Inhibition of Antibacterial Activity of Cycloserine by Alpha-Alanine.* (23452)

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The antibacterial properties of cycloserine have been described by various groups of investigators(1-3). This compound, identified as D-4-amino-3-isoxazolidone(4), has its greatest activity against Gram-positive cocci and the tubercle bacillus. Evidence has been presented(2) which indicates that cycloserine may be more active *in vivo* than *in vitro*. This observation suggested the possibility that certain ingredients of culture media used for testing its activity might be inhibitory(5). Consequently, a study was undertaken to determine the potential interference of the antibacterial properties of this uniquely simple compound by various growth stimulating compounds. This study revealed that the amino acid, alpha-alanine, specifically inhibited the antibacterial action of cycloserine on staphylococci.

Methods. Various strains of staphylococcus were selected as test organisms. Previous studies(6) have shown that members of this group of organisms may be cultivated in fluid defined media containing salts, vitamins, and 8 to 10 amino acids. The minimal inhibitory concentration (MIC) of cycloserine† against various strains of staphylococci in such media was determined. Simultaneous determinations of the MIC were also made in the same defined media to which had been added, separately, varying quantities of vitamins, amino acids, purines, and pyrimidines not essential for growth. The potential effect on cycloserine of growth factors essential for growth was determined by testing the activity of the antibiotic in media in which the concentration of each essential factor was increased 10 to 100 fold over that required for growth. Growth measurements were made after incubation at 37°C for 48 hours. These were made either on the Evelyn photoelectric colorimeter, or by gross determination of the presence or absence of growth. End points of 90 to 100% inhibition of growth were used in determining the MIC of the antibiotic. All strains of staphylococci studied had been isolated from clinical infections. These were transferred monthly on trypticase soy agar slants and stored in the refrigerator between experiments.

Results. An exhaustive study of most of the more common growth-promoting compounds revealed that alpha-alanine was the only compound which specifically inhibited activity of cycloserine on staphylococci. In Table I are shown the concentrations of cycloserine required for inhibition of growth of 3 strains of staphylococci grown in fluid defined medium containing varying amounts of alanine. Except for minor differences in susceptibility, the results with all 3 strains are quite similar. With each strain, the ratio of the concentration of cycloserine required for inhibition of growth to that of alanine over a wide range

TABLE I. Concentration of Cycloserine Required for Inhibition of Staphylococci in the Presence of Alanine.

Staph.	Alanine, μg/ml	Cycloserine,* μg/ml	Inhibition index†
7739R	0	.32	25
	.06	1.60	
	.32	8.0	
	1.6	40.0	
	8.0	200.0	
	40.0	1000	
	200	1000	
2255	0	1.0	20
	.5	10	
	5.0	100	
	50	1000	
Van	0	.2	10
	.1	1.0	
	1.0	10	
	10	100	
	100	1000	

* Minimal conc. producing 90 to 100% inhibition of growth.

† Ratio of $\frac{\text{Cycloserine conc.}}{\text{Alanine conc.}}$

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TABLE II. Inhibition of Growth of Various Organisms by Cycloserine in Presence and Absence of Alanine.

Culture	Alanine, μg/ml	Cycloserine, μg/ml 0	.32	1.6	8	40	200
		% inhibition of growth					
Staph. 2255	0	0	50	100	100	100	100
	50	0	0	0	0	0	0
E. coli	0	0	0	0	0	50	100
	50	0	0	0	0	0	100
S. flexneri	0	0	0	0	0	100	100
	50	0	0	0	0	0	100
B. cereus	0	0	0	0	0	0	100
	50	0	0	0	0	0	100

of concentrations of both compounds is remarkably constant. Only in the presence of large quantities of cycloserine, 1000 μg/ml or greater, does this appear not to be true.

The ability of alpha-alanine to interfere with activity of cycloserine does not appear to be true in the case of certain other species of organisms tested. By a similar procedure, the activity of cycloserine on single strains of *E. coli, Shigella flexneri and B. cereus* was determined in defined media in the presence and absence of alpha-alanine, 50 μg/ml. A culture of staphylococcus was likewise tested for comparative purposes. In Table II are shown the results of this experiment. The strain of staph. tested was considerably more susceptible than the other species of organisms. Furthermore, alanine in a concentration, 50 μg/ml, which definitely reduced the activity of cycloserine on the strain of staph., had a negligible effect on the activity of the antibiotic against the latter organisms.

TABLE III. Comparison of Inhibition of Cycloserine by Alanine and That by Related Compounds.

Compound	Inhibition of cycloserine,* % of alanine activity†
DL-alpha-alanine	100
L-alpha-alanine	100
D-alpha-alanine	75-90
Phenylalanine	0
Beta-alanine	0
Sodium pyruvate	0
Sarcosine	0
Alanylglycine‡	50
Glycylalanine‡	50
Serine	0
Alpha aminobutyric acid	1.0
Beta " "	0

* Staph. 7739 used as test strain.
† Comparisons made on wt basis.
‡ Other peptides active to extent of alpha-alanine content by wt.

The observation that alanine competitively antagonizes the activity of cycloserine on staphylococci indicated the need for studies to determine whether other compounds related to or structurally similar to alpha-alanine and/or cycloserine had similar antagonistic properties. In Table III are shown the results of such experiments comparing the activity of alpha-alanine with several such compounds. Both the D and L isomers of alpha-alanine antagonized the activity of the antibiotic. Aside from the peptides which were active to the extent of their alanine content, the only other compound with alanine-like activity on cycloserine was alpha-aminobutyric acid which had 1% the activity. It is evident that inhibition of cycloserine is a relatively specific function of alpha-alanine. Any major deviation from its normal structure results in complete loss of activity. Quite surprising was the observation that the amino acid, DL-serine, a degradation product of cycloserine, had no effect upon the antibiotic.

TABLE IV. Effect of Alanine on Antistaphylococcal Activity of Various Antibiotic Agents.

Agent	MIC of agent, μg/ml* In basal medium	In basal medium + alanine, 50 μg/ml
Cycloserine	.2	100
Penicillin	.04	.08
Magnamycin	.4	.2
Erythromycin	.1	.1
Tetracycline	3.1	6.2
Chloromycetin	6.2	6.2
Neomycin	.45	.45
Streptomycin	.8	.8
Oleandomycin	3.1	3.1
Novobiocin	3.1	1.6
Vancomycin	.4	.8

* Penicillin, units/ml.

In an attempt to determine whether the action of alanine was specific only for cycloserine, similar experiments were carried out with several of the other antibiotic agents. As shown in Table IV, alanine failed to affect the activity of any of the other agents. Under the same experimental conditions, however, the concentration of cycloserine required for inhibition of a strain of staphylococcus

was 500 times greater in the presence of alpha-alanine, 50 μg/ml, than in its absence.

Discussion. Several features of the phenomenon described in this report are quite significant. The ability of alpha-alanine to antagonize the action of cycloserine on staphylococci and not that on other organisms tested is not surprising. This may be attributed to the fact that the nutritional requirements of staphylococci are more complex. Of considerable significance is the fact that the alanine molecule must be unaltered for it to antagonize the antibiotic. Minor changes in the structure of the amino acid results in its failure to antagonize. Of particular significance in this respect is the complete loss of activity resulting from the changing of the amino group to the beta-position. In a similar fashion, any substitution on the methyl group results in complete loss of activity. As yet, we have been unsuccessful in locating a compound capable of relieving the inhibition of cycloserine by alanine.

The constancy of the ratio of the two compounds over a fairly broad range of concentrations is characteristic of that of a competitive type of inhibition(7). This lends support to the belief that cycloserine acts on staphylococci by virtue of its ability to interfere in some way with the metabolism of alanine. No doubt the greater susceptibility of staphylococci to the antibiotic than that of the other species studied is related to the specific interference in the metabolism of alanine by this group of organisms.

Summary. Alpha-alanine inhibits antibacterial activity of cycloserine on staphylococci. The inhibition appears to be of a competitive nature. This amino acid does not inhibit activity of the antibiotic on other organisms studied. Alpha-alanine does not interfere with the action of other antibiotic agents.

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