Erythromycin and Azithromycin Transport into *Haemophilus influenzae* ATCC 19418 under Conditions of Depressed Proton Motive Force (ΔµH)

JOHN O. CAPOBIANCO* and ROBERT C. GOLDMAN

Anti-Infective Research Division, Abbott Laboratories, Abbott Park, Illinois 60064-3500

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The effect of collapsing the electrochemical proton gradient (ΔµH) on [3H]erythromycin and [14C]azithromycin transport in *Haemophilus influenzae* ATCC 19418 was studied. The proton gradient and membrane potential were determined from the distribution of [3H]dimethylhenone and rubidium-86, respectively. ΔµH was reduced from 124 to 3 mV in EDTA-valinomycin-treated cells at 22°C with 150 mM KCl and 0.1 mM carbonyl cyanide m-chlorophenylhydrazine. During the collapse of ΔµH, macrolide uptake increased. Erythromycin efflux studies strongly suggested that this increase was not due to an energy-dependent efflux pump but was likely due to increased outer membrane permeability. These data indicated that macrolide entry was not a ΔµH-driven active transport process but rather a passive diffusion process.

Macrolide antibiotics such as erythromycin and azithromycin bind to the 50S subunit of bacterial ribosomes (22) and increase the dissociation of peptidyl-tRNA from the ribosomes (14), thus inhibiting protein synthesis. Since the ribosome target is intracellular and macrolides must cross one (gram-positive) or two (gram-negative) membrane barriers, the mechanism of uptake into bacteria is of central importance to macrolide action on bacteria. Previous studies have demonstrated that the intracellular accumulation of erythromycin in several gram-positive organisms is unaffected by metabolic inhibitors (3, 5, 12); however, measurements of the proton motive force or electrochemical proton gradient (ΔµH) under conditions of drug transport were not made. In addition, higher incubation temperatures and alkaline conditions tended to increase macrolide uptake and lower the MIC, thus implicating energy-independent uptake (5, 14, 20). In contrast, macrolides concentrate severalfold in susceptible bacteria because of internal binding sites, and in some cases, resistance to macrolides is believed to involve impermeability at the level of the cell membrane (6, 11). The latter two observations are more consistent with an active uptake process.

The outer membrane permeability barrier of gram-negative bacteria restricts the entrance of macrolide antibiotics (12, 16). However, *Haemophilus* spp. are more susceptible to these antibiotics than are most gram-negative bacteria (7). The increased susceptibility of *H. influenzae* may be due to the lack of an O-antigenic side chain (9), which would increase the permeability of the outer membrane to hydrophobic agents (7, 16). The presence of larger pores, reported to have a molecular weight exclusion limit of 1,400 (24), may also contribute to the susceptibility differences observed.

Previous studies on the accumulation of macrolide antibiotics in gram-negative bacteria were limited to *Escherichia coli*, and minimum data were generated on the mechanism of transport. The role of ΔµH in the transport of macrolides into *H. influenzae* or any other bacteria has not been explored. The emergence of two new macrolides, clarithromycin (R. Fujii and T. Nishimura, Program Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 668, 1988) and azithromycin (18), with potential for general use against *H. influenzae* required a closer look at the macrolide transport process. In particular, clarithromycin contains one nitrogen and azithromycin contains two nitrogenous which are protonated at physiological pH, raising the question of the role of membrane potential (Δψ) (inside negative) in uptake. We have investigated the effects of ΔµH on macrolide accumulation and the possibility of a macrolide efflux pump in *H. influenzae* ATCC 19418. Our findings are consistent with the hypothesis that these antibiotics passively enter bacterial cells and accumulate intracellularly with the assistance of the internal ribosomal binding site.

*H. influenzae* ATCC 19418 was grown in brain heart infusion broth plus 5% Fildes enrichment or Mueller-Hinton broth plus 0.5% yeast extract, 0.2% glucose, 15 μg of β-NAD per ml, and 15 μg of hematin per ml at 37°C to a cell density of 2 × 10⁸ to 6 × 10⁸ CFU/ml. Cells were harvested by centrifugation (5,000 × g for 10 min) and washed once with Mueller-Hinton medium in the drug efflux experiments or with 120 mM Tris hydrochloride (pH 8.0) in the ΔµH studies. For the concurrent study of macrolide transport and protein synthesis inhibition, mid-log cells (2 × 10⁸ CFU/ml) in Mueller-Hinton medium were incubated at 37°C for 3 h with 4 μg of [N-methyl-3H]erythromycin (39 mCi/mmol) per ml or 1.5 μg of [N-methyl-14C]azithromycin (7 mCi/mmol) per ml. These concentrations represent three times the MICs for this organism. MICs were determined by broth dilutions with inocula of 10⁵ and 10⁶ CFU/ml at 37°C for 24 h. In the transport studies, cells (1 ml) were removed from incubation at 10- to 20-min time intervals, and the cells were separated from medium on glass fiber filters (GFF; Whatman, Inc.). Trapped cells were washed three times with 10 ml of 0.9% saline, placed in scintillation vials, and solubilized with 0.5 ml of NCS tissue solubilizer (Amersham Corp.) at 50°C for 30 min. Instagel (10 ml; Packard Instrument Co., Inc.) was added, and samples were counted in a Tri-carb 300 scintillation counter (Packard). At various time points, cell-associated radioactivity was adjusted for counts at time zero (cells plus label at 4°C for <1 min). Cell volume was determined from ³H₂O uptake with cells separated from
medium by velocity-gradient centrifugation (13,000 × g for 1 min) through silicone oil (Dow Corning 550 and 560; 6:7, vol/vol) in microfuge tubes (5, 10). In the presence of methionine, cells incubated with unlabeled macrolide were removed at appropriate time intervals and labeled with [35S]methionine (150 μCi/mmolar) at 37°C for 5 to 9 min. Triplicate 0.4-ml samples were mixed with 5 ml of cold 5% trichloroacetic acid containing 10 mg of unlabeled methionine per ml. Samples were heated at 100°C for 10 min and then cooled at 4°C. Finally, samples were collected on glass fiber filters and processed as outlined above for whole cells, except for the omission of the solubilizer.

The two components of ΔμH, the proton gradient (ΔpH) and the Δψ, were determined by methods previously used for E. coli cells (17). Haemophilus cells were washed with 120 mM Tris hydrochloride (pH 8.0) and then suspended either in 5 mM Tris HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.5) containing 150 mM choline chloride or in the wash buffer. The latter culture was treated with 1 mM EDTA at 37°C for 10 min (23). Some EDTA-permeabilized cultures were also treated with the ionophore valinomycin (2 μg/mg of protein) during EDTA treatment (25). Treated cells were washed and suspended at 1.5 × 10^11 CFU/ml in 5 mM Tris HEPES buffer, pH 7.5, containing 150 mM choline chloride. Total energy depletion was accomplished in EDTA-valinomycin-treated cells by incubating with 0.1 mM carbonyl cyanide m-chlorophenylhydrazone (CCCP) at 22°C for 4 min prior to ΔμH determination in the presence of 150 mM KCl.

ΔpH was determined from the distribution of a weak radiolabeled acid, [2-14C]dimethylamine, across the cell membrane (1, 8, 19). H. influenzae cells (1.5 × 10^10 CFU/ml) were incubated at 22°C for 5 min in 5 mM Tris HEPES (pH 7.5) containing 16 mM succinate, 10 μM to 150 mM KCl, and 0.5 mM [14C]dimethylamine (5 μCi) in a final volume of 1 ml. Triplicate cell samples (0.2 ml) were processed by velocity-gradient centrifugation. Intracellular volumes were calculated from total pellet volume ([H2O] and extracellular volume ([14C]cinulin) determinations (8).

Δψ was determined from 86Rb+ distribution in the presence of the ionophore valinomycin (1, 2). Since valinomycin does not penetrate the outer membrane of gram-negative bacteria, cells were permeabilized with EDTA prior to ionophore introduction. Reaction conditions with 86Rb+ (0.2 μCi, 0.1 mM) were the same as those outlined above for ΔμH determinations.

Macrolide uptake in cells (3 × 10^10 CFU/ml) under conditions of progressively reduced ΔμH were done with 1 μg of [3H]erythromycin per ml or 0.5 μg [14C]azithromycin per ml at 22°C for 16 min. Transport rates but not ΔμH were also measured at 37°C for 16 min. Uptake rates were determined in the initial 4 min of incubation and expressed as picomoles of macrolide per microliter of cells per minute.

Macrolide efflux was determined by the decrease in intracellular [3H]erythromycin from preloaded cells. H. influenzae cells (5 × 10^10 CFU/ml) in Mueller-Hinton medium or EDTA-valinomycin-treated cells in 5 mM Tris hydrochloride (pH 7.5) containing 1 mM KCl, 16 mM succinate, and 150 mM choline chloride were incubated with 1.5 μg of [3H]erythromycin per ml at 37°C for 30 min (maximum saturation). Preloaded cells were centrifuged (5,000 × g for 5 min at room temperature) and suspended in fresh Mueller-Hinton medium or Tris hydrochloride-buffered medium containing 1.5 μg of unlabeled erythromycin per ml with or without 0.1 mM CCCP (Mueller-Hinton medium) or with 1 or 150 mM KCl (Tris hydrochloride-buffered medium).

TABLE 1. Proton electrochemical gradient across the cell membrane of H. influenzae ATCC 19418

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Cell Treatment</th>
<th>ΔpH (mV)</th>
<th>Δψ (mV)</th>
<th>ΔμH (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>EDTA-valinomycin</td>
<td>-0.22</td>
<td>138</td>
<td>125</td>
</tr>
<tr>
<td>1</td>
<td>EDTA-valinomycin</td>
<td>0.44</td>
<td>98</td>
<td>124</td>
</tr>
<tr>
<td>15</td>
<td>EDTA-valinomycin</td>
<td>0.60</td>
<td>49</td>
<td>14</td>
</tr>
<tr>
<td>150</td>
<td>EDTA-valinomycin-CCCP</td>
<td>0.18</td>
<td>14</td>
<td>3</td>
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</table>

a 5 mM Tris HEPES (pH 7.5)-16 mM succinate.
b 0.1 mM CCCP.

cubations at 37°C were continued for 70 min; triplicate cell samples (0.2 ml) were processed at appropriate intervals by the velocity-gradient centrifugation method. Data was plotted as ln (CL/CLo) versus incubation time (t), where CL is cellular label at time t and CLo is cellular label at zero time (first sample after suspension). The half-life (1/2) values were calculated from the slope (b) (15, 26).

Concurrent uptake and protein synthesis determinations were done in order to demonstrate drug entry into cells. Both the rate of accumulation and net accumulation of erythromycin and azithromycin were similar at three times their MICs during the initial 30-min incubation period. Macrolide levels reached 10 pmol/μl of cell volume at 30 min, and protein synthesis concurrently decreased to 15 to 20% of control values. The intracellular volume for H. influenzae 19418, determined from [3H]erythromycin uptake experiments, was 6.6 × 10^-10 μl per cell; therefore, the number of macrolide molecules bound within the cell was calculated to be 3,970. The actual number of ribosomes per cell, for cells isolated from two strains of H. influenzae, ranged from 1,870 to 3,490 (unpublished data). These data demonstrate that macrolides were transporting into the cells and not associating by nonspecific surface binding.

ΔμH and its components were measured in EDTA-valinomycin-treated H. influenzae cells, and the results are presented in Table 1. At KCl concentrations of 10 μM to 1 mM, the ΔμH was maintained at 124 to 125 mV with Δψ as the major component. At low external KCl (10 μM), cellular pH was about 7.3 and the (Rb)i/(Rb)out ratio was 233 (138 mV). When external KCl was increased to 1 mM, the (Rb)i/(Rb)out ratio dropped to 48 (98 mV) while the proton pump was compensated by increasing internal pH to 7.9. At 150 mM external KCl concentration, the (Rb)i/(Rb)out ratio decreased dramatically to 1.7 (14 mV), but the ΔμH (internal pH, 8.1) could not compensate for Δψ (ΔμH, 49 mV). Collapse of ΔμH (3 mV) was achieved by preincubating cells with the energy uncoupler CCCP.

Macrolide uptake was determined under conditions of normal and depressed ΔμH. The active transport of [3H]methionine into H. influenzae was used as a positive control for our system. Under conditions of depressed ΔμH, methionine accumulation was inhibited by 91 to 98% (data not shown). Rates of erythromycin and azithromycin transport into H. influenzae were determined at 22 and 37°C (Table 2). Treatment with or without EDTA did not significantly alter the uptake rates for either antibiotic at 22°C (92 and 90 fmol/μl per min, with and without EDTA, respectively, for azithromycin; 38 and 65 fmol/μl per min, with and without EDTA, respectively, for erythromycin). However, there was a three- to fourfold increase in rates when the macrolides were tested at 37°C after EDTA treatment. The increase in uptake rates observed at 37°C over rates observed at 22°C in untreated controls may reflect membrane
TABLE 2. ΔμH and macrolide uptake in 
*H. influenzae* ATCC 19418

<table>
<thead>
<tr>
<th>Concentration (mM) of KCl added to medium(^a)</th>
<th>Cell treatment</th>
<th>ΔμH at 22°C (mV)</th>
<th>Uptake (pmol/μl per min) of:</th>
<th>Azithromycin(^b) at:</th>
<th>Erythromycin(^c) at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>22°C</td>
<td></td>
<td>22°C</td>
<td>37°C</td>
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<tr>
<td>1</td>
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<td>0.07</td>
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</tr>
<tr>
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</tr>
<tr>
<td>150</td>
<td>EDTA-valinomycin</td>
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<td>0.44</td>
<td>&gt;4.1</td>
<td>0.36</td>
</tr>
<tr>
<td>150</td>
<td>EDTA-valinomycin-CCCP(^d)</td>
<td>3</td>
<td>0.63</td>
<td>&gt;3.7</td>
<td>0.55</td>
</tr>
</tbody>
</table>

\(a\) 5 mM Tris HEPES (pH 7.5)-16 mM succinate.

\(b\) Azithromycin was tested at 0.5 μg/ml.

\(c\) Erythromycin was tested at 1.0 μg/ml.

\(d\) 0.1 mM CCCP.

Fluidity changes which allow for greater penetration of lipophilic agents (21). The rate increases observed between the 22 and 37°C incubations of EDTA-treated cells may be due to Tris EDTA-mediated outer membrane structural damage (16) occurring at 37°C. Uptake rates increased five- to eightfold for both macrolides at 22°C when KCl concentrations were increased to 150 mM without (0.44 pmol/μl per min for azithromycin; 0.36 pmol/μl per min for erythromycin) or with (0.63 pmol/μl per min for azithromycin; 0.55 pmol/μl per min for erythromycin) CCCP pretreatment. At 37°C, uptake was so rapid that only minimum rates could be established. These data demonstrated that at 22°C, under conditions of ΔμH collapse, macrolide uptake did not decrease; therefore, drug influx was not an active transport process. The increase in macrolide uptake as ΔμH was reduced may indicate an energy-dependent efflux system or an increase in outer membrane permeability due to a decreased Donnan potential across this barrier (16).

We investigated the possibility of a ΔμH-driven macrolide efflux pump in *H. influenzae*. Erythromycin was used to

![Graph showing erythromycin efflux from CCCP-treated and untreated *H. influenzae* ATCC 19418 cells.](http://aac.asm.org/)

**FIG. 1.** Erythromycin efflux from CCCP-treated and untreated *H. influenzae* ATCC 19418 cells. Cells (5 × 10^10 CFU/ml) in Mueller-Hinton medium were incubated at 37°C for 30 min with 1.5 μg of [3H]erythromycin per ml. Labeled cells were harvested (arrow) and suspended in fresh medium (37°C) containing unlabeled antibiotic. Symbols: □, control; •, 0.1 mM CCCP. Results are expressed as the mean ± standard deviation (n = 3). (Inset) Macrolide efflux plot of ln (CL/CLo) versus t. The efflux data were plotted to determine the drug efflux t1/2. CL, Cellular-label concentration at time t; CLo, cellular label concentration at zero time (40-min sample). t1/2, 0.693/slope.
measure drug efflux in this organism, because erythromycin has a faster ribosomal binding off rate than azithromycin (R. Goldman, unpublished data), allowing for the observation of any decrease in efflux on deenergizing. Drug efflux was measured in Mueller-Hinton medium with or without the energy uncoupler CCCP in intact cells preloaded with 1.5 μg of [3H]erythromycin per ml at 37°C for 30 min (Fig. 1). The zero time for kinetic analysis was 40 min into the incubation, since this represented the first measurable time period following centrifugation and suspension (30- to 40-min time points). This processing period allowed for the decoupling effect of CCCP. The differences in initial intracellular macrolide concentrations between CCCP-treated and untreated cells at the 40-min time point may be due to cell loss during the processing period. This discrepancy was not germane to the analysis of the dissociation rate constant. The efflux of drug followed first-order kinetics for the untreated control cells with a t1/2 of 9.8 min, determined from a plot of ln (CL/CLo) versus t (Fig. 1, inset). Similar observations were made with CCCP-treated cells for the initial 30 min (t1/2, 10.8 min), after which time the rate of efflux appears to decrease.

The last two time points on the efflux curve (Fig. 1) gave measurements which were close to background and therefore not reliable for kinetic analysis. Drug efflux was also measured in EDTA-valinomycin treated cells preloaded with [3H]erythromycin, as described above (Fig. 2). Because of the permeabilized outer membrane caused by EDTA treatment, the efflux rates were higher than in intact cells; however, the efflux of drug still followed first-order kinetics, with t1/2's of 5.1 and 4.6 min for cells in 1 and 150 mM KCl, respectively (Fig. 2, inset). The t1/2 data for the EDTA-valinomycin-treated cells approach values observed in ribosome-binding studies (R. Goldman, unpublished data). Although our observations did not detect a macrolide efflux pump in this organism, we cannot rule out the existence of an inefficient efflux system masked by the ribosome binding off rate. There have been efflux pumps detected in E. coli for tetracycline (13) and norfloxacin (4), but as yet there are no published data for macrolides.

In summary, the driving force for the accumulation of erythromycin and azithromycin into H. influenzae was not dependent upon ΔμH but, rather, was dependent on intra-
cellular binding to ribosomes. Therefore, macrolide transport into this organism was by a passive process. Since an energy-dependent macrolide efflux pump was not detected, the increase observed in antibiotic uptake under conditions of depressed ΔH was likely the result of increased outer membrane permeability. The inhibition of protein synthesis paralleled macrolide uptake, confirming drug entry into these cells.

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LITERATURE CITED


