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Effect of Various Conditions on Accumulation of Oxytetracycline in *Escherichia coli*

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ABSTRACT

IZAKI, KAZUO (University of Tokyo, Tokyo, Japan), AND KEI ARIMA. Effect of various conditions on accumulation of oxytetracycline in *Escherichia coli*. J. Bacteriol. **89**:1335-1339. 1965.—Accumulation of large amounts of oxytetracycline occurred in *Escherichia coli* when the cells were incubated with high concentrations of oxytetracycline (100 to 400 μg/ml) in nutrient broth or in a medium containing glucose, K_2HPO_4, and $MgSO_4$. In the absence of glucose or $MgSO_4$, the accumulation was very small. The optimal *p*H for accumulation was about 6.5. Manganous ion could replace Mg^{++} in promoting the accumulation, though with decreased effectiveness. Malate and succinate were effective promoters of accumulation as well as glucose. Accumulation was inhibited at low temperatures or in the presence of metabolic inhibitors such as 2,4-dinitrophenol or sodium azide.

In a previous paper (Arima and Izaki, 1963), we described the accumulation of large amounts of oxytetracycline in cells of *Escherichia coli* incubated with high concentrations of the antibiotic. We also reported on the disappearance of this accumulation in the cells of an oxytetracycline-resistant strain of *E. coli* (Izaki and Arima, 1963). The accumulation seemed to be dependent on an energy-yielding system, since the presence of glucose was required for the accumulation, and inhibitors such as 2,4-dinitrophenol or sodium azide inhibited the accumulation at conditions below the lowest level at which oxidation of glucose by washed cells was inhibited. This report describes the effects of various conditions which promote or inhibit the accumulation of oxytetracycline.

MATERIALS AND METHODS

Chemicals. Aqueous solutions of oxytetracycline hydrochloride were freshly prepared in each experiment. The concentration of oxytetracycline was usually expressed as the hydrochloride.

Bacterial strain. E. coli B-151-1 and K-12 were the strains mainly used. The cultures were maintained on slants of nutrient agar.

Growth conditions. For experiments in which relatively large amounts of cells were required, bacteria were usually grown aerobically in nutrient broth medium. Cells were incubated for 15 to 16 hr at 30 C in a 500-ml shaken flask. To examine the effects of oxytetracycline on bacterial growth, bacteria, which were cultured overnight, were inoculated into 10 ml of medium in a 30-ml Monod tube. The tube was shaken at 30 C in a water bath. Growth was measured by use of a Kotaki nephelometer.

Accumulation test. The test for accumulation of oxytetracycline was usually carried out at 30 C, by use of a Monod test tube on a shaking machine. Nutrient broth and a nitrogen-free medium containing glucose (5.5×10^{-2} M), K_2HPO_4 (10^{-2} M; *p*H 6.5), and $MgSO_4$ (4.0×10^{-4} M) were used as incubation media. Accumulation of oxytetracycline was usually determined from both the turbidity increase and the value of optical density at 340 mμ of the boiled extracts of cells which had been allowed to accumulate oxytetracycline. As described in a previous report (Arima and Izaki, 1963), the cells were centrifuged after incubation and washed once with distilled water. The cells were suspended in 5 ml of distilled water and were heated for 5 min in boiling water. We estimated the accumulated oxytetracycline by measuring the optical density at 340 mμ of this boiled extract and comparing its value (A) with that of a standard oxytetracycline solution which was also heated under identical conditions. The endogenous value (B) was determined with boiled extracts of cells which were incubated without oxytetracycline; this value was subtracted from the value (A), giving the amount of oxytetracycline accumulated (A − B). In some cases, as described later, decrease in optical density of the medium at 360 or 270 mμ (caused by uptake of oxytetracycline from the medium) was measured. Increase in turbidity was measured by a Kotaki nephelometer, and optical densities in the range of 200 to 400 mμ were measured with a Hitachi EPU-2A type spectrophotometer. Oxygen uptake was measured by

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use of a conventional Warburg respirometer at 30 C.

RESULTS

Effect of oxytetracycline on growth of E. coli B-151-1. Growing cells of *E. coli B-151-1* were inoculated into 10 ml of nutrient broth in 30-ml Monod test tubes containing various concentrations of oxytetracycline. The tubes were shaken at 30 C, and the rate of bacterial growth was observed by measuring the turbidity of the culture. *E. coli* did not grow for several hours in the presence of 0.5 to 5 μg/ml of oxytetracycline (Fig. 1). At a concentration of 50 μg/ml, a decrease in turbidity was observed, which may be caused by lysis of cells. On the other hand, the turbidity rapidly increased at high concentrations of oxytetracycline (100 to 500 μg/ml), as if the growth were promoted. Microscopic observation, however, showed that the number of cells did not increase during incubation, and the viable cell count rapidly decreased at these high concentrations of oxytetracycline. As described in our previous report, it is evident that this increase in turbidity was due to the accumulation of large amounts of oxytetracycline in the cells of *E. coli*. Such cells became yellow due to this accumulation. They also were found to have a higher electron density than normal cells when examined in the electron microscope (Fig. 2A, B).

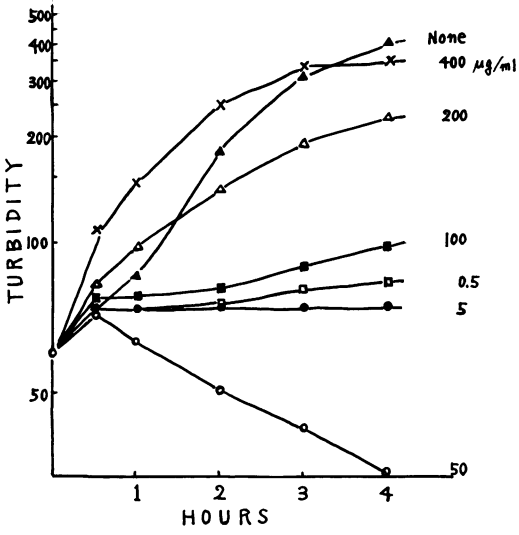

FIG. 1. *Effect of oxytetracycline on turbidity of Escherichia coli B-151-1. Cultures were grown in nutrient broth (pH 6.8) at 30 C under aerobic conditions.*

Effect of composition of reaction mixture on oxytetracycline accumulation. To clarify the accumulation phenomenon, a simple nitrogen-free medium (described in Materials and Methods) was used for the accumulation test instead of a nutrient broth medium. The accumulation was greatest when glucose, K_2HPO_4, and $MgSO_4$ were present (Fig. 3 and Table 1). Without glucose or $MgSO_4$, accumulation was greatly reduced. In agreement with these results, the decrease of oxytetracycline in a reaction mixture was found to be largest when glucose, $MgSO_4$, and K_2HPO_4 were present. These results were obtained by measuring the decrease in the optical density of the reaction mixture at 360 or 270 mμ. As described in a previous report, heat-treated cells did not accumulate any appreciable amount of oxytetracycline. The K_2HPO_4 did not seem necessary for the accumulation, since a large amount of accumulation also occurred in the presence of only glucose and $MgSO_4$, if the *p*H was adjusted between 6.0 and 7.0. We usually used K_2HPO_4 in the accumulation studies to take advantage of its

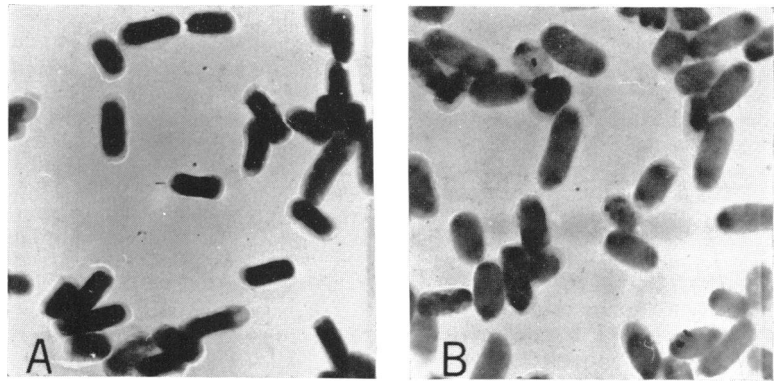

FIG. 2. *Electron micrograph of cells incubated in the presence (A) of 400 μg/ml of oxytetracycline and, in the absence (B) of oxytetracycline.* ×5,000.

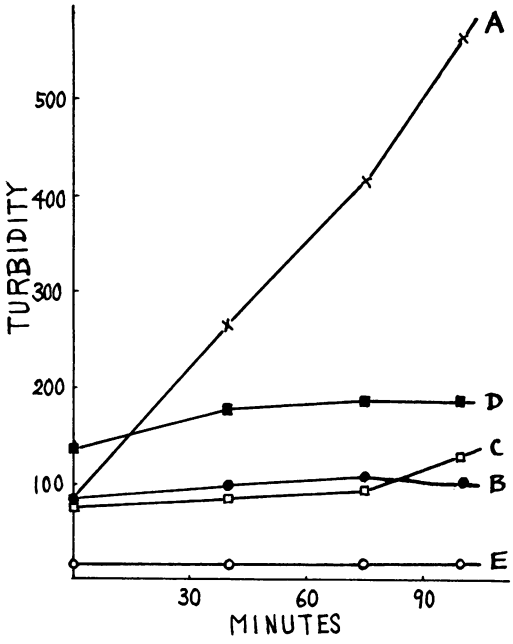

FIG. 3. *Accumulation of oxytetracycline in the cells of Escherichia coli B-151-1. The reaction mixture contained (in a total volume of 10 ml): 1 ml of cell suspension (1.6 mg, dry weight), 1 ml of oxytetracycline hydrochloride solution (8.0 × 10^{-3} M), and 1 ml each of 5.5 × 10^{-1} M glucose, 10^{-1} M K_2HPO_4 (pH 6.5), 4 × 10^{-3} M $MgSO_4 \cdot 7H_2O$. A, complete medium; B, without $MgSO_4$; C, without glucose; D, complete (heat-treated cells); E, complete (without cells).*

TABLE 1. *Accumulation of oxytetracycline in the cells of Escherichia coli B-151-1**

Medium	Oxytetracycline accumulation (μg/mg of cells)	
	40 min	100 min
A (complete)	305	464
B (Mg^{++} omitted)	35	99
C (glucose omitted)	70	75

* Incubation conditions were the same as described in Fig. 3. The amount of oxytetracycline was measured from the value of the optical density at 340 mμ of boiled extracts of the cells which had accumulated oxytetracycline.

buffering capacity in this *p*H range. The optimal *p*H for the accumulation was about 6.5 (Fig. 4). Glucose (5.5 × 10^{-5} M) and $MgSO_4$ (4 × 10^{-4} M) were sufficient to permit accumulation to continue for 2 hr.

Effect of various energy sources. We tested the

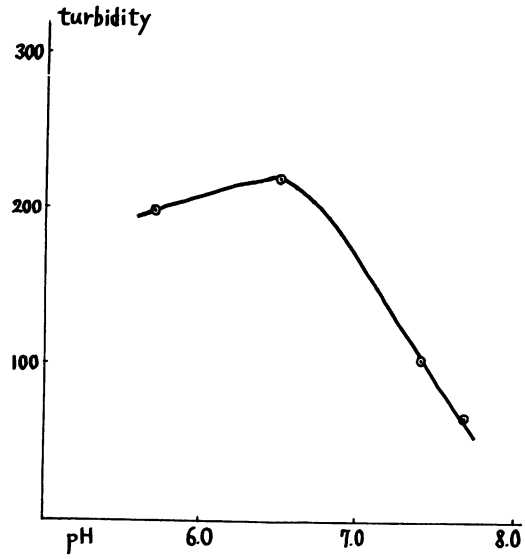

FIG. 4. *Effect of pH on the accumulation of oxytetracycline in the cells of Escherichia coli. The reaction mixture was the same as described in the legend for Fig. 3, except that the 1 ml of cell suspension in this mixture was 1 mg, dry weight. Incubation was carried out at 30 C for 2 hr.*

TABLE 2. *Effect of energy sources on accumulation of oxytetracycline*

Energy source	Turbidity increase	Oxytetracycline accumulated per mg of cells
		μg
None	6	43
Glucose	74	305
Succinate	27	155
Citrate	−24	20
Malate	66	298
Fumarate	4	68
Glutamate	11	88
Alanine	8	48

* The reaction mixture contained (in a total volume of 10 ml): cell suspension (0.8 mg, dry weight), oxytetracycline (8.0 × 10^{-4} M), energy source (5.5 × 10^{-3} M), K_2HPO_4 (10^{-2} M, *p*H 6.5), $MgSO_4 \cdot 7H_2O$ (4.0 × 10^{-4} M). Incubation was carried out at 30 C for 90 min.

effect of various energy sources, such as glucose, organic acids, and amino acids, on the accumulation of oxytetracycline. Malate was almost as effective as glucose in promoting action, but, of the others, only succinate showed some activity (Table 2).

Effect of metal ions. Effects of various metals on oxytetracycline accumulation were examined.

TABLE 3. *Effect of metal ions on the accumulation of oxytetracycline**

Metal	Turbidity increase	Oxytetracycline accumulated per mg of cells
		μg
$MgSO_4$	110	348
$MnSO_4$	80	223
$CoSO_4$	43	168
$CaSO_4$	33	129
$ZnSO_4$	30	125
$NiSO_4$	27	118
$CuSO_4$	23	95
$FeSO_4$	16	55
None	22	80

* The reaction mixture contained (in a total volume of 10 ml): cell suspension (0.8 mg, dry weight), oxytetracycline (8.0×10^{-4} M), glucose (5.5×10^{-2} M), K_2HPO_4 (10^{-2} M, *p*H 6.5). Incubation was carried out at 30 C for 90 min.

TABLE 4. *Effect of temperature on accumulation of oxytetracycline**

Condition	Turbidity increase	Oxytetracycline accumulated per mg of cells
		μg
Shaking, 30 C	140	328
No shaking, 30 C	100	296
No shaking, 2 C	2	30

* Reaction mixture contained (in a total volume of 10 ml): cell suspension (1.0 mg, dry weight), oxytetracycline (8.0×10^{-4} M), glucose (5.5×10^{-2} M), K_2HPO_4 (10^{-2} M, *p*H 6.5), $MgSO_4 \cdot 7H_2O$ (4.0×10^{-4} M). Incubation was carried out for 60 min.

Mg^{++} was most effective, Mn^{++} was next, and Cu^{++} and Fe^{++} had no promoting action (Table 3).

Effect of temperature and various inhibitors. At low temperatures, such as 2 C, the accumulation was slight (Table 4).

Since glucose was needed for the accumulation of oxytetracycline, energy seemed to be necessary for the accumulation. This speculation was supported by the experiment with inhibitors (Table 5). Metabolic inhibitors, such as 2,4-dinitrophenol (10^{-4} M) and sodium azide (10^{-2} M), inhibited the accumulation, but they did not inhibit the oxidation of glucose by washed cells. Moreover, they promoted the accumulation at these concentrations, although the rate of oxygen uptake by the resting cells dropped to about 50% of the control in the presence of oxytetracycline (8.0×10^{-4} M). Similar stimulation of the rate of glucose oxidation by 2,4-dinitrophenol or sodium azide was also observed when oxytetracycline was omitted from the reaction mixture. From these results, it would seem that a source of energy is required for the accumulation of oxytetracycline.

TABLE 5. *Effect of inhibitors on accumulation of oxytetracycline and oxygen uptake in Escherichia coli**

Strain of *E. coli* used	Inhibitor	Inhibitor concn	Inhibition of accumulation	Inhibition of oxygen uptake†
		M	%	%
B-151-1	Azide	10^{-4}	8	6
		10^{-3}	29	−8
		10^{-2}	81	−4
K-12		10^{-2}	78	−40
B-151-1	2,4-Dinitrophenol	10^{-4}	47	−12
		10^{-3}	98	46
K-12		2.5×10^{-4}	80	−32
		5.0×10^{-4}	100	16

* The accumulation test was carried out as in Fig. 3 in a medium containing: glucose (5.5×10^{-2} M), K_2HPO_4 (10^{-2} M, *p*H 6.5), $MgSO_4 \cdot 7H_2O$ (4.0×10^{-4} M), bacterial cells (1 to 2 mg, dry weight), and oxytetracycline (8.0×10^{-4} M). Oxygen uptake was measured by a Warburg respirometer, and the vessel contained the above medium and inhibitors, as indicated, in a total volume of 2 ml.

† The negative percentages indicate promotion of oxygen uptake.

TABLE 6. *Effect of composition of reaction mixture and temperature on both the accumulation of oxytetracycline and the number of viable cells**

Incubation medium	Temp	Oxytetracycline accumulated per mg of cells	Viable cells (per cent of control)
	C	μg	
Glucose, K_2HPO_4,	30	190, 181	51, 21
$MgSO_4 \cdot 7H_2O$	0	11	77
Glucose, K_2HPO_4	30	53	81
K_2HPO_4, $MgSO_4 \cdot 7H_2O$	30	27	90

* The reaction mixture contained (in a total volume of 10 ml): cell suspension (1 mg, dry weight; 2×10^8 or 3×10^8 viable cells), oxytetracycline (1.0×10^{-4} M), glucose (5.5×10^{-2} M), K_2HPO_4 (10^{-2} M, *p*H 6.5), and $MgSO_4 \cdot 7H_2O$ (4.0×10^{-4} M). Incubation was carried out at 30 C for 90 min. Viable cells were counted by the usual plate-culture method.

Decrease in number of viable cells during the incubation with oxytetracycline. Experiments were undertaken to determine whether there was a

decrease in viable-cell counts under those conditions which promoted the accumulation of oxytetracycline. The incubation conditions and the results are shown in Table 6. At 30 C, viable cells decreased 50% in 90 min in the presence of glucose, K_2HPO_4, and $MgSO_4$. Viable-cell counts decreased slowly at low temperature or in the absence of glucose. From these results, it is apparent that viable cells decreased most rapidly under those conditions which promoted the accumulation of oxytetracycline. However, even these high levels of oxytetracycline failed to kill the cells rapidly and completely.

DISCUSSION

Accumulation of oxytetracycline occurred when cells were incubated with a high concentration of oxytetracycline (100 to 400 μg/ml) in nutrient broth or the medium containing glucose, K_2HPO_4, and $MgSO_4$. At a concentration of 50 μg/ml of oxytetracycline, similar promotion of antibiotic accumulation by glucose has been observed. The promotion was not as great at the lower level as in the range of 100 to 400 μg/ml described in this report. It is not known whether accumulation occurs in low concentrations of oxytetracycline, such as 1 to 10 μg/ml, at which bacterial growth is inhibited. It is difficult to measure the accumulation in such low concentrations with unlabeled oxytetracycline. At low concentrations of oxytetracycline, promotion of accumulation by glucose may not be observed if the endogenous energy sources are sufficient for the accumulation, since the amount of energy required for the accumulation would be slight. This point should be clarified by improved sensitive methods.

Stimulation of oxytetracycline accumulation by glucose may be explained by the possibility that oxytetracycline combines with a metabolic product of glucose in the cells. Alternatively, a supply of energy might be necessary for the accumulation of Mg^{++} in the cells, but not for oxytetracycline accumulation. For the resolution of these questions, further experiments are required. We assume that free oxytetracycline or its Mg^{++} chelate compound accumulated in the cells at a high enough concentration to precipitate. If, indeed, a supply of energy is necessary for the entry and accumulation of oxytetracycline, it is interesting that bacteria use energy to take an antibiotic into the cells. Perhaps this occurs by mistake due to an inability to discriminate between the antibiotic and a certain nutrient, as is the case with amino acids and their analogues.

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