Canaline Carbamoyltransferase in Human Liver as Part of a Metabolic Cycle in Which Guanidino Compounds Are Formed

Samuel Natelson, Anthony Koller, Hsli-Yu Tseng, and Richard F. Dods

This and previous papers examine the reasons for the relationship between the concentrations of guanidinosuccinate and guanidinoacetate in human urine. With the demonstration here that extracts of human liver tissue can mediate ureidohomoserine formation from canaline [(-2-amino-4-aminoxy)-butyric acid] and carbamoyl phosphate, all steps in a cycle proposed for the production of guanidinosuccinate and guanidinoacetate have been documented. This includes synthesis of canavaninosuccinate from aspartate and ureidohomoserine, reductive cleavage of canavaninosuccinate to form guanidinosuccinate and homoserine, or, alternatively, lytic action on canavaninosuccinate to form fumarate and canavanine, and transamination to glycine to form guanidinoacetate, regenerating the canaline. We propose that canaline originates from aspartate, but the precise mechanism by which canaline is formed needs to be elucidated.

Additional Keyphrases: human intermediary metabolism - enzyme activity and kinetics - guanidinosuccinate and guanidinoacetate - metabolic pathways - preparation of canaline carbamoyltransferase and measurement of its activity

Earlier reports from this laboratory have suggested a metabolic pathway for the formation of guanidinosuccinate and guanidinoacetate in mammalian liver and kidney (1-5). In support of this proposed mechanism, the following steps have been demonstrated with human liver: the reductive cleavage of canavaninosuccinate to guanidinosuccinate and homoserine (3, 4), the alternative conversion of canavaninosuccinate to canavanine and fumarate by a lyase (2), the synthesis of canavaninosuccinate from aspartate and ureidohomoserine (1), and the transamination of canavanine with glycine to form guanidinoacetate (2).

This paper describes further studies of the proposed metabolic pathway, and demonstrates the feasibility of the condensation of carbamoyl phosphate with canaline to form ureidohomoserine. This completes the metabolic cycle from canaline to ureidohomoserine to canavaninosuccinate to canavanine and finally canaline (Figure 1).

Materials and Methods

Reagents

Triethanolamine buffer (0.2 and 0.02 mol/liter, pH 8.0 except as noted): Dissolve 53.3 ml of triethanolamine (Fisher Scientific Co., Fairlawn, N.J. 07410) in 1 liter of water, adjust to the required pH with HCl (1 mol/liter), and dilute to 2 liters with water. Dilute to make the 0.02 mol/liter buffer.

Ornithine solution (5 mmol/liter): Make a stock solution by dissolving 84.5 mg of L-ornithine-HCl (Sigma grade; Sigma Chemical Co., St. Louis, Mo. 63178) in 100 ml of triethanolamine buffer (0.2 mol/liter, pH 8.0 or pH 8.4).

Carbamoyl phosphate solution (10 mmol/liter): Dissolve 18.8 mg of dilithium carbamoyl phosphate (Sigma) in 10 ml of 0.2 mol/liter triethanolamine buffer, pH 8.0 or 8.4. To this solution add 7.27 mg of urease (260 U, mmol/min) Type III, Sigma) just before use, if urea is present. Omit the urease in those cases where tentative solutions or eluates from the column are being assayed.

Canaline: Prepare this from canaline dipicrate (Sigma) as described before for the synthesis of ureidohomoserine (1). Combine and lyophilize the canaline solutions from the ion-exchange chromatographic step. Redissolve the residue in a minimum of hot water (0.5 ml) and add 4 ml of cold ethanol, with mixing. Refrigerate the solution at 4 °C overnight. Filter the white crystalline precipitate, wash it with cold ethanol, and recrystallize it from water--ethanol, using the same volumes as for the initial crystallization. Dry the material over sodium hydroxide, under reduced pressure. The melting point should be 202--205 °C (with decomposition).

Ureidohomoserine: Prepare DL-O-ureidohomoserine as described elsewhere (1).

Perchloric acid (1 mol/liter): Dilute 10 ml of 70% perchloric acid with 90 ml of water.

Canaline solution (30 mmol/liter): Dissolve 105 mg of canaline (prepared above) in 25 ml of 0.2 mol/liter triethanolamine buffer, pH 8.0. Alternatively, dissolve the lyophilized residue from the column, used in preparing canaline, in a small amount of buffer, assay it

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against crystalline canaline as a standard, and dilute to 30 mmol/liter. The solution is stable at 4 °C for at least one month.

Citrulline reagent: Dissolve 1.22 g of antipyrine (Sigma) and 203 mg of ferric chloride in 62.5 ml of phosphoric acid (85%) and 37.5 ml of water. This reagent is indefinitely stable at room temperature.

Diacetyl monoxime, 30 g/liter: Dissolve 3 g of diacetyl monoxime (2,3-butanedione monoxime; Matheson Coleman Bell Chemical Co., Norwood, Ohio 45212) in 100 ml of water. Store in a brown bottle. When ready to use, dilute 100 ml to 300 ml with water to make 10 g/liter diacetyl monoxime reagent.

Ureidohomoserine reagent (6): Dissolve 38 mg of N-phenyl-p-phenylenediamine HCl (Eastman Kodak, Rochester, N.Y. 14650), recrystallized from hot water, in a solution of 15 ml of ethanol/water (10/90 by vol). Add 15 ml of the diacetyl monoxime, 30 g/liter. Prepare freshly each day, and keep in a brown bottle in the dark.

Biuret reagent: Dilute 2 g of CuSO₄·5H₂O to 100 ml with water. On the day of the test add NaOH solution (25 g/100 ml) to this copper sulfate solution in a volume ratio of 5/1 (e.g., 25 ml of NaOH and 5 ml of copper sulfate solution).

Ornithine carbamoyltransferase (EC 2.1.3.3) from S. faecalis: This preparation (Sigma) had a specific activity of 600 μmol of citrulline produced per minute per milligram of protein. Dissolve the enzyme in 20 mmol/liter triethanolamine buffer, pH 8.4, at a concentration of 0.4 μg of protein per 25 μl of buffer.

Liver extraction buffer (20 mmol/liter): To 100 ml of the 0.2 mol/liter triethanolamine buffer, pH 8.0, add 372 mg of disodium ethylenediaminetetraacetate (Fisher Scientific), stir, and add 500 ml of water. Adjust the pH to 8.0 with NaOH solution (100 g/liter) and dilute to 1.0 liter with water.

Preparation of canaline carbamoyltransferase (no EC no. assigned) from human liver: All extraction and fractionation procedures were done in the cold room at 4 °C.

Human-liver acetone powder was prepared as described before (1–5) and 15 g was homogenized in 200 ml of liver extraction buffer with a Polytron homogenizer (Brinkmann Instruments, Westbury, N.Y. 11590) by using three 10-s bursts. The solution was then stirred for 1.5 h.

The homogenate was centrifuged (7200 × g, 30 min), the precipitate was discarded, and the supernate (177 ml) was fractionated as follows.

While stirring, add ammonium sulfate during 30 min, until the desired concentration is reached. Continue stirring for an additional 30 min. Centrifuge the solution at 7200 × g for 20 min. Separate the supernate and save the residue. To the supernatant fluid, add additional ammonium sulfate as before to achieve the next higher concentration. Again centrifuge the solution to obtain a supernate and residue.

The volumes obtained and the amount of ammonium sulfate added in each case are shown in Table 1.

In each case, dissolve the residue in 5 ml of the extraction buffer. Dialyze aliquots of the two supernatant fractions and the solutions of the residues at 4 °C against the 20 mmol/liter triethanolamine buffer in continuous flow for 24 h, using a 600 ml/h flow rate of buffer.

The protein content of each tenate solution (dialysate) was assayed with biuret solution, and its enzymatic activity determined, with canaline and ornithine as substrates.

The residue from the 70%-saturated ammonium sulfate fraction, which had been dialyzed, was fractionated on a column containing Sephadex.

Fractionation by chromatography Sephadex: A 75 × 2.5 cm column was loaded with Sephadex G-150. Three milliliters of the dialyzed liver extract precipitated by 70%-saturated ammonium sulfate, containing 138 mg of protein, was applied to the column. The developing buffer was the 20 mmol/liter triethanolamine buffer. Fractions of 2.6 ml were collected for a total of

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**Table 1. Fractionation of Liver Extract by Precipitation with Ammonium Sulfate.**

<table>
<thead>
<tr>
<th>% Saturation</th>
<th>0</th>
<th>25</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supematant volume, ml</td>
<td>177</td>
<td>172</td>
<td>172</td>
<td>172</td>
<td>182</td>
<td>182</td>
<td>180</td>
<td>183</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ added, g</td>
<td>0</td>
<td>24.4</td>
<td>12.2</td>
<td>12.2</td>
<td>11.1</td>
<td>11.6</td>
<td>11.9</td>
<td>0</td>
</tr>
</tbody>
</table>

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230 ml. Flow rate was 0.44 ml/min. The dead volume was determined by use of Blue Dextran 2000 (Pharmacia Inc., Piscataway, N.J. 08854) to be 110 ml.

The eluate from the column was monitored continuously at 280 nm for its protein content. The protein content in the major fractions was determined by the biuret reaction: 1 ml of eluate plus 1 ml of biuret reagent was read vs. a blank of 1 ml of buffer plus 1 ml of biuret reagent, and a set of standards prepared with bovine serum albumin. The enzymatic activity of each fraction was assayed as described below.

Assay Procedures

**Measurement of canaline carbamoyltransferase activity (6-12):** The final volume in each reaction mixture was 300 µl: 100 µl of ornithine or canaline solution, 100 µl of carbamoyl phosphate solution, 50 µl of human liver extract, and 50 µl of the 0.2 mol/liter triethanolamine buffer. When inhibitors were used to determine inhibitor constants (K_i) they were dissolved in the 50 µl of buffer. Blanks, containing all components except enzyme, were also incubated with the reaction mixtures. Incubation was for 5 min at 37 °C except where otherwise indicated. For determination of Michaelis constants (K_m), the concentration of ornithine, canaline, or carbamoyl phosphate solutions was varied. The carbamoyl phosphate solution was kept in an ice bath and was added immediately before the reaction was started by adding the human liver extract. The reaction was stopped by adding 250 µl of perchloric acid (1 mol/liter) and immersing the reaction tube in ice. The reaction mixtures were centrifuged and the supernate was removed. The reaction rate was followed by measuring the rate at which citrulline or ureidohomoserine were produced.

Citrulline was assayed by using the above-described citrulline reagent (9). Aliquots (50-250 µl) of the centrifuged, perchloric acid-treated reaction mixtures were used. One milliliter of the citrulline reagent was added, followed by 250 µl of the 10 g/liter discetyl monoxide solution. The reaction mixture was mixed, heated at 100 °C for 20 min, cooled in an ice bath in the dark for 10 min, and the absorbance measured at 460 nm.

Ureidohomoserine was determined by using the ureidohomoserine reagent (6). Aliquots (250-500 µl) of the centrifuged, perchloric acid-treated reaction mixtures were mixed with 500 µl of the reagent, and 250 µl of concentrated sulfuric acid was added. The reaction mixtures were mixed, heated at 100 °C for 3 min, cooled in an ice bath for 10 min, and the absorbance was measured at 542 nm within 45 min.

**Results**

Figure 2 shows the pH–activity curves for human liver extract and a highly purified ornithine carbamoyltransferase from *Streptococcus faecalis* when ornithine or canaline is used as substrate.

With ornithine as a substrate (Figure 2A), a broad peak with a maximum at pH 7.6 is shown for the human liver extract. For the bacterial enzyme the optimum pH is at 8.5. With canaline as the substrate (Figure 2B), the optimum pH for the human liver extract is at 7.9 and for the bacterial enzyme at 8.4.

In subsequent experiments comparing the ornithine and canaline carbamoyltransferase activities of human liver extract, we chose a pH of 7.8 as a compromise because it was desirable to use the same pH when comparing activities. In the case of ornithine carbamoyltransferase there is only a 3% difference in activity from the optimum pH of 7.6.

Table 2 lists the Michaelis–Menten constants for the purified enzyme preparation from human liver. Where ornithine and canaline were varied, the carbamoyl phosphate concentration was held constant at 1.0 and 1.5 mmol/liter. Results for a typical experiment are shown for canaline in Figure 3. When ornithine was held constant and carbamoyl phosphate was varied, the ornithine concentration was held at 0.5 and 0.25 mmol/liter. Figure 4 shows a typical curve for canaline where canaline was held constant at 10 mmol/liter and carbamoyl phosphate was varied.

Figure 5 shows the relative reaction rates for ornithine carbamoyltransferase and canaline carbamoyltransferase, for the purified human liver enzyme preparation from the Sephadex column. The protein concentration was 192 mg/liter, canaline and ornithine each were 5 mmol/liter, and carbamoyl phosphate was 0.5 mmol/liter in the experiment illustrated.

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**Table 2. Comparison of Michaelis Constants (K_m) for Human Liver, with Canaline and Ornithine as Substrates**

<table>
<thead>
<tr>
<th>Substrate being varied</th>
<th>Ornithine</th>
<th>Canaline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbamoyl phosphate (ornithine)</td>
<td>0.35</td>
<td>4.50</td>
</tr>
<tr>
<td>Carbamoyl phosphate (canaline)</td>
<td>0.54</td>
<td>0.035</td>
</tr>
</tbody>
</table>

*When carbamoyl phosphate concentration varied the reaction was set up with ornithine or canaline concentration as constant.*
Figure 3. Curves demonstrating the Michaelis constant for canaline carbamoyltransferase activity with purified human liver extract
Carbamoyl phosphate concentration at 1.0 and 1.5 mmol/liter

Table 3 summarizes the data for fractions of the initial liver extract precipitated by various concentrations of ammonium sulfate. Most of the activity was obtained with the fraction precipitating between 60 and 70% saturation with ammonium sulfate. About half of the ornithine transferase and 24% of the canaline transferase activity was lost during the fractionation, but the specific activity was increased by 41 and 126%, respectively, in the process.

The fractionation of the 60-70% ammonium sulfate fraction on the Sephadex column is shown in Figure 6. Two major peaks were obtained when protein was measured; most of the protein in the fraction is inert, as demonstrated by the activity curves. Canaline and ornithine carbamoyltransferases were not clearly resolved.

Table 4 lists the effect of certain substances on the initial reaction velocities of the activity of canaline carbamoyltransferase. It will be noted that ornithine does not greatly inhibit this activity (only by 13%). However, the product of the reaction, ureidohomoserine, is a strong inhibitor (72%).

Parallel experiments performed with ornithine as the substrate indicated that citrulline inhibits ornithine carbamoyltransferase activity by 87%, while canaline inhibits the ornithine system by only 15%. Thus in both cases the product of the reaction has a greater effect than does the competing substrate. Note that citrulline will inhibit the canaline system by 25%. With the ornithine system, ureidohomoserine produced 46% inhibition. Thus both citrulline and ureidohomoserine can compete with either substrate for the active site.

Figure 7A shows graphically the strong competitive inhibiting action of ureidohomoserine on the canaline carbamoyltransferase system. To obtain inhibition of the ornithine carbamoyltransferase system by canaline (Figure 7B), a more than 10-fold higher concentration is required. The $K_i$ for ureidohomoserine is 0.65, as compared to 11.2 for canaline.

**Discussion**

As can be seen from Figure 1 and the references, all the steps in the guanidino cycle have now been documented as taking place with human liver enzyme preparations (1-5). Figure 1 illustrates the alternative
production of guanidinoacetate or guanidinosuccinate. It also indicates that canaline is the key substance that cycles, carrying an aspartate molecule, stripping it of ammonia and generating fumarate. It is apparent that for this cycle to continue, any canaline lost by attrition needs to be replaced. Its position in this cycle is analogous to that of oxaloacetate in the urea cycle.

We postulate that the canaline originates from aspartate. This suggestion is based on the observation that there is no transamidination to aspartate, and that radioactive aspartate, administered to humans, will give rise to some (because aspartate can follow other metabolic pathways, it would be expected to be little) radioactive guanidinosuccinate in the urine. Thus the

Table 3. Fractionation of Human Liver Carbamoyltransferase with Ammonium Sulfate

<table>
<thead>
<tr>
<th>Ammonium sulfate fraction</th>
<th>% Activity</th>
<th>Total protein, mg</th>
<th>Total acy., umol/min</th>
<th>Specific acy., nmol/mg protein per min</th>
<th>Specific acy., ratio (Orn/Can)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Orinithine</td>
<td>Canallne</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>100</td>
<td>100</td>
<td>4087</td>
<td>1560.8</td>
<td>381.9</td>
</tr>
<tr>
<td>25% ppt. b</td>
<td>0.30</td>
<td>0.30</td>
<td>91</td>
<td>5.5</td>
<td>60.0</td>
</tr>
<tr>
<td>40% ppt.</td>
<td>1.20</td>
<td>2.20</td>
<td>623</td>
<td>19.9</td>
<td>31.9</td>
</tr>
<tr>
<td>50% ppt.</td>
<td>2.60</td>
<td>4.90</td>
<td>825</td>
<td>41.3</td>
<td>50.1</td>
</tr>
<tr>
<td>60% ppt.</td>
<td>7.40</td>
<td>8.20</td>
<td>880</td>
<td>116.0</td>
<td>131.8</td>
</tr>
<tr>
<td>70% ppt.</td>
<td>32.6</td>
<td>52.4</td>
<td>948</td>
<td>509.3</td>
<td>537.2</td>
</tr>
<tr>
<td>80% ppt.</td>
<td>6.0</td>
<td>8.2</td>
<td>356</td>
<td>93.4</td>
<td>262.3</td>
</tr>
<tr>
<td>Final supernate</td>
<td>trace</td>
<td>trace</td>
<td>10</td>
<td>trace</td>
<td>trace</td>
</tr>
<tr>
<td>Total</td>
<td>50.3</td>
<td>76.4</td>
<td>3733</td>
<td>785.3</td>
<td>286.3</td>
</tr>
</tbody>
</table>

* The ratio of the specific activity of the various fractions for ornithine/canaline is shown in the last column. The original weight of the acetone powder was 15 g.

b Precipitate at percentage saturation shown.
reaction forming guanidinosuccinate could be formulated as follows:

\[ 2 \text{ aspartate} + \text{carbamoyl phosphate} \rightarrow \text{guanidinosuccinate} + \text{fumarate} \]

A question that needs answering is whether the enzyme catalyzing the formation of ureidohomoserine from canaline and carbamoyl phosphate is the same enzyme that catalyzes the formation of citrulline. Figure 2 demonstrates that the optimum pH for citrulline formation is 7.6, for ureidohomoserine, 7.9. These figures are too close to suggest that we are dealing with two different enzymes.

The affinity of canaline for the enzyme is substantially less than that for ornithine. This can be seen in Table 2, where the \( K_m \) with canaline as the substrate is over 10-fold that when ornithine is the substrate. Once the substrates are bound, the canaline complex has a stronger affinity for carbamoyl phosphate. Figure 5 shows that under the same conditions citrulline is formed 2.6 times more rapidly than ureidohomoserine.

Attempts to separate the two activities by ammonium sulfate fractionation were inconclusive. From Table 3 it is apparent that most of the activity in both cases is associated with the precipitate formed at 70% saturation with ammonium sulfate. However, the ratio of specific activity of the two activities in this fraction (2.6) was only a little more than half that noted in the initial and 60%-saturation fraction. This indicates that the 70% precipitate was more effective than the 60% or initial fraction in forming ureidohomoserine, as compared to citrulline. This could indicate that more than one enzyme is present, that an inhibitor selective for ureidohomoserine formation has been removed, or both.

Fractionation on Sephadex did not resolve the two activities. It is apparent from Figure 6 that there are at least five overlapping peaks in the ornithine carbamoyltransferase activity curve, each showing some activity. The peak with most of the ornithine carbamoyltransferase activity, at 170 ml, showed the molecular weight to be about 120 000, as calculated from reference compounds run on the same column. The major activity peak for both canaline and ornithine are at this location. It is interesting to note that, in Escherichia coli, the molecular weight of the enzyme corresponding to our human liver preparation has been shown to be 105 000 (13).

We also studied the effect of heat, to see whether we could destroy one of the activities without the other. At 55 °C the denaturation of both enzyme activities followed a similar course so that at 25 min 61% of the ornithine carbamoyltransferase activity and 54% of the

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**Table 4. Effects of Certain Compounds on the Initial Velocities of the Formation of Ureidohomoserine**

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Conc, mmol/liter</th>
<th>Acty, ( \mu \text{mol} / \text{liter per min} )</th>
<th>Inhibition, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>46.3</td>
<td>—</td>
</tr>
<tr>
<td>Ureidohomoserine</td>
<td>1.2</td>
<td>13.0</td>
<td>72</td>
</tr>
<tr>
<td>Citrulline</td>
<td>1.2</td>
<td>34.9</td>
<td>25</td>
</tr>
<tr>
<td>Inorganic phosphate</td>
<td>1.2</td>
<td>38.7</td>
<td>16</td>
</tr>
<tr>
<td>Ornithine</td>
<td>1.2</td>
<td>40.3</td>
<td>13</td>
</tr>
</tbody>
</table>

* Canaline was the substrate; its concentration was 1.2 mmol/liter; carbamoyl phosphate concentration was 5.6 mmol/liter.
canaline carbamoyltransferase activity had been destroyed.

With both enzymes the products of the reaction, citrulline and ureidohomoerine, are strong competitive inhibitors (Figure 7A), and it appears that both products have a strong affinity for the active site. It would be expected that canaline and ornithine would inhibit the action of the enzymes on each other, but this is not the case, except at high concentration (Figure 7B). This would support the argument that the same site is not being occupied by these two substrates. This requires further study.

Our studies on the cycle shown in Figure 1 are now complete, but they bring up the question of how canaline is formed initially. No mechanism, either in plants or animals, has been demonstrated for its formation. It requires replacement of the carboxyl group of aspartate by the —CH2—O—NH2 linkage. The demonstration of this reaction would place canaline as a significant intermediate in human metabolism.

Canavanine will transamidinate to ornithine to produce arginine and canaline (14). The reaction is irreversible. Thus the cycle shown in Figure 1 interlocks the urea cycle.

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References

Addendum
In a recent report it was claimed that guanidinosuccinate is formed by transamidination from arginine to aspartate, by either the perfused rat liver or the intact rat. The guanidinosuccinate was not characterized, except by its position on elution from a column (15). This overlapped the position of creatine. It is significant that the peak was obtained in the absence of aspartate, and that the peak would not form from canavanine, which transamidinates ornithine to form arginine. These phenomena need to be explained.