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Resistance of *Escherichia coli* to Tetracyclines

CHANGES IN PERMEABILITY TO TETRACYCLINES IN *ESCHERICHIA COLI* BEARING TRANSFERABLE RESISTANCE FACTORS

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1. When strains of *Escherichia coli*, bearing transferable factors for resistance to the tetracyclines (R-factors), and previously cultured in the absence of the tetracyclines, are grown for 15–30min. in a low, subinhibitory, concentration (10μg./ml.) of oxytetracycline or tetracycline, there is a rapid and striking increase in resistance to oxytetracycline or tetracycline, this being associated with a marked fall in the absorption of the drug by the cells. 2. Very short preincubation (1min.) with oxytetracycline, followed by growth for 15–30min. in drug-free medium, produces a marked fall in the absorption of the drug by the resistant cells. Preincubation for 30min. with very low concentrations (0·05μg./ml.) of oxytetracycline produces a similar effect. 3. β-Apo-oxytetracycline, which has very little antibacterial activity, also induces a decreased absorption of oxytetracycline. 4. The ability to exclude oxytetracycline is retained by preincubated resistant cells after growth for 2hr. in drug-free medium. However, after growth for 16hr. in drug-free medium, the cells absorb oxytetracycline freely. 5. Chloramphenicol and proflavine inhibit the adaptive decrease in tetracycline absorption. 5-Fluorouracil has only a slight effect. 6. Spheroplasts prepared from resistant cells show an impaired response to preincubation with tetracycline, compared with intact cells. 7. The relevance of these results to the probable mechanism of tetracycline resistance in R-factor-bearing *E. coli* is discussed.

Izaki & Arima (1963) demonstrated that strains of *Escherichia coli* bearing a factor for transferable multiple resistance to drugs including the tetracyclines (R-factor) absorb much less oxytetracycline than a sensitive strain of *E. coli*. Franklin & Godfrey (1965) found a similar difference in the absorption of chlortetracycline and tetracycline between a sensitive strain of *E. coli* and a resistant strain selected from the sensitive parent by serial passage through increasing concentrations of chlortetracycline. More recently, Izaki, Kiuchi & Arima (1966) discovered that there was a marked fall in the absorption of tetracycline by R-factor-bearing *E. coli*, resistant to the tetracyclines, previously cultured in drug-free medium, when the cells were grown for 3hr. in the presence of subinhibitory concentrations of oxytetracycline, chlortetracycline or tetracycline. This decrease in the absorption of tetracyclines was accompanied by a significant increase in the resistance of the cells to the drugs. Some evidence was presented by Izaki *et al.* (1966) that the effect reflected an adaptive change in the cells, and was not due to a selection of more resistant cells from a heterogeneous population. Rachmeler & Unowsky (1966) reported that there was a similar fall in tetracycline absorption when *E. coli* bearing an R-factor that mediates chromosomal transfer was preincubated with tetracycline, and that this effect was not observed with *E. coli* carrying an R-factor that inhibits chromosomal transfer.

The work described in this paper confirms the results of Izaki *et al.* (1966) and supports the hypothesis that the increase in resistance that occurs when strains of *E. coli* bearing transferable factors for tetracycline resistance are exposed to low concentrations of tetracycline antibiotics is associated with a diminished absorption of these drugs. Evidence is presented that suggests that this diminished absorption may be due to the induction of an enzyme system by low concentrations of the tetracyclines, this resulting in the inhibition of the active transport of tetracyclines into the bacterial cell. A preliminary account of this work has been given (Franklin, 1967).

MATERIALS AND METHODS

Radioactive chemicals. L-[G-^{14}C]Leucine (6·6mc/m-mole) and [7-^{3}H]tetracycline (409mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks.

Chemicals. Oxytetracycline hydrochloride and proflavine hemisulphate were products of Imperial Chemical Industries Ltd. Tetracycline hydrochloride was obtained from Chas. Pfizer and Co., Sandwich, Kent, and chloramphenicol from Parke, Davis and Co., Hounslow, Middlesex. 2,4-Dinitrophenol (recrystallized twice from ethanol) and iodoacetic acid were purchased from British Drug Houses Ltd., Poole, Dorset, and 5-fluorouracil was purchased from Calbiochem Ltd., London, W. 1.

Dr R. H. B. Galt kindly provided a sample of β-apo-oxytetracycline. This was free of oxytetracycline, but contained a trace of the α-isomer.

Strains of E. coli *and culture conditions.* The sensitive strains were: (1) *E. coli* A.T.C.C.112299/198 (S_1); (2) *E. coli* 4018/62 (S_2). The resistant strains were as follows. (1) Strain R_1; this was the strain described by Franklin & Godfrey (1965), and was isolated by the serial passage method of English & Gelwicks (1951). This strain was stored as a freeze-dried preparation, and on reconstitution was found to be capable of transferring tetracycline resistance to a streptomycin-resistant chromosomal mutant of *E. coli* 4018/62, as described by Watanabe (1964). It is not known whether strain R_1 possesses a true resistance-transfer factor or whether a resistance-determining fragment of the main chromosome has separated from the chromosome with an F-factor, i.e. an F-prime organism. (2) Strain R_2: this was *E. coli* 4018/62 bearing an R-factor conferring resistance to sulphonamide, kanamycin, neomycin, chloramphenicol and streptomycin as well as to the tetracyclines. All strains were maintained by periodic subculture on nutrient agar. Cells were obtained for experiment by loop transfer to the appropriate medium, and culturing for 6–9hr. at 37°. The resistant strains were cultured in medium containing 100μg. of tetracycline/ml. at intervals of about 2 weeks.

The media used were as follows: Medium *A* contained in 1l.: 5·4g. of KH_2PO_4; 1·2g. of $(NH_4)_2SO_4$; 12g. of glucose; 0·4g. of $MgSO_4,7H_2O$; adjusted to pH7·1 with NaOH. Medium *B* contained the same constituents as medium *A*, and in addition 10g. of Difco Bactopeptone/l. and 1g. of Difco yeast extract/l.

The cells were harvested from the media by centrifuging at 15000*g* for 10min., washed twice and finally resuspended in the appropriate medium to give E_{500} 0·8 (Unicam SP. 600 spectrophotometer).

Preparation of spheroplasts. These were obtained by the penicillin method of Kaback & Stadtman (1966), except that medium *B* was used instead of Difco Penassay Broth. The spheroplasts were washed and suspended in medium *B* supplemented with 20% (w/v) sucrose to give E_{500} 0·8.

Determination of uptake of tetracyclines by E. coli. In some experiments, the uptake of oxytetracycline by *E. coli* cells was determined by a modification of the spectrophotometric method of Izaki & Arima (1965). After incubation of the cells at 37° for 1hr. with the drug (200μg./ml.), they were harvested by centrifugation and washed by resuspension in a large volume (50ml./10ml. of original culture) of 0·9% NaCl. After the cells had been resedimented, the oxytetracycline was extracted with 0·5N-$HClO_4$ (4ml./10ml. of original culture) at 0° for 30min. This method gave a slightly less efficient extraction of oxytetracycline than the method of Izaki & Arima (1965), but also gave much lower blank values from cells incubated without oxytetracycline. An equal volume of N-NaOH was added to the acid extract and the oxytetracycline content was determined by measuring E_{380}. Because of its insensitivity, this method of determining the uptake of the drug was only useful when the cells were cultured with 50μg. or more of oxytetracycline/ml. The use of [^{3}H]-tetracycline enabled the drug uptake to be followed at much lower concentrations and by highly resistant cells. The cells were usually incubated with 0·02μc of the radioactive drug/ml. and an additional quantity of unlabelled tetracycline (see the Results section). The cells were then harvested and washed as described above. The radioactive drug was then extracted from the cells by heating an aqueous suspension of the cells (5ml.) at 100° for 10min. This extracted 95–98% of the bound drug from the cells into the water. The denatured cells were removed by centrifugation and retained for protein determination. The supernatant (2·5ml.) was mixed with 17·5ml. of the scintillator solution described by Franklin & Godfrey (1965). The mixture was cooled to 0–1° and counted in a Packard Tri-Carb automatic liquid-scintillation spectrometer (model 526). The efficiency of counting, determined by the addition of an internal standard, was about 16% for ^{3}H. Non-specific absorption of the tetracyclines was determined by keeping the cell suspensions at 0–1° with the appropriate concentration of either oxytetracycline or [^{3}H]tetracycline.

Uptake of [^{14}C]leucine by E. coli. Cells suspended in medium *A* containing 1μc of [^{14}C]leucine/10ml. were incubated with shaking for 20min. at 37°. The suspension was then chilled in ice and samples (0·1ml.) were applied to strips of Whatman no. 120 filter paper for the determination of the incorporation of radioactivity into protein, as described by Franklin (1963).

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

RESULTS

Increase in resistance of resistant strains of E. coli *after preincubation with tetracyclines.* Strains R_1 and R_2 were incubated for 30min. in medium *B* with or without oxytetracycline or tetracycline (10μg./ml.). The cells were harvested, washed twice in a large volume of drug-free medium *A* and finally resuspended in medium *A* to give E_{500} 0·8. The effects of various concentrations of oxytetracycline or tetracycline on the incorporation of leucine into protein by the cells in 20min. was determined. Fig. 1 shows that preincubation of both the resistant strains with the tetracyclines produced a striking fall in the subsequent inhibition of protein biosynthesis by these drugs. Presumably this indicated a marked increase in cellular resistance to tetracyclines. Neither of the sensitive strains of *E. coli*, however, showed any increase in resistance to tetracyclines when preincubated with oxytetracycline or tetracycline.

Uptake of [^{3}H]tetracycline by sensitive and resistant E. coli. There were no significant differences in the uptake of radioactivity between sensitive and resistant strains, preincubated in the absence of the drug, when the tetracycline concentration was

200 μg./ml. in the second incubation. At 100 μg. and less of tetracycline/ml. in the second incubation, however, the resistant cells absorbed much less radioactivity than the sensitive cells (Table 1) Preincubation with tetracycline (10 μg./ml.) produced a marked fall in the subsequent absorption of radioactivity by both resistant strains. In contrast, the uptake of radioactivity by the sensitive cells was unaffected by preincubation with tetracycline. Thus the increase in resistance of the resistant cells produced by preincubation with tetracycline was accompanied by a striking fall in the absorption of the drug.

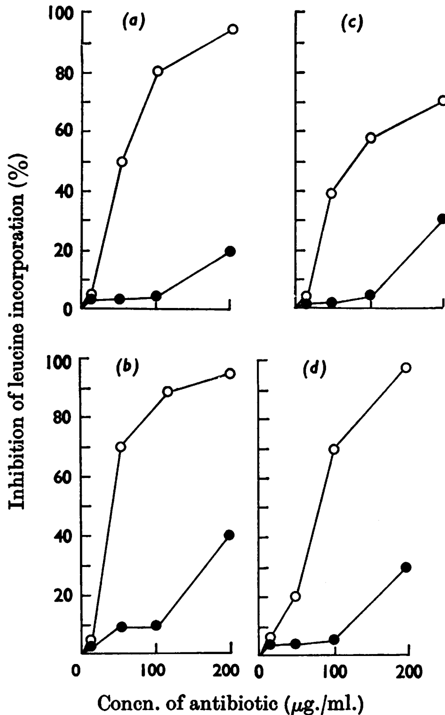

Fig. 1. Increase in resistance of resistant strains of *E. coli* after preincubation with tetracyclines. Strains R_1 (*a* and *b*) and R_2 (*c* and *d*) were preincubated for 30 min. in medium *B* (E_{500} 0·8, total vol. 10 ml.) with (●) or without (○) oxytetracycline (*a* and *c*) or tetracycline (*b* and *d*). After the cells had been washed, the ability of the drugs to inhibit [^{14}C]leucine incorporation into protein by cell suspensions in medium *A* in 20 min. was determined as described in the Materials and Methods section.

Fig. 2 indicates that the uptake of the drug by both sensitive and resistant cells not preincubated with tetracycline, in media containing 200 μg. of tetracycline/ml., was approximately linear. The absorption was completely inhibited by 2,4-dinitrophenol (1 mM) as noted by Izaki & Arima (1965), and also by iodoacetic acid (0·1 mM). Assuming a cell volume for *E. coli* of 10^{-12} ml., and also assuming that there are about 10^{10} cells/mg. of protein (Luria, 1960), it was calculated that, at 200 μg. of tetracycline/ml. in the medium, both sensitive cells and resistant cells not preincubated with the drug reached an intracellular concentration of tetracycline 40–50-fold greater than that of the medium in 30 min. At 10 μg. of the drug/ml., the sensitive cells achieved a concentration of tetracycline about 20-fold greater than that of the medium in 30 min. At 10 μg./ml., however, strain R_1 absorbed very little of the drug in 30 min.

Effect of length of preincubation time and drug concentration on the adaptive response of resistant E. coli. The experiments of Izaki *et al.* (1966) and Rachmeler & Unowsky (1966) involved exposure of resistant cells to tetracycline for at least 2 hr. in order to demonstrate a subsequent decrease in the absorption of the drug. The use of such long exposures to tetracycline increases the risk of selecting more resistant cells from a heterogeneous population. The present experiments described above involved preincubation with tetracycline for 30 min. only. No further increase in resistance of

Table 1. *Uptake of* [^{3}H]*tetracycline by sensitive and resistant strains of* E. coli

Cells were incubated with or without tetracycline (10 μg./ml.) for 30 min. (first incubation) in medium *B*; (E_{500} 0·8, total vol. 10 ml.). Tetracycline was then added to bring the concentration to that indicated in the Table, together with [^{3}H]tetracycline (0·02 μc/ml.), and the uptake of radioactivity in 30 min. (second incubation) was determined as described in the Materials and Methods section.

Concn. of tetracycline in second incubation (μg./ml.)	Tetracycline absorbed in second incubation (μg./mg. of protein)					
	First incubation without tetracycline			First incubation with tetracycline		
Strain ...	R_1	R_2	S_1	R_1	R_2	S_1
200	98	96	91	2·25	1·77	96
100	39	35	61	0·04	0·03	63
50	7	8	17	0·02	0·01	25
10	0·09	0·06	2	0·03	0·02	2

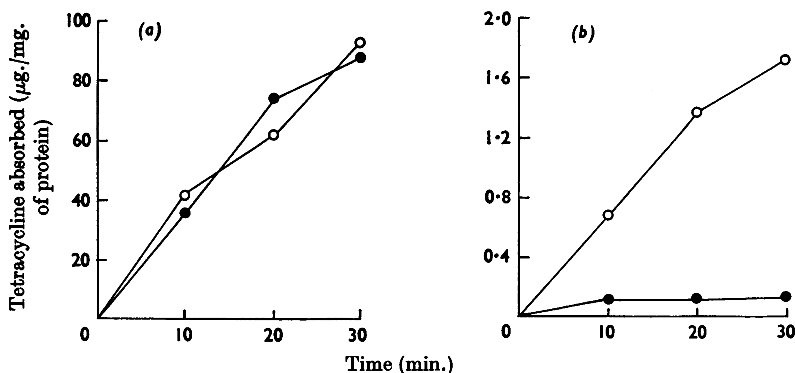

Fig. 2. Uptake of [³H]tetracycline by sensitive and resistant strains of *E. coli*. Strains S_1 (○) and R_1 (●) were suspended in medium *B* (E_{500} 0·8, total vol. 10 ml.) containing 200 μg. of tetracycline/ml. (*a*) or 10 μg. of tetracycline/ml. (*b*), and also 0·02 μc of [³H]tetracycline/ml. in both cases. After the appropriate interval the cells were harvested and the uptake of radioactivity was determined as described in the Materials and Methods section. The radioactivity values were converted into μg. of tetracycline.

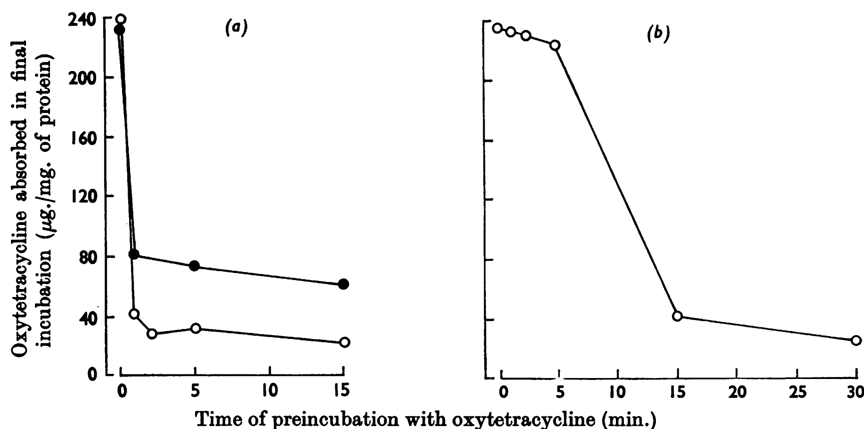

Fig. 3. Effect of length of preincubation with oxytetracycline on the adaptive response of resistant *E. coli*. (*a*) R_1-strain (○) and R_2-strain (●) cells were incubated in medium *B* (E_{500} 0·8, total vol. 10 ml.) containing 10 μg. of oxytetracycline/ml. for the indicated time (*t* min.). After being harvested and twice washed in drug-free medium, the cells were incubated in drug-free medium for a further $30-t$ min., and 200 μg. of oxytetracycline/ml. was then added for the determination of the uptake of the drug in 60 min. (*b*) R_1-strain cells were incubated in medium *B* containing 10 μg. of oxytetracycline/ml.; oxytetracycline was immediately added to give a concentration of 200 μg./ml. The uptake of oxytetracycline in 60 min. was determined as described in the Materials and Methods section.

the resistant strains could be detected on further incubation in medium *B*, though occasionally, in experiments in which the minimal medium *A* was used, the increase in resistance was not complete until after 60 min. incubation with drug. It was decided to determine the minimum period of exposure of resistant cells to antibiotic in medium *B* that was necessary to produce a significant fall in the subsequent absorption of drug by the cells. The results given below were obtained with oxytetracycline both in the preincubation period and in the period during which the absorption of the drug was measured. Very similar results have been obtained with [³H]tetracycline. Two different types of experiments were carried out. In the first series the resistant cells were incubated in medium *B* containing 10 μg. of oxytetracycline/ml. for various periods (*t* min.). The cells were then harvested, washed twice in drug-free medium and incubated in drug-free medium for $30-t$ min. Oxytetra-

cycline (200 μg./ml.) was then added, and the uptake of the drug by the cells in 60 min. was determined. Fig. 3(*a*) shows that exposure to oxytetracycline for 1 min. markedly depressed the subsequent absorption of the drug by R_1-strain cells. Strain R_2 required a somewhat longer period of preincubation with oxytetracycline to produce a similar effect.

In the second series of experiments, the resistant cells (R_1 strain) were incubated in medium *B* containing 10 μg. of oxytetracycline/ml. for various periods followed by the immediate addition of further oxytetracycline to give a final concentration of 200 μg./ml. The uptake of the drug in 60 min. was again determined. Fig. 3(*b*) shows that preincubation with oxytetracycline for 5 min. or less, immediately before the uptake period, resulted in little change in the ability of the cells to absorb the drug. Preincubation for 15 min., however, produced a marked fall in the absorption of oxytetracycline.

Table 2 shows that preincubation of R_1-strain cells for 30 min. in medium containing 0·05 μg. of oxytetracycline/ml. resulted in a significant fall in the subsequent absorption of the drug. The effect obtained with 0·5 μg. of oxytetracycline/ml. was as great as that with 10 μg./ml. It is noteworthy that an acid-degradation product of oxytetracycline, β-apo-oxytetracycline, which has less than 10% of the antibacterial activity of oxytetracycline (T. J. Franklin, unpublished work), also induced a striking fall in the absorption of oxytetracycline by R_1-strain cells (Table 2) and a corresponding increase in resistance to the inhibition of protein biosynthesis by oxytetracycline (not shown). The effect of this derivative on R_2-strain cells was not studied.

Persistence of decreased oxytetracycline absorption in preincubated resistant cells. Strain-R_1 cells were incubated in medium *A* containing 10 μg. of oxytetracycline/ml. for 30 min. The cells were harvested, washed twice in a large volume of drug-free medium and resuspended in drug-free medium *A* to give E_{500} 0·8. The uptake of oxytetracycline was then determined either immediately or after a further 1 hr. or 2 hr. incubation without the drug. Before the drug (200 μg./ml.) was added at 1 hr. or 2 hr., the E_{500} values of the cultures were determined and adjusted to 0·8 by dilution with medium *A* at 37°. Table 3 shows that, even after 2 hr. incubation in drug-free medium after preincubation with oxytetracycline, there was no significant increase in the uptake of drug by the resistant cells. In a further experiment, however, resistant cells that had been preincubated with oxytetracycline were washed twice with drug-free medium and subcultured (5%, v/v, inoculum) in drug-free medium *A* for 16 hr. at 37°. After 16 hr. the cells absorbed as much oxytetracycline as resistant cells not preincubated with drug (Table 3). The ability of the cells subcultured for 16 hr. in the absence of oxytetracycline to respond to preincubation with the drug, however, remained unimpaired.

Table 2. *Effect of variation in the concentrations of oxytetracycline and β-apo-oxytetracycline on the subsequent absorption of oxytetracycline by resistant* E. coli

R_1-strain cells suspended in medium *B* (E_{500} 0·8, total vol. 10 ml.) were incubated for 30 min. with the indicated concentrations of tetracycline derivatives. The subsequent uptake of oxytetracycline (200 μg./ml. of medium) by the cells in 60 min. was determined.

Compound in first incubation	Concn. in first incubation (μg./ml.)	Oxytetracycline absorbed in second incubation (μg./mg. of protein)
None	—	210
Oxytetracycline	10	19
	5	24
	0·5	14
	0·1	35
	0·05	134
β-Apo-oxy-tetracycline	10	9
	5	30
	0·5	92
	0·1	225
	0·05	231

Table 3. *Effect of incubation of resistant* E. coli *in drug-free medium after preincubation with oxytetracycline*

R_1-strain cells were preincubated for 30 min. in medium *A* (E_{500} 0·8, total vol. 10 ml.) with or without oxytetracycline (10 μg./ml.). After being washed, the cells were incubated in drug-free medium for the indicated times before the addition of 200 μg. of oxytetracycline/ml. for the determination of the absorption of the drug in 60 min. The 16 hr. value was obtained by subculturing washed preincubated cells for 16 hr. in drug-free medium *A* (5%, v/v, inoculum); the cells were then harvested, washed and resuspended in medium A containing 200 μg. of oxytetracycline/ml.

Time grown in drug-free medium after preincubation (hr.)	Oxytetracycline absorbed in final incubation (μg./mg. of protein)	
	Cells preincubated with drug	Cells preincubated without drug
0	37	167
1	18	156
2	19	156
16	186	165

Effects of chloramphenicol, 5-fluorouracil and proflavine on the response of resistant cells to preincubation with tetracycline. (*a*) Chloramphenicol. Preliminary experiments showed that 50 μg. of this antibiotic/ml. completely inhibited protein biosynthesis in R_1-strain cells. R_1-strain cells were therefore incubated in medium *B* containing 10 μg. of tetracycline/ml. with or without 50 μg. of chloramphenicol/ml. The cells were harvested and washed twice by resuspension in 0·9% sodium chloride, and then incubated in medium *A* containing 200 μg. of tetracycline/ml. and 0·02 μc. of [^{3}H]tetracycline/ml. Table 4 shows that the uptake of radioactivity in 30 min. by cells preincubated with tetracycline in the presence of chloramphenicol was not significantly different from that by cells preincubated in drug-free medium. Thus chloramphenicol effectively blocked the response of resistant cells to pretreatment with tetracycline.

(*b*) 5-Fluorouracil. This compound is said to be incorporated to some extent into messenger RNA (Horowitz & Chargaff, 1964), and, when added to *E. coli* cells in the presence of inducers of β-galactosidase, may result in the production of a protein that is serologically related to β-galactosidase but without its enzymic function (Bussard, Naono, Gros & Monod, 1960). R_1-strain cells were preincubated for 30 min. in medium *A* containing 10 μg. of tetracycline/ml. with or without 50 μg. of 5-fluorouracil/ml. After being harvested and washed with 0·9% sodium chloride, the cells were incubated in medium *A* containing 200 μg. of tetracycline/ml. and 0·02 μc of [^{3}H]tetracycline/ml. for 30 min. at 37°. Table 4 shows that 5-fluorouracil produced only a slight depression in the adaptive response of the cells to preincubation with tetracycline.

(*c*) Proflavine. A number of workers have found that this compound suppresses DNA-directed RNA synthesis and induced enzyme formation in bacteria (Waring, 1966; Kepes, 1963). In the present investigation the effect of proflavine on the ability of R_1-strain cells to respond to preincubation with tetracycline was tested. After 30 min. preincubation in medium *A* containing 10 μg. of tetracycline/ml. with or without proflavine (20 μg./ml.), the cells were harvested and washed twice before being resuspended in medium *A* containing 200 μg. of tetracycline/ml. and 0·02 μc of [^{3}H]tetracycline/ml. Proflavine almost completely prevented the adaptive response of the cells to preincubation (Table 4).

Uptake of [^{3}H]*tetracycline by spheroplasts from resistant cells.* Spheroplasts prepared from R_1-strain cells (preparation A) were preincubated in medium *B* supplemented with 20% (w/v) sucrose, with or without 10 μg. of tetracycline/ml., for various times before the addition of 200 μg. of tetracycline/ml. and 0·02 μc of [^{3}H]tetracycline/ml. Preincubation

Table 4. *Effects of chloramphenicol, 5-fluorouracil and proflavine on the response of resistant* E. coli *to preincubation with tetracycline*

R_1 strain cells were incubated for 30 min. in medium *A* (E_{500} 0·8, total vol. 10 ml.) containing the indicated additions. The cells were harvested, washed and incubated for a further 30 min. in medium *A* containing 200 μg. of tetracycline/ml. and 0·02 μc of [^{3}H]tetracycline/ml.

Expt. no.	Additions to preincubation	Tetracycline absorbed in second incubation (μg./mg. of protein)
I	None	60·2
	Tetracycline (10 μg./ml.)	5·3
	Chloramphenicol (50 μg./ml.)	63·2
	Tetracycline (10 μg./ml.) + chloramphenicol (50 μg./ml.)	59·7
II	None	103·0
	Tetracycline (10 μg./ml.)	4·1
	5-Fluorouracil (50 μg./ml.)	120·2
	Tetracycline (10 μg./ml.) + 5-fluorouracil (50 μg./ml.)	8·6
	Proflavine (20 μg./ml.)	66·1
	Tetracycline (10 μg./ml.) + proflavine (20 μg./ml.)	57·1

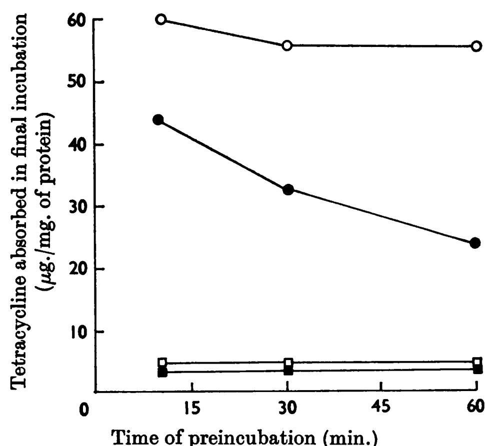

Fig. 4. Uptake of [^{3}H]tetracycline by spheroplasts from resistant *E. coli*. Spheroplasts from R_1-strain cells were preincubated in medium *B* (E_{500} 0·8, total vol. 10 ml.) supplemented with 20% (w/v) sucrose for the indicated time with (●) or without (○) 10 μg. of tetracycline/ml. before the addition of 200 μg. of tetracycline/ml. and 0·02 μc of [^{3}H]tetracycline/ml. for the determination of the uptake of the radioactive drug in 30 min. Uptake of [^{3}H]-tetracycline by spheroplasts prepared from R_1-strain cells incubated with 10 μg. of tetracycline/ml. before and during conversion into spheroplasts and subsequently preincubated with (■) and without (□) 10 μg. of tetracycline/ml. immediately before the addition of 200 μg. of tetracycline/ml. and 0·02 μc of [^{3}H]tetracycline/ml. is also shown.

of the spheroplasts with tetracycline resulted in a decline in the subsequent uptake of the radioactive drug (Fig. 4), though the adaptive response developed much more slowly than in corresponding experiments with intact R_1-strain cells. Spheroplasts prepared from R_1-strain cells that had been incubated with $10\mu g$. of tetracycline/ml. for 2hr. before and also during the $2\frac{1}{2}$hr. treatment with penicillin G (preparation B) absorbed little of the drug when incubated for 30min. with $200\mu g$. of tetracycline/ml. and $0{\cdot}02\mu c$ of [^{3}H]tetracycline/ml. Preincubation of this preparation of spheroplasts with $10\mu g$. of tetracycline/ml. had no effect on the subsequent uptake of the radioactive drug.

DISCUSSION

The work described in this paper supports the view that resistance to the tetracycline antibiotics in *E. coli* bearing transferable resistance markers (R-factors), either for the tetracyclines alone or in an association with those for a number of other drugs, depends on a decreased permeability for the tetracyclines compared with sensitive cells. I have also confirmed the observation of Izaki *et al.* (1966) that, in *E. coli* bearing an R-factor for tetracycline resistance, incubation with low concentrations of tetracyclines results in a considerable increase in cellular resistance to these drugs. The increase in resistance was associated with a marked fall in the absorption of tetracyclines by the cells.

The resistant cells thus exhibited two levels of resistance. When they were cultured for an extended period in the absence of the tetracyclines and then challenged with these antibiotics they exhibited what may be termed 'low-level' resistance, i.e. a concentration of $10\mu g$. of tetracycline/ml. had little or no effect on protein biosynthesis, and one of $50\mu g$./ml. produced about 50% inhibition. At concentrations of tetracycline of $100\mu g$./ml. and below, the resistant cells absorbed much less of the drug than did sensitive cells. After incubation with subinhibitory concentrations of tetracyclines for short periods, the resistant cells became very much more resistant, $200\mu g$. of the drug/ml. then producing less than 45% inhibition of protein biosynthesis. The amount of drug absorbed (at $200\mu g$./ml.) was six- to ten-fold less than that by cells preincubated without tetracyclines.

The effectiveness of extremely low concentrations of the drug and very short incubation times with oxytetracycline in producing adaptation provides good evidence against the possibility that more resistant cells were being selected from a heterogeneous population of cells of differing degrees of resistance.

The results obtained with chloramphenicol, which inhibits protein biosynthesis directly, and proflavine, which prevents the transcription of DNA into RNA and hence inhibits protein biosynthesis indirectly, suggest that the decreased uptake of tetracyclines after preincubation with these antibiotics may be the result of the induction of an enzyme or enzymes that inhibit the tetracycline-absorptive mechanism of the unadapted cells. Since even unadapted resistant cells absorbed much less of the tetracyclines at low drug concentrations than did sensitive cells, there may be a certain amount of constitutive synthesis of this proposed enzyme. It is therefore suggested that, when resistant cells are incubated with low concentrations of the tetracyclines, sufficient of the drug enters the cells to induce further enzyme synthesis, though the drug concentration does not rise to an inhibitory level. The enzyme system would presumably be encoded for in the DNA of the resistance-determining R-factor. The structural features of the tetracycline molecule required for the induction of increased resistance differ from those required for the inhibition of cell growth, since a biologically inactive derivative, β-apo-oxytetracycline, was also effective in bringing about the adaptation of resistant cells.

The explanation of the persistence of the decreased absorption of oxytetracycline by resistant cells after growth for 2hr. in drug-free medium is uncertain. Very small quantities of antibiotic bound to the cells, despite thorough washing in drug-free medium, could have resulted in continued induction of the proposed tetracycline-transport-antagonizing enzyme system. However, preliminary experiments have been carried out in which tetracycline-adapted resistant cells were cultured for 2hr. in tetracycline-free medium containing either chloramphenicol or proflavine. The cells cultured in the presence of these inhibitors of enzyme production showed no increase in the subsequent uptake of [^{3}H]tetracycline. It therefore seems likely that the tetracycline-induced enzyme system has a fairly long half-life, though further evidence on this point is required. Possible persistence of traces of oxytetracycline bound to washed cells might, however, explain the apparent effectiveness of very short exposures (5min. or less) of resistant cells to the drug in bringing about adaptation when the cells were allowed to grow for a further period in drug-free medium.

The failure of 5-fluorouracil to affect significantly the adaptive response of resistant cells to tetracycline may have been due to inadequate incorporation of 5-fluorouracil into messenger RNA; the use of a uracil-requiring auxotroph bearing an R-factor for tetracycline resistance might be an advantage in this context. Alternatively, even if the analogue

was appreciably incorporated into messenger RNA by R_1-strain cells, this does not necessarily result in the production of enzymes with significantly diminished activity (Brockman & Anderson, 1963).

The conversion of intact bacterial cells into the spheroplast form involves a certain amount of damage to the cell membrane (Bishop, Roche & Nisman, 1964). If the membrane is involved in the adaptive response of resistant cells to treatment with tetracycline, membrane damage could explain the impaired response of R_1-strain spheroplasts to preincubation with tetracycline compared with that of intact cells. Since the ability to exclude tetracycline remained high in spheroplasts derived from cells treated with the drug before and during conversion into spheroplasts, it appears that, once induced, the enzyme system that antagonizes tetracycline absorption is unaffected by any damage suffered by the membrane.

The mechanism of the active absorption of the tetracyclines by sensitive cells and by unadapted resistant cells remains obscure. More information is urgently needed about this fascinating phenomenon, which can result in the intracellular accumulation of very large amounts of tetracyclines. Such information may lead to an understanding of the antagonism of tetracycline absorption that occurs in resistant cells, and this in turn offers the prospect of eventually overcoming this type of bacterial resistance to tetracyclines.

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