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Interaction of the Fluoroquinolone Antimicrobial Agents Ciprofloxacin and Enoxacin with Liposomes

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The interaction between ciprofloxacin and enoxacin and phospholipid-containing bilayers was examined as the initial step in transmembrane diffusion processes. By using cosedimentation, maximal association of liposomes and ^{14}C-labeled enoxacin and ciprofloxacin was detected at acidic and neutral pHs. Aqueous solubility of ciprofloxacin, enoxacin, and norfloxacin was poorest at neutral pH and greater at alkaline or acidic pHs. These investigations suggest that the interaction occurs because of ionic and hydrophobic forces and is nonsaturable up to 20 μg/ml.

Fluoroquinolones have been reported to reach their intracellular target, DNA gyrase, in *Escherichia coli* by a simple diffusion process through the outer and inner membranes (2). The lack of a requirement for energy for cell uptake has been recently confirmed by two other studies (4, 6). The porin pathway through the outer membrane has been shown to be a major route of entry because mutations affecting porins or presumed porins cause resistance to fluoroquinolones (1, 2, 7–9). In some cases, these mutations have been associated with lower levels of uptake of the drugs (1, 2, 3a, 6) and reduced rates of diffusion of enoxacin (2).

More recently, Chapman and Georgopapadakou reported an increase in surface hydrophobicity of *E. coli* and sensitization to sodium dodecyl sulfate-mediated lysis after exposure of cells to fleroxacin (4). They proposed that fleroxacin could chelate magnesium located in the lipopolysaccharide layer, progressively creating hydrophobic patches in the outer membrane, and enter through these patches. Although fleroxacin has a slightly higher hydrophobicity, as revealed by the partition coefficient in *n*-octanol–0.1 M phosphate buffer (pH 7.2) (0.08), than enoxacin (0.007), norfloxacin (0.01), or ciprofloxacin (0.02), it has a lower hydrophobicity than many other potentially clinically important fluoroquinolones (8). Therefore, it seems likely that for many clinically important fluoroquinolones, a hydrophobic route of permeation exists in both outer and inner membranes of *E. coli*. The process of diffusion of compounds with low partition coefficients through phospholipid-containing bilayers is not well understood. An initial interaction between the compound and the lipid bilayer is likely the first step in the diffusion process. In the present study, we used liposomes to determine if an interaction between fluoroquinolones with relatively low partition coefficients and a lipid bilayer could be demonstrated. We also examined the effect of pH on this interaction and the nature of the equilibrium between the drugs and the liposomes.

Enoxacin was obtained from Parke, Davis & Co., Toronto, Ontario, Canada; norfloxacin was from Merck & Co., Inc., Rahway, N.J.; and ciprofloxacin hydrochloride was from Miles Pharmaceuticals, Toronto, Ontario, Canada. All other reagents and the liposome preparation kit were from Sigma Chemical Co., St. Louis, Mo. [^{14}C]enoxacin (4.8 Ci/mol) and [^{14}C]ciprofloxacin (14.8 Ci/mol) were generously provided by Parke, Davis & Co. and A. Dalhoff, Bayer AG, Pharma Research Center, Wuppertal, Federal Republic of Germany, respectively. Stock solutions of quinolones for solubility studies were prepared at 10 mg/ml in water. A few drops of sodium hydroxide were added to enoxacin and norfloxacin to ensure complete solubilization. A sample (100 μl) was diluted in 800 μl of 0.1 M MES [2-(*N*-morpholino) ethane-sulfonic acid]–NaOH and adjusted to a specific pH in the range of 3 to 10 with 1 N HCl or NaOH, and the volume was brought to 1 ml. These preparations were held at room temperature for 1 h to ensure complete precipitation of nonsolubilized drug and centrifuged for 5 min at 15,000 × *g*. The supernatants were removed and diluted 1:200 with the same buffer, and the optical densities at 260, 273, 267, and 270 nm were measured for nalidixic acid, norfloxacin, enoxacin, and ciprofloxacin, respectively. The concentrations were determined with a standard curve relating concentration to the optical density established at each pH.

Liposomes were prepared from a commercial liposome kit containing phosphatidylcholine, dicetylphosphate, and cholesterol in a ratio of 100:28.6:14.3, respectively. A portion of phosphatidylcholine (6.2×10^{-6} mol) was dried in a conical tube previously acid washed under a nitrogen gas stream to remove chloroform. This preparation was incubated for at least 1 h in a desiccator under vacuum at room temperature. A fine glass rod was used to scrape the lipid film from the wall of the tube, 100 μl of 0.1 M MES–NaOH (pH 7.0) was added, and vesicles were formed by agitation in a vortex mixer. A 10-μl sample of this suspension was diluted in 200 μl of 0.1 M glycine buffer (pH 4.0), 0.1 M MES–NaOH (pHs 6.0 and 7.0) or 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid) buffer (pHs 8.0, 9.0, and 10.0) containing either 2 μg of [^{14}C]enoxacin or 1.23 μg of [^{14}C]ciprofloxacin at their original specific activities per ml. The final concentration of phosphatidylcholine was 3.1×10^{-6} mol/ml. This preparation was centrifuged at 100,000 × *g* for 5 min in an airfuge (Beckman Instruments, Inc., Fullerton, Calif.). The supernatant was discarded, and the pellet was suspended to the same volume in the same buffer without an antimicrobial agent and centrifuged again at 100,000 × *g* for 5 min. The pellet was suspended, and 5 ml of PCS solubilizer was added to determine radioactivity. No counts were detected in controls without liposomes treated in the same manner. For studies on the effects of quinolone concentration, the assays were performed at pH 7.0. Con-

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centration ranges were 1 to 5 μg/ml for [^{14}C]enoxacin and 0.62 to 3.10 μg/ml for [^{14}C]ciprofloxacin. To determine the effects of lipid concentration, 3.1×10^{-6} to 24.8×10^{-6} mol of phosphatidylcholine per ml was examined with either 2 μg of [^{14}C]enoxacin or 1.23 μg of [^{14}C]ciprofloxacin per ml. All samples were corrected for quenching by lipid by adding labeled antimicrobial agents to scintillation fluid containing the range of phosphatidylcholine described above.

We examined the cosedimentation of [^{14}C]enoxacin or [^{14}C]ciprofloxacin with liposomes at various pHs to determine the extent of a liposome-drug interaction and its relationship to aqueous solubility of the drugs (Fig. 1A and 1B). These results showed maximal association of both agents with liposomes at pHs below 8, corresponding to the protonated and uncharged forms of the drugs. Reduced association of the drugs with liposomes was observed with increasing pH, which correlated with an increase in their hydrophilicity as well as their electronegativity (Fig. 1A and 1B).

The aqueous-solubility pattern of another amphoteric fluoroquinolone, norfloxacin, resembled those of enoxacin and ciprofloxacin, but all were distinctly different from that of nalidixic acid (Fig. 1C). Our lack of radiolabeled norfloxacin and nalidixic acid prevented measurement of their association with liposomes. However, on the basis of similar solubility profiles, it seems reasonable to assume that norfloxacin may interact with liposomes in a manner similar to that of enoxacin and ciprofloxacin.

The amount of ciprofloxacin and enoxacin associated with liposomes relative to drug concentration was found to be a linear function (Fig. 2A). The amount of ciprofloxacin associated with lipid was 1.4 times higher than the amount of enoxacin, as determined from the slopes of the plots in Fig. 2, and correlated with its slightly higher partition coefficient. The association was nonsaturable (Fig. 2A insert) at drug concentrations of up to 20 μg/ml.

The percentage of drug associated with 1 μmol of phosphatidylcholine (Fig. 2A) was independent of fluoroquinolone concentration in the range of 0.62 to 5 μg/ml, with values of 0.422% ± 0.015% for ciprofloxacin and 0.344% ± 0.030% for enoxacin. The values of drug bound to liposomes at equilibrium 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. A time course experiment showed that this equilibrium was established within 5 min and was stable within 30 min (data not shown). Contrary to expectation, the amount of drug associated with liposomes at equilibrium was found to decrease as the lipid concentration increased (Fig. 2B). We propose that this phenomenon reflects the diminution of surface area available for drug binding, due to liposome aggregation, as observed under light microscopy at high lipid concentrations.

The binding of enoxacin and ciprofloxacin to liposomes was maximal when the majority of the drug population was predicted to carry a net positive charge or no net charge. This indicates that the binding is the result of two different processes. One of these is ionic binding between negatively charged phosphate groups of the phospholipid and the positively charged piperazine ring at the C-7 position of the quinolone. Klopman et al. concluded that cell permeability of fluoroquinolones was predominantly controlled by the nature of the C-7 substitution (10). The second form of binding is likely to be by hydrophobic forces between the lipid bilayer and enoxacin and ciprofloxacin.

The major phospholipid in the liposomes used in our studies was phosphatidylcholine, which would behave in a manner somewhat similar, regarding charge, to that of phosphatidylethanolamine, the major bacterial phospholipid. Both have ionic groups over a wide pH range, with phosphatidylethanolamine being slightly more acidic than phosphatidylcholine. The charge states range from 2− and 1+ at low pH to 0 or 1− at pH 10. These do not change our conclusions that the drug-liposome interaction is explained

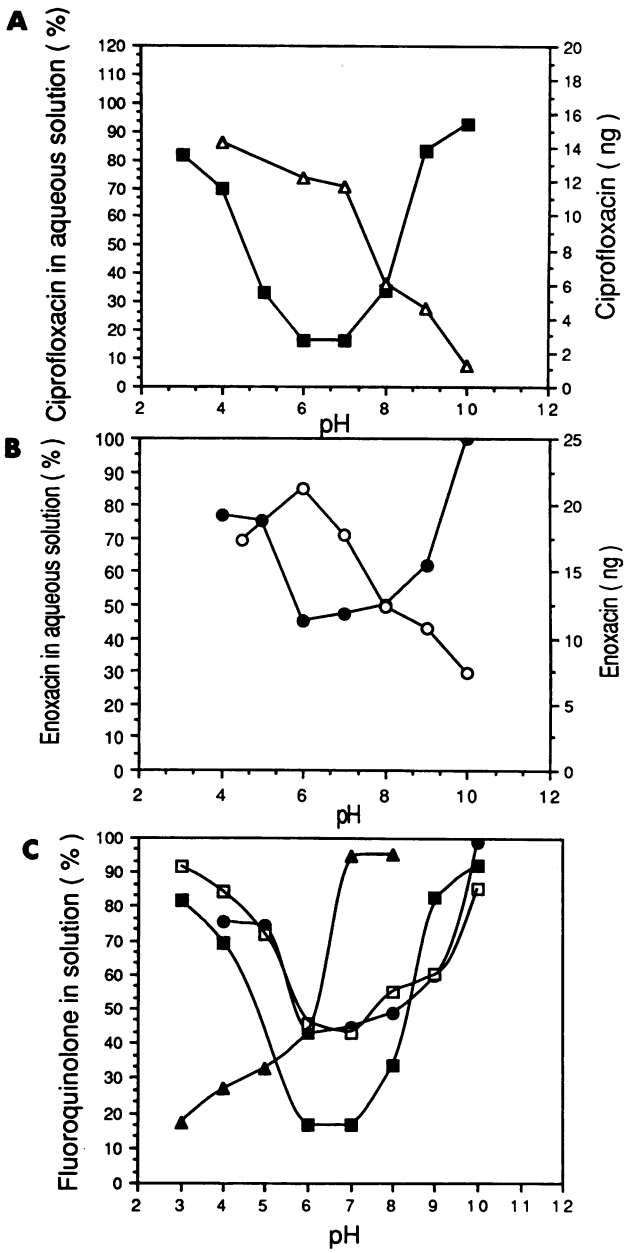

FIG. 1. (A) Correlation between the amount of [^{14}C]ciprofloxacin (1.23 μg/ml) associated with liposomes (3.1×10^{-6} mol of phosphatidylcholine per ml) (△) and ciprofloxacin solubility in 0.1 M MES–NaOH as a function of pH (■). (B) Correlation between the amount of [^{14}C]enoxacin (2 μg/ml) associated with liposomes (3.1×10^{-6} mol of phosphatidylcholine per ml) (○) and enoxacin solubility in 0.1 M MES–NaOH as a function of pH (●). (C) Percentages of enoxacin (●), ciprofloxacin (■), norfloxacin (□), and nalidixic acid (▲) in aqueous solution (0.1 M MES–NaOH) as a function of pH at an initial concentration of 1 mg/ml.

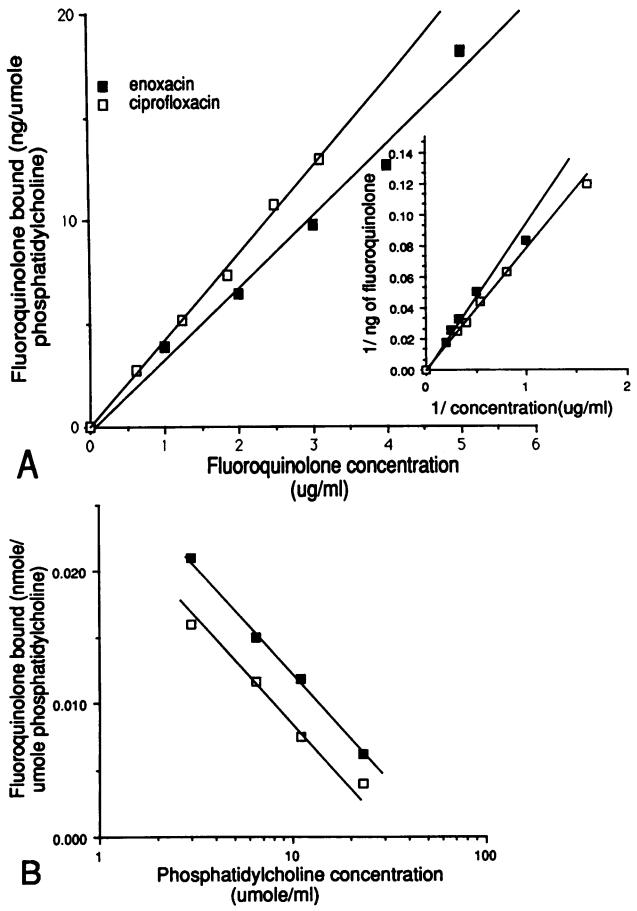

FIG. 2. (A) Effects of enoxacin (■) and ciprofloxacin (□) concentrations on the amount of drug associated with liposomes per micromole of phosphatidylcholine. The assay was performed at pH 7.0 in 0.1 M MES–NaOH. Insert, Lineweaver-Burk plot of 1/ng of fluoroquinolone bound per μmol of phosphatidylcholine versus 1 per concentration of the fluoroquinolone. (B) Amount of enoxacin (2 μg/ml) (■) and ciprofloxacin (1.23 μg/ml) (□) associated per micromole of phosphatidylcholine per milliliter as a function of phosphatidylcholine concentration. The assay was performed in 0.1 M MES–NaOH (pH 7.0).

by a mainly ionic interaction at low pH and a mainly hydrophobic interaction at neutral pH.

Enoxacin and ciprofloxacin are frequently regarded as highly hydrophilic compounds because of their partition characteristics in *n*-octanol–phosphate buffer (8). However, it is clear from poor aqueous solubilities at pHs 5 to 9 that these compounds have complex hydrophobicity and hydrophilicity characteristics. We feel it is an oversimplification to predict the tendency of these agents to interact with phospholipid bilayers on the sole basis of the partition coefficients. Rather, a direct measure of the interaction is necessary because of the complexity of the various interactions between these drugs and a lipid bilayer. A phospholipid bilayer does not bind drugs simply on the basis of hydrophobicity or charge, but likely on the basis of some component of each. In addition, ciprofloxacin, enoxacin, and norfloxacin consist of a wide range of charged species over a narrow pH range, and they all possess hydrophobic components, particularly at the N-1 position. This hydrophobicity may be very prominent when these drugs carry no net or a small net charge and may promote solubilization within the phospholipid bilayer.

The relevance of liposome binding by ciprofloxacin and enoxacin to whole bacterial activity of these compounds is not clear. In general, the activity of these two antimicrobial agents tends to be less at low pHs, although this varies with different bacteria (3, 5, 11). This trend is not reflected in the results of the liposome-binding assay. However, other factors may be involved in determining the pH effect of a quinolone on whole cells, including binding to DNA (12, 13), activity of DNA gyrase at different pHs, rate of cell growth, activity of the efflux system (3a, 6), and the magnitude of other binding sites on the cell, which may obscure the effect of phospholipid binding. We believe liposomal binding is relevant to an understanding of diffusion of fluoroquinolones across phospholipid bilayers as one component of uptake into whole cells. We propose that binding represents the initial step in diffusion. Thereafter, partitioning in the bilayer occurs particularly at neutral pHs, at which aqueous solubility is lowest. With a concentration gradient from the outer to the inner face, the drug would move to the cell interior. Inside the cell, the inner face of the membrane interfaces with slightly alkaline cell water (under most conditions), favoring release of the fluoroquinolone and maintaining the cross membrane gradient until steady state is achieved.

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