Uptake of \(^3\)H-Tetracycline by Resistant and Sensitive \textit{Escherichia coli}

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The uptakes of \(^3\)H-tetracycline by 12 tetracycline-sensitive and 24 tetracycline-resistant \textit{Escherichia coli} hospital cultures were found to be 270 and 75 nmoles of tetracycline per milliliter of cell water per 20 min, respectively. This confirms reports by other investigators who, by using only one or two cultures, suggested a relationship between tetracycline uptake and tetracycline resistance. However, minimum inhibitory concentrations of tetracycline for the cultures bore no relation to the tetracycline uptake values, suggesting that loss of tetracycline uptake may not be the primary cause of resistance. In addition there were three resistant cultures with uptake values greater than 140 and two sensitive cultures with uptakes lower than 180, raising the question of how these tetracycline-resistant cultures could grow with tetracycline at concentrations nearly as high as those found to inhibit growth of sensitive organisms. Of the tetracycline-resistant cultures, 15 were able to transfer tetracycline resistance to a recipient organism and 9 were not. Two of the cultures transferred TC-resistance to a recipient with no modification-restriction system (\textit{E. coli} C) but did not transfer resistance to a recipient with a known modification-restriction system (\textit{E. coli} K-12).

The uptake of oxytetracycline (and presumably all the related tetracyclines) by \textit{Escherichia coli} was first shown by Arima and Izaki (2) to have some of the characteristics of an active transport process. At high oxytetracycline concentration the cells accumulated large quantities of the drug; accumulation was dependent on glucose added to the medium and was inhibited by dinitrophenol or azide. They (7) then found that a strain of \textit{E. coli} K-12 which exhibited multiple drug-resistance accumulated much less oxytetracycline than a sensitive strain. De Zeeuw (4) found that although tetracycline (TC) uptake by \textit{E. coli} at high TC concentrations was energy dependent, uptake at low TC concentrations was not. Using a strain of \textit{E. coli} isolated from poultry, but with which TC-resistance transfer could not be demonstrated, De Zeeuw (4) found that the minimum concentration necessary to initiate energy-dependent uptake was higher for the resistant than for a sensitive strain. Franklin (6) has confirmed and extended the observation of Izaki, Kiuchi, and Arima (8) that uptake of TC by an R factor bearing TC-resistant \textit{E. coli} was decreased by incubation of the cells with TC prior to the uptake measurement. Franklin used two resistant strains, one bearing an R factor and the other obtained by serial passage through increasing concentration of chlorotetracycline.

Whereas the above observations suggest that TC resistance is related to TC uptake, information is unclear as to whether or not some of the cultures studied possessed episomal or chromosomal factors which might control resistance. The objectives of this investigation were to establish whether or not there is a causal relationship between loss of uptake and TC resistance with a number of clinical isolates and, in addition, to estimate the extent of R factor mediation in the resistant strains.

MATERIALS AND METHODS

Cultures and chemicals \textit{E. coli} (ATCC 14948) was obtained from the American Type Culture Collection. \textit{E. coli} 14948NA-1 was obtained by serial passage of \textit{E. coli} 14948 in nalidixic acid (NA) to a level of 1,000 \(\mu\)g/ml. \textit{E. coli} W53 was provided by K. Paigen, Roswell Park Memorial Institute, Buffalo, N.Y. \textit{E. coli} W53NA-1 was a mutant of \textit{E. coli} W53 resistant to NA at 100 \(\mu\)g/ml prepared by isolation of single colonies from Penassay Agar plates containing NA at increasing concentrations. \textit{E. coli} CSH-2(222) was obtained from S. Falkow, Georgetown University School of Medicine, Washington, D.C., and carried the R factor 222 originally isolated by T. Watanabe who reported that R factor 222 conferred resistance to TC, streptomycin, chloramphenicol (C), and the
sulfonamides. Clinical cultures of either fecal or urinary origin were provided by N. O'Connell, Sisters of Charity Hospital, Buffalo, N.Y., and K. Wichier, E. J. Meyer Memorial Hospital, Buffalo, N.Y. All cultures included in this study were verified as E. coli by standard fermentation tests or by the 20 tests for identification of Enterobacteriaceae marketed by Analytab Products, Inc., New York. They were maintained on Penassay Agar (Difco antibiotic medium 2) and transferred before use to Penassay Broth (Difco antibiotic medium 3). 3H-tetracycline was obtained from New England Nuclear Corp., Boston, Mass., and TC was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, as the hydrochloride and weighed as such. We obtained 1, 4-bis-2-[(4-methyl-5-phenylazoxyyl)-benzene (POPOP) and 2, 5-diphenylxazole (PPO) from Packard Instrument Co., Inc., Downers Grove, III. Nalidixic acid was a gift from F. Nachod, Sterling-Winthrop Research Institute, Rensselaer, N.Y.

Uptake measurements. Cells grown overnight in shake culture were transferred and grown to mid-log phase (3 to 4 hr) and washed with C medium in grams per liter: NH4Cl, 2; Na2HPO4, 6; KH2PO4, 3; NaCl, 3; MgCl2, 0.010; Na2SO4, 0.026; and glucose, 10 (see ref. 9). The uptake flask consists of H-TC (10 μCi/ml; 0.25 μmole/ml), washed cells, and C medium adjusted to an absorbance (600 μm) of about 0.500 in a final volume of 10 ml. The uptake was run at 37 C with shaking for 20 min. Duplicate 2-ml samples of the cell suspension were filtered with Millipore filters (0.45 μm), and the filter pads containing the cells were counted directly with 10 ml of scintillation fluid [toluene-ethanol (2:1) containing POPOP (50 mg/liter) and PPO (4 g/liter)] in a liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Counts of the uptake medium were used to calculate specific activity, and pads were counted as above after filtration of medium containing H-TC but no cells. By using the pad counts to correct for H-TC addition to the pads and calculating cell water from A600 readings (ref. 9, page 5), the uptake of TC was calculated in terms of nmols per ml of bacterial cell water per 20 min. Absorbance measurements were made with 0.5-inch tubes in a Spectronic 20 (Bausch & Lomb, Inc., Rochester, N.Y.). A few of the hospital cultures were found to filter very slowly, not reaching dryness in less than 5 min, and these were excluded from the study.

Resistance transfer. Transfer of resistance was carried out essentially as described by Shaw (10). The various hospital cultures and 14948(222)-1 were the donor strains, and 14948NA-1 and W35NA-1 were the recipients. The mixture of donor and recipient was plated on agar containing per milliliter: 4 μg of TC and 500 μg of NA for 14948NA-1 and 8 μg of TC and 50 μg of NA for W35NA-1. Resistance was considered to be transferred if any colonies appeared on the TC plus NA-containing plates. The resultant colonies were then cultured in broth at the same concentrations of TC and NA as used in the plates. Prior to use in the sensitivity test, the resultant cultures were grown overnight in drug-free medium and then again to mid-log phase in drug-free medium (see above).

Transfer of resistance was carried out twice for each strain with 14948NA-1 as the recipient. Those strains which failed to transfer resistance to 14948NA-1 were then tested twice with W35NA-1 as the recipient.

Antibiotic sensitivities were determined by using discs (Baltimore Biological Laboratory, Div. of B-D Laboratories, Inc., Balt., Md.) on Penassay Agar. Minimum inhibitory concentrations (MIC) were determined by 16-hr growth of cells in tubes containing various concentrations of TC. The concentrations used for sensitive strains were 0.5, 1.0, 2.0, and 4.0 μg/ml and for resistant strains were 25, 50, 100, and 200 μg/ml.

RESULTS AND DISCUSSION

The results of uptake of H-TC by a TC-sensitive (14948) and by an R factor-bearing, TC-resistant (14948(222)-1) E. coli are shown in Fig. 1. Uptake by the TC-sensitive E. coli was found to reach equilibrium at about 10 min when the TC concentration was 12.5 μM but did not reach equilibrium even at 20 min at high TC concentrations (200 μM). In other experiments, not shown in Fig. 1, it was found that the uptake at high concentration continued for 30 to 60 min and was markedly reduced by 10^-2 M azide. The uptake at low concentration was proportionately less affected by azide at 10^-2 M. The uptake at 20 min at 12.5 μM and 200 μM can be seen to achieve cell per medium ratios of 27 and 51, respectively. These results, which suggest an active uptake system for TC, are consistent with the findings of De Zeeuw (4). In contrast, the TC-resistant E. coli exhibited much less ability to take up TC at both high and low concentrations; the percent of reduction in uptake was more pronounced at low than at high concentration. For this reason and because the low concentration corresponded more closely to an achievable therapeutic blood level, the low concentration (12.5 μM) was chosen for the remainder of the uptake experiments in which it was desired to contrast uptake by sensitive and resistant cells.

Thirty-six bacterial isolates were collected from two Buffalo, New York, hospitals, of which 12 were sensitive to TC (MIC < 1.0 μg/ml) and 24 were resistant to TC (MIC > 25 μg/ml). There was no selection of isolates except to limit them to those E. coli with which H-TC uptake could be measured, and thus the organisms fell into two well-defined groups, sensitive and resistant. In addition, the resistant organisms could be subdivided into two groups, those which could be shown to transfer TC-resistance to another organism (E. coli 14948NA-1) and those which could not.

Uptake of H-TC and the MIC of TC for these three groups of organisms are listed in Tables 1 through 3. The mean uptake for the sensitive
UPTAKE OF *H-TETRACYCLINE BY E. COLI

TABLE 1. Uptake of *H-tetracycline (TC) and minimum inhibitory concentration (MIC) of TC in TC-sensitive Escherichia coli

| E. coli strain | *H-TC uptake* (nmoles/ml) | MIC (μg/ml) |
|----------------|-----------------------------|-------------|
| 730            | 371                         | 0.5         |
| 924            | 373                         | 0.5         |
| 1267           | 324                         | 0.5         |
| 1343           | 290                         | 1.0         |
| 1365B          | 281                         | 1.0         |
| 1366           | 164                         | 0.5         |
| 1396           | 346                         | 0.5         |
| 1541           | 178                         | 0.5         |
| 1542           | 256                         | 0.5         |
| 1556           | 224                         | 0.5         |
| 1566           | 228                         | 0.5         |
| 1661           | 202                         | 1.0         |

* Mean ± standard error of the mean for *H-TC uptake was 270 ± 21 nmoles/ml.

TABLE 2. Uptake of *H-tetracycline (TC) and minimum inhibitory concentration (MIC) of TC in Escherichia coli

| E. coli strain | *H-TC uptake* (nmoles/ml) | MIC (μg/ml) |
|----------------|-----------------------------|-------------|
| 590            | 60                          | >200        |
| 1179           | 44                          | 100         |
| 1291           | 2                           | 200         |
| 1430           | 0                           | 100         |
| 1459           | 98                          | >200        |
| 1821           | 7                           | >200        |
| 1860           | 106                         | >200        |
| 1888           | 76                          | >200        |
| 3438           | 16                          | >200        |
| 3510           | 146                         | 100         |
| 3516           | 117                         | >200        |
| 3536           | 64                          | >200        |
| 3654           | 56                          | >200        |
| 4643           | 0                           | >200        |
| 4928           | 150                         | >200        |

* Strains which transfer TC resistance.

organisms (Table 1) was 270 ± 21 nmoles/ml ranging from 164 to 373 nmoles/ml. Since the uptakes were performed at a concentration of 12.5 nmoles/ml, this represented a range of concentration gradients of 13 to 30 between cell water and medium. The uptake by *E. coli* 14948 was 337 nmoles/ml. This confirmed previous results from other laboratories (2) which indicated that uptake of TC by *E. coli* was a concentrative process.

The TC uptakes of TC-resistant *E. coli* in which transfer of TC-resistance could and could not be
dium concentration of 12.5 nmoles/ml (6.0 μg/ml) would require 200 μg/ml for growth inhibition. Further question is raised by the fact that there was a resistant strain (E. coli 4928: MIC, >200) with uptake of 150 nmoles/ml while a sensitive strain (E. coli 1366: MIC, 0.5) had an uptake of 164 nmoles/ml. Thus, two strains with nearly identical levels of TC uptake exhibited far different MIC values.

The negative finding that TC resistance could not be transferred from 11 of the resistant strains to E. coli 14948NA-1 did not definitely exclude the possibility of R factor mediation of resistance. One possibility was that the donor organisms listed in Table 3 had competent resistance determinants but defective transfer factors (13). However, an alternative explanation could be based on the fact that E. coli 14948 was a K-12 strain with a known deoxyribonucleic acid restriction-modification system. To investigate the possibility that transfer of R factor was taking place, but restriction-modification was preventing ex-
pression of resistance, a culture of *E. coli* C (W53) was obtained which had no known restriction-modification system. An NA mutant of W53 was obtained and used as the recipient for those strains which did not exhibit transfer to *E. coli* 14940NA-1. Two strains (1860 and 1888) did transfer TC-resistance to W53NA-1, suggesting that the restriction-modification system (1) may have inhibited the expression of resistance by R factors when the R factors were transferred to 14940NA-1. This points to the necessity for careful selection of recipient strain for transfer experiments and suggests a mechanism for loss of resistance to antibiotics other than TC during transfer of TC-resistance.

Table 4 shows the sensitivities of those cultures which were able to transfer their TC-resistance. The greatest changes in sensitivity pattern occurred with streptomycin and ampicillin where five of seven resistant cultures and four of four resistant cultures did not transfer resistance to the recipient.

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