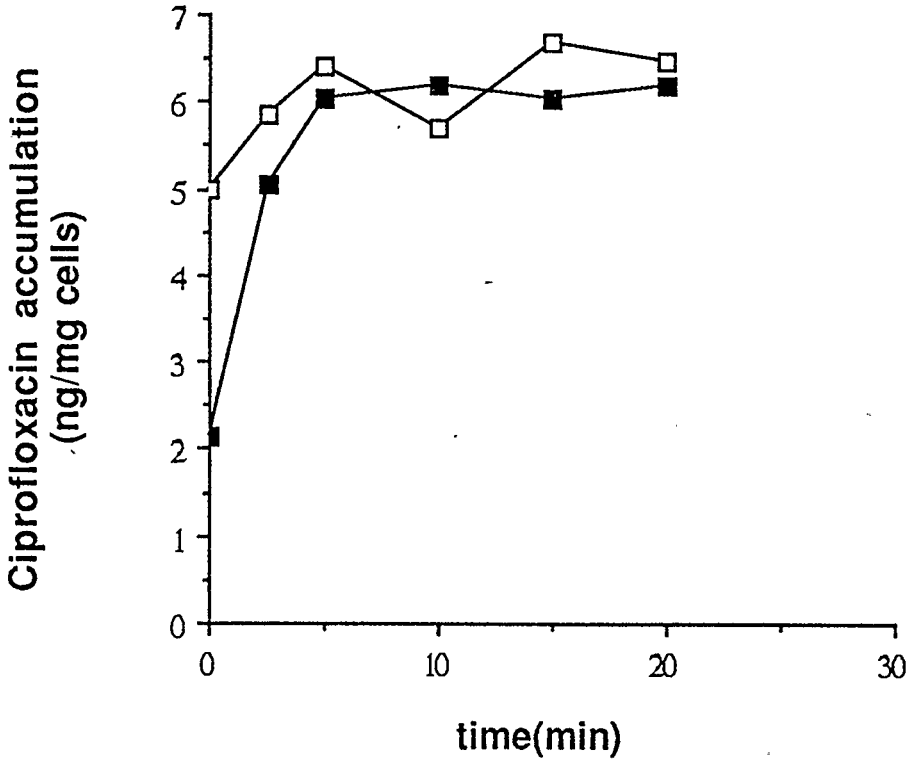
FIG. 20: Comparative study of the accumulation profile of ciprofloxacin at a concentration of 0.154 μg/ml by *P. aeruginosa* clinical isolates Y 4449 (pre-therapy), Y 4492 (during therapy), and Y 4516 (post-therapy) with time at 35°C in Nutrient Broth under 2-ml washing conditions. Cells were pretreated with 20 μM CCCP 2 min before drug addition. Symbols: ■, *P. aeruginosa* Y 4449; □, Y 4492; o, Y 4516.


Ciprofloxacin accumulation
(ng/mg cells)
7
6
5
4
3
2
1
0
10
20
30
time(min)

3.2.2.3 Role of LPS in the ciprofloxacin accumulation process in *P. aeruginosa*.

In *P. aeruginosa*, since data on ciprofloxacin uptake and washing studies was consistent with the drug being more firmly bound to an extracytoplasmic site, the potential role of lipopolysaccharide structure as a binding component for fluoroquinolones was investigated. It is already known that LPS do not act as a barrier for hydrophilic quinolones such as ciprofloxacin in *E. coli* and *S. typhimurium* (Hirai *et al.*, 1986a). The larger amount of drug associated with *P. aeruginosa* in NB compared to *E. coli* after 2-ml and 10-ml washes (Figure 16) does not correlate with the higher ciprofloxacin MIC of 0.15 μg/ml observed with *P. aeruginosa* as compared to 0.006 μg/ml for *E. coli* in NB (Table 2a). To try to clarify this situation, accumulation profile studies of ciprofloxacin were performed in a set of smooth and rough *P. aeruginosa* strains, PAO 503 and PAO 503-18, respectively (Bryan *et al.*, 1984). *P. aeruginosa* PAO 503-18 was obtained by transduction of the gentamicin-resistance phenotype from a *P. aeruginosa* clinical isolate to PAO 503 using a phage E79.tv2. The higher accumulation of drug observed particularly at zero time for PAO 503-18 suggested that more binding sites were available in the rough strain (Figure 21). Such binding sites might be located within the lipid A portion of the LPS structure at the phosphorylated glucosamine

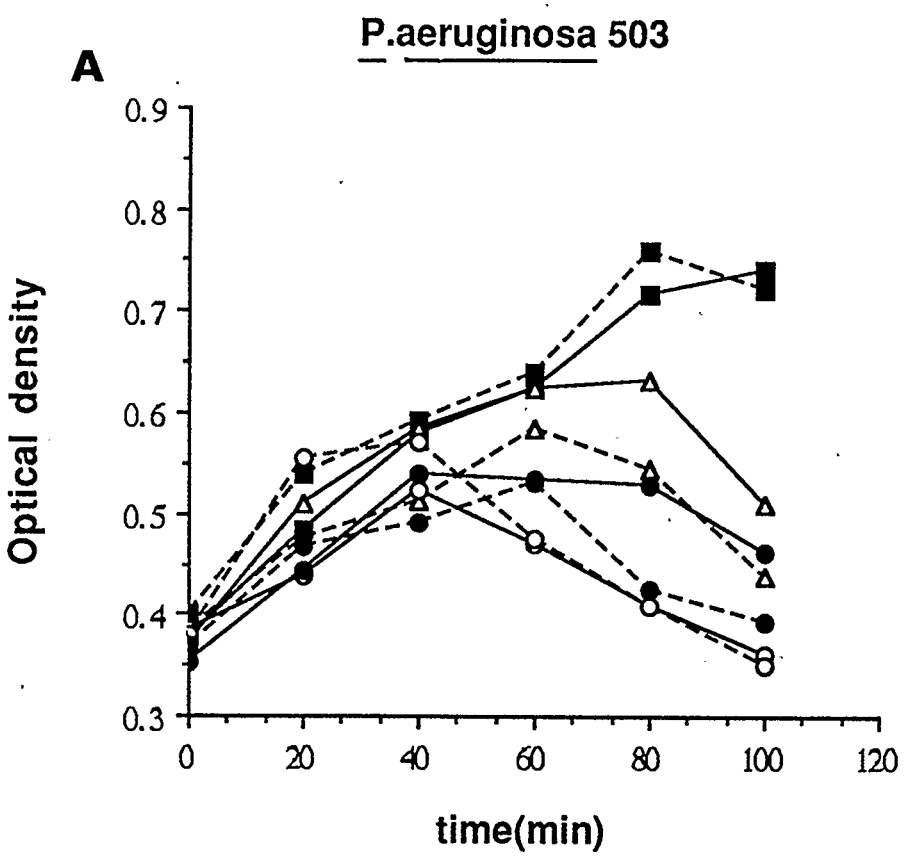
FIG. 21: Comparative study of the accumulation profile of ciprofloxacin at 0.154 μg/ml by *P. aeruginosa* PAO 503 (smooth) and 503-18 (rough) with time at 35°C in Nutrient Broth in presence of 20 μM CCCP under 2-ml washing conditions. Symbols: ■ , *P. aeruginosa* PAO503; □ , *P. aeruginosa* 503-18.


Ciprofloxacin accumulation
(ng/mg cells)
0
1
2
3
4
5
6
7
0
10
20
30
time(min)

disaccharide unit sites noncovalently cross-bridged by divalent cations especially magnesium. The same phenomenon was observed with regard to the accumulation profile of ciprofloxacin in a set of smooth and rough *Salmonella typhimurium* isogenic strains (data not shown). In a rough strain, the accessibility to this region would be facilitated. However, the equilibrium achieved in both PAO 503 and PAO 503-18 did not show significant differences. It should be noted that cells were pre-treated with 20 μM CCCP for a period of 2 min before drug addition. In another set of experiments, the susceptibility to SDS-induced cell lysis of *P. aeruginosa* PAO 503 and 503-18 by various quinolones was compared (Figures 22a, b, 23a, b). As expected, PAO 503-18 was found to be more susceptible by 0.05% SDS lytic effect than the parent PAO 503, since in a rough strain, the lipid bilayers of the outer membrane would be more available to the action of detergents such as SDS. Therefore, a concentration of 0.01% SDS was used with PAO 503-18.

With respect to hydrophilic quinolones such as ciprofloxacin, norfloxacin, and enoxacin at a concentration of 10 μg/ml, an inhibitory effect on cell growth was observed after 40 - 60 min drug exposure for PAO 503 (Figure 22a). A period of 60 to 80 min was required for PAO 503-18 with norfloxacin and enoxacin; for ciprofloxacin, an incubation time of 40 min was required to

FIG. 22 (A) (B): Effect of enoxacin, ciprofloxacin, and norfloxacin at a concentration of 10 μg/ml on SDS-induced cell lysis of **(A)** *P. aeruginosa* PAO 503 (smooth) and **(B)** PAO 503-18 (rough) in Nutrient broth at 35°C with shaking with and without 0.05% and 0.01% SDS for PAO503 and PAO503-18, respectively. Symbols:——, no SDS; ---, 0.01% or 0.05% SDS; ■, control, no antibiotic added; o, ciprofloxacin; △, enoxacin; ●, norfloxacin.


P.aeruginosa 503
A
Optical density
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0
20
40
60
80
100
120
time(min)

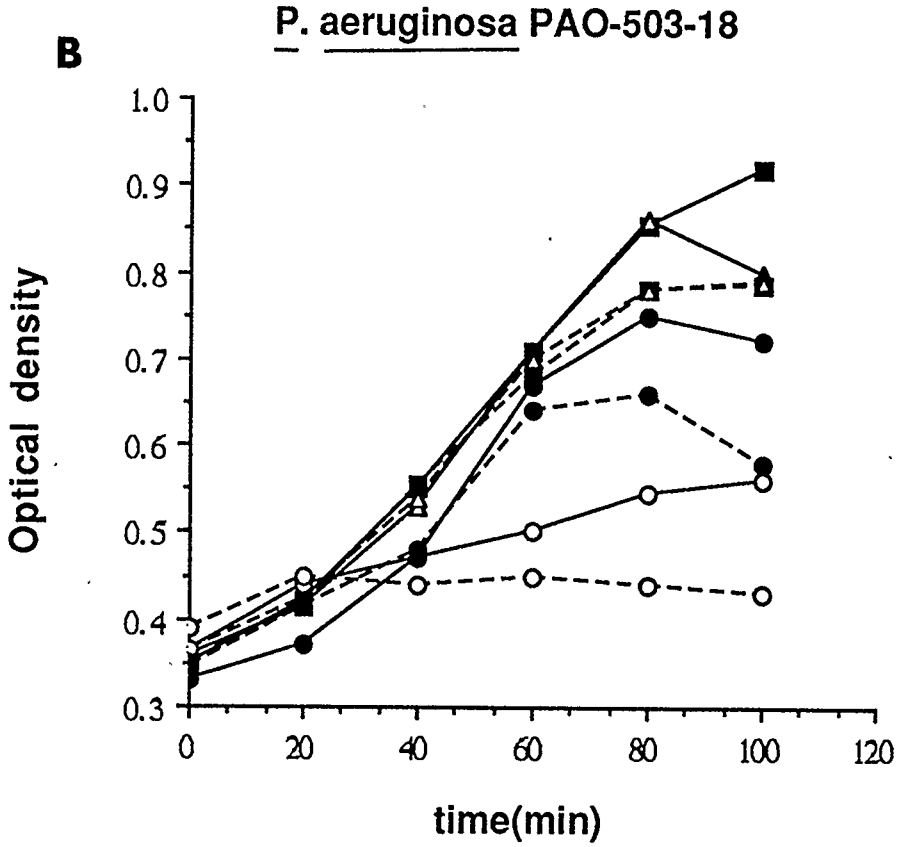
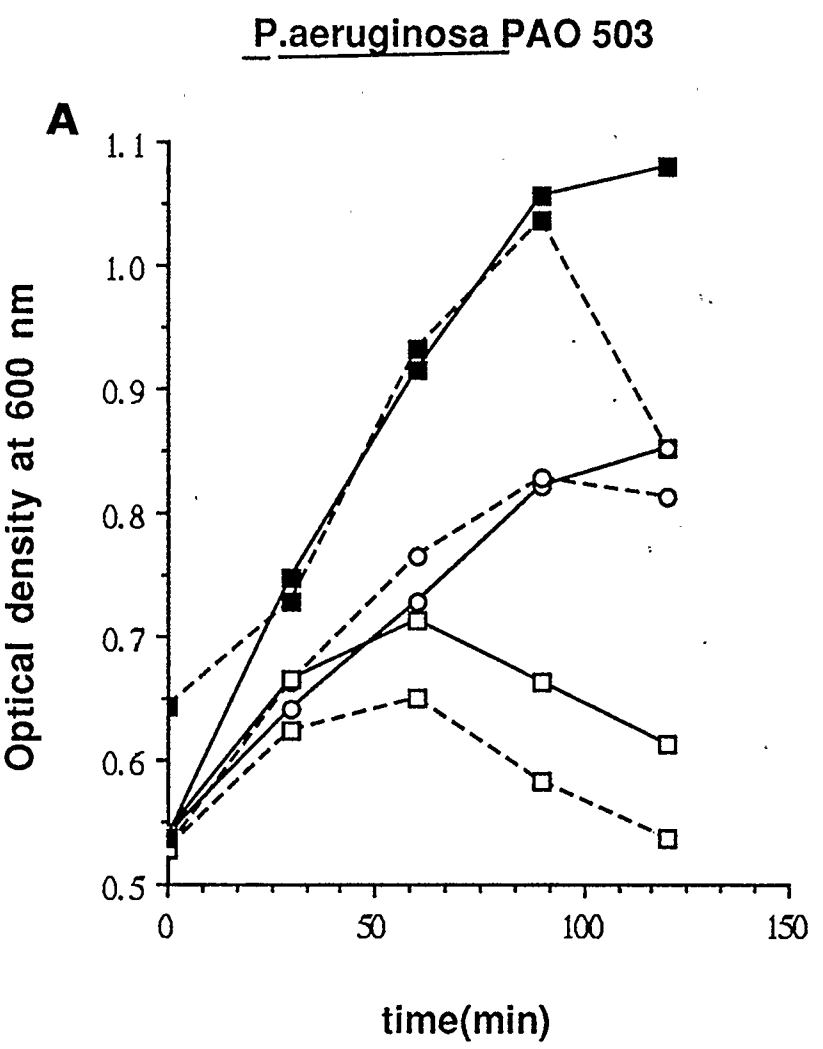
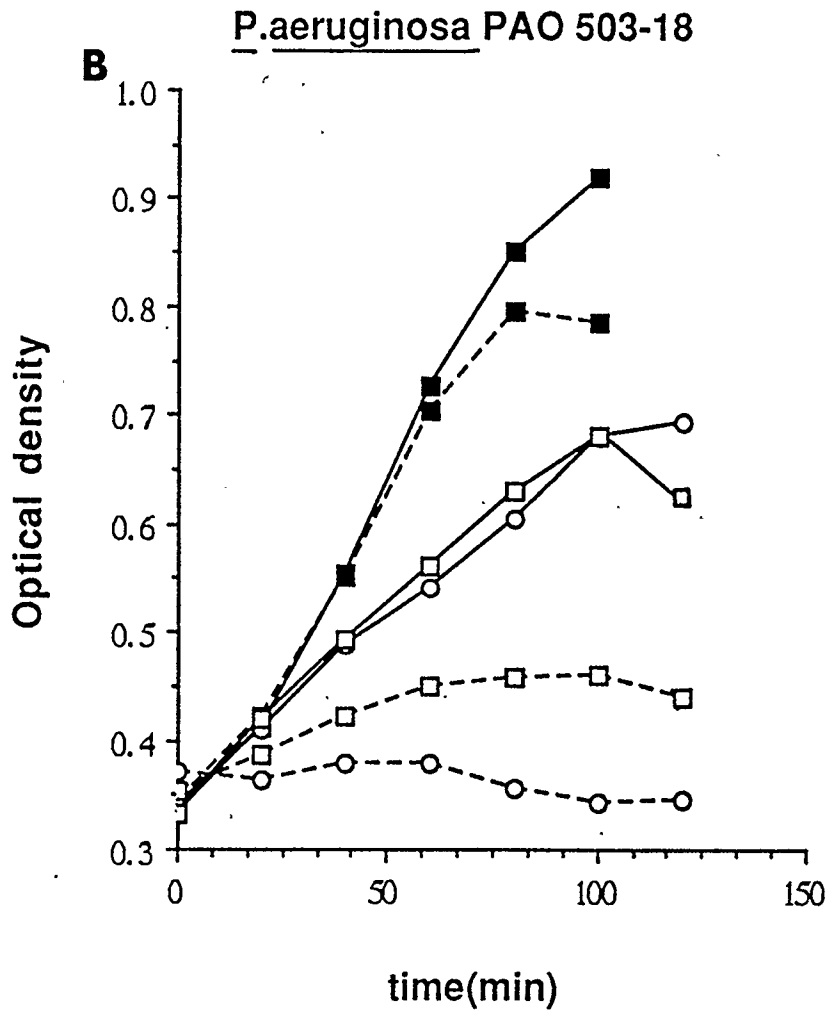

B
P. aeruginosa PAO-503-18
Optical density
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0
20
40
60
80
100
120
time(min)

FIG. 23 (A) (B): Effect of ofloxacin and fleroxacin at a concentration of 10 μg/ml on SDS-induced cell lysis of **(A)** *P. aeruginosa* PAO 503 (smooth) and **(B)** PAO 503-18 (rough) in Nutrient broth at 35°C with shaking in the presence and absence of 0.05% and 0.01% SDS for PAO503 and PAO503-18, respectively. Symbols:——, no SDS; ---, 0.01% or 0.05% SDS; ■ , control, no antibiotic added; o, fleroxacin; □ , ofloxacin.


P.aeruginosa PAO 503
A
Optical density at 600 nm
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0
50
100
150
time(min)


P.aeruginosa PAO 503-18
B
Optical density
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0
50
100
150
time(min)

obtain an antibacterial effect (Figure 22b). Interestingly, a higher magnitude of cell lysis was observed with hydrophilic quinolones for PAO 503 compared to PAO 503-18 after an incubation period of 80 min. This suggests that quinolone-LPS interaction might have a greater destabilization effect on the outer membrane integrity. Cell growth inhibition with hydrophilic quinolones was independent of the addition of 0.05 and 0.01% SDS for PAO 503 and PAO 503-18, respectively, since similar patterns were observed. Generally, the presence of SDS with hydrophilic quinolones did not induce earlier growth inhibition in *P. aeruginosa* compared to bacterial cells incubated with drug alone.

With regard to relatively hydrophobic quinolones such as fleroxacin and ofloxacin used at a concentration of 10 μg/ml (Figure 23a, b), the presence of 0.01% SDS significantly reduced the growth of PAO 503-18 compared to cells incubated with these antimicrobial agents alone (Figure 23b). Such phenomena were not observed for PAO 503 to the same extent as with either 0.01% (not shown) or 0.05% SDS (Figure 23a). Thus, the presence of SDS with hydrophilic quinolone did not generally cause a significant difference in the cell growth rate for PAO 503 and PAO 503-18 compared to cells incubated with drugs alone. A similar observation was noted for PAO 503 in the presence of hydrophobic quinolones with 0.05% SDS. However, the presence of fleroxacin and ofloxacin at 10

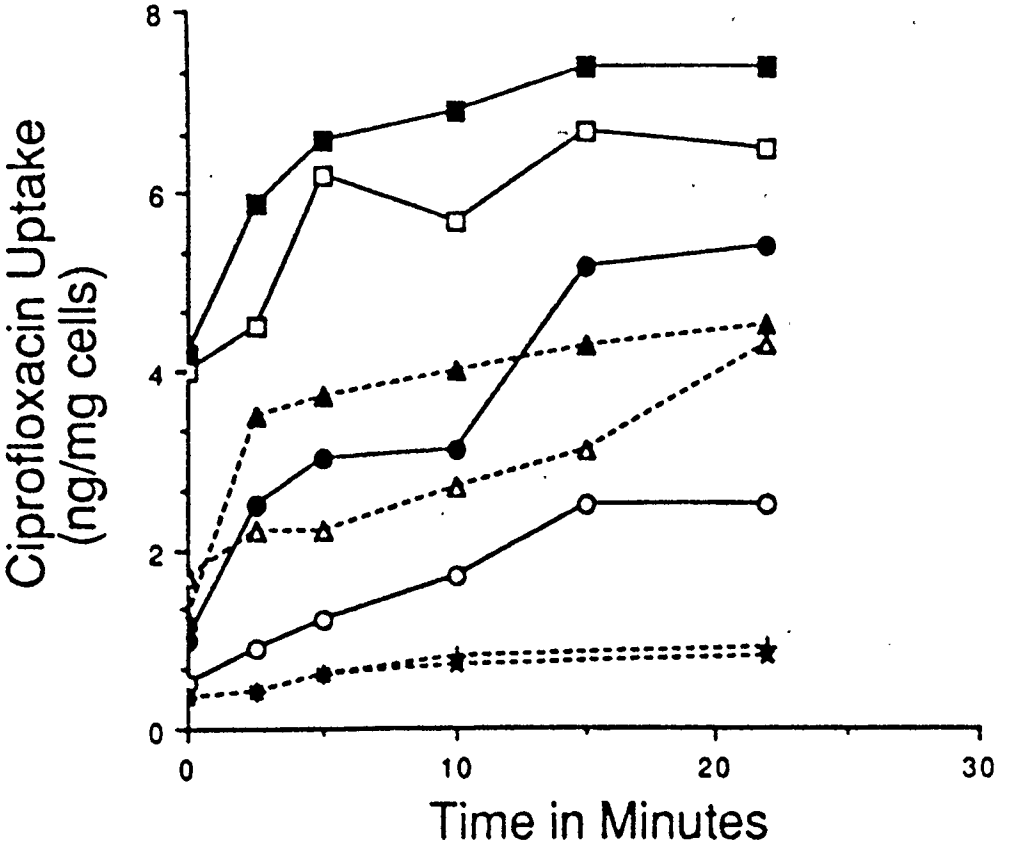
μg/ml did potentiate the action of SDS on cell membranes of rough *P. aeruginosa* strain within the time range studied. It should be noted that a cell clumping effect was observed with *P. aeruginosa* cells inoculated with SDS in the presence of fluoroquinolones. This suggests that increased cell surface hydrophobicity was obtained as a consequence of LPS release.

In summary, these results are consistent with the view that LPS structures are important in determining susceptibility of *P. aeruginosa*, especially to relatively hydrophobic quinolones.

3.2.2.4 Studies of ciprofloxacin uptake in *A. faecalis*.

Ciprofloxacin accumulation was also studied in *A. faecalis* (Figure 24). The amount of drug associated with cells was found to be influenced by the nature of medium used, since lower uptake was observed in BHIB compared to NB. The energy inhibitor CCCP had a significant effect on drug uptake in NB, whereas no pronounced effect was detected in BHIB for this organism. Various washing regimens did not produce differential effects on the amount of drug associated with *A. faecalis* as opposed to *P. aeruginosa*. The amount of [^{14}C] ciprofloxacin removed was in direct relationship with the volume of washing regimens used. The greater the volume or the number of wash steps used, the smaller the amount of ciprofloxacin

FIG. 24: Accumulative profile of ciprofloxacin at a concentration of 0.2 μg/ml with time at 35°C under 2-ml washing conditions by *E. coli* J5-3 and *A. faecalis* ATCC 19018 in Nutrient broth and Brain Heart Infusion broth with and without 20 μM CCCP. Symbols: □ , *E. coli* in NB without 20 μM CCCP; ■ , *E. coli* in NB with 20 μM CCCP; o, *E. coli* in BHIB without 20 μM CCCP;●*E. coli* in BHIB with 20 μM CCCP;△*A. faecalis* in NB without 20 μM CCCP; ▲ , *A. faecalis* in NB with 20 μM CCCP; x, *A. faecalis* in BHIB without 20 μM CCCP; +, *A. faecalis* in BHIB with 20 μM CCCP.


Ciprofloxacin Uptake
(ng/mg cells)
8
6
4
2
0
0
10
20
30
Time in Minutes

was associated with *A. faecalis*. Ciprofloxacin accumulation was consistently lower in *A. faecalis* known to possess outer membrane diffusion pores of smaller size than *E. coli* (Ishii and Nakae, 1988). Although quantitative differences were observed, the same pattern of ciprofloxacin uptake as observed for *E. coli* was obtained with *A. faecalis*.

3.2.3 Quinolone interaction with liposomes.

3.2.3.1 Studies of drug effect on liposome integrity and on liposome-liposome interactions.

Studies noted earlier showed that the fluoroquinolones enoxacin and ciprofloxacin use simple diffusion to permeate bacterial cells. Therefore, we investigated whether or not interactions existed between these antimicrobial agents and lipid bilayers. As an *in vitro* model, liposomes composed of multilayers of phosphadidylcholine, dicetylphosphate, and cholesterol in a ratio of 100:28.6:14.3, respectively, were used. Two sets of experiments were used to investigate effects on liposome integrity occurring at high drug concentrations. Liposomes exposed to 6 mg/ml ciprofloxacin and enoxacin demonstrated a significant increase of optical density at pH 4.5 only (Table 5). Observation by light microscopy confirmed the liposome clumping which occurred only at this pH. Physiological concentrations of drugs failed to induce such detectable phenomenon. Liposome clumping

TABLE 5. EFFECTS OF DRUG CONCENTRATION AND pH ON LIPOSOMAL CLUMPING.

Drug Concentration[a] (μg/ml)	Optical Density (450 nm)					
	pH 4.5		pH 7.0		pH 10.0	
	cipro	enox	cipro	enox	cipro	enox
6000	1.12	0.89	0.48	0.48	0.44	0.41
3000	1.07	0.85	0.45	0.44	0.42	0.38
1500	0.95	0.81	0.41	0.40	0.42	0.39
750	0.86	0.72	0.41	0.41	0.43	0.36
375	0.57	0.57	0.42	0.44	0.44	0.39
188	0.51	0.52	0.40	0.42	0.42	0.39
94	0.47	0.53	0.41	0.42	0.38	0.39
47	0.45	0.51	0.39	0.42	0.45	0.38
0	0.46	0.51	0.41	0.41	0.41	0.38

[a]prepared in 5 mM MES-NaOH

might result from the neutralization of negative charges carried by the phosphate head of phosphatitylcholine molecules at the liposomal periphery by positively charged fluoroquinolones. Therefore, reduced electro-repulsive forces would favor hydrophobic interactions among liposomes. An intermolecular drug-drug interaction of drug molecules located at the liposomal surface might also induce liposome aggregations. From this study, it was concluded that ionic forces are likely to be involved in the fluoroquinolone interactions with lipid bilayers. In another study, the drug effect on liposome integrity was investigated at different pHs (pH 4.5, 7.5, 10) by measuring the amount of [^{14}C] ATP retained by liposomes after exposure to 0.02 M concentrations of various quinolones exhibiting different pH-dependent aqueous solubility profiles (Figure 25). A significant reduction of [^{14}C] ATP associated with liposomes as compared to the control was observed at pH 7.5 for the amphoteric quinolones enoxacin, ciprofloxacin, norflox-acin, and ofloxacin. This pH corresponded to the zwitterionic state of these molecules, which therefore exhibited their highest hydrophobicity in the buffer used in this experiment (Figure 26). A significant effect on liposome integrity was noted at pH 4.5 for the acidic quinolone, nalidixic acid consistent with a higher hydrophobicity at this pH. At pH 10, none of the quinolones tested showed any effect since the amount of

FIG. 25: Effect of pH on the aqueous solubility of enoxacin, ciprofloxacin, norfloxacin, and nalidixic acid in 0.1 M Mes-NaOH. The initial drug concentration used was 1 mg/ml. Results indicated as a percentage of total drug in solution. Symbols: ●, enoxacin; ■ , ciprofloxacin, □ , norfloxacin; ▲ , nalidixic acid.

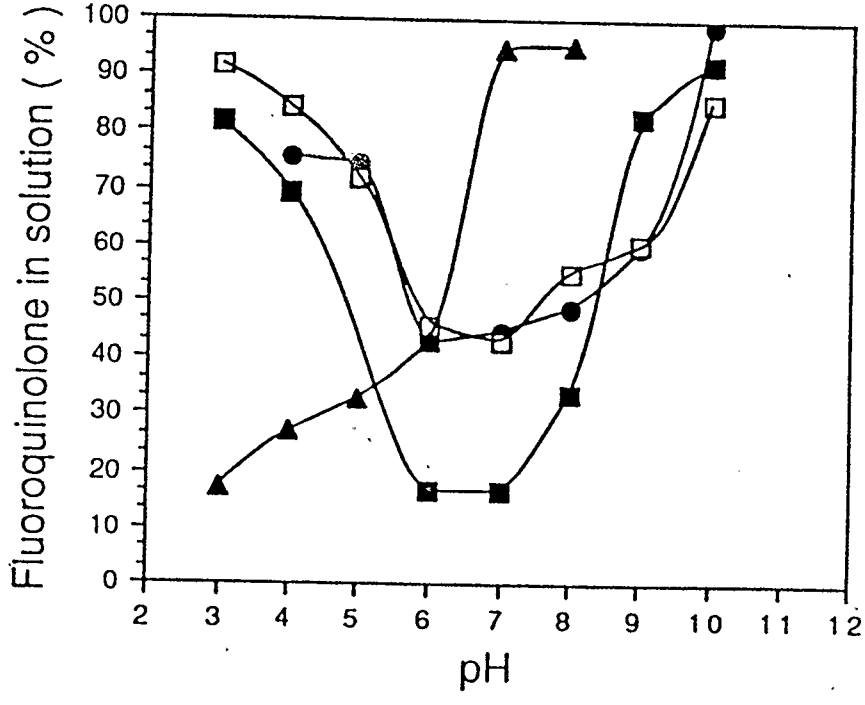
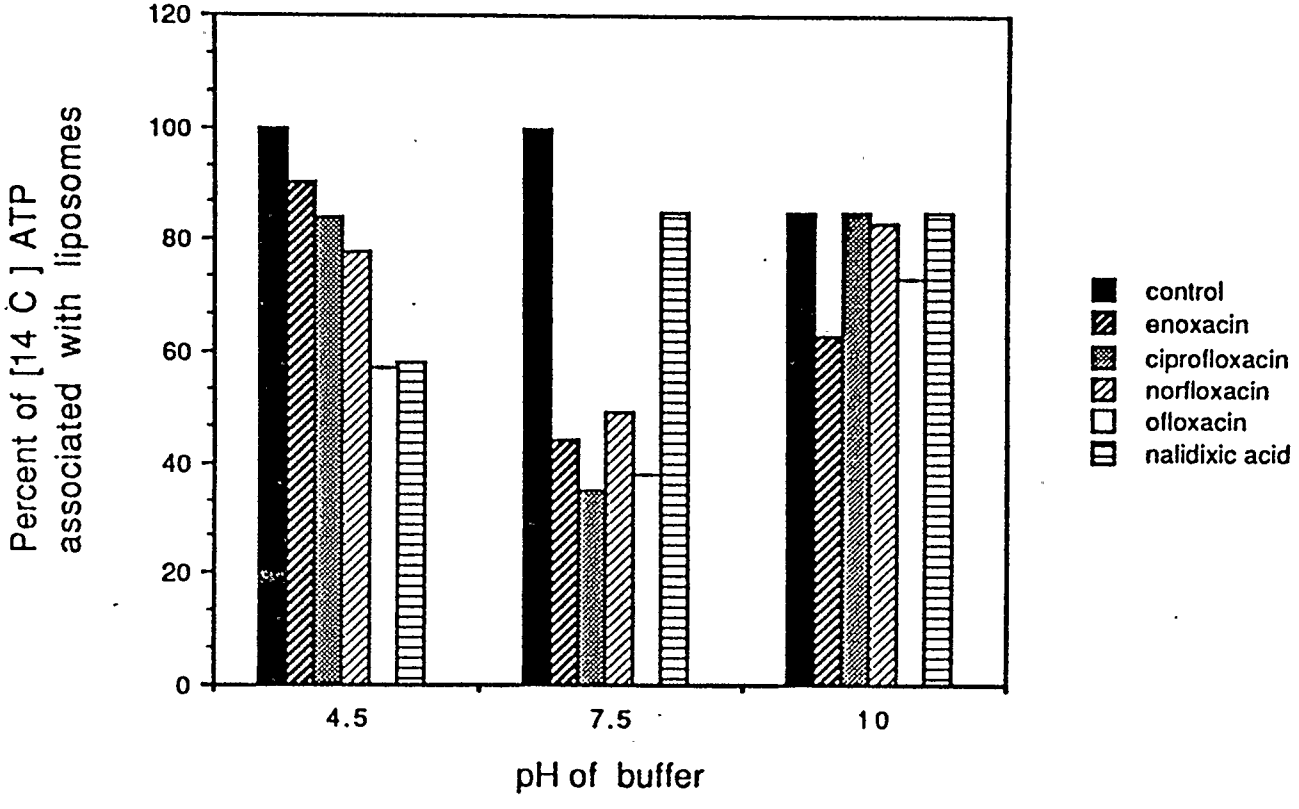

Fluoroquinolone in solution (%)
100
90
80
70
60
50
40
30
20
10
0
2
3
4
5
6
7
8
9
10
11
12
pH

FIG. 26: Effects of enoxacin, ciprofloxacin, norfloxacin, ofloxacin, and nalidixic acid on the amount of [^{14}C] ATP retained by liposomes (3.1 x 10^{-6} mole phosphatidylcholine/ml) as a function of pH. The drug concentration used was 0.02 M in 5 mM Mes-NaOH, and the time of drug-liposome treatment was 10 min at room temperature.


Percent of [14 C] ATP
associated with liposomes
120
100
80
60
40
20
0
4.5
7.5
10
pH of buffer
control
enoxacin
ciprofloxacin
norfloxacin
ofloxacin
nalidixic acid

[^{14}C] ATP retained was similar to the control value. At this pH liposomes were found to be less stable since the control value at pH 10 showed a 15% decrease in the amount of [^{14}C] ATP retained as compared to control values at pH 4.5 and 7.5. This study suggests that liposome integrity is most likely influenced by the state of hydrophobicity of quinolones at high concentrations. Despite ionic fluoroquinolone liposome association at low pHs (see next section), this process by itself seems ineffectual in achieving destablilization of liposomes.

3.2.3.2 Studies on pH, drug and liposome concentrations on liposome-fluoroquinolone interactions.

Characteristics of liposome-fluoroquinolone interactions were investigated using radiolabelled [^{14}C] enoxacin and [^{14}C] ciprofloxacin in order to increase the sensitivity of our measurements enabling us to study this phenomenon at relevant physiological drug concentrations. Measurements of the amount of enoxacin and ciprofloxacin associated with liposomes at various pHs were performed using a co-sedimentation technique. Maximal association of ciprofloxacin and enoxacin with liposomes occurred at acidic and neutral pHs (Figures 27, 28) which correspond to the protonated and zwitterionic forms of these two fluoroquinolone molecules. Moreover, these antimicrobial agents exhibit their highest hydrophobicity at pHs close to neutrality (Figure 25). At basic pHs, a significant

FIG. 27: Correlation between the amount of [^{14}C] ciprofloxacin at a concentration of 1.23 μg/ml associated with liposomes (3.1 x 10^{-6} mol of phosphatidylcholine per ml) and ciprofloxacin solubility in 0.1 M Mes-NaOH as a function of pH. Symbols: ■, ciprofloxacin aqueous solubility; △, ciprofloxacin association with liposomes.

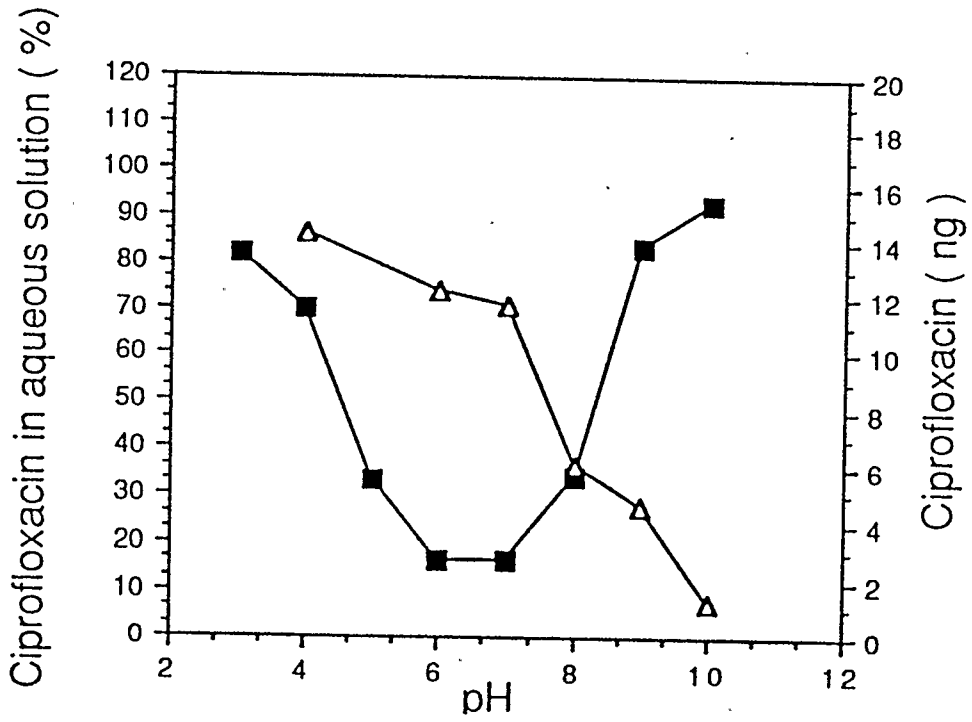
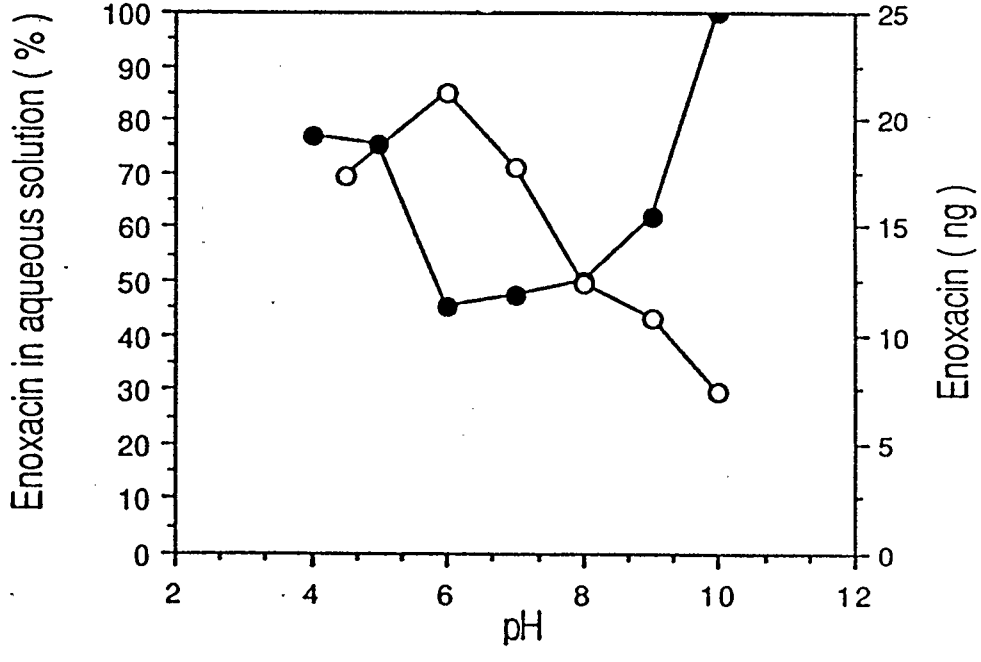
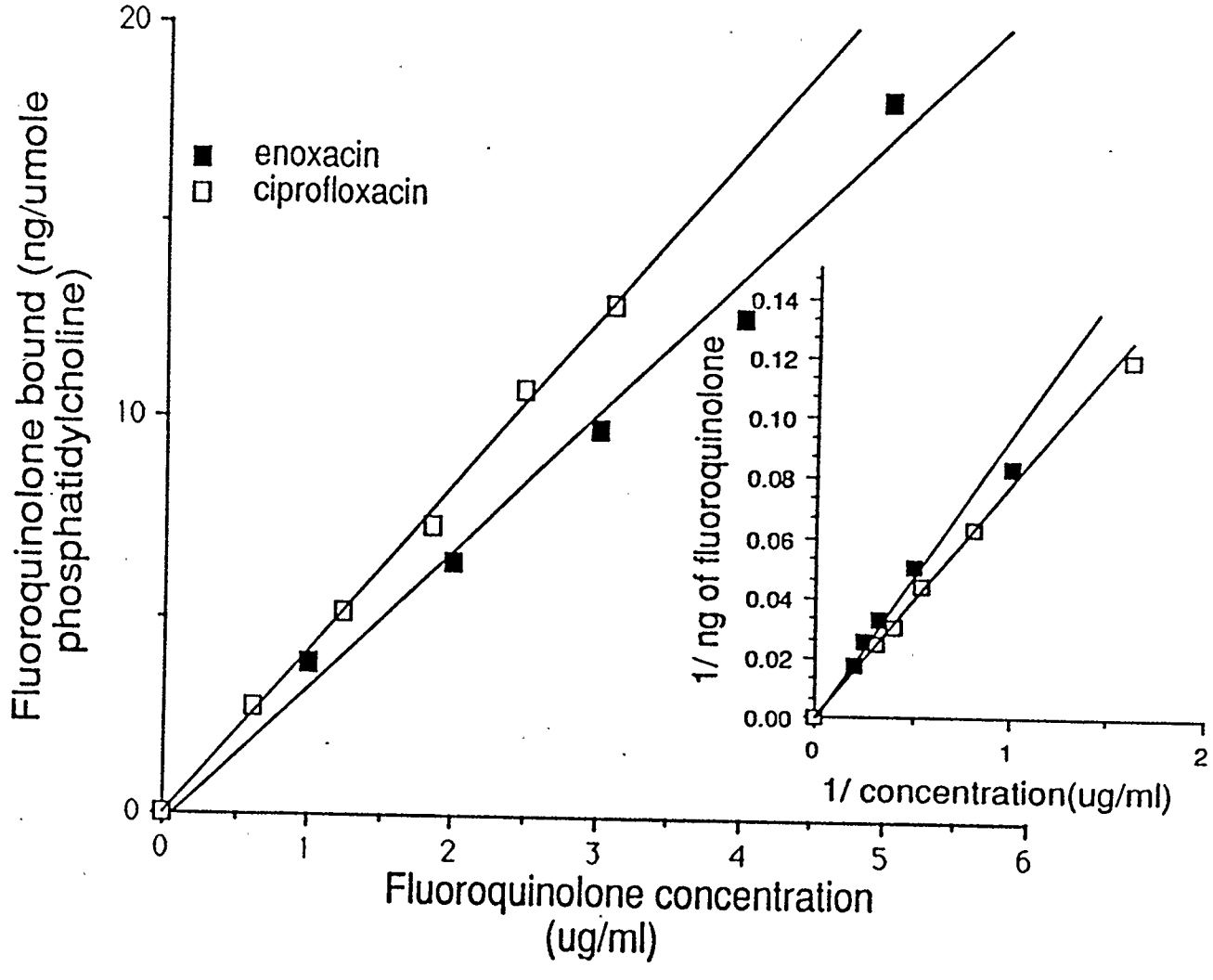

Ciprofloxacin in aqueous solution (%)
120
110
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
pH
20
18
16
14
12
10
8
6
4
2
0
Ciprofloxacin (ng)

FIG. 28: Correlation between the amount of [^{14}C] enoxacin at a concentration of 2 μg/ml associated with liposomes (3.1 x 10^{-6} mol of phosphatidylcholine per ml) and enoxacin solubility in 0.1 M Mes-NaOH as a function of pH. Symbols: ●, enoxacin aqueous solubility; o, enoxacin association with liposomes.


Enoxacin in aqueous solution (%)
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
pH
25
20
15
10
5
0
Enoxacin (ng)

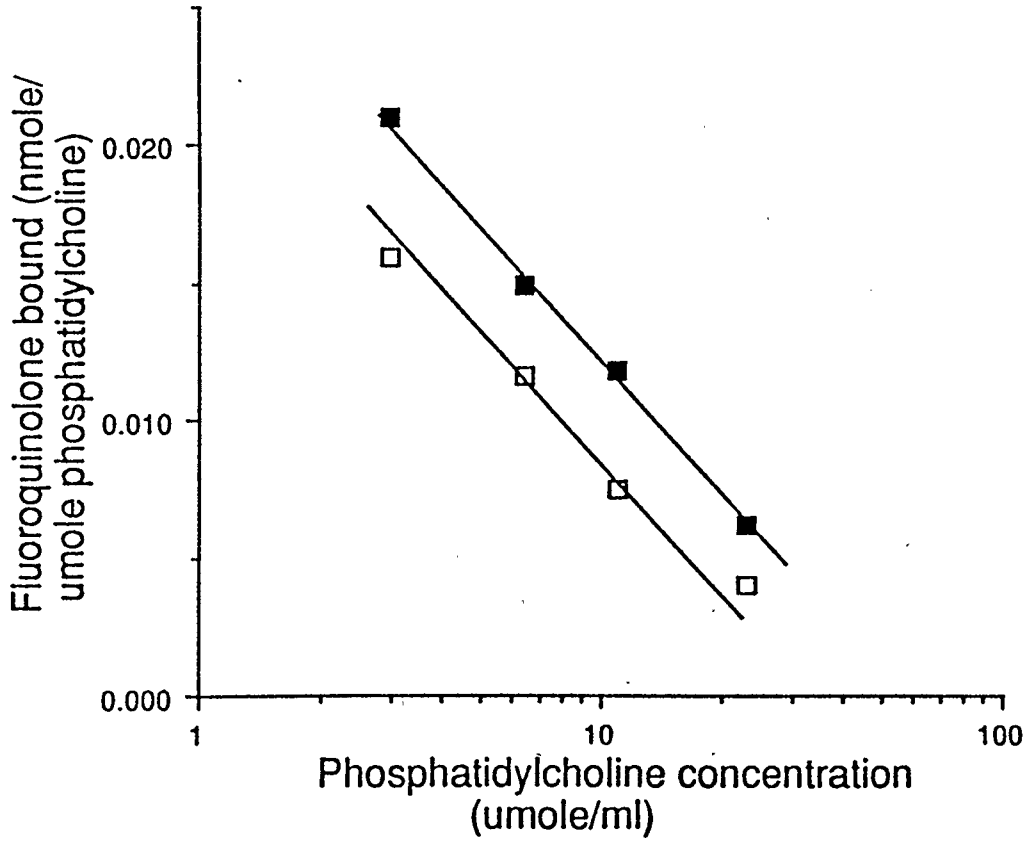
reduction of the amount of enoxacin and ciprofloxacin molecules associated with liposomes was observed, correlating with a reduced hydrophobicity and acquisition of a net negative charge for these antimicrobial agents. Over the pH range used in our experiments, phosphatidylcholine possesses ionic groups carrying two negative and one positive charge at pH 4.5 and 0 to one negative charge at pH 10.0. Similar behavior regarding the net charge can be attributed to phosphatidylethanolamine, the major bacterial phospholipid. The effects of enoxacin and ciprofloxacin concentrations, as well as increasing concentration of phosphatidylcholine on the amount of drug associated with liposomes was investigated at pH 7.0. The amount of enoxacin and ciprofloxacin associated with liposomes was found to be a linear relationship with drug concentration (Figure 29), the amount of ciprofloxacin being 1.4 times greater than enoxacin at equivalent drug concentrations. This result is consistent with the slightly higher partition coefficient of ciprofloxacin in n-octanol-phosphate buffer, pH 7.2 (Hirai *et al.*, 1986a). The percentage of drug associated with one μmole of phosphatidylcholine was independent of fluoroquinolone concentration in the range of 0.62 to 5 μg/ml, with values of 0.422% plus/minus 0.015% for ciprofloxacin and 0.344% plus/minus 0.030% for enoxacin. This phenomenon was found to be nonsaturable at drug concentrations up to 20 μg/ml since Lineweaver-Burk plots

FIG. 29: Studies of the effect of [^{14}C] ciprofloxacin and [^{14}C] enoxacin concentrations on the amount of drug associated with liposomes per micromole of phosphatidycholine.


20
10
0
Fluoroquinolone bound (ng/umole phosphatidylcholine)
enoxacin
ciprofloxacin
0
1
2
3
4
5
6
Fluoroquinolone concentration (ug/ml)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
1/ ng of fluoroquinolone
0
1
2
1/ concentration(ug/ml)

intercepted at zero value (Figure 29, insert). Contrary to expectations, increasing amounts of phosphatidylcholine/ml caused a reduction in the amount of enoxacin and ciprofloxacin associated per micromole of phosphatatidylcholine in liposomes (Figure 30). At high liposome concentrations used (20 μmole/ml), aggregation was observed under light microscopy, which might explain the decreased amount of drug bound. Under these circumstances, the total surface area available for drug binding would be diminished. These experiments were performed at equilibrium which was found to be established within 5 min and was stable for at least 30 min (data not shown). The value of drug bound to liposomes at equilibrium at pH 7.0 using 3.1×10^{-6} mole phosphatidylcholine with 2 μg of enoxacin and 1.23 μg of ciprofloxacin per ml were 2.15×10^{-5} and 1.57×10^{-5} mol of enoxacin and ciprofloxacin, respectively, per mol of phosphatidylcholine. The higher value observed for enoxacin is due to a higher concentration used which was obviously below the saturation level. From these studies, it can be concluded that both ionic and hydrophobic properties of fluoroquinolones participate as contributing factors allowing interactions to occur between these antimicrobial agents and lipid bilayers.

FIG. 30: Studies of the effect of phosphatidylcholine concentration on the amount of [^{14}C] enoxacin (2 μg/ml) and [^{14}C] ciprofloxacin (1.23 μg/ml) associated with lipsomes per micromole of phosphatidylcholine per ml. Symbols: ■, enoxacin at 2 μg/ml; □ , ciprofloxacin at 1.23 μg/ml.

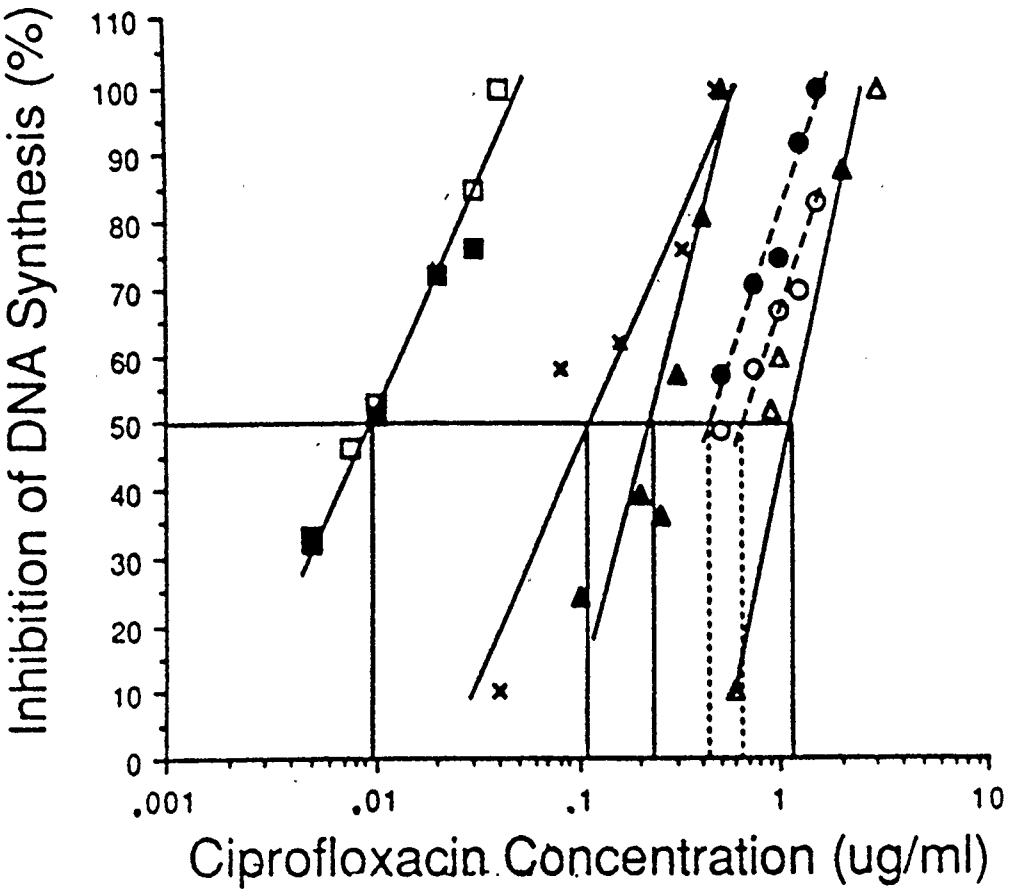

Fluoroquinolone bound (nmole/
umole phosphatidylcholine)
0.020
0.010
0.000
1
10
100
Phosphatidylcholine concentration
(umole/ml)

3.3 Study of Factors Determining Bacterial Cell Susceptibility to Fluoroquinolones: DNA Synthesis Inhibition Induced by Ciprofloxacin in *E. coli*, *A. faecalis* and *P. aeruginosa*, Wild-Type and Isogenic Mutants.

3.3.1 Study of the inhibition of DNA synthesis in *E. coli* and *A. faecalis* bacterial strains.

Different methods of measurement for the inhibition of DNA synthesis induced by ciprofloxacin were used in order to reassure ourselves of the validity of data obtained. In the case of *E. coli* strains, the method of Chow *et al.* (1988) was used because it does not involve a step of cell permeabilization since this organism is able to incorporate thymidine into DNA. Studies with permeabilized *E. coli* cells were performed using two methods: (i) the method of Chow *et al.* (1988) with cells pretreated with 10 mM EDTA. EDTA at this concentration did not reduce the rate of DNA synthesis; however, *E. coli* cells were found to be susceptible to the lytic effect of 0.05% SDS, showing that the integrity of the LPS permeability barrier was reduced or lost; (ii) the method of Rella and Haas (1982) which involves the use of ether instead of toluene (Moses and Richardson, 1970) as a permeabilizing agent was also used in DNA synthesis inhibition studies for *E. coli* and *A. faecalis* strains.

FIG. 31: Studies of the inhibitory effect of ciproflox-acin at various concentrations on the DNA synthesis for *E. coli* J5-3 derivatives and *P. aeruginosa* PAO503 using the methods of Chow *et al.* (1988) and Benbrook and Miller (1986), respectively. Symbols: □ , *E. coli* J5-3 without 10 mM EDTA; ■ , *E. coli* J5-3 with 10 mM EDTA; X, *E. coli* SA 1306 without 10 mM EDTA; o, *E. coli* JB-5R without 10 mM EDTA; ●, *E. coli* JB-5R with 10 mM EDTA; △ , *P. aeruginosa* PAO503 without 2.5 mM EDTA; ▲ , *P. aeruginosa* PAO503 with 2.5 mM EDTA.


Inhibition of DNA Synthesis (%)
110
100
90
80
70
60
50
40
30
20
10
0
.001
.01
.1
1
10
Ciprofloxacin Concentration (ug/ml)

Various concentrations of ciprofloxacin were used to induce inhibition of DNA synthesis and dose response curves were obtained showing a linear relationship between inhibition of DNA synthesis and ciprofloxacin concentration (Figure 31). The values obtained for the ID_{50} of *E. coli* J5-3 and SA 1306 strains correlated with their MICs to ciprofloxacin (Table 6). Treatment of cells with EDTA or ether did not significantly change the ID_{50} values. *E. coli* JB-5R, a mutant SA 1306 derivative known to have a permeability defect with regard to the F porin (Bedard *et al.*, 1987) exhibited an ID_{50} and MIC value higher than J5-3, but closer to SA 1306 known to possess a *gyr*A mutation. EDTA treatment of JB-5R did cause a small reduction in the ID_{50} (Figure 31, Table 6). Values obtained for ID_{50} were in agreement among the two methods used (Table 6). These studies show that the main factor determining *E. coli* bacterial cell susceptibility to ciprofloxacin is the ability of this antimicrobial agent to induce inhibition of DNA synthesis.

For *A. faecalis*, an ID_{50} value of 0.015 μg/ml obtained after ether permeabilization treatment was very similar to values obtained with *E. coli* strains; however, as opposed to *E. coli*, the ID_{50} value was about 16.5- to 63-fold less than the corresponding MIC (Table 6). It should be mentioned that for *A. faecalis*, a maximum of 60% inhibition of DNA synthesis could be obtained with ciprofloxacin concentrations ten or more times the ID_{50};

TABLE 6. CONCENTRATIONS OF CIPROFLOXACIN REQUIRED TO INHIBIT DNA SYNTHESIS IN E. COLI, P. AERUGINOSA, AND A. FAECALIS STRAINS.

Bacterial Strains	MIC[a]	I.D.$_{50}$ (µg/ml) Chow et al.[b] 1988 (^{3}H-thymidine)	Benbrook and Miller,[b] 1986 (^{3}H-adenine)	Rella and Haas,[b] 1982 ([^{3}H]dTTP)
E. coli J5-3	0.03	0.01	ND	0.015
J5-3 (EDTA-treated)		0.01	ND	ND
E. coli JB-5R	0.5-1.0	0.65	ND	ND
JB-5R (EDTA-treated)		0.45	ND	ND
E. coli SA 1306	0.125	0.1	ND	0.15
P. aeruginosa PAO 503	0.125	ND	1.0	0.1
P. aeruginosa PAO 503 (EDTA-treated)		ND	0.2	ND
P. aeruginosa PAO 236	4.0	ND	ND	4.0
P. aeruginosa PCC 1989	1.0	ND	2.0	ND
P. aeruginosa PC1989 (EDTA-treated)		ND	1.0	ND
A. faecalis ATCC 19018	0.25-0.95	ND	ND	0.015

[a]MIC values are given for the medium used for inhibition of DNA synthesis studies except for E. coli JB-5R and A. faecalis strains which are the MIC ranges in the three media used

[b]Methods used to measure DNA synthesis

this extent of inhibition was set at 100%. This study shows that for *A. faecalis*, poor permeability associated with the small pore size of the outer membrane (Ishii and Nakae, 1988) is the major determinant of ciprofloxacin susceptibility.

3.3.2 Study of the inhibition of DNA synthesis in *P. aeruginosa* strains.

The ID_{50} values obtained for *P. aeruginosa* strains were consistently higher than ID_{50} values found for *E. coli* and *A. faecalis* (Table 6), suggesting that the DNA gyrase activity is more refractory to the inhibitory effects by fluoroquinolones. This is consistent with a previously published study with nalidixic acid (Miller and Scurlock, 1983). Moreover, *P. aeruginosa* PAO 236 and PCC 1989 carrying a *gyr*A mutation were less susceptible to inhibition of DNA synthesis compared to *P. aeruginosa* PAO 503. Ether and EDTA were used as permeabilizing agents. Since *P. aeruginosa* is more sensitive to EDTA treatment, a lower concentration of 2.5 mM instead of 10 mM was used. At this concentration, cells were susceptible to 0.05% SDS-induced lysis, but the rate of DNA synthesis was found unaffected. The amount of ciprofloxacin required to obtain an ID_{50} in *P. aeruginosa* 503 permeabilized cells was significantly reduced compared to untreated cells using the two methods mentioned above (Table 6). The method of Chow *et al.* (1988) was not used

for *P. aeruginosa* and *A. faecalis* since our laboratory has observed that these organisms do not incorporate exogenous thymidine into DNA. These studies suggest that there are two factors contributing to the higher MIC observed in *P. aeruginosa* compared to *E. coli*. Firstly, the *P. aeruginosa* outer membrane acts as a permeability barrier to ciprofloxacin; secondly, the reduced susceptibility of DNA synthesis to inhibition induced by ciprofloxacin is also a determinant factor.

D I S C U S S I O N

4.0 DISCUSSION

4.1 Overall Uptake Process: Fluoroquinolone Association With Intact Bacterial Cell and Liposomes: Proposed Model.

The mechanisms of the fluoroquinolone permeation process in bacterial cells have been investigated using several approaches. Investigations of quinolone association with *E. coli* cells were performed by studying [^{14}C] enoxacin displacement by structural analogs, calcium and piperazine. The use of samples taken at zero time and rapid washing allowed more precise study of the binding of the drugs. However, despite these precautions, it is almost certain that a small proportion of drug was already within the cell due to the time required to handle the samples. Accumulation profiles of enoxacin and ciprofloxacin were investigated in whole bacterial cells and vesicle membranes. The effects of various external conditions in uptake assays were defined as well as the relative importance of some cell components such as porins, LPS, and DNA gyrase, which were studied by using sets of isogenic mutants. More emphasis was given to the LPS structure with studies of SDS-induced cell lysis in the presence of various quinolones which had different hydrophobicity levels. A major role is attributed to the LPS in *P. aeruginosa* with regard to the permeation process of quinolones.

A potential permeability barrier to hydrophobic quinolones has been attributed to the LPS structure in *E. coli* and *S. typhimurium* (Hirai *et al.*, 1986a). Chapman and Georgopapadakou (1988) proposed self-promoted uptake (Hancock *et al.*, 1981) of fleroxacin, a relatively hydrophobic quinolone, in *E. coli*. Their model suggested a progressive exposure of hydrophobic patches at the external surface of the outer membrane, supported by the observation of increased surface hydrophobicity, in the presence of this antimicrobial agent. This phenomenon resulted from LPS release induced by the formation and removal of magnesium-quinolone complexes. Thereafter, it was proposed that fleroxacin would partition into the lipid portion and diffuse toward the periplasmic space. This model is consistent with the mechanism of simple diffusion previously proposed for the enoxacin accumulation process in *E. coli* (Bedard *et al.*, 1987). Studies of the accumulation profile of [^{14}C] ciprofloxacin in a set of isogenic strains, *P. aeruginosa* PAO 503 (smooth) and PAO 503-18 (rough) demonstrated a higher binding phase for the rough strain, most probably the result of increased accessibility of ciprofloxacin to the phosphate residues of LPS. Results of studies of SDS-induced *P. aeruginosa* lysis in the presence of quinolones are consistent with the conclusion that LPS does not protect against hydrophilic quinolones under the conditions examined. Cell lysis of different magnitude

after a relatively long period of drug exposure was observed in the presence of 10 μg/ml ciprofloxacin, norfloxacin, and enoxacin for PAO 503 and PAO 503-18 with or without 0.05% and 0.01% SDS, respectively. These observations do not clearly show the absence or presence of drug interaction with LPS. However, the possibility of such an interaction has not been excluded since it has been reported that ciprofloxacin was able to induce LPS release in *E. coli* (Cohen and McConnell, 1986). With regard to ciprofloxacin and other hydrophilic quinolones, it is not excluded that the outer membrane destabilization could occur at the inner leaflet level caused by the presence of fluoroquinolone molecules in the periplasmic space. Cell lysis is probably caused by the destabilization of the outer membrane, in part due to drug partition into the lipid portion of the membrane. This is consistent with the immediate growth inhibition observed for PAO 503-18 when exposed to 0.01% SDS in the presence of ofloxacin and fleroxacin, two relatively hydrophobic quinolones. These results are consistent with the hypothesis that the hydrophobicity of a fluoroquinolone determines the relative importance of self-promoted uptake on the overall fluoroquinolone uptake process in *E. coli* (Chapman and Georgopapakakou, 1988). Alterations of the LPS structure in deep rough enteric bacteria are usually associated with changes of the phospholipid content and their location within the

outer membrane (Nikaido and Vaara, 1985). Such modification of the outer membrane would result in structural discontinuity (nonbilayer structures), membrane blebs, or regions of enhanced fluidity (Hancock, 1984). *P. aeruginosa* PAO 503-18 increased sensitivity to the detergent action of SDS is most likely caused by weaker interactive forces between LPS structures and the reduction of the surface aqueous layer associated with longer chain polysac-charide. The apparent inability of hydrophilic quino-lones to induce cell lysis to the same extent as hydrophobic quinolones in a rough *P. aeruginosa* strain in the presence of SDS does not exclude the possibility of diffusion through the outer membrane by a hydrophobic pathway. Greater outer membrane instability induced by hydrophobic quinolones is probably caused by their diffusion at a faster rate and in larger amounts through the hydrophobic pathway compared to hydrophilic quinolones. It should be remembered that all fluoroquinolones studied in this thesis are associated with ionic and chelating properties.

Studies of [^{14}C] enoxacin displacement observed consistently in the presence of increasing concentrations of structural analogs and by calcium also suggest that the chelation activity associated with quinolones is an important property involved in fluoroquinolone interaction with bacterial cells. The presence of calcium would enhance formation of complexes of calcium-quinolone

resulting in a reduced number of drug molecules available for chelation of magnesium positioned within the LPS structure. Similar results were obtained in the ciprofloxacin uptake assays in the presence of magnesium, which at a concentration of 10 mM caused a significant reduction of ciprofloxacin accumulation by bacterial cells.

The less effective displacement of [^{14}C] enoxacin observed with nalidixic acid compared to that seen with the fluoroquinolones examined, demonstrates that the ionic property exhibited by these antimicrobial agents is another factor involved in drug interactions with bacterial cells. Nalidixic acid, a nonamphoteric molecule, unlike fluoroquinolones does not possess a piperazine substitution at C-7 position which allows the latter to be protonated at acidic pHs. This contention is also consistent with the higher binding observed at pH 4.5 for [^{14}C] enoxacin with whole cells and for [^{14}C] enoxacin and [^{14}C] ciprofloxacin with liposomes, compared to pH 7.0. Most probably, since fluoroquinolones do not carry a net charge at neutral pH, the contribution of this type of ionic interaction might not be as important in determining the overall number of drug molecules bound with bacterial cells, but would be necessary to the diffusion process through membranes. Shen *et al.* (1989c) proposed that drug-drug interactions involving stacking of naphthyridine rings in a flip-over position was very

important in the quinolone-DNA binding process with regard to the cooperativity observed. This was described for nalidixic acid but such a conclusion can be extended to fluoroquinolones. This type of interaction might also occur with the LPS structure through the positively charged amino groups of the piperazine ring instead of the 3-carboxyl and 4 keto groups which are involved in DNA binding. The former would establish an ionic interaction with the negatively charged phosphate residues of the LPS structure (Figure 32). This would be consistent with the partial [^{14}C] enoxacin displacement observed with piperazine. Studies of the structure of quinolone antibacterial agents using computer-automated technology (Klopman *et al.*, 1987) have suggested that the piperazine substitution at C-7 position would be the predominant factor controlling cell permeability to these compounds. Therefore, despite the fact that LPS structure offers a permeability barrier to hydrophobic quinolones, their ionization and chelation properties coupled with relatively high hydrophobicity would enable them to overcome this barrier and partition through the lipid portion of the outer membrane.

The increased amount of [^{14}C] enoxacin observed with *E. coli* SA 1306 cells at acidic pH (pH 4.5) most likely represents in large proportion the binding component of the drug at extracytoplasmic sites, since cell susceptibility was found to be reduced at this pH, consistent

FIG. 32. Proposed model of fluoroquinolone permeation process in gram-negative bacteria:

(A) chelation process exhibited by a fluoroquinolone molecule;

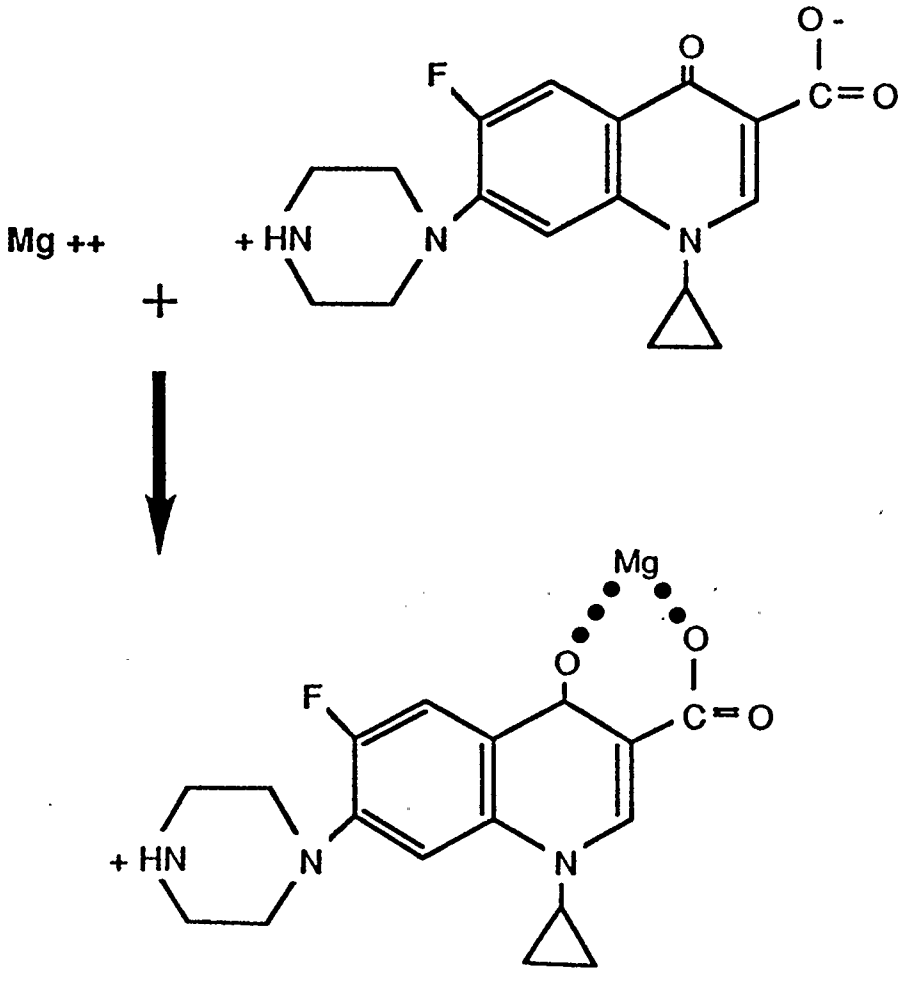
(B) structure of LPS molecules (Jawetz *et al.*, 1987);

(C) fluoroquinolone association with LPS structures;

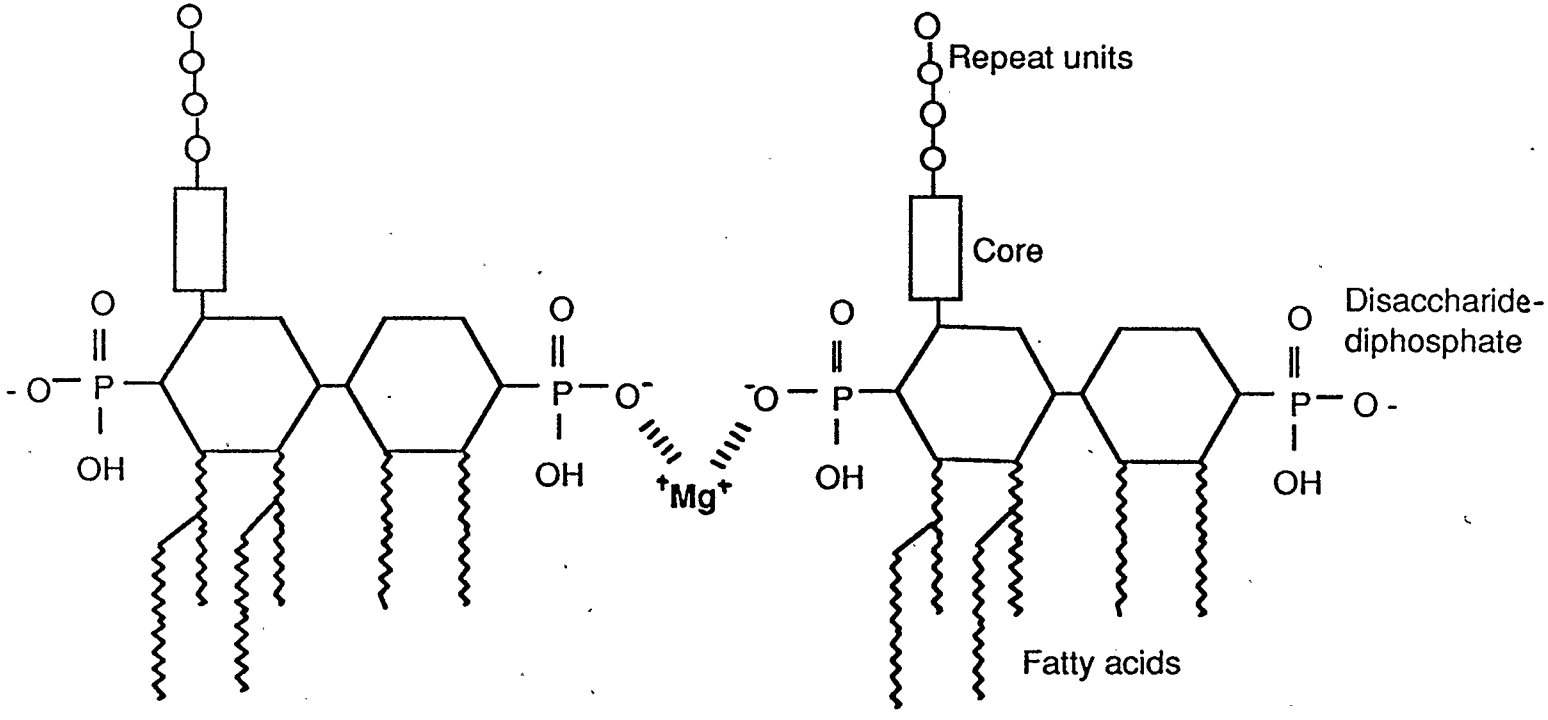
(D) fluoroquinolone association with phospholipids (structure of phospholipids from A.L. Lehninger, 1975);

(E) overall process of fluoroquinolone accumulation in gram-negative bacteria.

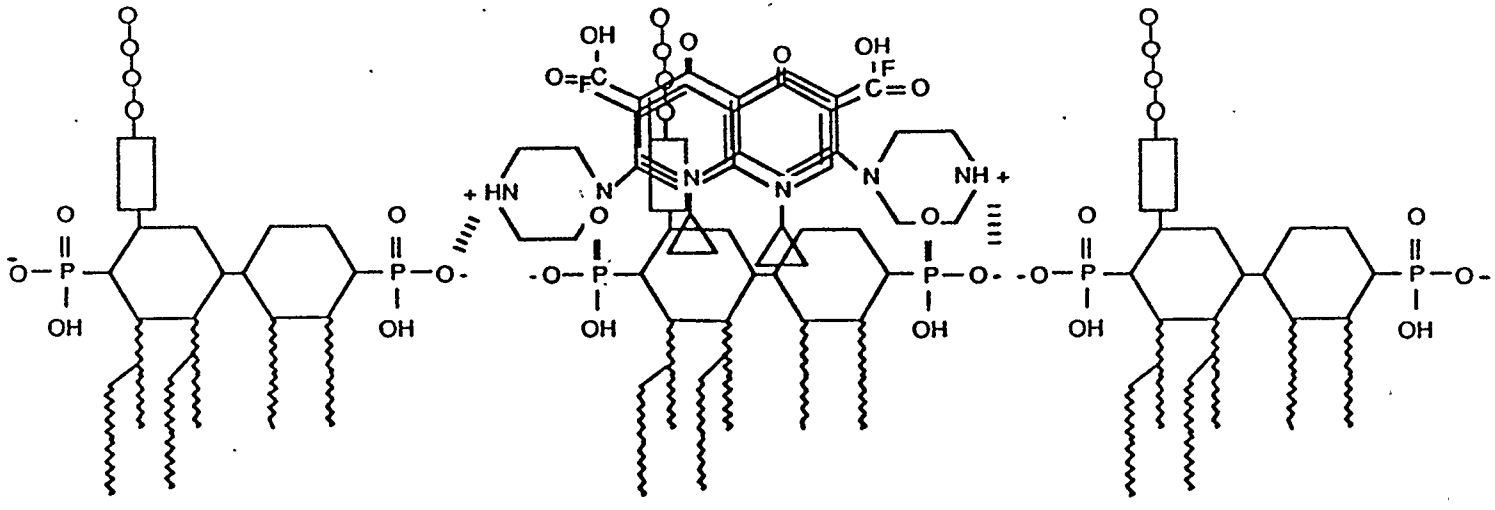
A.



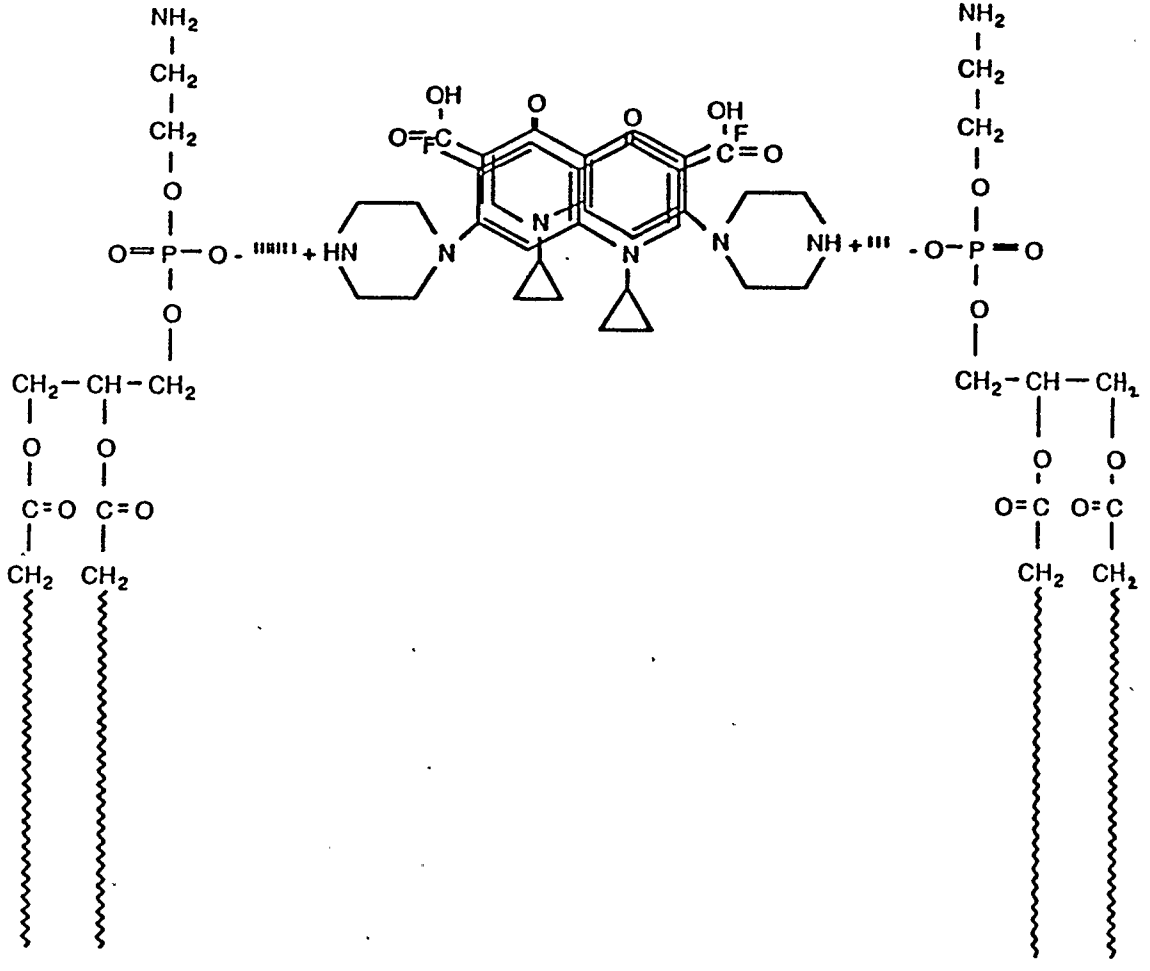
B.


Repeat units
Core
Disaccharide-
diphosphate
Fatty acids
OH
OH
OH
OH
+Mg+

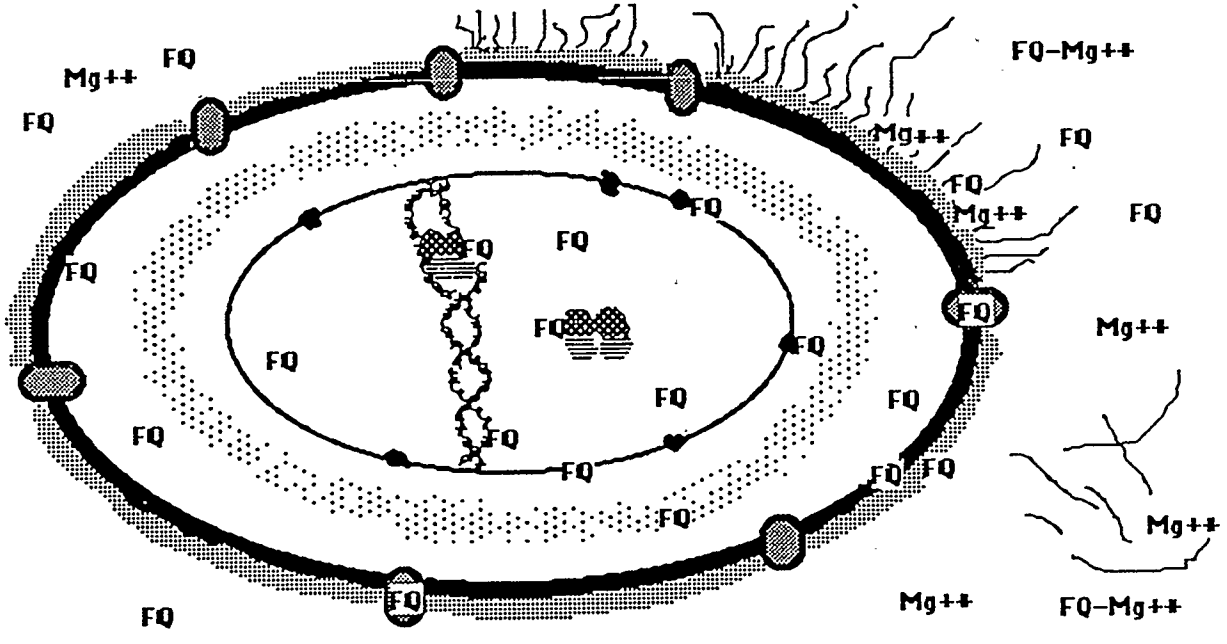
C.



D.



E.



FQ :Fluoroquinolone

:Lipopolysaccharide

:Porin

: Carrier protein(efflux system)

:DNA gyrase(A subunit)

:DNA gyrase(B subunit)

:Inner membrane

:Peptidoglycan

:Outer membrane

:DNA

with a reduction in the intracytoplasmic uptake of protonated enoxacin. This would be consistent with previously published data from Yoshimura and Nikaido (1985) which demonstrated that β-lactams diffuse more rapidly through *E. coli* porin channels in their zwitterionic state compared to corresponding molecules in protonated form. Such a phenomenon could also be true for fluoroquinolones. Studies performed by Hooper *et al.*, 1989 confirmed this hypothesis since in their uptake assay, the equilibrium level of norfloxacin accumulation was found to be 2 to 2.5-fold decreased at pH 6.0 compared to pH 7.0.

With regard to fluoroquinolone association with lipid bilayers, initial interaction would involve ionic binding of a positively charged piperazine ring of these antimicrobial agents with the negatively charged phosphate residue located at the "head" of phospholipids. Thereafter, drug partitioning into the lipid bilayer would occur utilizing the lipophilic tail (Figure 32). The higher association of [^{14}C] ciprofloxacin and [^{14}C] enoxacin observed with liposomes at acidic pHs compared to basic pHs, and the liposome clumping observed at acidic pH in the presence of a high concentration (6 mg/ml) of these two antimicrobial agents did not correlate with their ability to affect the integrity of vesicle membranes. This phenomenon was found to be dependent on the drug concentration and was

maximal when drug hydrophobicity was highest. At pH 7, fluoroquinolones exhibit a neutral state of charge, and maximum interaction with liposomes occurs. This suggests that both ionic and hydrophobic forces are involved in fluoroquinolone-lipid interactions. The ionic interaction would be predominant at a low pH, and hydrophobic association would occur mainly at the neutral pH. Such interactions would represent the first step in a diffusion process through lipid bilayers.

The *E. coli* F porin was found to be a very important factor in determining cell susceptibility and uptake process of fluoroquinolones in this bacterium. Data presented in this thesis were confirmed by several other investigators (Aoyama *et al.*, 1987; Hooper *et al.*, 1989, Hirai *et al.*, 1986b). *E. coli* JF-derivatives 694 ($OmpC^-$,F^-) and 701 ($OmpC^-$) were still susceptible to various fluoroquinolones, as well as the parent strain, JF-568. This shows that additional nonporin routes exist consistent with lack of fleroxacin-resistant mutants found with F porin mutation being solely responsible for the drug resistance observed (Chapman *et al.*, 1988). For hydrophilic quinolones such as enoxacin, ciprofloxacin and norfloxacin, the porin pathway is more relevant to the permeation process through the outer membrane of *E. coli* than for relatively hydrophobic quinolones such as ofloxacin and fleroxacin. It is interesting to note that the *E. coli* F porin mediates cell susceptibility to a

variety of unrelated antimicrobial agents such as tetracycline and chloramphenicol (Chopra and Eccles, 1978), some aminoglycosides (Foulds and Chai, 1978), and some β-lactams such as carbenicillin, ticarcillin, and benzyl penicillin (Harder *et al.*, 1981). The clinical significance of this observation is yet to be defined. An F porin pathway preferentially used in *E. coli* by fluoroquinolones could be highly relevant to an *in vivo* situation. For instance, studies of porin expression of a clinical isolate of *Salmonella typhimurium* in a high osmolarity medium with osmotic activity similar to that in a patient's tissues demonstrated that the synthesis of OmpF porin was repressed (Madeiros *et al.*, 1987). This could be a detrimental factor to the success of antimicrobial therapy, since in the case mentioned above, resistance to cephalosporins arising during therapy was attributed, in addition to the absence of expression *in vivo* of F porin, to the loss of the OmpC porin.

However, the major outer membrane protein of *P. aeruginosa*, protein F, does not seem to be an important cell component contributing to the susceptibility or resistance to fluoroquinolones. This is consistent with data obtained from uptake studies with *P. aeruginosa* PCC 23 strain associated with mutated F protein and also from uptake studies with a set of clinical isolates from a cystic fibrosis patient. There is also new evidence that this protein may not have solely or even significant

porin function in the whole bacterium (Gotoh *et al*, 1989), despite pore-forming activity observed *in vitro* (Hancock *et al.*, 1979) with isolated porin protein. This view is further supported by the lack of relationship of quinolone resistance and the presence or absence of F protein reported very recently from our laboratory (Chamberland *et al.*, 1990).

The investigations presented in this thesis demonstrate that fluoroquinolones bind reversibly to various cell components including the cell target DNA (Shen and Pernet, 1985; Tornaletti and Pedrini, 1988) or a DNA-DNA gyrase complex (Shen *et al.*, 1989c). This is supported by cell growth studies in the presence of enoxacin at various concentrations and time exposures which demonstrated cell recovery upon washing treatment. Despite a very rapid uptake process observed for enoxacin, a period of 40 to 50 minutes was required for enoxacin to exert its antibacterial activity. The bacteriostatic effect observed probably results from inhibition of DNA gyrase activity. The process was independent of increased drug uptake (unsaturable process) since the time required to obtain growth inhibition was not reduced at enoxacin concentrations up to 80 μg/ml. It seems that a large amount of drug is not required to saturate cell target(s).

Uptake studies of enoxacin and ciprofloxacin demonstrated that the nature of drug permeation into *E. coli*, *P. aeruginosa*, *A. faecalis*, and *B. subtilis* bacterial cells is simple diffusion. This is consistent with (i) rapid equilibrium achieved within one to two and one-half minutes; (ii) lack of saturability proven by Lineweaver-Burk plot studies; (iii) no competition observed with structural analogs; (iv) effect of temperature; (v) energy-independent process since no reduction of uptake was observed with cell energy inhibitors; (vi) the reversibility of the drug accumulation process. The concept of the fluoroquinolone uptake process being a simple diffusion mechanism in *E. coli* was later supported by the work of Cohen *et al.*, 1988a, Chapman and Georgopapadakou, 1988, and Hooper *et al.*, 1989. The same conclusion was reported later for *P. aeruginosa* by Celesk and Robillard, 1989.

As mentioned in the Results section, uptake of ciprofloxacin in *P. aeruginosa* was observed at concentrations below MIC, suggesting that this antimicrobial agent is being bound at an extracytoplasmic site, most likely at the outer membrane or at the periplasmic space. This was associated with evidence of ciprofloxacin being more firmly bound in *P. aeruginosa*, since increased washing regimens were relatively ineffectual at removal of the drug compared to *E. coli*. The cellular concentration of [^{14}C] ciprofloxacin using a 10-ml washing regimen in the

uptake protocol is, on the average, about four times the extracellular concentration (0.154 μg/ml) for *E. coli* J5-3, and about seven times under the same conditions for *P. aeruginosa* PAO 503. There are several possible explanations for this set of observations: (i) The LPS structure of the outer membrane of *P. aeruginosa* is known to be rich in phosphate residues (Nikaido and Hancock, 1986), therefore, additional sites for chelation and binding with the positively charged piperazine substitution would be available, increasing the probability of fluoroquinolone interaction with the outer membrane. However, a monovalent cation like the positively charged fluoroquinolone bound to phosphate residues would be dissociated or displaced by divalent cations present in the medium. This suggests that there are several events occurring at the same time including ionic interactions, chelation of magnesium, diffusion through hydrophilic and hydrophobic pathways. (ii) Another possibility would reside in the relatively low permeability of *P. aeruginosa*'s outer membrane due to the presence of small hydrophilic diffusion pores (Yoneyama and Nakae, 1986). This is in agreement with reduced uptake of some compounds observed in *P. aeruginosa* compared to *E. coli*. The relative contribution of a hydrophilic route involving the porin pathway is most likely to be of little importance in this organism for all quinolones. However, ciprofloxacin, a relatively hydrophilic

quinolone, is still the most active quinolone drug against *P. aeruginosa* (Wolfson and Hooper, 1985). This suggests that the non-porin pathway (Hancock *et al.*, 1981, Chapman and Georgopapadakou, 1988) might be more important in *P. aeruginosa* strain compared to *E. coli*, perhaps even for relatively hydrophilic quinolones. As suggested to me by R.E.W. Hancock, once in the periplasm, ciprofloxacin would chelate magnesium and this newly formed complex would be a poor substrate for a further permeation process across the cytoplasmic membrane, or for the non-porin pathway across the outer membrane.

Interestingly, uptake studies demonstrated that CCCP caused a small but consistent increase of fluoroquinolone accumulation, presumably due to the presence of an efflux system (Cohen *et al.*, 1988a). It is rather difficult to assess the relative importance of the efflux system in the overall accumulation of fluoroquinolones in the bacterial cell. The activity of this efflux system, as revealed by the addition of CCCP was found to vary among types of bacteria studied, the nature of media utilized, and the type of energy inhibitor used (Bryan *et al.*, 1989). It is also possible that increased enoxacin and ciprofloxacin accumulation seen in the presence of 20 μM CCCP could be the result of an enhancement of the outer membrane permeation process for these antimicrobial agents. It has been proposed that cell de-energization might result in alteration of outer membrane

permeability, since increased fluorescence of hydrophobic fluorescent probes such as N-phenyl naphthylamine (suggesting lipid partition) was observed in de-energized bacterial cells (Hancock, 1984). Interestingly, a differential effect of CCCP and 2,4-DNP, two energy inhibitors which dissipate the proton motive force, has been observed by Diver *et al.* (1990). In their studies, increased quinolone uptake in *E. coli* was observed with CCCP, whereas cells in presence of 2,4-DNP, accumulated a significantly reduced amount of drug. In our case, studies of [^{14}C] enoxacin accumulation profiles in the presence of 2,4-DNP demonstrated no inhibitory effect on the uptake process.

Usually gram-positive bacteria require a higher concentration of fluoroquinolones to exhibit antibacterial activity (Wolfson and Hooper, 1985) despite the increased uptake observed for *B. subtilis* compared to *E. coli*. This situation most likely results from reduced potencies of quinolones against DNA gyrase enzymes of gram-positive bacteria. In fact, determinations of supercoiling inhibition constants of various quinolones for *Micrococcus luteus* DNA gyrase revealed that, on the average, quinolones were 79-fold more potent against *E. coli* DNA gyrase than *M. luteus* enzyme (Shen *et al.*, 1989c). The same authors suggested that the lower sensitivity observed for *M. luteus* DNA gyrase resides in the

greater distance between the two separated DNA strands at the site of DNA-DNA gyrase activity which would not be spanned by quinolones.

A model of fluoroquinolone accumulation in gram-negative bacteria has been undertaken from studies performed in this thesis (Figure 32) as well as investigations from other researchers. It is believed that the fluoroquinolone permeation process involves several routes of entry: (i) Hydrophilic pathways represented by porins in *E. coli* and perhaps as-yet-unknown outer membrane proteins associated with pore channel forming activity in *P. aeruginosa*; (ii) Another route resides in uptake by binding of drug through chelation and ionic forces to LPS followed by destabilization of the outer membrane from LPS release; (iii) A third route operates through partitioning of drug through hydrophobic residues into the lipid portion of the outer or inner membranes following the binding to LPS as given in (ii) or to polar heads of phospholipids. The importance of the last pathway depends on the relative hydrophobicity of the quinolones, but the cumulated evidence indicates all fluoroquinolones would diffuse to various extents by partitioning within lipid bilayers. In all cases, diffusion is most likely to be bidirectional.

The amount of quinolones associated with the cell at equilibrium is dependent upon several factors. Fluoroquinolones would initially interact with LPS by chelating magnesium, which once removed exposes negatively charged phosphate residues of the lipid bilayer of the outer membrane. Ionic interaction would occur thereafter with the positively charged piperazine ring at C-7 position of the quinolone nucleus. This process would allow a progressive influx of drug molecules closer to the lipophilic portion of the outer membrane, followed by drug partition and diffusion toward the periplasmic space. Diffusion through the inner membrane would once again involve ionic and hydrophobic interactions. Once within the cytoplasm, the drug would be subject to active efflux.

4.2 Factors Determining Cell Susceptibility to Fluoroquinolones.

Cell susceptibility to quinolones is determined by several factors which are in part obviously related to: (i) the molecular structure of these antimicrobial agents; (ii) the type of bacteria involved; and (iii) the composition of the extracellular environment.

(i) It is interesting to note the striking effect of a piperazine substitution at the C-7 position of nalidixic acid which most likely increases the rate of the drug uptake process as well as the affinity for the

DNA-DNA gyrase complex. The latter is demonstrated by higher ID_{50} values for inhibition of DNA synthesis found for nalidixic acid in *P. aeruginosa* (6 - 16 μg/ml) (Rella and Haas, 1982) compared to ID_{50} values of ciprofloxacin found with the same organism (0.1 μg/ml) (Bedard *et al.*, 1989). Similar differential results for ID_{50} values of these two antimicrobial agents, being >100 μg/ml for nalidixic acid and 0.24 μg/ml for ciprofloxacin, were obtained with the *Staphylococcus aureus* DNA gyrase (Takahata and Nishino, 1988). Altogether, these results are consistent with the proposed model of Shen *et al.* (1989c), that the piperazine ring is one of the domain(s) involved in drug-DNA gyrase interactions. The increased drug potency observed with ciprofloxacin compared to norfloxacin which results from a cyclopropyl substitution at N-1 position (Figure 1) might result in a higher cooperativity in the drug-DNA binding process since hydrophobic interactions are required for the drug-drug self association process (Shen *et al.*, 1989c).

(ii) The type of bacteria would govern several factors related to target affinity and relative permeability to quinolones. For instance, *P. aeruginosa* DNA gyrase is most likely more refractory to the inhibitory effect of ciprofloxacin (Bedard *et al.*, 1989) as is *S. aureus* (Takahata and Nishino, 1988), and *Micrococcus luteus* (Shen *et al.*, 1989c) compared to *E. coli* DNA gyrase (Bedard *et al.*, 1989; Chow *et al.*, 1988). More-

over, the decreased susceptibility of *P. aeruginosa* to fluoroquinolones compared to *E. coli* might also be related in part to a different cascade of events induced in these two organisms in the presence of these antimicrobial agents. For instance, recovery of DNA synthesis preceded by the initial inhibition of DNA synthesis induced by quinolones in *P. aeruginosa* was not found to be related to an SOS-DNA repair system which is involved in *E. coli* (Benbrook and Miller, 1986). In their studies, *P. aeruginosa* cells were infected with bacteriophage D_3 which has been previously exposed to UV radiation. No increased capacity to repair UV-induced damage to phage D_3 was noted in the presence of norfloxacin or nalidixic acid. However, the DNA synthesis recovery induced in *P. aeruginosa* by the presence of quinolones was found to be modulated with protein synthesis. Such a phenomenon was attributed to induction of DNA gyrase. With regard to cell permeability, studies presented in this thesis demonstrated that the outer membrane plays a major role in determining bacterial cell susceptibility to fluoroquinolones, specifically for *A. faecalis* and *P. aeruginosa*.

(iii) Composition of the extracellular environment will also be a determining factor which will affect the antimicrobial activity of quinolones by determining the net charge and degree of hydrophobicity and extent of chelating ability, and by modulation of phenotypic

expression of cell structures (porin, LPS) mediating the permeation process of these antimicrobial agents. For instance, the expression of porins in enteric bacteria is known to be regulated by osmotic pressure and temperature (Nikaido and Vaara, 1985). Cell growth rate might also be affected in an environment offering poor growth conditions. It is well known that the bactericidal activity of quinolones is reduced in viable nonreplicative bacteria which might reflect a lower activity of DNA gyrase.

In conclusion, it can be stated that fluoroquinolone permeation processes, as well as factors determining the overall bacterial cell susceptibility to these antimicrobial agents, presents a set of processes of high complexity.

The exact lethal events triggered by fluoroquinolones in bacterial cell, as well as antitumor drugs targeting eukaryotic topoisomerase II, remain unknown since the exact mechanism of inhibition of topoisomerase II activities at the molecular level is yet to be defined. Such problems will be the subject of my post-doctoral studies.

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