Effect of Osmotic Shock on Tetracycline Resistance in *Escherichia coli* Bearing an R-Factor

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(Received 27 August 1970)

1. *Escherichia coli* with an R-factor conferring resistance to tetracycline was induced to high-degree resistance by pre-exposure to the antibiotic. The degree of resistance was drastically lowered by subjecting the cells to osmotic shock. 2. Resistance to tetracycline was rapidly restored by incubating the shocked cells in a glucose-salts medium containing shock proteins prepared from tetracycline-sensitive and -resistant cells. Resistance was also restored by incubating the cells in a complex medium without shock protein. 3. The initial recovery of resistance was followed by a secondary fall in resistance when the cells were cultured in complex medium; this secondary fall was largely prevented by the addition of a low concentration (10 µg/ml) of tetracycline to cells. The secondary fall was significantly less in shocked *E. coli* cells harbouring a mutant R-factor in which tetracycline resistance is largely constitutive. 4. Tetracycline resistance was also transiently depressed by treating R-factor-bearing cells with EDTA in tris buffer. 5. The significance of these results in relation to the mechanism of tetracycline resistance in R-factor-bearing cells is discussed.

There is evidence that tetracycline resistance determined by R-factors in *Escherichia coli* may be due to a specific antagonism of the tetracycline-accumulation process that occurs in tetracycline-sensitive cells; the anti-accumulation mechanism is apparently partially inducible by the tetracycline antibiotics (Izaki, Kiuchi & Arima, 1966; Franklin, 1967). In the uninduced state the R-factor-bearing cells (R^+) are partially resistant to tetracycline and absorb less antibiotic than sensitive (R^-) cells. Exposure to subinhibitory concentrations of tetracycline results in a prompt increase in resistance which is associated with a further fall in the accumulation of tetracycline. The uptake of tetracycline by sensitive cells, which may be dependent on a membrane-associated process, is unaffected by 'osmotic shock' (Franklin & Higginson, 1970), a procedure which liberates lipoproteins, lipopolysaccharides and various proteins from the surface layers of Gram-negative bacteria (Heppel, 1968; Winshell & Neu, 1970). However, it was conceivable that the resistance mechanism in R^+ cells might be damaged by osmotic shock if it were located either on the outer face of the membrane or in the superficial layers of the cell. In the present paper we report on the depression and subsequent recovery of tetracycline resistance in R^+ *E. coli* after osmotic shock.

MATERIALS AND METHODS

Radioactive chemicals. L-[1- 14 C]Leucine (312 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Chemicals. Tetracycline hydrochloride was obtained from Pfizer Ltd., Sandwich, Kent, U.K.

Strains of *E. coli* and culture conditions. *E. coli* strain R_1 was the strain described by Franklin & Godfrey (1965) and carries an R-factor for tetracycline resistance. Strain MR_1 was the mutant form of strain R_1 used by Franklin & Cook (1971). The tetracycline-sensitive strain, 4018/62, was that used by Franklin (1967). The organisms were maintained on nutrient agar. 'Starter' cultures were obtained by loop transfer to the appropriate medium followed by incubation for 6-9 h at 37°C. Subsequent cultures were made by using 5% (v/v) inocula from 'starter' cultures into fresh media. The media used were as follows. Medium A contained in 1 litre: 5.4 g of KH_2PO_4 ; 1.2 g of $(NH_4)_2SO_4$; 12 g of glucose; 0.4 g of $MgSO_4 \cdot 7H_2O$; adjusted to pH 7.1 with NaOH. Medium B contained the same constituents as medium A and in addition 10 g of Difco Bacto-peptone/l and 1 g of Difco yeast extract/l. The cells were harvested by centrifuging at 15000 g for 10 min.

Osmotic shock. This was carried out as described by Heppel (1968) for cells in the exponential phase of growth. This procedure has little or no effect on cell viability.

Uptake of [14 C]leucine by *E. coli*. Cells, suspended in medium A to give E_{500} 0.8, were incubated with 0.1 µCi of [14 C]leucine/ml and unlabelled leucine to give 0.2 mM for

20 min at 37°C. Samples (0.2 ml) were removed at intervals and applied to strips of Whatman no. 120 filter paper for the determination of the incorporation of radioactivity into protein as described by Franklin (1963). Under these conditions the incorporation of radioactivity into protein was linear for at least 60 min.

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

Preparation of shock protein. A large volume (6 litres) of medium B was inoculated (5%, v/v) with the appropriate strain of cells and growth permitted until mid-log phase. The cells were harvested and shocked (Heppel, 1968) and the shock fluid, i.e. the fluid remaining after harvesting the cells from dilute $MgCl_2$ solution, was concentrated to approximately one-tenth of its original volume by using a Diaflo apparatus with a PM10 membrane. The concentrated shock fluid was freeze-dried and the solid material dissolved in a small volume of water to give a protein concentration of about 10 mg/ml.

RESULTS

Effect of osmotic shock on the resistance of E. coli strain R₁ to tetracycline. *E. coli* strain R₁ cells were induced to high-degree tetracycline resistance by growing them to mid-exponential phase in the presence of 10 µg of tetracycline/ml of medium B. The effect of osmotic shock on the degree of resistance was tested by measuring the inhibition of protein synthesis produced by high concentrations of tetracycline after resuspending the cells in medium A. Table 1 shows that unshocked cells were only slightly inhibited by 50 and 100 µg of tetracycline/ml; in contrast the shocked cells were markedly inhibited by these concentrations of drug. The amount of protein synthesis in the absence of tetracycline was sharply depressed by osmotic shock although the extent of the depression varied considerably in different experiments. In contrast, the increase in sensitivity to tetracycline after osmotic shock was relatively constant; thus the percentage inhibition of protein synthesis in shocked cells by

100 µg of tetracycline/ml over 20 min varied between 70 and 95%.

Effect of shock protein on the degree of tetracycline resistance in shocked cells. Anraku (1967) reported that the addition of protein obtained from the shock fluid restores the uptake of galactose by osmotically shocked *E. coli* cells. This implies that a protein involved in galactose uptake that is removed from the cells by the procedure of osmotic shock spontaneously reassociates with specific cellular sites to re-establish the transport process. It was therefore of interest to determine whether shock protein had any effect on tetracycline resistance in shocked cells. Table 2 shows that shock protein isolated from strain R₁ cells partially restored the resistance of shocked strain R₁ cells to 100 µg of tetracycline/ml. Preincubation at 37°C was essential for this effect. However, it was also found that shock protein isolated from tetracycline-sensitive R⁻ *E. coli* was equally effective in restoring tetracycline resistance to shocked strain R₁ cells. Clearly therefore, the ability of the shock protein to re-establish resistance was apparently not due to a specific component determined by the tetracycline-resistance genes of the R-factor harboured by strain R₁ cells.

Effect of recovery in growth media on the degree of tetracycline resistance in E. coli strain R₁ after osmotic shock. It was noted that in addition to restoring tetracycline resistance, preincubation with the shock-protein preparations from both tetracycline-sensitive and -resistant cells stimulated protein synthesis in the shocked cells. In the absence of shock protein, the composition of the recovery medium was found to have a marked effect upon the rate of restoration of tetracycline resistance. Thus Table 2 also shows that there was a marked increase in tetracycline resistance in cells recovering from osmotic shock in medium B alone. Fig. 1 compares the rate of recovery of tetracycline resistance in cells incubated in media A and B. The

Table 1. *Effect of osmotic shock on tetracycline resistance in R-factor-bearing E. coli cells*

After osmotic shock, strain R₁ cells preinduced to high-degree tetracycline resistance were resuspended in medium A to give E_{500} 0.8 and containing [¹⁴C]leucine and the indicated concentrations of tetracycline. The amount of radioactivity incorporated into protein was determined after 20 min incubation at 37°C.

	Concn. of tetracycline (µg/ml)	Radioactivity incorporated into protein in 20 min (c.p.m./mg)	% inhibition of incorporation of radioactivity
Control cells	0	24 963	—
	50	23 607	5.5
	100	21 846	12.5
Shocked cells	0	4983	—
	50	3030	39.1
	100	944	81.0

Table 2. *Effect of shock protein on the tetracycline resistance of osmotically shocked E. coli cells*

Shock protein prepared from both R_1 strain cells and also tetracycline-sensitive R^- cells was added (500 $\mu\text{g/ml}$) to osmotically shocked strain R_1 cells suspended in medium A (E_{500} 0.8) and the suspension incubated at 0 or 37°C for 15 min. Tetracycline (100 $\mu\text{g/ml}$) and [^{14}C]leucine were then added and the uptake of radioactivity into cell protein was determined after 20 min at 37°C. In systems 10 and 11 the shocked cells were incubated in medium B for 15 min and then resuspended in medium A for challenge with tetracycline. The values in parentheses indicate the system against which the percentage inhibition by tetracycline was calculated.

System	Shock protein and source	Preincubation temp. (°C)	Tetracycline in second incubation	Radioactivity incorporated into cell protein (c.p.m./mg)	% inhibition of incorporation of radioactivity
1	—	37	—	3004	—
2	—	37	+	884	70.6 (1)
3	—	0	+	650	78.2 (1)
4	+ R_1	37	—	10850	—
5	+ R_1	37	+	6232	42.5 (4)
6	+ R_1	0	+	493	83.4 (4)
7	+ R^-	37	—	11100	—
8	+ R^-	37	+	6480	41.6 (7)
9	+ R^-	0	+	380	84.1 (7)
10	— in medium B	37	—	12240	—
11	— in medium B	37	+	7260	40.0 (10)

rate of recovery of tetracycline resistance appeared to be associated in some way with the ability of the medium to stimulate protein synthesis in the shocked cells. It seems likely therefore that the ability of the shock protein to accelerate the restoration of tetracycline resistance may have been secondary to a stimulation of the biosynthetic activity of the shocked cells.

Effect of prolonged incubation on tetracycline resistance in strain R_1 cells after osmotic shock. We extended the period of recovery of shocked strain R_1 cells in medium B to 4 h. The initial recovery of resistance which was complete after about 30 min in medium B, was followed by a marked decline in resistance, over the next 3.5 h (Fig. 2). When the E_{500} of the culture was measured during the recovery phase, a lag was observed during the first 30 min after shock with a slight fall in E_{500} . Thereafter the E_{500} of the culture increased rapidly with a period of exponential growth. Growth ceased about 4 h after osmotic shock (Fig. 3). It seemed possible that the decline in tetracycline resistance after the initial recovery after osmotic shock might be explained in the following way. If it is assumed that the initial recovery of resistance depends on the completion of the components of the resistance mechanism partially synthesized before shock and the translation of mRNA synthesized before shock, the subsequent decline in resistance might be due to a progressive dilution of the resistance components, i.e. the gene products of the tetracycline-resistance marker, as the cells grow and divide. Osmotic shock may remove trace amounts of

Fig. 1. Influence of composition of medium on recovery of tetracycline resistance in osmotically shocked *E. coli* cells. After osmotic shock, the cells were resuspended to give E_{500} 0.8 in either medium A (●) or medium B (○) and incubated at 37°C. At the indicated intervals the cells were harvested, resuspended in fresh medium A and the effect of 100 μg of tetracycline/ml on [^{14}C]leucine incorporation into protein was determined over 20 min.

tetracycline from the cells, thus precluding the continued induction of the tetracycline-resistance genes during the recovery process. We have attempted to test this hypothesis in several ways.

Reinduction of tetracycline resistance in strain R_1

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Fig. 2. Effect of prolonged incubation in a complex growth medium on tetracycline resistance in osmotically shocked *E. coli* cells. After shocking, the cells were resuspended to give E_{500} 0.8 in medium B and incubated at 37°C. At the indicated intervals, the cells were harvested, resuspended in medium A and the effect of 100 µg of tetracycline/ml on protein synthesis was measured as before. The arrow indicates the degree of inhibition produced by this concentration of tetracycline in unshocked cells.

Fig. 3. Growth of osmotically shocked *E. coli* cells in a complex medium. After shocking the cells were resuspended to give E_{500} 0.8 in medium B and then incubated at 37°C.

cells by tetracycline during recovery from osmotic shock. Osmotically shocked strain R₁ cells were incubated in medium B for 30 min. The culture was divided into two and tetracycline (10 µg/ml) was added to one half. The incubation was continued for 3.5 h and the tetracycline resistance of the two cultures was compared at intervals. Fig. 4 shows that the decline in resistance was markedly decreased by the addition of tetracycline after 30 min.

Effect of osmotic shock on E. coli bearing a mutant R-factor. Further evidence that R-factor-mediated tetracycline resistance is inducible is provided by the isolation of an apparently constitutive type of resistance determined by a mutant R-factor (Franklin & Cook, 1971). *E. coli* cells bearing this R-factor exhibit tetracycline resistance at a high degree even without pre-exposure to low concentrations of tetracycline. Fig. 5 shows the effect of subjecting *E. coli* cells bearing this mutant R-factor to osmotic shock. As with the wild-type R-factor, osmotic shock drastically depressed the resistance of the cells to tetracycline and on transfer to medium B there was a rapid recovery of resistance. Prolonged incubation of the cells in medium B resulted in a decline in resistance only to the level observed in unshocked

Fig. 4. Effect of the addition of an inducing concentration of tetracycline on tetracycline resistance in *E. coli* cells recovering from osmotic shock. After shocking the cells were resuspended to give E_{500} 0.8 in medium B and incubated at 37°C for 30 min. The vertical arrow indicates the addition of 10 µg of tetracycline/ml to half of the culture (●). At various intervals the cells were resuspended in medium A and the inhibition of protein biosynthesis by 100 µg of tetracycline/ml was measured as before. The horizontal arrow indicates the degree of inhibition by this concentration of tetracycline in unshocked cells.

Fig. 5. Effect of osmotic shock on tetracycline resistance in *E. coli* cells bearing a mutant R-factor. Cells harbouring an R-factor, in which repressor control over tetracycline resistance is apparently incomplete (mutant MR₁, see the Materials and Methods section), were osmotically shocked, resuspended to give E_{500} 0.8 in medium B and incubated at 37°C. At intervals the cells were harvested, resuspended in medium A and the amount of inhibition of protein biosynthesis produced by 100 µg of tetracycline/ml was measured as before. The arrow indicates the amount of inhibition produced by this concentration of tetracycline in unshocked cells.

Fig. 6. Effect of EDTA-tris treatment on the amount of inhibition of protein synthesis by tetracycline in R-factor-bearing *E. coli* cells. The cells were preinduced to high-degree tetracycline resistance and half of the culture was subjected to the EDTA-tris treatment of Heppel (1968). The cells were then resuspended in medium A and the effect of various concentrations of tetracycline on protein synthesis was measured as before. ○, Untreated cells; ●, cells treated with EDTA-tris.

cells. The initial recovery of resistance after osmotic shock appeared to overshoot the degree of resistance in unshocked cells. However, we have found that the degree of resistance in cells bearing the mutant R-factor varied somewhat in different experiments.

Effect of EDTA treatment on tetracycline resistance in *E. coli*. It was possible that the initial EDTA-tris-sucrose stage in the osmotic-shock procedure may have had a damaging effect on tetracycline resistance, since EDTA is known to increase the sensitivity of *E. coli* to a number of antibiotics to which it is intrinsically resistant (Leive, 1965). Exponential-phase *E. coli* strain R₁ cells were subjected to the EDTA treatment described by Heppel (1968). After resuspension in medium A the rate of incorporation of [¹⁴C]leucine was measured in the presence of various concentrations of tetracycline. Fig. 6 shows that EDTA treatment strikingly depressed the resistance of the R-factor-bearing cells to the antibiotic. Recovery of resistance also occurred in EDTA-treated cells when they were resuspended in medium B although there was no subsequent decline in resistance after 1 h in this medium (not shown).

DISCUSSION

The effects of the combined EDTA-osmotic-shock procedure on Gram-negative bacteria are undoubtedly many and complex. The procedure was devised by Neu & Heppel (1965) to release so-called 'surface' or periplasmic enzymes from bacterial cells and has been exploited by a number of workers in the isolation of proteins thought to be involved in the accumulation by cells of various solutes (Anraku, 1968; Pardee, 1968). The enzyme that catalyses the transfer of an adenylyl residue to streptomycin in R-factor-bearing *E. coli* cells resistant to this antibiotic is also released by osmotic shock (Ozanne, Benveniste, Tipper & Davies, 1969).

The preliminary treatment of the cells with EDTA and tris buffer apparently releases significant amounts of lipid and lipopolysaccharide (Winshell & Neu, 1970). The subsequent dispersal into an ice-cold dilute solution of magnesium chloride releases further lipid and lipopolysaccharide from the cells and also a number of proteins. By using polyacrylamide-gel electrophoresis we have detected at least 17 components in the concentrated shock fluid, i.e. the supernatant obtained from

centrifuging the suspension of *E. coli* cells in magnesium chloride solution (T. J. Franklin & S. J. Foster, unpublished work).

It has been shown that EDTA-osmotic shock has no significant effect on the uptake of tetracycline by *E. coli* cells sensitive to tetracycline (Franklin & Higginson, 1970). However, in view of the probable involvement of the cell membrane in the uptake of tetracycline by *E. coli* (T. J. Franklin, unpublished work) it was conceivable that this process might be antagonized in tetracycline-resistant cells by some factor(s) either loosely associated with the membrane in the periplasmic space or with the surface layers of the cell, which would therefore be susceptible to EDTA-osmotic shock. The present results clearly show that EDTA-osmotic shock markedly interferes with R-factor-mediated tetracycline resistance. Since pretreatment of the R-factor-bearing cells with EDTA in the presence of tris buffer also increased their sensitivity to tetracycline it seems likely that the initial EDTA-sucrose-tris stage of the osmotic shock was mainly responsible for the increased tetracycline-sensitivity of the osmotically shocked cells. While this work was in progress Neu & Winshell (1970) reported that EDTA did not significantly increase the sensitivity of *Salmonella* bearing an R-factor to tetracycline as measured by the growth of the cells on plates and in liquid cultures. However, the conditions of their experiments would have precluded the detection of the transient decrease in resistance that we have observed. The nature of the specific damage to the cells brought about by EDTA-osmotic shock which causes an increase in tetracycline resistance remains unknown. The EDTA stage might for example cause the loss of an anionic effector substance, shielding cationic sites on the cell envelope that may be involved in the uptake of tetracycline by *E. coli* cells. In addition the osmotic shock stage may release enzymes concerned with the biosynthesis of the effector substance.

The rapid initial increase in tetracycline resistance when the shocked cells were transferred to a medium encouraging a high rate of protein synthesis is consistent with the known ability of such cells to recover their metabolic activity in suitable growth media (Heppel, 1968). The subsequent restoration of tetracycline resistance, often indeed overshooting the pre-shock degree of resistance, implies a rapid synthesis of the components of the resistance mechanism. The evidence we have obtained so far suggests that the eventual decline of resistance as the cells begin exponential growth may be due to the progressive dilution of the resistance mechanism, i.e. the products of the gene(s) of the R-factor conferring tetracycline resistance

by other cellular constituents. Thus the secondary decline of resistance was markedly decreased when an inducing concentration of tetracycline was added to the cells in the recovery medium, possibly because further transcription and translation of the tetracycline-resistance genes occurred. Further, the secondary fall in resistance was much less in shocked cells harbouring a mutant R-factor in which repressor control over tetracycline resistance appears to be incomplete (Franklin & Cook, 1971). In this case, it is suggested that, since the tetracycline-resistance genes are always partially switched on, the dilution of the resistance-exponential products during growth is much less extensive than in cells bearing the wild-type R-factor.

The decline in resistance in cells bearing the wild-type R-factor after the initial recovery from EDTA-osmotic shock argues against the possibility that this procedure merely produces a non-specific increase in cell permeability resulting in a decrease in tetracycline resistance. We consider that the EDTA-osmotic shock may deplete the R-factor-bearing cells of the R-factor gene products specifically involved in antagonizing the specific uptake of tetracycline. This permits the re-entry of the drug in the immediate post-shock phase. A transient resynthesis of the tetracycline-resistance gene products antagonizing tetracycline transport is then followed by a progressive fall in resistance as these products are diluted out. We have obtained no evidence to suggest that the R-factor may be lost from the shocked cells.

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