The Mechanism of the Inhibition of Aminotransferases by Salicylate

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SUMMARY

Salicylate inhibits pig heart alanine and aspartate aminotransferase activities *in vitro*. The inhibitions are reversible, involving competition with α-oxoglutarate and alanine for the alanine aminotransferase and with α-oxoglutarate and aspartate for the aspartate aminotransferase. The results are consistent with a binary mechanism for the action of the aminotransferases. In addition they show that salicylate inhibits alanine aminotransferase much more strongly than aspartate aminotransferase. These results are discussed with reference to some of the *in vivo* actions of the drug.

INTRODUCTION

It has been shown that salicylates caused an increased incorporation of radiocarbon from ^{14}C-labeled pyruvate into glutamic acid in chopped preparations of rat tissues (1). This result suggested that salicylate interfered with the further metabolism of the amino acid, and it has been found that salicylate inhibits several pathways of glutamate metabolism. These include the conversion to glutamine (2), the incorporation into proteins (3), the decarboxylation to γ-aminobutyrate and the oxidative deamination to α-oxoglutarate (4), and the conversion to proline (5). However, the most important quantitative pathway for glutamate metabolism in animal tissues is that controlled by the various aminotransferases. Salicylate was found to inhibit both alanine and aspartate aminotransferase activities in rat serum (6) and in extracts of rat organs (7), and later work (8) has shown that this inhibition extends to at least eighteen aminotransferase activities present in mammalian tissues. The present paper is concerned with an investigation of the mechanism of the inhibition using purified alanine and aspartate aminotransferases.

MATERIALS AND METHODS

Materials. Pig heart alanine aminotransferase (L-alanine: 2-oxoglutarate aminotransferase, EC 2.6.1.2), aspartate aminotransferase (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1), malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37), rabbit-muscle lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27), and $NADH_2$ were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Other chemicals were of analytical grade and deionized water was used throughout. Before use, the commercial enzyme preparations were dialyzed against the buffer used in the subsequent assay, the dialyzing medium being replaced three times over a period of 24 hr.

Enzyme assays. Alanine and aspartate aminotransferase activities were determined by the techniques described by Wróblewski and La Due (9) and by Karmen (10). Mixtures containing substrates, buffer, lactate or malate dehydrogenase, $NADH_2$ and salicylate, when present, were allowed to come to thermal equilibrium in a 1-cm cell fitted into a constant-temperature cell housing. Full experimental details are given in

Tables 2 and 3. The reaction was started by adding a freshly diluted solution of aminotransferase and followed by measuring the changes in optical density at 365 mμ in a Unicam SP800 recording spectrophotometer. Fresh dilutions of the aminotransferase were used in each experiment to avoid the loss of activity which occurred in stored dilute solutions of the enzyme. Measurements of extinction were made at 365 mμ, not at 340 mμ, to avoid interference caused by the absorption of salicylate at the lower wavelength. The initial rates (v) were determined from the tracings obtained with an external recorder.

Dialysis experiments. In general the enzyme solutions, in either the absence or the presence of salicylate, were placed in cellulose tubing (inflatable diameter 6 mm, Visking Co.) and dialyzed at 0° against the appropriate buffer, which was changed several times. The enzyme activities were measured before and after dialysis. Full experimental details are given in Tables 4 and 5.

Analysis of kinetic data. Previous workers (11–15) have suggested that the mechanism of action of alanine and aspartate aminotransferases involves a binary complex. This binary mechanism assumes that the enzyme undergoes interconversion between the pyridoxal and pyridoxamine forms in a cyclic process. Equations 1 and 2 show the intermediates involved and Eq. 3 gives the initial rate. The symbols refer to the following: A is either alanine or aspartate, Ox is either pyruvate or oxalacetate, α is α-oxoglutarate, and G is glutamate. E and E′ are the functionally undissociated complexes of pyridoxal and pyridoxamine phosphates with the enzyme and EA, E′Ox, E′α, and EG are used to represent the intermediates.

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E'Ox \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E' + Ox \qquad (1)$$

$$E' + \alpha \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E'\alpha \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} EG \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} E + G \qquad (2)$$

Initial rate equation

$$\frac{1}{V} = \frac{1}{K[E]}\left\{1 + \frac{K_A}{[A]} + \frac{K_\alpha}{[\alpha]}\right\} \qquad (3)$$

where

$$K = \frac{k_2k_5k_9k_{11}}{k_9k_{11}(k_2 + k_4 + k_5) + k_2k_5(k_9 + k_{10} + k_{11})}$$

$$K_A = K\left(\frac{k_2k_4 + k_2k_5 + k_3k_5}{k_1k_3k_5}\right)$$

$$K_\alpha = K\left(\frac{k_2k_{10} + k_9k_{11} + k_9k_{11}}{k_7k_9k_{11}}\right)$$

Competitive inhibition by an inhibitor I, may be expressed in terms of the inhibitor occupying a single site on the enzyme that is used by all four substrates. Only two inhibited complexes, EI and E′I; those formed by the inhibitor with the amino and aldehyde forms of the enzyme–coenzyme complex, respectively, need be considered. The rate constants in the appropriate equations are as follows;

$$E + I \underset{k_{14}}{\overset{k_{13}}{\rightleftharpoons}} EI \qquad (4)$$

$$E' + I \underset{k_{16}}{\overset{k_{15}}{\rightleftharpoons}} E'I \qquad (5)$$

Their inclusion in the rate equation leads to

$$\frac{1}{V} = \frac{1}{K[E]}\left\{1 + \frac{K_A}{[A]}\left(1 + \frac{[I]}{K_I}\right) + \frac{K_\alpha}{[\alpha]}\left(1 + \frac{[I]}{K'_I}\right)\right\} \qquad (6)$$

where

$$K_I = \frac{k_{14}}{k_{13}} \quad \text{and} \quad K'_I = \frac{k_{16}}{k_{15}}$$

The above equations can be used to construct a primary plot ($1/v$ against 1/[substrate]) by the method of Lineweaver and Burk (16) but the theoretical lines in either the presence or the absence of inhibitor do not intercept on the $1/v$ axis. In order to calculate the kinetic constants, secondary plots are necessary, i.e., the slopes and intercepts from the primary plots are plotted against inhibitor concentration. From Eq. 6 it can be shown that for a constant concentration of α-oxoglutarate [α_c] and for several concentrations of inhibitor, the slopes and intercepts on the $1/v$ axis of the Lineweaver-Burk primary plot can be represented by the following equations:

$$\text{Slopes of primary plot} = \frac{K_A}{K[E]}\left(1 + \frac{[I]}{K_I}\right) \qquad (7)$$

TABLE 1

Interpretation of secondary plots from Lineweaver-Burk primary plots

The following expressions are derived from Eqs. 7–10. The numbers in parentheses will subsequently be used in the text to represent the appropriate expressions.

		Slopes of secondary plots		Intercepts of secondary plots	
$[\alpha_c]$	Slopes of primary plots/[I].	$\frac{K_A}{K[E]K_I}$	(1)	$\frac{K_A}{K[E]}$	(5)
	Intercepts of primary plots/[I].	$\frac{K_\alpha}{[\alpha_c]K[E]K'_I}$	(2)	$\frac{1}{K[E]}\left(1+\frac{K_\alpha}{[\alpha_c]}\right)$	(6)
$[A_c]$	Slopes of primary plots/[I].	$\frac{K_\alpha}{K[E]K'_I}$	(3)	$\frac{K_\alpha}{K[E]}$	(7)
	Intercepts of primary plots/[I].	$\frac{K_A}{[A_c]K[E]K_I}$	(4)	$\frac{1}{K[E]}\left(1+\frac{K_A}{[A_c]}\right)$	(8)

Intercepts of primary plot

$$= \frac{1}{K[E]}\left\{1+\frac{K_\alpha}{[\alpha_c]}\left(1+\frac{[I]}{K'_I}\right)\right\} \quad (8)$$

Similarly for a constant concentration of either alanine or aspartate $[A_c]$ the following equations can be derived:

$$\text{Slopes of primary plot} = \frac{K\alpha}{K[E]}\left(1+\frac{[I]}{K'_I}\right) \quad (9)$$

Intercepts of primary plot

$$= \frac{1}{K[E]}\left\{1+\frac{K_A}{[A_c]}\left(1+\frac{[I]}{K_I}\right)\right\} \quad (10)$$

The reinterpretation of the slopes and intercepts of the secondary plots are summarized in Table 1; from this table the following kinetic parameters can be calculated:

$$K_I = \frac{(5)}{(1)} \text{ or } \frac{(5)}{(4)[A_c]}; \quad K'_I = \frac{(7)}{(3)} \text{ or } \frac{(7)}{(2)[\alpha_c]}$$

$$\frac{1}{K[E]} = (6) - \frac{(7)}{[\alpha_c]} \text{ or } (8) - \frac{(5)}{[A_c]}$$

$$K_A = (5) \times K[E]; \quad K_\alpha = (7) \times K[E]$$

RESULTS

The results given in Tables 2 and 3 show that salicylate inhibits the activities of pig heart alanine and aspartate aminotransferases and that the degree of inhibition increases with salicylate concentration.

The order in which the components of the reaction mixtures were added, even in the presence of salicylate, did not alter the reaction rates. Preincubation of the reaction mixtures, excluding either alanine or aspartate, with salicylate for time intervals up to 15 min prior to starting the reactions by the addition of the amino acid, did not alter the degree of inhibition produced by salicylate. More conclusive proof that the inhibition caused by salicylate is reversible is provided by the results given in Tables 4 and 5, which show that complete reactivation of the

TABLE 2

Effect of salicylate on alanine aminotransferase activity

All solutions were prepared in 0.1 M-imidazole-HCl buffer, pH 7.5, and the pH was adjusted to 7.5 with KOH solution if necessary. Mixtures containing α-oxoglutarate, 1.1 mM; L-alanine, 33.3 mM; lactate dehydrogenase, 5 μg; $NADH_2$, 0.44 mM; and potassium salicylate, 0–5 mM, contained in a total volume of 3 ml, were allowed to come to thermal equilibrium at 25° in a 1-cm silica cell. The reaction was started by the addition of 10 μl of a solution containing approximately 2 μg of alanine aminotransferase. The results are expressed as the means of three separate determinations ± standard deviations.

Salicylate (mM)	Initial velocity ΔE_{365}/min	% Inhibition
0	0.111 ± 0.006	—
0.2	0.101 ± 0.005	9.0
0.5	0.094 ± 0.005	15.3
1.0	0.089 ± 0.002	19.8
1.5	0.081 ± 0.002	27.0
2.0	0.076 ± 0.007	31.6
2.5	0.066 ± 0.001	40.5
5.0	0.060 ± 0.003	46.0

TABLE 3

Effect of salicylate on aspartate aminotransferase activity

All solutions were prepared in 0.1 M-potassium phosphate buffer, pH 7.5, and the pH was adjusted to 7.5 with KOH solution if necessary. Mixtures containing α-oxoglutarate, 0.5 mM; L-aspartate, 5.0 mM; malate dehydrogenase, 6 μg; $NADH_2$, 0.22 mM; and potassium salicylate, 0–50 mM, contained in a total volume of 3 ml, were allowed to come to thermal equilibrium at 25° in a 1-cm silica cell. The reaction was started by the addition of 10 μl of a solution containing approximately 0.5 μg of aspartate aminotransferase. The results are expressed as the means of three separate determinations ± standard deviations.

Salicylate (mM)	Initial velocity ΔE_{365}/min	% Inhibition
0	0.062 ± 0.001	—
2.5	0.060 ± 0.002	3.0
5.0	0.055 ± 0.002	11.2
7.5	0.048 ± 0.002	22.5
10.0	0.043 ± 0.003	30.5
15.0	0.036 ± 0.003	41.8
20.0	0.029 ± 0.001	53.2
50.0	0.015 ± 0.002	75.8

inhibited aminotransferases occurred after dialysis.

The results of varying the concentrations of α-oxoglutarate and salicylate in the presence of a constant concentration of alanine on the reaction rate of alanine aminotransferase are shown in Fig. 1. Both the primary and secondary plots (see section on analysis of kinetic data) are presented in this figure and in Fig. 2, which gives the results of similar experiments in which the concentrations of alanine and salicylate were varied and that of α-oxoglutarate remained constant.

The results of similar experiments with aspartate aminotransferase in which constant concentrations of either aspartate or α-oxoglutarate were maintained are given in Figs. 3 and 4.

The inhibitor constants, calculated from the data contained in Figs. 1–4, are given in Table 6.

DISCUSSION

The Michaelis constants calculated from the results obtained in the present work (Table 6) agree with those reported by other workers for alanine aminotransferase (11, 12) and for aspartate aminotransferase (14, 15). The slopes of the primary plots when plotted against salicylate concentration gave straight lines (Figs. 1–4, part c). This result is consistent with a binary mechanism of action of both transaminases because a ternary mechanism would demand curved plots.

Salicylate inhibits both aminotransferase activities *in vitro*. The inhibitions are reversible and competitive with α-oxoglutarate and alanine for alanine aminotransferase and with α-oxoglutarate and aspartate for the aspartate aminotransferase. A binary mechanism for the action of the aminotransferase means that salicylate must also compete with the other substrates, glutamate, pyruvate, and oxalacetate. The inhibitor constants for salicylate and both substrates

TABLE 4

Effect of dialysis on inhibition of alanine aminotransferase by salicylate

Experimental details were as in Table 2 except that the reaction was started by the addition of 0.5 ml of 0.2 M L-alanine. Equal volumes of enzyme solution (16 μg in 2.0 ml) and 2.0 ml of either 0.1 M imidazole–HCl buffer pH 7.5, or the buffer containing 60 mM salicylate were dialyzed against 200 ml of the buffer at 0°, the dialyzing medium being replaced three times over a period of 4 hr. The enzyme activity was estimated on 0.5-ml samples of the various enzyme solutions before and after dialysis. An additional experiment was made in which the enzyme activities were measured in similar mixtures stored at 0° for 5 hr without dialysis. The results are expressed as ΔE_{365}/min and represent the means ± standard deviations. The number of observations is given in parentheses.

Sample	Control	Salicylate
Before dialysis	0.107 ± 0.001 (4)	0.055 ± 0.002 (4)
After dialysis	0.085 ± 0.004 (8)	0.087 ± 0.001 (8)
After storage without dialysis	0.106 ± 0.004 (4)	0.053 ± 0.002 (4)

TABLE 5

Effect of dialysis on inhibition of aspartate aminotransferase by salicylate

Experimental details were as in Table 3 except that the reaction was started by the addition of 0.2 ml of L-aspartate, 75 mM. Equal volumes of enzyme solution (4 μg in 2.0 ml) and 2.0 ml of either 0.1 M-potassium phosphate buffer, pH 7.5, or the buffer containing 0.2 M salicylate were dialyzed against 200 ml of the buffer at 0°, the dialysing medium being replaced three times over a period of 4 hr. The enzyme activity was estimated on 0.5-ml samples of the various enzyme solutions before and after dialysis. An additional experiment was made in which the enzyme activities were measured in similar mixtures stored at 0° for 5 hr without dialysis. The results are expressed as ΔE_{365}/min and represent the means ± standard deviations. The number of observations is given in parentheses.

Sample	Control	Salicylate
Before dialysis	0.055 ± 0.002 (4)	0.031 ± 0.001 (4)
After dialysis	0.049 ± 0.002 (8)	0.050 ± 0.002 (8)
After storage without dialysis	0.049 ± 0.002 (4)	0.028 ± 0.002 (4)

with alanine aminotransferase (Table 6) show that salicylate resembles other inhibitors of aminotransferases, such as aliphatic dicarboxylic acids, which show a higher affinity for either the pyridoxal or pyridoxamine form of the enzyme (17). However, this is less evident with aspartate aminotransferase where the difference between the inhibitor constants for salicylate with the two substrates is much smaller. The inhibitor constants also show that salicylate is a considerably more powerful inhibitor of alanine aminotransferase than of the aspartate aminotransferase with particular reference to α-oxoglutarate.

The *in vitro* results observed in the present work may have important implications with respect to certain of the *in vivo* actions of salicylate. It has been shown that the drug inhibits a number of aminotransferase activities in animal tissues (8). If the mechanism of inhibition established for the two aminotransferases used in the present experiments extends to the remainder, then salicylate may interfere with aminotransferase activities *in vivo* in both a widespread

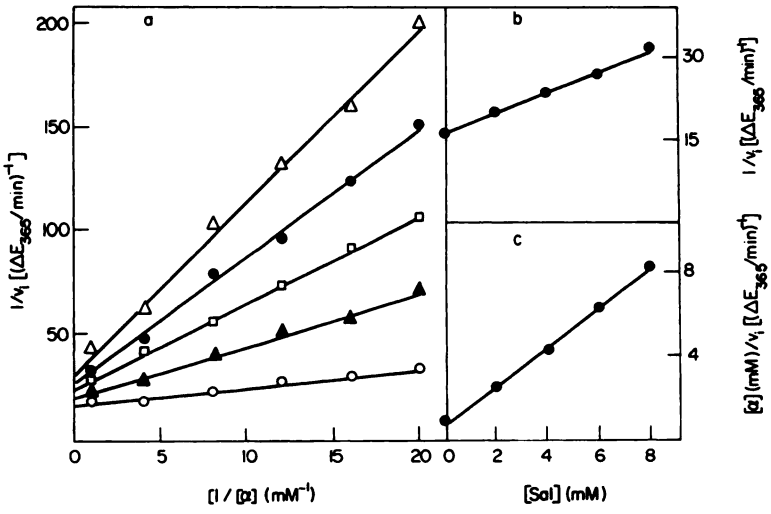

FIG. 1. *Alanine aminotransferase, kinetics with a constant concentration of alanine*

The experimental conditions were the same as in Table 2 except that the alanine concentration was 20 mM and the α-oxoglutarate [α] and salicylate [Sal] concentrations were varied as shown. (a) Primary plot. ○, Control; ▲, 2 mM salicylate; □, 4 mM salicylate; ●, 6 mM salicylate; △, 8 mM salicylate. (b) Secondary plot of intercepts of (a). (c) Secondary plot of slopes of (a).

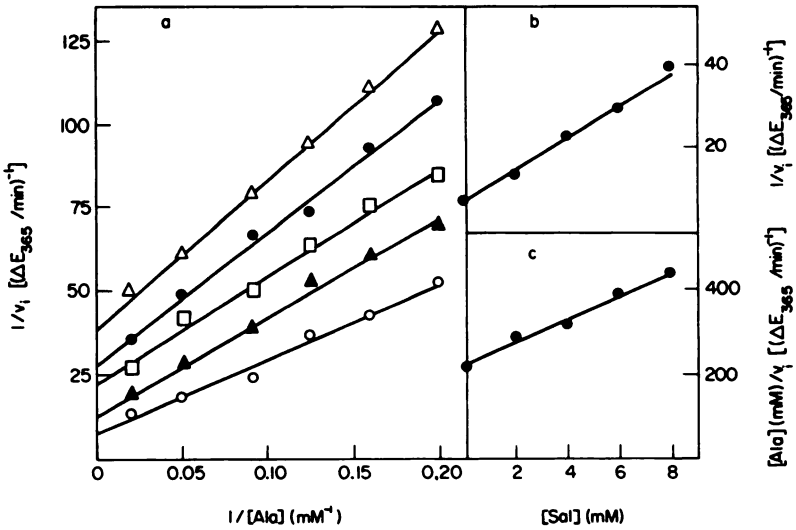

FIG. 2. *Alanine aminotransferase, kinetics with a constant concentration of α-oxoglutarate*

The experimental conditions were the same as in Table 2 except that the α-oxoglutarate concentration was 0.25 mM and the alanine [Ala] and salicylate [Sal] concentrations were varied as shown. (a) Primary plot. ○, Control; ▲, 2 mM salicylate; □, 4 mM salicylate; ●, 6 mM salicylate; △, 8 mM salicylate. (b) Secondary plot of intercepts of (a). (c) Secondary plot of slopes of (a).

and differential manner. The degree of inhibition of any particular aminotransferase would depend, at least in part, on the endogenous concentrations of the substrates of the enzyme. In addition the more marked inhibition of alanine aminotransferase by salicylate suggests that the enzymes may exhibit a spectrum of varying sensitivity to the drug *in vivo*. Salicylate could therefore produce differential inhibitory actions on aminotransferase reactions *in vivo* depending on the particular salicylate concentration achieved in the tissues and on the concentration of α-oxo acids and amino acids present in individual tissues.

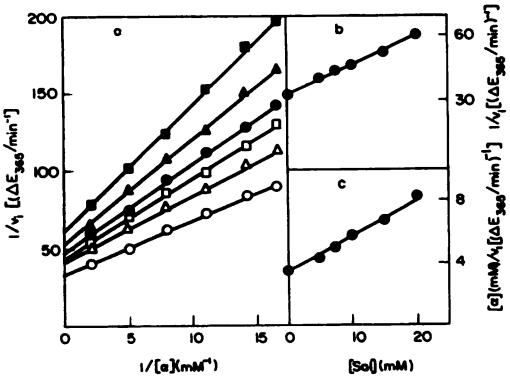

FIG. 3. *Aspartate aminotransferase, kinetics with a constant concentration of aspartate.*

The experimental conditions were the same as in Table 3 except that the aspartate concentration was 2.0 mM and the α-oxoglutarate [α] and salicylate [Sal] concentrations were varied as shown. (a) Primary plot. ○, control; △, 5 mM salicylate; □, 7.5 mM salicylate; ●, 10 mM salicylate; ▲, 15 mM salicylate; ■, 20 mM salicylate. (B) Secondary plot of intercepts of (a). (c) Secondary plot of slopes of (a).

The expected results of such actions of salicylate would be an interference with the interconversion of amino acids in the body leading to disturbed patterns and altered pool sizes of amino acids in the tissues. It has been reported (18) that the injection of salicylate in the intact rat causes a 2-fold increase in the amounts of glutamate and alanine present in the liver. An increased intracellular accumulation of such amino acids would be expected to enhance their entry into the circulation, and this would lead to an "overflow" type of aminoaciduria. An aminoaciduria of this type has been found to occur in rats receiving large doses of salicylate (19) and in salicylate-intoxicated patients (20).

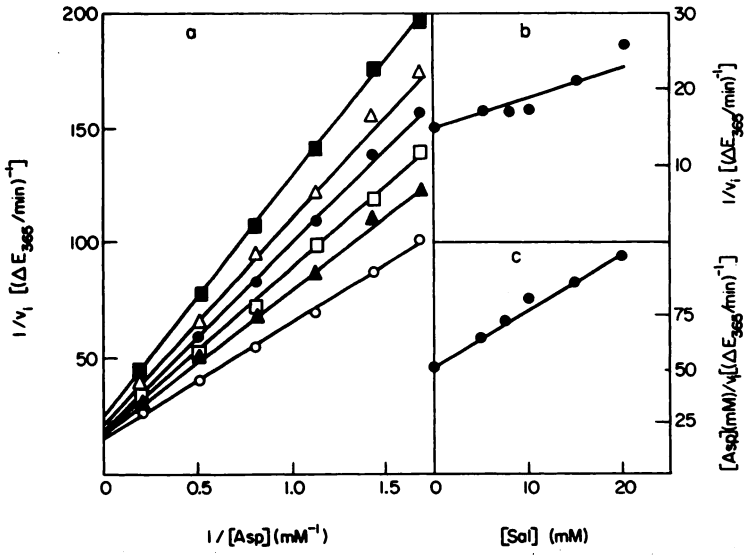

FIG. 4. *Aspartate aminotransferase, kinetics with a constant concentration of α-oxoglutarate*

The experimental conditions were the same as in Table 3 except that the α-oxoglutarate concentration was 0.5 mM and the aspartate [Asp] and salicylate [Sal] concentrations were varied as shown. (a) Primary plot. ○, Control; ▲, 5 mM salicylate; □, 7.5 mM salicylate; ●, 10 mM salicylate; △, 15 mM salicylate; ■, 20 mM salicylate. (b) Secondary plot of intercepts of (a). (c) Secondary plot of slopes of (a).

TABLE 6

Kinetic parameters of alanine and aspartate aminotransferases

The values given here have been calculated from the constants defined under "Analysis of kinetic data." The inhibitor constants were calculated by both expressions given in the text and represent the mean values.

Aminotransferase	Substrate	Michaelis parameters (mM)	Inhibitor constants (mM)
Alanine	Alanine	$K_A = 43.50$	$K_I = 7.2$
	α-Oxoglutarate	$K_\alpha = 0.11$	$K'_I = 0.6$
Aspartate	Aspartate	$K_A = 6.20$	$K_I = 18.2$
	α-Oxoglutarate	$K_\alpha = 0.42$	$K'_I = 15.9$

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