# Energetics of tetracycline efflux system encoded by Tn10 in *Escherichia coli*

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Tritiated tetracycline was actively accumulated in inverted membrane vesicles prepared from *Escherichia coli* W3104$^{rf}$, which has a transposon, Tn10, on the plasmid, R388, by means of a protonmotive force when NADH was added as an energy source. The tetracycline accumulation was reduced to about one-half the full value on the addition of a cation/proton-exchange ionophore, nigericin. In contrast, remarkable stimulation of the tetracycline accumulation was observed with a $K^+$-specific ionophore, valinomycin. The accumulation of [$^3$H]tetracycline could also be driven by an artificially imposed interior-acidic pH gradient ($\Delta$pH), but not, however, by an artificially imposed interior-positive membrane potential ($\Delta\psi$). These results strongly indicate that the plasmid-encoded tetracycline transport was mainly due to an electrically neutral proton/tetracycline antiport system.

*Tetracycline resistance* *Tetracycline transport* *Inverted membrane vesicle* (Escherichia coli)

## 1. INTRODUCTION

The tetracyclines are broad spectrum antibiotics. Their bacteriostatic action is due to their inhibition of protein synthesis on ribosomes. Bacterial resistance to tetracyclines is widespread, especially in the Enterobacteriaceae, and is commonly specified by plasmids (reviews [1,2]). The resistance is generally induced by subinhibitory amounts of tetracyclines [3]. The tetracycline resistance determinants are classified into 4 classes on the basis of their DNA-DNA hybridization as well as their levels of resistance to tetracyclines and their hydrophobic analogs [4]. Among them, class B or Tn10-type determinants, which show the highest level of resistance to tetracyclines, occur most frequently [5]. The 2 kinds of proteins associated with the resistance, i.e. the specific inner membrane protein (Tet protein) with an apparent molecular mass of 36 kDa [6] and the repressor protein of 23 kDa [7], are encoded on Tn10. The nucleotide sequence [8,9], organization [7] and regulation of expression [10] of the genes involved have been reported. The other plasmid-mediated determinants of tetracycline resistance were also shown to produce inner membrane proteins similar to the Tet protein [11,12].

The resistance is related to a decrease in tetracycline accumulation [13,14]. Energy-dependent efflux of tetracycline was proposed by Levy and McMurry [15] on the basis of an energy-dependent decrease in accumulation of the drug in resistant cells and the energy-dependent uptake of tetracycline by inverted membrane vesicles prepared from resistant cells [16]. This active efflux was assumed to be a function of the Tet protein, however, direct evidence of this has not yet been reported.

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*Abbreviations:* ANS, 8-anilino-1-naphthalenesulfonate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $\Delta$pH and $\Delta\psi$, pH gradient and electrical potential, respectively



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Here, we report the nature of the driving force of the tetracycline efflux system of Tn10-bearing tetracycline resistant *E. coli* using inverted membrane vesicles.

## 2. MATERIALS AND METHODS

### 2.1. *Bacterial strains and growth conditions*

The *E. coli* K-12 derivative strains, W3104$^{rif}$ (galT$_{12}$, $\lambda^-$, rifampicin resistance) and W3104$^{rif}$/R388::Tn10, were used in this study as sensitive and resistant strains to tetracycline, respectively. The Tn10-bearing plasmid, R388::Tn10, encodes the resistance to trimetoprim and sulfonamide in addition to that to tetracycline.

These strains were cultured in medium A [16] supplemented with 0.2% glucose and 0.1% casamino acid with vigorous shaking at 37°C. In the case of the resistant cells, the medium was further supplemented with 4 $\mu$g tetracycline per ml for induction of the tetracycline resistance.

### 2.2. *Preparation of inverted membrane vesicles*

Inverted membrane vesicles were prepared from cells lysed in a French pressure cell according to McMurry et al. [16]. The vesicles were then frozen in dry ice/ethanol and stored at −80°C.

### 2.3. *Tetracycline uptake by the vesicles*

Tetracycline uptake was assayed as described by McMurry et al. [16] with some modifications. Vesicles were thawed and diluted to 7 mg protein per ml in 50 mM $KPO_4$/10 mM $MgSO_4$, pH 7.5, at 4°C unless otherwise stated, and then the suspension was put into glass tubes followed by preincubation at 30°C. To 45 $\mu$l of the same buffer in a small glass tube, [$^3$H]tetracycline was added to a final concentration of 2 $\mu$M, with or without $\beta$-NADH (at a final concentration of 2.5 mM), and with or without an ionophore, as described in the text. The ionophores were used at the following concentrations: CCCP, 10 $\mu$M; valinomycin, 2 $\mu$g/ml; nigericin, 2 $\mu$g/ml. After 20 min preincubation at 30°C, 5 $\mu$l vesicles was added to each mixture followed by agitation by means of hand shaking. At various times, samples were mixed with 2 ml of 0.1 M LiCl/0.1 M $KPO_4$, pH 7.5, unless otherwise stated, followed by filtering through Millipore HAWP 0.45 $\mu$m pore membrane filters and washing twice with 2 ml of the same buffer. Radioactivity remaining on the filters was measured with a liquid scintillation counter.

### 2.4. *Other procedures*

ANS fluorescence was measured as described by Kobayashi et al. [17] with a fluorescence spectrophotometer (Hitachi MPF4) with excitation at 370 nm and emission at 480 nm. Protein was determined by the method of Lowry et al. [18] with bovine serum albumin as a standard.

### 2.5. *Reagents*

[$^3$H]Tetracycline was purchased from New England Nuclear. Valinomycin, nigericin and CCCP were obtained from Sigma. ANS was purchased from Wako, Japan. $\beta$-NADH was purchased from Oriental Yeast, Japan.

## 3. RESULTS AND DISCUSSION

The inverted membrane vesicles prepared from the Tn10-bearing tetracycline resistant cells showed NADH-dependent uptake of [$^3$H]tetracycline (fig.1A), while the vesicles prepared from isogenic sensitive cells did not show such tetracycline uptake (fig.1B). This indicates that the NADH-dependent tetracycline uptake system was encoded by the resistance determinant as reported

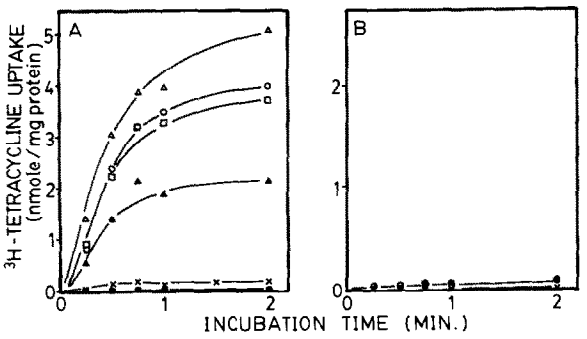


Fig.1. NADH-dependent accumulation of [$^3$H]-tetracycline in inverted membrane vesicles prepared from resistant (A) and sensitive (B) strains. The uptake of [$^3$H]tetracycline was assayed in the presence (○) and absence (●) of NADH, and in the presence of NADH and various ionophores (valinomycin, △; nigericin, ▲; CCCP, ×) or ethanol (□) as described in section 2. 0.5 $\mu$l of ethanolic solutions of the ionophores and ethanol were added.

by McMurry et al. [16]. They showed that the tetracycline uptake by inverted membrane vesicles prepared from resistant cells was inhibited by the addition of 2,4-dinitrophenol, and thus they concluded that the resistant cells should possess an energy-dependent efflux system for tetracycline. We also observed that NADH-driven uptake of tetracycline was completely inhibited by the addition of a proton conductor, CCCP. This clearly indicates that this uptake system was driven by a protonmotive force. This observation was consistent with the results for intact cells reported by Curiale et al. [19], who reported that the level of accumulation of tetracycline in Tn10-bearing cells was remarkably stimulated by the addition of CCCP.

To examine the nature of the driving force of this uptake system, we used 2 ionophores, valinomycin and nigericin. Under the experimental conditions in which the membrane vesicles were energized by the NADH supplied in the potassium phosphate buffer, it was expected that valinomycin should dissipate the membrane potential ($\Delta\psi$) by mediating electrogenic potassium influx, and nigericin should dissipate the chemical gradient of protons ($\Delta$pH) through electrically neutral exchange of $K^+$ and $H^+$. The effects of these ionophores on $\Delta\psi$ and $\Delta$pH were examined on the basis of the changes in fluorescence of ANS (for $\Delta\psi$) and quinacrine (for $\Delta$pH). The use of such indirect assays for $\Delta\psi$ and $\Delta$pH is widely accepted, although the quantitative relationships between $\Delta\psi$ or $\Delta$pH and the fluorescence changes of such indicators remain unclear. The fluorescence quenching of quinacrine by NADH indicated the formation of a respiration-dependent interior-acidic proton gradient. The quenching was completely prevented by nigericin. In contrast, the degree of quenching was drastically increased by valinomycin, probably due to stimulation of respiration-driven proton uptake (not shown). Fluorescence enhancement of ANS, used as a measure of the interior-positive membrane potential, was detected with dl-lactate as an energy source, because the fluorescence change of ANS could not be detected in a NADH solution because of overlapping of the wavelength of the fluorescence of ANS and that of NADH. dl-Lactate-driven fluorescence enhancement of ANS was completely prevented by valinomycin, however, enhancement of ANS fluorescence was still observed in the presence of nigericin (not shown).

The effects of valinomycin and nigericin on the energy-dependent accumulation of tetracycline in inverted membrane vesicles prepared from Tn10-bearing cells are shown in fig.1A. Valinomycin showed no inhibition of [$^3$H]-tetracycline accumulation. Instead, it rather accelerated the accumulation, indicating that the accumulation did not require a membrane potential. On the other hand, nigericin hindered the NADH-induced accumulation of [$^3$H]tetracycline, a level of only about one-half the full value being attained. Such behavior of the [$^3$H]tetracycline accumulation apparently paralleled the behavior of the pH gradient when nigericin or valinomycin was added. These results strongly indicated that the driving force of [$^3$H]tetracycline accumulation in the inverted membrane vesicles was the proton gradient across the membrane (interior acidic).

To investigate whether $\Delta$pH alone can drive the [$^3$H]tetracycline accumulation or not, we performed the following experiments using an artificially imposed $\Delta$pH and $\Delta\psi$. As shown in fig.2,

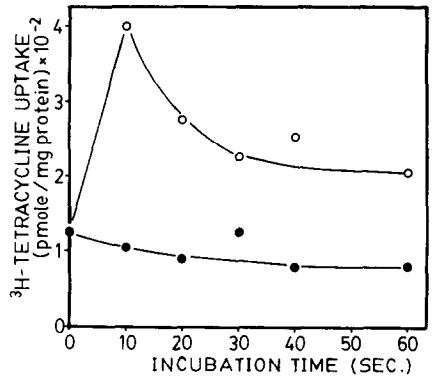


Fig.2. Accumulation of [$^3$H]tetracycline in inverted membrane vesicles driven by an artificial $\Delta$pH (interior acidic). Vesicles were prepared in 50 mM $KPO_4$ buffer from resistant cells and then diluted 10-fold in 50 mM Tris-HCl/10 mM $MgSO_4$ (pH 7.5) containing [$^3$H]-tetracycline (2 $\mu$M), followed by incubation at 30°C for 2 min before nigericin was added. Starting from the addition of nigericin, the suspension was incubated at 30°C for the indicated periods, and then [$^3$H]tetracycline transport was stopped by dilution of the suspension with 2 ml of 0.1 M LiCl/0.1 M Tris-HCl buffer (pH 7.5) (○). Accumulated [$^3$H]tetracycline was assayed as described in section 2. As a control, the assay was performed in the absence of nigericin (●).

when nigericin was added to a potassium-preloaded vesicle suspension, a rapid burst of [$^3$H]-tetracycline accumulation was observed that corresponded to the rapid generation of an artificial interior-acidic proton gradient ($\Delta$pH) resulting from an electrically neutral $K^+/H^+$ exchange mediated by nigericin.

On the other hand, the valinomycin-induced interior-positive membrane potential, which was generated by valinomycin-mediated $K^+$ influx into Tris-preloaded vesicles, could not drive the [$^3$H]tetracycline transport (fig.3A), although valinomycin-induced generation of an artificial membrane potential was certainly detectable with the fluorescence enhancement of ANS (fig.3B).

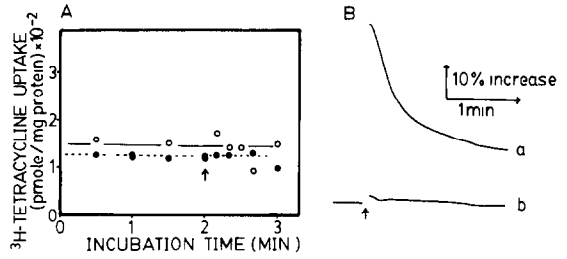


Fig.3. Effect of an artificially imposed membrane potential on [$^3$H]tetracycline transport in inverted membrane vesicles. Inverted membrane vesicles were prepared from resistant cells in 50 mM $KPO_4$ buffer (pH 6.6) and then diluted with excess 50 mM Tris-HCl buffer (pH 7.5), followed by centrifugation at 150000 × *g* for 1 h at 4°C. Then the pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.5) to a concentration of 7 mg protein per ml, followed by incubation for 2 h at 4°C. (A) [$^3$H]Tetracycline accumulation. At zero time, vesicles prepared as above were diluted 10-fold in 50 mM $KPO_4$/10 mM $MgSO_4$ buffer (pH 7.5) containing [$^3$H]tetracycline (2 $\mu$M) at 30°C. After incubation for 2 min, valinomycin was added to the mixture (indicated by the arrow), followed by incubation for the indicated periods (○). The accumulation of [$^3$H]tetracycline was measured at different times as described in section 2. As a control, valinomycin was omitted (●). (B) Generation of an artificial membrane potential. Vesicles were diluted 200-fold in 50 mM $KPO_4$/10 mM $MgSO_4$ buffer (pH 7.5) in a cuvette, followed by the addition of ANS to a final concentration of 2 $\mu$g/ml. At the time indicated by the arrow, valinomycin was added to the suspension, and then the change in fluorescence intensity of ANS was followed in the absence (a) and presence (b) of CCCP as described in section 2.

Therefore, it is obvious that the plasmid-encoded [$^3$H]tetracycline transport could be driven by an artificially imposed $\Delta$pH but not by an artificially imposed $\Delta\psi$. These results confirmed the conclusion that the driving force of the [$^3$H]tetracycline transport is the proton gradient ($\Delta$pH). This conclusion also suggested that the [$^3$H]tetracycline transport was carried out by an electrically neutral antiport system of protons and a cationic form of the tetracycline. If this is the case, the efflux of tetracycline in intact cells should only occur when the medium pH is lower than the intracellular pH. We found that the level of resistance of the Tn10-bearing strain to tetracycline increased with a decrease in the medium pH of from 8.0 to 6.0, although the tetracycline susceptibility of the wild strain remained unchanged (in preparation).

It remains unclear why [$^3$H]tetracycline was still accumulated in the vesicles, about one-half the full value being attained, even in the presence of nigericin (fig.1A), although the NADH-induced proton gradient ($\Delta$pH) was negligible under these conditions. There are 2 possible explanations for this. One of them is that the tetracycline transport system can gain some protons in competition with nigericin. The other possibility is that the number of protons translocated by the tetracycline carrier is not equal to that of tetracycline molecules, so the system can also be driven electrically even though the efficiency of the membrane potential as a driving force for tetracycline transport is less than that of the proton gradient. An example of the latter case is the $Na^+/H^+$ system in *E. coli* [20]. The $Na^+/H^+$ antiport system can be driven by $\Delta$pH alone as well as $\Delta\psi$ alone under certain conditions, thus it is believed that the system includes an electrogenic exchange mechanism. If the tetracycline transport system is also driven by a membrane potential, the efficiency should be much less than that of a proton gradient because the addition of valinomycin to respiring vesicles rather accelerated the accumulation of a tetracycline.

The Tet protein encoded by the tetracycline resistance determinant is apparently responsible for the efflux of tetracyclines. The Tet protein has the unique characteristic that its structural gene consists of 2 domains which complement each other, intracistronically [19]. The 2 domains of the Tet protein may represent 2 distinct active sites for

protons and tetracycline, respectively. Further experiments are being performed to determine the characteristics of the efflux system for tetracyclines and the functions of the Tet protein.

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