BIOSYNTHESIS OF ARGinine FROM CANAVANINE AND ORNITHINE IN KIDNEY

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Borsook and Dubnoff (1, 2) and Bloch and Schoenheimer (3) have demonstrated that the amidine moiety of arginine is the precursor of the corresponding group of glycocyamine; in mammals this reaction is carried out primarily in the kidney. Fuld (4) has recently observed that the arginine-glycine transamidination reaction is reversible. By means of group transfer the high chemical potential of the amidine moiety is conserved. Since other metabolites containing the guanidino group occur in nature, it is of interest to determine whether they also participate in transamidination reactions. One such compound that might be investigated is canavanine, a naturally occurring amino acid which possesses a terminal guanidinoxyl group.

In this paper data will be presented which show that in the presence of an enzyme from hog kidney canavanine donates an amidine group to ornithine, with the formation of arginine and canalin.

Materials—L-Canavanine sulfate, L-ornithine dihydrochloride, L-arginine monohydrochloride, and beef liver arginase were purchased from the Nutritional Biochemicals Corporation. Preliminary experiments were carried out with canavanine isolated from jack bean meal as the thrice recrystallized flavianate; canavanine was regenerated from the flavianate with barium hydroxide.

Analytical Methods—The ascending paper chromatographic procedure of Williams and Kirby was employed (5, 6). Paper chromatograms were sprayed with ninhydrin to detect primary amino groups or alkaline ferri-cyanide-nitroprusside to detect guanidino groups (6). Since canavanine does not react significantly with the Sakaguchi reagent, arginine could be determined colorimetrically in the reaction mixture with this reagent. The modified procedure of Albanese and Frankston (7) was employed. The components were mixed at 5°, and the optical densities at 540 mμ were read within 10 minutes with an Evelyn colorimeter. The stipulated hypochlorite concentration was met by using commercial Clorox, diluted 1:1.

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Results

Since mammalian kidney was already known to contain an enzyme which catalyzes a reversible arginine-glycine transamidination reaction, it was logical that hog kidney should be the tissue first examined for activity in a canavanine-ornithine transamidination. The low arginase activity of mammalian kidney was considered to be another favorable characteristic of this tissue, as far as interpretation of results was concerned. Although it was probable that such a transamidination, if it occurred, would be reversible, analytical considerations indicated that the reaction might be most conveniently studied in the following direction:

\[
\begin{align*}
\text{HOOC-CH-CH_2-CH_2-O-NH-C-NH_2} + & \\
\text{Canavanine} & \\
\text{NH} \\
\text{NH}_2
\end{align*}
\]

\[
\begin{align*}
\text{H}_2\text{N-CH_3-CH_2-CH_2-CH- COOH} \rightarrow & \\
\text{Ornithine} & \\
\text{NH}_2
\end{align*}
\]

\[
\begin{align*}
\text{HOOC-CH-CH_2-CH_2-O-NH_4} + & \\
\text{Canalin} & \\
\text{NH}_2
\end{align*}
\]

\[
\begin{align*}
\text{H}_2\text{N-C-NH-CH_3-CH_2-CH_2-CH-COOH} \rightarrow & \\
\text{Arginine} & \\
\text{NH}_2
\end{align*}
\]

With this postulated reaction under consideration, the search for an active enzyme preparation was started.

Enzyme Preparation—Acetone-dried hog kidney (8) was extracted for 2 hours with 0.05 M potassium phosphate buffer, pH 7.4, at 5°. Protein was precipitated by the dropwise addition of a saturated solution of ammonium sulfate to 60 per cent saturation. The precipitate was redissolved in 0.02 M buffer, and the solution was added dropwise with stirring to 50 volumes of cold acetone. The precipitate was collected by suction filtration, washed with acetone, and the acetone removed in vacuo.

It was found by paper chromatographic analysis, and confirmed by the Sakaguchi test for arginine, that this preparation catalyzed the reaction.
as postulated above. In an attempt to concentrate the activity, the acetone powder was dissolved in 0.05 M phosphate buffer, pH 7.0, and refractionated with ammonium sulfate. A clean fractionation of the enzymatic activity was not achieved in this manner, but the fraction precipitating between 30 and 40 per cent saturation had the highest specific activity. Dialysis of this fraction for 24 hours at 3–5° against three changes of 400 volumes of 0.01 M phosphate buffer, pH 7.0, resulted in a gain, rather than a loss, in activity. Similarly, the addition of the chelating agent ethylenediaminetetraacetate increased the activity slightly. Consequently subsequent dialyses were carried out against buffer plus ethylenediaminetetraacetate. After dialysis, an acetone powder of the 30 to 40 per cent fraction was prepared and stored in the cold.

Reaction Characteristics—Activity of the dialyzed preparations was not enhanced by the addition of cysteine, magnesium, manganese, or concentrates of liver or yeast coenzymes (Sigma). The reaction proceeds at a significant rate between pH 6.2 and 8.2, with an optimum at pH 7.2 under the conditions employed.

The effect on the reaction of omitting one of the components is shown in Table I. It can be seen that the reaction does not proceed unless all components are present. If the enzyme is inactivated by heating the reaction mixture for 5 minutes at 100°, no arginine is formed upon subsequent incubation. Lysine will not substitute for ornithine in this reaction; the results obtained with glycine will be discussed later. Although the reaction products could be detected after 2 hours incubation at room temperature, in this experiment the reaction mixtures were incubated for 43 hours, 11 hours after the arginine concentration had reached its maximal value in Reaction Mixture 1. This value represents a 42 per cent conversion of ornithine into arginine. In Reaction Mixture 5, in which the canavanine concentration was doubled, the conversion was increased to 67 per cent. Whether the arginine concentration obtained in Reaction Mixture 1 represents the equilibrium value under these conditions will not be known until a more purified enzyme preparation is available.

Identification of Reaction Products—The fact that the reaction product which gives a red color with the Sakaguchi reagent is indeed arginine was confirmed by paper chromatography, in which either a ninhydrin spray was employed for the primary amino group or an alkaline ferricyanide-nitroprusside spray to detect the guanidino moiety (6). For this purpose the most satisfactory solvent system was water-saturated phenol, with an ammonia atmosphere. Arginine spots were obtained only from those reaction mixtures in which all components were present. Additional confirmation was furnished by microbiological assays for arginine with L. mesenteroides P-60 (Table I). This organism was chosen because it
responds to arginine and not to ornithine or citrulline (9), since it lacks the enzyme argininosuccinase (10). Of particular importance for the purpose of this assay is the fact that the growth of this organism is not affected by canavanine (9). Consequently in a reaction mixture which contains canavanine, ornithine, canaline, and arginine, *L. mesenteroides*

### Table I

**Results of Colorimetric and Microbiological Assays for Arginine Formation**

Each tube contained 15 mg. of an acetone powder of a dialyzed, 30 to 40 per cent ammonium sulfate fraction of kidney extract as the enzyme source, plus 1.6 ml. of 0.125 M potassium phosphate buffer, pH 7.0, and other components as shown below. Incubation at room temperature, under toluene, for 43 hours, 11 hours after maximal arginine production was obtained. The reactions were stopped by heating at 100° for 3 minutes and the mixtures centrifuged. Analyses of supernatant solutions for arginine were carried out both colorimetrically with the Sakaguchi reagent against an arginine standard and microbiologically with *Leuconostoc mesenteroides* P-60.

<table>
<thead>
<tr>
<th>Reaction mixture No.</th>
<th>Substrates</th>
<th>Arginine produced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Colorimetric</td>
</tr>
<tr>
<td></td>
<td></td>
<td>assay μmoles</td>
</tr>
<tr>
<td>1</td>
<td>Canavanine, 76 μmoles; ornithine, 78 μmoles</td>
<td>33</td>
</tr>
<tr>
<td>2</td>
<td>“ 76 “</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>Ornithine, 78 μmoles</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Canavanine, 76 μmoles; lysine, 78 μmoles</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>“ 153 μmoles; ornithine, 78 μmoles</td>
<td>52</td>
</tr>
<tr>
<td>6</td>
<td>“ 76 μmoles; ornithine, 78 μmoles;</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>boiled 5 min. at 0 time</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Canavanine, 76 μmoles; glycine, 80 μmoles</td>
<td>16*</td>
</tr>
<tr>
<td>8</td>
<td>Glycine, 80 μmoles</td>
<td>1</td>
</tr>
</tbody>
</table>

*The Sakaguchi color is reported in arginine equivalents, but, as the microbiological assay and paper chromatography indicate, the color is due not to arginine, but to glycocyamine.

It can be seen from Table I that the colorimetric and microbiological assays for arginine are in close agreement.

The other reaction product, canaline, was identified by a specific and sensitive paper chromatographic method developed in this laboratory. In this method advantage was taken of the fact that canaline is an O-substituted hydroxylamine capable of reacting with carbonyl groups (11), with an α-amino group which can be detected with ninhydrin. Consequently the *R* values of the oxime type complexes of canaline with various carbonyl compounds should serve to identify canaline in complex reaction mixtures. Preliminary experiments were performed to establish
the corresponding $R_F$ values. Beef liver arginase was incubated with canavanine to form canaline. After the reaction was complete, various carbonyl compounds were added to aliquots of the deproteinized solution. The solutions were chromatographed on paper with a solvent of water-saturated phenol, ammonia atmosphere, and sprayed with ninhydrin. The $R_F$ values of the complexes of canaline with several carbonyl compounds are listed in Table II. In an adjacent column are given the $R_F$ values of the ninhydrin spots obtained when the same carbonyl compounds were added to aliquots of the deproteinized canavanine-ornithine reaction mixture. These latter spots were not observed when either canavanine,

<table>
<thead>
<tr>
<th>Carbonyl compound added</th>
<th>$R_F$ value of oxime</th>
<th>$R_F$ value of reaction mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribose-5-phosphate</td>
<td>0.05</td>
<td>0.04</td>
</tr>
<tr>
<td>Ribose</td>
<td>0.43</td>
<td>0.43</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.40</td>
<td>0.38</td>
</tr>
<tr>
<td>Oxalacetate</td>
<td>0.11</td>
<td>0.10</td>
</tr>
<tr>
<td>$\alpha$-Ketoglutarate</td>
<td>0.19</td>
<td>0.20</td>
</tr>
</tbody>
</table>

ornithine, or the enzyme was omitted during incubation. From these data it is apparent that canaline is a product of the transamidination reaction. Further confirmation was obtained by isolating the complexes obtained with pyruvate and $\alpha$-ketoglutarate by means of paper chromatography. The respective paper strip eluates were subjected to treatment with palladium black and hydrogen gas at room temperature and atmospheric pressure for 3 hours and were rechromatographed on paper. It was found that the pyruvate complex yielded homoserine and alanine, while the $\alpha$-ketoglutarate complex yielded homoserine and glutamic acid, as determined by their $R_F$ values in solvents of water-saturated phenol, ammonia atmosphere, and butanol-acetic acid-water (4:1:1). These are the products which would be expected from a reduction of an oxime linkage between canaline and the respective keto acid.

Reversibility of Reaction—That the canavanine-ornithine transamidina-
tion reaction is reversible was indicated by the results obtained in the following experiment. A mixture of 25 mg. of canavanine sulfate and 3 ml. of 0.1 M phosphate buffer was adjusted to pH 7.5, and 15 mg. of arginase were added. The mixture was incubated in the presence of toluene for 23 hours at room temperature to assure the complete hydrolysis of canavanine to canaline, and then heated for 5 minutes at 100°. The mixture was centrifuged to remove coagulated protein. To 1.2 ml. of the canaline-containing supernatant solution were added 20 mg. of L-arginine monohydrochloride and 10 mg. of the dialyzed transamidinase preparation. After incubation for 20 hours at room temperature in the presence of toluene, the reaction mixture was deproteinized by heating at 100° for 3 minutes. Paper chromatograms of the supernatant solution, with water-saturated phenol, ammonia atmosphere, as the solvent, were sprayed with alkaline ferricyanide-nitroprusside reagent. Below the red arginine spot, at the predicted Rf value, could be distinguished the violet spot characteristic of canavanine. This spot was not present in the canavanine hydrolysate before arginine and enzyme were added. No canavanine was formed when either arginine or transamidinase was omitted from the reaction mixture.

Evidence for Canavanine-Glycine Transamidination—Preliminary experiments indicate that glycine also can act as an amidine acceptor when canavanine is the donor. In the experiment described in Table I, when glycine was substituted for ornithine, it was observed that a compound was produced which reacted in the Sakaguchi test, but did not support growth of L. mesenteroides in a medium lacking only arginine. Paper chromatographic analysis, with butanol-acetic acid-water (4:1:1) as solvent and an alkaline ferricyanide-nitroprusside spray, provided strong evidence that the compound produced was glycoxyamine. This compound was not produced in the absence of canavanine. When the compound was eluted and hydrolyzed with alkali, a ninhydrin-reactive compound was formed which corresponds with glycine on paper chromatograms.

DISCUSSION

It is not known at present whether canavanine and canaline are normal metabolites of higher animals. However, it is known that plants containing canavanine are ingested by higher animals. Consequently the metabolic pathways in which canavanine can participate are of interest. In mammals, canavanine may combine with fumarate to form canavaninosuccinic acid (12), be hydrolyzed to canaline and urea (13), or undergo transamidination, with the formation of canaline. It is interesting to speculate about the rôle of this transamidination reaction in metabolism.
parently *L. mesenteroides* lacks such an enzyme, since its arginine requirement cannot be met by a combination of canavanine and ornithine. If avian kidney can perform this transamidination, then in those birds which cannot synthesize citrulline from ornithine it is possible that canavanine might replace arginine in the diet, provided that sufficient endogenous or exogenous ornithine is present.

The mechanism of the canavanine-ornithine transamidination reaction reported here is not known. A cofactor requirement was not shown by these experiments. Since arginine-handling enzymes of many organisms apparently cannot distinguish completely between arginine and canavanine, it is tempting to speculate that reactions similar to the following may occur.

\[
\text{Canavanine} + \text{enzyme} \rightleftharpoons \text{enzyme} \sim \text{urea} + \text{canaline} \quad (2) \\
\text{Enzyme} \sim \text{urea} + \text{ornithine} \rightleftharpoons \text{arginine} + \text{enzyme} \quad (3) \\
\text{Sum.} \quad \text{Canavanine} + \text{ornithine} \rightleftharpoons \text{arginine} + \text{canaline} \quad (4)
\]

In this interpretation of the results reported in this paper, a single enzyme would be involved in the activation of both arginine and canavanine, and the same enzyme-amidine complex would be formed from either substrate. If this scheme is correct, the amidine group of arginine may be considered to be in a state of dynamic metabolic equilibrium in mammalian kidney, as well as in other biological systems which contain this enzyme (Reaction 3).

The postulated enzyme-amidine complex may be considered to be a form of "active urea." If such a complex is formed, it is possible that in some organisms a mechanism might exist for the synthesis of that complex from urea plus an energy source. A similar mechanism was earlier postulated to account for the utilization of urea as the sole nitrogen source for growth of organisms which apparently lack urease (14). Because of the wide-spread use of urea in foliar sprays and as a nitrogen source for ruminants, such a proposal warrants further study.

As an interesting by-product of this investigation, it might be noted that the reaction of canaline with various carbonyl compounds of biochemical interest could serve as a useful means of identification of those carbonyl compounds in complex biological mixtures. Canaline can be readily formed from commercially available canavanine and arginase. The procedure would involve simply adding the canavanine hydrolysate, containing canaline, to the unknown mixture and chromatographing the solution on paper. Use of the the sensitive ninhydrin reagent would permit the identification of small quantities of such compounds as pyruvate, \(\alpha\)-ketoglutarate, oxalacetate, and ribose (Table II).
SUMMARY

An enzyme preparation from hog kidney was found to catalyze a transfer of the amidine moiety of canavanine to ornithine, with the formation of arginine and canaline. No cofactor requirement was observed; the optimal pH is 7.2. Preliminary experiments indicated that the reaction is reversible. The same crude enzyme preparation apparently catalyzes a canavanine-glycine transamidination, glycocyamine being tentatively identified as a reaction product. It is suggested that the canavanine-ornithine transamidination reaction involves the reversible formation of the same enzyme-amidine ("active urea") complex from both canavanine and arginine. If this concept is correct, the amidine group of arginine may be considered to be in a state of dynamic equilibrium in mammalian kidney.

As a by-product of this investigation, a previously unreported method for identifying carbonyl compounds of biological interest was described. An arginase hydrolysate of canavanine, containing canaline, a ninhydrin-reactive O-substituted hydroxylamine, is added to the unknown to be identified, and the resulting solution is chromatographed on paper. The oxime type complexes formed between canaline and such compounds as pyruvate, α-ketoglutarate, and oxalacetate can be readily identified by their characteristic $R_F$ values, as detected with ninhydrin.

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BIBLIOGRAPHY

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