The Enzymatic Reduction of Hydroxyguanidine*

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This laboratory first became interested in hydroxyguanidine as a result of our studies on the mechanism of action of transamidination enzymes. We have found that transaminases from *Streptomyces griseus* (2), mammalian pancreas (3) and kidney (4), and chicken and frog liver (5) can catalyze a transfer of the terminal formamidine group of arginine to hydroxylamine, with the formation of hydroxyguanidine.

\[
\text{NH}_2
\]

Arginine + \( \text{NH}_2\text{OH} \) → ornithine + \( \text{H}_2\text{N}--\text{C}--\text{NH}--\text{OH} \)

In addition, arginine-glycine transaminase can catalyze the synthesis of hydroxyguanidine from guanidinoacetate and hydroxylamine (3).

Early in our investigations, a search of the literature revealed that practically nothing was known of the chemical or biological properties of hydroxyguanidine. Consequently a chemical synthesis of hydroxyguanidine sulfate was developed (3), and an investigation of the biological activity of this compound was undertaken.

It was found that after hydroxyguanidine sulfate was injected intraperitoneally into rats, its reduction product, guanidine, appeared in the urine. Later it was discovered that *Escherichia coli* B, while growing on a glucose-salts synthetic medium, converted hydroxylamine (3).

The solvent used was 95 per cent methanol:5 per cent water. To avoid streaking of the hydroxyguanidine, the applied solutions should be made acid. After development, papers were sprayed with an alkaline ferricyanide-nitroprusside reagent (7); with this reagent hydroxyguanidine gives a purple spot at an \( R_f \) of 0.60, and guanidine gives an orange spot at an \( R_f \) of 0.55.

Enzyme Assays with Reduced Riboflavin Phosphate as Donor

The complete system consisted of enzyme suspension; 250 \( \mu \)moles of potassium phosphate buffer, pH 7.4; 42 \( \mu \)moles of hydroxyguanidine (as the sulfate); 16 \( \mu \)moles of riboflavin phosphate, Na salt; 82 \( \mu \)moles of \( \text{Na}_2\text{SO}_4 \); and water to give a final volume of 2.0 ml. (For reproducible results it is desirable to add the riboflavin phosphate plus \( \text{Na}_2\text{SO}_4 \) last. Equal volumes of freshly prepared double-strength solutions of riboflavin phosphate and \( \text{Na}_2\text{SO}_4 \) in 0.1 M phosphate buffer, pH 7.4, were mixed rapidly, and the desired aliquot, e.g., 0.4 ml., was immediately pipetted into the reaction mixture containing all other components.)

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Incubations were carried out in 13 × 100 mm. stoppered test tubes at 25° for 30 minutes. The tubes were agitated twice during this interval to keep the enzyme particles in suspension. Variations from this protocol are noted in the individual experiments. The reaction was stopped with 0.5 ml of 30 per cent trichloroacetic acid. After 10 minutes the tubes were centrifuged, and the supernatant solutions were assayed, after suitable dilution, for the remaining hydroxyguanidine or for guanidine.

Assays for hydroxyguanidine were carried out with trisodium pentacyanotetramminoferrate (Fisher Scientific, S-659) as described previously (2), except that a wave length of 525 mµ was employed to minimize interference from riboflavin phosphate. Guanidine was assayed by a diacetyl method (8); hydroxyguanidine interferes somewhat with this assay, whereas guanidine does not interfere with the hydroxyguanidine assay.

Experiments with E. coli—E. coli B was grown on a glucose-ammonium-salts basal medium, as described elsewhere (6), in capped test tubes containing 5 ml of medium. Preliminary experiments had established that neither hydroxyguanidine nor guanidine could be utilized as a nitrogen source by this organism. Hydroxyguanidine sulfate was sterilized by filtration and added to the cooled sterile growth medium. After an incubation period of 21 hours at 34°, the cultures were centrifuged, and the media were analyzed as described above for residual hydroxyguanidine (at 480 mµ) and for guanidine. For the isolation of guanidine, E. coli was grown in 500 ml of medium in a 1-l. Erlenmeyer flask at 34°. Hydroxyguanidine sulfate was added to a concentration of 1 mg per ml. After 21 hours the culture was centrifuged, and the supernatant solution was acidified with hydrochloric acid and evaporated to dryness. Guanidine hydrochloride was extracted from the residue with methanol, and the solution was evaporated to dryness. The residue was taken up in a minimal volume of water, and a saturated solution of picric acid was added. The precipitated guanidine picrate was collected and recrystallized from water.

**RESULTS**

Metabolism of Hydroxyguanidine by Intact Rat—In these experiments 200-gm. male rats were given an intraperitoneal injection of a solution of hydroxyguanidine sulfate (50 mg in 2 ml of water), and the urine was collected for analysis by paper chromatography. It was found that, after a small transient excretion of the administered compound had occurred, a new compound appeared in the urine. Excretion of this new compound, identified as guanidine, reached a plateau approximately an hour after hydroxyguanidine injection. These results suggested that a precursor-product relationship existed between the two compounds.

Preliminary Experiments in vitro—Attempts were next made to obtain an in vitro system for hydroxyguanidine reduction. Preliminary experiments revealed that homogenates of rat or guinea pig liver catalyzed the disappearance of hydroxyguanidine, coupled with the appearance of guanidine. This reaction was markedly stimulated by the addition of DPN plus riboflavin phosphate, and was inhibited by cyanide. Endogenous DPN-reducing substrates did not appear to be limiting (the animals were not starved before sacrifice); succinate apparently could not act as a donor in the reaction. The observation that riboflavin phosphate was stimulatory suggested that the system might be simplified by employing chemically reduced riboflavin phosphate as the electron donor, and this was found to be the case.

Enzymatic Reduction with Reduced Riboflavin Phosphate—Reduced riboflavin phosphate, formed by reduction of riboflavin phosphate with dithionite, was found to be an excellent donor for the enzymatic reduction of hydroxyguanidine. The reaction can be followed by measuring (a) the increase in guanidine, or more conveniently (b) the decrease in hydroxyguanidine concentration. Employing the latter assay, hydroxyguanidine reductase activity was found to reside in a washed particulate fraction of liver. Guinea pig liver was found to have a higher activity than rat liver and was employed for the experiments described in this paper. The enzymatic nature of the reaction and the requirements for the individual reaction components are indicated in Table I and Figs. 1 to 4. (The basal assay conditions for all experiments are described in the "Experimental" section.)

From these data it is apparent that the rate of hydroxyguanidine reduction is (a) proportional to the enzyme concentration (Fig. 1), (b) approximately linear with time (Fig. 2), and (c) dependent upon the concentration of riboflavin phosphate over the range shown in Fig. 3. Fig. 4 demonstrates that the quantity of hydroxyguanidine reduced is proportional to the amount of dithionite added, when the reaction is allowed to proceed to completion. From these experiments it may be calculated that the liver particulate preparation can catalyze the reduction of 170 μmoles of hydroxyguanidine per hour per mg. of nitrogen. The enzyme complex is labile to heat, and its activity is inhibited

**Table I**

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Hydroxyguanidine reduced</th>
<th>Guanidine formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>42</td>
<td>41</td>
</tr>
<tr>
<td>Complete system - enzyme</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Complete system - riboflavin phosphate</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Complete system - Na₂S₂O₃</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Complete system, enzyme heated 5 min. at 60°</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

![Fig. 1. Rate of reduction of hydroxyguanidine by reduced riboflavin phosphate as a function of enzyme concentration. HG is hydroxyguanidine.](http://www.jbc.org/issue/234/6/1482/article-pdf/234/6/1482/1041567/234-06-1482.pdf)
FIG. 2. Reduction of hydroxyguanidine by reduced riboflavin phosphate as a function of time. Enzyme concentration equivalent to 0.25 mg. of nitrogen per tube. HG is hydroxyguanidine.

FIG. 3. Rate of reduction of hydroxyguanidine by reduced riboflavin phosphate as a function of reduced riboflavin phosphate concentration. Enzyme concentration equivalent to 0.4 mg. of nitrogen per tube. HG is hydroxyguanidine; FMNH₂, reduced riboflavin phosphate.

50 per cent by $2 \times 10^{-4}$ M cyanide. Additional experiments have established that leucosafranine, but not leucomethylene blue, can serve as an electron donor in this enzymatic reaction.

**Distribution of Hydroxyguanidine Reductase Activity**—Reductase activity has been observed in mammalian liver and kidney, pigeon liver acetone powder (Sigma Chemical Company), S. griseus acetone powder, and E. coli B. In the case of E. coli, it was established that this organism, while growing on a glucose-ammonium-salts synthetic medium, quantitatively reduces added hydroxyguanidine to guanidine (Table II). Since guanidine is not further metabolized by this organism, it can be readily isolated as the picrate (see "Experimental").

C₇H₈S₅Oₛ

Calculated: C 29.17, H 2.80, N 29.17
Found: C 28.49, H 2.49, N 28.83

**DISCUSSION**

It is remarkable, considering the ease of synthesis and stability of hydroxyguanidine, that this simple 1-carbon compound has until recently escaped detailed scrutiny by chemists and biochemists: literature references to this compound before 1956 are surprisingly rare (10, 11). However, with the introduction of the simple synthetic procedure described in this paper, there is nothing to prevent more sophisticated research on the biological properties of hydroxyguanidine and the pharmacologically interesting compounds which may be synthesized from it. Derivatives of hydroxyguanidine are known to occur in nature; canavanine (12) and canavaninosuccinic acid (7) are two such derivatives. The O-N bond in all three of these compounds can be reduced by hydrogen plus palladium (7). It is of interest that Kihara et al. (13) have reported the reduction of canavanine to homoserine and guanidine by certain bacteria, as well as the hydrolysis of canavanine to homoserine and hydroxyguanidine by a soil pseudomonad (6).

Speculations on the nature and the physiological substrates of the enzyme exhibiting hydroxyguanidine reductase activity must await further purification; such studies are in progress.

**SUMMARY**

1. Rats have been found to convert injected hydroxyguanidine to guanidine. Studies in vitro have shown that hydroxyguanidine reductase activity occurs in a washed particulate fraction of guinea pig liver. An assay for this enzyme has been devised which utilizes reduced riboflavin phosphate as the electron donor. Under the experimental conditions employed, the enzyme preparation catalyzes the reduction of 170 μmoles of hydroxyguanidine per hour per mg. of nitrogen. The enzyme is heat labile and is inhibited by cyanide.

2. Growing cultures of Escherichia coli B convert added hydroxyguanidine to guanidine. Guanidine has been isolated from the medium and identified as the picrate. Hydroxyguanidine
reductase activity has also been found in mammalian kidney, pigeon liver, and *Streptomyces griseus*.

3. A rapid and convenient synthesis of hydroxyguanidine sulfate is described.

REFERENCES


The Enzymatic Reduction of Hydroxyguanidine
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