OXYTETRACYCLINE STIMULATED INCORPORATION OF CYSTEINE AND TRYPTOPHAN INTO AN IN VITRO PROTEIN SYNTHESIZING SYSTEM FROM ESCHERICHIA COLI

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A reputed primary mode of action of the tetracycline family of antibiotics in vivo is inhibition of protein synthesis (Gale and Folkes, 1953; Nakaya and Treffers, 1959; Hash et al., 1964). In in vitro experiments an inhibition of incorporation of the following radioactive amino acids or mixture of labeled amino acids has been observed: glycine (Gale and Folkes, 1957); leucine (Franklin, 1963); phenylalanine (Laskin and Chan, 1964; Day, 1966); lysine, proline, phenylalanine (Hierowski, 1965); and ^{14}C-algal protein hydrolysate (Okamoto and Mizuno, 1964; Maxwell, 1967). It has often been assumed that the incorporation of other labeled amino acids in in vitro systems would also be inhibited by antibiotics of the tetracycline family, but the present paper presents evidence to the contrary.

In a cell-free amino acid incorporating system derived from E. coli we have observed an apparent stimulation of incorporation of cysteine and tryptophan by the antibiotic oxytetracycline (OTC) under conditions in which the incorporation of the other amino acids is inhibited. The degree of stim-

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ulation of incorporation of the above amino acids is increased with increasing concentrations of OTC but is not inhibited at low levels of the antibiotic.

MATERIALS AND METHODS

E. coli 21 (a gift from Chas. Pfizer and Co., Inc., Groton, Conn.) was grown in the minimal salts and glucose(0.5%) media of Roberts et al. (1955) less the NaCl, at 37° in 10-liter batches in a Model 314 New Brunswick Fermentor (100 rpm and aeration rate of 2 liter/min). Early log phase cells were rapidly cooled by the addition of crushed ice and collected by a Sharples centrifuge. Cells were subsequently washed in the standard buffer of Nirenberg and Matthaei (1961), and suspended in a volume of standard buffer equal to 3 times the wet weight of cells. Disruption of cells was accomplished by a single pass through a precooled (-30°) French Press Cell at 10,000-15,000 p.s.i. The suspension was then centrifuged at 20,000 x g for 20 minutes, 30,000 x g for 30 minutes and finally 30,000 x g for 1 hour; each time the pellet was discarded, and in the final centrifugation the supernatant fraction was aspirated off to within 1/2 inch of the pellet. The supernatant fraction was then dialyzed overnight at 5° against 500 volumes of standard buffer with one change of buffer after the initial 3 hours of dialysis. Aliquots of the dialyzate (termed S-30) were quick-frozen in a dry ice and acetone bath and stored at -30° until needed.

The amino acid incorporating system is derived from that of Nirenberg and Matthaei (1961) containing the following in a volume of 1 ml: 100 μmoles Tris, pH 7.8 at 37°; 10 μmoles $Mg(C_2H_3O_2)_2$; 50 μmoles KCl; 6.0 μmoles $HS(CH_2)_2OH$; 1.0 μmoles ATP, Na salt; 5.0 μmoles phosphoenolpyruvate, K salt; 20 μg pyruvate kinase as an $(NH_4)_2SO_4$ suspension (Mann Research Laboratories, Inc., New York, N. Y.); 0.03 μmoles GTP, Na salt; 50 μg OTC

where indicated (a gift from Chas. Pfizer and Co., Inc.); 0.18 ml of S-30 dialyzate containing 0.33-0.37 mg of protein depending on the preparation; 0.05 μmoles of each of 19 cold L-amino acids and 0.05 μmoles of one ^{14}C-amino acid. The corresponding non-radioactive amino acid was omitted when a ^{14}C-amino acid was added. Radioactive amino acids were uniformly labeled L-amino acids from Nuclear Chicago, Des Plaines, Ill., made up to a constant specific activity of 5 μC/μM except for the following: 10 μC/μM DL-cysteine-3-^{14}C, DL-cystine-3-^{14}C, 2 μC/μM L-histidine (ring-2-^{14}C), DL-leucine-1-^{14}C, L-methionine-methyl-^{14}C, 2 μC/μM L-α-tryptophan-^{14}C, also 10 μC/μM DL-trytophan-2-^{14}C (ring labeled) (Schwarz Bio Research, Inc., Orangeburg, N. Y.). The S-30 dialyzate was in all cases the last component added to the incubation mixture. The tubes were then incubated with shaking for 1 hour at 37^0 and the incorporation terminated by the addition of an equal volume of 10% trichloroacetic acid. All experiments included a "zero time blank" for each amino acid. The "zero time blank" was made by adding trichloroacetic acid before the S-30 dialyzate, and the counts obtained were subtracted from the experimental. The precipitates were then washed by the method of Siekevitz (1952) in a clinical centrifuge. The air-dried protein was then weighed and dissolved in 1 ml of 0.1 N NaOH and counted in a Packard Tri Carb Liquid Scintillation Spectrometer Model 2002 with an average counting efficiency of 60% using a scintillation solvent of toluene:Triton X-100, 2:1 (Patterson and Greene, 1965). An internal standard was added to determine the counting efficiency.

RESULTS AND DISCUSSION

The results appearing in Table 1 summarize the findings of several experiments using different cell-free preparations as indicated. Each number

TABLE 1

Summary of Amino Acid Incorporation in the Absence and Presence of Oxytetracycline

Expt.	S-30 prep	^{14}C Amino Acid added	Without OTC $\frac{\mu\,\mu moles}{mg\ protein}$	With OTC $\frac{\mu\,\mu moles}{mg\ protein}$	$\frac{With\ OTC}{Without\ OTC}$
1	A	Leucine	92	59	.64
		Phenylalanine	68	43	.63
		Valine	36	24	.67
2	B	Leucine	61	40	.66
		Glutamic acid	25	13	.52
		Lysine	31	11	.35
		Tryptophan	95	138	1.45
3	B	Leucine	63	39	.62
		Alanine	12	6	.50
		Cysteine	29	39	1.34
		Serine	35	21	.60
4	C	Leucine	67	44	.66
		Glycine	31	19	.61
		Cystine	12	22	1.83
		Threonine	28	16	.57
5	C	Leucine	65	45	.69
		Aspartic acid	18	12	.67
		Glutamine	29	16	.55
		Isoleucine	23	13	.57
6	D	Leucine	124	92	.74
		Proline	20	9.3	.47
		Tyrosine	12	8.9	.74
		Arginine	35	17	.49
7	D	Leucine	133	88	.66
		Methionine	20	14	.70
		Histidine	4.4	2.5	.57
		Cysteine	23	28	1.22

The incubation mixture was as described in the materials and methods section.

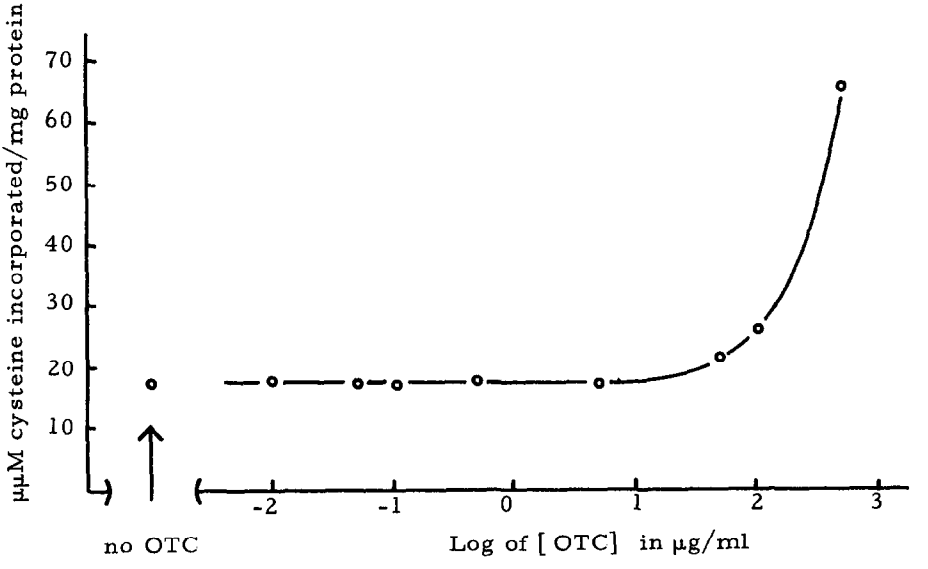
The incorporation of cysteine and cystine were figured on the basis of what was added to the incorporating mixture.

represents the average of results from duplicate tubes. The incorporation of leucine with and without OTC serves as a control in each experiment. The incorporation of several of the amino acids including tryptophan and cysteine has been determined several times with different cell-free preparations. The same stimulatory effect of OTC on incorporation of cysteine and tryptophan has been observed along with inhibition of incorporation of the others. To our knowledge, the effect of OTC on the incorporation of cysteine and tryptophan, as well as several other amino acids, has not been reported previously.

The stimulatory effect of OTC is dependent on its concentration (Fig. 1). It is important to note that there is no inhibition of cysteine incorporation at low levels of OTC. The same general trend was seen in the dose response curve for tryptophan (not shown), although the curve was less reproducible.

FIGURE 1

Dose Response Curve for Cysteine



This phenomenon is reminiscent of the mode of action of streptomycin in producing misreadings of the genetic code (Davies et al., 1964). In this light it may be no coincidence that both cysteine and tryptophan are coded for by similar codons not shared by other amino acids, namely UGU, UGC, and UGG. One also notes that since tryptophan has been implicated in active sites and cysteine in the tertiary structure of enzymes, their incorporation in place of another amino acid due to OTC might be expected to alter the enzyme's capacity for catalysis sufficiently to cause bacteriostasis apart from any general inhibition of protein synthesis which might follow. Since our system has presumably utilized endogenous messenger RNA as opposed to synthetic polynucleotides, it is not possible to speculate further at this time as to the full significance of these findings, especially with respect to their relation to the action of streptomycin in codon misreading.

In view of the work of Kaji et al. (1965) on a soluble amino acid incorporating system from E. coli, it is possible that the stimulatory effect observed by us is due to this system. This possibility is enhanced since, of the amino acids tested in the soluble system, the levels of incorporation of leucine, phenylalanine, and possibly tryptophan were high enough to represent a significant fraction of the counts incorporated by a ribosomal system. The incorporation of cysteine in the soluble system was not reported.

Since the tetracycline family of antibiotics has always behaved qualitatively the same, it is expected that the other tetracycline antibiotics will show the same activity. However, this, as well as the other aspects mentioned above, is presently being investigated in our laboratory.

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