of tissues was not achieved in all animals.

Reversal of positive tuberculin reactions in animals subjected to single or multiple drug therapy postinoculation has been reported by several investigators(1,11-13). Observation of reverters following cessation of treatment indicated that a positive reaction may again occur(12,14). In the present study, a positive reaction (following a previous reversal) occurred in only 2 guinea pigs. The possibility that some of the animals may have reverted as a result of repeated tuberculin tests cannot be definitely answered. The data in this study, however, would not support this concept.

Culturable organisms were not demonstrated in tissues of animals with negative gross pathology although histologic evidence of tuberculosis was found in 2 guinea pigs. Viable tubercle bacilli have been recovered from grossly negative tissues(12). The observation that drug-treated animals with negative or intermittent weak reactions may still exhibit macroscopic disease(13) is in agreement with the findings reported here.

Summary. Reversal of the tuberculin reaction occurred in 70% of animals treated with INH following development of a positive test. One year of INH treatment failed to sterilize the tissues in 15% of the animals. Evidence is presented that INH completely protected guinea pigs from tuberculosis when treatment was initiated prior to inoculation of a minimal dose. All non-treated controls developed visceral tuberculosis.

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Antagonism of the *D*-Alanine Reversal of *D*-Cycloserine Action by *L*-Alanine in *Mycobacterium acapulcensis*. (30096)

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During a study of the effect of *D*-cycloserine on growth of *Mycobacterium acapulcensis* it was found that *D*-alanine reversed the growth inhibition produced by this antibiotic, but surprisingly enough *DL*-alanine was not so effective. This paper reports on the characteristic of the antagonism of *L*-alanine over the *D*-alanine reversal of *D*-cycloserine action. In addition, a mutant resistant to *D*-cycloserine was isolated and studied.

Methods. *D*-cycloserine was kindly supplied by Hoffmann-LaRoche (Basel, Switzerland). *D*-alanine and *DL*-alanine were purchased from California Corp. for Biochemical

Research. All chemicals were of reagent grade.

The composition of the basal medium were as follows (in g/liter): L-asparagine 5; Na_2HPO_4, 2.5; $KH_2PO_4 \cdot 7H_2O$, 0.20; $FeCl_2 \cdot 4H_2O$, 0.0005; Triton WR-1339, 0.5; KCl, 0.01; and glycerol 20 ml; initial pH, 6.8-7.2. Neutralized solutions of D-cycloserine and isomers of alanine were sterilized by Seitz filtration and were added to previously autoclaved Erlenmeyer flasks containing the basal medium.

Mycobacterium acapulcensis 102, a rapid and homogeneously growing mycobacterium (1) selected for this study, was maintained on Löwenstein-Jensen's slants. From these, inocula were prepared by seeding flasks of basal medium. The flasks were incubated at 37°C and shaken twice daily; 4 days later the cells were harvested by centrifugation, washed twice with sterile saline solution and resuspended in basal medium to a standardized optical density reading.

A cycloserine resistant mutant of *M. acapulcensis* was isolated as follows: a heavy inoculum of *M. acapulcensis* that was sensitive to 20 μg/ml of D-cycloserine was seeded into basal medium containing 100 μg/ml of D-cycloserine and was incubated at 37°C. The whole inoculum was transferred at 3-day intervals to fresh medium containing the antibiotic, until growth was observed. The resulting growth was then transferred to agar plates from which a mutant resistant to 100 μg/ml but sensitive to 200 μg/ml of D-cycloserine was isolated.

Growth was measured turbidimetrically, using a Coleman Jr. Spectrophotometer at a wavelength of 650 mμ.

N-acetyl aminosugar determinations were carried out by the procedure of Reissig *et al* (2). Morphological observations were made by phase-contrast microscopy.

Results. The growth rate of *M. acapulcensis* was similar either in the absence or presence of different concentrations of L, D or DL-alanine (Fig. 1). Growth of *M. acapulcensis* was inhibited by 20 μg/ml of D-

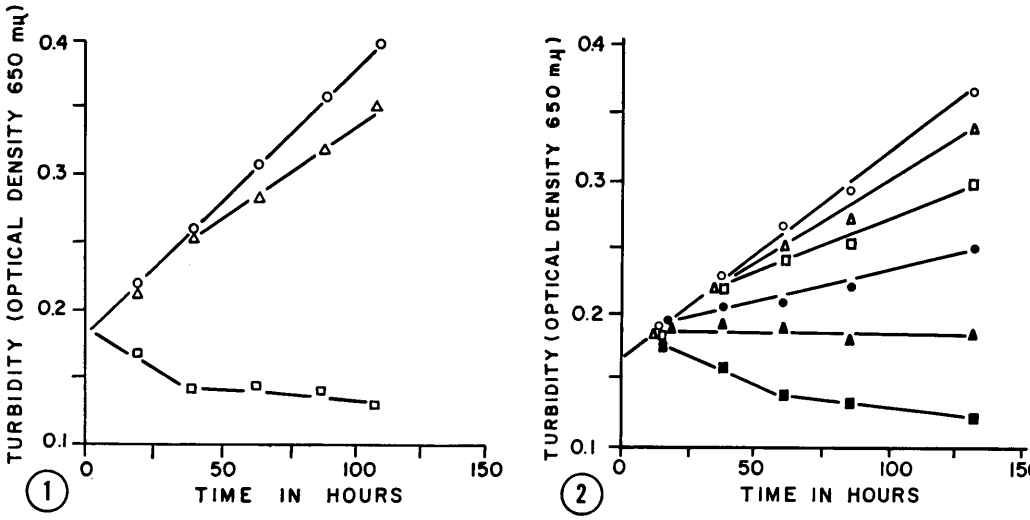

FIG. 1. Effect of different concentrations of D-, L-, and DL-alanine on growth of *M. acapulcensis* with or without D-cycloserine. ○ = Type of curve obtained without additions or with 25, 50, 250 and 500 μg/ml of L-alanine or D-alanine or DL-alanine; △ = Curves obtained with 20 μg/ml D-cycloserine and either 25, 100, 200 or 500 μg/ml of D-alanine; □ = Curves obtained with 20 μg/ml of D-cycloserine or 20 μg/ml of D-cycloserine plus either 25, 100, 200 or 500 μg/ml of L-alanine.

FIG. 2. Effect of DL- and mixtures of D- and L-alanine on growth of *M. acapulcensis*. Characteristic curves are given. ○ = Control; △ = 20 μg/ml of D-cycloserine + 25 μg/ml of L-alanine and 500 μg/ml of D-alanine or 20 μg/ml of D-cycloserine + 1000 μg/ml of DL-alanine; □ = 20 μg/ml of D-cycloserine + 25 μg/ml of L-alanine and 200 μg/ml of D-alanine; ● = 20 μg/ml of D-cycloserine + 25 μg/ml of L-alanine and 50 μg/ml of D-alanine; ▲ = 20 μg/ml of D-cycloserine + 50 μg/ml of DL-alanine or 20 μg/ml of D-cycloserine + 25 μg/ml of D-alanine and 500 μg/ml of L-alanine; ■ = 20 μg/ml of D-cycloserine.

TABLE I. Effect of *D*-cycloserine and *D*-alanine in Accumulation of N-acetyl aminosugar by *M. acapulcensis*.

Strain	Addition to basal medium, μg/ml		μmoles of N-acetyl aminosugar per liter at optical density 1.0 (650 mμ), Days, incubation							
	D-cycloserine	*D*-alanine	0	2	4	6	8	10	12	17
Wild type,	0	0	1.0	2.0	3.1	4.2	6.6	—	6.7	—
D-cycloserine	20	0		10.0		12.0	—	—	—	—
—sensitive	100	0		8.8	9.0	12.0	32.0	—	36.0	—
	100	100		2.8	1.6	2.1	2.8	—	3.4	—
Mutant	0	0	11.0	11.8	—	11.8	—	10.8	—	10.0
D-cycloserine	20	0		11.4	—	12.0	—	13.3	—	—
—resistant	50	0		10.2	—	19.2	—	20.0	—	—
	100	0		10.4	—	22.8	—	28.1	—	40.0
	100	100		—	—	—	—	—	—	24.0

cycloserine with some accompanying lysis of organisms occurring as evidenced by a decrease in the optical density readings. The antibiotic activity was almost completely reversed by 25 μg/ml of *D*-alanine. *L*-alanine was totally inactive when used at similar concentrations. Similar results have been reported by Zygmunt(3) using another species of *Mycobacterium*.

DL-alanine displayed an unexpected effect. Fifty μg/ml of this compound were only partially effective in reversing the action of 20 μg/ml of *D*-cycloserine. A similar effect was observed with a mixture of 500 μg/ml of *L*-alanine and 25 μg/ml of *D*-alanine. Only 500 μg/ml of *D*-alanine in the presence of 25 μg/ml of *L*-alanine, or 1000 μg/ml of *DL*-alanine, afforded almost complete protection against 20 μg/ml of *D*-cycloserine (Fig. 2).

Filamentous and globular forms of *M. acapulcensis* were observed when the cells were incubated for 72 hours in the presence of 20 μg/ml of *D*-cycloserine; with 0.15 *M* sucrose a protective effect was observed, optical density of the culture increased, although at a reduced rate.

The sensitive strain of *M. acapulcensis* accumulated a mucopeptide even in the absence of *D*-cycloserine. This accumulation increased in the presence of 20 μg/ml of the antibiotic. This effect was completely antagonized when *D*-alanine was added to the medium in a similar concentration as the antibiotic. Furthermore the accumulation of the mucopeptide is less than in the absence of the antibiotic (Table I).

The resistant mutant grows at a slower rate than the wild strain and contains a 10 times greater amount of the mucopeptide per cell weight. This mutant accumulates N-acetyl aminosugar only in the presence of 50 μg/ml of *D*-cycloserine. An increase in the accumulation was observed as the concentration of the antibiotic was raised. *D*-alanine when used in an equimolecular concentration as *D*-cycloserine was unable to reverse completely the accumulation of the mucopeptide (Table I).

Discussion. The fact that a mucopeptide was accumulated by cells of *M. acapulcensis* in the presence of *D*-cycloserine, and the reversal of this effect by *D*-alanine, strongly suggests that this mucopeptide is a normal precursor of the mycobacterial cell wall, and that *D*-cycloserine competes with the formation or the utilization of *D*-alanine for cell wall synthesis. This is in accord with the protection afforded by sucrose when cells of *M. acapulcensis* were grown in the presence of *D*-cycloserine, and also with the appearance of filamentous and globular forms. However, it should be noted that "protoplast" forms were not observed.

Up to now, 3 enzymic sites in bacterial cells have been described in which *D*-cycloserine competes with *D*-alanine. These are: the alanine racemase, the *D*-alanyl-*D*-alanine synthetase(4), and the permease that carries *D*-alanine into the cell(5). The fact that *D*-cycloserine inhibits the growth of *M. acapulcensis* in a minimal medium shows that this antibiotic competes inside the cell with formation or utilization of *D*-alanine.

L-alanine does not reverse the inhibition

caused by *D*-cycloserine; however, this amino acid (25 μg/ml) is able to compete in the protection afforded by *D*-alanine (from 25 to 500 μg/ml) against *D*-cycloserine (20 μg/ml). A competition for a common extracellular site between these 2 diastereoisomers is not enough to explain this effect, since high concentrations of *L*-alanine do not inhibit the protection afforded by *D*-alanine. Moreover, it would be difficult to explain why 500 μg/ml of *D*-alanine were required to protect against the effect of 25 μg/ml of *L*-alanine as well as against the effect of 500 μg/ml of *L*-alanine.

Mora and Snell(5) have shown that in spite of the fact that extracellular *L*-alanine is able to slow down the penetration of *D*-alanine in protoplasts of *Streptococcus faecalis*, pyridoxal was only able to stimulate the transport of *L*-alanine; this probably indicates that more than a simple competition is involved at the receptor site in the membrane. Studies on the uptake of radioactive amino acids and peptides by *Pediococcus cerevisiae* lead Shankman *et al*(6) to postulate the existence of 2 physically different sites in the membrane interacting at a distance.

In regard to the accumulation of a mucopeptide by the resistant strain of *M. acapulcensis* to *D*-cycloserine, the results are in accordance with data obtained by Park(7) in cells of *Staphylococcus aureus* resistant to penicillin.

Summary. Growth of *Mycobacterium acapulcensis* is inhibited by *D*-cycloserine. This effect is completely reversed by equimolecular concentrations of *D*-alanine. *L*-alanine is totally inactive while *DL*-alanine is only effective at higher concentrations. *L*-alanine antagonized the protection afforded by *D*-alanine, a fact that cannot be explained by a simple competition between these diastereoisomers at the cell membrane level. In the presence of *D*-cycloserine a mucopeptide accumulates in the sensitive strain and also in the resistant mutant when allowed to grow at high antibiotic concentrations.

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Histochemistry LXXVIII. Ascorbic Acid in Normal Mast Cells and Macrophages and in Neoplastic Mast Cells.* (30097)

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The role of ascorbic acid in the mast cell remains to be delineated, but some interesting observations of possible influences have appeared. Relative depletion of mast cells from guinea pig skin due to ascorbic acid deficiency was reported by von Numers(1), and Pettersson(2) who confirmed this also observed a similar effect in aortic adventitia. The possibilities indicated are that ascorbic acid is necessary, either directly or indirectly, for formation of mast cells, or for their maintenance once formed, or both. Another point of interest is whether ascorbic acid plays a role in hydroxylation of tryptophan (TP) as the first step in the biosynthesis of 5-hydroxytryptamine (serotonin, 5HT), since the pos-

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