Active Uptake of Tetracycline by Membrane Vesicles from Susceptible *Escherichia coli*

LAURA M. McMURRY,¹* JOANNE C. CULLINANE,¹ RICHARD E. PETRUCCI, JR.,¹ AND
STUART B. LEVY^{1,2}

Department of Molecular Biology and Microbiology¹ and Department of Medicine,² Tufts University School of Medicine, Boston, Massachusetts 02111

Received 13 March 1981/Accepted 3 June 1981

A major portion of tetracycline accumulation by susceptible bacterial cells is energy dependent. Inner membrane vesicles prepared from susceptible *Escherichia coli* cells concentrated tetracycline 2.5 to 5 times above the external concentration when the electron transport substrate D-lactate or reduced phenazine methosulfate was added. This stimulation was reversed by cyanide, 2,4-dinitrophenol, and carbonyl cyanide *m*-chlorophenyl hydrazone. These vesicle data showed that proton motive force alone could energize tetracycline uptake. The lactate-dependent uptake had a pH optimum of 6.9 and a magnesium optimum of 1 mM and was not saturable up to 400 μ M tetracycline. Although the vesicles were not as active as cells in concentrating tetracycline, they were less active to a similar extent in concentrating proline, the transport of which is known to be solely proton motive force dependent. Therefore, we concluded that the active uptake of tetracycline in susceptible cells was largely, if not solely, energized by proton motive force.

Antibiotics of the tetracycline family inhibit protein synthesis by interfering with aminoacyl-transfer ribonucleic acid binding to the ribosomal A site in microorganisms (14, 33, 36). To reach the ribosomes, the antibiotic must pass through the cell membranes. This transport of tetracycline in susceptible *Escherichia coli* has both an energy-independent (25) and an energy-dependent (2, 9, 25) component, distinguishable by the use of inhibitors. The mechanism of the energy-dependent transport of tetracycline in susceptible *E. coli* cells is the subject of this communication.

In general, there are three categories of energy-dependent transport known in *E. coli* and other bacteria (30): (i) those, such as glutamine transport, requiring adenosine triphosphate (ATP) or a related compound (16) as primary energy source (periplasmic binding proteins may be involved); (ii) those which modify the substrate during its transport, using membrane and cytoplasmic enzymes (well-known examples are the systems in which transported hexoses are phosphorylated specifically by phosphoenolpyruvate); and (iii) those, such as lactose and proline transport, which depend only on a proton motive force across the inner membrane; the proton motive force is an electrochemical gradient of hydrogen ions which can form in vivo due to expulsion of hydrogen ions both during

electron transport and by the membrane-bound adenosine triphosphatase during its hydrolysis of ATP (12, 30).

Active uptake of tetracycline is completely blocked by arsenate (which inhibits ATP synthesis), cyanide (which inhibits the cytochrome oxidase reaction), anaerobiosis (which inhibits respiration), and 2,4-dinitrophenol (DNP), which destroys proton motive force (17, 25). It was not possible on the basis of the inhibition data to place energy-dependent tetracycline uptake into one of the three categories. Therefore, we used spheroplasts and inner membrane vesicles to examine the roles played by outer membrane and periplasm and by inner membrane and cytoplasm in active tetracycline uptake. Damage of outer membrane and loss of periplasm is characteristic of spheroplasts formed by treatment of cells with ethylenediaminetetraacetic acid (EDTA) and lysozyme (13). Upon osmotic lysis of spheroplasts in the manner described (18), membrane vesicles are formed which are largely free of outer membrane, periplasm, and cytoplasm, and in which the remaining inner membrane is oriented as in cells ("right side out"). Since the electron transport system of cells is located in this membrane, the vesicles can form a proton motive force by using added electron transport substrates such as D-lactate and reduced phenazine methosulfate (PMS)

(19). We report here that tetracycline was actively concentrated in such membrane vesicles from susceptible *E. coli* cells upon addition of electron transport substrates. Thus, proton motive force alone was sufficient for active transport of tetracycline into susceptible bacterial cells.

MATERIALS AND METHODS

Bacterial strain. *E. coli* strain ML308-225 (*lacI lacY⁺ lacZ) (38) was obtained from P. D. Bragg.*

Media and chemicals. Medium A is a minimal medium described previously (26); it was supplemented with 0.2% glucose or 0.5% glycerol. Tetracycline hydrochloride, D(-)-lactic acid (lithium salt), phenazine methosulfate (PMS), carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), ribonuclease A (type 1-A), and L-ascorbic acid (sodium salt) were purchased from Sigma Chemical Co., St. Louis, Mo. Lithium chloride and potassium cyanide were purchased from Fisher Scientific Co., Pittsburgh, Pa. 2,4-Dinitrophenol (DNP) came from General Biochemicals, Chagrin Falls, Ohio. Deoxyribonuclease I came from Calbiochem, La Jolla Calif., and lysozyme (salt-free) was from Millipore Corp., Bedford, Mass. [7-³H]-tetracycline (0.6 to 1 Ci/mmol) was obtained as the powdered free base from New England Nuclear Corp., Boston, Mass. A portion was freshly dissolved each week in a small volume of methanol and diluted into water. L-[³H]proline (5 Ci/mmol), ⁴⁵CaCl₂ (16.7 mCi/mg), [¹⁴C]dextran (1.1 μCi/mg; average molecular weight, 20,000), and ³H₂O (1.31 μCi/ml) were obtained from New England Nuclear.

Preparation of membrane vesicles. An overnight culture of ML308-225 grown in supplemented medium A was inoculated to an absorbancy at 530 nm (*A*₅₃₀) of 0.1 (Coleman 101 spectrophotometer) and grown at 37°C to an *A*₅₃₀ of 0.8 (late logarithmic phase). Preparation of EDTA-lysozyme spheroplasts and vesicles followed the procedure of Kaback (18) with the following details or changes: 0.3 mg of lysozyme per ml was used, spheroplasts and vesicles were suspended gradually with a spatula or stirring rod during addition of small increments of buffer, 1 liter of lysis buffer (pH 6.9) per liter of harvested culture was used, 3 μg of deoxyribonuclease and ribonuclease per ml (final concentration) was used, the lysates were centrifuged at 18,000 × *g* for 1 h, and the pellets were suspended in 0.1 M KPO₄-10 mM EDTA (pH 6.9) and centrifuged at 2,300 × *g* for 15 min to remove cells. Vesicles were then sedimented at 40,000 × *g* for 30 min and suspended in 50 mM KPO₄ (pH 6.9) at an *A*₅₃₀ of 5, and equal portions were quickly frozen in a dry ice-ethanol bath and stored at -70°C.

Protein determinations. Protein was determined by the method of Lowry et al. (24), with bovine serum albumin as a standard.

Uptake of [³H]tetracycline and [³H]proline. (i) By vesicles. Vesicles were diluted to 0.7 mg of protein per ml in cold 50 mM KPO₄-1 mM MgSO₄ assay buffer at the desired pH. Portions of 0.15 to 0.5 ml were incubated with rapid shaking at 30°C for 10 to 30 min before addition of [³H]tetracycline at 5 μM (5 μCi/ml)

or [³H]proline at 10 μM (5 μCi/ml) unless otherwise specified. Energy sources (20 mM lithium D-lactate; 0.1 mM PMS with the reducing agent 20 mM sodium ascorbate; or 0.2% glucose) and inhibitors were added at times specified. After various intervals, 50-μl samples were withdrawn, mixed with 10 ml of 0.1 M LiCl-0.1 M KPO₄ buffer at 22°C at the pH of the assay, filtered through a Gelman Metrical GN6 0.45-μm membrane filter, and washed with 4 ml of the same buffer; the filters were dried and counted in Betafluor (National Diagnostics, Somerville, N.J.) by a scintillation counter. Radioactivity trapped by the filters in the absence of vesicles was subtracted. The counting efficiencies of tritium in aqueous samples in Ultrafluor (National Diagnostics) and on filters with Betafluor were 30 and 25%, respectively.

(ii) By cells. When cells growing at 37°C in medium A reached an *A*₅₃₀ of 0.8, they were pelleted at 37°C (1,500 × *g*, 10 min), washed twice with assay medium warmed to 37°C, and resuspended in assay medium at an *A*₅₃₀ of 3 to 4. They were assayed for tetracycline uptake at 30 or 37°C by the same method used for the vesicles; 80 μg of chloramphenicol per ml was added to inhibit protein synthesis.

Uptake of calcium. Accumulation of ⁴⁵Ca²⁺ was measured as previously described (26).

Volumes of cells and vesicles. The internal volume of cells was found to be 1.1 μl/*A*₅₃₀ unit as reported (26). The volume of vesicles was determined by methods described previously (31). In one case, total water was determined by weight; in the other, it was determined by tritiated water; excluded water was determined by [¹⁴C]dextran. By both methods, 5 μl (internal volume) per mg of vesicle protein was found. Values in the literature for the volumes of vesicles range from 1 to 4 μl/mg of vesicle protein (15, 20, 22). For the present work, we used 5 μl/mg.

RESULTS

Parameters affecting uptake of tetracycline by cells. The energy-dependent uptake of tetracycline by cells was characterized in assay buffer to compare it subsequently with uptake by vesicles. Energy-dependent uptake in cells was defined as the difference in accumulation without and with 1 mM DNP, measured at steady state (values at 10, 20, and 30 min after tetracycline addition were averaged). A steady-state measurement was chosen rather than initial rate because the former was more easily determined on vesicles (see below). The active uptake by glycerol grown cells at 37°C increased as the pH of the assay decreased. At pH 8.0 uptake was negligible, at pH 6.9 it averaged 70 μM, and at pH 6.0 it averaged 150 μM. At pH 6.0, uptake at 30°C was about half that at 37°C. Low levels of magnesium cation (0.01, 0.1, or 1 mM MgSO₄) had no effect on active uptake, whereas 10 mM inhibited uptake by 60% (assayed at pH 6.0, 37°C).

Uptake of tetracycline by spheroplasts. To see if the cell wall changes accompanying formation of EDTA-lysozyme spheroplasts would affect the uptake of tetracycline, we compared uptake in such spheroplasts with that in cells. Sucrose (20%) was necessary to prevent the lysis of spheroplasts, and so cells were also assayed in sucrose for comparison. Uptake was not less in spheroplasts than in cells (Fig. 1). Therefore, periplasm and intact outer membrane appeared unnecessary for normal tetracycline uptake.

Uptake of tetracycline by inner membrane vesicles. We next asked whether inner membrane vesicles alone, using proton motive force, could concentrate tetracycline. We prepared such vesicles from *E. coli* cells by osmotic lysis of EDTA-lysozyme spheroplasts (see Materials and Methods). The vesicles were virtually free of cells and unlysed spheroplasts as determined by phase microscopy and viable cell count (<0.001% of vesicle protein was contributed by viable cells).

In the absence of energy substrates, uptake of tetracycline by the vesicles was $9 \pm 2.5 \mu\text{M}$

(mean $\pm$ standard deviation; 19 determinations on 3 preparations) at an external concentration of $5 \mu\text{M}$. This uptake was not lowered by DNP, and these vesicles did not concentrate proline above its external concentration in the absence of energy. These findings indicated that endogenous energy sources were not present. Moreover, since the uptake of $9 \mu\text{M}$ was greater than the $5 \mu\text{M}$ that would result from a simple equilibration of free tetracycline across the unenergized membrane, there was presumably some energy-independent binding of tetracycline by the vesicles.

When the electron transport substrate D-lactate or ascorbate-PMS was added, uptake was stimulated (Fig. 2). The loss of accumulated tetracycline from vesicles stimulated with ascorbate-PMS presumably resulted from ascorbate depletion (21, 22) since addition of fresh ascorbate led to reaccumulation of tetracycline (Fig. 2). Glucose, although an energy source for cells, is not an electron transport substrate and did not stimulate uptake. That energized vesicles were actually concentrating tetracycline was indicated by the magnitude of the lactate-dependent accumulation. This accumulation after 20 min of uptake at pH 6.9 was generally 2.5 to 5 times the external concentration of tetracycline.

FIG. 1. Uptake of tetracycline by cells and spheroplasts. Spheroplasts were prepared from glycerol-grown cells as described in the text. Cells were sedimented after washing in cold 10 mM tris(hydroxymethyl)aminomethane, and spheroplasts were sedimented after their formation. Both were resuspended to $A_{530} = 4$ in 50 mM potassium phosphate, pH 6.9, containing 10 mM MgSO_4 and 20% sucrose and were assayed with 20 mM lactate with and without 1 mM DNP as described in the text. The wash buffer included 20% sucrose. Symbols: $\bullet$, cells alone; $\circ$, cells with DNP; $\blacktriangle$, spheroplasts alone; $\triangle$, spheroplasts with DNP.

FIG. 2. Uptake of tetracycline by membrane vesicles energized by electron transport substrates. Vesicles from glucose-grown cells were assayed at pH 6.9 as described in the text; external tetracycline was $10 \mu\text{M}$. Ascorbate and PMS were added within 1 min before tetracycline; lactate was added 10 min prior. At the arrow, fresh ascorbate was added to 20 mM to the ascorbate-PMS assay.

CCCP and DNP are both organic acids that eliminate the proton motive force by rendering the membranes proton permeable (12). When DNP and CCCP were added at 6.5 and 13 min, respectively, after addition of tetracycline to vesicles energized by lactate, tetracycline exited from the vesicles until the energy-independent level seen in the absence of lactate was reached (Fig. 3). Potassium cyanide (20 mM) had the same effect (data not shown). These results showed that proton motive force indeed was energizing tetracycline uptake in vesicles.

Characterization of tetracycline transport system in vesicles. Lactate-dependent uptake of tetracycline at steady state occurred at all pH values tested (from pH 5.5 to 8.2) and had a broad optimum at about pH 6.9 (Fig. 4). The pH dependence of the initial rate of lactate-dependent uptake (measured at 1 min) was more difficult to determine because incorporation was close to background at this early time; however,

FIG. 3. Effect of uncouplers added after initiation of lactate-stimulated tetracycline uptake in membrane vesicles. Fresh vesicles from glucose-grown cells were assayed at pH 5.9 as described in the text, with 10 mM $MgSO_4$. [3H]tetracycline was added at time zero, and 1 mM DNP or 10 μM CCCP was added at times shown. Symbols: $\circ$, lactate plus DNP; $\bullet$, lactate plus CCCP; $\blacktriangle$, no lactate.

FIG. 4. Effect of pH on steady-state tetracycline accumulation in membrane vesicles. Vesicles from glucose-grown cells were diluted into 50 mM potassium phosphate buffer (1 mM $MgSO_4$) to give the final pH values specified. After preincubation at 30°C for 15 min, lactate was added to one of two tubes at each pH. After 15 min more, [3H]tetracycline was added. Uptakes at 12, 21, and 30 min were averaged. Accumulation with lactate minus that without lactate at each pH defined "lactate-dependent" uptake ($\bullet$); accumulation without lactate is shown as "no energy" ($\circ$).

the pH dependence was similar to that of the steady state (not shown).

Tetracycline forms chelates with magnesium (27), and although tetracycline uptake in cells was not enhanced by externally added magnesium (7; this report, but see reference 2), there is evidence that membrane-associated magnesium may nevertheless be involved in tetracycline transport (7). We therefore examined the effect of magnesium ion on the steady-state lactate-dependent uptake in membrane vesicles. In the absence of $MgSO_4$ in the assay, vesicles displayed only a small amount of lactate-dependent tetracycline uptake. In contrast to results with cells, however, $MgSO_4$ increased this active uptake. At the optimal concentration of 1 mM, active uptake was two to four times that without magnesium (determined from a concen-

tration series of 0.05 to 10 mM MgSO_4 at both pH 6.0 and pH 6.9). This was true even after vesicles were washed in 50 mM potassium phosphate to remove any EDTA still remaining after vesicle preparation. That the effect of magnesium was not simply to increase membrane energization was shown by the fact that proline uptake was not stimulated by magnesium. Calcium ion (CaCl_2) stimulated uptake of tetracycline in only some vesicle preparations at concentrations between 0.05 and 3 mM at pH 6.0 or 6.9, and maximal uptake in the presence of calcium was $\leq 50\%$ that with magnesium.

No evidence for saturation of active tetracycline transport in vesicles was seen when the external tetracycline concentration was varied from 2 to 400 μM . Both the 1-min and the steady-state active uptake values increased linearly with the external tetracycline concentration (data not shown).

Quantitative comparison of cells and vesicles. To determine whether the proton motive force-dependent (lactate-stimulated) tetracycline uptake of vesicles made a major contribution to the energy-dependent (DNP-inhibited) uptake of whole cells, we compared these two active uptakes quantitatively. To estimate how much loss in ability to generate proton motive force occurred during the process of making vesicles from cells, we measured uptake of proline in both cells and vesicles. Proline transport is known to be exclusively proton motive force dependent (5, 15, 23).

The vesicles used were made from glycerol-grown cells. Accumulation of tetracycline and proline at steady state was measured. All assays were done at 1 mM MgSO_4 . Choice of assay pH and temperature influenced the comparisons of cells with vesicles because optimal conditions for tetracycline transport were not the same for cells and vesicles. When vesicle assay conditions were used (pH 6.9, 30°C), the active uptake of tetracycline by vesicles was typically 18 μM , which amounted to at least 20% and as much as 60% of that of cells under the same assay conditions. When the assay was done under conditions better for cells (pH 6.0, 37°C), vesicle active uptake was approximately 10% of the 150 μM typically achieved by cells. Steady-state active uptake of proline in cells was relatively insensitive to the above differences in pH and temperature. Under vesicle assay conditions, cells concentrated proline to 850 μM , whereas vesicles achieved 20% of this value when assayed at 1 mM MgSO_4 and about 35% when assayed at 10 mM MgSO_4 .

These results indicated that vesicles retained 10 to 60% of the ability of cells to concentrate tetracycline and 20 to 35% of their ability to

concentrate proline. Since proline transport is completely dependent on proton motive force (5, 15, 23), vesicles had evidently lost some ability to perform proton motive force-dependent transport in general. We found that part of the loss in vesicle transport ability was because some everted (inside-out) vesicles were present in the membrane preparations. Everted vesicles are not able to concentrate either proline or tetracycline (26) and so would lower the activity of the membrane preparations. Everted vesicles can be detected by calcium uptake supported by lactate or ascorbate-PMS (1). Using vesicles prepared as described in Materials and Methods, we found an ascorbate-PMS-dependent calcium uptake of 60 to 100 nmol/mg of vesicle protein (in our hands, lactate does not serve as an energy source for calcium uptake [26]).

It was likely, therefore, that the lower tetracycline (and proline) transport in vesicles as compared with cells reflected in part the less effective generation of proton motive force in vesicles due to some vesicle eversion, rather than use of an additional source of energy (besides proton motive force) in cells for tetracycline transport.

DISCUSSION

Transport of tetracycline into susceptible *E. coli* cells has two components, one energy independent and the second energy dependent (25). In nutrient broth (L broth), the two components are distinguishable kinetically (25). In phosphate buffer, both uptakes have similar kinetics but are separable by energy inhibitors.

We have shown here, by using inner membrane vesicles from susceptible *E. coli* cells, that proton motive force alone can energize active uptake of tetracycline against a concentration gradient. Although comparisons of cell and vesicle activities were problematic for the reasons explained (see Results), the vesicles did not appear to be as active as cells in concentrating tetracycline. However, the vesicles were deficient to about the same degree in ability to concentrate proline, whose transport is known to be solely dependent on proton motive force (5, 15, 23). Thus, we concluded that the major portion of energy-dependent transport of tetracycline in susceptible cells was proton motive force dependent. Some of the loss of activity upon conversion from cells to vesicles can be explained by (i) damage during conversion, in particular, formation of everted vesicles whose presence was suggested by ascorbate-PMS-dependent uptake of calcium by our vesicle preparations (other workers have also reported mosaic or everted vesicles from osmotically lysed

spheroplasts [1, 11]); (ii) loss in activity upon freezing (up to 30% for tetracycline transport); (iii) possible errors in internal vesicle and cell volumes which would affect calculations of internal concentrations; or (iv) possible suboptimal assay conditions for vesicles.

Previous reports with membrane vesicles failed to show any dependence of tetracycline uptake on added energy substrates (8, 10). The failure may have been due to the use of incorrect substrates or to treatment of the vesicles with Triton and by sonication, which can increase eversion (11).

The fact that maximally inhibitory concentrations of DNP, cyanide, arsenate, and anaerobiosis all inhibit tetracycline uptake in whole cells to the same extent (25) is surprising. Except for DNP, none of these should completely eliminate proton motive force. Using uptake of proline as an indicator of the magnitude of proton motive force in prestarved ML308-225 cells, Berger (5) showed that some proton motive force was generated with glucose in the presence of arsenate by respiration and, in the absence of respiration, by ATP hydrolysis by the membrane adenosine triphosphatase; only uncouplers such as DNP completely destroyed proton motive force. In agreement with these findings, we found (using unstarved, glycerol-grown cells) that both cyanide (20 mM) and anaerobiosis inhibited DNP-sensitive proline uptake only 20 to 40%. However, in the same experiment, cyanide and anaerobiosis inhibited DNP-sensitive tetracycline uptake 85% (not shown; assays done at 37°C at pH 6.1 in the presence of 20 mM lactate). One hypothesis to explain these results is that the active uptake of tetracycline may require a higher proton motive force than does uptake of proline, perhaps as a threshold. All the inhibitors used in cells may, by affecting respiration or ATP hydrolysis, directly or indirectly lower the proton motive force below a critical value.

Tetracycline is a hydrophilic molecule (6, 28) with three ionizable groups and a net negative charge at physiological pH (3). It probably crosses the outer membrane through pores formed by porin proteins (25, 28). The simplest explanation for how it crosses the inner membrane and concentrates within the cell is that an inner membrane carrier (perhaps one containing magnesium) is involved. The only evidence for such a carrier in bacterial cells has come from fluorometric assays, which have shown saturation of tetracycline uptake rate in *Rhodospseudomonas sphaeroides* ($K_m = 400 \mu\text{M}$) (37), *Staphylococcus aureus* ($K_m = 254 \mu\text{M}$) (7), and *E. coli* ($K_m = 38 \mu\text{M}$) (32). However, when radioactive tetracycline has been used to measure transport, saturation has not generally been

observed (this report; 10, 25, 29, 34; but see reference 37). Evidence for a carrier for tetracycline transport is therefore presently inconclusive.

Plasmid-mediated tetracycline resistance is associated with an active, carrier-mediated efflux of tetracycline (4, 26). That the plasmid merely reverses the direction of a carrier-catalyzed tetracycline transport used in susceptible cells is unlikely since the carrier in the efflux is easily saturable ($K_m = 6 \mu\text{M}$) (26).

Magnesium at 1 mM increased the energy-dependent uptake of tetracycline in vesicles but not in cells. The inclusion of 10 mM EDTA in the final wash during vesicle preparation may have depleted the vesicle membranes of magnesium; however, they either were not totally depleted or were not totally dependent upon this ion, since in its absence some active uptake was found. That magnesium affected tetracycline uptake specifically rather than enhanced membrane energization was suggested by the failure of the ion to increase proline uptake. The inhibition of tetracycline uptake in cells by externally added high levels of magnesium (this work; 7, 35) may mean that only at this level does external magnesium begin to compete effectively with membrane-bound magnesium (or other cation) for unchelated tetracycline molecules (7). The different pH optima for active tetracycline uptake by cells as opposed to vesicles is unexplained but may involve the requirement for passage of the drug across the outer membrane and periplasm in cells.

In conclusion, we have shown that the major part of active tetracycline uptake in susceptible *E. coli* cells was energized by proton motive force. The complete inhibition of this uptake in cells by conditions which presumably only reduced but did not eliminate proton motive force was curious; a threshold hypothesis has been offered. It remains to be determined whether tetracycline crosses the inner membrane via a low-affinity carrier (presumably meant for another substrate) or whether it might even cross the membrane by diffusion followed by equilibration as a cation (e.g., complexed with magnesium) with the electrical potential or as an acid with the pH gradient across the membrane (see reference 30).

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