Active Accumulation of Tetracycline by *Escherichia coli*

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1. At low concentrations of tetracycline (10 $\mu\text{g/ml}$) net accumulation of the drug by *Escherichia coli* cells ceased after 7-10 min. 2. At higher concentrations of tetracycline (>30 $\mu\text{g/ml}$) the period of net accumulation of the drug was significantly extended. 3. The efflux of tetracycline from *E. coli* cells transferred from medium containing 10 μg of tetracycline/ml to drug-free medium was a rapid temperature-dependent process and was accelerated by 2,4-dinitrophenol. 4. As the concentration of tetracycline in the preloading phase was increased, the rate of subsequent efflux of the drug progressively declined. The efflux of drug from cells preloaded in medium containing 200 μg of tetracycline/ml was negligible, although efflux was readily provoked by 2,4-dinitrophenol, by *N*-ethylmaleimide or by omission of glucose from the medium. 5. The initial rate of uptake of tetracycline by *E. coli* cells was linearly proportional to the concentration of tetracycline in the medium up to the maximum concentration of drug obtainable under the experimental conditions used (400 $\mu\text{g/ml}$, 0.83 mM). 6. Although *N*-ethylmaleimide strongly inhibited the accumulation of tetracycline by *E. coli*, no evidence was obtained for the direct involvement of thiol groups in the transport process. It was concluded that *N*-ethylmaleimide inhibited accumulation by interruption of the energy supply of the cells. 7. Osmotic shock of *E. coli* cells did not significantly affect the influx of tetracycline, but promoted both efflux of tetracycline and cell lysis in cells treated with a high concentration of tetracycline. 8. A study of the distribution of tetracycline among the subcellular fractions of penicillin-induced spheroplasts preincubated with various concentrations of tetracycline indicated that 60-70% of the accumulated tetracycline was in the high-speed supernatant fraction. Sephadex chromatography showed that the tetracycline of this fraction was present as the free drug. Sephadex chromatography of a detergent extract of the membrane fraction, however, indicated that a significant proportion of the tetracycline radioactivity of this fraction was apparently bound to some macromolecular component. 9. Cellulose phosphate paper chromatography of cold-acid extracts of spheroplasts preloaded with tetracycline indicated that the accumulated drug was chemically unchanged. 10. Membrane preparations isolated from osmotically lysed penicillin-induced spheroplasts showed a temperature-dependent binding of tetracycline that was not energy-dependent and was not inhibited by *N*-ethylmaleimide. The binding process was stimulated by omitting Mg^{2+} from the medium, but conversely was profoundly inhibited by EDTA. 11. The relevance of these findings to the probable mechanism of active tetracycline accumulation by *E. coli* is discussed.

The accumulation of oxytetracycline by *Escherichia coli* was described by Arima & Izaki (1963). They showed that this process is inhibited by azide and 2,4-dinitrophenol and is dependent on an energy source in the medium. A requirement for Mg^{2+} was also demonstrated (Izaki & Arima, 1965). All of these studies were carried out with very high concentrations of oxytetracycline (>50 $\mu\text{g/ml}$ of medium). Franklin & Godfrey (1965) found that both tetracycline and chlortetracycline were ab-

sorbed by *E. coli* at much lower concentrations in the medium (1-10 $\mu\text{g/ml}$ of medium) by an energy-dependent process. Izaki & Arima (1963) and Franklin & Godfrey (1965) showed that resistance to the tetracyclines in *E. coli* is associated with a marked fall in the ability of the cells to accumulate these antibiotics. Exposure of strains of *E. coli* bearing transferable resistance factors for tetracycline to subinhibitory concentrations of tetracyclines results in a striking increase in the resistance

associated with an equally striking decrease in the accumulation of drug by the cells (Izaki, Kiuchi & Arima, 1966; Franklin, 1967). These changes are suppressed by inhibition of protein and RNA synthesis, but not by inhibition of DNA synthesis (Franklin, 1967; T. W. Young & T. J. Franklin, unpublished work).

Clearly the extent of uptake of the tetracyclines by *E. coli* is a major factor in determining sensitivity and resistance to these drugs. We have therefore extended previous work on the phenomenon of tetracycline accumulation by sensitive *E. coli*. The work reported below suggests the possibility of the involvement of a membrane-associated carrier process. Some of this work has been described previously (Franklin & Higginson, 1969).

METHODS

Chemicals. Chemicals were obtained from the following sources: [^3H]tetracycline hydrochloride (144 mCi/mmol) from The Radiochemical Centre (Amersham, Bucks., U.K.); tetracycline hydrochloride from Chas. Pfizer and Co. (Sandwich, Kent, U.K.); ATP (disodium salt), ADP (trisodium salt), AMP (disodium salt) and NADH (disodium salt) from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany); deoxyribonuclease 1 from the Sigma Chemical Co. (St Louis, Mo., U.S.A.); NEM* from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., U.K.); 2,4-dinitrophenol (recrystallized twice from ethanol) from British Drug Houses Ltd. (Poole, Dorset, U.K.); and Triton X-100 from Charles Lennig and Co. (London W.C.1, U.K.). Penicillin G (sodium salt) is a product of Imperial Chemical Industries Ltd.

Culture conditions. *E. coli* 4018/62 was maintained by periodic subculture on nutrient agar. 'Starter' cultures were prepared by loop transfer into a medium containing in 1 litre: 5.4 g of KH_2PO_4 , 1.2 g of $(\text{NH}_4)_2\text{SO}_4$, 12 g of glucose, 0.4 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 g of Difco Bacto-peptone and 1 g of Difco yeast extract, adjusted to pH 6.9 with NaOH (medium 1). Incubation at 37°C without aeration was continued for 6–9 h. Subsequent cultures were obtained by 5% (v/v) subculture from the 'starter' culture into fresh medium 1 and incubation was continued for 5 h (late exponential phase). The cells were harvested by centrifugation at 15000g for 10 min at 20–25°C; centrifugation at 0–4°C resulted in subsequent erratic uptake of tetracycline. The cells were resuspended in the medium 1 at 20–25°C to give E_{500} 0.8 (approx. 10^9 cells/ml).

Preparation of spheroplasts. The cells were grown until late exponential phase in medium 1 and then diluted six-fold with medium 1 at 37°C containing 20% (w/v) of sucrose and 1000 units of penicillin G/ml. Incubation at 37°C was continued for 2½–3 h, and the cells were centrifuged at 15000g for 10 min and resuspended in medium 1 containing 20% (w/v) of sucrose.

The spheroplasts were lysed by suspending the cells in 10 vol. of a medium containing 0.1 M-tris-HCl buffer, pH 7.0 (at 0°C), 10 mM-MgCl₂ and 20 µg of deoxyribonuclease 1/ml. The suspension was kept at 0°C for 30 min with frequent vigorous swirling.

Fractionation of lysed spheroplasts. The lysates were centrifuged at 4°C for 15 min at 40000g. The sediment, which consisted largely of the cell-membrane fraction and unlysed cells, was washed twice by resuspension and centrifugation in 0.1 M-tris-HCl buffer, pH 7.0, containing 10 mM-MgCl₂. The supernatant fraction remaining after the first centrifugation after lysis was centrifuged at 151000g for 60 min to give the ribosomal pellet and high-speed supernatant fraction.

Determination of uptake of [^3H]tetracycline. (a) Uptake by whole cells. The cells were incubated in medium 1 containing 0.02 µCi of [^3H]tetracycline/ml and the appropriate concentration of unlabelled antibiotic. The radioactivity bound to the cells was measured as described by Franklin (1967) except that the scintillator solution used was that described by Scales (1967). Unless otherwise indicated, the value for tetracycline bound to the cells at 0°C was subtracted from the subsequent values obtained after incubation at 37°C.

(b) Uptake by cell-membrane fraction. The membrane fraction obtained by centrifugation for 15 min at 40000g of spheroplasts lysed at 0°C in 0.1 M-tris-HCl buffer, pH 7.0, containing 10 mM-MgCl₂, was washed by resuspension in this buffer and again centrifuged at 40000g for 15 min. The sediment was finally resuspended (0.6 mg of protein/ml) in 10 mM-tris-HCl buffer, pH 7.5, containing 10 mM-MgCl₂, 0.1 M-NaCl and 20 µg of deoxyribonuclease 1/ml. The particles were completely dispersed by incubation at 37°C with rapid shaking for 10 min before the addition of [^3H]tetracycline (0.02 µCi and 0.06 µg/ml). Samples (5 ml) were taken at required intervals and chilled in ice, followed by dilution with ice-cold 10 mM-tris-HCl buffer, pH 7.5, containing 10 mM-MgCl₂, 0.1 M-NaCl and 100 µg of non-radioactive tetracycline/ml. The particles were sedimented at 40000g for 15 min and the supernatant was discarded. The walls of the tubes were carefully dried and the sediment was resuspended in water (3 ml). A portion (2.5 ml) was mixed with the scintillator solution (Scales, 1967) containing 50 mg of Degussa Aerosil (Deutsche Gold und Silber Scheideanstalt vormals Roessler, Frankfurt/Main, Germany)/ml and the radioactivity determined in a Packard Tri-Carb model 3320 liquid-scintillation spectrometer. The efficiency of counting, determined by the addition of internal standards was 15–25%.

(c) Distribution among spheroplast fractions. After lysis, a portion of the lysate (2.5 ml) was mixed with the scintillator-silica suspension described above. The membrane and ribosomal fractions were resuspended in a convenient volume of water. These suspensions and the high-speed supernatant fraction were also mixed with the scintillator-silica suspension and the radioactivity was determined.

Chromatography. (a) Sephadex chromatography. Sephadex G-25 (fine grade) was used in columns (1.5 cm × 25 cm) equilibrated with 10 mM-tris-HCl buffer, pH 7.5 (at 0°C), containing 5 mM-MgCl₂ and 0.1 M-NaCl. In some experiments the buffer also contained 1 mM-ATP and 2% (v/v) of Triton X-100. Elution was carried out at 4°C with the corresponding buffer mixture at a flow rate of 15 ml/h. Samples (1 ml) of each fraction (2 ml) were mixed with scintillator solution (7 ml) and the radioactivity was determined.

(b) Ion-exchange paper chromatography. Derivatives of tetracyclines are conveniently separated by descending

* Abbreviation: NEM, *N*-ethylmaleimide.

chromatography on Whatman cellulose phosphate paper (P81) with 0.1% NH_4Cl as the developing reagent (Addison & Clark, 1963). Development was carried out for 5–8 h at 4°C to minimize the possibility of degradation of any unstable tetracycline derivatives.

Osmotic shock of *E. coli* cells. The procedure described by Anraku & Heppel (1967) was used. The cells were harvested from medium 1 by centrifuging at 3°C and washed twice in 0.01 M-tris-HCl buffer, pH 8.0, containing 0.03 M-NaCl (40 ml of buffer/g wet wt. of cells). The cells were then dispersed in 80 vol. of 20% (w/v) sucrose containing 0.035 M-tris-HCl buffer, pH 8.0, and 0.1 mM-EDTA at room temperature (22–24°C) and shaken gently for 10 min. After centrifugation the cells were dispersed in 80 vol. of 0.5 mM-MgCl₂ and shaken at 4°C for 10 min. After centrifugation the cells were resuspended in medium 1 at room temperature to give E_{500} 0.8 and used immediately.

Determination of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used, with crystalline bovine plasma albumin as a standard.

Determination of ATP content of *E. coli* cells. The cells, suspended in medium 1 (5 ml) (E_{500} 0.8), were chilled and centrifuged at 0°C for 5 min at 20000g and washed by re-suspension and centrifugation in ice-cold 0.14 M-NaCl. The cells were then suspended in water (2 ml) and heated at 100°C for 10 min and then centrifuged. The ATP content of the supernatant was determined by the method of Strehler & McElroy (1957) with firefly-lantern extract (FLE50) purchased from the Sigma (London) Chemical Co. Ltd. (London S.W.6, U.K.). Fluorescence measurements were carried out in a Locarte spectrofluorimeter connected to a pen recorder. As little as 0.1 nmol of ATP is measurable by this method. NEM (1 mM) had no effect on the assay procedure.

RESULTS

Dependence of uptake of [³H]tetracycline by *E. coli* on external drug concentration. The progress of the uptake of tetracycline by *E. coli* cells in medium 1 changed markedly as the concentration of drug in the medium was increased. At the lowest concentration of tetracycline (10 µg/ml) the drug accumulated rapidly for 7–10 min (in a few experiments this phase was extended to 30 min); thereafter a plateau was reached and there was little further increase in the net accumulation of tetracycline by the cells. As the concentration of tetracycline in the medium was increased, the period of net accumulation of drug by the cells progressively lengthened; at 100 µg of tetracycline/ml net accumulation frequently continued for 1 h (longer incubations were not performed).

Outward transport of [³H]tetracycline from *E. coli*. In many examples of active transport, efflux of the accumulated solute from the cells also occurs; net accumulation of the solute ceases when the rate of outward transport, V_e , equals that of inward transport, V_i . Outward transport can be conveniently studied by preloading the cells with tetra-

Fig. 1. Dependence of the rate of efflux of tetracycline from *E. coli* cells on the concentration of tetracycline in the preloading phase. The cells were preincubated at 37°C for 30 min in medium 1 containing 10 (○), 75 (●) 100 (□) and 200 (▲) µg of tetracycline/ml and 0.02 µCi of [³H]tetracycline/ml. The cells were transferred to drug-free medium 1 and the loss of tetracycline was determined as described in the text.

Fig. 2. Dependence of the rate of efflux of tetracycline from *E. coli* cells on the length of preincubation with 200 µg of tetracycline/ml in medium 1 (0.02 µCi of [³H]tetracycline/ml). The cells were preincubated at 37°C for 5 (○), 10 (●), 15 (△) and 30 (▲) min before being transferred to drug-free medium 1.

Fig. 3. Effect of temperature and 2,4-dinitrophenol on the efflux of tetracycline from *E. coli* cells preloaded in medium 1 containing $10\mu\text{g}$ of tetracycline/ml. The cells were incubated at 37°C for 30 min in medium 1 containing tetracycline ($0.02\mu\text{Ci}$ of $[^3\text{H}]$ tetracycline/ml). The cells were then transferred to tetracycline-free medium 1 at 0 ($\circ$ and $\bullet$), 25°C ($\triangle$ and $\blacktriangle$) and 37°C ($\square$ and $\blacksquare$) respectively in the absence ($\circ$, $\triangle$ and $\square$) and presence ($\bullet$, $\blacktriangle$ and $\blacksquare$) of 1 mM-2,4-dinitrophenol.

cycline and following the subsequent loss of the drug in tetracycline-free medium. Accordingly, *E. coli* cells were preincubated in medium 1 for 30 min with various concentrations of tetracycline. The cells were centrifuged for 5 min at $20\,000g$ at 25 – 30°C (centrifugation at 0°C resulted in subsequent variable losses of tetracycline) and then resuspended in tetracycline-free medium 1 at 37°C . Fig. 1 illustrates a striking inverse relationship between the net outward flux of tetracycline and the concentration of the drug used in the preloading phase. The loss of drug from cells preloaded in $10\mu\text{g}$ of tetracycline/ml was rapid and extensive; no increase in cell protein occurred during this phase. The rate of net flux from cells preloaded in concentrations of tetracycline greater than $50\mu\text{g}/\text{ml}$ was significantly depressed, and at a preloading concentration of $200\mu\text{g}$ of tetracycline/ml the subsequent net flux of drug from the cells was negligible, although in some experiments small losses of tetracycline of up to 10% were noted. It appears therefore that as the total cellular concentration of tetracycline rises the net loss of the drug is progressively diminished. This effect is also illustrated in Fig. 2. In this experiment the cells were preincubated for increasing periods in medium 1 containing $200\mu\text{g}$ of tetra-

Fig. 4. Effect of interruption of energy supply on efflux of tetracycline from *E. coli* cells preloaded in medium 1 containing $200\mu\text{g}$ of tetracycline/ml. The cells were incubated at 37°C for 30 min in medium 1 containing tetracycline ($0.02\mu\text{Ci}$ of $[^3\text{H}]$ tetracycline/ml). The cells were then transferred to tetracycline-free medium 1. $\bullet$, Medium 1 at 37°C ; $\blacktriangle$, medium 1 minus glucose at 37°C ; $\circ$, medium 1 containing 1 mM-2,4-dinitrophenol at 37°C ; $\triangle$, medium 1 containing 1 mM-2,4-dinitrophenol at 0°C .

cycline/ml. On transference to tetracycline-free medium the rate of loss of drug was inversely related to the length of the preloading phase and therefore to the amount of drug accumulated by the cells. Thus the increasing period of net accumulation of tetracycline by the cells as the concentration of drug in the medium was increased may have been due to the progressive decrease in net outward flux of the drug.

Additional factors influencing the outward transport of $[^3\text{H}]$ tetracycline. (1) Energy production. The rapid loss of tetracycline from cells preloaded in $10\mu\text{g}$ of tetracycline/ml was significantly accelerated by 1 mM-2,4-dinitrophenol added at the start of the second incubation (Fig. 3). 2,4-Dinitrophenol or omission of glucose also rapidly provoked loss of tetracycline from *E. coli* cells preloaded in medium 1 containing $200\mu\text{g}$ of tetracycline/ml (Fig. 4).

(2) Temperature. The net efflux of tetracycline from cells preloaded in $10\mu\text{g}$ of tetracycline/ml of medium 1 was significantly depressed at 25°C and was negligible at 0°C (Fig. 3). The effect of 2,4-dinitrophenol on this exit reaction was also markedly temperature-dependent and the compound did not induce release of tetracycline at 0°C from cells preloaded in medium 1 containing $200\mu\text{g}$ of tetracycline/ml (Fig. 4).

Fig. 5. Reciprocal plots of the initial rate of uptake of tetracycline by *E. coli* cells (v) against the concentration of tetracycline in medium 1 (i). (a) Results obtained over the concentration range 0.1–0.5 μg of tetracycline/ml of medium; (b) results obtained over the concentration range 10–400 μg of tetracycline/ml. The concentration of [^3H]tetracycline was 0.02 μCi /ml. v is expressed as μg of tetracycline accumulated/min per mg of protein, and i as μg of tetracycline/ml.

Inability of tetracycline to saturate inward transport. If the transport of tetracycline is mediated by some sort of carrier mechanism it might be expected that the initial rate of uptake would be dependent on the external concentration of drug up to a value where all the available carrier sites are saturated with tetracycline. Further increases in tetracycline concentration would then produce no further increase in transport rate. Accordingly *E. coli* cells were sampled 5 and 7 min after mixing with various concentrations of [^3H]tetracycline in medium 1. The initial rate of transport was determined by extrapolation and the results were plotted against the external concentration of tetracycline. Figs. 5(a) and 5(b) show that there was no tendency towards a saturation effect up to the maximum concentration of tetracycline that can be obtained under these conditions, i.e. 400 μg /ml (0.83 mM).

Effect of NEM on the transport of tetracycline. Fox & Kennedy (1966) have made ingenious use of the thiol-alkylating agent NEM to identify a membrane-associated protein (M protein), which is apparently involved in the transport of β -galactosides in *E. coli*.

Table 1. Effect of NEM on the ATP content of *E. coli* cells

The cells were suspended in medium 1 (E_{500} 0.8) and incubated at 37°C for 15 min with the indicated concentrations of NEM or 2,4-dinitrophenol. The suspensions (5 ml) were chilled, centrifuged and washed once by resuspension and centrifugation in ice-cold 0.9% NaCl. The ATP content of the cells was then measured as described in the Methods section.

Addition	ATP content of cells after 15 min incubation (nmol/mg of protein)
None	2.43
1 mM-NEM	0.72
0.1 mM-NEM	1.23
1 mM-2,4-Dinitrophenol	0.54

Fig. 6. Effect of NEM on the uptake (○) and release (●) of tetracycline by *E. coli* cells. In the uptake experiment the cells were suspended (E_{500} 0.8) in medium 1 containing 200 μg of tetracycline and 0.02 μCi of [^3H]tetracycline/ml and the indicated concentrations of NEM. After 30 min incubation at 37°C the uptake of tetracycline was determined as described in the text; the left-hand ordinate indicates the inhibition of uptake. In the release experiment *E. coli* cells were preincubated at 37°C for 30 min in medium 1 containing 200 μg of tetracycline and 0.02 μCi of [^3H]tetracycline/ml. The cells were then transferred to drug-free medium 1 containing the indicated concentrations of NEM. The right-hand ordinate indicates the tetracycline remaining bound to the cells after 30 min.

We therefore decided to study the effects of NEM on tetracycline transport. Fig. 6 indicates that NEM is a powerful inhibitor of the accumulation of tetracycline by *E. coli*. However, NEM is a general metabolic poison and would therefore be expected to inhibit the energy-generating mechanisms of the

cell. A study of the effects of NEM on the ATP concentrations in *E. coli* cells has shown that this is in fact the case (Table 1). This effect on NEM is reflected in the ability of the inhibitor to provoke release of tetracycline from cells preloaded in medium 1 containing 200 μg of tetracycline/ml (Fig. 6). NEM did not retard the efflux of tetracycline from preloaded cells treated with 2,4-dinitrophenol (not shown).

Attempts were made to protect the tetracycline-accumulation mechanism against NEM (cf. Fox & Kennedy, 1966) by preincubating the cells with 200 μg of tetracycline/ml of medium 1 for 15 min. NEM was then added at a concentration of 0.1 mM, since this concentration had a minimal effect on the ATP content of the cells and was also less effective in provoking release of tetracycline from preloaded cells than in inhibiting tetracycline accumulation. No conclusive evidence for protection against the inhibition by NEM of subsequent influx of [^3H]-tetracycline was obtained in a number of experiments. This was not unexpected in view of the inability of tetracycline to saturate the transport mechanism.

Effect of osmotic shock on the transport of tetracycline. The numerous reports describing the effects of procedures known as 'osmotic shock' on the transport and accumulation of various substances by bacterial cells have been reviewed by Pardee (1968).

The cells are first treated with solutions containing tris, EDTA and a high concentration of sucrose and then dispersed in very dilute magnesium chloride at 0°C. This treatment releases a number of membrane-associated proteins into the hypo-osmotic solution and depresses the ability of the osmotically shocked cells to transport various solutes. Further, transport can be restored by adding the shock fluid back to the cells. Certain proteins isolated from the shock fluid have specific affinities for the transported solutes and are thought to be involved in membrane transport phenomena. It was therefore decided to determine whether osmotic shock had any effect on the ability of *E. coli* to accumulate tetracycline. The rate of uptake of tetracycline and also the maximum intracellular concentration of antibiotic achieved by *E. coli* cells in medium 1 containing 10 μg of tetracycline/ml were not significantly affected by prior osmotic shock. The rate of uptake of tetracycline by shocked cells suspended in medium 1 containing 200 μg of tetracycline/ml was, however, somewhat lowered.

Cells that were shocked after preincubation for 30 min in medium 1 containing 200 μg of tetracycline/ml rapidly lost the drug on transfer to drug-free medium 1 (Fig. 7); in addition, considerable cell lysis occurred. It seems likely therefore that the diminished accumulation of tetracycline by shocked

Fig. 7. Effect of osmotic shock on *E. coli* cells preincubated in medium containing 200 μg of tetracycline/ml (0.02 μCi of [^3H]tetracycline/ml). The cells were preincubated at 37°C for 30 min in medium 1 containing tetracycline, shocked as described in the text and transferred to drug-free medium 1 at 37°C. The loss of tetracycline (○ and ●) and protein (△ and ▲) from control (○ and △) and shocked cells (● and ▲) is illustrated.

cells in 200 μg of tetracycline/ml of medium was due to an enhanced net efflux of tetracycline from the cells under these conditions.

Uptake of [^3H]tetracycline by penicillin-induced spheroplasts. Penicillin-induced spheroplasts from *E. coli* accumulate tetracycline (Franklin, 1967). We have now studied the distribution of tetracycline among various cell fractions after osmotic lysis of spheroplasts previously loaded with tetracycline. Spheroplasts were incubated with various concentrations of tetracycline and 0.02 μCi of [^3H]tetracycline/ml of medium 1 containing 20% (w/v) of sucrose for 30 min. The cells were then chilled, centrifuged and washed by resuspension in 0.14 M-sodium chloride containing 20% (w/v) of sucrose at 0°C. The cells were osmotically lysed and then fractionated by differential centrifugation (see the Methods section). The 40 000g sediment, comprising the cell-membrane fraction and some intact spheroplasts, was extracted for 15 min at 0°C with 10 mM-tris-HCl buffer, pH 7.4, containing 2% (v/v) of Triton X-100, 5 mM-magnesium chloride and 0.1 M-sodium chloride and centrifuged at 100 000g for 60 min. The radioactivity of the following fractions was determined: (1) cell lysate; (2) 151 000g supernatant; (3) ribosomes; (4) supernatant; (5) sediment from Triton X-100 extraction of membranes. Table 2 indicates that the 151 000g supernatant contained 60–70% of the total cellular tetracycline. Treatment of the membranes with Triton X-100 released 70% of the total tetracycline of this fraction.

Table 2. *Distribution of tetracycline among subcellular fractions of penicillin-induced spheroplasts of E. coli*

The spheroplasts were suspended in medium 1 containing 20% (w/v) of sucrose and 0.02 μ Ci of [3 H]tetracycline/ml and the indicated concentrations of unlabelled tetracycline. After incubation for 30 min at 37°C the suspensions were chilled, the cells were harvested and the distribution of radioactivity among the subcellular fractions was determined as described in the Methods section.

Fraction	Concn. of tetracycline in medium (μ g/ml)	Tetracycline accumulated in 30 min (μ g/mg of protein)	% of total cell-bound tetracycline accumulated by fraction
Lysate	10	0.94	100
	50	7.31	100
	100	21.4	100
	200	36.1	100
	400	63.1	100
Triton X-100 supernatant	10	0.83	27.3
	50	6.02	23.8
	100	17.6	16.0
	200	29.1	18.8
	400	46.9	17.4
Triton X-100 sediment	10	0.30	6.9
	50	1.61	6.1
	100	6.04	5.8
	200	7.79	5.0
	400	18.2	5.9
151 000g supernatant	10	1.24	64.0
	50	9.36	70.0
	100	28.9	63.0
	200	38.3	67.0
	400	67.8	60.5
Ribosomes	10	0.48	1.4
	50	2.60	1.3
	100	6.65	1.0
	200	12.5	1.0
	400	24.6	1.2

The ribosomal fraction contained less than 2% of the total cellular tetracycline. However, since electron microscopy of the membrane fraction by the method of Kaback & Stadtman (1966) revealed a significant number of ribosome-like particles in this fraction the values for total binding by the ribosomes are an underestimate of the true value.

Chromatography of tetracycline accumulated by penicillin-induced spheroplasts. Spheroplasts in medium 1 (200 ml) (E_{500} 0.8) containing 20% (w/v) of sucrose and 200 μ g of [3 H]tetracycline/ml were incubated for 60 min at 37°C. After centrifugation at 0°C the cells were washed in cold 0.1M-sodium chloride containing 20% (w/v) of sucrose followed by lysis in 10mM-tris-HCl buffer, pH 7.5, containing 0.1M-sodium chloride and 5mM-magnesium chloride

Fig. 8. Elution profile on Sephadex G-25 of the high-speed supernatant fraction from *E. coli* spheroplasts preincubated with 200 μ g of tetracycline and 0.02 μ Ci of [3 H]-tetracycline/ml. Penicillin-induced spheroplasts suspended in medium 1 containing 20% (w/v) of sucrose and tetracycline were incubated for 60 min at 37°C before osmotic lysis and fractionation as described in the text. The 151 000g supernatant fraction was chromatographed at 4°C as described in the text and the radioactivity of each fraction measured.

(10 ml) at 0°C. The lysate was fractionated as described above. The Triton X-100 supernatant fraction from the membranes and the 151 000g supernatant fraction were chromatographed on Sephadex G-25 at 4°C (see the Methods section). Fig. 8 shows that less than 5% of the radioactivity of the 151 000g supernatant fraction was eluted in the void volume of the column, suggesting that most of the tetracycline was present as free, although not necessarily chemically unchanged, drug. With the Triton X-100 extract a considerably greater proportion of the total radioactivity of the extract (23%) was eluted in the void volume, suggesting the possibility of attachment of tetracycline to some high-molecular-weight component removed from the membrane fraction by treatment with Triton X-100 (Fig. 9). An attempt to assess the chemical nature of the tetracycline accumulated by the cells was made by using the ion-exchange cellulose phosphate paper. Spheroplasts preincubated for 60 min in medium 1 containing 200 μ g of [3 H]tetracycline/ml were extracted with ice-cold 5% (w/v) trichloroacetic acid. After removal of the acid, the aqueous phase was freeze-dried, redissolved in water and chromatographed (see the Methods section). Of the total radioactivity on the chromatogram, over 90% was associated with the unchanged tetracycline and 8.3% with an area corresponding to anhydrotetracycline. No significant radioactivity was found in the area corresponding to epitetracycline.

Binding of tetracycline by membrane preparations. The demonstration by Kaback & Stadtman (1966)

Fig. 9. Elution profile on Sephadex G-25 of the Triton X-100 extract of the membrane fraction from *E. coli* spheroplasts preincubated with 200 μ g of tetracycline and 0.02 μ Ci of [3 H]tetracycline/ml. Penicillin-induced spheroplasts suspended in medium 1 containing 20% (w/v) of sucrose and tetracycline were incubated for 60 min at 37°C before osmotic lysis and fractionation as described in the text. The detergent extract was chromatographed at 4°C as described in the text and the radioactivity of each fraction measured.

Fig. 10. Binding of tetracycline by membrane preparation from *E. coli* spheroplasts. The membrane suspension (0.6 mg of protein/ml) was incubated at 37°C with [3 H]-tetracycline (0.02 μ Ci and 0.06 μ g/ml) in 10mM-tris-HCl buffer, pH 7.5, containing 10mM-MgCl₂, 0.1M-NaCl and 20 μ g of deoxyribonuclease/ml (○) plus 1mM-NEM (●) or 1mM-succinate (□) or 1mM-NADH⁺ (■). Δ, Control system at 0°C. The radioactivity bound by the membrane preparation was measured as described in the text.

that a membrane preparation obtained from penicillin-induced spheroplasts of *E. coli* was capable of energy-dependent accumulation of proline *in vitro* prompted a study of the binding of tetracycline to this type of preparation. Binding of [3 H]tetracycline to these membranes occurred more or less instantaneously at 0°C and no further binding occurred on incubation for 60 min at 37°C with or without added glucose (T. J. Franklin & B. Higginson, unpublished work). Subsequently we examined the binding of tetracycline *in vitro* to the membranes prepared from penicillin-induced spheroplasts by the method described in the Methods section. Fig. 10 indicates that this preparation also bound tetracycline at 0°C. In addition, however, there was on incubation at 37°C a progressive binding of antibiotic that reached a plateau in about 20 min. No dependence of the binding on added energy sources could be detected and there was no inhibition by NEM. Omission of Mg²⁺ from the incubation medium resulted in a marked increase in the amount of [3 H]tetracycline taken up by the membranes (Fig. 11). Further, when the Mg²⁺ concentration was increased from 10–100mM there was a marked decrease in the amount of antibiotic bound (Fig. 11); EDTA (in the absence of Mg²⁺) was unexpectedly strongly inhibitory to the binding of [3 H]tetracycline (Fig. 11). EDTA and Mg²⁺ (100mM) also decreased the amount of drug bound to the membranes at 0°C. In these experiments unlabelled tetracycline was not added to the incubation mixtures. Table 3 shows the effect on the binding of

tetracycline by a membrane preparation of adding various concentrations of unlabelled drug in the absence of Mg²⁺. Although the absolute amount of tetracycline bound, both at 0 and 37°C, increased with increasing concentrations of added, unlabelled drug, the proportional increment between the radioactivity values at 0 and 37°C decreased above 1 μ g of unlabelled tetracycline/ml. Thus there was a tendency towards saturation of the temperature-dependent uptake with increasing concentration of unlabelled tetracycline. Subsequent experiments were therefore carried out without addition of unlabelled tetracycline to the incubation mixtures.

Electron microscopy (Kaback & Stadtman, 1966) of the membrane preparations showed that they also contained a number of apparently unlysed cells. *E. coli* cells were therefore washed in 10mM-tris-HCl buffer, pH 7.5, containing 10mM-magnesium chloride and 0.1M-sodium chloride at 0°C and then resuspended in this medium (E_{500} 0.8). Incubation of this suspension at 37°C with [3 H]tetracycline for 20 min did not increase the amount of radioactivity bound to the cells compared with that bound at 0°C (Table 4). Omission of Mg²⁺, however, greatly stimulated the binding at 37°C, and neither 2,4-dinitrophenol nor NEM had any significant effect on this process (Table 4). The specific radioactivity of the whole-cell preparation after 20 min incubation with [3 H]tetracycline was much less than that of the membrane preparation after incubation with the

Fig. 11. Effects of Mg^{2+} and EDTA on the binding of tetracycline to a membrane preparation from *E. coli*. The conditions of this experiment were as described in Fig. 10. $\circ$, 10mM- Mg^{2+} ; Δ , 100mM- Mg^{2+} ; $\bullet$, minus Mg^{2+} ; $\square$, 10mM-EDTA, minus Mg^{2+} .

Table 3. Dependence of uptake of tetracycline in vitro by a membrane preparation from *E. coli* on the concentration of unlabelled tetracycline

The membranes were suspended in a medium containing 10mM-tris-HCl buffer, pH 7.5, and 0.1M-NaCl to give a protein concentration of about 0.4mg/ml. $[^3H]$ Tetracycline was added to give specific radioactivity 0.02 μCi /ml. The values for the absolute amount of tetracycline bound were obtained from the radioactivity values after determination of the efficiency of counting by the addition of an internal standard.

Concn. of tetracycline in medium (μg /ml)	Tetracycline bound by membranes in 20 min (per mg of protein)			
	At 0°C		At 37°C	
	(c.p.m.)	(μg)	(c.p.m.)	(μg)
0.1	1213	0.012	9621	0.110
0.5	1033	0.048	9114	0.466
1.0	1201	0.125	9832	1.130
10.0	566	0.569	2752	2.883
50.0	599	3.126	1632	8.512

antibiotic (Table 4). Further, when the membrane preparation was purified free from unlysed cells and debris by density centrifugation as described by Kaback & Stadtman (1966) (the buffer system used by these workers was replaced by 0.1M-tris-HCl buffer, pH 7.0, containing 0.1M-sodium chloride), the specific radioactivity of the membranes after incubation for 20min with $[^3H]$ tetracycline in 10mM-tris-HCl buffer, pH 7.5, containing 0.1M-sodium chloride was markedly higher than that of

Table 4. Uptake of $[^3H]$ tetracycline by whole-cell and membrane preparations from *E. coli* in tris-buffered media

The suspension medium was 10mM-tris-HCl buffer, pH 7.5, containing 0.1M-NaCl and 10mM- $MgCl_2$, where indicated. The cells were suspended to give E_{500} 0.8 and the membrane preparations to give approx. 0.2mg of protein/ml. 'Crude' membranes were prepared as described in the Methods section and the 'purified' membranes by density centrifugation of the crude preparation as described by Kaback & Stadtman (1966). The concentration of $[^3H]$ tetracycline was 0.02 μCi and 0.06 μg /ml and the binding results refer to the radioactivity bound to cells in a 20 min incubation at 37°C, after subtracting the value for radioactivity bound at 0°C.

Preparation	$[^3H]$ Tetracycline bound (c.p.m./mg of protein)
Whole cells plus 10mM- $MgCl_2$	267
Whole cells	2990
Whole cells plus 10mM-2,4-dinitrophenol	2840
Whole cells plus 10mM-NEM	2701
Crude membranes	10000
Purified membranes	21000

Fig. 12. Loss of radioactivity from a membrane preparation preincubated with $[^3H]$ tetracycline. The membrane preparation was preincubated for 20min at 37°C with $[^3H]$ tetracycline (0.02 μCi and 0.06 μg /ml). The particles were then transferred to fresh buffer (10mM-tris-HCl buffer, pH 7.5, containing 10mM- $MgCl_2$ and 0.1M-NaCl) with ($\bullet$) or without ($\circ$) 100 μg of tetracycline/ml and incubation at 37°C was continued; Δ indicates labelled membranes kept in fresh buffer (no tetracycline) at 0°C for 20 min.

both the whole-cell and crude membrane preparations (Table 4).

Membranes that were incubated with $[^3H]$ tetracycline for 20min and then resuspended in fresh

buffer progressively lost label on incubation at 37°C. Addition of unlabelled tetracycline (100 µg/ml) in the second incubation had little effect on the rate of loss of label from the membrane preparation (Fig. 12).

DISCUSSION

The active accumulation by bacterial cells of a toxic substance not obviously related to any known nutrient is a remarkable phenomenon and it has been found to have a number of unusual features. The initial rate of accumulation of tetracycline did not reach a maximum even when the external concentration of tetracycline was 0.83 mM. This might suggest that the transport process does not involve a saturable carrier mechanism. However, it should be remembered that such linear kinetics do not preclude the possibility of carrier-mediated transport if it is assumed that the affinity of the postulated carrier for the transported solute is low, i.e. if the K_m of the carrier is much greater than the working concentration range of the transported solute (Wilbrandt & Rosenberg, 1961). Since the uptake of tetracycline by bacterial cells is likely to be fortuitous, the affinity of the postulated carrier for tetracycline may well be very low. While this work was in progress, Hutchings (1969) described the active accumulation of tetracycline by *Staphylococcus aureus*. At low external concentrations of tetracycline he was able to demonstrate Michaelis-Menten-type kinetics with K_m 4.76 µM, although at high concentrations of the tetracycline the transport kinetics became linear.

In the presence of low concentrations of tetracycline we have clearly demonstrated transport in both directions across the cell membrane, resulting in an equilibrium between intracellular and extracellular tetracycline concentrations. With increasing external concentrations of tetracycline the period of net accumulation of drug by the cells was considerably extended and in addition the rate of efflux of tetracycline progressively declined. After preloading the cells in a medium containing 200 µg of tetracycline/ml it was difficult to detect any subsequent loss of drugs from the cells, provided that an energy source was available. The reason for this curious phenomenon is uncertain. Increasing intracellular concentrations of tetracycline could conceivably progressively inhibit efflux of the drug, although if the same carrier is used for both influx and efflux it is difficult to see how such asymmetry would be achieved.

However, an alternative, kinetic, model may be proposed. This assumes that in the absence of ATP the kinetic parameters of influx and efflux are identical, i.e. no accumulation against a concentration gradient can occur under these conditions. In the presence of intracellular ATP, however, the

V_{max} of efflux, $V_{max(e)}$, would be very much less than the V_{max} of influx, $V_{max(i)}$, although the K_m values would be identical. To account for a maximum value for the intracellular concentration of tetracycline of 20 times the external concentration, which is achieved at an external tetracycline concentration of 10 µg/ml (Franklin, 1967), we assume that $V_{max(i)} = 20 V_{max(e)}$. At a critical external tetracycline concentration, C_e , the resulting internal concentration of tetracycline, C_i , will saturate the efflux reaction. Thus up to C_e equilibrium can be achieved between internal and external tetracycline concentration such that $V_i = V_e$. Above C_e , however, tetracycline will continue to be accumulated by the cells, since V_i will always be greater than V_e . The progressive fall in the rate of net loss of tetracycline from cells preloaded in increasing concentrations of tetracycline on transferring to drug-free medium is more difficult to interpret although it might be suggested that the lipoprotein, lipopolysaccharide and murein layers external to the membrane trap sufficient tetracycline close to the external face of the membrane to maintain V_i greater than V_e for some time.

The lack of effect of osmotic shock on the influx of tetracycline into *E. coli* cells from medium containing a low concentration of tetracycline suggests that, if a carrier exists, it may be tightly bound to the membrane. Osmotic shock combined with incubation of *E. coli* with a high concentration of tetracycline results in leaky fragile cells.

Unlike the M protein, which is implicated in β -galactoside transport (Fox & Kennedy, 1966), the mechanism for tetracycline transport appears not to involve essential thiol groups. Although NEM inhibited net accumulation of tetracycline it seems certain that this was an indirect effect due to the inhibition of energy-generating processes in the cell. Two pieces of evidence argue in favour of this explanation. First, if the same carrier is involved in efflux of tetracycline then NEM might have been expected to retard efflux of tetracycline; no such effect was observed. Secondly, if the temperature-dependent binding of tetracycline by isolated membranes is relevant to the phenomenon of active accumulation by intact cells, then the lack of effect of NEM on the membrane binding may indicate that the thiol groups are not directly involved in the binding of tetracycline with membrane-associated carrier molecules.

The fractionation of spheroplasts preloaded with tetracycline indicates that the larger part of the accumulated tetracycline was present as free chemically unchanged drug, although significant amounts were bound to the ribosomes and to the membranes. The binding of some 20% of the tetracycline solubilized by Triton X-100 from the membrane fraction to material excluded from Sephadex

G-25 may be of relevance to the problem of trans-membrane transport of the drug. The precise significance of the temperature-dependent binding of tetracycline to the isolated membrane preparations is somewhat uncertain. The inhibitory effect of EDTA in the absence of Mg^{2+} on the one hand and the stimulating effect of the omission of Mg^{2+} on the other suggests that tetracycline may bind to the preparation through membrane-associated cations; conceivably Mg^{2+} in the medium may compete with the membrane-associated cations for tetracycline. It is possible that the uptake of tetracycline by the isolated membrane preparations may be due to a carrier-mediated transport of the antibiotic into membrane vesicles. Since no dependence on an energy source was observed, clearly the transport could not result in accumulation against a concentration gradient.

The binding of tetracycline to *E. coli* cells suspended in tris-sodium chloride medium was unaffected by either 2,4-dinitrophenol or NEM, but was inhibited by Mg^{2+} , and therefore resembles the binding by the isolated membrane preparations. It therefore seems likely that the presence of tris facilitates binding of tetracycline to some cellular receptor, possibly associated with the membrane, although under these conditions the cells do not

accumulate tetracycline against a concentration gradient.

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