GUANINE DEGRADATION BY CLOSTRIDIUM ACIDIURICI

II. ISOLATION AND CHARACTERIZATION OF GUANASE

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Received for publication November 1, 1954

In the work on the anaerobic purine degradation by Clostridium acidiurici, Beck (1950) postulated that xanthine was the key intermediate in the breakdown of guanine and uric acid. Beck (1951) found additional evidence that xanthine was the intermediate in uric acid attack. Later Rakosky and Beck (1955) pointed to the probable existence of a guanine deaminase necessary in the conversion of guanine into xanthine. The latter was supported by manometric studies, the deminization of 8-azaguanine and 1-methyl guanine to form the xanthine analogues, and the apparent inhibition of guanine breakdown by 0.1 N NaF. Further proof to corroborate the assumption that xanthine is an intermediate in guanine utilization is the isolation and characterization of the enzyme guanase. The preparation and study of guanase from C. acidiurici will be presented in this paper.

METHODS

The composition of the broth in which cells were grown was altered from that originally described (Rakosky and Beck, 1955) so that a maximum yield of enzyme could be secured repeatedly.

The medium employed was the following composition: Guanine, 0.50 g; K2HPO4, 0.25 g; K2CO3, 0.25 g; yeast extract, 0.10 g; FeCl3, trace; MgSO4, 0.10 g; glycine, 0.25 g; gelatin, 0.25 g; distilled water, 500 ml; adjusted pH 7.3.

1 This investigation was supported in part by a research grant (no. C648) from the National Cancer Institute and as a Research Fellowship from the National Institute of Microbiology, National Institutes of Health, the Department of Health and Welfare, Bethesda, Maryland.

2 Authorized for publication on September 21, 1954, as paper no. 1906 in the journal series of the Pennsylvania Agricultural Experiment Station.

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The procedure used in making this medium was similar to that described by Rakosky and Beck (1955), with the exception that both MgSO4 and the gelatin solution were sterilized separately and added aseptically to the sterilized medium after cooling. The broth, which was contained in 500 ml glass stoppered bottles, was inoculated with 10 ml of a 24 hr semisolid guanine agar culture. After sealing, the bottles were incubated at 33 C with frequent mixing of the contents. Although the exact length of time of incubation was determined by visual inspection of the inoculated medium, this time usually approximated 24 hr. (The two criteria used for determining the incubation end point were a disappearance of guanine and a deposition of MgNH4PO4 crystals on the interior surface of the culture bottle.) After incubation, the bottle was refrigerated (10 C) for 18 hr.

During refrigeration there was an apparent increase in MgNH4PO4 crystals as well as an apparent decrease in residual guanine. Following storage at 10 C, the broth was centrifuged to remove all cells and debris, and the pH of the supernatant fluid adjusted to 6.7 with dilute sulfuric acid. Free ammonia and total nitrogen determinations were made on 1 ml aliquots, whereas enzymatic activity was assayed on a 5 ml aliquot.

Enzyme precipitation. Four hundred ml of the supernatant broth were placed in an ice bath and cooled to about 1 C. The cooled supernatant broth was added to 400 ml of 95 per cent ethyl alcohol which had been precooled to -25 C. In spite of the precooling, the temperature rise necessitated further cooling, with slow stirring, until -10 C was reached. One-half ml of a saturated NaCl solution was added and the entire mixture allowed to remain at -10 C from ½ to 1 hour. The mixture was then centrifuged at approximately 3,000 xG for 20 minutes at -10 C. Upon completion, the tubes containing the precipitate were placed immediately in a H2SO4 Hempel desiccator and a vacuum drawn to about
1 cm of mercury. The tubes were allowed to remain in the desiccator for approximately 18 hours, and portions of the dried active powder obtained from this procedure were assayed for activity by dissolving them in 0.02 M potassium phosphate buffer (pH 7.2). (The buffer solution also contained 10 mg per ml of freshly added and neutralized cysteine.)

**Measurement of activity.** Although ammonia determinations could be made to measure activity, convenient use was made of optical density ratios which are noted as "Ex". These readings were taken at 250 mλ and 270 mλ using the Beckman spectrophotometer, Model DU. Preliminary tests revealed an almost linear relationship in ratio change when readings were taken on various mixtures of purified guanine and xanthine. Table 1 shows the values obtained. In calculating the per cent guanine converted into xanthine, the following equations were used:

\[
\begin{align*}
(1) & \quad Ex = \frac{D \times (250 \text{ mλ})}{D \times (270 \text{ mλ})} \\
(2) & \quad \frac{Ex \text{ (initial)} - Ex \text{ (final)}}{0.9} \times 100 = \text{ per cent conversion}
\end{align*}
\]

In the complete conversion of guanine into xanthine, the Ex values changed from 1.59 to 0.65 or a difference of 0.91. The latter figure was rounded off to 0.9 in the above equation. The results obtained spectrophotometrically were checked periodically by free ammonia determinations.

**Method of conducting test.** The substrate of 0.01 N colloidal guanine was prepared by adding approximately 10 to 15 ml of 0.5 per cent gelatin solution to 76 mg of guanine in a 50 ml volumetric flask. The solution was cleared by adding dropwise with swirling 30 per cent NaOH to dissolve all the guanine. The solution was then partially neutralized with dilute sulfuric acid and 30 to 40 ml more of gelatin solution were added. The solution was adjusted to approximately pH 7.3 with dilute sulfuric acid using several drops of phenol red as an indicator, and the volume brought up to 50 ml with the gelatin solution. This colloidal solution of guanine was kept in the refrigerator for not more than one week.

The supernatant broth was tested for activity by adding to a Thunberg tube 2 ml of buffer (0.02 M potassium phosphate, pH 7.3), 2 ml of guanine, 1 ml penicillin (20,000 units), and 5 ml of the supernatant broth. (Since Hobby et al. (1942) noted that penicillin appeared to inhibit proliferating cells, this method was used with success to inhibit multiplication of the few C. acidurici cells remaining in the supernatant broth.) As soon as the contents were mixed, 1 ml was removed for control purposes and immediately placed in a tube containing 1 ml of 20 per cent perchloric acid solution.

The Thunberg tube was then closed, evacuated, filled with nitrogen, and placed in a 40 C water bath for 5 or 24 hours. At the end of the test period, the tube contents were thoroughly mixed, another 1 ml sample taken and placed in the perchloric acid. After allowing the perchloric acid mixture to stand several minutes, to precipitate protein, 16 ml of distilled water were added and mixed. The solutions were then filtered and readings taken in the Beckman spectrophotometer. The blank contained a ratio of 1 part perchloric acid solution to 17 parts of water by volume. In cases where the purine was less concentrated, less water was added to the perchloric acid solution. It should be noted that no matter what dilution was used, the ratio D_{560}:D_{270}, or the Ex, always remained the same.

The activity tests on the active dried powder were conducted in a similar manner as that executed on the supernatant broth. Here no penicillin was used; instead, the following were mixed: Two ml buffer, 2 ml guanine, and 2 ml of enzyme extract (usually in 0.02 M phosphate buffer). At zero time and at other designated times, samples were taken and treated as de-

### Table 1

**Optical densities and ratios obtained in mixtures of guanine and xanthine**

<table>
<thead>
<tr>
<th>Percentage</th>
<th>Guanine</th>
<th>Xanthine</th>
<th>D∞/D270</th>
<th>D∞/D250</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>1.238</td>
<td>0.791</td>
<td>1.56</td>
</tr>
<tr>
<td>90</td>
<td>10</td>
<td>1.175</td>
<td>0.831</td>
<td>1.45</td>
</tr>
<tr>
<td>80</td>
<td>20</td>
<td>1.110</td>
<td>0.829</td>
<td>1.34</td>
</tr>
<tr>
<td>70</td>
<td>30</td>
<td>1.059</td>
<td>0.855</td>
<td>1.23</td>
</tr>
<tr>
<td>60</td>
<td>40</td>
<td>1.001</td>
<td>0.876</td>
<td>1.14</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>0.938</td>
<td>0.889</td>
<td>1.05</td>
</tr>
<tr>
<td>40</td>
<td>60</td>
<td>0.882</td>
<td>0.910</td>
<td>0.97</td>
</tr>
<tr>
<td>30</td>
<td>70</td>
<td>0.826</td>
<td>0.936</td>
<td>0.88</td>
</tr>
<tr>
<td>20</td>
<td>80</td>
<td>0.759</td>
<td>0.949</td>
<td>0.80</td>
</tr>
<tr>
<td>10</td>
<td>90</td>
<td>0.712</td>
<td>0.975</td>
<td>0.73</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>0.648</td>
<td>0.994</td>
<td>0.65</td>
</tr>
</tbody>
</table>

Values were obtained on purified guanine and xanthine.
scribed above. At first these tests were conducted anaerobically, but later aerobic conditions were employed when it was discovered that enzymatic activity decreased only slightly in the presence of air. To offset the slight loss of activity from the utilization of aerobic conditions, cysteine was added to the test mixture.

**RESULTS AND DISCUSSION**

In the early phases of this work attempts at isolation of the enzyme from the cells failed while success was achieved in isolations from broth. Since guanine was always in an insoluble state at the pH of the medium, it was felt that in all probability guanase was an extracellular enzyme. To test this postulate, the following experiment was designed:

A test tube containing 5 ml of guanine semisolid agar was sterilized, cooled, and inoculated. Over the surface of the medium was placed 1 ml of 2 per cent agar (no nutrients). This was allowed to harden, after which approximately 3 ml of guanine semisolid agar were added. This also was permitted to solidify. Finally, a cap of 1 ml of 2 per cent agar was placed over the semisolid medium. The tube was allowed to incubate for 5 days. The disappearance of guanine and the appearance of alkalinity in the second semisolid plug were indicative of extracellular guanase activity. A gram stain revealed a few cells in this portion. However, their paucity ruled out the possibility of any major role played by an intracellular guanase.

Several methods of precipitation were employed to obtain the enzyme from the supernatant broth with a 1:1 ratio of ethanol to supernate adjusted to pH 6.7 proving most effective. The active dried powder obtained through this procedure usually increased in purity over the supernate by 3 to 6-fold. The time allotted before removing the precipitate from the ethanol supernate mixture was critical past the first hour, even though the entire mixture was held at -10 C. Activity was lost to the extent that a 43 per cent decrease occurred when removing the precipitate was put off for as long as 18 hours.

It was necessary to add gelatin prior to precipitation to prevent inordinate amounts of enzyme inactivation. Of the three "protective agents" tried, only gelatin fulfilled its role. No protection was offered by gum arabic or blood albumin.

According to Schmidt (1932) guanase is inactivated when held at a temperature of 65 C for 10 minutes and is greatly weakened when held for the same length of time at 60 C. Although the lower temperature was not used, the bacterial guanase was also inactivated when held at 65 C for 10 minutes. In another paper Schmidt and Engel (1932) reported that 0.1 N NaF inhibited their guanase preparation. In our hands NaF apparently had no effect on our active preparation under the conditions of the test, while in the manometric studies (Rakosky and Beck, 1955) NaF appeared to decrease the activity of the resting cells on guanine.

In those cases where the enzyme concentration was varied, proportional activity was observed. This can be seen in figure 1. In this particular instance 6 tubes were set up, each containing 2 ml of colloidal guanine and varying amounts of buffer, so that when the enzyme was added, the total volume was 6 ml. The enzyme solution was made up in phosphate buffer so that each ml of the solution contained 1.1 mg of total nitrogen, and the tubes in this test were incubated at 33 C.

For most enzymes activity is high at an optimum pH while few cases exist where this activity

![Figure 1. The effect on activity of varying enzyme concentration as measured with a 5 and 24 hr period of incubation.](image-url)
Three different high remains varying in used at mum 23 hour period this vicinity the versions: 32.2, 28.9, 34.4, and Roush if following the 2 figure activity was optimum after activity falls off inactivated hours. deaminated Roush and Norris work 8-azaguanine and acid, 8-azaguanine, 1-methyl guanine, studies (Rakosky powder, Michaelis-Menten constant (Km) found by Hitchings and Falco
was found to be highly specific by Schmidt (1932), Hitchings and Falco (1944), and Roush and Norris (1950). In spite of this specificity 8-azaguanine and 1-methyl guanine are deaminated to the xanthine analogues. In this work specificity tests were conducted on guanyclic acid, 8-azaguanine, 1-methyl guanine, and guanosine. It was found that the active dried powder, like the whole cells in the analogue studies (Rakoisky and Beck, 1955), deaminated 8-azaguanine and 1-methyl guanine.

Attempts were made to determine the Michaelis-Menten constant (Km) of guanase. In these experiments the amount of substrate was varied from 1 to 40 μM. The test was set up and allowed to incubate for 1 hour in a 40°C water bath. At the end of the test a sample was taken, placed in perchloric acid, diluted, filtered, and read in the Beckman spectrophotometer. The readings were corrected and calculations made to obtain the amount of guanine converted in the 1 hour test period (see table 2).

These values were plotted as in the method of Lineweaver and Burke (1934). A plot of 1/v against 1/S gave points that did not fall into a straight line. The best possible line was determined by the method of least squares resulting in the equation:

\[
\frac{1}{v} = 3.03 \frac{1}{S} + 0.233
\]

v was found to be 4.292 while Km equalled 1.3 × 10^{-4} M as compared to the value of 5 × 10^{-4} M found by Roush and Norris (1950) using animal guanase. It is possible that the differences in Km may be attributable to inherent differences of the enzyme source material or differences in enzyme purity. Undoubtedly a higher degree of purification of bacterial guanase would have yielded a more accurate evaluation of Km.

In a test to determine the stability of the enzyme, fresh bacterial guanase was prepared and its activity determined while a second portion was stored in a calcium chloride desiccator at room temperature under atmospheric conditions for six days. A 43 per cent loss of activity occurred during this time period.

**SUMMARY**

A guanase-like enzyme was isolated from the broth in which Clostridium acidiurici had been grown. The enzyme was found to be similar in several respects to the animal guanase described
by earlier workers. Its isolation, along with the data of Rakosky and Beck, corroborate the postulation of Beck that xanthine is an intermediate in anaerobic guanine degradation by _C. aciduri_

REFERENCES


**Beck, S. M.** 1951 Xanthine as a possible intermediate in uric acid catabolism by _Clostridium aciduri_. Thesis, the Pennsylvania State University, State College, Pennsylvania.


