The Enzymatic Cleavage of Canavanine to Homoserine and Hydroxyguanidine*

G. D. KALYANKAR, MIYOSHI IKAWA AND ESMOND E. SNELL

From the Department of Biochemistry, University of California, Berkeley, California

(Received for publication, July 14, 1958)

Previous studies have shown canavanine to be a potent antago-

nist of arginine in Neurospora (1) and in some bacteria, but not

in others (2, 3). Streptococcus faecalis belongs to the resistant
group of bacteria and has been shown to degrade canavanine in
two distinct ways: by reductive cleavage to guanidine and homo-

serine (4) and, to a lesser extent, by hydrolytic cleavage to am-

monia and O-ureidohomoserine (5).

In an extension of these studies, a pseudomonad capable of
utilizing canavanine as a sole source of carbon and nitrogen was
isolated from soil by the enrichment culture technique. The
initial attack on canavanine by this organism was found to
consist in a hitherto undescribed cleavage of this amino acid to
yield hydroxyguanidine and homoserine. The present paper
deals with the demonstration of this reaction.

**EXPERIMENTAL**

Culture Medium—The medium used both for isolation and
subsequent culture of the organism contained per l: 2.0 gm.
of L-canavanine, 1.0 gm. KH₂PO₄, 0.5 gm. of MgSO₄·7H₂O,
3.0 mg. of CaCl₂ and 3.0 mg. of FeCl₃·6H₂O. It was adjusted
to pH 7.0 with potassium hydroxide and sterilized by autoclaving
at 15 pounds of pressure for 10 minutes. Canavanine is some-
what labile to autoclaving. It was sterilized by filtration and
added after the remainder of the medium was autoclaved. Stock
cultures of the isolated organism were maintained on slants of
the same medium solidified with 2 per cent of purified agar.

Isolation of Organism—Erlenmeyer flasks (50 ml.) containing
10 ml. of the basal medium were inoculated with 100 to 500 mg. of
mud from San Francisco Bay and incubated without shaking
at 30°. After 48 hours, when abundant turbidity had developed,
0.01 ml. aliquots were transferred to fresh medium and incuba-
tion was continued in the same way. After three such succes-
sive transfers, the culture was streaked onto solid basal medium.
The single predominating colony type was replated, and an
isolated colony picked for further study. The organism forms a
yellow-green fluorescent pigment, and from its microscopic
appearance and cultural characteristics appears to be a species
of the genus *Pseudomonas*.

Quantitative Methods—Homoserine and canavanine were
estimated by the quantitative paper chromatographic method of
Giri et al. (6). Canavanine also was estimated by a slightly
modified Archibald procedure described previously (4). Hy-
droxyguanidine was estimated by the method of Walker (7).
Homoserine, guanidine, and ammonia were found not to inter-
fere in the determination of hydroxyguanidine. Canavanine,
however, gave a color value equivalent to 13 per cent of that of
hydroxyguanidine on the molar basis; accordingly, the value
found for hydroxyguanidine was corrected for the amount of
unchanged canavanine present.

Synthesis of Hydroxyguanidine Hydrochloride—The reaction
originally described by Prátorius-Seidler (8) and recently
utilized by Adams et al. (9) was used for preparing hydroxy-
guanidine. Since the isolation of hydroxyguanidine in pure
form has not been previously described, its method of prep-
RESULTS

Degradation of Canavanine by Growing Cultures—After various times of incubation in the described medium, all cultures were centrifuged and the supernatant solutions concentrated in a vacuum and examined by paper chromatography. After only 2 hours of growth, the medium showed traces of homoserine and an unknown substance which did not react with ninhydrin but gave a characteristic green color with α-naphthol-diacetyl spray (12). The amounts of both substances increased during the first few hours of incubation; thereafter, homoserine decreased while the unknown continued to increase in amount. The homoserine was identified by paper chromatographic comparisons with authentic homoserine in pyridine-water (80:20), in 77 per cent ethanol, and in n-butanol-acetic acid-water (40:10:50). The identification was confirmed by eluting the substance from a paper chromatogram, heating the eluate with dilute hydrochloric acid, and chromatographing the resulting solution on paper. In addition to homoserine, a compound was formed that gave the same yellowish-brown color with ninhydrin as homoserine lactone and also migrated on paper identically with an authentic sample of homoserine lactone. The unknown substance was identified as hydroxyguanidine by paper chromatographic comparisons with synthetic hydroxyguanidine in absolute ethanol-water-acetic acid (77:23:1) (RF, 0.59), in absolute ethanol-1 M ammonium hydroxide (77:23) (RF, 0.59), and in n-butanol-acetic acid-water (4:1:1) (RF, 0.23). Both natural and synthetic samples gave the same green color when the chromatograms were sprayed with α-naphthol-diacetyl reagent, and the same purple color when sprayed with alkaline ferricyanide-nitroprusside reagent. The green color formed with the α-naphthol-diacetyl reagent seems to be quite characteristic of hydroxyguanidine since both canavanine and guanidine give a pink color. The identity with hydroxyguanidine of the unknown substance was further confirmed by its isolation, as described later.

After 18 hours of incubation, both canavanine and homoserine had disappeared from the medium. The concentration of hydroxyguanidine increased until all canavanine had disappeared, then remained constant even after 30 hours of incubation, showing that it was not further metabolized by the organism. It was found that the test organism grew equally as well when homoserine, arginine, citrulline, ornithine, alanine, or serine was substituted for canavanine as the sole source of carbon and nitrogen, but did not grow when hydroxyguanidine was substituted. The cleavage reaction apparently serves the organism solely as a means for formation of homoserine, which serves as the actual substrate for growth.

Isolation of Hydroxyguanidine—A culture grown in 500 ml. of medium was centrifuged after 12 hours of incubation and the supernatant liquid concentrated in a vacuum to 25 ml. An equal volume of ethanol was added, the precipitated salts were filtered, the filtrate was further concentrated, and the concentrate placed on a Dowex 50 (H+) column (2.6 × 45 cm., 200 to 400 mesh). The column was washed with 11 l. of water, then developed with 1 N hydrochloric acid. The compound giving the green color with α-naphthol-diacetyl spray was obtained in the 2000 to 2500 ml. fraction of the acid effluent from the column. This fraction was concentrated to dryness in a vacuum and the residue recrystallized by dissolving it in 2 ml. of ethanol and slowly adding 20 ml. of ether. An 11 mg. sample of the slightly impure product yielded 25 mg. of colorless t-dinitrophenyl derivative which, on recrystallization from ethanol, gave 14 mg. of pure product melting at 198–199° (corrected) with decomposition, and which showed no depression in decomposition point when mixed with authentic dinitrophenylhydroxyguanidine.

Degradation of Canavanine by Cell-Free Extracts—Cells from an 18 hour culture were collected by centrifuging and washed twice with distilled water. The washed cells were suspended in 0.02 M phosphate buffer, pH 7.2 (18.5 mg. dry weight of cells per ml.), and broken by treating them for 20 minutes in a Raytheon 9 kc sonic oscillator. The resulting opalescent solution was centrifuged, and the supernatant solution dialyzed against deionized water in the cold room. The dialyzed preparation degraded canavanine to homoserine and hydroxyguanidine with an optimal pH near 7 (Table I). Homoserine and hydroxyguanidine are formed in equimolar amounts (Table I). The over-all reaction thus corresponds to a simple hydrolytic cleavage of canavanine.

DISCUSSION

The enzymatic reactions in which canavanine is known to participate are: (a) reductive cleavage to homoserine and guanidine by Streptococcus faecalis (4); (b) hydrolysis to O-ureido-homoserine and ammonia by the arginine desiminase of S. faecalis (5); (c) hydrolysis to canaline and urea by arginase (13); (d) oxidation by L-amino acid oxidase of Neurospora, presumably to the corresponding keto acid and ammonia (14); (e) condensation with fumaric acid to yield canavaninosuccinic acid catalyzed by arginosuccinase (15); and (f) transamidination with ornithine (or other amidine acceptors) to yield canaline and arginine (or other guanidine compounds) by preparations from kidney (16) and Streptomyces griseus (7). The present investigation has revealed a third hydrolytic pathway which results in the formation of hydroxyguanidine and homoserine from canavanine (Reaction (g)). These known enzymatic transformations of canavanine are represented structurally in Diagram 1. Of these, Reactions (a) and (g) are the only ones that specifically require canavanine (or related derivatives of hydroxyamine) as substrates. Reactions (b) through (f) are all catalyzed either by enzymes for which arginine is a much more effective, and probably, therefore, the primary substrate, or by nonspecific enzymes such as the L-amino acid oxidase.

The possibility that reductive cleavage of canavanine by

TABLE I

Stoichiometry and pH effects on degradation of canavanine by cell-free extracts

<table>
<thead>
<tr>
<th>Experiment</th>
<th>pH</th>
<th>Buffer</th>
<th>Homoserine formed</th>
<th>Hydroxyguanidine formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td></td>
<td></td>
<td>µmoles</td>
<td>µmoles</td>
</tr>
<tr>
<td>1*</td>
<td>5.5</td>
<td>M/20 acetic</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>2†</td>
<td>6.8</td>
<td>M/60 phosphate</td>
<td>9.2</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>8.2</td>
<td>M/60 phosphate</td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>M/50 phosphate</td>
<td>4.2</td>
<td>4.0</td>
</tr>
</tbody>
</table>

* Incubation for 20 minutes.
† Incubated for 1 hour. A more dilute enzyme preparation was employed in this independent experiment.
**Diagram 1**

\[
\begin{array}{c}
\text{Canavanine} \quad \text{H}_2\text{NCONH}_2\text{CH}_2\text{CHCOOH} \\
\text{(a)} \\
\text{NH}_2 + \text{NHCNHOCH}_2\text{CHCHCOOH} \\
\end{array}
\]

\[
\begin{array}{c}
\text{Fumaric acid} \quad \text{HOOC}\text{CH}_2\text{HNHCNHOCH}_2\text{CHCHCOOH} \\
\text{(b)} \\
\text{HOOCCH}_2\text{CHNHCHNOCH}_2\text{CHCHCOOH} \\
\end{array}
\]

\[
\begin{array}{c}
\text{Ornithine} \quad \text{H}_2\text{NCNHCH}_2\text{CHCHCOOH} + \text{H}_2\text{NOCH}_2\text{CHCHCOOH} \\
\text{(c)} \\
\text{NH}_2 + \text{NH}_2 \\
\text{NH}_2 + \text{NH}_2 \\
\end{array}
\]

\[
\begin{array}{c}
\text{H}_2\text{NCNH}_2 + \text{HOCH}_2\text{CHCHCOOH} \\
\text{(d)} \\
\text{NH}_2 + \text{NHCNHOCH}_2\text{CHCHCOOH} \\
\text{NH}_2 + \text{NH}_2 \\
\end{array}
\]

\[
\begin{array}{c}
\text{Ornithine} \quad \text{H}_2\text{NCNHCH}_2\text{CHCHCOOH} + \text{H}_2\text{NOCH}_2\text{CHCHCOOH} \\
\text{(e)} \\
\text{NH}_2 + \text{NH}_2 \\
\text{NH}_2 + \text{NH}_2 \\
\end{array}
\]

\[
\begin{array}{c}
\text{Fumaric acid} \quad \text{HOOC}\text{CH}_2\text{HNHCNHOCH}_2\text{CHCHCOOH} + \text{NH}_2 \\
\text{(f)} \\
\text{HOOCCH}_2\text{CHNHCHNOCH}_2\text{CHCHCOOH} \\
\end{array}
\]

S. faecalis (Reaction (a)) may consist in the hydrolytic cleavage (Reaction (g)) followed by reduction of hydroxyguanidine to guanidine has been considered. Despite the characteristic color formed when paper chromatograms of hydroxyguanidine are sprayed with the α-naphthol reagent, no formation of this product was observed in the previous work (4, 5) with growing cultures, with resting cells, or with cell-free extracts of S. faecalis. Attempts to show its intermediate formation in Reaction (a) were made during the present study, but again failed. In the presence of glucose, however, resting cell suspensions of S. faecalis destroy hydroxyguanidine, and, although guanidine is not a prominent product of this reaction, the possible relationship of the two pathways has not been excluded.

Hydroxyguanidine has not previously been recognized as a naturally-occurring product. The compound is formed, however, when hydroxylamine is added as a trapping agent during enzymatic transamination (16).

The organism studied here apparently does not metabolize hydroxyguanidine further, but utilizes the homoserine formed in Reaction (g) for growth purposes. The metabolic reactions concerned in degradation of homoserine have not been studied in detail. Pyruvic acid was isolated (as its 2,4-dinitrophenylhydrazone) as one product of its metabolism.

**SUMMARY**

A pseudomonad was isolated from soil that grows readily in synthetic medium with L-canavanine as sole source of carbon and nitrogen. Growing cultures or cell-free preparations of the organism degrade canavanine by a previously undescribed route to yield hydroxyguanidine and homoserine in stoichiometric amounts. The former product accumulates in growing cultures of the organism, and the latter is utilized for growth purposes.

Procedures for isolation of hydroxyguanidine in pure form are described. This product has not been recognized previously as occurring in nature. Although not further metabolized by the organism that forms it, hydroxyguanidine is degraded to unidentified products by resting cells of Streptococcus faecalis in the presence of glucose.

**REFERENCES**
