ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1996, p. 2865–2873 Vol. 40, No. 12
0066-4804/96/$04.00+0

Correlation of Trimethoprim and Brodimoprim Physicochemical and Lipid Membrane Interaction Properties with Their Accumulation in Human Neutrophils

MASSIMO FRESTA,[1] PIO M. FURNERI,[2] ELENA MEZZASALMA,[1] VITO M. NICOLOSI,[2] AND GIOVANNI PUGLISI[1]*

Department of Pharmaceutical Sciences[1] and Institute of Microbiology,[2] University of Catania, Catania, Italy

Received 22 March 1996/Returned for modification 2 July 1996/Accepted 29 September 1996

Dipalmitoylphosphatidylcholine vesicles were used as a biological membrane model to investigate the interaction and the permeation properties of trimethoprim and brodimoprim as a function of drug protonation. The drug-membrane interaction was studied by differential scanning calorimetry. Both drugs interacted with the hydrophilic phospholipid head groups when in a protonated form. An experiment on the permeation of the two drugs through dipalmitoylphosphatidylcholine biomembranes showed higher diffusion rate constants when the two drugs were in the uncharged form; lowering of the pH (formation of protonated species) caused a reduction of permeation. Drug uptake by human neutrophil cells was also investigated. Both drugs may accumulate within neutrophils; however, brodimoprim does so to a greater extent. This accumulation is probably due to a pH gradient driving force, which allows the two drugs to move easily from the extracellular medium (pH ~7.3) into the internal cell compartments (acid pH). Once protonated, both drugs are less able to permeate and can be trapped by the neutrophils. This investigation showed the importance of the physicochemical properties of brodimoprim and trimethoprim in determining drug accumulation and membrane permeation pathways.

Folate biosynthesis is one of the best-known enzymatic pathways operating in many organisms (6, 12, 22). Thus, the folic acid pathway of bacteria can be a suitable target for the selective toxicity of some synthetic antibacterial agents; this is the case for 2,4-diaminobenzylpyrimidines (3, 6, 27, 29).

The high safety and efficacy of trimethoprim, an antimicrobial agent belonging to the 2,4-diaminobenzylpyrimidine class and active against a wide spectrum of gram-negative and -positive strains, lie in its selective inhibition of bacterial dihydrofolate reductase (DBFR) compared with the mammalian enzyme (18, 21, 34, 45, 56). In fact, the 7 nM drug concentration required to reduce the activity of the *Escherichia coli* enzyme by 50% is similar to that required for DHFRs from other bacteria susceptible to inhibition, e.g., *Proteus vulgaris* and *Staphylococcus aureus* (13, 51). The 50% inhibitory concentrations required for the various mammalian species examined and for human enzymes are 4 to 5 orders of magnitude greater (4, 24). Therefore, trimethoprim seems to be a very attractive antibacterial drug since it is highly active against strains resistant to other antibiotics frequently used, e.g., β-lactam antibiotics (35, 59). This drug is also used and marketed in combination with sulfamethoxazole in an attempt to exploit this interaction. Since sulfonamides inhibit dihydropteroate synthetase, a stage slightly earlier than DHFR in the anabolic process, the blocking of these two enzymes was believed to have a synergistic effect (17).

The continued development of this class of antibiotics has been directed to solving two main problems: (i) how to broaden the spectrum, as some species remain inherently resistant (2, 38), and (ii) how to improve the pharmacokinetics. For this purpose, a series of trimethoprim analogs have recently been prepared (35). The most effective of these compounds is brodimoprim (62), in which the 4-methoxy group on the benzyl ring of trimethoprim is replaced by bromine (Fig. 1). The effect of this substitution is to improve the binding of the drug to DHFR, as can be demonstrated by measuring the degree of enzyme inhibition with known inhibitor concentrations (1). Particularly, brodimoprim showed an inhibiting capacity against bacterial DHFRs two times higher than that of trimethoprim (59). This drug was also more active against DHFRs that had been relatively insensitive to trimethoprim, such as those from *Pediococcus cerevisiae*, *Bacteroides fragilis*, and *Nocardia restricta*. Preferential binding of brodimoprim with regard to trimethoprim was also found with the plasmid-encoded DHFRs that mediate trimethoprim resistance (59). These findings and perspectives have recently prompted further investigations in experimental microbiology, biochemistry, and clinical pharmacology to fully evaluate the structure-activity relationship, the drug-membrane interaction, and the therapeutic potentials of these drugs (7, 8, 11, 14, 44).

In the evaluation of the antimicrobial effectiveness of trimethoprim or brodimoprim, the efficiency of DHFR inhibition does not represent an infallible parameter of and/or guide to likely antibacterial activity. The capability of the drug to reach the target is also important (15, 26, 28, 30, 58) to both the target pathogenic bacterium itself in the host and the target enzyme inside the pathogen, as the DHFR is an intracytoplasmic enzymatic system.

It seems likely that for 2,4-diaminobenzylpyrimidines, a hydrophobic pathway of bacterial or cellular penetration is responsible for drug passage through either outer or inner membranes. The diffusion of trimethoprim and brodimoprim through the phospholipid bilayer structures of the membranes is not well-known. Probably, an initial interaction between the compound and the lipid bilayer is the first phenomenon in the

* Corresponding author. Mailing address: Istituto di Chimica Farmaceutica e Tossicologica, Università di Catania, Viale Andrea Doria 6, I-95125 Catania, Italy. Phone: 39 95 22.22.15. Fax: 39 95 22.22.39.

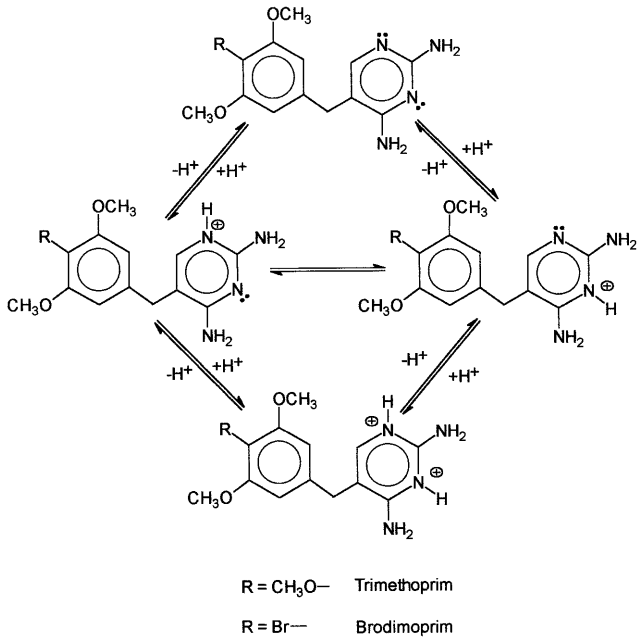

FIG. 1. Structural formulas of trimethoprim and brodimoprim. The presence of a 2,4-diaminopyrimidine ring in the molecules of both drugs can lead to different protonation and deprotonation equilibria as a function of the pH of the environment.

diffusion process. Structure-activity relationships for 2,4-diaminobenzylpyrimidines have been reported (9, 20, 39, 52), showing the influence of structure modifications on biological activity. However, few studies have dealt with biologically important physicochemical parameters. The knowledge of these characteristics is important for a thorough understanding of adsorption, transport, and receptor binding of these drugs at the molecular level.

In this study, we used lipid vesicles to determine whether an interaction between trimethoprim or brodimoprim with a certain partition coefficient and a lipid bilayer could be demonstrated. The effect of pH on this interaction was also investigated. The drug-membrane interaction was studied by means of differential scanning calorimetry (DSC). The usefulness and correctness of using DSC analysis to investigate interactions between drugs and the lipid model membrane are well established (36, 37, 47–50). Dipalmitoylphosphatidylcholine (DPPC) was used to prepare lipid vesicles to study the interaction phenomenon. This phospholipid represents a suitable compound for the creation of a membrane model, that is, it is able to mimic many aspects of biological membranes, being one of their most abundant constituents. Considering that DPPC has well-defined thermotropic properties (19, 31), any changes in these properties may be easily related to the type of drug-membrane interaction (32). Drug permeation of membranes and the drug partition coefficient were related to DSC data. Furthermore, the accumulation of trimethoprim and brodimoprim in human neutrophils was studied and correlated with the physicochemical characteristics of these drugs.

MATERIALS AND METHODS

Materials. Synthetic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine monohydrate (DPPC) was purchased from Fluka Chemical Co. (Buchs, Switzerland). The purity of the phospholipid (>99%) was assessed by bidimensional thin-layer chromatography on silica gel plates (E. Merck, Darmstadt, Germany) loaded with a solution of the lipid in chloroform-methanol (3:1, vol/vol). Bidimensional thin-layer chromatography was performed by eluting the plate first with a solvent system consisting of chloroform-methanol–5 N ammonium hydroxide (60:3:5, vol/vol) and then with chloroform-methanol-acetic acid-water (12:60:8:2.5, vol/vol). The chromatographic spots of DPPC were stained with Dragendorff's reagent. The phosphorus content of the phospholipid was determined as inorganic phosphate by the method described by Bartlett (5). Hanks balanced salt solution (HBSS) without red phenol and Histopaque-1077 are Sigma Chemical Co. (St. Louis, Mo.) products.

The drugs 2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine (trimethoprim) and 2,4-diamino-5-(4-bromo-3,5-dimethoxybenzyl)pyrimidine (brodimoprim) were kindly provided by Master Pharma S.r.l. The purity of the drugs was greater than 99.5% as assayed by high-pressure liquid chromatography (HPLC) analysis. Double-distilled, pyrogen-free water was used. All of the other reagents and solvents used were of analytical grade (Carlo Erba, Milan, Italy).

Partition coefficient determination. The partition coefficients of trimethoprim and brodimoprim were determined with octanol–0.1 M phosphate buffer at pH values ranging from 3 to 10. The organic solvent and the aqueous buffer were poured together into a glass flask and then thoroughly stirred for 24 h at a thermostat-controlled temperature of 37°C to allow them to fully presaturate each other. An aliquot (1 mg) of the drug was added to 10 ml of octanol layered on 10 ml of aqueous buffer. This mixture was immersed in a thermostat-equipped water bath at 37°C and mixed by mechanical stirring (~1,000 rpm) for at least 3 h. The mixture was then centrifuged at 3,000 × *g* for 15 min, achieving complete phase separation. Aliquots were removed from the organic and aqueous phases and then assayed by spectrofluorimetric analysis at the drug λ_{max} (Uvikon 820; Kontron Instruments, Zurich, Switzerland), and the data reported were analyzed with respect to a calibration straight line determined at each pH value. The partition coefficient (log *P*) was expressed as the logarithm of the ratio between the amount of the antimicrobial drug in the organic phase and that in the aqueous phase. The drug recovery during determination of the partition coefficient was always higher than 98.9%.

Phospholipid membrane preparation. DPPC was dissolved in chloroform in a round-bottom flask containing 20 g of glass beads (2- to 3-mm mean size) (Carlo Erba). The purpose of the glass beads was to increase the surface area of the dried lipid film, thus enhancing the phospholipid contact with the aqueous solution. In this way, lipid hydration and dispersion were facilitated. The final volume of this solution was 20 ml. The organic solvent was evaporated at 35°C on a rotary evaporator until the lipids were dried and distributed as a thin film on the wall of the vessel and on the surface of the glass beads. Any trace of chloroform was eliminated by storage for 24 h at 40°C under high vacuum (Buchi T-50). The lipid films (10 mg) were hydrated by adding 200 μl of isotonic buffer at different pH values containing a certain amount of the drugs to obtain a molar fraction of 0.03. The flask was alternatively vortexed and warmed in a water bath at 50°C for 5 min. The temperature was higher than that of the DPPC gel-liquid crystal phase transition to allow complete phospholipid hydration. The procedure was repeated four times. The antibacterial agents were added to the lipid sample before vesicle formation to determine the extent of the effect of the association with lipid head groups. Finally, the liposome suspension was left at room temperature for 1 h to anneal the bilayer structure. All of the procedures were carried out under nitrogen to prevent oxidation of the lipid.

The resulting lipid vesicles were extruded by means of a stainless steel extrusion device (Lipex Biomembranes, Vancouver, British Columbia, Canada) equipped with a 10-ml water-jacketed thermobarrel connected to a thermostat, which allowed extrusion at 50°C. The vesicle suspensions were equilibrated at the selected temperature for at least 20 min prior to extrusion. Extrusion was carried out through two (stacked) polycarbonate filters (25-mm diameter; Nuclepore Corp., Pleasanton, Calif.). The extrusion procedure was performed at nitrogen pressures of up to 5,600 kPa. To obtain large, unilamellar vesicles, the extrusion procedure consisted of 10 passages of the lipid vesicle suspension through 400-nm-pore-size polycarbonate filters, followed by another cycle of 10 passages through 200-nm-pore-size filters. Phospholipid recovery after the extrusion procedure was determined to be higher than 94%.

DSC analysis. The various samples (40 μl containing ~2 mg of lipid material) were sealed in an aluminum pan and submitted to DSC analysis. The calorimetric experiments were performed with a Mettler DSC12E apparatus equipped with a Haake D8-G apparatus. Indium was used to calibrate the instrument, that is, the temperature scale and enthalpy changes (Δ*H*). The detection system was a Mettler Pt100 sensor. The sensor presented a thermometric sensitivity of 56 μV/°C, a calorimetric sensitivity of about 3 μV/mW, and a noise level lower than 60 nV (<20 μW) peak to peak. The baseline reproducibility was <3 μV (<1 mW). Each DSC scan presented an accuracy of ±0.4°C with a reproducibility and a resolution of 0.1°C. The reference was an aluminum pan containing 40 μl of isotonic phosphate buffer. Reference and sample pan masses were always matched to within 5.3% of the total mass and usually to within 1.8%. The various samples were submitted to heating and cooling cycles (three times) in the temperature range of 20 to 60°C at a scanning rate of 1°C/min. The data coming from the first scan were always discarded to avoid mixing artifacts. The necessity of periodic recalibration of the system over the course of these experiments may obviate direct comparison between groups of results. For this reason, a control sample containing the basic phospholipid preparation without any drug accompanied each set of studies. The endotherm obtained during the second scan of this control sample was used as a reference template for analysis of the accom-

panying experiments. ΔH values were calculated from peak areas by a Mettler TA89E and FP89 system software (version 2.0).

Drug permeation of membrane. Brodimoprim and trimethoprim permeation of DPPC biomembranes was evaluated by monitoring drug leakage as a function of time. The untrapped drugs were removed from DPPC vesicles by gel permeation chromatography. An aliquot (1 ml) of DPPC vesicles was loaded onto a Sepharose 4B fine column (1.5-cm diameter, 50-cm length; Pharmacia, Uppsala, Sweden). Gel permeation chromatography was carried out at room temperature with pH 7.4 isotonic phosphate buffer as the mobile phase. The flow rate was set at 250 μl/min with a peristaltic pump, and fractions of 3 ml were collected. Detection was carried out both at 550 nm to assay sample turbidity and at the drug λ_{max} (283 and 288 nm for brodimoprim and trimethoprim, respectively) to monitor free-drug elution. Encapsulation was evaluated by destroying DPPC vesicles with Triton X-100 (5%, wt/vol) and determining the drug amount by HPLC.

The drug-loaded DPPC biomembranes prepared at different pH values were dispersed in 200 ml of pH 7.4 isotonic phosphate buffer and incubated at 37 ± 0.01°C (F3-R; Haake, Berlin, Germany) under gentle mechanical stirring. At appropriate intervals, 2-ml portions of the various DPPC biomembrane suspensions were withdrawn, transferred into Whatman VectaSpin3 ultrafiltration membrane cones (molecular weight cutoff, 20,000; Whatman International Ltd., Milan, Italy), and centrifuged at 500 × *g* for 5 min. The filtrate containing the released drug was assayed spectrofluorimetrically: λ_{ex} of 283 nm and λ_{em} of 335 nm (r = 0.9991) for brodimoprim and λ_{ex} of 288 nm and λ_{em} of 339 nm (r = 0.9987) for trimethoprim. No drug-membrane association was observed.

Drug uptake by human neutrophil cells. The experiment on drug accumulation within neutrophils was carried out as reported by Climax and coworkers (16), with some modifications. Blood (25 ml) was withdrawn from healthy volunteers by venipuncture and 100 μl of preservative-free heparin (3,000 U/ml) was added. An aliquot (5 ml) of Emagel (Behring Werke A.G., Marburg, Germany) was added to the blood. This mixture was incubated for 15 min at 37°C, allowing for sedimentation. The leukocyte-rich supernatant was either washed in HBSS three times and the leukocyte count was adjusted to 10^7 cells per ml or it was layered on density gradient reagent (Histopaque-1077) and centrifuged at 400 × *g* for 30 min. The neutrophils were harvested from the bottom of the tube (10). Contaminating erythrocytes were eliminated by a lysing procedure at 37°C with 0.83% (wt/vol) ammonium chloride. Cell viability was >97% as determined by dye exclusion with acridine orange (0.01%, wt/vol). The cells were then washed three times with HBSS and adjusted to a concentration of 10^7/ml with a neutrophil fraction greater than 90%.

Trimethoprim or brodimoprim was dissolved in HBSS (0.1 mg/ml). The cell suspensions were incubated at 37°C for 1 h with 100 μl of the drug solution in a total volume of 2.1 ml. After incubation, the various samples were cooled in an ice bath and then centrifuged at 800 × *g* for 15 min at 4°C. Aliquots of the supernatants were withdrawn, and the drug amount was determined by HPLC. To determine drug accumulation, the cell pellets were washed in warm HBSS and lysed with chilled, double-distilled water (2 ml for each well). These suspensions were then frozen and thawed three times and sonicated for 10 min.

HPLC determination of drugs. The aqueous samples containing the lysed cells were sonicated for 15 min at 45°C and filtered through a 0.22-μm-pore-size Teflon membrane (Sartorius, Göttingen, Germany). An appropriate amount (20 μl) of an internal standard solution (0.5 mg of pyrimethamine per ml of 0.01 M HCl) was added to 500 μl of the various aqueous samples. These samples were mixed with 500 μl of pH 9 phosphate buffer, layered with 2 ml of an organic mixture of $CHCl_3$-CH_3CN (95:5, vol/vol), and thoroughly mixed in screw-cap glass test tubes for 15 min. After 2 h of incubation at room temperature, 1 ml of the organic solution was withdrawn, evaporated to dryness at 40°C under a gentle nitrogen stream, and submitted to HPLC analysis following solubilization of the dry residue in 300 μl of the mobile phase. The recovery efficiencies of this extraction procedure on samples spiked with known amounts of brodimoprim and trimethoprim were 98.7% ± 2.4% and 101.3% ± 1.7%, respectively.

The chromatographic apparatus was a Hewlett-Packard 79852A solvent delivery pump, a Rheodyne 7125 loading injection valve (Rheodyne, Cotati, Calif.) with a 100-μl loop, a Hewlett-Packard 79853 variable-wavelength UV-visible light detector, and a Hewlett-Packard 3395 reporting integrator. Chromatographic separation was carried out with a CN silica Merck 250-4 Hibar column (5-μm-particle-size column, 250 by 4.6 mm [inner diameter]; Merck). The mobile phase, 5 mM, pH 7 phosphate buffer containing 1% triethylamine (Merck)-acetonitrile (75:25 [vol/vol]; LiChrosolv; Merck), was delivered at a flow rate of 1.0 ml/min with a mean pressure of 100 atm (10,129 kPa). The eluent was filtered through a 0.2-μm-pore-size Teflon membrane (Spartan-3; Schleicher & Schuell, Keene, N.H.) and deaerated by ultrasonication prior to use. Detection was carried out at 288 and 283 nm during the determination of trimethoprim and brodimoprim, respectively. For biological samples, internal standardization was used. Chromatography was carried out at room temperature. Trimethoprim, brodimoprim, and pyrimethamine presented retention times of 5.27, 6.61, and 7.64 min, respectively. The reproducibility of the assay was evaluated by repetitive analysis of human neutrophil cell suspensions spiked with a known amount of the two drugs. Within-day reproducibilities were 98.7% ± 2.6% and 97.4% ± 3.1% (assay accuracy ± relative standard deviation; n = 9) for trimethoprim and brodimoprim, respectively. Day-to-day reproducibilities were 98.9% ± 2.3% and 99.2% ± 2.6%, respectively. The lower limit of detection for the assay in human neutrophils was ~10 ng/ml for both drugs with a signal/noise ratio of 5:1. In the chromatographic analysis, no interference from the other components present in the various samples was observed.

RESULTS

The compounds belonging to the 2,4-diaminobenzylpirimidine class, such as trimethoprim and brodimoprim, have in their molecular structure sites which can be promoted as a function of the environmental pH value. In fact, both trimethoprim and brodimoprim contain the two nitrogen atoms of the pyrimidine ring that are easily protonatable (Fig. 1). Protonation of the molecule occurs at the level of N_1 and N_3 of the pyrimidine ring rather than over the two amino groups, because the lone pairs of these groups are conjugated with the aromatic ring and hence the basicity of the amino groups is decreased. This conjugation, leading to an increase of the electron density on the nitrogen atoms of the pyrimidine ring, make the pyrimidine moiety more basic because of the greater availability of the lone pairs of this molecule. These considerations show how the pH of the medium is the most important factor influencing the physicochemical properties of trimethoprim, brodimoprim, and the other molecules of the 2,4-diaminobenzylpyrimidine class. As a consequence, the interaction between trimethoprim or brodimoprim, and the biological substrate can be influenced by pH.

The acid-base properties of these molecules are very important and may be depicted both at the molecular level in terms of macrospeciation and at the submolecular level in terms of microspeciation (42, 43).

The overall acid-base properties of trimethoprim and brodimoprim, and hence macrospeciations, were evaluated in terms of the octanol-water partition coefficient (log P) as a function of the pH value of the aqueous environment. In fact, the partition coefficient is a biologically important physicochemical parameter, particularly for molecules that have to permeate the cellular membrane to reach the intracellular target site, the enzyme DHFR. As show in Fig. 2, there is no great difference between the two antimicrobial drugs. Both drugs showed the same trend of the partition coefficient as a function of pH, although brodimoprim was more hydrophobic than trimethoprim. In particular, the two drugs presented a hydrophilic character at low pH values, where the molecules are protonated and hence charged. With an increase in the pH, drug protonation decreases, and as a consequence, the hydrophobic character increases up to a pH of ~7. Beyond this value, no variation in the partition coefficient was observed because of the absence of acid groups in the molecules of brodimoprim and trimethoprim.

The brodimoprim and trimethoprim microspeciations and their affinities for the lipid membrane bilayers are very important in determining drug passage. This particular aspect was investigated by DSC employing DPPC biomembranes as models and evaluating the drug-membrane interaction as a function of the environmental pH.

Phospholipid biomembranes, when submitted to DSC, present an endothermal peak due to the transition from the gel state to the liquid crystal phase. This phase transition is characterized by a perturbation of the ordered structure of the bilayer leading to a less packed, and hence more fluid, state. The thermotropic properties, i.e., enthalpy change (ΔH), entropy change (ΔS), and transition temperature (T_m), are determined by the following energetic contributions: (i) hydrogen bond energy among phospholipid polar heads, (ii) hydrophobic interaction between the phospholipid alkyl chains (these two contributions increase the T_m and ΔH values), and (iii) elec-

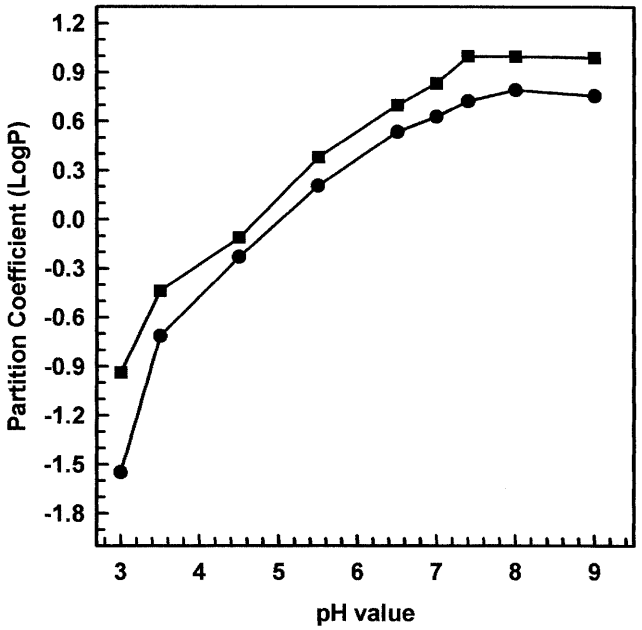

FIG. 2. Partition coefficients in octanol (log P_{oct}) of trimethoprim (●) and brodimoprim (■) as a function of the pH of the aqueous buffer. The experiments were carried out at 37°C. Each value represents the average of six different experiments ($P < 0.01$, t test).

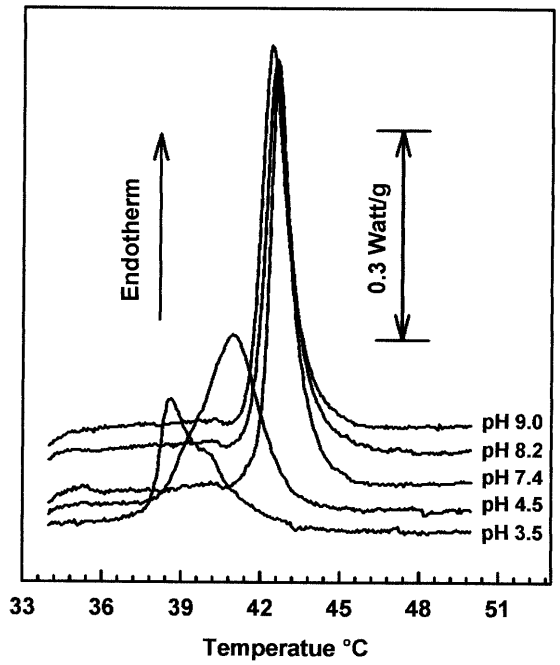

FIG. 3. DSC curves determined in the heating mode of 200-nm unilamellar DPPC biomembrane colloidal suspensions prepared in the presence of brodimoprim dispersed in different-pH buffers. The thermal analysis scanning rate was 1°C/min. Trimethoprim-DPPC biomembrane interaction is similar to that shown by these DSC scans.

trostatic and repulsion energy leading to a reduction of ΔH values. In addition to the main transition, DPPC biomembranes also present a pretransition peak due to the mesophasic transition from the gel state to the ripple phase. Therefore, the presence of a biologically active molecule in the DPPC biomembranes may influence the thermotropic behavior of the vesicles depending on its own macrospeciation and microspeciation properties.

The interaction of brodimoprim and trimethoprim with DPPC biomembranes was influenced by the pH of the environment and hence by the protonation of the drug (Fig. 3). In fact, the DSC studies allowed a more suitable interpretation of the interaction between the two drugs and the DPPC biomembranes as a function of drug microspeciation related to pH.

The presence of the two drugs caused a depression of the pretransition peak of DPPC biomembranes at all pH values, due to a slight interaction at the level of the polar head of phospholipids, while the perturbation of the main transition peak is related to the protonation, and hence to the microspeciation, of the drugs. As shown in Fig. 3, no particular variation of the shape of the main peak of DPPC vesicles was observed in the presence of brodimoprim in the pH range of 7 to 9. Similar thermograms were obtained in the presence of trimethoprim. Under these conditions, the two drugs are not protonated and hence present a certain hydrophobic character, which is not sufficient to confer lipophilic behaviors allowing an interaction with the ordered structures of the alkyl chains of the DPPC bilayers. This hypothesis was confirmed by the thermotropic parameters of the main transition peak; that is, the transition temperature, the transition range temperature, and the enthalpy values were not influenced by the presence of the drugs in the pH range of 7 to 9 with respect to pure DPPC biomembranes (Table 1). Thus, the drugs are distributed between the liposome aqueous compartments and the lipid bilayers. Upon protonation of the molecules by lowering of the pH, noticeable perturbation of the DPPC transition was observed for both drugs. These changes are due to the interaction of trimethoprim and brodimoprim with DPPC vesicles, because the thermotropic parameters of pure DPPC are not influenced by the pH of the aqueous buffer (data not reported). As shown in Fig. 3, for brodimoprim at pH 4.5, the main transition was broadened and shifted to a lower temperature (Table 1). At pH 3.5, the DPPC transition peak was centered at 37.7°C with a shoulder at a higher temperature.

The broadening and shift of the DPPC main transition peak were probably due to a strong interaction of brodimoprim at the level of the hydrophilic part of the bilayers. In fact, the

TABLE 1. Thermotropic parameters of the interaction between brodimoprim[a] and DPPC biomembranes with regard to the phase transition from the gel to the liquid crystal state as a function of pH[b]

pH	ΔH (J/g)	T_m (°C)[c]	$T10\%$ (°C)[d]	$T95\%$ (°C)[e]	$\Delta T_{1/2}$ (°C)[f]
Blank[g]	52.7	42.2	41.7	43.5	1.9
3.0	31.2	37.9	37.5	41.4	3.9
4.5	47.2	40.2	38.6	42.0	3.4
7.4	50.6	41.9	41.5	43.5	2.1
8.2	50.5	41.8	41.3	43.5	2.2
9.0	49.1	41.6	41.2	43.3	2.1

[a] 0.03 M fraction.
[b] Each value is the average of three different experiments.
[c] T_m, transition peak temperature.
[d] $T10\%$, melting range temperature when 10% of the sample underwent the phase transition.
[e] $T95\%$, melting range temperature when 95% of the sample underwent the phase transition.
[f] $T_{1/2}$, width half-height of the transition peak.
[g] DPPC vesicles were prepared in the absence of the drug in pH 7.4 isotonic phosphate buffer. No variation of thermotropic properties was observed in the pH range of 3 to 9.

TABLE 2. Thermotropic parameters of the interaction between trimethoprim[a] and DPPC biomembranes with regard to the phase transition from the gel to the liquid crystal state as a function of pH[b]

pH	ΔH (J/g)	T_m (°C)[c]	$T10\%$ (°C)[d]	$T95\%$ (°C)[e]	$\Delta T_{1/2}$ (°C)[f]
Blank[g]	52.7	42.2	41.7	43.5	1.9
3.0	35.4	38.8	38.1	41.2	3.4
4.5	47.7	40.7	39.9	42.6	2.9
7.4	50.7	41.8	41.2	43.4	2.1
8.2	50.1	41.7	41.5	43.5	1.9
9.0	50.3	41.9	41.4	43.3	2.0

[a] 0.03 M fraction.
[b] Each value is the average of three different experiments.
[c] T_m, transition peak temperature.
[d] $T10\%$, melting range temperature when 10% of the sample underwent the phase transition.
[e] $T95\%$, melting range temperature when 95% of the sample underwent the phase transition.
[f] $T_{1/2}$, width half-height of the transition peak.
[g] DPPC vesicles were prepared in the absence of the drug in pH 7.4 isotonic phosphate buffer. No variation of thermotropic properties was observed in the pH range of 3 to 9.

protonation of brodimoprim, leading to a more hydrophilic form of the drug, allowed an interaction with the head groups of the DPPC vesicles, triggering an electrostatic repulsion between the positively charged pyrimidine ring of the drug and the choline moiety of DPPC, while the lipophilic part of the molecule (the benzyl ring) partially anchors brodimoprim via hydrophobic interaction to DPPC biomembranes. When the pH was lowered, this situation was enhanced, leading to a phase segregation within DPPC biomembranes, namely, the appearance of a shoulder that showed the presence of brodimoprim-rich and brodimoprim-poor domains in the vesicle bilayers. This kind of interaction between protonated brodimoprim and DPPC vesicles caused both a loss of cooperativity in the ordered structure of the bilayers, as shown by the width half-height values ($\Delta T_{1/2}$) and the melting range ($T10\%$ to $T95\%$) in Table 1, and a reduction of the packing energy due to increased repulsion at the level of the phospholipid head groups (decreased ΔH values [Table 1]).

A similar situation was observed for trimethoprim (Table 2). In this case, the T_m values were shifted less to lower temperatures with respect to brodimoprim and no phase segregation was observed. The main transition peaks were less broad (melting range and $\Delta T_{1/2}$ [Table 2]) and the ΔH values were slightly higher than those of brodimoprim. These differences showed that an electrostatic interaction with the DPPC headgroups may also be observed for trimethoprim but at an outer level of the bilayers with respect to brodimoprim. Considering that the charged moiety for both drugs is absolutely the same, this different interaction is due mainly to the hydrophobic part of the molecules. In fact, replacement of a methoxyl group with a bromide group renders the brodimoprim molecule more hydrophobic than trimethoprim, particularly at a low pH (Fig. 2). Therefore, the benzyl ring of trimethoprim does not anchor the molecule to the DPPC bilayer and hence the electrostatic interaction is confined to the surface of the bilayers, triggering a lower perturbation of the packing order of the DPPC bilayer.

The abilities of trimethoprim and brodimoprim to permeate biological membranes as a function of protonation were studied by evaluating the release of these drugs from DPPC biomembranes with an internal aqueous compartment at different pHs (the external medium was always pH 7.4 phosphate buffer). The in vitro release of the two drugs was observed, and the results are shown in Fig. 4. Comparing the results with the partition coefficient data reported in Fig. 2 makes it apparent that lipophilic forms of trimethoprim and brodimoprim having comparatively larger partition coefficients were released much more rapidly than the protonated (hydrophilic) drugs, which were released extremely slowly. Straight lines were obtained by plotting the release data on a semilog scale, at least in the early phase of drug leakage (data not reported). Thus, drug release

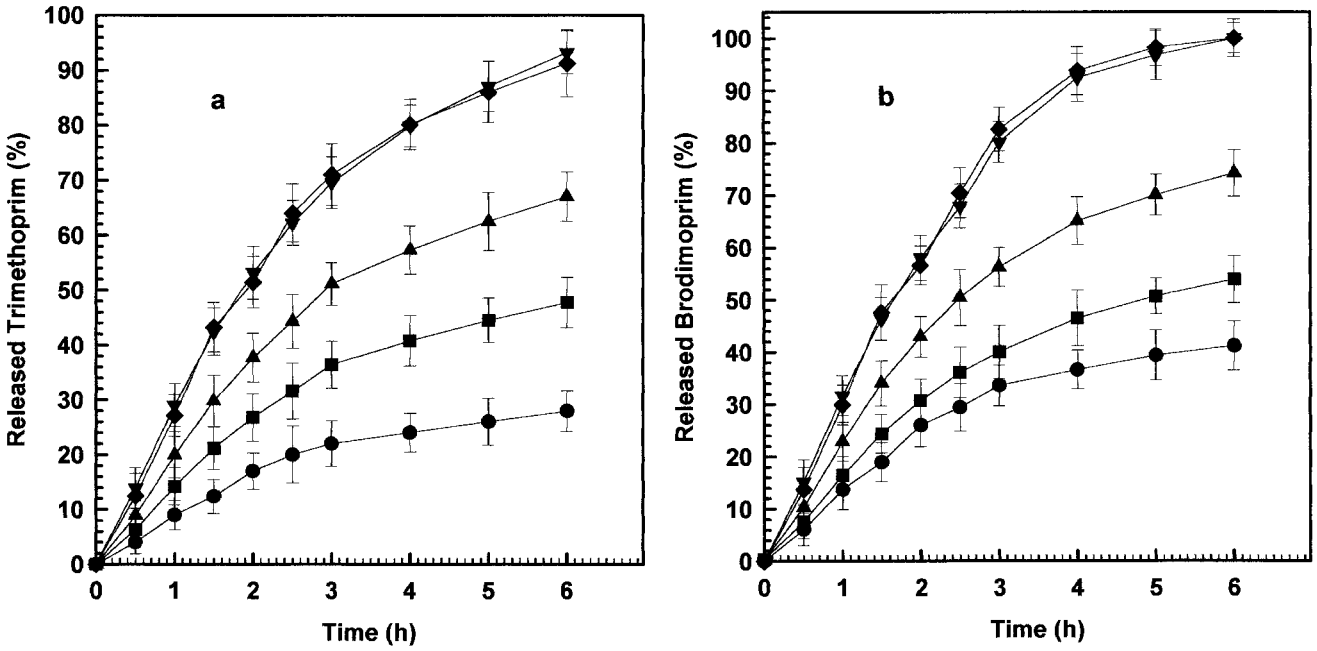

FIG. 4. Trimethoprim (a) and brodimoprim (b) leakage from unilamellar DPPC biomembranes suspended in pH 7.4 phosphate buffer. The release experiments were carried out at 37 ± 0.2°C. Drug-loaded DPPC biomembranes were prepared in the presence of different-pH buffers. Symbols: ●, pH 3.5; ■, pH 4.5; ▲, pH 5.5; ▼, pH 7.4; ◆, pH 9.0. Each point is the average of five experiments. Error bars indicate standard deviations.

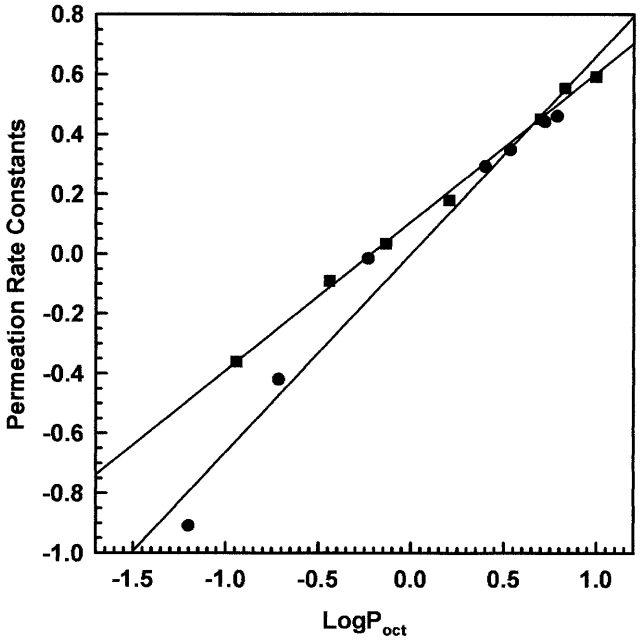

FIG. 5. Correlation between permeation rate constants obtained from the release of trimethoprim (●) and brodimoprim (■) through DPPC biomembranes prepared at different pHs and the partition coefficients of the two drugs at the different pHs. The experiments were carried out at 37°C. The correlation coefficients for trimethoprim and brodimoprim were $r = 0.97491$ and $r = 0.99649$, respectively.

under these experimental conditions was calculated to follow first-order kinetics. The results showed that the partition of the two drugs from the inner aqueous phase of the DPPC biomembranes into the membrane matrix is one of the main factors controlling drug release. Therefore, the first-order drug release suggested that drug transport occurred mainly by a diffusion-controlled mechanism (25).

The first-order rate constants were calculated for trimethoprim and brodimoprim at different pHs. As shown in Fig. 5, the rate constant for the initial release phase was plotted against the partition coefficient on a log-log scale. The results showed that the smaller the partition coefficient of the drug, the slower the release rate. However, it is worth noting that trimethoprim was less able to permeate than brodimoprim in the pH range of 4.5 to 6.5, although both drugs presented very similar partition coefficients (Fig. 2). This slower release might be due to the difficulty of trimethoprim partitioning into the membrane matrix of liposomes. Therefore, drug microspeciation should also be taken into account in the case of drug permeation through membranes.

In this study, accumulation of trimethoprim and brodimoprim in human neutrophils was also investigated. The time courses of trimethoprim and brodimoprim accumulation in neutrophils are shown in Fig. 6. Both drugs presented typically biphasic accumulation curves, that is, a first period of very fast drug entrance into cells, followed by more gradual uptake. The phase of rapid accumulation occurred within 15 min. Despite the similar drug accumulation profiles, a small but significant ($P < 0.01$) difference between trimethoprim and brodimoprim was observed. The first phase of rapid accumulation was a little more rapid for brodimoprim than for trimethoprim, namely, 6 and 10 min, respectively. Also, the maximum drug concentration within cells was greater for brodimoprim than for trimethoprim: 0.78 and 0.91 $\mu g/10^7$ cells, respectively. The slight difference between the molecular structures of the two drugs also triggered different accumulation profiles in human neutrophils.

DISCUSSION

A number of antimicrobial agents show a certain lipophilicity, giving them the ability to diffuse through the lipid bilayer region of any biological membranes (57). Some antibiotics, such as 2,4-diaminobenzylpyrimidines (including trimethoprim and brodimoprim), contain functional groups that may be protonated or deprotonated at physiological pH. As a consequence of the protonation and the molecular charge distribution, the diffusion rates and the final equilibrium distribution of the various compounds across the membrane bilayer may be greatly influenced. In fact, it is generally assumed that only the uncharged forms of chemotherapic agents are able to diffuse through lipid membrane bilayers, which are essentially impermeable to charged ions.

In the case of trimethoprim and brodimoprim, the influence of pH, and hence of molecular protonation, on permeation of a DPPC biomembrane model was observed (Fig. 4). The in vitro permeation of these two drugs showed good linearity when plotted against the square root of time, showing that diffusion of the drugs from the membrane matrix of DPPC biomembranes (vesicles) is the main rate-determining step in molecule permeation. Therefore, drug release by partitioning to the lipid membranes follows first-order kinetics. According to the literature (60), the first-order rate constant, which is equivalent to the permeability constant of the membrane (k), may also be derived from the equation $k = (D \times A \times P_c)/(V_o \times h)$, where D is the diffusion coefficient of the drug in the lipid membrane, A is membrane area, V_o is the inner aqueous volume, h is membrane thickness, and P_c is the partition coefficient of the drug. Since D is not normally affected by the drug

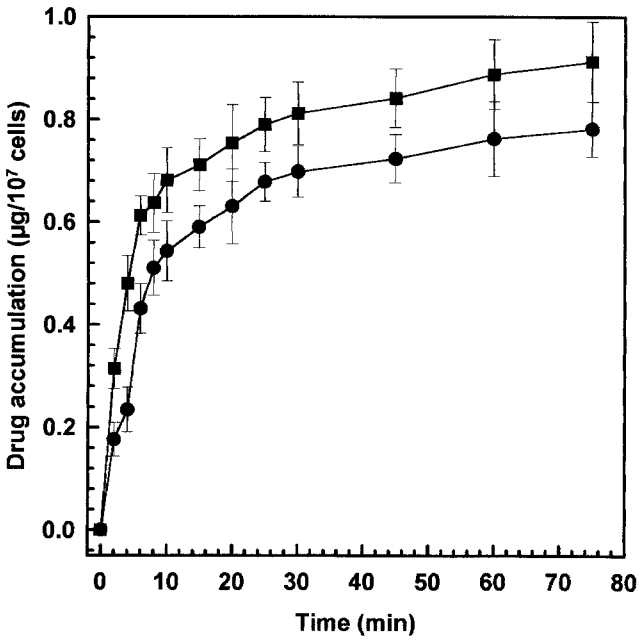

FIG. 6. Time course of 2,4-diaminobenzylpyrimidine accumulation in human neutrophil cells. The biological assay was carried out under temperature control (37 ± 0.2°C) by adding 100 µl (0.1 mg/ml) of trimethoprim (●) or brodimoprim (■) to human neutrophil cell suspensions. The accumulated drug was determined by HPLC. Each point represents the average of nine different experiments ± the standard deviation.

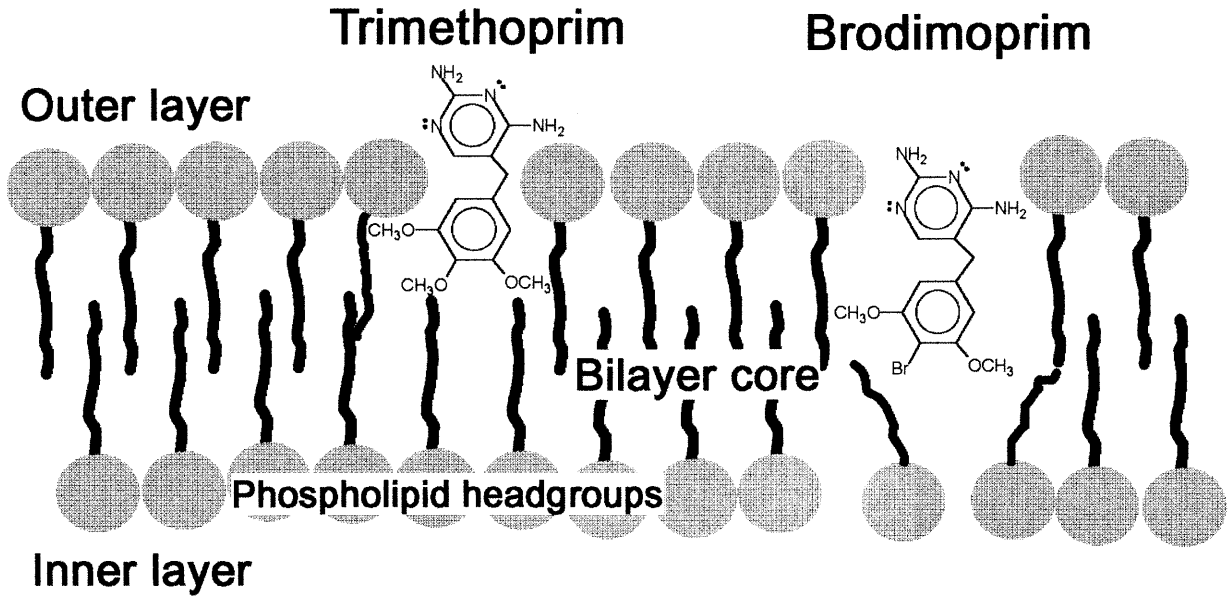

FIG. 7. Schematic representation of possible interactions of trimethoprim and brodimoprim with the bilayer structure of DPPC biomembranes.

and A, V_o, and h are related to the kind of membrane, the P_c values should greatly influence k. Therefore, under these conditions, the protonation of trimethoprim and brodimoprim could be the rate-determining step of drug permeation through phospholipid membranes.

The permeation properties of trimethoprim and brodimoprim are determined not only by their acid-base properties but also by their own submolecular domains (microspeciation parameters), which achieved different permeation profiles, even for very similar molecules. This finding could be due to the difference in the interactions of trimethoprim and brodimoprim with DPPC biomembranes (Fig. 7). The first step in the permeation of a biological membrane by a molecule implies an interaction between the two entities. In particular, brodimoprim, with its benzyl moiety, seems to be able to penetrate the bilayer structure of DPPC biomembranes to a certain extent because of the hydrophobicity conferred by the bromine substituent.

The higher affinity of brodimoprim for the bilayer structure of membranes is reflected in its greater accumulation than that of trimethoprim within human neutrophils. These findings are in agreement with the in vitro MIC of brodimoprim, which was lower than that of trimethoprim (1). Furthermore, the higher uptake of brodimoprim by neutrophil cells has some important implications. In fact, certain bacteria, such as *Mycobacterium tuberculosis*, *Listeria* sp., *Brucella* sp., and *S. aureus*, may survive after phagocyte uptake, leading to chronic or recurring infections since viable intracellular organisms are protected from the action of antimicrobial agents. This aspect should be considered not only for facultative intracellular parasites but also for extracellular bacteria such as *S. aureus*. Therefore, any means able to increase the intracellular concentration of antibiotics within infected cells would enhance bacterial killing and elimination of infections.

The greater uptake of brodimoprim than trimethoprim by human neutrophils is very important in the prophylaxis and treatment of infection in chronic granulomatous diseases, which are normally treated with sulfamethoxazole-trimethoprim (23, 53). In fact, suitable therapy of infections in chronic granulomatous diseases requires the use of antibiotics which are able to permeate the cellular membrane of phagocytic cells, accumulate within cells in an active form, and exert their antimicrobial action by killing the surviving intracellular microorganisms. It was reported (33, 41, 61) that lipid-soluble antibiotics are able to penetrate biological membranes and may be taken up by mammalian cells, in contrast to water-soluble antibiotics, which penetrate poorly. Our findings are in agreement with this concept, also demonstrating the importance of drug-membrane interaction.

Both trimethoprim and brodimoprim can accumulate within human neutrophils, achieving concentrations in cells greater than those in the surrounding extracellular environment. The driving force responsible for 2,4-diaminobenzylpyrimidine accumulation may be partition along a pH gradient which is able to direct weak, lipid-soluble bases into the acid compartment. Thus, compounds with base characteristics, such as trimethoprim and brodimoprim, could be taken up into the acid compartments of neutrophils. Indeed, the mechanism leading to trimethoprim and brodimoprim accumulation would be based on the fact that at a plasma pH of 7.3, the two drugs are mostly in their nonprotonated (lipid-soluble) form (Fig. 2). Considering that only lipid-soluble molecules are able to freely permeate the matrix of a lipid bilayer (Fig. 4) in this condition, trimethoprim and brodimoprim may readily diffuse across the lipid cell membrane of neutrophils. Once inside, both molecules will be protonated in the presence of acid granules or acid phagocytic vacuole fluid. In addition, it should be remembered that pH values lower than those in these fluids may exist in neutrophil compartments; that is, the pH inside lysosomes is 4.8 (46) and the pHs within phagocytic vacuoles are 6.0 and 5.5 in normal and pathological cells, respectively (54). When both drugs are in the protonated (water-soluble) form, they are unable to diffuse back across the membrane. In fact, as demonstrated in Fig. 4, the permeation of DPPC biomembranes by both drugs was less intense and much slower in acid medium because of a decrease of the permeation rate constant (Fig. 5). In this case, both drugs were trapped within neutrophils, the influx rate being much higher than the efflux rate (Fig. 8). Since equilibrium is reached when the cytoplasmic concentration of the nonprotonated form becomes equal to that of the same

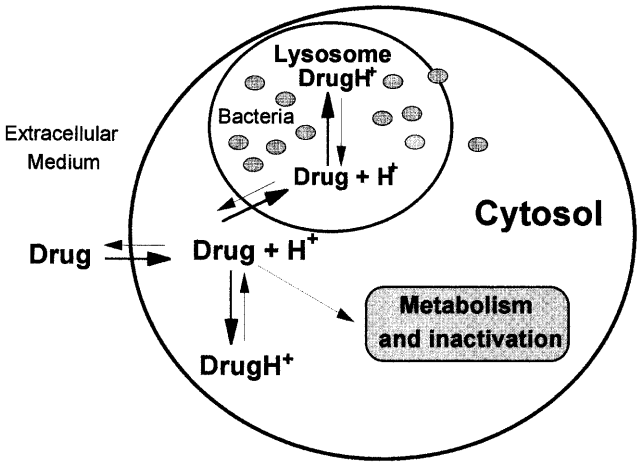

FIG. 8. Proposed mechanism for the accumulation of brodimoprim and trimethoprim in cells and for their partition between the cytosol and lysosomes. A weak base at physiologic pH is in the unprotonated form, so that it can easily pass through cellular membranes. In the cytosol, slight drug protonation may occur because of a decrease of about 0.5 pH unit. The unprotonated form (Drug) can still penetrate lysosomes and be entrapped by low-pH conditions. In this case, the drug is mainly in the protonated form ($DrugH^+$) and thus it is unable to diffuse back through the lysosomal membrane. Therefore, the $DrugH^+$ forms of basic drugs, i.e., trimethoprim and brodimoprim, are accumulated in the corresponding compartments in relation to the proton concentration difference (see reference 61 for a complete analysis). The bold arrows indicate the main direction of movement by which equilibrium is maintained.

form present in the extracellular medium, the total amount of the cytoplasmic (protonated plus nonprotonated) drug will be greater in the intracellular compartment as a result of the ion-trapping mechanism (55). The total amount of the effective agent is also much greater inside the neutrophil than in the blood plasma, because both the protonated and nonprotonated forms of trimethoprim and brodimoprim present antibacterial activity. This finding shows the beneficial effect on neutrophil function as the two antimicrobial agents, in their active form, came into contact with ingested bacteria.

The slightly faster and greater accumulation of brodimoprim than trimethoprim could be due to the different interactions with the matrix of the membrane bilayer rather than to their partition properties, which were similar in the pH range of 7 to 10. In fact, the interaction of the benzyl ring of brodimoprim with the hydrophobic part of the bilayer may render the molecule similar to the lipid constituent of the bilayers. In this case, the permeation of the biological membranes may ensure permeation via a fast flip-flop mechanism common to lipidic material (31) besides via a single process of diffusion through the bilayer structure, as for trimethoprim.

Other mechanisms may also be responsible for trimethoprim or brodimoprim penetration of and accumulation within neutrophils, i.e., encapsulation of extracellular fluid containing the drug during ingestion of bacteria.

In conclusion, evidence is presented on the importance of the physicochemical properties of two antimicrobial agents, trimethoprim and brodimoprim, in determining the intracellular accumulation and the membrane permeation pathways. The findings presented here show that intracellular pharmacokinetics and activity should not be neglected or studied only in a very late stage of drug development. In fact, intracellular infections are of importance not only for obligatory intracellular parasites but also for facultative intracellular parasites, whose intracellular survival or even multiplication can play a significant role in the pathogenesis of the diseases these organisms cause (40). Therefore, increased attention should be focused on the intracellular accumulation properties of antibiotics such as trimethoprim and brodimoprim. Furthermore, the intracellular accumulation of the two drugs investigated in this study suggests that neutrophils may function as active carriers of these agents in vivo. Considering the secretory nature of neutrophils (16), there is also the possibility that accumulated intracellular drugs may be released at the site of infection. The development of antimicrobial agents with the ability to enhance natural host defenses is of considerable clinical significance. In particular, antibiotics, such as trimethoprim and brodimoprim, which are accumulated intracellularly by neutrophils are of great importance in clinical situations in which intracellular pathogens cause chronic and recurrent infections.

ACKNOWLEDGMENTS

We are very grateful to Master Pharma S.r.l. for technical and financial support. This work was also supported by Italian C.N.R.

REFERENCES

1. **Amyes, S. G. B.** 1993. Comparative antibacterial spectrum of trimethoprim and brodimoprim. J. Chemother. **5:**417–421.
2. **Amyes, S. G. B., and K. J. Towner.** 1990. Trimethoprim resistance, epidemiology and molecular aspects. J. Med. Microbiol. **31:**1–19.
3. **Baccanari, D. P., S. Daluge, and R. W. King.** 1982. Inhibition of dihydrofolate reductase: effect of reduced nicotinamide adenine dinucleotide diphosphate on the selectivity and affinity of diaminobenzyl-pyrimidines. Biochemistry **21:**5068–5075.
4. **Baccanari, D. P., and L. F. Kuyper.** 1993. Basis of selectivity of antibacterial diaminopyridines. J. Chemother. **5:**393–399.
5. **Bartlett, G. R.** 1959. Phosphorous assay in column chromatography. J. Biol. Chem. **234:**466–468.
6. **Blakley, R. L.** 1984. Dihydrofolate reductase, p. 191–253. *In* R. L. Blakley and S. J. Benkovic (ed.), Folates and pteridines, vol. I. John Wiley & Sons, Inc., New York.
7. **Blaney, J. M., C. Hansch, C. Silipo, and A. Vittoria.** 1984. Structure-activity relationships of dihydrofolate reductase inhibitors. Chem. Rev. **84:**333–407.
8. **Bowden, K., A. D. Hall, B. Birdsall, J. Feeney, and G. C. K. Roberts.** 1989. Interaction between inhibitors of dihydrofolate reductase. Biochem. J. **258:** 335–342.
9. **Bowden, K., N. V. Harris, and C. A. Watson.** 1993. Structure-activity relationships of dihydrofolate reductase inhibitors. J. Chemother. **5:**377–388.
10. **Boyum, A.** 1976. Isolation of lymphocytes, granulocytes and macrophages. Scand. J. Immunol. **5:**9–15.
11. **Braunsteiner, A. R., and F. Finsinger.** 1993. Brodimoprim: therapeutic efficacy and safety in the treatment of bacterial infections. J. Chemother. **5:** 507–511.
12. **Burchall, J. J.** 1983. Dihydrofolate reductase, p. 55–74. *In* G. H. Hitchings (ed.), Inhibition of folate metabolism in chemotherapy. Springer-Verlag KG, Berlin.
13. **Burdeska, A., M. Ott, W. Bannwarth, and R. L. Then.** 1990. Identical genes for trimethoprim-resistant dihydrofolate reductase from *Staphylococcus aureus* in Australia and central Europe. FEBS Lett. **266:**159–162.
14. **Careddu, P., C. Bellosta, P. Tonelli, and A. Boccazzi.** 1993. Efficacy and tolerability of brodimoprim in pediatric infections. J. Chemother. **5:**543–545.
15. **Chapman, J. S., and N. H. Georgopapadakou.** 1988. Routes of quinolone permeation in *Escherichia coli*. Antimicrob. Agents Chemother. **32:**438–447.
16. **Climax, J., T. J. Lenehan, R. Lambe, M. Kenny, F. Caffrey, and A. Darragh.** 1986. Interaction of antimicrobial agents with human peripheral blood leucocytes: uptake and intracellular localization of certain sulphonamides and trimethoprims. J. Antimicrob. Chemother. **17:**489–498.
17. **Darrell, J. H., L. P. Garrod, and P. M. Waterworth.** 1968. Trimethoprim: laboratory and clinical studies. J. Clin. Pathol. **21:**202–209.
18. **Edman, J. C., U. Edman, M. Cao, B. Lundgren, J. A. Kovacs, and D. V. Santi.** 1989. Isolation and expression of the *Pneumocystis carinii* dihydrofolate reductase gene. Proc. Natl. Acad. Sci. **86:**8625–8629.
19. **Estep, T. N., D. B. Mountcastle, R. L. Biltonen, and T. E. Thompson.** 1978. Thermal behaviour of synthetic sphingomyelin-cholesterol dispersion. Biochemistry **18:**2112–2117.
20. **Feeney, J.** 1990. NMR studies of interactions of ligands with dihydrofolate reductase. Biochem. Pharmacol. **40:**141–152.
21. **Fling, M. E., J. Kopf, and C. A. Richards.** 1988. Nucleotide sequence of the dihydrofolate reductase gene of *Saccharomyces cerevisiae*. Gene **63:**165–174.
22. **Freisheim, J. H., and D. A. Matthews.** 1984. The comparative biochemistry of dihydrofolate reductase, p. 70–131. *In* F. M. Sirotnak, J. J. Burchall, W. B. Ensminger, and J. A. Montgomery (ed.), Folate antagonist as therapeutic

agents, vol. I. Academic Press, Inc., Orlando, Fla.
23. **Gmünder, F., and R. Seger.** 1981. Chronic ganulomatous disease: mode of action of sulfamethoxazole/trimethoprim. Pediatr. Res. **15:**1533–1537.
24. **Harris, N. V., C. Smith, and K. Bowden.** 1992. Antifolate and antibacterial activities of 6-substituted 2,4-diaminoquinazolines. Eur. J. Med. Chem. **27:** 7–18.
25. **Higuchi, T., S. J. Desai, A. P. Simonelli, and W. I. Higuchi.** 1965. Investigation of factors influencing release of solid drug dispersed in insert matrices. J. Pharm. Sci. **54:**1459–1464.
26. **Hirai, K., H. Aoyama, T. Irikura, S. Iyobe, and S. Mitsuhashi.** 1986. Differences in susceptibility to quinolones of outer membrane mutants of *Salmonella typhimurium* and *Escherichia coli*. Antimicrob. Agents Chemother. **29:** 535–538.
27. **Hitchings, G. H.** 1989. Selective inhibitors of dihydrofolate reductase (Nobel lecture). Angew. Chem. Int. Ed. Engl. **28:**879–885.
28. **Huncock, R., V. Roffle, and T. Nicas.** 1981. Involvement of the outer membrane in gentamicin and streptomycin uptake and killing in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **19:**777–785.
29. **Ivanetich, K. M., and D. Santi.** 1990. Bifunctional thymidylate synthase-dihydrofolate reductase in protozoa. FASEB J. **4:**1591–1597.
30. **Jaffe, A., Y. Chabiert, and O. Semonin.** 1982. Role of porin proteins OmpH and OmpC in the permeation of β-lactams. Antimicrob. Agents Chemother. **22:**942–948.
31. **Jain, M. K.** 1988. Properties of bilayer, p. 86–121. *In* M. K. Jain (ed.), Introduction to biological membranes. John Wiley & Sons, Inc., New York.
32. **Jain, M. K.** 1988. Order and dynamics in bilayers and solute in bilayers, p. 122–146. *In* M. K. Jain (ed.), Introduction to biological membranes. John Wiley & Sons, Inc., New York.
33. **Johnson, J., W. Hand, J. Francis, N. King-Thompson, and R. Corwin.** 1980. Antibiotic uptake by alveolar macrophages. J. Lab. Clin. Med. **95:**429–439.
34. **King, R. D., S. Muggleton, R. A. Lewis, and M. J. E. Sterberg.** 1992. Drug design by machine learning: the use of inductive logic programming to model the structure-activity relationships of trimethoprim analogues binding to dihydrofolate reductase. Proc. Natl. Acad. Sci. USA **89:**11322–11326.
35. **Kompis, J., R. Then, A. Wick, and M. Montavon.** 1980. 2,4-Diamino-5-benzyl pyrimidines as inhibitors of dihydrofolate reductase, p. 177–189. *In* U. Brodbeck (ed.), Enzyme inhibitors. Verlag Chemie, Weinheim, Germany.
36. **La Rosa, C., D. Grasso, M. Fresta, C. Ventura, and G. Puglisi.** 1992. Phospholipid vesicles as a drug delivery system. Part I. Interaction of cytidine-5′-diphosphate choline with charged and zwitterionic phospholipids. Thermochim. Acta **195:**139–148.
37. **La Rosa, C., D. Grasso, M. Fresta, C. Ventura, and G. Puglisi.** 1992. Phospholipid vesicles as drug delivery systems. Part II. A study on kinetic fusion between vesicles containing CDP-choline and dipalmitoylphosphatidylcholine vesicles. Thermochim. Acta **198:**181–190.
38. **Lyon, B. R., J. W. May, and R. A. Skurray.** 1983. Analysis of plasmids in nosocomial strains of multiple-antibiotic-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **23:**817–826.
39. **Matthews, D. A., J. T. Bolin, J. M. Burridge, D. J. Finman, K. W. Volz, and J. Kraut.** 1985. Dihydrofolate reductase: the stereochemistry of inhibitor selectivity. J. Biol. Chem. **260:**392–399.
40. **Moulder, J. W.** 1985. Comparative biology of intracellular parasitism. Microbiol. Rev. **49:**298–337.
41. **Nikaido, H., and D. G. Thanassi.** 1993. Penetration of lipophilic agents with multiple protonation sites into bacterial cells: tetracyclines and fluoroquinolones as examples. Antimicrob. Agents Chemother. **37:**1393–1399.
42. **Noszàl, B.** 1986. Group constant: a measure of submolecular basicity. J. Phys. Chem. **90:**4104–4110.
43. **Noszàl, B.** 1986. Microspeciation of polypeptides. J. Phys. Chem. **90:**6345–6349.
44. **Nyffenegger, R., D. Riebenfeld, K. H. Bandau, M. E. Nouri, U. Schirmböck, and M. Streppel.** 1993. A multicenter comparative study of brodimoprim and amoxicillin therapy in the treatment of tonsillopharyngitis in adults. J. Chemother. **5:**512–516.
45. **Ohemeng, K. A., and B. Roth.** 1991. Receptor-based design of novel dihydrofolate reductase inhibitors: benzimidazole and indole derivatives. J. Med. Chem. **34:**1383–1394.
46. **Ohkuma, S., and B. Poole.** 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. USA **75:**3327–3331.
47. **O'Leary, T. J., P. D. Ross, and I. W. Levin.** 1986. Effect of cyclosporin A on biomembranes. Vibrational spectroscopic, calorimetric and hemolysis studies. Biophys. J. **49:**795–801.
48. **Papahadjopoulos, D., K. Jacobson, G. Poste, and G. Shephard.** 1975. Effect of local anesthetics on membrane properties. I. Changes in the fluidity of phospholipid bilayers. Biochim. Biophys. Acta **394:**509–519.
49. **Puglisi, G., M. Fresta, C. La Rosa, C. A. Ventura, A. M. Panico, and G. Mazzone.** 1992. Liposomes as a potential drug carrier for citicoline (CDP-choline) and the effect of formulation conditions on encapsulation efficiency. Pharmazie **47:**211–215.
50. **Puglisi, G., M. Fresta, C. Ventura, G. Mazzone, and M. A. Vandelli.** 1995. Methotrexate interaction with a lipid membrane model of DPPC. J. Therm. Anal. **44:**1287–1299.
51. **Roos, D. S.** 1993. Primary structure of the dihydrofolate reductase-thymidylate synthase gene from *Toxoplasma gondii*. J. Biol. Chem. **268:**6268–6280.
52. **Roth, B., D. P. Baccanari, C. W. Sigel, J. P. Hubbel, J. Eaddy, J. C. Kao, M. E. Grace, and B. S. Rauckman.** 1988. 2,4-Diamino-5-benzylpyrimidines and analogues as antibacterial agents. 9. Lipophilic trimethoprim analogs as antigonococcal agents. J. Med. Chem. **31:**122–129.
53. **Rubin, R., and M. Swartz.** 1980. Trimethoprim/sulfamethoxazole. N. Engl. J. Med. **303:**426–432.
54. **Segal, A., M. Greisow, R. Garcia, A. Harper, and R. Miller.** 1981. The respiratory burst of phagocytic cells is associated with a rise in vacuolar pH. Nature (London) **290:**406–409.
55. **Seger, R. A., S. Baumgartner, and F. K. Gmünder.** 1982. Chronic granulomatous diseases: effect of sulfamethoxazole/trimethoprim on neutrophil microbicidal function, p. 96–105. *In* H.-U. Eickenberg, H. Hahn, and W. Opferkuch (ed.), The influence of antibiotics on the host-parasite relationship. Springer-Verlag KG, Berlin.
56. **Selassie, C. D., R.-L. Li, M. Poe, and C. Hansch.** 1991. On the optimization of hydrophobic and hydrophilic substituent interactions of 2,4-diamino-5-(substituted benzyl)pyrimidines with dihydrofolate reductase. J. Med. Chem. **34:**46–54.
57. **Stein, W. D.** 1967. The movement of molecules across cell membranes. Academic Press, Inc., New York.
58. **Then, R. L., E. Böhni, P. Angehrn, G. Plozza-Nottebrock, and K. Stoeckel.** 1982. New analogues of trimethoprim. Rev. Infect. Dis. **4:**372–377.
59. **Then, R. L., and F. Hermann.** 1984. Properties of brodimoprim as an inhibitor of dihydrofolate reductases. Chemotherapy **30:**18–25.
60. **Tsukada, K., S. Ueda, and R. Okada.** 1984. Preparation of liposome-encapsulated anti-tumor drugs; relationship between lipophilicity of drugs and *in vitro* drug release. Chem. Pharm. Bull. **32:**1929–1935.
61. **Tulkens, P. M.** 1991. Intracellular distribution and activity of antibiotics. Eur. J. Microbiol. Infect. Dis. **10:**100–106.
62. **Wüst, J., and J. Schwarzenbach.** 1983. Activity of brodimoprim and metioprim alone and in combination with sulfonamides against anaerobic bacteria. Antimicrob. Agents Chemother. **23:**490–492.