Effects of Antibiotics on Synthesis of Lysine Decarboxylase in *Escherichia coli.** (24032)

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This study is an extension of an earlier report that antibiotic chlortetracycline impairs, *in vivo* and *in vitro*, the elaboration of amines by intestinal microorganisms(1). Subsequently, others have noted similar effects (2-5). Since these amines arise through the action of the amino acid decarboxylases, a group of adaptive enzymes(6), and because a number of antibiotics inhibit the synthesis of proteins such as adaptive enzymes(7-10), it is conceivable that the induction of amino acid decarboxylases in bacteria is sensitive to certain antibiotics. The experimental work to follow supports this thesis, particularly insofar as chlortetracycline, oxytetracycline and chloramphenicol are concerned.

Methods. Production of cells. The bacterium, *Escherichia coli* (Crookes), was grown in a synthetic medium containing glucose (2%), KH_2PO_4 (0.1%), K_2HPO_4 (0.1%), NaCl (0.1%), $(NH_4)_2SO_4$ (0.4%), Na citrate (0.05%), $MgSO_4 \cdot 7H_2O$ (0.07%), ferric ammonium sulfate (1.56 mg%) and lactic acid (4 ml 80%/100 ml medium). The medium was adjusted to pH 6.8, dispensed in 100-ml lots in 250- or 500-ml Erlenmeyer flasks and sterilized at 114° for 10 min. The medium was usually inoculated with 0.1 ml of a 12-hr culture grown in the same menstruum. Unless otherwise stated, the inoculated medium was incubated at 25° for 18 hrs. To obtain cells with lysine decarboxylase activity, the medium had to be supplemented with L-lysine·HCl (0.02M). *Determination of enzymic activity.* The cells were harvested by centrifugation, washed once with distilled water and resuspended in 0.05M phthalate buffer, pH 4.5. Activity of lysine decarboxylase was determined according to Warburg technic by measuring CO_2 evolution from amino acid substrate at 30°. The side arm of each cup contained 0.5 ml 0.05M L-lysine·HCl in 0.05M phthalate buffer, while 1 ml of suitable density of cells (0.4 to 1 mg N) and sufficient buffer to achieve total volume of 3.2 ml were placed in the main compartment. The vessels were gassed for 7 min with a mixture of 95% nitrogen and 5% CO_2. After equilibration, the substrate was tipped into the main compartment and the increase in pressure noted at 5 or 10 min. up to 2 hrs. On occasion the cells, after being washed, were treated with toluene to increase permeability so that the effects of the coenzyme, pyridoxal phosphate, could be measured(11). This necessitated use of phthalate buffer at pH 5.5 instead of 4.5 for maximal activity. Pyridoxal phosphate was added at a level of 10 μg/cup. *Induction of lysine decarboxylase in resting cells. First method.* Cells were recovered from basal medium (no lysine present), washed and suspended in 0.05M phthalate (pH 4.5 or 5.5). To the main compartment of each Warburg vessel was added cells (0.4 mg N), glucose‡ (50 mg) and pyridoxal phosphate (10 μg); the sidearm received 0.5 ml 0.05M L-lysine·HCl. After gassing with nitrogen, lysine was tipped in from sidearm and appearance of lysine decarboxylase activity observed over a 3-hr period at 30°. *Second method.* Because of abundance of CO_2 which evolved from glucose when induction was measured by the first method, a method was adopted which eliminated glucose during manometric measurement of lysine

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‡ Mandelstam(11) found glucose to be essential for induction of lysine decarboxylase in resting cells of *Bacterium cadaveris*.

TABLE I. Effect of Oxytetracycline and Chlortetracycline on Formation of Lysine Decarboxylase in *Escherichia coli* during Growth.

Nature of cell suspension (.4 mg N)	pH in cup	Activity (μl CO_2/hr) in cells from medium with: Oxytetracycline	Chlortetracycline	No antibiotic (control)
Intact	4.5	190	225	297
Toluene-treated	5.5	266	404	536

decarboxylase. Unadapted cells were obtained as before and suspended in buffer at a density equivalent to approximately 0.5 mg cell N/ml. Test tubes (20 x 200 mm), fitted with sidearm which could be closed with a rubber stopper as used with serum bottles, were arranged in a series for gassing with nitrogen. The following materials were added to these tubes: 10% glucose (2 ml), 0.128M L-lysine · HCl (2 ml), distilled water or solution of antibiotic (1 ml), 0.05M phthalate buffer at pH 5.5 (21 ml) and cell suspension (4 ml). The tubes were placed in water bath at 30° and gassed with nitrogen for 5 min. Four 5-ml aliquots, including one at the very beginning, were removed and promptly refrigerated at measured intervals during 1 hr. As soon as possible, the cells were washed by centrifugation and resuspended in phthalate buffer, pH 5.5. One ml of each suspension, equivalent to 1 ml cell N, was used to determine, by the customary manometric procedure, the activity of lysine decarboxylase.

Results. The $Q_{CO_2}(N)$ of lysine decarboxylase in *E. coli* recovered from the lysine-supplemented defined medium was generally of an order of magnitude of 600. Toluenized cells were 2 to 3 times more active than intact resting cells. Pyridoxal phosphate increased activity of toluenized cells by less than 10%. Lysine decarboxylase, in either intact or toluene-treated cells, was completely stable in buffered, substrate-free suspensions of intact or toluene-treated cells for several hours in the Warburg vessel at 30° under nitrogen. This agrees with reported relative stability of this enzyme in cell-free preparations(12) and eliminated concern over spontaneous denaturation of the enzyme during subsequent tests. The tetracycline group of antibiotics had but slight effect on activity of lysine decarboxylase under conditions employed to measure the action of this enzyme. In fact, slight stimulation of activity was noted with intact resting cells.

Effects of chlortetracycline and oxytetracycline on formation of lysine decarboxylase in growing cells. Antibiotic was added to the lysine-fortified synthetic medium at a level which would not interfere with growth or acid production. This maximal non-inhibitory concentration of tetracyclines was 0.1 μg/ml. In one typical experiment (Table I), chlortetracycline · HCl or oxytetracycline · HCl, at a concentration of 0.04 μg/ml of medium, suppressed significantly the formation of the enzyme without a measurable influence on final pH or turbidity of the culture.

Effects of antibiotics on enzyme induction in resting cells. To determine the action of higher concentrations of antibiotic on formation of lysine decarboxylase necessitated the use of resting cells. Preliminary to studying effects of antibiotics on induction, however, the conditions optimum for induction of non-proliferating cells had to be established. Using the "first method," the effects of aeration and temperature during growth of unadapted cells as well as the pH of suspension during induction were tested. The organism was grown in the synthetic medium devoid of lysine on a rotary shaker at 30°, or without agitation at either 25° or 30°. The cells were recovered by centrifugation after 20 hrs of incubation, washed and resuspended in 0.05M phthalate buffer at pH 4.5 or 5.5. On the basis of results in Table II, cultivation without agitation at 25° and induction at pH 5.5 gave the best response. Under the conditions employed, the lag period for induction of this enzyme was less than 10 min. Also, it was learned that exogenous supply of pyridoxal phosphate was not influential on inductive process in resting cells except for a very slight reduction in length of lag period. The "first method" did not lend itself well to determin-

TABLE II. Effects of Aeration and Temperature during Growth on Subsequent Inducibility of Lysine Decarboxylase in Resting Cells of *Escherichia coli*.

Conditions during growth			
Extent of aeration	Temp.	pH in cup	Net activity (μl CO_2/3 hr)*
Agitation	30	4.5	8
"	"	5.5	24
Stationary	"	4.5	15
"	"	5.5	181
"	25	4.5	7
"	"	5.5	266

* .4 mg cell N/cup.

ing the influence of antibiotics on induction of lysine decarboxylase because of rapid production of CO_2 from the glucose present, which, incidentally, was impeded by chlortetracycline and oxytetracycline. The other method of testing the action of antibiotics on induction of lysine decarboxylase in resting cells was relatively successful. Unadapted cells were obtained from stationary cultures incubated at 25° and induced according to description of the "second method" (*cf. Methods.*). It is evident from the results in Table III that chlortetracycline and oxytetracycline discouraged induction of lysine decarboxylase. Of interest is the rapidity with which the cells responded to lysine as reflected in activity of control cells at zero time, presumably a result of adaptation while these cells were refrigerated to await removal of the last aliquot. In retrospect, this difficulty might have been alleviated by employing an inhibitor, *e.g.*, azide (11), one which would not affect subsequent activity of the enzyme induced. No alteration in the population of the cells could be detected turbidimetrically during the induction and holding periods.

TABLE III. Time Study of Action of Oxytetracycline and Chlortetracycline on Induction of Lysine Decarboxylase in Resting Cells of *Escherichia coli*.

Treatment	Length of induction period (min.)*			
	0	20	40	60
None (control)	79†	164	174	200
Oxytetracycline‡	48	58	61	44
Chlortetracycline‡	44	35	44	41

* An additional period of time elapsed between induction and measurement of activity. Undoubtedly this contributed to the final activity.

† Q_{CO_2} (N)

‡ Used at a conc. of 1 μg/ml during induction.

In further experiments, both anaerobic and aerobic conditions were maintained during induction period in testing a number of other antibiotics by the last-mentioned method. The results of these experiments (Table IV) indicate that chlortetracycline, oxytetracycline and, to a lesser degree, chloramphenicol interfered with induction of lysine decarboxylase. The other antibiotics were either slightly inhibitory or stimulatory to formation of this enzyme. Marked stimulation was observed with bacitracin and neomycin. Bacitracin caused pronounced clumping of the cells.

TABLE IV. Effect of Various Antibiotics on Induction of Lysine Decarboxylase in Resting Cells of *Escherichia coli*.

Antibiotic	Conc. (per ml)	Oxygenation during induction: Aerobic	Oxygenation during induction: Anaerobic
Chlortetracycline HCl	1 μg	21*	39
	10 "	20	23
Oxytetracycline HCl	1 "	17	10
	10 "	17	11
Chloramphenicol	10 units	76	123
	100 "	4	6
Streptomycin	1 "	285	245
	10 "	194	240
K penicillin G	10 "	249	250
	100 "	256	200
Neomycin	10 "	320	300
	100 "	166	146
Bacitracin	10 "	320	300
	100 "	276	415
Control, initial†		22	18
" 90 min. later		240	245

* Q_{CO_2} (N) (after a 90-min. exposure to antibiotic).

† See footnote (*) in Table III.

Discussion. The results of this study contribute to the mounting evidence that certain antibiotics interfere with the metabolism of proteins(10,12,13,14). Thus chlortetracycline and oxytetracycline were found to inhibit induction of lysine decarboxylase in cultures of *E. coli* at levels having no measurable effect on growth. These observations suggest that syntheses of these

proteins (enzymes) are singularly sensitive to the presence of such antibiotics. Also, exposure of resting cells to the 2 tetracycline compounds and chloramphenicol retarded induction of lysine decarboxylase; neomycin and bacitracin, under identical circumstances, were stimulatory to this process, while streptomycin and penicillin were without effect. Amino acid decarboxylases so affected need not be of the adaptive type since the production by *E. coli* of the constituitive enzyme, glutamic acid decarboxylase, has been reported to be inhibited by chloramphenicol as readily as the adaptive enzymes, arginine and lysine decarboxylase(2).

Although chlortetracycline, oxytetracycline and chloramphenicol blocked induction of lysine decarboxylase, it does not necessarily follow that they do so through an identical mechanism. The 2 tetracycline derivatives probably have a common mode of action(10). While chlortetracycline and oxytetracycline impaired the synthesis of this enzyme at comparable concentrations, chloramphenicol was active only at appreciably higher levels. The chelating property of tetracycline antibiotics may be significant since the inhibition of a number of enzyme systems by high concentrations of tetracyclines can be reversed by some divalent metal ions(15). Unfortunately the role of chelation was not studied, although low concentrations of these antibiotics were not inhibitory — rather, they were slightly stimulatory—to the activity of preformed lysine decarboxylase. In view of the hypothesis that amino acid decarboxylation results upon formation of a Schiff's base between pyridoxal phosphate and the substrate upon stabilization by a metal ion(16), higher concentrations of the tetracycline compounds might have reversed the activity of this enzyme. Sensitivity of the synthesis of lysine decarboxylase to these antibiotics may reflect interference with exergonic metabolism. Thus, tetracyclines have been reported to inhibit or uncouple oxidative metabolism(15,17,18) and to disrupt the electron transport system of DPN(19). It was noted in one phase of this study that the fermentation of glucose by resting cells was inhibited by the tetracyclines concomitant with interruption of the induction of lysine decarboxylase.

Efforts to divulge the "mode of action" of antibiotics are countless(20,21,22). While this study has not resulted in uncovering a mode by which some of the antibiotics inhibit the formation of adaptive enzymes, it does suggest avenues of approach which must await further developments in the field of the synthesis of inducible enzymes(14,23,24,25).

Summary. At concentrations not inhibitory to growth, the antibiotics chlortetracycline and oxytetracycline inhibited induction of lysine decarboxylase in proliferating cells of *Escherichia coli.* Induction of this enzyme in resting cells was readily inhibited by chlortetracycline, oxytetracycline and chloramphenicol. Penicillin and streptomycin were indifferent while bacitracin and neomycin enhanced induction of lysine decarboxylase in resting cells of this bacterium.

Addendum. After submission of this manuscript for publication, Bachrach, *et al.* (PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 874) reported tetracycline antibiotics to inhibit the formation of isoamylamine and isobutylamine (products of the adaptive enzymes, leucine and valine decarboxylases, respectively) in cultures of *Proteus vulgaris.* They reported also that high concentrations of tetracyclines interfered with the decarboxylation of tyrosine and phenylalanine in dried or cell-free preparations of *Streptococcus faecalis.*

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Temperature Effects on Botulinum A Toxin.* (24033)

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The physical and chemical properties of botulinum toxin(1,3,7,12,13,15,16,18,19, 20) and its physiological action(2,4,5,6,17) have been studied extensively because of the interest in the agent's high toxicity. Burgen *et al.*(4) found that toxin heated in a water bath at 100° for 15 minutes lost its ability to produce paralysis, and Ambache(2) showed that boiling it for 2 minutes destroyed its toxicity. In this report, more detailed data on the effect of temperature on type A botulinum toxin are presented.

Materials and methods. Five toxin preparations, supplied by Fort Detrick, Frederick, Md., were used. Two were partially purified and 3 had been crystallized. The initial studies were performed with the partially purified samples, designated S-1 and S-2. Inactivations at various temperatures and pH values were done with a crystalline sample labelled WA-19. The effect of heating on ultracentrifuge sedimentation patterns was determined with the remaining 2 crystalline samples. Toxicity was assayed in the goldfish, *Carassius auratus*, kept in aquaria at room temperature before and after inoculation. The technic has been described(5). The end points were calculated by modification(10) of the method of Reed and Muench (14) on the basis of mortality ratios observed after 36 hours. Thermal inactivations were carried out in a thermostatic water bath with temperature fluctuations of less than 0.1°. Since preliminary experiments showed that the toxin inactivated rapidly, the diluent, 0.1 M phosphate buffer at pH 6.9 containing 0.2% gelatin, was heated a few degrees above the desired temperature before mixing with the toxin, so that the mixture of toxin and diluent came to the inactivation temperature immediately. This eliminated the necessity of waiting for the mixture to come to thermal equilibrium before the first sample could be taken. Samples were removed from the reaction mixture at subsequent time intervals and tested in 10 goldfish. In this way, the effect of heating on the 50% lethal dose was determined.

Results. Preliminary experiments with

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