ENZYMOLOGY OF MYCOBACTERIA
VII. DEGRADATION OF GUANIDINE DERIVATIVES*

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Several strains of Mycobacteria are able to attack the guanidine groups of arcanine (1,4-diguanidinobutane) and of agmatine (1-amino-4-guanidinobutane). The guanidine-decomposing enzyme (GD) responsible for this reaction is different from several other hitherto characterized guanidine-splitting systems, and has been obtained in a soluble form (2). Since urease is present in the strains used for these investigations, the liberation of ammonia in the enzymic hydrolysis of guanidine derivatives mentioned above gives no indication as to whether ammonia is formed directly or indirectly through the degradation of urea, which in turn is set free from the guanidine derivative. Thus further evidence, as presented in this paper, was required to define the mechanism of action of GD found in Mycobacteria.

Materials and Methods

The strains of Mycobacterium smegmatis1 and Mycobacterium phlei,2 and the suspensions and soluble enzyme preparations obtained from them, as well as a description of the general experimental conditions and of the measurement of guanidine groups and ammonia, have been described previously (24). Urea was determined by a manometric procedure, with acetate buffer at pH 5.0 (5). In order to release all gaseous carbon dioxide

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1 Strain I, streptomycin-sensitive; Strain II, streptomycin-resistant; Strain III, streptomycin-dependent (Karlson); Strain IV, isoniazid-sensitive; and Strain V, isoniazid-resistant.

2 We are indebted to Dr. A. G. Karlson, Mayo Foundation, Rochester, Minnesota, for providing us with a stock culture of M. smegmatis, Strain III; to Hoffmann-La Roche, Inc., Nutley, New Jersey, for stock cultures of Strains IV and V; and to Dr. W. B. Sutton, Lilly Research Laboratories, Indianapolis, Indiana, for a stock culture of M. phlei.
evolved from urea, dilute sulfuric acid was tipped into the manometric vessel from a second side arm. Urease was prepared from commercial jack bean meal by fractionating a water extract with acetone (6). Degradation of guanidine derivatives under standard conditions involved incubation in \( \tfrac{m}{30} \) phosphate buffer, pH 8.0, and at 38°. All concentrations were calculated for the final volume of the enzymic system. In all cases, enzyme blanks and usually substrate blanks were carried through the same procedure as for the complete mixtures. The blanks were subtracted from the actual results.

**Results**

**Occurrence of Urease**—In all the strains of *M. smegmatis* and *M. phlei* so far tested, urease was present. The \( Q \) values (micromoles of decomposed urea per gm. of wet, packed cells per hour; 38°) for various strains range from 180 to 1270 for whole cells and from 90 to 920 for the corresponding amount of the supernatant fluid, obtained by breaking up the bacteria by sonic oscillations and centrifugation at 105,000 \( \times g \) for 30 minutes (2). The activity of this enzyme was also measured under conditions employed for the evaluation of GD; namely, with phosphate buffer, pH 8.0, and 0.01 M substrate. The \( Q \) values of whole cells and soluble preparations were found to lie between 10 and 20.

**Inactivation of Urease**—Urease of whole cells and of the supernatant fluid was inactivated by 0.1 mM mercuric chloride, as tested with phosphate buffers, pH 7.2 and 8.0. Heating for 15 minutes at 80° in phosphate buffer, pH 8.0, also destroys urease, while under these circumstances GD remains intact (2).

**Formation of Urea**

**Liberation of Ammonia**—In long term experiments with whole cells, 2 to 3 molecules of ammonia are generally set free per guanidine groups decomposed (2). However, in the presence of a soluble preparation, and with short incubation periods, less than 1 molecule per molecule of substrate decomposed may appear.

**Effect of Mercuric Chloride on Ammonia Formation**—With 0.1 mM mercuric chloride a slight inhibition of the enzymic decomposition of guanidine groups of agmatine and arcaine took place, while the production of ammonia was almost completely suppressed (Table I).

**Effect of Heating of Enzyme Preparation on Ammonia Formation**—Previous heating of the soluble enzyme preparation at 80° did not affect its GD activity (2), but prevented effectively the liberation of ammonia (Table II). However, when urease was added afterwards, 2 moles of ammonia per mole of substrate appeared.
Isolation and Determination of Urea—In the presence of the supernatant fluid obtained from 0.2 gm. of bacteria (Strain I) and 0.1 mM mercuric chloride, 20 μM of agmatine were incubated in a total volume of 10 ml. of m/30 phosphate buffer, pH 8.0. After 21 hours of incubation at 37°, the Sakaguchi reaction was negative and no ammonia could be detected. The solution was treated with hydrogen sulfide and distilled in vacuo to dryness. The residue was refluxed with 10 ml. of ethanol, and the latter was distilled and the residue taken up in 5 ml. of water and decomposed by jack bean urease in 0.03 M acetate buffer. The urease liberated 47 per cent of the theoretical amount of ammonia; a control sample, before decomposition, gave 45 per cent of the theoretical amount of nitrogen by the Kjeldahl method.

TABLE I

Influence of Mercuric Chloride on Deamination of Guanidine Derivatives

The vessels contained 0.1 ml. of wet packed cells (Strain I) in a total volume of 2 ml. of 0.04 M phosphate buffer, pH 7.2, and 1.0 mM substrates. Incubation, 20 hours at 37°.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>HgCl</th>
<th>Guanidine equivalents hydrolyzed</th>
<th>Ammonia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>μeq.</td>
<td>μM</td>
</tr>
<tr>
<td>Agmatine, 2 μeq.</td>
<td>0</td>
<td>1.9</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1.5</td>
<td>0.6</td>
</tr>
<tr>
<td>Arcaine, 4 μeq.</td>
<td>0</td>
<td>4.0</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>2.6</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Formation of Amines

Microbiological Determination of Putrescine—The degradation product of 6.0 μM of agmatine had the same growth-promoting property of Hemophilus parainfluenzae (7) as did 6.6 μM of putrescine.  

Manometric Determination of Tyramine—It has been shown that 1-guanidino-2-(p-hydroxyphenyl)ethane is attacked by GD.  

The product of this reaction may be tyramine, a substrate of monoamine oxidase. In order to test this assumption, this guanidine derivative was incubated with soluble GD and washed hog liver cell mitochondria, a potent source of monoamine oxidase (8). While neither GD nor monoamine oxidase catalyzed any oxidative deamination, the combination of both enzymes proved to be effective, indicating that through the action of GD a substrate of mono-

3 We thank Dr. E. J. Herbst, University of Maryland, Baltimore, for carrying out the microbiological determination of putrescine.


5 The experiments were performed in this laboratory by Mrs. Elaine R. Berman.
amine oxidase was formed (Table III). These results furnish the basis of a manometric determination of GD.

Isolation and Identification of Putrescine 0.214 gm. of agmatine sulfate was incubated under standard conditions with 2 gm. of soluble GD preparations (Strain V) in a volume of 147 ml. until the Sakaguchi reaction became negative. The pH of the solution was changed to 4.0 with acetic acid and boiled briefly. After removal of the proteinaceous precipitate, the volume of the solution was reduced to 25 ml. by distillation in vacuo. To the boiling solution, 10 ml. of a boiling solution of 0.5 gm. of picric acid were added. The collected crystals were washed three times with 2 ml. of ethanol and freely with ether. The yield was 0.39 gm. (71 per cent of the theoretical amount). Darkening of the crystals began at 255°, and decomposition at 268°; recrystallization from water did not

TABLE II  

Influence of Heating of Enzyme on Ammonia Liberation

A soluble preparation (Strain V) was heated in m/15 phosphate buffer, pH 8.0, for 15 minutes at 80°; 0.8 ml. of this material, equivalent to 0.08 ml. of wet packed cells, was incubated for 1 hour at 38° with 8 μM of agmatine sulfate in a total volume of 8 ml. of m/30 phosphate buffer; after removal of 0.7 ml. for analytical purposes, 1.4 ml. of jack bean extract (1:10) were added and the mixture was kept for another hour at the same temperature; the percentage was calculated on the basis of 2 moles of ammonia per mole of substrate.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Guanidine equivalents hydrolyzed</th>
<th>Ammonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heated preparation</td>
<td>8.0</td>
<td>1.6</td>
</tr>
<tr>
<td>Same + urease</td>
<td>8.0</td>
<td>15.0</td>
</tr>
</tbody>
</table>

TABLE III  

Enzymic Degradation of 1-Guanidino-2-(p-hydroxyphenyl)ethane

The vessels contained mitochondria, isolated from 0.25 gm. of hog liver by differential centrifugation with 0.25 m sucrose (9, 10) and washed once with sucrose solution and resuspended in phosphate buffer, and a soluble preparation (Strain V) corresponding to 0.1 gm. of wet packed bacteria in a total volume of 3 ml. of m/15 phosphate buffer at pH 8.0 and 3.3 mM substrates (10 μM). Incubation for 6 hours in oxygen-filled Warburg type manometric vessels at 38°.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Oxygen uptake</th>
<th>Ammonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mitochondria + bacteria</td>
<td>3.3</td>
<td>8.9</td>
</tr>
</tbody>
</table>
change these temperature points. The picrate from authentic putrescine, synthesized from succinyl nitril, darkened at 255° and decomposed at 270°. Part of the picrate was converted into the hydrochloride by extracting a suspension of the powdered crystals in hydrochloric acid in the Steudel-Kutscher apparatus for 60 hours. After that, the solution was concentrated and the residual picro acid removed with Norit. With this solution and benzene sulfonyl chloride, an N,N'-disulfonylelamide was obtained in the conventional way and the latter compound was converted with dimethyl sulfate into an N,N'-dimethyl-N,N'-disulfonylelamide, which was recrystallized from ethanol-ether. The same products were prepared from authentic putrescine. The uncorrected melting points were determined with a Fisher-Johns melting point apparatus (Table IV).

<table>
<thead>
<tr>
<th>Table IV</th>
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<tbody>
<tr>
<td><strong>Melting Points of Putrescine Derivatives</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Isolated product</td>
</tr>
<tr>
<td>Authentic material</td>
</tr>
<tr>
<td>Mixed melting point</td>
</tr>
<tr>
<td>Wrede et al. (11)</td>
</tr>
</tbody>
</table>

**Degradation of Agmatine under Anaerobic Conditions**

While usually air was not excluded from the reaction vessels, some experiments were carried out anaerobically. Warburg vessels were filled with nitrogen. The residual oxygen in commercial nitrogen was removed by washing the gas through a solution of chromous chloride. The same reducing agent was placed in the center cup of the reaction chamber. After incubation, sulfuric acid was tipped from the second side arm to the enzymic system to stop the reaction before admitting air. In 30 minutes a soluble preparation, obtained from 0.06 gm. of bacteria (Strain I) in a total volume of 6 ml., under standard conditions completely decomposed 6 μM of agmatine.

**DISCUSSION**

Previously the existence of urease in *Mycobacteria* had been established only with qualitative methods (12, 13). In this paper quantitative data in the form of Q values are given and the liberation of urease from *Mycobacteria* is described for the first time. In so far as it has been tested, this bacterial enzyme has the same properties as the classical jack bean urease.
Ammonia is not an immediate product of the guanidine-decomposing agent (GD) of Mycobacteria, since the formation of ammonia is suppressed whenever urease is inactivated, while the hydrolysis of guanidine groups continues. However, 2 µM of ammonia appear per guanidine equivalent of substrate when jack bean urease acts on the reaction product. Urea, therefore, is one of the products of the GD action.

The other split-product was recognized as the amine corresponding to the guanidine compound, as established by preparative, microbiologic, and enzymologic methods. If the fact is taken into consideration also that no oxygen is required, the GD reaction can be described by the following equation:

\[
RNHC(:NH)NHz + H_2O = RNH_2 + \text{urea}
\]

In spite of the fact that this mechanism is the same as that known for arginase, this enzyme is quite different from GD, as shown in the preceding paper (2).

When a mixture of putrefactive bacteria acted on arcaine, minute quantities of urea could be isolated (14), indicating a similar mechanism as to that described in this paper. With agmatine, no urea but 1-amino-4-ureidobutane was found (15), so that the liberation of urea was considered to be only a side reaction as compared with the formation of the ureido compound. However, an enzyme present in Escherichia coli seems to attack agmatine in the same way as GD of Mycobacteria (16), but the evidence presented in this case is not complete.6

With the elucidation of the mechanism of GD action, a complete sequence of reactions leading to arginine degradation could be established for some Mycobacteria. L-Arginine is first decarboxylated7 and the resulting agmatine is hydrolyzed by GD to form urea and putrescine. The former is attacked by urease, the latter by diamine oxidase (3, 4, 17, 18). Thus GD, urease, and diamine oxidase may represent links in the metabolism of L-arginine in M. smegmatis. The deamination of putrescine and agmatine by diamine oxidase is also responsible for the occasional appearance of more than 2 moles of ammonia per guanidine equivalent or for the production of some ammonia even after blocking of urease by mercuric chloride (Table I).

6 Nakamura (16) incubated washed bacteria (a cell-free preparation of the guanidine decomposing agent of E. coli could not be obtained) at 37° for 7 days with agmatine. From the obnoxious smelling suspension a picrate with a melting point of 250° was isolated. Since no other data of this product were given, the identification of putrescine from this melting point alone, which actually is a decomposition point, leaves room for some doubt.

SUMMARY

1. Urease in fairly high activity is found in five strains of *Mycobacterium smegmatis* and in one strain of *Mycobacterium phlei*. A considerable part of the urease activity of the cells appears in soluble form after treatment of the bacteria in a sonic oscillator. Some of the properties of this urease are investigated.

2. Ammonia is produced from certain guanidine derivatives by the action of *Mycobacteria*. This liberation of ammonia is a function of urease activity and is not related to the primary attack of the guanidine-decomposing enzyme (GD) on its substrates.

3. The formation of tyramine from 1-guanidine-2-(p-hydroxyphenyl)-ethane is demonstrated by means of monoamine oxidase, and the appearance of putrescine after the incubation of agmatine is proved with enzymologic and microbiologic methods, and by the isolation of its dipicrate, which is converted into the N,N'-disulfonylputrescine and N,N'-dimethyl-N,N'-disulfonylputrescine.

4. The enzymic degradation of guanidine derivatives takes place in the absence of oxygen. It consists of a hydrolytic splitting of the substrate into urea and the corresponding amine.

5. The possible rôle of GD, urease, and diamine oxidase in the degradation of L-arginine is discussed.

BIBLIOGRAPHY

ENZYMEOLOGY OF MYCOBACTERIA: VII. DEGRADATION OF GUANIDINE DERIVATIVES
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