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Two Transport Systems for Tetracycline in Sensitive *Escherichia coli*: Critical Role for an Initial Rapid Uptake System Insensitive to Energy Inhibitors

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Escherichia coli sensitivity to tetracycline involves transport and accumulation of the antibiotic within the cell by two different uptake systems: an initial rapid uptake, which occurs over the initial 6 min of contact of the cell with tetracycline, and a slower uptake system, which continues indefinitely and whose rate of uptake is 1/10 that of the rapid system. Only the slow uptake system is blocked by inhibitors of energy-driven systems; it appears to be particularly dependent upon energy from oxidative phosphorylation. Although both uptake systems lead to accumulation of intracellular tetracycline and contribute to the cell's sensitivity, the rapid uptake system appears to be the more important. While these studies confirm active transport of tetracycline into the cell, they demonstrate that a critical uptake system which appears insensitive to metabolic inhibitors occurs initially.

The tetracyclines are effective bacteriostatic antimicrobial agents. They were introduced in the beginning of the 1950s and received widespread acceptance because of their broad-spectrum activity against gram-negative as well as gram-positive bacterial infections (17). The drugs inhibit protein synthesis by interfering with the binding of aminoacyl tRNA to the ribosome-mRNA complex (36). The site of action of the drug appears to be codon-anticodon recognition (19).

Studies of tetracycline metabolism have demonstrated active uptake of the drug into sensitive cells (1, 15). However, the amounts of drug taken up by different strains of *Escherichia coli* did not correlate with levels of sensitivity (32). We have been particularly interested in this property because we are concerned with the mechanism of resistance to tetracycline, particularly that mediated by plasmids (24, 25, 27). For this reason we have investigated in more detail the mechanism by which tetracycline is accumulated in sensitive cells. From these studies we have determined that the sensitive cell has two uptake systems for tetracycline, only one of which appears sensitive to metabolic inhibitors. An initial uptake occurs, which appears to reach an equilibrium with the tetracycline in the medium after 6 min. A slow, second uptake system then is evident, which accumulates tetracycline over a period of hours. It is the slow uptake system that is sensitive to energy inhibitors. Experiments with mutants uncoupled in oxidative phosphorylation confirmed the presence of two uptake systems and indicated that sensitivity to the drug can be conferred by the rapid uptake system alone.

MATERIALS AND METHODS

Bacterial strains and media. DO-1 is our subculture of *E. coli* K-12 strain CSH-1, received from T. Watanabe in 1964. This strain has a requirement for proline and methionine. Wild-type *E. coli* AN-180 and its mutants AN-120 (*uncA*) and AN-283 (*uncB*) uncoupled in oxidative phosphorylation (9) were received from J. Davies. They are designated DO-21, DO-22, and DO-23 respectively. L-broth (10 g of tryptone [Difco], 5 g of yeast extract, 5 g of NaCl, and 1 g of glucose per liter, adjusted to pH 7) (22) was used for most of these studies. Where noted, defined C medium (11) and buffered saline (8.5 g of NaCl, 0.6 g of Na_2HPO_4, and 0.1 g of gelatin per liter) (10) were used.

Tetracycline uptake studies. Uptake of tetracycline was assessed by uptake of [7-^{3}H]tetracycline, which was received as a powder (0.6 to 1.0 Ci/mmol) from New England Nuclear Corp. Fresh solutions of the radioactive drug in methanol were prepared each week, since breakdown products accumulate in solution and preferentially bind to cell walls (26, 27). For uptake measurements in L-broth, cells were grown to mid to late log phase (about 5×10^8 cells per ml) at 37°C, centrifuged, and resuspended in fresh L-broth at 37°C at 1.3×10^9 to 2.0×10^9 cells per ml. After a 5- to 15-min preincubation at 37°C with shaking, [^{3}H]tetracycline was added at specified amounts and shaking continued. Two methods were used to determine uptake.

Method 1. At different times after tetracycline addition, 100-μl samples were removed, diluted 1:17 in cold buffered saline (4°C), and centrifuged at 4°C within 10 min to remove the cells from the free tetracycline in the medium. The resultant cell pellets were resuspended in buffered saline, and samples were used for measurement of absorbance at 530 nm (A_{530}; with a Coleman 101 spectrophotometer) and for measurement of radioactivity (samples dissolved in Aquasol from New England Nuclear Corp. were counted in a Beckman LS-235 scintillation counter). From these two determinations, the amount of [^{3}H]tetracycline per A_{530} unit (5×10^8 cells per ml) was calculated. Counting efficiency was 35%. When the [^{3}H]tetracycline content of cells diluted into cold buffered saline was compared with that of cells centrifuged at 37°C out of labeling medium, only a minimal difference was found, which could be accounted for by removal of trapped media. If, however, cells were diluted or washed in buffered saline at 23°C, 20% or more of the accumulated radioactivity was lost with each washing. Washing centrifuged cells in L-broth at 4°C also resulted in significant loss of labeled tetracycline from the cells.

Method 2. A 100-μl sample of cells was diluted 1:25 in cold buffered saline and filtered within 10 min through 0.4-μm Nucleopore polycarbonate filters, dried, and counted in Liquifluor (New England Nuclear Corp.) in toluene. Previous studies showed that background absorption of [^{3}H]tetracycline is low for these filters, whereas it is high on Millipore mixed cellulose acetate-cellulose nitrate filters, and on Millipore polyvinyl chloride filters. The number of A_{530} units of the filtered cells was determined from the A_{530} of the labeling culture at the time of sampling. Counting efficiency was 20%.

Results were the same (with corrections for counting efficiency) whether uptake was measured by method 1 or 2. Background for uptake was determined by adding [^{3}H]tetracycline to cells at 0°C and immediately diluting them into cold buffered saline and taking them through the procedure. Background was 5% or less of the 6-min uptake and is represented by a point at time zero on graphs. Most of the background was due to binding of [^{3}H]tetracycline to the filters, not to the cells. Uptake is expressed as counts per minute per A_{530} unit. To compare experiments in which slightly different amounts of radioactivity were added to the medium, the uptake has been normalized in all experiments to a standard concentration of 10^6 cpm/ml added. To obtain the amount (in micrograms) of tetracycline taken up per microgram of cell protein, one can use the specific activity of the tetracycline added (this value must be multiplied by 20/35 to allow for quenching on the filters) and the conversion factor of 300 μg of protein per A_{530} unit of cells. Specific activities given in figure legends were determined by the radioactivity measured in Aquasol. Intracellular concentration of tetracycline was calculated assuming that 1 mg of dry cells was equivalent to a 2.7-μl internal volume (38). For DO-1 this value corresponded to 1.2 μl per A_{530} unit.

To examine the uptake kinetics of cells grown in enriched medium and assayed in minimal medium as per Del Bene and Rogers (11), we harvested cells from an overnight stationary-phase culture grown in L-broth and resuspended them at A_{530} = 0.1 or 4.0 in C medium. Uptake was determined by method 2 except that the samples removed for A_{530} = 0.1 were 0.5 ml. Results were the same if uptake was measured in cells grown in Trypticase soy broth and assayed as described by Del Bene and Rogers (11).

Analysis of [^{3}H]tetracycline taken up in 6 min. DO-1 cells grown in L-broth to A_{530} = 1.4 were collected by centrifugation and resuspended in L-broth at 37°C at different cell concentrations (A_{530} = 4, 8, 11.7). [^{3}H]tetracycline was added to give 1.3×10^6 cpm/ml (1.1 μg/ml), and the cultures were incubated with aeration. At 6 min 0.25 ml of each culture was diluted into 2.5 ml of cold L-broth, and the cells were centrifuged out of solution and washed once in 2.5 ml of cold L-broth. The cells were then resuspended in buffered saline, and the radioactivity per A_{530} unit was determined. After 10 min of incubation, the remainder of the culture at A_{530} = 11.7 was chilled, and the cells were sedimented by centrifugation. The supernatant, containing [^{3}H]tetracycline, was added to freshly harvested cells at an A_{530} of 3.3; after 6 min, the [^{3}H]-tetracycline taken up by these cells was determined. The cells sedimented from the above A_{530} = 11.7 culture were washed twice in cold L-broth, and the tetracycline was extracted from the cells with butanol-10% acetic acid-methanol (4:2:1) at 37°C for 30 min. The extract, containing 86% of the [^{3}H]tetracycline in the cells, was dried under N_2, and the residue was resuspended in L-broth. Fresh cells were added at A_{530} = 5.7, and [^{3}H]tetracycline uptake was determined at 6 min as above.

Calculation of permeability coefficient. The initial rate of uptake (velocity) of tetracycline in nanograms per minute per A_{530} unit was converted to micromoles per second per square centimeter by assuming that 1 A_{530} unit equals 0.45 mg (dry weight) of cells, and that 130 cm^2 of *E. coli* membrane surface area equals 1 mg (dry weight) of cells, as is true for *Salmonella typhimurium* (35). The initial internal tetracycline concentration was assumed to be zero, and the permeability coefficient was then the velocity divided by the external tetracycline concentration in micromoles per cm^3 (20, 29).

Drugs and chemicals. Tetracycline-hydrochloride was obtained from Sigma; 2,4-dinitrophenol (DNP) was from General Biochemicals. Sodium arsenate and sodium cyanide were obtained from Fisher. Concentrations of DNP, arsenate, and cyanide used were those which inhibited cell growth at least 80%.

Sensitivity to tetracycline. (i) By growth rate. Sensitivity to tetracycline was assessed in cells growing in L-broth or defined media. Growing cells were subjected to challenge with different concentrations of the drug. The lowest concentration that resulted in no visible turbidity by eye after 15 h of growth at 37°C with shaking, starting with an inoculum of 10^4 cells per ml, was designated the minimal inhibitory concentration. That concentration which showed a 50% inhibition of growth rate after 90 min (starting with 2×10^7 to 3×10^7 cells per ml) was also determined and designated IC_{50}.

(ii) By rate of protein synthesis. At time zero, 20 to 35 μCi of [^{35}S]methionine (New England Nuclear,

550 Ci/mmol) per ml was added to cells resuspended in L-broth at 37°C at A_{530} = 1.2 to 4.0. Samples of 25 μl were removed at various times (every 2 min until 25 min; thereafter, every 5 min) and spotted onto 2.3-cm Whatman 3 or 3MM paper disks which had been pretreated with trichloroacetic acid. Tetracycline was then added to part of the culture, and sampling continued. After drying at room temperature for 30 to 60 min, all disks were processed simultaneously as described (23) to evaluate [^{35}S]methionine incorporated into trichloroacetic acid-precipitable protein.

RESULTS

Biphasic uptake of tetracycline in sensitive *E. coli*. *E. coli* DO-1 cells grown to mid to late logarithmic phase in L-broth were centrifuged, resuspended at A_{530} = 2 to 4 (1×10^9 to 2×10^9 cells per ml), and incubated in the presence of [^{3}H]tetracycline. We emphasized experiments in nutrient medium both to evaluate uptake in a medium in which optimal growth is obtained and to use the same medium in which the cells had been grown. High cell density was chosen to limit cell growth so that the uptake would be seen in cells not undergoing extensive cell division. Uptake of the drug demonstrated biphasic kinetics: a rapid uptake, which ceased at the end of approximately 6 min and a slow uptake, which then became evident and continued for at least 2 h (Fig. 1A). The average rate of the rapid uptake, evaluated from seven experiments, was 1.6 ng of tetracycline per min per A_{530} unit when the tetracycline concentration in the medium was 0.5 μg/ml. This corresponded to a permeability coefficient (see above) of approximately 0.8×10^{-6} cm/s. Both rapid and slow uptakes were observed in cells preincubated in nonradioactive tetracycline (Fig. 1A); both uptakes have also been seen in logarithmically growing cells. Since sensitivity to tetracycline was greater in minimal medium (Table 1), and since uptake studies of tetracycline have been done in different laboratories using both enriched and minimal media, we tested uptake in minimal medium C as well. Biphasic uptake kinetics could also be demonstrated, but only at the lower cell density of A_{530} = 0.1 (Fig. 1B). At higher cell densities (A_{530} = 4) in minimal medium C, the rapid uptake system was greater than in enriched medium and the slow uptake system was absent or only minimally present; this finding was seen for cells harvested from L-broth or C medium. Furthermore, in minimal medium the rapid uptake appeared to last longer (10 to 12 min) than usually seen in L-broth (Fig. 1B).

Although paper chromatography (26) of the labeled tetracycline showed only one species, tetracycline, we were concerned that the biphasic kinetics might merely reflect uptake of two different compounds, one a minor impurity that had not been detected. To show that the rapid uptake was transporting tetracycline and not an impurity, we did two experiments (as described in Materials and Methods: analysis of [^{3}H]tetracycline taken up in 6 min).

Experiment 1. When cells at different con-

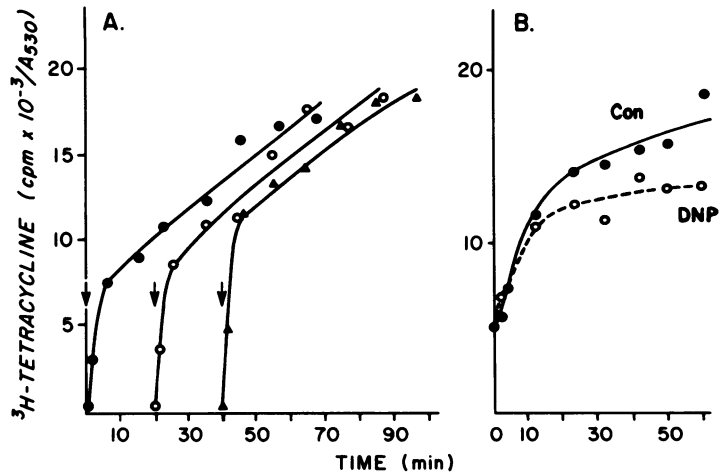

FIG. 1. *Uptake of [^{3}H]tetracycline by sensitive E. coli. (A) Resuspended DO-1 cells (A_{530} = 3.5) were preincubated in L-broth with 0.5 μg of unlabeled tetracycline per ml for 0 (●), 20 (○), or 40 min (▲) prior to the addition (at the arrow) of 0.55 μg of [^{3}H]tetracycline (specific activity, 1.8 × 10^6 cpm/μg) per ml. The preincubations were begun so that [^{3}H]tetracycline was actually added to all cell preparations at the same time. Uptake was assayed by the filtration method. (B) DO-21 cells were preincubated in C medium (A_{530} = 0.1) without (●) or with (○) 1 mM DNP for 10 min prior to addition of [^{3}H]tetracycline to the concentration of 0.6 μg/ml (specific activity, 1.8 × 10^6 cpm/μg).*

TABLE 1. *Sensitivity to tetracycline in various media*

E. coli strain	Characteristics	IC_{50}[a] (L-broth)	MIC[b]	
			L	C
DO-1	*pro, met*	0.8	1.0	0.75
DO-21	Wild type, *arg, thi*	0.45	1.0	0.75
DO-22	*uncA, arg, thi*	1.1	1.7	
DO-23	*uncB, arg, thi*	1.6	2.0	
DO-23H	*uncB*, tetracycline resistant	11.5	10.0	

[a] IC_{50}, Concentration showing 50% inhibition of growth rate after 90 min; see text. Expressed in micrograms of tetracycline per milliliter.

[b] MIC, Minimal inhibitory concentration, in micrograms of tetracycline per milliliter. L, L-broth (22); C, C medium (11).

centrations were incubated with [^{3}H]tetracycline in L-broth, the amount of tetracycline accumulated by the rapid uptake system was proportional to the cell concentration (for A_{530} of 3.8, 7.4, and 11.7, the percentage of total tetracycline taken up was 6.6, 10.5, and 15.8, respectively). At the highest cell concentration used (A_{530} = 11.7), the uptake was 16% of total [^{3}H]-tetracycline, a value well above that of an impurity detectable by chromatographic methods. When fresh cells (A_{530} = 3.3) were added to the medium in which the A_{530} 11.7 cells had been incubated, yet another 4.1% of total tetracycline was incorporated by the rapid uptake system, again exactly proportional to the cells present. Thus no "impurity" was being removed from the medium by the rapid uptake system.

Experiment 2. Alternatively, the radioactivity taken up in 6 min was extracted from the cells and given to fresh *E. coli* at A_{530} = 5.6. If this extracted radioactivity had presented an impurity removed from the medium by 6 min, 100% of it should have been incorporated within 6 min by the fresh cells. However, only 7% was taken up rapidly. Again, the amount was directly proportional to the number of cells present. In all instances, 1.37% of added labeled tetracycline was taken up per A_{530} unit (about 5×10^8 cells). These results indicated that the rapid uptake kinetics were not manifesting an impurity in the radioactively labeled tetracycline. Similar experiments showed that the slow uptake system also represented a large fraction of the total labeled material and was therefore not an impurity.

Effect of inhibitors and cell mutations upon the biphasic uptake of tetracycline. The concentration of tetracycline reached within the cell by 4 min (assuming an internal cell volume of 2.3×10^{-9} μl per cell based on 2.7 μl of cell water per mg of dry weight) (38) was 5 to 10 times higher than that in the medium. Thus, tetracycline appeared to be concentrated in the cell by both the rapid and slow uptake systems. The energy dependence of both rapid and slow uptake systems was therefore investigated.

First we tested the effects of energy inhibitors on uptake. Arsenate (an inhibitor of the synthesis of high-energy phosphates) (21), DNP (an uncoupler of oxidative phosphorylation from electron transport) (21), and cyanide (an inhibitor of electron transport) (21) all decreased or stopped the slow tetracycline uptake in L-broth while, unexpectedly, the rapid uptake was unchanged (Fig. 2). These effects were seen, for

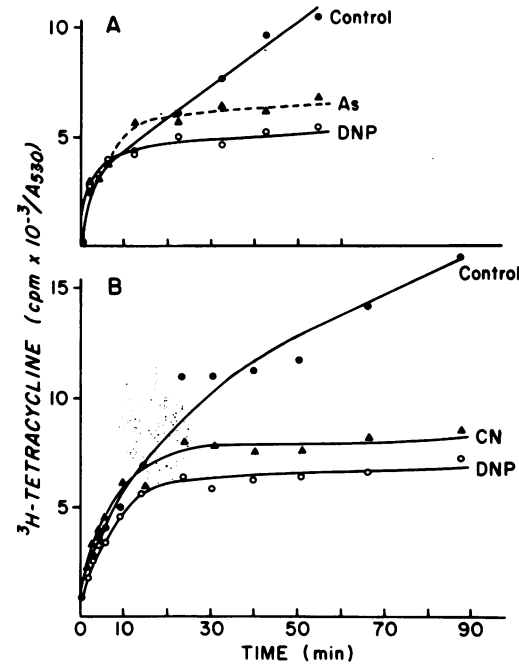

FIG. 2. *Effect of energy inhibitors on tetracycline uptake by DO-1 cells in L-broth. (A) DO-1 cells in L-broth (A_{530} = 4.6) were preincubated for 13 min without any inhibitor (●) or with 0.7 mM DNP (○) or 10 mM sodium arsenate (▲). [^{3}H]tetracycline (specific activity, 2.1 × 10^5 cpm/μg) was added to give 10 μg/ml final concentration. At 34.5 min, 7 mM more arsenate was added to the arsenate tube because the effectiveness of arsenate as assayed by growth inhibition declined by 35 to 40 min. Uptake was assayed by the filtration method. (B) DO-1 cells in L-broth (A_{530} = 2.6) were preincubated for 18 min without any inhibitor (●) or with 0.7 mM DNP (○) or 2 mM sodium cyanide (▲). [^{3}H]tetracycline (specific activity, 1.8 × 10^6 cpm/μg) was added to give 0.63 μg/ml. Uptake was assayed by the centrifugation method, but values have been multiplied by 20/35 to correct to the lower counting efficiency of the filter method. In this experiment, a clear distinction between rapid and slow uptake was not observable, and the rapid uptake as drawn appears more prolonged than usual.*

DNP at least, at both high and low concentrations of tetracycline, at values at, below, or 10-fold above the minimal inhibitory concentration (Fig. 2). As in L-broth, the initial uptake in C medium was insensitive to DNP, whereas the second uptake was sensitive (Fig. 1B). The two uptake systems were thus clearly separable. Only the slow uptake system was affected by energy inhibitors; it appeared to require oxidative phosphorylation and electron transport.

We next examined tetracycline uptake in two cell mutants uncoupled for oxidative phosphorylation, so-called *unc* mutants (9). The *uncA* mutant lacks the Mg^{2+} Ca^{2+}-dependent adenosine triphosphatase activity that is necessary for oxidative phosphorylation; the *uncB* mutant, while possessing this activity, is defective in a part of the adenosine triphosphatase-membrane complex which couples electron transport to ATP synthesis (18). Although the parent strain of these mutants exhibited typical biphasic uptake kinetics (Fig. 1B), the *unc* mutants had only a rapid uptake, as exemplified by *uncB* in Fig. 3. This finding further confirmed that oxidative phosphorylation was involved in the slow uptake system only.

Loss of accumulated tetracycline. When cells labeled with tetracycline for 5 min were filtered and resuspended in L-broth at 37°C without tetracycline, there was a rapid loss of the drug from the cells, about half being lost within 2 to 5 min. A two- to four-times-slower loss was seen upon resuspension after 60 min of uptake. Rapid efflux was also observed when cells were diluted into medium containing unlabeled tetracycline. Greater than 90% of all accumulated [^{3}H]tetracycline was lost from the cells. Thus a rapid exchange of tetracycline between the medium and the cells occurs continuously.

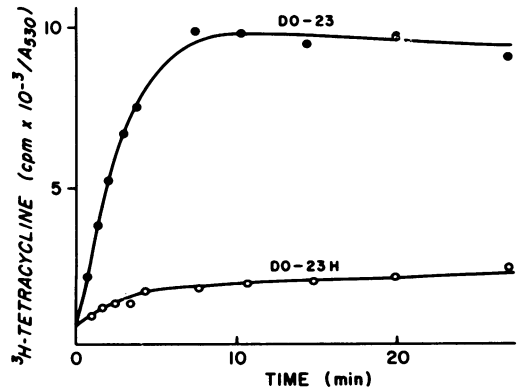

FIG. 3. *Tetracycline uptake in DO-23 and in DO-23H, a mutant of DO-23 resistant to higher tetracycline levels. Cells were resuspended in L-broth at A_{530} = 4.0. [^{3}H]tetracycline was added to give 0.8 μg/ml (1.8 × 10^6 cpm/μg), and uptake was measured by the filter method.*

Relationship of the two tetracyline uptake systems to inhibition of protein synthesis. We found that both tetracycline uptake systems, the one energy dependent and the other energy independent, were contributing to the drug's inhibition of cellular protein synthesis.

First we measured the rate of protein synthesis in DO-21 cells in the presence or absence of 0.5 μg of tetracycline per ml. [^{35}S]methionine was added to the culture, and its incorporation into protein with time was measured as described in Materials and Methods. At the same time, the culture was examined for kinetics of uptake of tetracycline. We found that about 30% inhibition of protein synthesis occurred within 6 min in accord with tetracycline entering the cell by the rapid uptake system (Fig. 4). A slowly increasing inhibition occurred thereafter, reaching about 70%. These results demonstrated that the rapid uptake system was effective in putting tetracycline into its antibacterial site in the cell, and that the slow uptake does eventually contribute to inhibition of protein synthesis.

Second, we evaluated protein synthesis in DO-1 cells by pulsing them for 2 min with [^{35}S]-methionine at various times after addition of 1.4 μg of tetracycline per ml. Once again, inhibition of protein synthesis occurred within 3 min of tetracycline contact, and this inhibition thereafter increased slowly with time. With this concentration of tetracycline, the initial inhibition was 50%.

Third, we examined tetracycline effect on growth and protein synthesis in the *uncB* mutant, which lacked the slow uptake system. Despite absence of the slow uptake system, the *unc* mutants are only about twofold less sensitive to tetracycline (Table 1). This sensitivity clearly indicated that the rapid uptake of tetracycline led to intracellular antimicrobial activity and was not the result of nonspecific adherence of drug to the cell wall. When the effect of tetracycline on protein synthesis in *uncB* (DO-23) was examined, we saw that, in contrast to results with wild type (DO-21), the inhibition of protein synthesis remained constant for over 60 min (the time of the experiment) (Fig. 4), in agreement with a lack of slow uptake in *uncB* (Fig. 3).

Uptake as a function of tetracycline concentration. Previous investigators (16, 31) have found tetracycline uptake in *E. coli* to be unsaturable, thus implying no carrier transport molecule. Having discovered that two tetracycline systems were involved in uptake, we examined them separately for evidence of saturability.

Uptake of total tetracycline by the rapid system deviated only slightly from being strictly proportional to the tetracycline concentration in

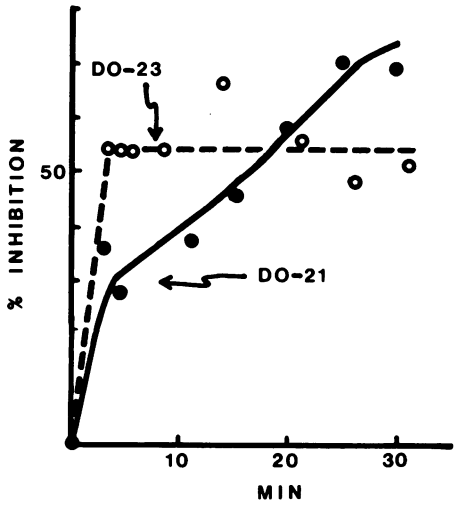

FIG. 4. *Inhibition of protein synthesis after addition of tetracycline to DO-21 and DO-23 cells. The rate of protein synthesis for both control and tetracycline-treated cells at a given time was defined as the slope of the [^{35}S]methionine incorporation curve at that time. It was approximated by finding the slope of the best straight line through four points bracketing that time, two on either side, using the method of least squares. Percent inhibition = 100% − 100% (slope of tetracycline treatment/slope of control). The time is shown in minutes after addition of tetracycline. The amounts of tetracycline were chosen to inhibit the two strains equally: DO-21 (●), 0.53 μg/ml; DO-23 (○), 2.6 μg/ml.*

the medium from 0.34 to 176 μg/ml (Fig. 5), and in other experiments up to 380 μg/ml (0.8 mM). The rapid uptake system was thus not saturable, at least up to 0.8 mM. Using the same method on the slow uptake system, we found that the rate of slow uptake between 45 and 105 min after tetracycline addition remained proportional to the tetracycline concentration over a range from 1 to 400 μg/ml; however, at earlier time points after tetracycline addition (10 to 40 min), the rate of slow uptake appeared to show some saturation. These results for the slow uptake are as yet, therefore, inconclusive.

Effect of inhibitors of protein and ribonucleic acid synthesis upon uptake. There was no effect in DO-1 cells on either rapid or slow uptake systems by chloramphenicol (25 μg/ml) or rifampin (200 μg/ml), which completely inhibit cellular protein synthesis and RNA synthesis, respectively, at these concentrations. These inhibitors were added 30 min before the tetracycline.

Isolation of cellular mutants resistant to high levels of tetracycline. Since *unc* mutants appeared to have only one uptake system, we postulated that cellular tetracycline resistance mutants might be more easily isolated. We have isolated many such mutants with resistance to up to 10 μg of tetracycline per ml by selection for spontaneous mutations through stepwise dilution into increasing amounts of tetracycline. These mutants are presumably multistep mutants. By this method, no such mutants were isolatable from the parent DO-21.

We studied the uptake of tetracycline in one of the high-level resistance mutants, DO-23H. There was a marked reduction in uptake by the rapid uptake system (Fig. 3), which correlated with the reduced sensitivity of this strain to tetracycline. At 7.5 min the ratio of uptakes was 6; the ratio of sensitivities was about 5 (Fig. 3, Table 1). The results were further confirmation that the rapid uptake system was critical to cellular sensitivity to tetracycline.

DISCUSSION

Sensitivity to tetracycline in *E. coli* has been linked previously to an active accumulation of the drug within the cell (1, 15). Our studies, however, suggest that while some active accumulation does occur and represents about half the uptake in 40 min in L-broth, a critical uptake occurs within 6 min by a rapid system which appears to be unaffected by any energy inhibitors used.

Quantitative reexamination of earlier work suggests that it may not be completely divergent

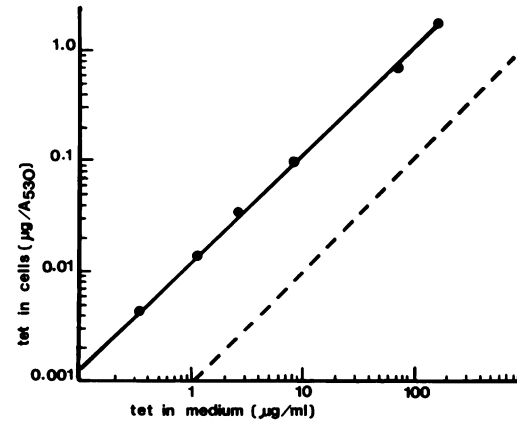

FIG. 5. *Rapid uptake of tetracycline (tet) as a function of tetracycline concentration in medium. [^{3}H]-tetracycline at different specific activities was added to DO-1 cells suspended at A_{530} = 4.4 in L-broth. Tetracycline uptake after 4 min was determined and is plotted here as a function of the tetracycline concentration in the medium using log-log coordinates. The dotted line represents an intracellular concentration equal to the concentration in the medium, assuming an intracellular volume of 1.2 μl per 5 × 10^{8} cells (A_{530} unit) (see the text).*

from the present observations. Del Bene and Rogers saw a nonlinear, probably biphasic uptake in C medium (11), and found that the total uptake in a 30-min period was inhibited only 30% by 0.1 mM azide. This is similar to the DNP-effected 25% inhibition after 30 min that we saw in C medium (Fig. 1B). Franklin and Godfrey (15) did not measure uptake kinetics; the uptake after 1 h in a minimal medium, after subtraction of the zero time value, had an absolute requirement for glucose. This result might be explained if the zero time value was very large and included tetracycline accumulated by the rapid uptake system. Franklin and Higginson (16) described an uptake which ceased after 7 to 10 min in an enriched medium. They may have been observing only the rapid uptake system in their medium; no studies on energy requirements for the uptake were presented. We have noted lack of detectable slow uptake in some enriched as well as minimal media (unpublished data). Shipley and Olsen (34) reported that for *E. coli* and *S. typhimurium* in various media, DNP and azide never inhibited tetracycline uptake more than 50%. In general, then, earlier experiments are not in contradiction to the existence of an energy-independent rapid uptake in *E. coli*. It is also possible that the different media used for uptake measurements by different groups in conjunction with different methods of washing and determining the amount of tetracycline in cells have previously obscured the existence of two uptake systems.

Our studies here indicate that both the energy-insensitive rapid uptake and the energy-dependent slow uptake systems transport tetracycline to cellular sites which cause inhibition of protein synthesis. Both systems are in constant flux, as is shown by the rapid loss of tetracycline from cells diluted into drug-free media containing unlabeled drug. Such rapid release of drug corresponds to the known reversibility of the antibacterial activity of tetracycline. We have previously shown that about 75% of tetracycline accumulated by both systems in 45 min is located within the cell, 15% is in the periplasm, and 7% is bound to the outer cell membrane (27).

A few observations on the mechanism of tetracycline uptake can be made. It seems possible that the mildly hydrophilic tetracycline molecule passes through the outer membrane of the cell by diffusion through protein-lined "pores" (28) rather than through the lipid membrane layer. This idea results from the finding that cell mutants missing most of the lipopolysaccharide of the outer membrane ("deep rough mutants") do not have increased sensitivity to tetracycline (2, 28) or altered uptake (unpublished data); these mutants do have increased sensitivity and permeability to lipophilic substances which enter presumably by diffusion directly through the more exposed phospholipids of the membrane (and not through pores). Furthermore, the *tolF* mutants, which have little or no outer membrane porin 1a, show a 1.5- to 2.0-fold decreased sensitivity to tetracycline (3, 8, 13).

Since the rapid uptake is insensitive to energy inhibitors, it probably occurs via either passive or facilitated (carrier-mediated) diffusion. Within 6 min an equilibrium of binding of tetracycline to internal cellular components present in great excess may be attained. The apparent 5- to 10-fold concentration of tetracycline within the cell may be due to such binding rather than to an energy-dependent gradient of free tetracycline. The rate of uptake of tetracycline by the rapid system, with a permeability coefficient of about 0.8×10^{-6} cm/s, is not out of line with permeability coefficients observed for passive diffusion of polar compounds through artificial bilayer lipid membranes (20) or through outer-membrane pores (29); on the other hand, the rate of uptake by the rapid system is not incompatible with facilitated (i.e., carrier-mediated) diffusion. Therefore, the rate of the rapid uptake does not help in distinguishing between passive and facilitated diffusion as the uptake mechanism.

Lack of saturability of the rapid uptake also does not distinguish between the two mechanisms. Generally one expects a facilitated mechanism to exhibit saturability with increasing substrate concentrations, while passive diffusion will not. In our case, however, a lack of saturability up to 0.8 mM may only mean that a much higher concentration is needed, as would be expected if tetracycline were using the uptake system of another substrate. In this regard also, technical difficulties should be noted, involving the insolubility of tetracycline above this value unless the medium is acidic and the leakage of molecules from the cells at high tetracycline levels (30).

A rapid uptake for chlorotetracycline has also been observed in *Staphylococcus aureus*, using an increase in chlorotetracycline fluorescence as a measure of its uptake (12). This reaches a maximum by 10 min at 25°C. The *S. aureus* uptake, however, is unlike that described here for *E. coli* in that it can be inhibited significantly by energy inhibitors such as azide, cyanide, and omission of glucose and is saturable with a K_m of 107 μM for chlorotetracycline and 254 μM for tetracycline (12).

The difficulty in isolating chromosomal mutants resistant to high levels of tetracycline may relate to the fact that there are normally two

uptake systems for the drug. Mutants in either one of the uptake systems would be unselected since tetracycline would be accumulated by the other uptake system. This may explain our ability to isolate mutants with higher-level resistance to tetracycline from *unc* mutants, which lack a slow uptake system. The nature of the mutation(s) affecting rapid uptake is under investigation. It may result from altered diffusion rates of tetracycline entry and exit (e.g., due to a decrease in numbers of carrier molecules or pores), decreased internal binding constants, or decreased numbers of binding sites.

It would appear somewhat paradoxical that bacteria have retained through evolution an active uptake of a substance that could inhibit their growth. Thus it seems more reasonable to find that the initial uptake of tetracycline does not apparently include such an active process. Transport of some antibiotics, however, has been linked to active uptake systems (6, 14), including the secondary uptake of tetracycline as shown here. It is probable that the uptake systems used by antibiotics are those required also for important cell metabolites, although no evidence for this was seen for the uptake of aminoglycoside antibiotics (7).

The slow uptake system for tetracycline appears to be unlike any previously described transport system in *E. coli*. Like that for glutamine and several other amino acids (5, 37), it appears to be lost upon osmotic shock of cells (unpublished data) and is sensitive to arsenate and the *unc* mutation under aerobic conditions. Glutamine uptake, however, was only partially lowered in *unc* mutants and by DNP or cyanide in wild-type cells if glucose was the substrate, suggesting that both glycolytic ATP and ATP from oxidative phosphorylation could be used for glutamine uptake (4, 5). In the case of tetracycline, however, the complete inhibition of the aerobic slow tetracycline uptake system by DNP or cyanide in the presence of glucose in normal cells and by the *unc* mutation suggests that ATP derived only from oxidative phosphorylation is used for the slow tetracycline transport. In support of this, preliminary experiments showed no slow tetracycline uptake to be present under anaerobic conditions (unpublished data). The apparent functional distinction between glycolytic ATP and ATP from oxidative phosphorylation suggested by our data is unprecedented and requires further work for verification; it is possible that side effects of the inhibitors (see 5, 33) or unknown properties of the *unc* mutations have obscured the precise energy requirements of the slow uptake system. Whatever the mechanism, the slow uptake system requires energy, whereas the rapid uptake system does not. Under anaerobic conditions the rapid system, unlike the slow, is present (unpublished data); this rapid uptake system could, therefore, be the only one relevant to inhibition of sensitive *E. coli* residing in the anaerobic environment of the avian and mammalian intestine.

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