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Accumulation of Tetracyclines by *Escherichia coli*

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The net accumulation of tetracyclines by *Escherichia coli* as a function of concentration was shown to be biphasic. At concentrations less than the bacteriostatic levels, the mode of uptake was not azide-sensitive and was considered to be physical adsorption on the cell surface. At concentrations above the minimal inhibitory level, a second, azide-sensitive, uptake component was functional in addition to the surface adsorption process. This second energy-requiring mode was judged to represent penetration of the cytoplasmic membrane by tetracycline molecules to their sites of inhibitory action. Each mode for a given tetracycline and culture is expressed algebraically by a characteristic Freundlich equation. Resistance in *E. coli* is shown to be a result of diminished transport of antibiotic. However, this resistance was due not to a reduction or loss of a transport mechanism but rather to a requirement for higher antibiotic concentrations before the second mode of uptake could become operative.

The physiological basis for the development of bacterial resistance to the clinically important tetracyclines is little understood. It has been reported that a tetracycline sorption mechanism is somehow diminished in resistant strains (5, 6) Izaki and Arima (6) demonstrated that a resistant *Escherichia coli* strain accumulated much less oxytetracycline than did a susceptible strain. Their procedures, however, allowed only for relative comparisons at antibiotic concentrations which were several-fold higher than bacteriostatically effective levels. Franklin and Godfrey (5) worked at lower drug concentrations by the use of labeled chlortetracycline and tetracycline. They also observed that resistant cells accumulated much less of the tetracyclines than did susceptible cells, but quantitation was difficult.

Indirectly, recent studies, which establish that therapeutic levels of tetracyclines act by their inhibition of protein biosynthesis (4), also suggest that resistance to these antibiotics is due to decreased penetration of the drugs to the site of their inhibitory action. When cell-free extracts from susceptible and resistant *E. coli* cells were compared, tetracycline inhibition of amino acid incorporation into polypeptides was found to be equivalent in both preparations (5, 10, 13). This demonstration of crypticity indicated to all three groups that resistance is probably due to an inadequacy in drug permeation.

An aberration in the cell-transport process is one of seven possible biochemical mechanisms proposed for drug resistance by Davis and Maas (3). To detail this mechanism, a program was initiated to quantitate tetracycline transport by use of both resistant and susceptible strains of *E. coli*. Attention was focused particularly on those concentrations of different tetracyclines which are characterized as the minimal inhibitory levels. In this paper, the methodology used and the two concepts which were derived from experiments aimed at model development are described. Results with resting-cell populations of only two *E. coli* strains are reported, and the uptake values are those of an equilibrium or a steady state and do not reflect kinetic phenomena. The conditions for the incubation experiments were based on the work reported by Izaki and Arima (7).

MATERIALS AND METHODS

E. coli cultures. The tetracycline-susceptible strain of *E. coli* used was Crook's culture (ATCC 8739). The resistant strain was culture 3-85 isolated from a Mississippi poultry flock and provided by A. R. English (Chas. Pfizer & Co., Inc.). Both cultures were maintained by periodic transfer on a nutrient agar medium.

Preparation of resting-cell population. Portions (25-ml) of an inoculated nutrient broth contained in 300-ml Bellco nephaloflasks were incubated at 37 C with gyratory shaking. Growth was followed turbidimetrically in a Klett-Summerson colorimeter (KF-66 filter). When the population was in the middle of its exponential growth period, the flasks were refrigerated and held at 4 C for 30 min. The cells were then recovered by centrifugation. Typically, the initial cell load was 10 Klett units, while at harvest the reading was 100 to 105 Klett units. The cells were washed twice with cold 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (*p*H 6.0). The final cell pellet was re-

constituted to 200 Klett units with the same MES buffer. This suspension was found to contain 8×10^8 cells per ml by both viable (plate) and total (Petroff-Hausser) counts. The cell mass was 800 μg per ml, as determined by the freeze-drying of a 2-ml portion of cell suspension which was impinged on a Metricel VM-6 filter.

Tetracyclines. All antibiotics were obtained from Pfizer stock as the hydrochloride salts. Aqueous solutions were prepared on the day needed by the dissolving of the antibiotics in 800 μM $MgSO_4 \cdot 7H_2O$ (with boiled-out water). The *p*H was adjusted to 6.0 with KOH. Just before use, the solution was passed through a membrane filter (type HA, Millipore Corp., Bedford, Mass.). Standard solutions for fluorometry were made up with barbital-calcium reagent. A 1.0 μM solution was prepared daily by dilution of a 200 μM stock standard. The stock standard remained stable for several weeks when stcred in an amber bottle at refrigerator temperature.

Barbital-calcium reagent. The stock solution contained 0.25 mole of barbital and 0.015 mole of $CaCl_2 \cdot 2H_2O$ per liter of distilled water. Barbital was recrystallized once from water. This solution was adjusted to *p*H 9.0 with KOH. The reagent was prepared by mixing 200 ml of barbital-calcium stock solution with 800 ml of aqueous *n*-propanol azeotrope. Distillation of *n*-propanol as the azeotrope permits simple purification to minimize fluorescent contamination. Care was exercised to exclude CO_2 from the basic solutions during preparation and storage.

Assay of tetracyclines. The procedure utilized to assay tetracyclines was an adaptation of the method described by Kohn (9). In place of a water-immiscible solvent, *n*-propanol was used. The aqueous *n*-propanol in the final reagent (approximately 50%, w/w) was an excellent solvent for the $CaCl_2$ and the barbital, as well as for the uncharged tetracycline complex that formed. Fluorometric measurement of the tetracycline content in barbital-calcium reagent solutions was performed with an Aminco-Bowman spectrophotofluorometer with an Hanovia 901C-1 Xenon lamp, a RCA 1P21 photomultiplier tube, slit arrangement No. 4, and the sensitivity set at 50. The optimal wavelengths for excitation and emission were 390 and 520 mμ, respectively. A fluorescence unit (FU) was defined as the product of the meter multiplier setting, the recorder deflection, and 1,000. The fluorescent peak height was a linear function of the tetracycline concentration over an instrument range of 20 to 20,000 FU. Typical fluorescence values of 1 μM solutions of the different tetracyclines are shown in Table 1. The usual experiments were conducted to test the quality of the assay. No evidence was found for quenching phenomena or other interferences associated with nontetracycline extractables.

Cellular uptake of tetracyclines. All experiments to measure celluar uptake of tetracyclines were carried out at 32 C in a Dubnoff shaker. Portions (8-ml) of the reaction mixture were added to 25-ml Erlenmeyer flasks fitted with loose glass tops. The reaction mixture had an initial *p*H of 6.0 and was composed of: D-glucose, 50 mM; MES, 20 mM; $MgSO_4 \cdot 7H_2O$, 200 μM; resting *E. coli* cells, 200 μg/ml; and a tetracycline, 5 to 2,500 μM. Uptake was initiated by the addition of the appropriate antibiotic stock solution and was continued for 2 hr. Preliminary time-course studies showed little or no antibiotic uptake after 2 hr of incubation. To stop the uptake, 1 ml of the reaction mixture was added to 10 ml of a cold special buffer, *p*H 6 (MES, 20 mM; dithiothreitol, 3 mM; L-cysteine·$HCl \cdot H_2O$, 1 mM; and $MgSO_4 \cdot 7H_2O$, 1 mM). This was then rapidly impinged on a 50% aqueous isopropanol-washed, 25-mm membrane filter (type HA). The filter disc and contents were then washed twice with cold 5-ml portions of the special buffer (*p*H 6). The filtrate and washings were collected and freeze-dried if the amount of nonsorbed antibiotic was to be determined.

TABLE 1. *Fluorescence of 1 μM standards*

Antibiotic	Mol wt of HCl salt	FU[a]
5-Oxytetracycline	497	5,980
6-Deoxy-5-oxytetracycline	481	3,360
Tetracycline	481	9,440
6-Demethyltetracycline	467	7,650
6-Deoxytetracycline	465	5,100
6-Demethyl-6-deoxytetracycline	451	2,820

[a] Fluorescence units (FU), corrected for the nonspecific background fluorescence of the barbital-calcium reagent (20 to 25 FU).

Extraction of tetracyclines. At room temperature, 10 ml of the barbital-calcium reagent was used to extract the antibiotic from a membrane filter with its impinged cells. The aqueous *n*-propanol solvent has the desired lipophilic properties to allow direct extraction of the cells without preliminary treatment. Extraction is almost immediate, but the disc was routinely allowed to stay in contact with the reagent for 30 min. The extract was clarified by centrifugation if it was necessary. Nonsorbed antibiotic in the lyophilized filtrate was dissolved in 50 ml of barbital-calcium reagent.

Antibiotic sensitivity testing. The minimal inhibitory concentration (MIC) of each tetracycline for the two *E. coli* strains was determined under conditions which paralleled those characteristic of the antibiotic uptake experiments. To use the uptake formulation as a growth medium, the MES buffer level was raised to 200 mM; 20 mM $(NH_4)_2SO_4$ and 200 μM KH_2PO_4 were added along with trace quantities of Fe^{++}, Mn^{++}, and Ca^{++}. Tubes which contained 5 ml of medium supplemented with a tetracycline were inoculated with 0.05 ml of a 10 Klett unit resting-cell suspension and incubated at 32 C until the control tube read approximately 100 Klett units. To establish the MIC, the growth in each tube as a percentage of control was plotted against the log of the antibiotic concentration. The data were extrapolated to zero growth graphically (Fig. 1). The assigned MIC values are listed in Table 2.

Chromatography. Cell extracts for paper chromatograms were prepared by a scaling-up of the uptake and extraction procedures described above. The cells

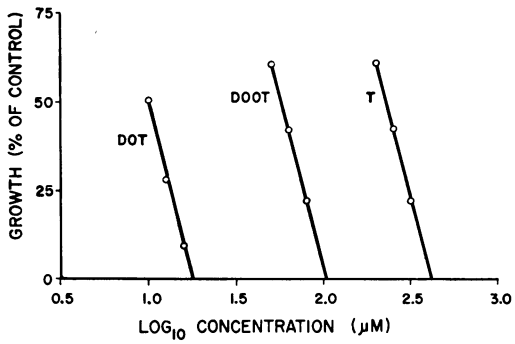

FIG. 1. *Growth of resistant Escherichia coli 3-85 as a function of the particular tetracycline (T, tetracycline; DOT, 6-deoxytetracycline; DOOT, 6-deoxy-5-oxytetracycline) and its concentration. Each point (○) is a single turbidimetric determination. Extension of the resulting lines to zero growth permitted assignments of minimal inhibitory concentrations.*

TABLE 2. *Minimal inhibitory concentrations*[a]

Antibiotic	Susceptible strain		Resistant strain	
	Concn (μg/ml)	Log_{10} μM	Concn (μg/ml)	Log_{10} μM
5-Oxytetracycline	5.6	1.05	130	2.41
6-Deoxy-5-oxytetracycline	4.0	0.92	49	2.01
Tetracycline	3.8	0.90	200	2.62
6-Demethyltetracycline	1.9	0.60	95	2.31
6-Deoxytetracycline	6.0	1.11	8.3	1.25
6-Demethyl-6-deoxytetracycline	3.4	0.88	5.7	1.10

[a] Average of duplicate determinations as described in Materials and Methods.

were recovered by centrifugation instead of filtration. After the cells were washed twice with cold special buffer (*p*H 6), they were lyophilized and extracted with a minimal volume of barbital-calcium reagent. The extract was clarified by centrifugation and then spotted on Whatman no. 4 paper. The three systems which were used in a standard manner at room temperature were: no. 33 (ethyl acetate saturated with water, descending); no. 60B (chloroform, nitromethane, pyridine, and water, 10:20:3:1, clarified by filtration through glass wool, descending); and the solvent of Last and Snell (11) (isobutyric acid and 0.5 N ammonium hydroxide, 5:3, ascending). For system no. 33, the paper was treated with McIlvain's buffer (*p*H 3.5) and dried before it was spotted. For system no. 60B the paper was similarly treated but was used wet. Tetracyclines were located on the paper by their fluorescence under ultraviolet light.

Radioactivity measurements. Tetracycline-7-^3H hydrochloride was used (New England Nuclear Corp. Boston, Mass.). Cell extracts or digests of 1-ml volumes were counted in a Packard Tri-Carb liquid scintillation spectrometer (model 314-DC). All counts observed were corrected for background. The efficiency factor for each sample was established by the internal standard technique (2). Results were compared as disintegrations per minute (DPM). Digests of membrane filter-impinged bacteria were prepared to measure total radioactivity by an extension of the formamide digestion procedure described by Neujahr and Ewaldsson (12). The filter suspended in 6.5 ml of formamide was held at 60 C for 2 hr under a nitrogen blanket. After this incubation, 3.5 ml of dimethylformamide, which contained 1.5% hydrogen peroxide, was added to dissolve the filter. If allowed to stand overnight at room temperature, the final digest was a clear, virtually colorless solution.

RESULTS

Recovery studies. Assessment of the methodology was keyed on oxytetracycline because Kohn (9) reported this to be the one tetracycline in the compound series whose barbital-calcium complex was incompletely extracted by his procedure. The principal studies were done at an initial incubation concentration of 200 μM, which is higher than the MIC for the susceptible strain but less than that for the resistant culture. The data from recovery experiments are shown in Table 3. Of the 20 individual recovery determinations, 18 were greater than 95%. These experiments also directly compared the ability of the susceptible and resistant cultures to take up oxytetracycline. The susceptible strain sorbed 36% of the total antibiotic available, whereas the resistant strain accumulated only 1%. The chemical profile of the oxytetracycline sorbed by the resistant strain was studied chromatographically. Only one significant fluorophor was found, the oxytetracycline. No evidence of cellular transformation of tetracyclines by resistant or susceptible strains was ever detected. Transformations to nonfluorescent moieties were ruled out since recoveries measured by fluorescence were essentially complete.

In similar studies in which the initial concentration of oxytetracycline was 20 μM, 18.7 ± 1.6 mμmoles (94%) of each ml was accounted for in the cell extract plus the filtrate.

The totality of the extraction of the filter-impinged cells by the barbital-calcium reagent was further investigated by use of tritium-labeled tetracycline and the resistant culture (Table 4). The amounts of radioactivity in cell extracts and cell digests were comparable, verifying that the reagent effectively extracts low levels of cell-associated tetracyclines.

Representative uptake determination. The raw fluorescence data from a typical 2-hr uptake

TABLE 3. *Recovery of oxytetracycline after uptake experiments*

Escherichia coli strain and condition	Oxytetracycline per ml of 2-hr incubation mixture[a]			
	Membrane filter[b] (mμmoles)	Filtrate (mμmoles)	Total[c] (mμmoles)	Recovery[c]
				%
Susceptible, resting	71.5	128.8	200.3 ± 5.15[d]	100.2
Resistant, resting	2.02	193.1	195.1 ± 4.71	97.6
Susceptible, nonviable[e]	2.09	185.9	188.0 ± 4.60	94.0
Resistant, nonviable	1.95	192.0	194.0 ± 2.89	97.0

[a] Protocol as described in Materials and Methods with 200 μM oxytetracycline in make-up. Each value is the average of five determinations.
[b] Antibiotic found after 1 ml of mixture was impinged.
[c] The average total was 195.0 ± 6.31 mμmoles; the average recovery was 97.5%.
[d] Standard deviation.
[e] Resting-cell population heated in a boiling-water bath for 15 min.

TABLE 4. *Efficacy of barbital-calcium reagent as an extractant*

Expt	Specific activity of tetracycline solution (DPM[a]/mμmole)	Uptake[b] by fluorescence (mμmoles/mg of cells)	DPM/mg of cells			DPM ratios	
			Expected by fluorescence	Found in extract	Found in cell digest	Extract / Digest	Extract / Expected
1	3.46×10^4	2.56	8.86×10^4	7.63×10^4	8.35×10^4	0.914	0.861
2	4.09×10^4	3.10	12.68×10^4	10.91×10^4	11.39×10^4	0.958	0.860
Avg	—	2.83	—	—	—	0.936	0.861

[a] DPM = disintegrations per minute.
[b] Protocol as described in Methods and Materials with 50 μM tetracycline in the make-up and with use of the resistant culture.

determination experiment are shown in Table 5. In the absence of cells, a small but readily measurable quantity of oxytetracycline became insoluble in the incubation mixture during the experimental time course. This material, impinged on the membrane filter, could not be removed by washing, but it was extracted by reagent. This was true of all tetracyclines studied. No other procedural problems were encountered. In agreement with Franklin and Godfrey (5), we found little loss of cell-associated tetracyclines during the washings. Therefore, tetracycline uptake was defined as the quantity of antibiotic retained by the washed cells and corrected for (i) the zero-time fluorescence of the complete incubation mix, and (ii) the quantity of nonsorbed antibiotic which became filter impingeable during the time course of uptake.

Mathematical relationship of uptake as a function of concentration. To partition the multiple effects of antibiotic concentration on net uptake, use was made of Arima and Izaki's (1) observation that 10^{-2} M sodium azide significantly hindered, but did not abolish, cellular accumulation of the tetracyclines. Over the entire concentration range evaluated, the portion of uptake not sensitive to azide inhibition could be expressed best by a Freundlich equation for an adsorption isotherm. While the Freundlich equation is an empirically derived relationship, it is excellent for use in compiling information and interpolation. In its logarithmic form it is written: log uptake = log α + β log concentration, where uptake has the units of millimicromoles per milligram of cells, and the concentration is the nonsorbed antibiotic in millimicromoles per milliliter. With this relationship established, the cellular accumulation of tetracyclines under standard conditions was recognized to be biphasic. From low to high antibiotic concentrations, the initial mode is indistinguishable from the uptake observed in the presence of azide, and it is expressed by the same isotherm equation. But, abruptly at a concentration characteristic for each tetracycline and each culture, there is the onset of a second mode of uptake which is azide-sensitive. This second phase is specified also by a Freundlich equation but by one with a much steeper slope (Fig. 2, 3).

Specification of cellular accumulation of different tetracyclines. The level of uptake and the particular concentration at which the azide-sensitive component of the process is initiated was established for a series of tetracyclines by use of both the susceptible and resistant strains. The individual isotherm equations were determined by ascertaining the uptakes in the presence and absence of 10^{-2} M azide over the one-log concentration range just after the respective minimal inhibitory concentrations. The individual intercepts were then calculated (Table 6, Fig. 4, 5).

Tetracycline adsorption by nonviable cells. Early studies suggested that the portion of tetracycline uptake which is not abolished by azide was synonymous with the uptake characteristic of heat-killed cells. To test this hypothesis, experiments were conducted with both the resistant and susceptible strains; oxytetracycline levels were examined at pre- and postintercept concentrations (Table 7). Clearly, the cellular uptake of oxytetracycline in the presence of azide is equivalent to the quantity of antibiotic adsorbed by nonviable cells.

TABLE 5. *Raw fluorescence data from a 50 μM oxytetracycline study with susceptible Escherichia coli*

Antibiotic used	Fluorescence units per ml of extract[a]			
	No cells		Susceptible *E. coli*	
	0 time	2 hr	0 time	2 hr
None	23	24	40	37
50 μM oxytetracycline	53	152	95	9,742

[a] Corrected only for the background fluorescence of the barbital-calcium reagent (25 fluorescence units).

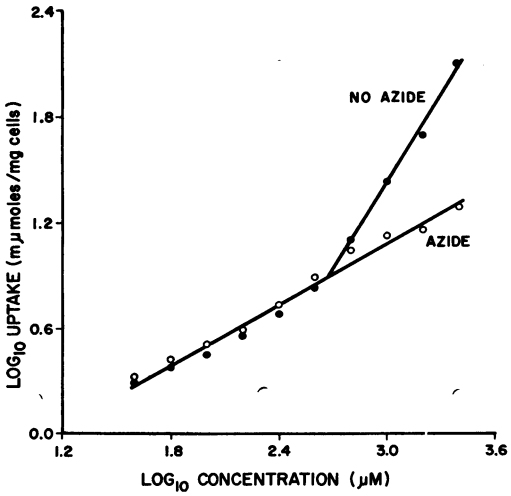

FIG. 2. *Tetracycline uptake by the resistant Escherichia coli as a function of concentration. Each point is the average of three determinations. Symbols:* ○ = *results from incubations supplemented with* 10^{-2} M *azide;* ● = *results from standard conditions.*

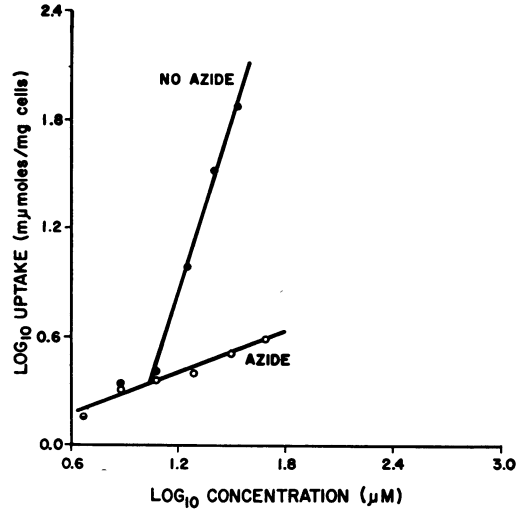

FIG. 3. *Oxytetracycline uptake by the sensitive Escherichia coli as a function of concentration. Each point is the average of two determinations. Symbols:* ○ = *results from incubations supplemented with* 10^{-2} M *azide;* ● = *results from standard conditions.*

DISCUSSION

Progress toward understanding the physiological basis for the development of bacterial resistance to the tetracyclines has been limited by (i) the lack of a simple assay procedure which is accurate and precise at the minimal bacteriostatic levels of these antibiotics, and (ii) by the absence of a specific biological model which may be subjected to experiment. This work was addressed to both of these problems.

The adaptation of Kohn's fluorometric procedure (9) proved a solution to the first problem. The method was convenient and applicable to all tetracyclines studied. The measurements were sufficiently sensitive to allow determination of cellular uptakes when the initial antibiotic concentrations were less than the minimal inhibitory levels. The recovery experiments and the isotope study of sampling effectiveness attest to the accuracy of the method.

The recovery studies and the chromatograms of the cell extracts showed that no significant quantity of tetracycline was degraded or trans-

TABLE 6. *Compilation of sorption data for various tetracyclines*

Escherichia coli strain	Tetracycline	Intercept of isotherms		Total uptake		Azide-insensitive uptake	
		Log_{10} uptake (mμmoles/mg)	Log_{10} concn (μM)	Isotherm slope	r^a	Isotherm slope	r^a
Resistant	5-Oxytetracycline	0.93	2.93	2.68	1.00	0.435	0.959
	6-Deoxy-5-oxytetracycline	0.84	2.11	2.52	0.990	1.27	0.982
	Tetracycline	0.90	2.67	1.58	0.986	0.558	0.993
	6-Demethyltetracycline	1.56	2.38	2.23	0.991	0.524	0.981
	6-Deoxytetracycline	0.68	1.41	1.35	0.990	0.439	0.984
	6-Demethyl-6-deoxytetracycline	0.22	0.63	1.43	0.983	1.08	0.997
Susceptible	5-Oxytetracycline	0.33	1.05	3.30	0.998	0.389	0.985
	6-Deoxy-5-oxytetracycline	0.72	1.54	3.48	0.996	0.418	0.971
	Tetracycline	0.25	1.00	0.867	0.997	0.212	0.953
	6-Demethyltetracycline	0.38	0.87	0.827	0.987	0.127	0.985
	6-Deoxytetracycline	0.82	0.99	2.53	0.975	0.345	0.978
	6-Demethyl-6-deoxytetracycline	0.84	0.98	1.76	0.995	0.179	0.979

[a] *r* is the correlation coefficient for the "best" line calculated by the minimizing of the uptake components of deviation.

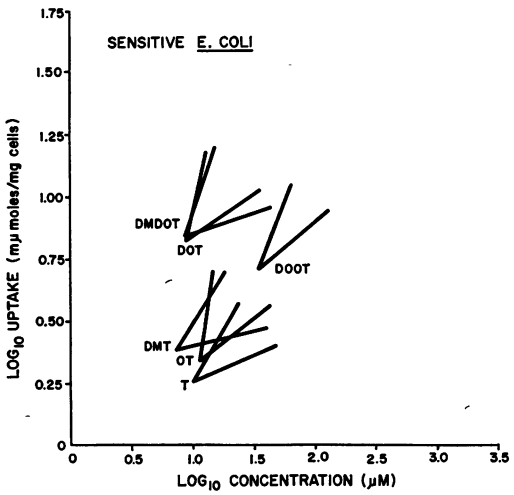

FIG. 4. *Sorption isotherms of the sensitive Escherichia coli for the different tetracyclines (T, tetracycline; OT, 5-oxytetracycline; DMT, 6-demethyltetracycline; DOT, 6-deoxytetracycline; DOOT, 6-deoxy-5-oxytetracycline; DMDOT, 6-demethyl-6-deoxytetracycline). The lines with the steeper slope are plots of the total uptake. The azide-insensitive portions of the uptake are shown by the lines with the shallower slopes.*

formed during the course of the experiments with either culture. These data are evidence that the resistant strain does not achieve its particular physiological state through metabolic modification of the tetracycline during its transport and accumulation processes. This knowledge, plus the crypticity phenomenon cited earlier, and Franklin and Godfrey's (5) observation that resistance is not attributable to super excretion of the antibiotic by resistant strains, completes a body of indirect evidence that tetracycline resistance results from decreased penetration of the drug to its inhibition sites.

The comparative data presented document, in detail, the several general reports which showed that, at concentrations which allow *E. coli* strains to be classified as resistant or susceptible to a particular tetracycline, the resistant culture accumulates much less of the antibiotic than does the susceptible one. However, the accumulation of a tetracycline as a function of concentration was found to follow an identical pattern whether the culture was resistant or susceptible. Only the placement of the pattern in two-dimensional space was highly specific for each culture and each compound. The discovery that the uptake data for a given tetracycline and culture could be expressed by two distinctive Freundlich equations allowed ready compilation and interpretation. The dependence of uptake on concentration is biphasic, and it changes from the first mode to the second very abruptly. The first phase is not dependent on functional cell machinery but the second is. The second mode is the principal mechanism of uptake at relatively high antibiotic concentrations and is undoubtedly the energy-dependent process described by Arima and Izaki (1).

The data which compare the two cultures have led to the conclusion that tetracycline-resistance development actually consists of the shift of the

bimodal uptake pattern into a region of higher concentration. Conceptually, a strain that becomes resistant simply requires higher external concentrations of tetracycline before the second mode of uptake is triggered to become functional. Although resistance to the tetracycline is clearly a transport anomaly, the genome-mediated modification of the cell is not the simple loss or reduction of the active transport mechanism.

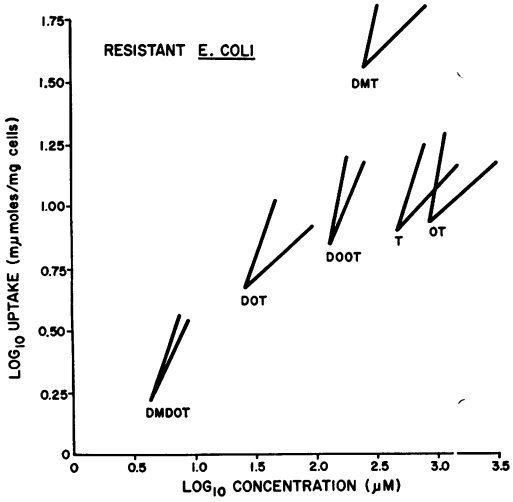

FIG. 5. *Sorption isotherms of the resistant Escherichia coli for the different tetracyclines (see legend to Fig. 4). The lines with the steeper slope are plots of the total uptake. The azide-insensitive portions of the uptake are shown by the lines with the shallower slopes.*

TABLE 7. *Influence of cell condition on the azide-insensitive oxytetracycline (OT) uptake*

Culture	Log_{10} initial OT concn (μM)	OT uptake[a, b] (mμmoles per mg of cells)			
		Resting cells		Nonviable cells[c]	
		No azide	10^{-2} M azide	No azide	10^{-2} M azide
Susceptible	0.60	1.29	1.28	1.34	1.27
	1.40	10.3	4.01	4.58	4.11
Resistant	2.20	5.35	5.15	4.85	4.75
	3.40	875	35.4	35.8	35.7

[a] The geometric means for the four conditions were 15.8 (resting, no azide), 5.53 (resting, 10^{-2} M azide), 5.73 (nonviable, no azide), and 5.46 (nonviable, 10^{-2} M azide).

[b] Standard procedure, single flask per determination. The calculated log_{10} intercept concentration for the isotherms for the susceptible culture was 1.05; for the resistant culture it was 2.93.

[c] Resting-cell population heated 15 min in a boiling-water bath.

Another and more obvious conclusion stems from the relationship observed between the concentration at which the uptake mode changes and the organism's susceptibility to the antibiotic. There is essentially a one-for-one correlation between the minimal inhibitory concentration and the concentration at which energy-dependent transport becomes functional. From a sample size of 12 (2 cultures $\times$ 6 tetracyclines), the correlation coefficient was 0.934 between the logarithms of these two concentrations. The correlation is remarkably good when one considers the dissimilarities of the two physical environments used for the respective determinations.

Based on the foregoing, and on the assumption that the double phasing of uptake is a manifestation of cell compartmentation, a model is advanced which suggests the sequence of events which occur in transport of tetracyclines and the consequences of genetic change toward resistance.

The first interaction between a tetracycline and *E. coli* is proposed to be a sorption process by active centers located in cell wall material and to be based on physical forces only. For a given strain and compound, the number and affinity of the active centers specifies the concentration effect. A particular level of tetracycline saturation or penetration of the cell by this first uptake mode must be achieved before molecules are spatially available for participation in the second mode. This second process, when it occurs, brings antibiotic molecules inside the permeability membrane by an energy-dependent push or pull transport mechanism and makes them available for binding at the sites inhibiting protein biosynthesis. These latter are the bacteriostatically effective tetracycline molecules. Those bound to cell wall centers are essentially irrelevant.

The model, as far as it has gone, is consistent with the facts. Available data support the concept that the first process is simply physical adsorption by a bulk constituent of the cell, specifically the cell wall. Franklin and Godfrey (5) showed that, at minimal bacteriostatic levels, the majority of the sorbed chlortetracycline was in the fraction consisting mainly of cell wall material. In my work, the process was shown to be azide-insensitive. It was also observed that uptake by this mode was identical whether the cells were viable or heat-killed. All the evidence supports the contention that no metabolic machinery is required for this first sorption process to occur, and that the adsorbent is exterior to the cytoplasmic membrane.

The proposition that not all tetracycline uptake

is relevant to bacteriostatic action is supported by studies which investigate the mechanism of action of these antibiotics. The tetracyclines most probably interfere with the transfer of amino acids from the aminoacyl-transfer ribonucleic acids to polypeptides on the ribosomes (4). L. E. Day (*unpublished data*) has shown that 25 μg per mg of ribosomal protein of the different tetracyclines used in these experiments was roughly equivalent in effectiveness. All inhibited 40 to 70% of polyuridylic acid-directed polyphenylalanine formation in a cell-free *E. coli* system. While the quantitative meaning of this effect is difficult to extrapolate to the intact cell, it is reasonable to assume that, within the cell, all of the tetracyclines under examination do not differ by more than twofold in their efficacy as inhibitors of protein biosynthesis. Yet, the uptake levels for the different tetracyclines at the MIC values vary sevenfold, which indicates that not all sorbed antibiotic is functional in the inhibitory process.

Since the tetracyclines exert their effect at the ribosomal level of cell organization, a second process must become operative to enable these antibiotics to penetrate beyond the cell wall. The observed second mode of uptake is energy-dependent. This satisfies at least one of the requirements for an active transport of tetracyclines across the cytoplasmic membrane.

The suggestion that the second mode represents the accumulation of bacteriostatically effective molecules is compatible with the observed effect of the tetracyclines on bacterial growth. The concentration correlation has already been mentioned. The onset of that portion of antibiotic accumulation which is azide-sensitive is very abrupt. This particular accumulation also rises extremely rapidly with small increases in external concentration (Fig. 6). The abrupt and rapid increase of this class of molecules as a function of concentration has a direct counterpart in the sharp and sudden diminution in growth response which was observed during the titration of minimal inhibitory concentrations.

The model also permits a mechanistic interpretation of the multistep, cumulative resistance pattern found for tetracyclines (*see* 14). Resistance development is viewed as a series of changes in cell wall chemistry which increase the quantity or alter the affinity, or both, of those sites which adsorb the tetracycline. These modifications exterior to the cytoplasmic membrane lead to requirements for higher extracellular antibiotic concentrations in order to initiate the second uptake process.

Additional genetic studies coupled with uptake determinations are warranted. In our uptake experiments with resting cells, resistant cells

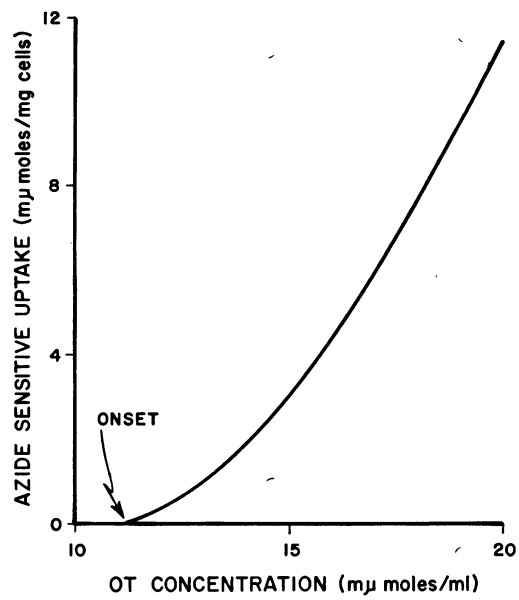

FIG. 6. *Azide-sensitive portion of oxytetracycline uptake by the susceptible Escherichia coli. The curve was determined from the difference between the isotherms for the total and the azide-insensitive uptakes detailed in Table 6.*

accumulated less antibiotic than susceptible ones even though they were grown in a medium with no tetracycline supplementation. This contrasts with the report of Izaki, Kiuchi, and Arima (8), who showed that, for a strain whose resistance factor was demonstrably extrachromosomal, the decreased uptake of tetracycline is dependent on the growth of the organism in the presence of the drug. The genetic factor in the resistant isolate used in my work could not be transferred to *E. coli* K-12 by cell-to-cell contact (A. R. English, *unpublished data*).

Obvious kinetic and cell fractionation data are necessary to prove and modify the model.

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