ASPARAGINASE FROM MYCOBACTERIA1, 2

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Asparagine has been used extensively as the nitrogen source in the cultivation of tubercle bacilli. Relatively little is known about the metabolism of asparagine by mycobacteria. Kirchheimer and Whitaker (1954) have described asparaginase in washed cells of Mycobacterium tuberculosis strains H37Ra, H37Rv, and BCG and in Mycobacterium smegmatis; little asparaginase activity was found in Mycobacterium phlei. Deamidation of asparagine was demonstrated with aspartic acid and ammonia as the products of the reaction. Cell-free preparations of M. tuberculosis and M. smegmatis also catalyze the deamidation. Halpern and Grossowicz (1957) described asparaginase activity in cell-free extracts of M. tuberculosis strain BCG and M. phlei. The stereospecificity of the enzymes was demonstrated for L-asparagine, with competitive inhibition by D-asparagine. Altenbern and Housewright (1954) have described asparaginase from extracts of Brucella abortus. They demonstrated the existence of two stereospecific asparaginases in their extracts. Asparaginase activity in rat liver homogenates has been described by Price and Greenstein (1947), Errera and Greenstein (1947), Greenstein and Price (1949), and Meister et al. (1952). Their findings can be summarized as follows. Activity was enhanced by added phosphate and α-keto acids. When homogenates were heated to 50 C, loss of activity occurred that could be restored by the addition of α-keto acids; phosphate had no effect on the heated extracts. Asparaginase activity stimulated by phosphate and relatively heat labile was designated asparaginase I. The relatively heat stable asparaginase requiring α-keto acids for activity was designated asparaginase II. Asparaginase II activity was demonstrated by Meister et al. (1952) to be due to two enzymatic activities: (1) a transamination reaction requiring the α-keto acid as the amino acceptor and (2) an omega deamidation reaction on α-ketosuccinamic acid formed from asparagine by the transamination reaction. The preparation of L-asparaginase from guinea pig serum has been described by Meister and Fraser (1954). Specificity was determined as well as pH optimum. No indication was given that phosphate or α-keto acids had any effect on this asparaginase preparation.

The purpose of the present investigation was to study in some detail the mechanism of breakdown of asparagine by mycobacteria and to compare it with asparaginase from other sources.

EXPERIMENTAL METHODS

Two strains of mycobacteria were used: (1) M. tuberculosis, strain H37Ra and (2) M. smegmatis. Both strains were grown as surface cultures on the Youmans and Karlson (1947) modification of Proskauer and Beck medium. M. smegmatis was grown on the basal medium; media for the growth of H37Ra contained in addition, 0.15 per cent serum albumin (bovine serum albumin, fraction V, Armour) and 0.015 per cent oleic acid.

Inocula for cultivation of large amounts of organisms were prepared on 100 ml of media in Erlenmeyer flasks by inoculation from stock cultures maintained on solid media. After appropriate incubation at 37 C (3 to 6 days for M. smegmatis and 3 to 4 weeks for H37Ra) several cm² of surface culture were transferred to 900 ml of media in a large Blake Bottle. M. smegmatis was incubated for 6 days at 37 C; H37Ra for 4 weeks at 37 C. Cells were harvested by filtration through a coarse sintered glass filter and washed with distilled water on the filter. The mass of cells was removed from the filter, weighed, and ground in a mortar with 1/2 volume of water (w/v) to form a suspension. These whole cell preparations were

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used immediately or stored at −20°C until tested or subjected to further treatment.

Whole cell preparations in quantities of 20 to 35 ml were subjected to treatment in a 9-kc Raytheon sonic oscillator for 55 min. After sonic treatment, cellular debris was removed by centrifugation at 15,000 × g at 0°C for 30 min. The supernatant from this centrifugation was used as the cell-free extract. Extracts were stored at −20°C.

Studies on oxygen uptake and carbon dioxide production were carried out with the conventional Warburg apparatus. Carbon dioxide formation was determined by the direct method.

Experiments were conducted at 37.6°C with air as the gas phase.

Enzymatic reactions were carried out in 25-ml flasks in a Dubnoff shaking incubator at 37°C. Reactions were terminated by the addition of acid. Precipitated protein was removed by centrifugation and the amount of ammonia determined in a sample of the supernatant.

The method used to determine ammonia was steam distillation in a Parnas-Wagner still using 50 per cent NaOH as the alkali. Ammonia was trapped in 1.0 ml of 0.1 N sulfuric acid, made to 10.0 ml and determined colorimetrically after nesslerization. Ammonia was formed from asparagine during steam distillation. With rapid distillation and collection of 7 to 8 ml of distillate, the amount of ammonia released from 10 μmoles of asparagine amounted to 5 to 7 μg. Appropriate corrections were applied for ammonia formed from asparagine by steam distillation.

The protein content of cell-free extracts was determined by the biuret reaction (Weichselbaum, 1946).

The presence of amino acids in reaction mixtures was determined by ascending paper chromatography using Whatman no. 1 paper and 80 per cent phenol as solvent. The amino acids were located by spraying with 1 per cent ninhydrin in acidified butanol and heating to 100°C for 10 min. Samples of reaction mixtures were spotted on the paper in amounts from 10 to 50 μl. They were neutralized on the paper with concentrated ammonium hydroxide before development.

RESULTS

Oxidation of asparagine. Whole cell preparations of H37Ra and M. smegmatis showed considerable endogenous oxygen uptake. Oxygen uptake was not appreciably increased in the presence of L-asparagine. The formation of carbon dioxide from asparagine was not detected. Cell-free preparations showed little endogenous gas exchange. The addition of asparaginase had no effect on these preparations. Experiments were carried out in phosphate buffer, pH 7.2, (hydroxymethyl)-aminomethane (tris) buffer, pH 7.4, and acetate buffer, pH 5.0.

Formation of ammonia from L-asparagine. Whole cell preparations and cell-free extracts from H37Ra and M. smegmatis formed ammonia with L-asparagine as substrate. Qualitative paper chromatography of reaction mixtures indicated the presence of aspartic acid in those reaction mixtures that formed ammonia from L-asparagine. Aspartic acid was not deaminated under the conditions of the experiment. The reaction appeared to be a hydrolytic deamination of asparagine as indicated in the following equation:

L-Asparagine + H₂O → aspartic acid + NH₃

Further studies on asparaginase activity were carried out with cell-free extracts.

pH Optimum. The rate of formation of ammonia from asparaginase was greatest at pH 8.5. The results of asparaginase determinations at various pH values for H37Ra and M. smegmatis are shown in figure 1. Histidine buffer (0.1 M) was used for all pH values. At pH 8.5, tris buffer gave the same results as the histidine buffer.
Figure 2. Formation of ammonia from L-asparagine as a function of concentration of enzyme. Experimental conditions: L-asparagine, 20 μmoles; tris buffer (pH 8.5), 100 μmoles; Mycobacterium tuberculosis extract (○), as indicated or Mycobacterium smegmatis extract (×), as indicated; total volume, 2.5 ml; time, 15 min; temperature, 37 C.

Figure 1 also shows that there was approximately twice as much asparaginase activity in extracts of H37Ra per mg of protein as in extracts from M. smegmatis.

Effect of increasing enzyme concentration. The effect of increasing the concentration of enzyme is shown in figure 2. The curve is linear within limits; i.e., linear for all concentrations of M. smegmatis tested but linear only up to approximately 4 mg of protein for H37Ra.

Standard test system and unit of activity. The system usually used for the determination of asparaginase activity contained: L-asparagine, 10 μmoles; tris buffer, pH 8.5, 200 μmoles; cell-free extract, 0.1 to 0.2 ml (1 to 4 mg of protein) in a total volume of 2.5 ml. The reaction mixture was incubated in a shaking incubator at 37 C for 15 min. The reaction was terminated by the addition of 0.5 ml of 15 per cent trichloroacetic acid or 10 per cent perchloric acid; precipitated protein was removed by centrifugation. A sample of the supernatant was used to determine the ammonia present. A unit of activity has been defined as the amount of enzyme that forms 1 μg of ammonia in 15 min at 37 C. Specific activity is defined as units per mg of protein.

Rate of reaction. The formation of ammonia from L-asparagine as a function of time is shown in figure 3. The reaction decreased with time following the linear portion of the curve but essentially went to completion in 90 min.

Kinetics. The formation of ammonia as a function of substrate concentration is shown in figure 4. From these data the Michaelis constant for L-asparagine with extract from H37Ra was calculated to be 1.6 × 10^{-4} M; that for M. smegmatis, 7.4 × 10^{-4} M. The constants were calculated by the method of Lineweaver and Burk (1934) using a plot of the reciprocal of the velocity versus the reciprocal of the substrate concentration. The best straight line fitting these data was calculated by the method of least squares.

Effect of dialysis. In an attempt to determine the possible presence of cofactors required for asparaginase activity, extract was dialyzed over-
inhibitors of phosphate, pyruvate, and a-ketoglutarate activity. The effect of phosphate, pyruvate, and a-ketoglutarate on asparaginase activity was tested. Neither the keto acids nor phosphate has any appreciable effect on asparaginase activity.

Effect of inhibitors. The effect of various types of inhibitors on asparaginase activity is shown in table 2. None of the materials tested showed any significant inhibition. Amounts of inhibitors listed in the table were the largest amounts tested. Lesser amounts gave the same results.

### TABLE 1

<table>
<thead>
<tr>
<th>Treatment and Additions</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mycobacterium tuberculosi</td>
</tr>
<tr>
<td>Untreated and no additions</td>
<td>20.4</td>
</tr>
<tr>
<td>Dialysis and no additions</td>
<td>14.2</td>
</tr>
<tr>
<td>Dialysis and:</td>
<td></td>
</tr>
<tr>
<td>Phosphate (50 μmoles)</td>
<td>15.9</td>
</tr>
<tr>
<td>Pyruvate (50 μmoles)</td>
<td>12.4</td>
</tr>
<tr>
<td>a-Ketoglutarate (50 μmoles)</td>
<td>13.0</td>
</tr>
<tr>
<td>Boiled extract*</td>
<td>13.8</td>
</tr>
<tr>
<td>Liver concentrate (Sigma 1:10)</td>
<td>12.1</td>
</tr>
<tr>
<td>Yeast extract (0.1%)</td>
<td>13.0</td>
</tr>
<tr>
<td>Pyridoxal phosphate (0.88 mg)</td>
<td>10.2</td>
</tr>
</tbody>
</table>

Experimental conditions: L-asparagine, 10 μmoles; tris buffer (pH 8.5), 240 μmoles; *M. tuberculosis* or *M. smegmatis* extract, dialyzed or untreated, as indicated; additions, as indicated; total volume, 2.5 ml; time, 15 min; temperature, 37 C.

* Boiled extract was untreated extract placed in boiling water bath for 10 min, precipitated protein removed by centrifugation, and the supernatant added in same volume as untreated extract used for asparaginase activity.

### TABLE 2

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Amount</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles</td>
<td>Mycobacterium tuberculosi</td>
</tr>
<tr>
<td>None</td>
<td>20.8</td>
<td>6.9</td>
</tr>
<tr>
<td>Chloromercuribenzaoate</td>
<td>0.3</td>
<td>19.8</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
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<td>20.4</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>7.5</td>
<td>19.6</td>
</tr>
<tr>
<td>Semicarbazide</td>
<td>10.0</td>
<td>21.2</td>
</tr>
<tr>
<td>Azide</td>
<td>100.0</td>
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</tr>
<tr>
<td>Cyanide</td>
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</tr>
<tr>
<td>2,4-Dinitrophenol</td>
<td>0.75</td>
<td>19.2</td>
</tr>
<tr>
<td>Arsenite</td>
<td>10.0</td>
<td>19.8</td>
</tr>
<tr>
<td>Arsenate</td>
<td>10.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>50.0</td>
<td>21.2</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetate</td>
<td>3.0</td>
<td>20.8</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>5.0</td>
<td>21.0</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>50.0</td>
<td>20.2</td>
</tr>
</tbody>
</table>

Experimental conditions: L-asparagine, 10 μmoles; tris buffer (pH 8.5), 200 μmoles; inhibitors, as indicated, preincubated for 10 min with extract before addition of extract; *M. tuberculosis* or *M. smegmatis* extract; total volume, 2.5 ml; time, 15 min; temperature, 37 C.

Effect of D-asparagine. No ammonia was formed when D-asparagine was incubated with cell-free extract. D-Asparagine inhibited the formation of ammonia from L-asparagine by extracts from *H37Ra*. A plot of the reciprocal of the velocity versus the reciprocal of the L-asparagine concentration showed a change in slope with essentially no change in intercept at various levels of d-asparagine. This type of plot is shown in figure 5. Slope and intercept were determined by the method of least squares. The inhibition constant for d-asparagine was determined for competitive inhibition by the method of Lineweaver and Burk (1934) from the increase in the apparent Michaelis constant. By this method an inhibition constant of 1.1 × 10⁻³ M was calculated for the 4 levels of d-asparagine. The standard deviation was 1 × 10⁻⁴ M.

Purification of asparaginase. Asparaginase activity was precipitated by ammonium sulfate between 25 and 50 per cent saturation. Specific activity was increased only about 20 per cent.
**Figure 5.** Effect of \( D \)-asparagine on velocity of asparaginase reaction. The reciprocal of the velocity (specific activity) is plotted against the reciprocal of the concentration of \( L \)-asparagine. Curve 1, no \( D \)-asparagine (●); curve 2, 2 \( \mu \)moles (○); curve 3, 3 \( \mu \)moles (△); curve 4, 5 \( \mu \)moles (▲); and curve 5, 8 \( \mu \)moles (●).

**TABLE 3**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mycobacterium tuberculosis</th>
<th>Mycobacterium smegmatis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein</td>
<td>Specific activity</td>
</tr>
<tr>
<td>Untreated</td>
<td>436</td>
<td>16</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>204</td>
<td>2</td>
</tr>
<tr>
<td>pH 4.5</td>
<td>132.7</td>
<td>12</td>
</tr>
<tr>
<td>pH 4.0</td>
<td>54.6</td>
<td>45</td>
</tr>
<tr>
<td>Remainder</td>
<td>45</td>
<td>2</td>
</tr>
<tr>
<td>Totals</td>
<td>436.3</td>
<td>5508</td>
</tr>
</tbody>
</table>

*Experimental conditions:* \( L \)-Asparagine, 10 \( \mu \)moles; tris buffer (pH 8.5), 100 \( \mu \)moles; *M. tuberculosis* or *M. smegmatis* extract, treated as indicated with acetic acid, then dissolved in tris buffer (pH 8.5); total volume, 2.5 ml; time, 15 min; temperature, 37 C.

Precipitation of protein with acetic acid and resolution in tris buffer, pH 8.5, showed greatest specific activity in the protein fraction precipitated between pH 4.0 and 4.5. Slightly less than a 3-fold increase in specific activity was achieved with H37Ra and about a 2-fold increase with *M. smegmatis*. Recovery of activity was not very satisfactory. Data on the specific activity and recovery are shown in table 3. The method used for the acid precipitation was as follows. Untreated extract, kept in an ice bath, was adjusted to pH 5.0 (determined by glass electrode) with 1 N acetic acid added slowly with stirring. The precipitate was removed by centrifugation at 15,000 \( \times \) g at 0 C for 15 min and redissolved in 0.1 M tris buffer, pH 8.5. The supernatant was then adjusted to pH 4.5 and precipitated protein treated as before. After the protein precipitated at pH 4.0 was removed, the supernatant was adjusted to pH 6.6 (original pH of extract) and constituted the extract indicated "remainder" in table 3.

Transamination reactions with \( L \)-asparagine. No evidence has been found for a transamination or transamidation reaction between \( L \)-asparagine and pyruvate or \( \alpha \)-ketoglutarate under the con-
conditions used to determine asparaginase activity. Neither alanine nor glutamate could be detected by paper chromatography. L-Glutamate was not deaminated by the extracts. No ammonia was formed from glutamine by the extracts under the conditions used for asparaginase assay.

**DISCUSSION**

The metabolism of asparagine by mycobacteria involves as a first step the deamidation of asparagine to form aspartic acid and ammonia. No cofactors have been demonstrated for the asparaginase. No difference has been detected between asparaginase from *M. smegmatis* and *M. tuberculosis* other than the amount present in the extracts. The specific activity of extracts from *M. tuberculosis* was usually about twice that in extracts from *M. smegmatis*. No significance is attached to this difference at the present time. Optimal activity occurred at pH 8.5 with appreciable activity between pH 7.0 and 10.0. These data agree with the findings of Kirchheimer and Whittaker (1954) and Halpern and Grossowicz (1957).

The effect of dialysis in decreasing activity requires further study. Halpern and Grossowicz (1957) reported that dialysis had no effect on extracts from *M. phlei*. The heat stability of the asparaginase reported here agrees with the findings with *M. phlei* (Halpern and Grossowicz, 1957) and with some properties of rat liver asparaginase. Asparaginase from mycobacteria, in contrast to asparaginase from rat liver, does not require phosphate or keto acids for activation, even after heat treatment.

The failure to decrease activity by the addition of various inhibitors indicates that the asparaginase is probably devoid of functional groups such as sulphhydryl groups, metal ions, or easily oxidized groups. The stereospecificity of asparaginase and competitive inhibition by D-asparagine are similar to the findings with *M. phlei* (Halpern and Grossowicz, 1957).

The addition of pyridoxal phosphate and to a lesser extent of pyruvate and α-ketoglutarate (table 1) to dialyzed extract showed some inhibition of activity. These data raise the possibility of a transamination or transamidation reaction with asparagine as the amino or amide donor. Further experiments are required to test this possibility.

From the results presented, it would appear that asparaginase functions as a source of ammonia in the cultivation of tubercle bacilli. As a general conclusion, asparaginase from mycobacteria appears to be essentially the same regardless of the strain of origin and to differ from asparaginase in rat liver homogenates.

**ACKNOWLEDGMENTS**

The author gratefully acknowledges the interest and helpful discussions of Dr. C. A. Colwell. The technical assistance of James R. Greco is also gratefully acknowledged.

**SUMMARY**

The enzymes from *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* responsible for the breakdown of asparagine were investigated. Formation of ammonia from L-asparagine was determined with cells and cell-free preparations. Aspartic acid was the other product of the reaction. Formation of ammonia from L-asparagine was proportional to enzyme concentration within limits. The rate of formation of ammonia was linear with time for the early part of the reaction. Complete deamidation of L-asparagine occurred after sufficient incubation. Optimal activity was found at pH 8.5. Dialysis against distilled water decreased activity; no restoration of activity could be achieved with any material tested. The enzyme was stable at 50 C for 10 min. With one exception various potential inhibitors including isoniazid had no effect on asparaginase activity; D-asparagine inhibited the asparaginase in a competitive manner. Two strains of mycobacteria showed only quantitative differences in asparaginase activity.

**REFERENCES**


Kirchheimer, W. F., and C. K. Whittaker 1954
Lineweaver, H., and D. Burk 1934 The
determination of enzyme dissociation con-
Meister, A., and P. E. Fraser 1954 Enzym-
ic formation of l-asparagine by transami-
Meister, A., H. A. Sober, S. V. Tice, and P. E.
Fraser 1952 Transamination and asso-
ciated deamidation of asparagine and gluta-
Price, V. E., and J. P. Greenstein 1947
Studies on the effect of pyruvate on the
desamidation of glutamine, asparagine and
7, 275–279.
Weichselbaum, T. E. 1946 An accurate and
rapid method for the determination of protein
in small amounts of blood serum and plasma.
Youmans, G. P., and A. G. Karlson 1947
Streptomycin sensitivity of tubercle bacilli.
Studies on recently isolated tubercle bacilli
and the development of resistance to strepto-
535.