Purification and Properties of Rat Liver Ornithine δ-Transaminase*

HAROLD J. STRECKER

From The Department of Biochemistry, Albert Einstein College of Medicine, Yeshiva University, New York 61, New York

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Ornithine δ-transaminase has been reported to be present in gram-positive bacteria and yeasts, in fungi, green algae, protozoa, higher plants, and animals (1, 2). Some properties of the enzyme have been described for preparations obtained from Neurospora crassa (3, 4), and from rat liver (5, 6). The enzyme from N. crassa has been reported to catalyze the reaction

\[ \text{Ornithine} + \alpha\text{-ketoglutarate} \rightarrow \text{glutamic } \gamma\text{-semialdehyde} + \text{glutamate} \] (1)

Pyruvate, oxaloacetate, and β-ketoglutarate were reported to be inactive (4). In contrast, preparations from rat liver were found to be most active with pyruvate, but α-ketoglutarate, glyoxylate, and a number of other α-keto acids also were reactive at appreciable rates (5, 6). The equilibrium for Reaction 1 was shown to be very far towards the right, presumably because of spontaneous conversion of glutamic γ-semialdehyde to the cyclized form, Δ⁰-pyrroline-5-carboxylate (6, 7). In spite of the difficulty in demonstrating reversibility of Reaction 1, it has been assumed generally that this reaction is one step in the metabolism of proline to ornithine (8–10).

As part of an ongoing study of the interconversion of glutamic acid, proline, and ornithine, rat liver ornithine δ-transaminase has been purified partially and some of its properties examined. These studies were facilitated by the previously reported rapid and convenient method of determination of one of the products of reaction, namely glutamic γ-semialdehyde (7, 11). During the course of this work a report appeared describing the purification of what appears to be the same enzyme from the same tissue source (12). However, there seem to be some differences between our own findings on ornithine δ-transaminase and those reported in the literature (5, 6, 12). These findings and some attempts to explain these apparent discrepancies are presented in this communication.

**EXPERIMENTAL PROCEDURE**

**Enzyme Assay**—As discussed in the text, both substrates and products of the reaction are inhibitors. At the comparatively low, noninhibitory concentration of α-ketoglutarate used in the assay, 20-minute incubations were sufficient to obtain linearity with respect to time, or enzyme concentration corresponding to an activity of at least 3.6 units of enzyme. A unit of enzyme is defined as that amount of enzyme which catalyzes the formation of 1 μmole of Δ⁰-pyrroline-5-carboxylic acid (= glutamic γ-semialdehyde, cf. Strecker (7)) per hour. The standard incubation mixture contained 70 μmoles of L-ornithine, 7.5 μmoles of potassium α-ketoglutarate, and 100 μmoles of potassium phosphate, pH 7.1, in a total volume of 2 ml. When glyoxylate was the substrate, 4 mg of EDTA were added to inhibit the metal-catalyzed, nonenzymatic reaction between ornithine and glyoxylate (13, 14). The enzyme was added last, and the mixture was incubated with shaking at 37°C for 20 minutes, except where otherwise stated. The pyrroline-5-carboxylate formed was determined by reaction with α-amino-naphthalene-2-sulfonylhydrazide as previously described. The absorbance was determined at 443 mμ, in a 1-cm light path, with the aid of the Zeiss model PMQ spectrophotometer. For calculation of the pyrroline-5-carboxylate concentration, the millimolar extinction coefficient of 2.71 was used (see text).

Protein concentration was determined by the method of Lowry et al. (15). When glutamic acid and ornithine were determined, the 2-ml incubation mixture was deproteinized by adding 0.5 ml of 20% perchloric acid. After centrifugation to remove the protein, the perchloric acid was removed by neutralization with KOH, followed by sedimentation of the potassium perchlorate. For determination of glutamic acid, the supernatant solution was passed into a column of Dowex I-acetate (1 × 11 cm), which was then washed with 25 ml of water to remove ornithine and Δ⁰-pyrroline-5-carboxylic acid. The glutamic acid was eluted with 25 ml of 0.5 M acetic acid, and the concentration determined with the ninhydrin reaction according to Rose (16). For ornithine determination, the supernatant solution, after perchlorate removal, was passed into a column (1 × 5 cm) of Dowex 50-X8 (NH₄⁺). The column was then eluted with 30 ml of 0.1 M ammonium formate to remove the Δ⁰-pyrroline-5-carboxylate. Ornithine was eluted with 30 ml of 0.3 M ammonium formate and determined by the method of Chinard (17). α-Ketoglutarate was determined on the deproteinized supernatant by reaction with 2,4-dinitrophenylhydrazine (18). Interference by Δ⁰-pyrroline-5-carboxylate in this reaction was eliminated by addition of KCN (1.25 × 10⁻³ M) to the solution before addition of the 2,4-dinitrophenylhydrazine (19).

All solutions were prepared in glass-distilled water. Commercial preparations of L-ornithine were found to be impure and were purified by ion exchange chromatography as described.

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† The abbreviation used is: pyrroline-5-carboxylate, Δ⁰-pyrroline-5-carboxylic acid.
above. All other reagents were of the highest purity commercially available.

RESULTS

Preparation of Enzyme—All procedures were performed at 4-7°C. Subcellular fractionation of homogenates of rat liver prepared in 0.25M sucrose solution indicated that more than 50% of the ornithine δ-transaminase activity was bound to the fraction sedimented between 1,000 × g and 10,000 × g, for 10 minutes; this fraction also had the highest specific activity. The same fraction also was highest in specific activity of succinic-cytochrome c reductase and of cytochrome c oxidase and thus was tentatively concluded to be mitochondrial in agreement with the findings of Peraino and Pitot (12). In order to obtain maximum yield of enzyme, however, the total homogenate was centrifuged at about 40,000 × g for 30 minutes, and the residue was resuspended to the original volume in 0.05M potassium phosphate solution, pH 7.4. The suspension was centrifuged as before, and the sediment once again resuspended in 0.05M potassium phosphate solution, pH 7.4. This suspension was homogenized in the Waring Blender at one-half maximum speed for 1 minute, pyridoxal phosphate was added to a final concentration of 4 μg per ml, and the mixture quickly frozen in a Dry-Ice-acetone bath. The frozen suspension was stored overnight at -15°C, thawed, and then centrifuged at 18,000 × g for 30 minutes. The supernatant contained 50 to 85% of the activity initially present.

(NH₄)₂SO₄, 0.3 g per ml, was added slowly with stirring. The suspension was centrifuged at about 40,000 × g for 30 minutes. The precipitate thus obtained was dissolved in 0.1 of the previous volume of 0.005M potassium phosphate solution, pH 7 to 7.4, containing 4 μg per ml of pyridoxal phosphate. This protein solution was dialyzed against 25 volumes of the same buffer solution containing 4 μg per ml of pyridoxal phosphate. The dialyzing solution was replaced every hour for 4 hours. A precipitate which formed occasionally was removed by centrifugation as above. A preparation made from 30g of rat liver, containing at this stage about 400 to 500 mg of protein, was passed through a column (2 × 15 cm) of DEAE-cellulose. The protein solution was washed into the column with small portions of 0.005M potassium phosphate solution, pH 7.5, and then eluted with a phosphate buffer gradient with the use of a two-chamber mixing device. The first chamber contained 150 ml of 0.05M potassium phosphate solution, pH 7.6, and the second contained 150 ml of 0.5M potassium phosphate solution, pH 7.6. Each solution also contained 4 μg per ml of pyridoxal phosphate. The effluent was collected at a flow rate of about 0.4 ml per minute in 4-ml portions. The peak of activity was obtained at a concentration of about 0.15M potassium phosphate.

In some preparations, two peaks of activity were observed.

Table I

Preparation of ornithine δ-transaminase

<table>
<thead>
<tr>
<th>Step</th>
<th>Total</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units</td>
<td>units/mg</td>
</tr>
<tr>
<td>Sucrose homogenate</td>
<td>3190</td>
<td>0.4</td>
</tr>
<tr>
<td>Buffer extract</td>
<td>1580</td>
<td>1.7</td>
</tr>
<tr>
<td>Ammonium sulfate fraction</td>
<td>1280</td>
<td>2.6</td>
</tr>
<tr>
<td>DEAE-cellulose eluate</td>
<td>550</td>
<td>12.6</td>
</tr>
</tbody>
</table>

The first peak, appearing after about 50 ml of eluate had been collected, was relatively minor, but showed activity and substrate specificity similar to that of the major peak appearing after about 120 ml of eluate had been collected. Investigation of the conditions which resulted in the two peaks of activity showed that the first material was produced apparently during storage of the ammonium sulfate fraction at -15°C. Preparations which were carried through all the stages of purification without delay showed only one chromatographic peak with activity corresponding to the second (major) peak. The contents of the tubes containing the enzyme were combined, frozen, and lyophilized. The dry residue was dissolved in the smallest possible volume of 0.005M potassium phosphate solution, pH 7.4, containing 4 μg per ml of pyridoxal phosphate and then dialyzed against a liter of the same solution for 4 hours, replacing the solution every hour. The dialyzed product was used for most of the experiments to be reported. Table I presents data for a purification of the enzyme from 30 g of rat liver. The over-all yield for this preparation was about 17% and represented a 30-fold purification. However, yields of up to 50% with specific activities up to 25 have been obtained.

Stability of Enzyme—Ornithine δ-transaminase activity in homogenates of rat liver made in sucrose or phosphate solution was stable for some weeks at -15°C. The activity in the soluble extract, however, decreased during storage at -15°C, so that in 6 days less than half of the initial activity remained. Pyridoxal phosphate, at a concentration of 4 μg per ml, preserved activity almost fully if added initially and restored activity if added to the partially inactive extract within the 6-day period. Storage for periods longer than a week at -15°C, in the absence of pyridoxal phosphate, resulted in a progressive irreversible loss of activity. EDTA, glutathione, α-ketoglutarate, or anaerobiosis had no preservative or restorative ability. The addition of ornithine to enzyme solutions in the absence of pyridoxal phosphate accelerated the decrease of activity. The presence of pyridoxal phosphate for maintenance of activity became more indispensable as the enzyme was purified. For this reason each of the purification steps and dialyses were conducted with added pyridoxal phosphate.

Substrate Specificity—Quastel and Witty (5) reported that mammalian and avian liver and kidney preparations catalyzed transamination from ornithine to pyruvate, and to a lesser extent to α-ketoglutarate or oxaloacetate. Using a partially purified preparation from rat liver, Meister found that ornithine transaminated with a number of α-keto acids including pyruvate, glyoxylate, α-ketoglutarate, α-ketobutyrate, α-ketoisocaproate, and α-ketoisovalerate. The rate of reaction was fastest with pyruvate and was slower with the other α-keto acids in the order listed (6). Peraino and Pitot purified ornithine δ-transaminase 7-fold from rat liver mitochondria and stated that this preparation showed specificity similar to that of the Neurospora enzyme studied by Vogel and Kopac (4), i.e. α-ketoglutarate acted as acceptor, whereas pyruvate and α-ketobutyrate were inactive (12).

The partially purified transaminase described in this report catalyzed transamination from ornithine to α-ketoglutarate, glyoxylate, and pyruvate. α-Ketocaprate, α-ketovalerate, α-ketobutyrate, α-ketoisocaproate, α-ketoisovalerate, and oxaloacetate were all inactive as amino group acceptors. Lysine, cadaverine, and putrescine were tested as amino group donors with α-ketoglutarate as acceptor. For these experiments, ac-
activity was determined by reaction with o-aminobenzaldehyde since the product of deamination of each of these amines is known to react with the o-aminobenzaldehyde reagent to form a colored product. No evidence of amino group transfer from these amines was obtained. n-Ornithine was also tested for activity by incubating limiting concentrations of n-ornithine with a-ketoglutarate and sufficiently high concentrations of enzyme to ensure that the reaction could go to completion. Δ-Δ-Pyrroline-5-carboxylate was determined to be formed equivalent to half of the n-ornithine originally added, indicating that n-ornithine was not a substrate for the reaction.

When the relative rate of reaction with each of the three active acceptor a-keto acids was determined, each was found to inhibit the reaction as its concentration was increased. Study of the reaction with glyoxylate was complicated by an appreciable nonenzymatic reaction between it and ornithine which has been reported previously (14). However, the interference by this nonenzymatic reaction was almost abolished by the addition of EDTA to the incubation mixture. EDTA had no effect on the enzymatic catalysis. Double reciprocal plots (20) of the rates of reaction with each of the active acceptor keto acids at a noninhibitory concentration of ornithine are shown in Figs. 1 to 3. Extrapolation of the linear portion of the curves to the ordinate provided Vmax data, which indicated that the rates were faster with a-ketoglutarate or glyoxylate than with pyruvate. The optimum concentration for a-ketoglutarate was considerably lower than for either of the other acceptors. The Vmax and Km values, the optimum concentrations of acceptors just short of the concentration causing inhibition, and the relative rates at these optimum concentrations are shown in Table II.

With each of the active acceptors, ornithine also was found to reach an inhibitory concentration. Double reciprocal plots of the rates of reaction versus ornithine concentration at constant concentrations of a-ketoglutarate, glyoxylate, and pyruvate were made as for Figs. 1 to 3. The concentrations of acceptor used were about optimum. The apparent Km values and the optimum concentration of ornithine with each acceptor present at or near optimum concentration are shown in Table III.

Stoichiometry and Equilibrium Constant—In the presence of an excess of enzyme, 52.5 μmoles of ornithine and 7.5 μmoles of a-ketoglutarate were incubated, and the accumulation of Δ-pyrroline-5-carboxylate acid was determined with time by means
of the reaction of aliquots with o-aminobenzaldehyde. Under the conditions used, the amount of product formed reached a constant value after 90 minutes and remained at this level. The addition, at this time, of 100 μmoles of the second product of the reaction, L-glutamate, did not cause disappearance of any pyrroline-5-carboxylate. In another experiment, L-glutamate was added at the beginning of the incubation and the amount of pyrroline-5-carboxylate formed in this experiment was the same as in the previous experiment. In a third experiment, 15 μmoles of α-ketoglutarate were incubated with 7.5 μmoles of ornithine. Again the amount of pyrroline-5-carboxylate accumulated was the same. In a fourth experiment, L-amino-benzaldehyde was added in excess at the beginning of the incubation in order to pull the reaction to completion. The amount of product formed was the same. These results indicated that the reaction was very much in the direction of Δ-pyrroline-5-carboxylate formation as previously reported (6). This was further confirmed by determination of glutamic acid formed in another experiment with ornithine and α-ketoglutarate as substrates. Within the limitations of the methods, 7.45 μmoles of glutamic acid were found when 7.5 μmoles of α-ketoglutarate and an excess of ornithine were used. On the assumption that 7.5 μmoles of pyrroline-5-carboxylate were also formed, the millimolar extinction coefficient of the α-aminobenzaldehyde-DL-pyrroline-5-carboxylic acid reaction product was calculated to be 2.71. In previous work, the millimolar extinction coefficient of the reaction product of α-aminobenzaldehyde with synthetic pyrroline-5-carboxylate has been reported to be 2.14 (7). However, reduction of the synthetic compound to proline (21) or oxidation to glutamic acid (22) indicated that it was not more than 80% pure. The present results confirm this estimation of purity.

In order to obtain an equilibrium constant for the reaction, we measured the formation of products from the reverse direction. Glutamic acid and pyrroline-5-carboxylate were incubated with sufficient quantities of the purified enzyme to attain equilibrium in a reasonable time. The results of these experiments are presented in Table IV. As noted, an average equilibrium constant of 71 was obtained for the reaction.

<table>
<thead>
<tr>
<th>Substrates added</th>
<th>Products formed</th>
<th>K (calculated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-glutamate</td>
<td>DL-Pyrroline 5-carboxylate</td>
<td>Ornithine</td>
</tr>
<tr>
<td>100 μmoles/ml</td>
<td>6.05</td>
<td>1.51</td>
</tr>
<tr>
<td>50 μmoles/ml</td>
<td>6.9</td>
<td>1.29</td>
</tr>
<tr>
<td>25 μmoles/ml</td>
<td>0.4</td>
<td>0.84</td>
</tr>
</tbody>
</table>

Calculation of the equilibrium constant was made by considering the concentration of ornithine formed to be the same as that of the determined α-ketoglutarate, since the estimation of the latter compound was considered to be more reliable. Since the synthetic Δ-pyrroline-5-carboxylate was a racemic mixture, the assumption was made that the d form was not used as a substrate. The concentration of Δ-pyrroline-5-carboxylate remaining at the end of the incubation period was calculated by subtracting the amount of product formed from the amount added initially. Because Δ-pyrroline-5-carboxylate undergoes a small degree of spontaneous and variable polymerization during the course of incubation, some error is unavoidable in the calculation. If it were assumed that the d and l forms disappeared nonenzymatically at equal rates (which is a gross and limiting assumption since the l form also was being utilized enzymatically) the equilibrium constant was calculated to be about 50. Thus, it appears that the correct value for the equilibrium constant lies between 50 and 70. A value in this range satisfactorily accounts for the completeness of the reaction in the forward direction as determined by the methods used, and specifically for the almost complete conversion of 7.5 μmoles of α-ketoglutarate to glutamate, when incubated with 52.5 μmoles of ornithine. The standard free energy change for the above reaction at 25° was calculated to be approximately -2600 calories.

Inhibitors—A number of structurally related compounds were tested for inhibition of the reaction between ornithine and α-ketoglutarate. Table V lists those compounds which were inhibitory. Compounds tested at 0.025 M concentration which inhibited less than 10% included L-proline, L-arginine, DL-α-amino butyrate, ε-aminocaproate, L-citrulline, D- and L-glutamate, glycine, L-salanine, D-leucine, hydroxy-L-proline, D-valine, DL-β-amino butyrate, glutamine, and L-lysine. Representatives of each of the classes of inhibitory compounds were used for determination of the nature of inhibition. Double reciprocal plots were made of the rates of reaction versus the concentration of either ornithine or α-ketoglutarate with and without inhibitor added. Three kinds of plots were obtained. Strictly competitive inhibition was seen only with canavanine against ornithine as shown in Fig. 4. A second kind of plot in which V_{max} de-
FIG. 4 (left). Plot of reciprocal velocity against reciprocal ornithine concentration with canavanine added. The concentration of α-ketoglutarate was 3.7 mM and that of canavanine 5 mM. All other conditions were as for Fig. 1.

FIG. 5 (right). Plot of reciprocal velocity against reciprocal ornithine concentration with norvaline added. The concentration of norvaline was 50 mM. All other conditions were the same as for Fig. 1.

FIG. 6. Plot of reciprocal velocity against reciprocal α-ketovalerate concentration with α-ketovalerate added. The concentration of ornithine was 35 mM and that of α-ketovalerate 43 mM. All other conditions were the same as for Fig. 1.

donated and for α-ketoglutarate, glyoxylate, and pyruvate as amino group acceptors. All substrates become inhibitory at some concentration. At optimum concentrations, α-ketoglutarate reacts at a rate slightly faster than that of glyoxylate, which in turn is greater than that of pyruvate. The relative order of the $V_{max}$ values is also the same. The optimum concentration for α-ketoglutarate is considerably lower than for the other two keto acids, suggesting that this compound may be the physiological substrate for ornithine δ-transaminase. At the concentrations used by Meister and by Quastel and Witty, α-ketoglutarate inhibits strongly.

Peraino and Pitot (12) found pyruvate and α-ketoisobutyrate to be inactive with a partially purified ornithine δ-transaminase from rat liver. Pyruvate in solution is unstable and forms other products (cf. Montgomery and Webb (23)). In our laboratory it was found necessary to prepare the pyruvate solutions fresh daily, since solutions kept in an ice bath for several hours, frozen overnight, were quite inhibitory when tested afterwards. Peraino and Pitot do not indicate the method or preparation of pyruvate and its solutions as employed in their experiments. The partially purified enzyme described here was inactive with α-ketoisobutyrate as well as with other keto acids. Indeed, these keto acids were inhibitory. It is possible that the activity with keto acids reported by Meister is indicative of the presence of another enzyme. The difficulty in obtaining and maintaining pure preparations of these keto acids makes it necessary to leave this question open for the present.

Peraino and Pitot reported that rat liver homogenates contained sufficient ornithine δ-transaminase to catalyze the formation of 51 to 73 μmoles of Δγ-pyrrolidine-5-carboxylate per g of fresh tissue per hour, and also found rat kidney to be about 70% more active than liver. Since these investigators used a millimolar extinction coefficient of 2.45 for the reaction product with α-aminobenzaldehyde, as compared with the value of 2.71 reported here, their value for transaminase activity should be corrected to 46 to 66 μmoles per g per hour. Under the conditions described here, rat liver homogenates catalyzed the formation of 67 to 107 μmoles per g per hour of Δγ-pyrrolidine-5-carboxylate. The lower values obtained by Peraino and Pitot probably are a consequence of the inhibitory concentrations of

**DISCUSSION**

Quastel and Witty (5), as well as Meister (6), reported pyruvate to be most active as acceptor for transamination with ornithine. The latter investigator found a number of α-keto acids to be active, including glyoxylate and α-ketoglutarate. The data reported here indicate specificity for ornithine as amino group donor and for α-ketoglutarate, glyoxylate, and pyruvate as amino group acceptors. All substrates become inhibitory at some concentration. At optimum concentrations, α-ketoglutarate reacts at a rate slightly faster than that of glyoxylate, which in turn is greater than that of pyruvate. The relative order of the $V_{max}$ values is also the same. The optimum concentration for α-ketoglutarate is considerably lower than for the other two keto acids, suggesting that this compound may be the physiological substrate for ornithine δ-transaminase. At the concentrations used by Meister and by Quastel and Witty, α-ketoglutarate inhibits strongly.

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ornithine and \( \alpha \)-ketoglutarate used in their experiments. Using preparations of specific activity which they reported as 1.7 \( \mu \)moles per mg per hour, these investigators calculated apparent \( K_m \) values for ornithine and \( \alpha \)-ketoglutarate of 7.2 mM and 1.1 mM, respectively. Our purified preparations of specific activity 12 to 13 \( \mu \)moles per mg per hour were used to obtain apparent \( K_m \) values of 2.8 mM and 0.28 mM, respectively, for ornithine and \( \alpha \)-ketoglutarate.

The determination of the equilibrium constant of the transamination between ornithine and \( \alpha \)-ketoglutarate, although subject to some experimental difficulties, yielded results which demonstrate the equilibrium to be considerably in the direction of pyrroline 5-carboxylic acid formation as has been reported previously (6). The data obtained here indicate the standard free energy of the reaction to be about \(-2500\) calories at \(25^\circ\). This negative value for the free energy change may indicate that this enzyme is concerned only with the catabolism of ornithine and permits proposals for the role of this enzyme in the known conversion of ornithine to glutamic acid and to proline in the animal (24). The formation of glutamic acid could be accomplished readily by coupling of ornithine \( \delta \)-transaminase to \( \Delta^1 \)-pyrroline-5-carboxylic acid dehydrogenase (22) viz.

\[ \text{Ornithine} + \alpha \text{-ketoglutarate} \rightleftharpoons \Delta^1 \text{-pyrroline-5-carboxylic acid} + \text{glutamate} \]

The determination of the equilibrium constant of the transamination between ornithine and \( \alpha \)-ketoglutarate permits proposals for the role of this enzyme in the known conversion of ornithine to glutamic acid and to proline in the animal (24). It is known, however, that ornithine can be synthesized from both glutamic acid and proline. Recent work from our laboratory provides some evidence for ornithine biosynthesis from proline in rat kidney by a pathway which is stimulated by aspartic acid (27). In Escherichia coli and other gram-negative organisms, ornithine biosynthesis has been shown to proceed via acetylated intermediates (28). The reported absence of ornithine \( \delta \)-transaminase in gram-negative organisms (1) leaves open the question of the mechanism of the conversion of ornithine to proline known to occur in these cells.

\section*{SUMMARY}

Ornithine \( \delta \)-transaminase was isolated from rat liver and purified about 30-fold. Activity of the purified enzyme was preserved by the addition of pyridoxal phosphate. The enzyme catalyzes the transamination of the terminal amino group of L-ornithine to \( \alpha \)-ketoglutarate, glyoxylate, or pyruvate. A number of amino acids and \( \alpha \)-keto acids tested for donor and acceptor specificity were not utilized. Each of the four active substrates became inhibitory at higher concentrations. Some preliminary kinetic data were obtained. The equilibrium constant for the reaction between ornithine and \( \alpha \)-ketoglutarate was found to be about 70. Several amino acids and keto acids, structurally related to the substrates, were inhibitory to the reaction. Both competitive and mixed inhibition were observed in the reactions studied.

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Purification and Properties of Rat Liver Ornithine δ-Transaminase
Harold J. Strecker


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