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Effect of L-Homoserine on the Growth of *Mycobacterium tuberculosis*

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L-Homoserine was observed to inhibit the growth of *Mycobacterium tuberculosis*. In the metabolism of *M. tuberculosis*, L-homoserine was found to be a precursor of threonine, isoleucine, and methionine. L-Homoserine-inhibited cells contained elevated levels of the enzyme acetohydroxy acid synthetase. In addition, washed cell suspensions of *M. tuberculosis* formed significant amounts of alpha-amino-*n*-butyric acid from supplements of L-homoserine. DL-Alpha-amino-*n*-butyric acid proved to be much more inhibitory for growth than L-homoserine. Growth antagonism by L-homoserine was reversed by L-lysine, L-threonine, and combinations of L-leucine with L-valine. At the cellular level, these amino acids reduced the amount of aceto-hydroxy acid synthetase in cells grown with L-homoserine and competed with DL-homoserine-*4*-^{14}C for entrance into the extractable cell pool. L-Isoleucine also antagonized the conversion of L-homoserine to alpha-amino-*n*-butyric acid. Available data indicated that the effect of L-homoserine was related to its conversion to alpha-amino-*n*-butyric acid which subsequently inhibited growth

During studies concerned with the pathways of amino acid synthesis in *Mycobacterium tuberculosis*, an inhibitory effect of L-homoserine upon the growth of this organism was noted. In the metabolism of *M. tuberculosis*, L-homoserine was found to function as a precursor of methionine, threonine, and isoleucine. As a key intermediate in the synthesis of these amino acids, it is apparent that the cellular concentration of L-homoserine could effectively determine the rate of growth. Reports of inhibition by L-homoserine of purified aspartokinases in extracts of various microorganisms (9, 11) have demonstrated how growth could be regulated by a pattern involving inhibition or repression. In the present study, this and other possibilities were considered in an attempt to determine the manner in which L-homoserine restricts the growth of *M. tuberculosis*. Data are presented which indicate that the principle effect of L-homoserine is related to its conversion to alpha-amino-*n*-butyric acid which then acts to inhibit growth.

MATERIALS AND METHODS

M. tuberculosis H37Rv was maintained by transfer in Middlebrook's 7H10 OA liquid media (7). For growth experiments, a basal medium consisting of 0.5% KH_2PO_4, 0.3% $(NH_4)_2SO_4$, 0.15% magnesium citrate, 0.5% Tween 80, 0.5% albumin fraction V, and 0.1% D-glucose was employed. Inocula consisted of log-phase cells washed with 0.05 M potassium phosphate buffer (*p*H 7.0) containing 0.5% Tween 80 and made up in suspension with buffer to contain 0.5 mg of cells (dry weight) per ml. Tubes (18 by 150 mm) containing 10 ml of basal medium received one drop of suspension from a 5-ml pipette. After incubation for 7 days at 37 C with constant agitation on a roller drum, growth was estimated by measuring optical density at 690 nm. Data from two to four replicate experiments were averaged for presentation in the tables.

Isotopic competition experiments (1) were carried out with the above media supplemented with D-glucose-*UL*-^{14}C. Cells were recovered by centrifugation, washed with buffer, and heated at boiling water temperature for 30 min in 5% trichloroacetic acid. The precipitated protein was washed with acidified ethanol, sealed in vacuo in tubes with 2 ml of 6 *N* HCl, and heated in an autoclave for 8 hr at 109 C. After removal of HCl, amino acids present in the hydrolysate were determined with a Technicon amino acid analyzer. Radioactivity present in individual amino acids was measured by shunting column effluent through a flow cell-liquid scintillation counter arrangement.

Except for some modifications, acetohydroxy acid synthetase was assayed in intact cells as previously described (6, 8, 13). A 1-ml amount of washed cells, suspended in 0.1 M potassium phosphate buffer (*p*H 7.0) containing 0.5% Tween 80, was added to tubes containing, in 1 ml buffer: 50 mg of sodium pyruvate, 0.02 ml of toluene, and 200 μg of sodium deoxycholate.

After mixing, the tubes were incubated at 37 C with shaking. At the completion of the assay, the reaction was stopped by the addition of 0.1 ml of 50% trichloroacetic acid or 0.1 ml of 2.5 N NaOH. The cells were packed by centrifugation, and the supernatant was sampled for acetoin determination (15). Supernatant from tubes receiving NaOH was assayed directly, whereas the supernatant from the acidified tubes was heated for 15 min at 60 C prior to analysis. There was no evidence of the direct conversion of pyruvate to acetoin.

In uptake experiments, 1 ml of washed cell suspension, made up in 0.1 M potassium phosphate-Tween 80 buffer (*p*H 7.0) to contain 25 mg dry cells per ml, was added to tubes containing 1 ml of buffer, 200 μg of chloramphenicol, 10 mg of D-glucose, 1 mg of L-homoserine, and 1 μCi of DL-homoserine-*4*-^{14}C. Two tubes served as controls while other tubes in duplicate contained either 4 mg of L-lysine HCl, 4 mg of L-isoleucine, or 2.5 mg of L-valine and 2.5 mg of L-leucine. After incubation at 37 C for 25 min with shaking, the tubes were chilled in an ice bath and the cells were recovered by refrigerated centrifugation. The cell pellet was washed with 2 ml of cold buffer and repacked by refrigerated centrifugation. The supernatant was discarded, and the cells were heated in 1 ml of buffer for 30 min at boiling water temperature. After a final centrifugation, the supernatant was sampled for radioactivity.

Acetoin was obtained from Eastman Organic Chemicals and purified by distillation. D-glucose-*UL*-^{14}C (specific activity, 3.1 mCi/mM) and DL-homoserine-*4*-^{14}C (specific activity, 11.5 mCi/mM) were obtained from New England Nuclear Corp. and Schwarz Bioresearch Inc., respectively.

RESULTS

The growth of *M. tuberculosis* in response to various levels of L-homoserine is shown in Table 1. Approximately 50 μg/ml was required to reduce maximum growth by one half. In separate experiments not shown here, only the L-isomer was found to be active. Moreover, strains of *M. tuberculosis* which were resistant to the antituberculosis drugs, *p*-aminosalicylic acid, streptomycin, or isoniazid, showed comparable susceptibility to growth inhibition by L-homoserine. Knowledge of the role homoserine plays in the metabolism of *M. tuberculosis* was obtained from isotopic competition experiments (1). With this technique, the inclusion of L-homoserine in growth media containing D-glucose-*UL*-^{14}C resulted in total suppression of carbon-14 in protein-bound threonine and methionine and partial suppression in isoleucine (Table 2). The suppression of radioactivity in these amino acids is in keeping with current knowledge of amino acid synthesis in other bacteria.

After these initial observations, attempts were made to reverse the effect of L-homoserine by adding various metabolites to the growth medium. Reversal was noted with casein hydrolysate, and the testing of individual amino acids showed that the effect of homoserine could be overcome by L-lysine, L-isoleucine, and combinations of L-leucine and L-valine (Table 3). These amino acids have the common property of being associated with the aspartic acid and related pathways in *M. tuberculosis*. Members of the aspartic acid pathway which showed no activity were L-threonine and L-methionine. The relationship between L-homoserine and combinations of L-valine with L-leucine is shown in Table 4. It is apparent that the relationship is mutualistic in that L-homoserine acts to suppress the growth antagonism observed at higher levels of L-leucine and L-valine. L-Isoleucine and L-lysine did not show a mutualistic effect with L-homoserine. To complement these observations, it was found that dipeptides containing lysine, valine, or leucine as component members did not reverse the homoserine

TABLE 1. *Effect of L-homoserine on the growth of Mycobacterium tuberculosis*

Amt (μg/ml) of L-homoserine	Optical density
0	0.73
25	0.70
50	0.38
100	0.13
250	0.04
500	0.03
1,000	0.03

TABLE 2. *Effect of exogenous L-homoserine on carbon-14 fixed in protein-bound amino acids by Mycobacterium tuberculosis*

Amino acid	Per cent of control[a]
Aspartate	0.96
Threonine	0.00
Serine	0.83
Proline	0.92
Glutamate	0.90
Glycine	0.94
Alanine	1.06
Valine	1.00
Methionine	0.00
Isoleucine	0.57
Leucine	1.01
Tyrosine	0.96
Phenylalanine	0.92
Lysine	0.99
Arginine	1.10

[a] Control cells grown in basal media containing 3.34×10^{-2} M D-glucose-*UL*-^{14}C, specific activity 0.14 mCi/mM). Radioactivity of control compared to radioactivity of cells grown in basal media supplemented with 5.06×10^{-3} M L-homoserine.

effect although *M. tuberculosis* possessed dipeptidases capable of hydrolyzing them.

Apart from growth-related phenomena, L-homoserine-inhibited cells were found to contain elevated levels of the enzyme acetohydroxy acid synthetase. In the presence of L-isoleucine, L-lysine, and L-leucine with L-valine, the level of this enzyme was reduced to that found in the control cells (Table 5). The elevated level of acetohydroxy acid synthetase apparently was in response to increased concentration of its substrate alpha-ketobutyric acid since washed cell suspensions of *M. tuberculosis* accumulated alpha-amino-*n*-butyric acid when incubated with supplements of L-homoserine (Table 6). Alpha-amino-*n*-butyric acid could be formed by transamination from alpha-ketobutyric acid, with the latter compound arising from theonine by the action of threonine deaminase (12). In keeping with isotopic competition experiments, increased amounts of threonine, isoleucine, and methionine were found. The possible significance of alpha-amino-*n*-butyric acid and alpha-ketobutyric acid on the growth of *M. tuberculosis* is illustrated in Table 7. Although both compounds are inhibitory for growth, DL-alpha-amino-*n*-butyric acid is approximately 10-fold more toxic than L-homoserine. Inhibition of *M. tuberculosis* by DL-alpha-amino-*n*-butyric acid has been noted previously (16). In separate experiments, it was found that the amino acids which restored the growth of L-homoserine-inhibited cells had no effect on growth inhibition by DL-alpha-amino-*n*-butyric acid. In this regard, the ability of L-isoleucine to restore growth may be related to inhibition of threonine deaminase (12). With washed cell suspensions of *M. tuberculosis* supplemented with L-homoserine, inhibition of alpha-amino-*n*-butyric acid formation occurred in the presence of L-isoleucine (Table 8). No comparable inhibition was observed with L-lysine or L-leucine and L-valine. However, these amino acids, along with L-isoleucine, antagonized the entrance of DL-homoserine-*4*-^{14}C into the extractable cell pool (Table 9).

TABLE 3. *Effect of various amino acids on the growth inhibition of Mycobacterium tuberculosis by* L-*homoserine*

Additions[a]	Optical density
None	0.94
L-Homoserine	0.26
L-Isoleucine and L-homoserine	0.71
L-Valine, L-leucine, and L-homoserine	0.93
L-Lysine HCl and L-homoserine	0.93

[a] L-Homoserine present at 100 μg/ml, L-isoleucine at 400 μg/ml, L-valine at 250 μg/ml, L-leucine at 250 μg/ml, and L-lysine HCl at 400 μg/ml.

DISCUSSION

Speculation that much of the growth antagonism shown by L-homoserine stems from its conversion to alpha-amino-*n*-butyric acid is supported by a variety of experimental facts. Among these may be cited the 10-fold greater toxicity of DL-alpha-amino-*n*-butyric acid over L-homoserine for the growth of *M. tuberculosis* and the accumulation of alpha-amino-*n*-butyric acid by *M. tuberculosis* cells in response to supplements of L-homoserine. Also in keeping with this contention is the finding that L-homoserine-inhibited cells contain elevated levels of the enzyme acetohydroxy acid synthetase, presumably in response to increased concentrations of its substrate alpha-ketobutyric acid. In addition, it is probably significant that the amino acids which reverse the inhibitory effect of L-homoserine have the unifying property of lowering the cellular level of acetohydroxy acid synthetase.

As for affecting the overall growth rate, L-homoserine apparently does not inhibit the aspartokinase reaction. If the synthesis of L-

TABLE 4. *Growth response of M. tuberculosis to combinations of* L-*leucine,* L-*valine, and* L-*homoserine*

Amt (μg/ml) of L-homoserine	Optical density with L-valine and L-leucine at						
	0 μg/ml	75 μg/ml	125 μg/ml	250 μg/ml	500 μg/ml	750 μg/ml	1,000 μg/ml
0	1.00	0.93	0.90	0.85	0.49	0.18	0.07
25	0.96	1.05	1.07	1.06	1.06	0.80	0.32
50	0.73	0.96	1.00	1.04	1.07	1.04	0.57
100	0.19	0.86	0.94	1.02	1.07	0.98	0.61
150	0.05	0.54	0.59	1.00	1.06	0.97	0.72
200	0.04	0.31	0.35	0.66	1.00	0.99	0.75
250	0.03	0.16	0.19	0.41	0.79	0.95	0.78
300	0.02	0.12	0.14	0.24	0.55	0.73	0.65

TABLE 5. *Acetohydroxy acid synthetase in M. tuberculosis cells grown with various amino acids*

Growth conditions[a]	Acetoin/ mg of dry cells
Control	26.2
L-Homoserine	56.9
L-Isoleucine and L-homoserine	31.5
L-Valine, L-leucine, and L-homoserine	27.0
L-Lysine HCl and L-homoserine	27.0

[a] L-Homoserine at 100 μg/ml, L-isoleucine at 400 μg/ml, L-valine 250 μg/ml, L-leucine 250 μg/ml, and L-lysine HCl at 400 μg/ml.

TABLE 6. *Amino acids present in extracts of M. tuberculosis after incubation with and without L-homoserine*

Amino acid	Without L-homoserine (μM/ml)	With L-homoserine[a] (μM/ml)
Aspartate	Absent	0.052
Threonine	Absent	1.622
Serine	0.029	0.207
Homoserine	0.278	NS[b]
Glutamate	0.884	NS[b]
Glycine	0.020	0.081
Alanine	0.019	0.047
Alpha-amino-*n*-butyrate	Absent	0.552
Valine	0.042	0.047
Methionine	Absent	0.026
Isoleucine	0.010	0.121
Leucine	0.012	0.026
Tyrosine	0.011	0.023
Phenylalanine	0.010	0.026

[a] Washed cells (25 mg) incubated for 16 hr in 3 ml of 0.1 M potassium phosphate-Tween 80 buffer (*p*H 7.0) containing 10 mg of L-homoserine. After 16 hr on a roller drum, tubes were made up to 5 ml with water and heated in a boiling water bath for 30 min.

[b] NS, large amount of homoserine masked glutamic acid peak.

TABLE 7. *Effect of DL-alpha-amino-n-butyric acid and alpha-ketobutyric acid on the growth of Mycobacterium tuberculosis*

Addition (μg/ml)	Optical density
None	0.76
Alpha-ketobutyric acid	
10	0.78
25	0.76
50	0.58
DL-Alpha-amino-*n*-butyric acid	
10	0.43
20	0.23

TABLE 8. *Effect of amino acids on the synthesis of alpha-amino-n-butyric acid by washed cell suspensions of M. tuberculosis*

Additions[a]	Alpha-amino-*n*-butyric acid (μmoles/ml)
Control	Not detected
L-Homoserine	0.416
L-Isoleucine and L-homoserine	0.180
L-Lysine HCl and L-homoserine	0.553
L-Valine, L-leucine, and L-homoserine	0.77

[a] Cells (24 mg) incubated with 3 ml of 0.1 M potassium phosphate-Tween 80 buffer (*p*H 7.0) containing, as indicated, L-homoserine (10 mg), L-isoleucine (50 mg), L-lysine HCl (40 mg), L-valine (25 mg), and L-leucine (25 mg). After 16 hr of incubation on a roller drum, 2 ml of water was added and the suspensions were heated in a boiling water bath for 30 min.

TABLE 9. *Effect of amino acids on the uptake of DL-homoserine-4-^{14}C into the extractable cell pool of M. tuberculosis*

Additions[a]	dpm[b]	Inhibition (%)
Control	27,409	
Isoleucine	18,116	34
L-Lysine HCl	23,520	15
L-Valine and L-leucine	18,127	34

[a] Experimental conditions described in text.

[b] Disintegrations per minute.

lysine were a limiting factor, the dipeptide L-lysylglycine, which is readily hydrolyzed by *M. tuberculosis* cells, should be as effective as L-lysine in restoring growth. In addition, L-threonine and L-methionine, other end products of the pathway leading from aspartic acid, have no effect on L-homoserine inhibition. The noninhibitory nature of L-threonine is difficult to explain since it lies on the pathway from L-homoserine to alpha-ketobutyric acid. Isotopic competition experiments showed exogenous L-threonine to suppress radioactivity in protein-bound threonine but its effect on isoleucine was not of the same order as that observed with L-homoserine. This could be explained on the basis of poor equilibration of exogenous L-threonine with the enzymatic pathway or by the synthesis of alpha-ketobutyric acid from L-homoserine through a pathway that did not involve L-threonine (5). The latter possibility, however, is not in keeping with the observed inhibitory effect of L-isoleucine on the conversion of L-homoserine to alpha-amino-*n*-butyric acid. Inhibition of threonine deaminase

(12) by L-isoleucine would cause a reduction in alpha-ketobutyric acid available for transamination and afford an explanation for the ability of L-isoleucine to reverse the growth antagonism shown by L-homoserine.

The greater effect of combinations of L-leucine and L-valine than their individual addition in reversing the growth inhibition by L-homoserine suggests that L-homoserine may have an affect on their synthesis. Since the enzyme acetohydroxy acid synthetase functions in pathways leading to the synthesis of isoleucine as well as leucine and valine by mediating the transfer of "active acetal" to alpha-ketobutyric acid and pyruvic acid, respectively (14), it is possible to speculate that an increase in alpha-ketobutyric acid might restrict the synthesis of valine and leucine through competition for "active acetal." The ability of L-homoserine to overcome the growth antagonism shown by leucine and valine may be related to reported inhibitory and suppressive effects of these amino acids on acetohydroxy acid synthetase (4, 10). Under such conditions, an increase in alpha-ketobutyric acid would give advantage to the isoleucine pathway. The principle effect of L-homoserine, however, would appear not to be on the synthesis of leucine or valine, since dipeptides of which they are component members had little effect on L-homoserine inhibition.

The lower values of acetohydroxy acid synthetase found in cells grown with L-homoserine and the growth-restoring amino acids in comparison with cells grown with L-homoserine alone may reflect the ability of these amino acids to exclude homoserine from the cell pool. Competition with homoserine for transfer across the cell membrane would have the effect of reducing the cellular level of alpha-ketobutyric acid available for transamination to alpha-amino-*n*-butyric acid. Although cellular accumulation of homoserine and the effect of the amino acids were not of a high order of magnitude, it is possible that, with the small number of cells used to inoculate a growth experiment, their effect would be of considerable significance. Supporting this is the observation that the degree of inhibition by L-homoserine in growth experiments was related to inoculum size. In one reported example of growth inhibition by L-homoserine (2), reversal by L-leucine was attributed to competition at the cell membrane (3).

On the basis of this work, animal experiments have been carried out in which rats were fed diets containing 4 and 8% DL-alpha-amino-*n*-butyric acid. After 7 days, plasma levels of alpha-amino-*n*-butyric acid in animals receiving the 8% supplement were found to be as high as 3.3 μmoles/ml. Theoretically, this is many times greater than the amount of DL-alpha-amino-*n*-butyric acid needed to inhibit growth of the standardized inoculum. Further studies designed to evaluate the potential of alpha-amino-*n*-butyric acid as an antituberculosis compound are under way.

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