

STUDIES IN METABOLIC SPECTRA

IV. EFFECTS OF TETRACYCLINES, SOME OF THEIR DERIVATIVES, AND CHLORAMPHENICOL ON ACCUMULATION OF GLUTAMIC ACID IN *ESCHERICHIA COLI*¹

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ABSTRACT

CHENG, LORRAINE (Radiobiochemistry Department, Chas. Pfizer & Co., Inc., Maywood, N. J.) AND J. F. SNELL. Studies in metabolic spectra. IV. Effects of tetracyclines, some of their derivatives, and chloramphenicol on accumulation of glutamic acid in *Escherichia coli*. J. Bacteriol. **83**:711-719. 1962.—*Escherichia coli* strain 21 was incubated in the Warburg apparatus at 37 C with sodium acetate-2-C¹⁴ and 0.1 μ mole/ml of various test compounds. Up to 1 hr, de novo C¹⁴-glutamic acid (synthesized from the C¹⁴-acetate precursor) accumulation in the fermentation broth was found to be a common phenomenon for the control cells and cells treated with oxytetracycline, chlortetracycline, tetracycline, and chloramphenicol. Subsequently, C¹⁴-glutamic acid continued to accumulate in the broth of the inhibited cells, but began to disappear from the broth of the control cells. During the first half hour, the rate of accumulation was most rapid in the presence of oxytetracycline. At 3 hr the total de nova C¹⁴-glutamic acid was found to be the same whether cells were treated with oxytetracycline or not. However, the distribution of this glutamic acid was different. In the oxytetracycline-treated cells, more than 87% of the total de nova C¹⁴-glutamic acid was in the broth, and only 13% was incorporated into the cell residue. In the control cells, no C¹⁴-glutamic acid was found in the broth, although 67% was in the cell residue. The possibility that the tetracyclines and chloramphenicol have dif-

ferent modes of action, and that oxytetracycline inhibits the incorporation of D-glutamic acid into the cell wall and membrane material in *E. coli* 21, was discussed.

During the investigation on the mode of action of oxytetracycline in this laboratory, evidence was gathered supporting the hypothesis that this antibiotic interferes with the normal utilization of D-glutamic acid by preventing its incorporation into the bacterial cell wall. This hypothesis was based on the following experimental observations: (i) the accumulation of endogenously synthesized, free D-glutamic acid in oxytetracycline-treated *Escherichia coli* (Snell and Cheng, 1959); (ii) the similarity of the A-ring of the tetracycline molecule to a glutamic acid moiety (Snell, 1960); and (iii) a decrease in radioactivity in the cell wall and associated residues paralleling an increase in radioactivity attributed to free glutamic acid in the broth (Snell and Cheng, 1958).

Sugano (1958) reported that tetracycline and chlortetracycline accelerated the increase of free glutamic acid within the cells of *Candida albicans* when incubated in the presence of L-glutamic acid. This, together with earlier studies by Gale and Paine (1951), Gale and Folkes (1953b), Porro and Soncin (1954), Grünberger, Škoda, and Šorm (1954), and Wong, Barban, and Aji (1953), points toward a definite relationship between the antibacterial activity of the tetracyclines and glutamic acid metabolism.

Oxytetracycline might be considered as an analogue of glutamic acid and thus compete with glutamic acid as a substrate. If this were the case, it would be logical to suppose the glutamic acid moiety (A-ring) of the tetracycline molecule to be basically responsible for its

¹ Part of the results of this investigation was published in a review by J. F. Snell and Lorraine Cheng, 1961. Studies on modes of action of tetracyclines (II), p. 107-132. *In* Developments in industrial microbiology. Plenum Press, New York.

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antibiotic activity. Ukita and Arakawa (1957) synthesized a compound with a structure analogous to the A-ring of tetracycline. This compound showed antibacterial activity against both *E. coli* and *Staphylococcus aureus*, although it was much less effective than tetracycline. Recently, Goldman (1960) found that, to permit the chelation of oxytetracycline with Mg^{++} and to become inhibitory, the A, B, and C rings of the tetracycline molecule had to be intact. McCormick et al. (1957, 1960) showed a lack of in vitro antibacterial activity against *S. aureus* when changes involving the A-ring of the tetracycline molecule were made, whereas no loss was caused by alteration of the C-ring.

The present study was initiated to test our hypothesis by comparing the effects of the tetracycline group of antibiotics and some of their derivatives, chloramphenicol, glutamic acid, and some of its derivatives, on the accumulation of endogenously synthesized glutamic acid in *E. coli*.

MATERIALS AND METHODS

Incubation. The procedure used in this study has been reported in detail (Snell and Cheng, 1958). Antibiotics and derivatives were compared on an equimolar basis (0.2 μ moles). This concentration is equivalent to an oxytetracycline concentration of 50 μ g/ml. All free bases were converted to the hydrochloride by combining stoichiometrically with hydrochloric acid. A molecular equivalent of hydrochloric acid was added to the nonbasic compounds.

Extraction. Extractions of the freeze-thawed cells were carried out successively with 0.5-ml volumes of distilled water, 5% (w/v) trichloroacetic acid (TCA) at room temperature for 30 min, 5% TCA in a boiling water bath for 30 min, acidified 75% ethanol, and ether.

Hydrolysis. The final cell residue was hydrolyzed with 1 ml of 6 N HCl in a sealed tube at 108 C for 16 hr. Hydrolysis was almost complete at this time interval (Roberts et al., 1957).

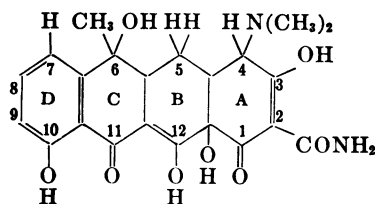
Chromatography and radioautography. All chromatography was carried out at 5 C with System P-II (tertiary amyl alcohol-water-88% formic acid, 3:3:1). The chromatograms were radioautographed and scanned, and glutamic acid identified by methods described previously (Snell and Cheng, 1958).

Determination of radioactivity. A sample of the material to be counted was spotted on a filter-

paper strip and scanned in a gas-flow paper-chromatogram scanner. The sample was applied in 2- μ liter portions, and dried between applications. This insured a uniform geometry as each radioactive spot was being counted. Radioactivity on the filter-paper strip and on the developed chromatograms was determined by measuring the peak areas on the scans and comparing with a standard curve prepared by plotting peak areas against known amounts of C^{14} .

Nitrogen determination. The method of Johnson as described by Umbreit, Burris, and Stauffer (1957) was used.

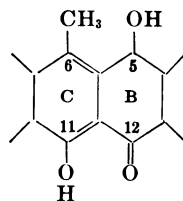
Tetracycline.



Oxytetracycline. One of the hydrogen atoms at C5 is changed to a hydroxyl (OH) group (Hochstein et al., 1953).

Chlortetracycline. The hydrogen atom at C7 is replaced by a chlorine atom.

Anhydrooxytetracycline. The B- and C-rings of the oxytetracycline molecule are changed to the following structure (Hochstein et al., 1953).

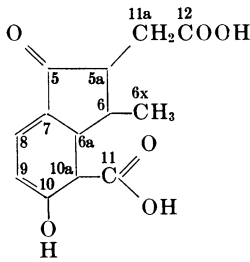
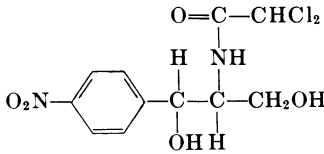


Dimethyloxytetracycline. The radicals at C3 and C12 of the oxytetracycline molecule are changed to OCH_3 (Hochstein, *personal communication*).

Desdimethylaminoxytetracycline. The dimethylamino group at C4 of the oxytetracycline molecule is replaced by a hydrogen atom (Hochstein et al., 1953).

2-Acetyl-2-decarboxamido-oxytetracycline. The radical at C2 of the oxytetracycline molecule is changed to $COCH_3$ (Hochstein et al., 1960).

Terracinoic acid. An alkaline degradation product of oxytetracycline, having the formula:

*Chloramphenicol.*

Glutamic acid and derivatives. D-Glutamic acid was obtained from the Amend Drug and Chemical Company. N:N-dimethylglutamic acid was obtained from A. J. Birch, Manchester University, Manchester, England, and γ -methyl-L-glutamic acid from General Mills, Inc., Central Research Laboratories, Minneapolis, Minn.

RESULTS

Effects of tetracyclines and related compounds on E. coli 21 at different time intervals. On an equimolar basis, all the tetracyclines and chloramphenicol inhibited the Q_{O_2} of *E. coli* 21 to approximately the same extent during the time intervals studied (Table 1). Desdimethylaminooxytetracycline showed the same degree of inhibition as the other tetracyclines at 0.5 and 1 hr, although the glutamic acid accumulation was much lower (Table 2). Terracinoic acid had no effect on Q_{O_2} as judged at 3 hr.

Figure 1 shows that all the characteristic compounds caused by oxytetracycline inhibition were present (Snell and Cheng, 1958). The differences between the effects of the tetracyclines and chloramphenicol were quantitative. The accumulation of most of the compounds occurred as a simple function of time.

Glutamic acid accumulation in the broth was a common phenomenon for the control cells and antibiotic-inhibited cells up to 1 hr; subsequently, glutamic acid continued to accumulate in the broth of the inhibited cells, but began to disappear in the broth of the control cells (Table 2). During the first 0.5 hr of contact, the rate of

TABLE 1. Q_{O_2} of *E. coli* 21 in presence of different compounds

Compounds (0.1 μ mole/ml)	Q_{O_2} (μ liters O_2 /hr with 10^8 cells) at different time intervals*		
	0.5 hr	1 hr	3 hr
Oxytetracycline HCl.....	7.3	7.7	18.8
Chlortetracycline HCl.....	5.9	6.3	19.6
Tetracycline HCl.....	8.9	9.1	18.3
Desdimethylaminooxytetracycline†.....	8.8	6.1	
Terracinoic acid‡.....			51.8
Chloramphenicol.....	8.8	8.8	21.1
None.....	13.9	18.6	54.4

* Averaged over the incubation period in presence of the compounds. Conditions of incubation same as in Fig. 1.

† Desdimethylaminooxytetracycline was studied at intervals of 0.5 and 1 hr only.

‡ Terracinoic acid was studied at the 3-hr interval only.

accumulation was most rapid in the presence of oxytetracycline.

Effects of derivatives of oxytetracycline and glutamic acid on E. coli. The effect of the various compounds on the Q_{O_2} of *E. coli* 21 is summarized as follows. Desdimethylaminooxytetracycline (an A-ring modified derivative) suppressed the Q_{O_2} of *E. coli* 21 to a greater extent than oxytetracycline. 2-Acetyl-2-decarboxamido-oxytetracycline was only slightly inhibitory. Dimethyloxytetracycline, D-glutamic acid, and the two glutamic acid derivatives were without effect. In 2-acetyl-2-decarboxamido-oxytetracycline and dimethyloxytetracycline, both of which caused a decrease of inhibitory effect on the Q_{O_2} of *E. coli*, a change in the A-ring of the oxytetracycline molecule was involved, but in desdimethylaminooxytetracycline, another A-ring modified compound, high inhibition occurred. The data also demonstrate that the glutamic acid moieties supplied were not sufficient for inhibitory action.

Table 3 shows how the different compounds affected the distribution of radioactivity in the various fractions. The radioactivity in the KOH fraction corresponded with the Q_{O_2} data, since it represented the respiratory $C^{14}O_2$. The lowest level of KOH radioactivity occurred in the presence of desdimethylaminooxytetracycline,

TABLE 2. *Glutamic acid radioactivity in Escherichia coli* 21 fermentation broths*

Compounds (0.1 μ mole/ml)	Per cent of radioactivity of C ¹⁴ -glutamic acid at different time intervals					
	0.5 hr		1 hr		3 hr	
	Added (25 μ c)	Broth	Added (25 μ c)	Broth	Added (25 μ c)	Broth
Oxytetracycline HCl.....	2.1	18.1	2.8	16.5	11.4	18.0
Chlortetracycline HCl.....	1.2	10.0	1.8	13.2	18.5	22.4
Tetracycline HCl.....	0.2	2.6	4.2	22.6	12.5	24.8
Desdimethylaminoxytetracycline†.....	0.1	0.8	0.8	7.1		
Terracinoic Acid‡.....					0.0	0.0
Chloramphenicol.....	0.1	0.8	3.9	20.3	18.1	25.3
None.....	1.1	8.9	3.6	27.3	0.0	0.0

* Conditions of incubation same as in Fig. 1.

† Desdimethylaminoxytetracycline was studied at intervals of 0.5 and 1 hr only.

‡ Terracinoic acid was studied at the 3-hr interval only.

35% of the control. The Q_{O_2} in this case was 41% of the control.

Although desdimethylaminoxytetracycline was most effective in inhibiting the respiration of *E. coli* 21, the highest retention of radioactivity in the broth occurred in the presence of oxytetracycline (an increase of more than eightfold over the control). Desdimethylaminoxytetracycline, anhydroxytetracycline, and 2-acetyl-2-decarboxamidoxytetracycline followed in decreasing order (eight, six, and fourfold increases, respectively). No significant increase was produced by dimethyloxytetracycline or the glutamic acid derivatives. A high retention of radioactivity in the broth was accompanied by a low incorporation of radioactivity into the cell extracts and cell residue.

The final cell residue must contain a significant amount of cell wall and membrane material (Snell, Radin, and Ikawa 1955; Park and Hancock, 1960). The data indicate a significantly lower level of radioactivity in the hydrolyzed cell residues from cultures inhibited by oxytetracycline and desdimethylaminoxytetracycline.

Chromatography of each of the culture fractions indicated that glutamic acid was present in the broth, the aqueous cell extract, and the hydrolyzed cell residue. Table 4 shows the levels of C¹⁴-glutamic acid in each of these fractions. Since the level is expressed in terms of total radioactivity, it represents the glutamic acid synthesized de novo from the C¹⁴-acetate precursor. The total de novo synthesis in the oxytetracycline-inhibited cells and in the control cells was the same. The difference lay in the distribution

of this glutamic acid. More than 87% of the total glutamic acid was in the broth of the oxytetracycline-treated cells, but only 13% was incorporated into the cell residue. In the control cells, no glutamic acid could be found in the broth, although 67% of the total glutamic acid was in the hydrolyzed cell residue; the remainder was in the aqueous extract.

In the cell residue from oxytetracycline-inhibited cultures, only 1.9% of the total radioactivity was incorporated as C¹⁴-glutamic acid (Table 5). In contrast, the control cells incorporated 10.2%. A comparison of the nitrogen contents and dry weights of the hydrolyzed cell residues also indicated lower values for the oxytetracycline-inhibited cells.

DISCUSSION

Our study shows that, on an equimolar basis, the tetracyclines and chloramphenicol produced similar responses on Q_{O_2} of *E. coli*. This differs from the finding of Ciak and Hahn (1958), who reported that chloramphenicol, when compared with the tetracyclines, was five times less active against growth (measured by turbidity) of *E. coli*. The different responses in these two studies indicate the basic difference, first pointed out by Gale (1953), in the sensitivities of various reaction systems in an organism to antibiotic concentration.

Though the chromatographic analysis of broths from chloramphenicol-inhibited *E. coli* and from cells inhibited by the tetracyclines also shows the same pattern (Fig. 1), a quantitative difference becomes evident upon closer observa-

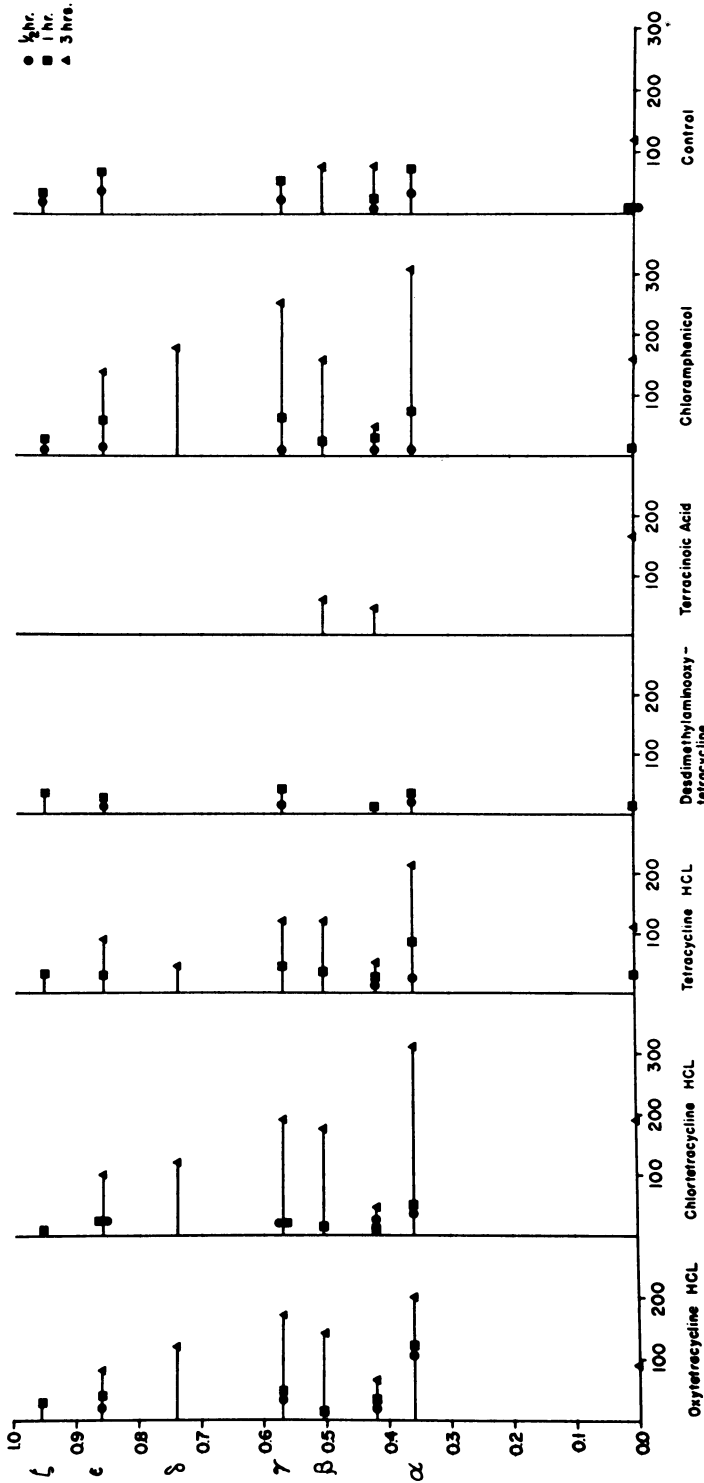


FIG. 1. Chromatographic analysis of *Escherichia coli* 21 fermentation broths in presence of different antibiotics. Incubation at 37.5 C in the Warburg apparatus; 10×10^8 cells in 2 ml of nutrient broth (Difco, pH 6.8) containing 0.05% glucose. Concentration of sodium acetate-2-C¹⁴, 12.5 μ c/ml. Concentration of antibiotics, 0.1 μ mole/ml. At the end of the indicated period, cells were separated from the broths by centrifugation and the broths chromatographed. Greek letters denote C¹⁴-labeled compounds accumulated due to the presence of the antibiotics. Compound α was glutamic acid. Ordinate: R_F values. Abscissa: relative radioactivity of accumulated compounds. Desdimethylaminoxytetracycline was studied at intervals of 0.5 and 1 hr only. Terracinoic acid was studied at the 3-hr interval only.

TABLE 3. Distribution of radioactivity in different fractions of *Escherichia coli* 21*

Compounds (0.1 μ mole/ml.)	Per cent of total added radioactivity (25 μ c) in fractions								Total recovery %
	KOH	Broth	Aqueous	Cold TCA	Hot TCA	Acidified 75% ethanol	Ether	Hydrolyzed cell residue	
Oxytetracycline HCl	19.4	84.0	3.6	2.6	2.7	6.0	<0.1	2.1	120.40
Anhydroxytetracycline HCl	27.8	57.2	6.7	2.0	4.4	8.0	0.29	4.1	110.49
Dimethyloxytetracycline HCl	29.5	14.0	6.2	6.2	7.1	17.0	0.35	14.0	94.35
Desdimethylaminoxytetracycline	12.2	77.6	4.6	0.6	1.4	7.0	0.14	2.4	105.94
2-Acetyl-2-decarboxamidoxytetracycline HCl	31.7	41.2	14.5	1.4	6.4	11.5	0.35	5.0	112.55
D-Glutamic acid HCl	35.5	10.4	5.7	8.0	7.4	21.5	0.50	21.0	110.00
N:N-dimethylglutamic acid HCl	36.0	11.6	5.4	9.0	7.6	15.8	0.36	16.6	102.36
γ -Methyl-L-glutamic acid HCl	36.9	17.2	5.4	9.7	7.3	12.2	0.26	16.0	104.96
None	35.1	10.0	5.3	8.8	8.0	14.5	0.43	18.7	100.83

* Incubation (3.5 hr.) at 37.5 C in conventional Warburg apparatus; compounds tipped at 30 min; 17×10^8 cells in 2 ml of nutrient broth (Difco, pH 6.8) containing 0.05% glucose; sodium acetate-2- C^{14} (12.5 μ c/ml; specific activity = 2 μ c/ μ mole) added.

TABLE 4. Distribution of de novo C^{14} -glutamic acid in *Escherichia coli* 21*

Compounds (0.1 μ mole/ml)	C^{14} -glutamic acid in fractions						Total C^{14} -glutamic acid†	
	Broth		Aqueous		Hydrolyzed cell residue			
	μ c	Total C^{14} -glutamic acid	μ c	Total C^{14} -glutamic acid	μ c	Total C^{14} -glutamic acid	μ c	%
Oxytetracycline HCl	3.08	%	0	%	0.48	13.5	3.56	100
Anhydroxytetracycline HCl	0.80	58.5	0	0	0.57	41.5	1.37	100
Dimethyloxytetracycline HCl	0.13	8.0	0	0	1.50	92.0	1.63	100
Desdimethylaminoxytetracycline	0.70	65.0	0	0	0.38	35.0	1.08	100
2-Acetyl-2-decarboxamidoxytetracycline HCl	0.21	25.6	<0.03	<3.7	0.58	70.7	<0.82	100
D-Glutamic acid HCl	<0.05	<1.8	0.08	2.9	2.62	95.3	<2.75	100
N:N-dimethylglutamic acid HCl	0.08	4.8	0.06	3.6	1.53	91.6	1.67	100
γ -Methyl-L-glutamic acid HCl	0.08	2.1	1.50	40.2	2.15	57.7	3.73	100
None	0	0	1.25	33.0	2.55	67.0	3.80	100

* Conditions same as for Table 3.

† Calculated by totaling the radioactivity from each of the three fractions.

tion. The accumulation of compound ζ during the first half-hour of contact with chloramphenicol and the quantitatively greater accumulation of compounds γ , δ , and ϵ at 3 hr seem to imply that a different mechanism or a different rate of inhibition (due to different rates of permeability and differences in enzyme affinity) is involved. The radioactive spots which reside at the base line

and the solvent front can represent a number of different compounds whose resolution might lead to an elucidation of the different modes of action.

That chloramphenicol and the tetracyclines have different modes of action is strengthened by the study of Hahn, Wissemann, and Hopps (1954). They observed that the L(+)-erythro-isomer of chloramphenicol inhibited the formation of

TABLE 5. Incorporation of C^{14} -glutamic acid into cell residues of *Escherichia coli* 21*

Compounds (0.1 μ mole/ml)	C^{14} -glutamic acid	
	Dry weight of hydrolyzate	Total radioactivity (25 μ c)
	μ c/mg	%
Oxytetracycline HCl.....	0.654	1.90
Anhydroxytetracycline HCl.....	0.564	2.26
Dimethyloxytetracycline HCl.....	0.606	6.00
Desdimethylaminoxytetracycline.....	0.411	1.53
2-Acetyl-2-decarboxamidoxytetracycline HCl.....	0.445	2.34
D-Glutamic acid HCl.....	1.414	9.60
N:N-dimethylglutamic acid HCl.....	0.715	6.13
γ -Methyl-L-glutamic acid HCl.....	0.958	8.47
None.....	1.491	10.20

* Conditions same as for Table 3.

D(-)glutamyl polypeptide in *Bacillus subtilis* but had no effect on growth, and the active antibiotic, the D(-)threo-isomer, inhibited growth of the organism but did not affect the formation of D(-)glutamyl polypeptide. Postulating that an analogous mechanism was involved, they suggested that the antibiotic inhibited protein synthesis by interfering with L-glutamyl polypeptide metabolism. Thus, one would expect L-glutamic acid to be accumulated in chloramphenicol-treated *E. coli*, as contrasted to the D-isomer in oxytetracycline-treated *E. coli* (Snell and Cheng, 1959).

Though evidence that chloramphenicol inhibits protein synthesis at bacteriostatic concentrations is not lacking in the literature (Gale and Folkes, 1953b; Harrington, 1958; Wisseman et al., 1954), the only work that concerns cell-wall synthesis in the presence of this antibiotic is by Hancock and Park (1958). They showed that chloramphenicol did not inhibit cell-wall synthesis at a level which completely inhibited protein synthesis.

Gale and Folkes (1953b) found that, at bacteriostatic concentrations of chlortetracycline and oxytetracycline, protein synthesis in *S. aureus* was almost completely inhibited. They (Gale and Folkes, 1953a) used the glutamate content of the hydrolyzate of the cold trichloroacetic acid-precipitable material from cell suspen-

sions as a measure of the protein present. This material must necessarily contain the cell walls and membranes. Therefore, what they estimated as "combined glutamate" did not differentiate between the glutamic acid incorporated into the cell wall and membrane fraction and that into the cytoplasmic protein fraction. Thus, the measure of "combined glutamate" does not indicate protein synthesis in the strict sense. Later, Gale (1958) did study the effect of penicillin on the incorporation of amino acids into the protein + wall fraction and the protein fraction alone after removing the wall with lysopeptidase, but no similar study has been done with the tetracycline antibiotics.

In the present experiment, lower values for C^{14} -glutamic acid, total dry weight, and total nitrogen content were obtained from the residues of oxytetracycline-inhibited cells, in comparison with those obtained from control cells. Assuming that cell-wall and membrane material must occupy a significant portion of the cell residue (Snell et al., 1955; Park and Hancock, 1960), these data, and those reported previously (Snell and Cheng, 1958, 1959), seem to strengthen our hypothesis that oxytetracycline interferes with the incorporation of D-glutamic acid into the cell-wall structure. Park (1958) demonstrated that the rate of incorporation of lysine (a cell-wall amino acid) into the wall is inhibited at least 85% by 50 μ g/ml (the level of tetracyclines used in the present study) of chlortetracycline.

During an infection, the selective action of the tetracyclines on bacterial cells as against host cells makes the interference of D-glutamic acid incorporation into the cell wall and associated structure an attractive hypothesis, since this type of metabolism has not been demonstrated in mammalian cells.

Data from the present study are not sufficient to pinpoint the active portion of the tetracycline molecule. Suffice it to say that (i) the glutamic acid moieties supplied were not sufficient for inhibitory action; (ii) of all oxytetracycline derivatives studied, only desdimethylaminoxytetracycline seemed to retain comparable activity; and (iii) 2-acetyl-2-decarboxamidoxytetracycline and dimethyloxytetracycline, derivatives with alterations in the A-ring of the oxytetracycline molecule, were much less inhibitory than oxytetracycline.

ACKNOWLEDGMENTS

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