Kinetics of Beef Heart Glutamic-Alanine Transaminase*

**BERNARD BULOS AND PHILIP HANDLER**

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(Received for publication, December 21, 1964)

It has now been clearly established that pyridoxal phosphate serves as the coenzyme of many transaminases. Several lines of evidence have indicated that the coenzyme acts alternately as acceptor and donor of amino groups, shuttling between the aldehyde (pyridoxal phosphate) and amino (pyridoxamine phosphate) forms (2-11). However, a detailed study of the kinetics of the transaminase reaction which might corroborate this concept has been lacking. A kinetic treatment of such shuttle or "ping-pong" mechanisms has been provided by Cleland (12). With this aim in mind, glutamic-alanine transaminase, EC 2.6.1.1) isolated from pig heart, and shuttle or "ping-pong" mechanisms has been provided by Cleland (12). With this aim in mind, glutamic-alanine transaminase, isolated from rat liver. The purity available from commercial sources and were the L isomers except where indicated. Alanine, citrulline, glutamine, lysine, norleucine, L-α-aminobutyric acid, M-α-aminobutyric acid, and M-β-α-aminobutyric acid were obtained from Mann Research Laboratories; α-ketoglutarate from Nutritional Biochemicals; glycylglycine, aspartic acid, arginine, histidine, isoleucine, leucine, phenylalanine, serine, threonine, and valine from Sigma Chemical Company; pyridoxal phosphate and ornithine from Calbiochem.

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**EXPERIMENTAL PROCEDURE**

**Materials**—Amino acids used in this study were of the highest purity available from commercial sources and were the L isomers except where indicated. Alanine, citrulline, glutamine, lysine, norleucine, α-aminobutyric acid, M-α-aminobutyric acid, and M-β-α-aminobutyric acid were obtained from Mann Research Laboratories; α-ketoglutarate from Nutritional Biochemicals; glycylglycine, aspartic acid, arginine, histidine, isoleucine, leucine, phenylalanine, serine, threonine, and valine from Sigma Chemical Company; pyridoxal phosphate and ornithine from Calbiochem; asparagine, glycine, sodium pyruvate, glutaric acid, and malic acid from Matheson, Coleman and Bell; DPNH and 2-acetylpyridine-DPNH from P-L Laboratories.

DEAE-cellulose was obtained from Brown and Company. Hydroxylapatite was prepared by the method of Tiselius, Hjerden, and Levin (14).

Rabbit muscle lactate dehydrogenase (EC 1.1.1.27), twice recrystallized, was obtained from Worthington Biochemical Corporation. Beef liver L-glutamate dehydrogenase (EC 1.4.1.2), recrystallized three times and free of ammonium sulfate, was obtained from C. F. Boehringer und Soehne, and Calbiochem.

**Purification of Glutamic-Alanine Transaminase**—The initial steps (Fractions 1 through 4) in the purification procedure were essentially those of Green, Leloir, and Nocito (15) for the preparation of the enzyme from pig heart. All operations, unless otherwise indicated, were carried out at 4°C; glass distilled water was used throughout.

Beef heart, 500 g, trimmed of fat, blood clots, and connective tissue, was homogenized in a Waring Blender-Disintegrator for 45 sec at low speed in 2 liters of water. The suspension was filtered through a layer of fine gauze and then centrifuged at 4000 rpm for 30 min. The supernatant fluid was decanted and saved. The sediment was re-extracted with 700 ml of water and centrifuged, and the supernatant fluids were pooled (Fraction 1).

Solid ammonium sulfate (300 g per liter) was added slowly to the extract, with stirring. The precipitate was collected by centrifugation at 20,000 × g for 20 min and dissolved in 250 ml of water (Fraction 2).

Fraction 2 was then adjusted to pH 7 with 5 N NaOH and heated to 60° in a boiling water bath. When the temperature reached 60°, the solution was rapidly cooled, kept at 4° for at least 15 min, and centrifuged; the residue was discarded (supernatant Fraction 3).

Ammonium sulfate (300 g per liter) was then added to the supernatant fluid (Fraction 3). The precipitate was collected by centrifugation at 20,000 × g for 20 min, dissolved in 65 ml of water and dialyzed overnight against 5 liters of water. Any precipitate formed on dialysis was removed by centrifugation.

The dialyzed solution (Fraction 4) was then applied to a column of DEAE-cellulose (4.8 × 13.5 cm) previously equilibrated with 0.005 M potassium phosphate, pH 7.5. The column was washed with 500 ml of 0.01 M phosphate, pH 7.5, and then a linear gradient of 0.01 to 0.08 M phosphate, pH 7.5, was applied. The total volume of eluting fluid was 1 liter; the flow rate was about 1.4 ml per min, and 10-ml fractions were collected. Tubes 66 through 85, containing the major portion of activity, were pooled, and protein was concentrated by the addition of ammonium sulfate (51.6 g/100 ml). The precipitate was collected by centrifugation, dissolved in 6 ml of water, and dialyzed overnight against 1 liter of water (Fraction 5).

Fraction 5 was then applied to a column of hydroxylapatite (2.2 cm × 13 cm), previously equilibrated with 0.001 M phosphate, pH 7.5, and eluted with a linear gradient of 0.01 to 0.08 M phosphate, pH 7.5. The total volume of eluting fluid was 250 ml. The flow rate was approximately 0.8 ml per min,
Excess so that Reaction 2 did not become rate-limiting. The most highly purified fraction of the transaminase activity of the enzyme was calculated from the linear portion. Absorbance changes were followed with either a Cary model 14 spectrophotometer with the temperature controls, lacking only the transaminase, were run simultaneously.

Lactate dehydrogenase, transaminase, 15 pmoles of α-ketoglutarate, 15 mmoles of n-alanine (pH 8.0), 0.147 unit of glutamic-alanine transaminase; 0.147 unit of glutamic-alanine transaminase; 0.147 unit of glutamic-alanine transaminase; the indicated concentrations of inhibitors where used, all in a total volume of 1.0 ml. A blank, containing all components except transaminase, was run simultaneously. All of the components except α-ketoglutarate were preincubated for 10 min at 25°, and then α-ketoglutarate was added to start the reaction.

To study the reverse reaction, transamination was coupled to glutamate dehydrogenase, with use of DPNH.

The possible oxidation of DPNH by pyruvate and glutamate dehydrogenase was corrected for by comparing the rate of the reaction with DPNH; 53.8 units of lactate dehydrogenase; 0.147 unit of glutamic-alanine transaminase (Fraction 6); the indicated concentrations of l-alanine, pH 8.0; and α-ketoglutarate, pH 8.0; as well as the indicated concentrations of inhibitors where used, all in a total volume of 1.0 ml. A blank, containing all components except transaminase, was run simultaneously. All of the components except α-ketoglutarate were preincubated for 10 min at 25°, and then α-ketoglutarate was added to start the reaction.

To study the reverse reaction, transamination was coupled to glutamate dehydrogenase, with use of DPNH.

α-Ketoglutarate + DPNH + NH₄⁺ ⇄

L-glutamate + DPN⁺ + H₂O (3)

The possible oxidation of DPNH by pyruvate and glutamate dehydrogenase was corrected for by comparing the rate of DPNH disappearance in the complete system against the rate in a sample containing all components except glutamic-alanine transaminase. With most samples of glutamate dehydrogenase the change in optical density of the blank was negligible. Inhibition of Reaction 3 by glutamate itself was overcome by the use of a high concentration of ammonium ion and an excess of glutamate dehydrogenase. The complete system contained 45 μmoles of glycylglycine, pH 8.0; 0.14 μmol of DPNH; 0.6 μmol of the 3-acetylpyridine analogue of DPN⁺; 24 units of glutamate dehydrogenase; glutamic-alanine transaminase; and 15 μmoles of α-ketoglutarate, pH 8.0. The final volume was 1.0 ml; the reaction was followed at 365 mμ against a blank containing everything but transaminase. The reaction was initiated by addition of α-ketoglutarate.

Assay for Enzyme Specificity Studies—In studies with amino acids other than alanine, activity was assayed by coupling the transamination reaction to that of glutamate dehydrogenase. 

The reaction was followed by measuring the extent of reduction of the 3-acetylpyridine analogue of DPN⁺ at 365 mμ in a Zeiss PM Q II spectrophotometer (22).

The assay system contained 45 μmoles of glycylglycine, pH 8.0; 12 μmoles of the L isomer of the test amino acid, pH 8.0; 0.6 m̄μol of the 3-acetylpyridine analogue of DPN⁺; 24 units of glutamate dehydrogenase; glutamic-alanine transaminase; and 15 μmoles of α-ketoglutarate, pH 8.0. The final volume was 1.0 ml; the reaction was followed at 365 mμ against a blank containing everything but transaminase. The reaction was initiated by addition of α-ketoglutarate.

Assay for Kinetic Studies—For the forward direction, as written in Equation 1, the standard system for kinetic studies contained 45 μmoles of glycylglycine, pH 8.0; 0.14 μmol of DPNH; 53.8 units of lactate dehydrogenase; 0.147 unit of glutamic-alanine transaminase (Fraction 6); the indicated concentrations of l-alanine, pH 8.0; and α-ketoglutarate, pH 8.0; as well as the indicated concentrations of inhibitors where used, all in a total volume of 1.0 ml. A blank, containing all components except transaminase, was run simultaneously. All of the components except α-ketoglutarate were preincubated for 10 min at 25°, and then α-ketoglutarate was added to start the reaction.

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α-Ketoglutarate + DPNH + NH₄⁺ ⇄ L-glutamate + DPN⁺ + H₂O (3)

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Analysis of Kinetic Data—The various kinetic constants were all determined from the experimental data. Initial velocities determined at varying concentrations of one substrate at several fixed levels of the second substrate were sufficient for the de-
termination of Michaelis constants and maximum velocities. Apparent maximum velocity \( V'_{\text{max}} \) and apparent Michaelis constants \( K'_{\text{m}} \) were obtained from Lineweaver-Burk double reciprocal plots (24). The limiting maximum velocity \( V_{\text{max}} \) and the limiting Michaelis constant \( K_{\text{m}} \) (12) were determined from secondary plots of \( 1/V'_{\text{max}} \) and \( K'_{\text{m}}/V'_{\text{max}} \) against the reciprocal of the cosubstrate concentration. The slope of the line in such a plot is the ratio of the \( K_{\text{m}} \) of the cosubstrate to \( V_{\text{max}} \); the vertical intercept of this line is equal to \( 1/V_{\text{max}} \), where \( V_{\text{max}} \) is the maximum velocity attainable when both substrates are present at saturating concentrations. Since \( K_{\text{m}} \) is that concentration of substrate necessary to give \( \frac{1}{2} V_{\text{max}} \), \( K_{\text{m}} \) represents the Michaelis constant of the particular substrate when the cosubstrate is present at its saturating concentration.

In product inhibition studies to determine the values of the inhibition constants \( K_{\text{a}1}, K_{\text{b}1}, K_{\text{a}2}, K_{\text{b}2} \), and \( K_{\text{a}3} \), the concentration of the keto acid substrates was varied at a fixed level of the cosubstrate amino acid and at various levels of the product amino acid inhibitor. The slopes and intercepts of the Lineweaver-Burk plots of these data were then plotted against the concentration of product inhibitor. The inhibition constants were then obtained from these secondary plots.

The inhibition constants for three substrate analogues (glutamate, malate, and \( \alpha \)-amino butyrate) were determined from experiments in which the concentration of one of the substrates was varied at a fixed level of cosubstrate, in the presence and absence of the inhibitor. The slopes and intercepts of the various lines of the Lineweaver-Burk plots were then plotted against the inhibitor concentration. In the present study \( K_{\text{a}1} \) and \( K_{\text{b}1} \) represented the concentrations of a given inhibitor at which the intercept or slope term of the rate equation, respectively, were doubled (25). In the present context, \( K_{\text{b}1} \) is the dissociation constant of the enzyme-rydixonal-phosphate-inhibitor complex while \( K_{\text{a}1} \) is the dissociation constant of the enzyme-rydixonal phosphatase-inhibitor complex. It should be noted that in subsequent discussions inhibition will be called competitive, uncompetitive, or noncompetitive, respectively, when the slope, ordinate intercept, or both, of Lineweaver-Burk plots are a function of the inhibitor concentration (25).

The initial velocity data were processed with computer programs for least square fit to a hyperbola and weighted least square fit to a straight line (26), kindly provided by W. W. Cleland. In the hyperbola program, the points are fitted to the curve, \( v = \frac{V'_{\text{max}} S}{K'_{\text{m}} + S} \), where \( v \) is the observed reaction velocity, measured at a known substrate concentration, \( S \), and at a fixed cosubstrate concentration. \( V'_{\text{max}} \) and \( K'_{\text{m}} \) are the apparent maximum velocity and the apparent Michaelis constant. The parameters that are found with this program are \( K'_{\text{a1}}, K'_{\text{b1}}, V'_{\text{max}}, K'_{\text{a2}}/V'_{\text{max}}, 1/V'_{\text{max}} \), their standard errors, and their weighting factors that are used in the straight line program.

In the straight line program, the points are fitted to the line, \( Y = mX + b \), where \( m \) and \( b \) are the slope and vertical intercept of the line, respectively; \( Y \) is the parameter determined from the hyperbola program, \( 1/V'_{\text{max}} \) or \( K'_{\text{m}}/V'_{\text{max}} \); and \( X \) is either the reciprocal of the cosubstrate concentration (in secondary plots of \( 1/V'_{\text{max}} \) against \( 1/S \)), the cosubstrate concentration (in secondary plots of \( K'_{\text{m}}/V'_{\text{max}} \) against \( S \)), or the inhibitor concentration (in secondary plots of \( 1/V'_{\text{max}} \) or \( K'_{\text{m}}/V'_{\text{max}} \) against \( I \)). The parameters found with this program are \( m \) and \( b \) and their standard errors.

### RESULTS AND DISCUSSION

#### Enzyme Specificity

The following amino acids were tested as possible substrates: di-\( \alpha \)-amino butyrate, di-\( \beta \)-amino butyrate, di-\( \alpha \)-amino isobutyrate, arginine, asparagine, aspartate, citrulline, glutamine, glycine, histidine, isoleucine, leucine, lysine, norleucine, ornithine, phenylalanine, serine, threonine, tryptophan, and valine. Of these, only di-\( \alpha \)-amino butyrate could replace alanine in agreement with the earlier findings of Green et al. (15). However, Segal, Beattie, and Hopper (27) reported that the rat liver enzyme is inactive with \( \alpha \)-amino butyrate as substrate. The apparent maximum velocity of the enzyme in the presence of \( \alpha \)-amino butyrate was found to be 2% that found with alanine (see Table II). The apparent Michaelis constant of \( \alpha \)-amino butyrate, however, was 50% that of alanine.

#### Kinetics of Transaminase Reaction

Lineweaver-Burk plots of initial velocity data from a series of experiments in which alanine was the variable substrate at four different fixed concentrations of \( \alpha \)-ketoglutarate are shown in Fig. 1A; Fig. 1B

### Table II

<table>
<thead>
<tr>
<th>Variable substrate</th>
<th>Amount of enzyme</th>
<th>( K'_{\text{m}} )</th>
<th>( V'_{\text{max}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>0.129</td>
<td>1.3</td>
<td>294</td>
</tr>
<tr>
<td>( \alpha )-Aminobutyrate</td>
<td>0.215</td>
<td>1.4</td>
<td>256</td>
</tr>
<tr>
<td>2.15</td>
<td>0.72</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>2.15</td>
<td>0.75</td>
<td>5.1</td>
<td></td>
</tr>
</tbody>
</table>

![Fig. 1. Lineweaver-Burk plots of initial velocities against amino acid concentration](image-url)
Fig. 2. Lineweaver-Burk plots of initial velocities against \( \alpha \)-ketoglutarate at a series of fixed concentrations of alanine. \( \alpha \)-Ketoglutarate (0.05 to 0.20 mM) was the variable substrate; assays were carried out as described in the legend of Fig. 1A. A, \( \alpha \)-alanine concentrations were: Line 1, 5.0 mM; Line 2, 6.25 mM; Line 3, 7.5 mM; Line 4, 10.0 mM; Line 5, 12.5 mM; Line 6, 25.0 mM; Line 7, 50.0 mM. B, \( \alpha \)-alanine concentrations were: Line 1, 0.1 mM; Line 2, 0.15 mM; Line 3, 0.2 mM; Line 4, 0.3 mM.

Fig. 3. Lineweaver-Burk plots of initial velocities against pyruvate at a series of fixed concentrations of glutamate. Pyruvate (0.092 to 0.25 mM) was the variable substrate. Assays were carried out as described in the legend of Fig. 1B. A, \( L \)-glutamate concentrations were: Line 1, 2.5 mM; Line 2, 3.75 mM; Line 3, 5.0 mM; Line 4, 6.25 mM; Line 5, 10.0 mM; Line 6, 12.5 mM. B, \( L \)-glutamate concentrations were: Line 1, 0.025 mM; Line 2, 0.05 mM; Line 3, 0.075 mM; Line 4, 0.10 mM.

Similarly summarizes experiments in which glutamate was the variable substrate at five different fixed concentrations of pyruvate. It is apparent from Fig. 1 that the family of lines within each graph is essentially parallel; increasing the concentration of fixed substrate affects the intercept of the lines only. The family of parallel lines shown in Fig. 1 is the pattern expected for an enzyme catalyzing a two-substrate reaction by a shuttle mechanism and is consistent, therefore, with Equations 4a and 4b, viz.,

\[
E-PMP + \alpha \text{-ketoglutarate} \rightleftharpoons E-PMP + \text{pyruvate} (4b)
\]

\[
E-PMP + \alpha \text{-ketoglutarate} \rightleftharpoons E-PMP + \text{pyruvate} (4a)
\]

Figs. 2 and 3 show the results from analogous experiments in which the keto acids were the variable substrates and the amino acids were the fixed substrates. Only at low concentrations of the fixed substrates (alanine in Fig. 2 and glutamate in Fig. 3) are the lines essentially parallel. As the concentration of the fixed substrate is increased, the lines become divergent; as the concentration of amino acid becomes larger, both the slopes and intercepts of the lines are affected. This pattern is not consistent with the shuttle mechanism, nor is it compatible with a sequential type of mechanism in which both substrates must be on the enzyme before a product can be released.

That this change from parallelism is due to an inhibition of the system by high concentrations of amino acid is shown in Fig. 4A. When the rate of transamination was measured at fixed levels of keto acid and at varying concentrations of amino acid, similarly summarizes experiments in which glutamate was the variable substrate and at five different fixed concentrations of pyruvate. It is apparent from Fig. 1 that the family of lines within each graph is essentially parallel; increasing the concentration of fixed substrate affects the intercept of the lines only. The family of parallel lines shown in Fig. 1 is the pattern expected for an enzyme catalyzing a two-substrate reaction by a shuttle mechanism and is consistent, therefore, with Equations 4a and 4b, viz.,

\[
E-PMP + \alpha \text{-ketoglutarate} \rightleftharpoons E-PMP + \text{pyruvate} (4b)
\]

\[
E-PMP + \alpha \text{-ketoglutarate} \rightleftharpoons E-PMP + \text{pyruvate} (4a)
\]

The abbreviations used are: E-PLP, enzyme-pyridoxal 5'-phosphate complex; E-PMP, enzyme-pyridoxamine 5'-phosphate complex.

Fig. 4. Effect of high concentrations of substrates on rate of reaction of glutamic-alanine transaminase. A, inhibitory effect of high concentrations of substrate amino acids. \( \Delta \), 0.10 mM \( \alpha \)-ketoglutarate at the indicated concentrations of \( L \)-alanine; lactate dehydrogenase assay system used as described in legend of Fig. 1A. \( \bigcirc \), 0.092 mM pyruvate at the indicated concentrations of \( L \)-glutamate; glutamate dehydrogenase system used as described in legend of Fig. 1B. \( \bigtriangleup \), lack of substrate inhibition by excess keto acids. \( \Delta \), 10 mM \( L \)-alanine at the indicated concentrations of \( \alpha \)-ketoglutarate; lactate dehydrogenase assay system used as described in the legend of Fig. 1A. \( \Delta \) and \( \bigcirc \), 6.25 mM and 2.5 mM \( L \)-glutamate, respectively, at the indicated concentrations of pyruvate; glutamate dehydrogenase assay system used as described in the legend of Fig. 1B. Note that the abscissa is a logarithmic scale.
acid, it was found that the amino acids were inhibitory at concentrations of less than 10 times their apparent Michaelis constants. It is apparent from Fig. 4A that glutamate caused a greater degree of substrate inhibition than did alanine. It should be noted that the large difference in peak velocities for alanine and glutamate are also due in part to the fact that, the concentration of α-ketoglutarate was near its $K_m$ value whereas the concentration of pyruvate was one-half of its $K_m$ value. Fig. 4B shows the results of an experiment in which the amino acid concentration was held fixed and the keto acid concentration varied over a 406-fold range. The keto acids were not inhibitory at concentrations as high as 106 times their apparent Michaelis constants. That the slopes of the lines shown in Figs. 2 and 3 are linear functions of the concentrations of alanine and glutamate, respectively, is shown in Fig. 5A.

One possible explanation for the substrate inhibition found when amino acids were present in high concentrations derives from the postulate that the mechanism of glutamic-alanine transaminase is, indeed, that shown in Equations 4a and 4b, but in addition, both amino acids can form abortive complexes with the pyridoxamine form of the enzyme, viz.

$$\text{E-PMP} + \text{alanine} \xrightarrow{k_a} \text{E-PMP-ala} \quad (4c)$$

$$\text{E-PMP} + \text{glutamate} \xrightarrow{k_{11}} \text{E-PMP-glu} \quad (4d)$$

The over-all rate equation for the postulated mechanism shown in Equations 4a through 4d was derived by the method of King and Altman (28) and is given in Equation 5, where $v$ is the observed initial velocity and $V_F$ and $V_R$ are the maximum velocities of the forward and reverse reactions. (ALA), (KG), (PYR), and (GLU) are the concentrations of alanine, α-ketoglutarate, pyruvate, and glutamate, respectively; $K_{\text{AL}, A}$, $K_{\text{KG}, A}$, $K_{\text{PYR}, A}$, and $K_{\text{GLU}}$ are the Michaelis constants; $K_{\text{ALA}}$, $K_{\text{KG}}$, $K_{\text{PYR}}$, and $K_{\text{GLU}}$ are the dissociation constants of the (E-PMP-Ala) and (E-PMP-Glu) complexes respectively; and $K_{\text{RO}}$ is a complex function of $K_{1A}$, $K_{1G}$, $K_{p}'$, $K_{s}'$, $K_{p}'$, and $K_{s}'$.

$$v = \frac{V_F V_R (\text{ALA})(\text{KG}) - (\text{PYR})(\text{GLU})/K_{eq}}{V_F K_{\text{RG}} \text{KG} + V_R K_{\text{KG}} (\text{PYR} + 2 \text{PYR}) + 2 \text{K}_{\text{PYR}} (\text{GLU}) + \text{K}_{\text{PYR}} (\text{GLU})}$$

$K_{eq}$ is the equilibrium constant of Reaction 1 and is related to the kinetic parameters through the relationships shown in Equation 6, which are of the type first obtained by Haldane (29). Thus $K_{eq}$ can be eliminated from Equation 5 by use of the proper Haldane relationship.

$$K_{eq} = \frac{V_F k_{\text{PYR}} k_{\text{GLU}}}{V_R K_{\text{AL}} K_{\text{KG}}} = \frac{V_F k_{\text{PYR}} k_{\text{GLU}}}{V_R K_{\text{AL}} K_{\text{KG}}}$$

Table III lists these kinetic parameters in terms of rate constants. With alanine and α-ketoglutarate as substrates, all terms in Equation 5 containing products (pyruvate and glutamate) are set equal to zero, and Equation 5 reduces to

$$\frac{1}{V_F} = \frac{1}{V_F} K_{\text{RO}} [1 + \frac{(\text{AL})}{K_{1A}}] = \frac{1}{V_F} K_{\text{RO}} [1 + \frac{(\text{AL})}{K_{1A}}] (1/V_F)$$

$$\frac{1}{V_F} = \frac{1}{V_F} K_{\text{RO}} [1 + \frac{(\text{AL})}{K_{1A}}] + \frac{1}{V_F} K_{\text{RO}} \frac{1 + \frac{(\text{AL})}{K_{1A}}}{(1/V_F)} (7a)$$

where $v_F$ is the observed reaction velocity measured in the forward direction and the other symbols have the same meaning as in Equation 5. Plots of 1/$v_F$ against the reciprocal of α-ketoglutarate at a fixed concentration of alanine should be linear, with the slope of the line equal to

$$\frac{K_{\text{RO}}}{V_F} [1 + \frac{(\text{AL})}{K_{1A}}]$$

and the vertical intercept equal to

$$\frac{1}{V_F} + \frac{K_{\text{RO}} (\text{AL})}{K_{1A}} (1/V_F)$$

Moreover, increasing the concentration of the fixed substrate
Kinetic parameters in terms of rate constants for the minimal mechanism of glutamic-alanine transaminase

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Rate constants</th>
<th>Experimental values</th>
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</thead>
<tbody>
<tr>
<td>$V_F$</td>
<td>$\frac{k_8k_7}{k_3 + k_7}$</td>
<td>0.041 μmole/min</td>
</tr>
<tr>
<td>$V_R$</td>
<td>$\frac{k_8k_8}{k_3 + k_8}$</td>
<td>0.035 μmole/min</td>
</tr>
<tr>
<td>$K_{ALA}$</td>
<td>$\frac{k_9(k_2 + k_2)}{k_3(k_2 + k_2)}$</td>
<td>$1.0 \times 10^{-2}$ M</td>
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<tr>
<td>$K_{KG}$</td>
<td>$\frac{k_9(k_3 + k_3)}{k_3(k_3 + k_3)}$</td>
<td>$1.2 \times 10^{-4}$ M</td>
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<td>$K_{PYR}$</td>
<td>$\frac{k_9(k_1 + k_1)}{k_3(k_1 + k_1)}$</td>
<td>$2.3 \times 10^{-4}$ M</td>
</tr>
<tr>
<td>$K_{GLU}$</td>
<td>$\frac{k_9(k_1 + k_1)}{k_3(k_1 + k_1)}$</td>
<td>$8.1 \times 10^{-3}$ M</td>
</tr>
<tr>
<td>$K_{a}^{1a}$</td>
<td>$\frac{k_9}{k_3}$</td>
<td>$8.2 \times 10^{-2}$ M</td>
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<td>$K_{a}^{1b}$</td>
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<td>$3.5 \times 10^{-4}$ M</td>
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<td>$2.3 \times 10^{-4}$ M</td>
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<td>$\frac{k_9}{k_3}$</td>
<td>$2.8 \times 10^{-3}$ M</td>
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<td>$K_{IA}$</td>
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</tr>
<tr>
<td>$K_{IG}$</td>
<td>$\frac{k_{12}}{k_{11}}$</td>
<td>0.096 M</td>
</tr>
<tr>
<td>$K_{KG}$</td>
<td>$\frac{k_9k_8k_8k_9(k_2 + k_2)}{k_8k_8k_8k_8k_8(k_2 + k_2)}$</td>
<td>2.2</td>
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</tbody>
</table>

Equation 7b predicts that plots of $1/V_F$ against the reciprocal of the alanine concentration at a fixed concentration of α-ketoglutarate would not be linear and that this deviation from linearity should be more pronounced at high concentrations of alanine or low concentrations of α-ketoglutarate. However, under the actual conditions of the experiment reported in Fig. 1A, the contribution of the underlined term in Equation 7b to $1/V_F$ is negligible (less than 3%) in comparison to that of the other terms in the equation. Hence Equation 7b reduces to Equation 8; the lines would then be linear, and the family of lines found by increasing the α-ketoglutarate concentration should be parallel.

$$\frac{1}{V_F} = \frac{K_{ALA}}{V_F} \left[ \frac{1}{(AL)} \right] + \left[ 1 + \frac{K_{KG}}{(RG)} \right] (1/V_F)$$

(8)

Indeed this was found experimentally (see Fig. 1A). Furthermore, the intercepts of these lines should be a linear function of the reciprocal of α-ketoglutarate concentration (see Fig. 5B).
The vertical intercept of the line plotting $1/V_R$ against $1/(KG)$ is equal to $1/V_R$ and from Fig. 5B $V_R$ was found to be equal to 0.041 μmole per min. The slope of this line is equal to $K_{KG}/V_F$ from which $K_{KG}$ was found to be equal to $1.2 \times 10^{-4}$ M. This is the value of $K_{KG}$ found from plotting the data according to Equation 7a, indicating that the assumption made in the use of Equation 8 is valid.

With pyruvate and glutamate as substrates, all terms in Equation 5 containing alanine and α-ketoglutarate are set equal to zero so that the rate equation becomes

\[
\frac{1}{V_R} = \frac{K_{PYR}}{V_F} \left[ 1 + \frac{(GLU)}{K_{GLU}} \right] \left( \frac{1}{V_R} \right) + \left[ 1 + \frac{K_{GLU}}{(PYR)} \right] \left( \frac{1}{V_F} \right) \quad (9a)
\]

\[
\frac{1}{V_P} = \frac{K_{GLU}}{V_P} \left[ 1 + \frac{K_{PYR}}{V_P K_{GLU}} \left( \frac{GLU}{PYR} \right) \right] + \left[ 1 + \frac{K_{PYR}}{V_P K_{GLU}} \right] \left( \frac{1}{V_F} \right) \quad (9b)
\]

The Lineweaver-Burk plots of $1/V_R$ against $1/(PYR)$ shown in Fig. 3 are consistent with Equation 9a; those for $1/v_P$ against $1/(GLU)$ shown in Fig. 1B are consistent with Equation 9b when the underlined term is disregarded (as discussed for Equation 7b). That this assumption is valid is shown by the following facts: (a) the lines in Fig. 1B are essentially parallel; (b) the secondary plot of the vertical intercepts of the lines in Fig. 1B against $1/(PYR)$ is linear (see Fig. 5B); (c) the lines in Fig. 3A are essentially parallel but only at low concentrations of glutamate; (d) the lines in Fig. 3B become divergent as the concentration of glutamate is increased further; (e) the secondary plot of the vertical intercepts of the lines in Fig. 3A against $1/(GLU)$ is linear (see Fig. 5C); (f) the secondary plot of the slopes of the lines in Figs. 3, A and B, against the glutamate concentration is linear (see Fig. 5A).

The results reported in Figs. 1 through 5 are consistent with the mechanism of glutamic-alanine transaminase, being of the shuttle type (Equations 4a and 4b) with glutamate and alanine, capable of forming abortive complexes with the phosphopyridoxamine form of the enzyme (Equations 4c and 4d). It should be noted that the mechanism depicted in these equations is the minimal mechanism necessary to describe the experimental results. It was only necessary to include one central complex in each half-reaction to derive the rate equation (Equation 5) for, as Velick and Vavra (8) have pointed out, isomerizations in each half-reaction to derive the rate equation (Equation 5) for the mechanism depicted in Equations 4a through 4d reduces to that shown in Equations 11a and 11b.

\[
\frac{1}{V_P} = \frac{K_{ALA}}{V_P} \left[ 1 + \frac{(GLU) + K_{GLU}^2}{(KA^2)_{KA}} \left( \frac{1}{V_P} \right) + \left[ 1 + \frac{K_{GLU}}{K_{ALA}^2} \right] \left( \frac{1}{V_R} \right) \quad (11a)
\]

\[
\frac{1}{V_F} = \frac{K_{GLU}}{V_F} \left[ 1 + \frac{K_{PYR} + K_{PYR}^2}{K_{PYR}^2} \left( \frac{1}{V_F} \right) + \left[ 1 + \frac{K_{PYR}}{K_{GLU}^2} \right] \left( \frac{1}{V_P} \right) \quad (11b)
\]

The ratio of the vertical intercept of this line to its slope is consistent with Equation 7c, indicating that the assumption made in the use of Equation 8 is valid.

The ratio of the vertical intercept of this line to its slope is consistent with Equation 7c, indicating that the assumption made in the use of Equation 8 is valid.
FIG. 6. Definition of the kinetic parameters in terms of rate constants for the expanded mechanism of glutamic-alanine transaminase. See the text for discussion.

Experimentally, however, it was found that (a) the product amino acid was a competitive inhibitor with respect to the substrate amino acids of the reaction (Figs. 7A and 8A); (b) the Lineweaver-Burk plots were linear; and (c) secondary plots of the slopes of the lines were a linear function of the product amino acid concentration. These results would seem to indicate that the mechanism proposed in Equations 4a through 4d is incorrect. However, it can be shown that, under the conditions of the experiments reported in the legends for Figs. 7A
and 8.4 (relatively low concentrations of alanine and glutamate, respectively), the contribution of the underlined terms to 1/v in Equations 11a and 11c is small. At the highest levels of amino acid used, the contribution of the underlined terms amounted to 5% of the total, and these terms, therefore, could be ignored. Thus, the patterns of product inhibition by the amino acids are in agreement with those predicted for the shuttle mechanism.

Further confirmation that the mechanism of glutamic alanine transaminase is of the shuttle type comes from studies on the patterns of product inhibition by the amino acids when the keto acids were the variable substrates. Plots of 1/v against 1/keto acid at various levels of the corresponding product amino acid should be linear, and the observed inhibition should be noncompetitive (Equations 11b and 11d). This, indeed, was found to be the case; the results are shown in Figs. 7B and 8B. Equations 11b and 11d also predict that the plots of the vertical intercept of each line against the product amino acid concentration should be linear while plots of slopes against product amino acid concentration should not be linear. However, when the data presented in Figs. 7B and 8B were plotted in such a way, both intercepts and slopes were found to be linear functions of the product amino acid concentration (Fig. 9).

As is the case with Equations 11a and 11c, Equations 11b and 11d hold only when the concentration of amino acids used is high; the underlined terms are significant only under these conditions. However, under the conditions of the experiments reported in the legends of Figs. 7B and 8B, the contribution of the underlined terms in Equations 11b and 11d to 1/v is less than 5% of the total at the highest level of product amino acid used. If these underlined terms are ignored, Equations 11b and 11d reduce to rate equations which can be shown to fit the mechanism depicted in Equations 4a through 4b.

If the underlined terms of Equation 11b are ignored, the slopes of the lines shown in Fig. 7B are given by the equation

\[
\text{Slope} = \frac{K_{\text{ALA}} K_{\text{ALA}}}{V_p(\text{ALA}) K_{\text{AL}}^4} (\text{GLU})
\]

and the vertical intercepts are given by

\[
\text{Intercept} = \frac{K_{\text{ALA}}}{V_p(\text{ALA}) K_{\text{AL}}^4} (\text{GLU}) \left[ 1 + \left( \frac{K_{\text{AL}}}{K_{\text{AL}}(\text{ALA})} \right) \right] \left( \frac{1}{V_p} \right)
\]

Plottedting the slopes of the lines in Fig. 7B against the glutamate concentration (Fig. 9B) yielded a straight line with a slope from Equation 12a equal to

\[
\text{Slope}_{\text{GLU}} = \frac{K_{\text{ALA}} K_{\text{ALA}}}{V_p(\text{ALA}) K_{\text{AL}}^4} (12a)
\]

The plot of the intercepts of the lines in Fig. 7B against the glutamate concentration yielded a straight line (Fig. 9A) with a slope from Equation 12b equal to

\[
\text{Slope}_{\text{GLU}} = \frac{K_{\text{ALA}}}{V_p(\text{ALA}) K_{\text{AL}}^4} (12b)
\]

The ratio of slope(8-GLU) to slope(2-GLU) is equal to \( K_{\text{AL}}^4 \). \( K_{\text{AL}} \) can then be calculated with Equation 13a or 13b. Similar analysis of Equation 11d and the data presented in Figs. 8B and 9B yield the values of \( K_{\text{PYR}} \) and \( K_{\text{GLU}} \). The values of these four inhibition constants are given in Table III.

**Equilibrium Constants of Partial and Over-all Reactions**—The equilibrium constant \( K_e \), for the conversion of the pyridoxal enzyme to the pyridoxamine form by the formation of pyruvate from alanine, depicted in Equation 4a, is given by Equation 14a. \( K_e \) was calculated to be 0.028.

\[
K_e = \frac{(\text{PYR})(\text{E}-\text{PLP})}{(\text{AL}(\text{E}-\text{PLP}))} = \frac{k_{\text{c}}}{k_{d}} = \frac{K_{\text{PYR}} V_p}{K_{\text{ALA}} V_p} = \frac{K_{\text{PYR}}}{K_{\text{ALA}}} \quad (14a)
\]

The equilibrium constant \( K_{e} \) for the reconversion of the pyridoxal enzyme to the pyridoxal enzyme by the addition of \( \alpha \)-ketoglutarate, depicted in Equation 4b, is given by Equation 14b; \( K_e \) was calculated to be 80. In both half-reactions, therefore, equilibrium favors the formation of the pyridoxal enzyme and free amino acid. This may be a result of attachment of the aldehyde group of pyridoxal phosphate to the enzyme in Schiff base linkage with the \( e \)-amino group of a lysyl residue, as proposed by Snell (30, 31).

The equilibrium constant for the over-all reaction (Equation 1) is given by the product of \( K_e \) and \( K_{e} \) (Equations 14a and 14b, respectively), as shown in Equation 6. \( K_{e} \), so calculated, was found to equal 2.2. Krebs (32) reported a value of 0.66 from direct equilibrium measurements with an enzyme preparation obtained from horse heart. Segal et al. (27) reported a value of 0.63 obtained by direct equilibrium measurements and a value of 0.38 calculated from kinetic measurements, with the equation

\[
K_{eq} = \frac{(V_p)^2}{V_p} \left( \frac{K_{\text{PYR}} K_{\text{GLU}}}{K_{\text{ALA}} K_{\text{AL}}} \right)
\]
of glutamic-alanine transaminase obtained from rat liver (13). No explanation can be offered for the relatively small difference between $K_{eq}$ as determined by these workers and the value of 2.2 reported herein.

Inhibition by Substrate Analogues—Jenkins, Yphantis, and Sizer (33) reported that various dicarboxylic acids, maleate and glutarate among them, form complexes with and inhibit glutamic-aspartic transaminase. The change in absorption spectrum of the enzyme induced by the addition of these inhibitory dicarboxylic acids resembled the change in absorption produced by the addition of the substrate keto acids, $\alpha$-ketoglutarate and oxalacetate. This suggested to these authors that the dicarboxylic acids bind to the enzyme by attachment at (or reaction with) the same groups as do the substrate keto acids. However, Velick and Vavra (8), with the same enzyme, reported that these dicarboxylic acids were noncompetitive inhibitors of both the keto and amino acid substrates, indicating that these analogues combined with both the pyridoxal and pyridoxamine forms of the enzyme. Similar results were obtained by Hopper and Segal (13), who studied the inhibition by maleate of glutamic-alanine transaminase of rat liver.

Inhibition of beef heart glutamic-alanine transaminase by glutarate and maleate was therefore studied to ascertain whether this enzyme behaves in a manner similar to the other transaminase preparations. Fig. 10 shows the results when glutarate was used as the inhibitor of the forward reaction. Glutarate was found to be a noncompetitive inhibitor with respect to both alanine (Fig. 10A) and $\alpha$-ketoglutarate (Fig. 10B). In addition, glutarate was found to be a noncompetitive inhibitor with respect to both substrates (glutamate and pyruvate) of the reverse reaction as well. Thus, glutarate combined with both forms of the enzyme, in confirmation of the findings of both Velick and Vavra (8) and Hopper and Segal (13). However, while these two laboratories reported that the inhibition constants for glutarate were identical when the reaction was studied in either direction, regardless of the form of enzyme, in the present studies it was found that $K_f$ for the dissociation from the pyridoxal form of the enzyme ($K_{f1} = 0.2 \times 10^{-3} M$) was 10 times less than that for the dissociation from the pyridoxamine form of the enzyme ($K_{f1} = 9.3 \times 10^{-3} M$).

Fig. 11 shows that maleate combined only with the pyridoxamine form of the enzyme; i.e. it was a competitive inhibitor ($K_{f1} = 8.4 \times 10^{-4} M$) with respect to $\alpha$-ketoglutarate (Fig. 11B) and an uncompetitive inhibitor with respect to alanine (Fig. 11A). It is unclear whether this apparent difference between our findings and those of Velick and Vavra (8) and Hopper and Segal (13) reflects the fact that these studies were carried out with different transaminase preparations. However, the present data are in accord with the spectral changes observed by Jenkins et al. (33) upon the addition of maleate to glutamic-aspartic transaminase.

Inhibition of the enzyme by $\alpha$-aminobutyrate was also studied. Fig. 12 shows that this analogue was a noncompetitive inhibitor with respect to both alanine (Fig. 12A) and $\alpha$-ketoglutarate (Fig. 12B), indicating that it combined with both forms of the enzyme ($K_{f1} = 0.039 M$ and $K_{f1} = 0.061 M$). The level of transaminase used in these experiments was lower than the amount of enzyme necessary to enable one to observe a detectable amount of transamination from $\alpha$-aminobutyrate. That $\alpha$-aminobutyrate could compete with alanine for the site of attachment on the pyridoxal form of the enzyme is not surprising. That it could also compete with $\alpha$-ketoglutarate for the site on the pyridoxamine form of the enzyme can best be explained by assuming that $\alpha$-aminobutyrate can act in a manner analogous to the substrate amino acids; viz. high levels of amino acid cause inhibition by combining with the pyridoxamine form of the enzyme.
of the enzyme (Equations 4c and 4d). In any case, it is noteworthy that, whereas alanine and glutamate bind to the pyridoxal form of the enzyme 57 and 34 times as strongly as they do to the pyridoxamine form, respectively, α-aminobutyrate binds to the pyridoxal form (pH 8.0): Line 1, no α-aminobutyrate; Line 2, 5.0 mm; Line 3, 10.0 mm; Line 4, 15.0 mm. B, Lineweaver-Burk plots of initial velocities as a function of pyruvate concentration (0.05 to 0.25 mm) at a fixed concentration of α-ketoglutarate (0.1 mM) in the presence of the following concentrations of L-α-aminobutyrate: Line 1, no α-aminobutyrate; Line 2, 5.0 mm; Line 3, 10.0 mm; Line 4, 15.0 mm.

**Table IV**

**Inhibition of glutamic-alanine transaminase by substrate analogues**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Variable substrate*</th>
<th>Dissociation constants for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amino acid</td>
<td>Keto acid</td>
</tr>
<tr>
<td>Glutamate</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Maleate</td>
<td>U</td>
<td>C</td>
</tr>
<tr>
<td>α-Aminobutyrate</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

* N, noncompetitive; U, uncompetitive; C, competitive.

The inhibitory effects of substrate analogues were also studied. Glutaric acid was found to be a noncompetitive inhibitor of all four substrates, indicating that it can combine with both the pyridoxal and pyridoxamine forms of the enzyme. However, glutarate was a 10-fold better inhibitor of the pyridoxal form than of the pyridoxamine form. Maleate was found to be an uncompetitive inhibitor with respect to alanine and a competitive inhibitor with respect to α-ketoglutarate, indicating that it combined with only the pyridoxamine form of the enzyme. α-Aminobutyrate was found to be a noncompetitive inhibitor of the reaction with respect to either alanine or α-ketoglutarate, indicating that it can combine with both forms of the enzyme.

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