Biochemical Pharmacology, Vol. 32, No. 4, pp. 679–689, 1983.
Printed in Great Britain.

0006-2952/83/040679-11 $03.00/0

INHIBITION OF GLUTAMATE-ASPARTATE TRANSAMINASE BY β-METHYLENE-DL-ASPARTATE*

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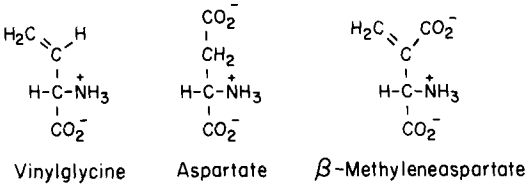
(*Received* 10 *April* 1982; *accepted* 2 *August* 1982)

Abstract—β-Methylene-DL-aspartate, a new β,γ-unsaturated amino acid, is an irreversible inhibitor of soluble pig heart glutamate-aspartate transaminase ($K_i \sim 3$ mM with respect to the L-form; limiting rate constant for inactivation $\sim 0.4\ min^{-1}$). The new amino acid is the most specific inhibitor of glutamate-aspartate transaminase thus far studied. It does not inactivate pig heart glutamate-alanine transaminase, soluble rat kidney glutamine transaminase K, γ-aminobutyrate transaminase (from *Pseudomonas fluorescens*), glutamate decarboxylase (*Escherichia coli*), snake venom L-amino acid oxidase, or hog kidney D-amino acid oxidase. In addition, the following enzymes were not inhibited by β-methylene-DL-aspartate in rat tissue homogenates: γ-aminobutyrate transaminase (brain), tyrosine transaminase (liver), glutamine transaminase L (liver), asparagine transaminase (liver), ornithine transaminase (liver) or branch-chain transaminase(s) (kidney). Intraperitoneal injection of β-methylene-DL-aspartate into mice decreased kidney and liver glutamate-aspartate transaminase activities but had no effect on liver glutamate-alanine transaminase activity.

It is generally believed that glutamate-aspartate transaminase is a catalyst of major metabolic significance. However, few studies have been carried out in which this enzyme is preferentially inhibited. Aminooxyacetate has often been used as an inhibitor of glutamate-aspartate transaminase; however, aminooxyacetate also strongly inhibits a large number of pyridoxal-P‡ enzymes. In the course of work designed to achieve a more useful inhibitor, we tested β-methylene-DL-aspartate as an inhibitor of glutamate-aspartate transaminase and found that β-methylene-DL-aspartate inhibits soluble pig heart glutamate-aspartate transaminase in a time-dependent fashion *in vitro* and that it does not inhibit pig heart glutamate-alanine transaminase [1, 2]. Moreover, unlike other β,γ-unsaturated α-amino acids, β-methylene-DL-aspartate is not converted to a saturated α-keto acid product. Although the selectivity of β-methylene-DL-aspartate is significantly higher than that of aminooxyacetate, the specificity is not absolute. Rat liver glutamate-alanine transaminase is inactivated *in vitro* (i.e. in homogenates incubated with β-methylene-DL-aspartate) but not *in vivo* (i.e. following intraperitoneal administration of β-methylene-DL-aspartate). Glutamate decarboxylase is also slowly inactivated in rat brain homogenates containing β-methylene-DL-aspartate. Nevertheless, β-methylene-DL-aspartate is useful, as shown here, in experiments on the inactivation of glutamate-aspartate transaminase.



EXPERIMENTAL PROCEDURES

Materials. β-Methylene-DL-aspartate was prepared as recently described [3]. Chromatographic analysis, using Whatman No. 1 paper, and the ascending technique of Fink *et al.* [4] yielded a single

* This investigation was supported by United States Public Health Service Grant AM-16739 (A. J. L. C.) and by Grant GM 19906 from the Institute for General Medical Sciences of the National Institutes of Health (P. D.). A. J. L. C. is a recipient of United States Public Health Service Career Development Award NS 00343. Part of this work has been presented at the Seventy-second Annual Meeting of the American Society of Biological Chemists, St. Louis, MO [S. M. Fitzpatrick, A. J. L. Cooper and T. E. Duffy, *Fedn Proc.* **40**, 1843 (1981)]. Preliminary results have been published [A. J. L. Cooper, S. M. Fitzpatrick, C. Kaufman and P. Dowd, *J. Am. chem. Soc.* **104**, 332 (1982)].

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‡ Abbreviations: pyridoxal-P, pyridoxal 5′-phosphate; pyridoxamine-P, pyridoxamine 5′-phosphate; GSH, glutathione; NEM, *N*-ethylmaleimide; and GABA, γ-aminobutyrate.

ninhydrin-positive spot in two chromatographic systems (Table 1). As has been our experience with other β,γ-unsaturated amino acids, β-methylene-DL-aspartate yielded a yellow spot on paper after spraying with ninhydrin reagent; the yellow spot slowly changed to blue-purple. γ-Methylene-DL-glutamate was a gift from Dr. Alton Meister (Department of Biochemistry, Cornell University Medical College). All other amino acids were obtained from the Sigma Chemical Co., St. Louis, MO. Soluble glutamate-aspartate transaminase (pig heart; sp. act. 200 units/mg) and glutamate-alanine transaminase (pig heart; sp. act. 80 units/mg) were obtained, as suspensions in 3.2 M ammonium sulfate, from Boehringer-Mannheim, Indianapolis, IN; the stated activities of these enzymes were verified. L-Amino acid oxidase (*Crotalus adamanteus*; 4.3 units/mg), D-amino acid oxidase (hog kidney; 17 units/mg in 3.2 M ammonium sulfate), "gabase" (*Pseudomonas fluorescens*; 25 units/ml in 50% glycerol), and glutamate decarboxylase (*Escherichia coli*; 25 units/mg solid) were purchased from the Sigma Chemical Co. Soluble glutamine transaminase K (rat kidney) was purified according to the method of Cooper [5].

Enzyme assays. Glutamate-aspartate transaminase was assayed spectrophotometrically by coupling oxaloacetate formation to NADH oxidation with malate dehydrogenase [6]. (Since in some experiments low levels of β-methylene-DL-aspartate were present in the assay mixture, it was important to determine whether β-methylene-DL-aspartate inhibits malate dehydrogenase. No effect on malate dehydrogenase was noted after 1 hr at 25° in the presence of 1 mM β-methylene-DL-aspartate, pH 7.1.) Glutamate-alanine transaminase was assayed spectrophotometrically by coupling pyruvate formation to NADH oxidation with lactate dehydrogenase [6]. *E. coli* glutamate decarboxylase was assayed by determining γ-aminobutyrate formation with "gabase" according to the method of Cozzani [7]. Soluble glutamine transaminase K was assayed by the L-phenylalanine-α-keto-γ-methiolbutyrate transaminase reaction [5]. L-Amino acid oxidase was assayed by the semicarbazone trapping technique of Hafner and Wellner; reaction mixtures contained L-amino acid oxidase (1–500 μg), catalase (10 μg), 100 mM semicarbazide and 100 mM Tris–HCl buffer (pH 8.1) in a final volume of 1.0 ml [8]. The rate of increase of absorbance at 248 nm was continuously monitored, and the extinction coefficient for each α-keto acid semicarbazone was assumed to be 1.07×10^4 [8]. (The semicarbazone technique cannot be used with D-amino acid oxidase. In the presence of 140 mM semicarbazide (25°; pH 8.2), enzyme activity is lost with a $T_{\frac{1}{2}}$ of 4 min.) "Gabase" (i.e. GABA transaminase and succinate dehydrogenase) was assayed by a modification of the GABA analysis procedure of Duffy *et al.* [9]. The reaction mixture (1 ml) contained 100 mM Tris–HCl buffer (pH 8.5), 10 mM 2-mercaptoethanol, 1 mM $NADP^+$, 1 mM α-ketoglutarate and 1 mM GABA; the rate of NADPH formation was measured by determining the rate of increase of absorbance at 340 nm. Asparagine-pyruvate [10], tyrosine-α-ketoglutarate [11], ornithine-α-ketoglutarate [12] and branch-chain-α-ketoglutarate transaminases [13] were assayed as described. Glutamine transaminase L was assayed by utilizing L-albizziin in place of glutamine [14]. L-Glutamate was determined spectrophotometrically with glutamate dehydrogenase by the method of Lowry and Passonneau [15]. All spectrophotometric measurements were carried out using a Zeiss QM 3 spectrophotometer.

In vivo *experiments with β-methylene-DL-aspartate.* Adult male mice (29–31 g; CD-1 strain) were purchased from the Charles River Breeding Colony, New York, and were allowed food and water *ad lib.* throughout the duration of the experiment. The mice were injected with 100 mM β-methylene-DL-aspartate in 0.9% sodium chloride. Controls were injected with saline only. The mice were killed by cervical dislocation. Brain, liver, kidneys, heart and thigh muscle were removed and homogenized in 5 vol. of ice-cold 5 mM potassium phosphate buffer (pH 7.2). Skeletal muscle was disrupted using a polytron homogenizer; other tissues were disrupted using a Potter–Elvehjem homogenizer. The homogenates were freeze-thawed twice and further diluted 5-fold with ice-cold water. After low-speed centrifugation (5000 *g*) for 15 min, aliquots of the supernatant fluid (1–5 μl) were assayed for glutamate-aspartate transaminase at 25° as described above.

Preparation of rat liver and kidney homogenates for various transaminase determinations. Adult male Sprague–Dawley rats were decapitated and exsanguinated. The organs were removed and homogenized in 5–25 vol. of ice-cold 10 mM potassium phosphate buffer (pH 7.2). The homogenate was freeze-thawed and centrifuged at 20,000 *g* for 1 hr, and the precipitate was discarded.

Preparation of rat brain homogenates for GABA transaminase and glutamate decarboxylase determinations. Brain extracts were prepared according to the method of Jung *et al.* [16]. Brain tissue was homogenized in a 10-fold excess of ice-cold glycerol (20%; v/v), Triton X-100 (0.13%; v/v), glutathione (100 μM), pyridoxal-P (100 μM), EDTA (1 mM) and 100 mM potassium phosphate buffer (pH 6.8). GABA transaminase was assayed by the coupled succinic semialdehyde dehydrogenase assay; the rate of NADH production was determined spectrophotometrically during the linear portion of the reaction [16]. Glutamate decarboxylase was assayed by the method of Jung *et al.* [16] (in which $^{14}CO_2$ production from L-[1-^{14}C]glutamate is measured), except that the volume of homogenate was 10–25% of that utilized by Jung *et al.*

Statistical analysis. In Tables 2–4, values are expressed as the means ± S.E. Significance values were determined using the two-tailed Mann–Whitney *U* test.

RESULTS

Inactivation of soluble pig heart glutamate-aspartate transaminase by β-methylene-DL-aspartate. Figure 1 shows that the enzyme was rapidly inactivated when incubated with β-methylene-DL-aspartate at 25° (pH 7.2). The β,γ-double bond appears to be essential for inactivation; no loss of activity was noted on prolonged incubation (90 min) with γ-methylene-DL-glutamate (18 mM) or with DL-β-

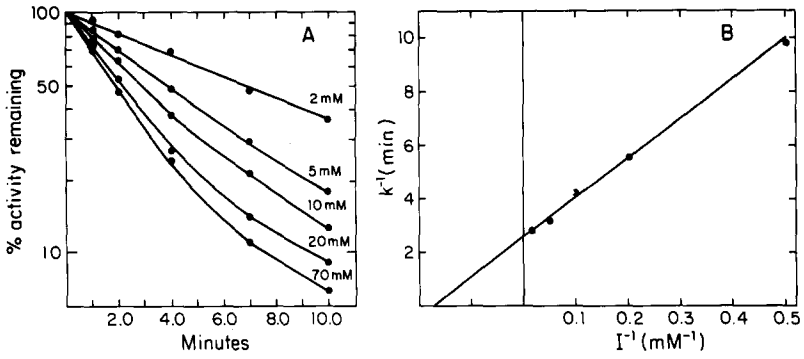

Fig. 1 (A) Inactivation of glutamate-aspartate transaminase in the presence of various concentrations of β-methylene-DL-aspartate. Enzyme (5 μg) was incubated in 0.1 ml of 100 mM potassium phosphate buffer (pH 7.2), containing various concentrations of β-methylene-DL-aspartate, 25°. At the times indicated, aliquots were withdrawn and immediately added to 0.8 ml of 100 mM potassium aspartate in 100 mM potassium phosphate buffer (pH 7.2) in order to quench inactivation. Residual activity was then determined by adding malate dehydrogenase, NADH and finally α-ketoglutarate to initiate the transaminase reaction. (B) Plot of reciprocal pseudo first-order rate constant (k^{-1}) versus reciprocal of β-methylene-DL-aspartate concentration [I^{-1}].

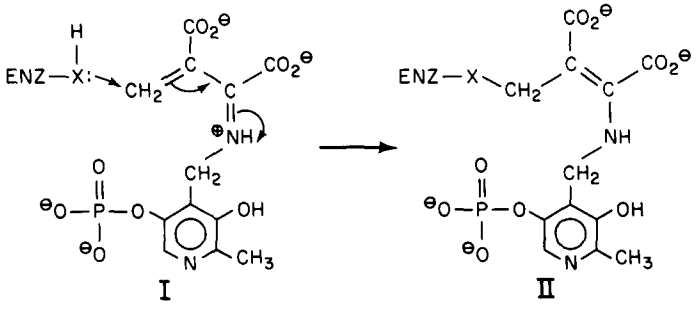

Fig. 2. Proposed mechanism for the inactivation of glutamate-aspartate transaminase by β-methylene-DL-aspartate (taken from Ref. 2). β-Methylene-L-aspartate reacts with pyridoxal-P at the active site to yield the ketimine (I). Nucleophilic attack on the methylene carbon of the conjugated ketimine (an excellent Michael acceptor) by a juxtaposed nucleophile leads to an inactive bridged structure (an enamine) (II). The enamine can then undergo double bond migration to an inactive ketimine.

methyl-DL-aspartate (40 mM).* From the data presented in Fig. 1, in which the reciprocal of the pseudo first-order rate constant (obtained from a semi-log plot of activity against time) has been plotted against the reciprocal of β-methylene-DL-aspartate concentration [17], we calculated that the K_i was about 6 mM and that the limiting rate constant for inactivation was about 0.4 min^{-1}.

The mechanism proposed for inactivation is shown in Fig. 2 [2]. The mechanism is similar to that originally proposed for the inactivation of glutamate-aspartate transaminase by DL-vinylglycine [18] and to that favored for inactivation of bacterial D-amino

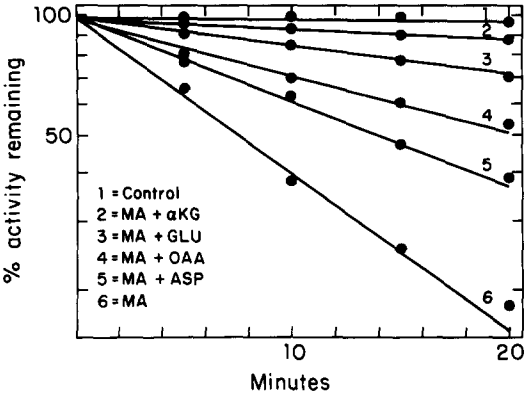

Fig. 3. Protection against inactivation by β-methylene-DL-aspartate with natural substrates. The reaction mixtures contained glutamate-aspartate transaminase (5 μg), 1.8 mM β-methylene-DL-aspartate and 2 mM substrate in 0.1 ml of potassium phosphate buffer (pH 7.2), 25°. At intervals, 2 μl portions were withdrawn and assayed as described in the legend to Fig. 1. Abbreviations: MA, β-methylene-DL-aspartate; OAA, oxaloacetate; GLU, glutamate; ASP, aspartate; and αKG, α-ketoglutarate. Control lacked both β-methylene-DL-aspartate and substrate.

* In fact, these compounds are poor substrates of glutamate-aspartate transaminase. Paper chromatography studies, using the ascending technique described in Experimental Procedures, revealed that transamination between 40 mM DL-β-methyl-DL-aspartate (or 18 mM γ-methylene-DL-glutamate) and 10 mM oxaloacetate was detectable but that it occurred at <1% the corresponding rate with 20 mM glutamate. Paper chromatography studies also revealed a slow transamination between 18 mM γ-methylene-DL-glutamate and 10 mM α-ketoglutarate.

acid transaminase by DL-vinylglycine [17]. Evidence in favor of this mechanism was obtained as outlined below.

Appreciable partitioning between inactivation and complete turnover in the presence of β-methylene-DL-aspartate was not detected. Enzyme (5 mg) was incubated with 2.0 mM β-methylene-DL-aspartate and 0.2 mM α-ketoglutarate in 0.7 ml of 100 mM potassium phosphate buffer (pH 7.2) for 24 hr at 23°. The solution was deproteinized by adding 0.2 ml of 3 M perchloric acid, followed by neutralization of the supernatant fluid with 2 M sodium bicarbonate. No glutamate was found in the neutralized extract. One complete turnover per fifty inactivation events could have been detected. That α-ketoglutarate (or oxaloacetate) did not accelerate the inactivation process (Fig. 3) also argues against appreciable transamination to pyridoxamine-P enzyme. Both oxaloacetate and α-ketoglutarate actually protected the enzyme against inactivation as did glutamate and aspartate (Fig. 3). The protective effects of α-ketoglutarate and oxaloacetate may be understood in terms of the well known observation that α-ketoglutarate [19–21] and oxaloacetate [19, 21] are able to form an abortive (dead-end) complex with the pyridoxal-P form of the enzyme. Also, α-ketoglutarate enhances inactivation of glutamate-aspartate transaminase by L-cysteine sulfinate but protects against inactivation by L-serine-*O*-sulfate [22].

The proposed mechanism of inactivation of glutamate-aspartate transaminase by β-methylene-DL-aspartate suggests that inactivated enzyme contains pyridoxamine-P in a Schiff's base linkage. Evidence for such a linkage was obtained as follows. Enzyme (750 μg) was separately dissolved in 20 μl of 100 mM potassium phosphate buffer (pH 7.2) containing (a) no addition or (b) 8.8 mM β-methylene-DL-aspartate. After incubation at 37° for 2 hr in a stoppered tube, the pyridoxal-P content of the enzyme was determined by the phenylhydrazone method [23]. The β-methylene-DL-aspartate-treated enzyme contained only 8% of the pyridoxal-P initially present in the enzyme. Similarly, a value of 5% pyridoxal-P was found after conversion of enzyme to the pyridoxamine-P form by treatment with 7 mM cysteine sulfinate (cf. Ref. 24). In another experiment, 5 mg of enzyme was incubated at 37° in a reaction mixture (50 μl) containing 5 mM potassium phosphate buffer (pH 7.2) and 1.2 mM β-methylene-DL-aspartate. After 6 hr, 4 μl of 1% picric acid was added. The sample was centrifuged, and all of the clear supernatant fluid was applied as a single spot (5 μl at a time) to Whatman No. 1 paper. After ascending chromatography in solvent system I, pyridoxamine-P was detected by its fluorescence under u.v. light and as an orange–gold spot with ninhydrin spray reagent, with an R_f of 0.14 (R_f pyridoxal-P, 0.37; R_f picric acid, 0.95). Similarly, pyridoxamine-P was detected in samples of glutamate-aspartate transaminase treated with 5 mM cysteine sulfinic acid but not in controls. It may be noted that pyridoxamine-P was found following paper electrophoresis of glutamate-aspartate transaminase inactivated with L-2-amino-4-methoxy-*trans*-3-butenoic acid [25].

Inactivation of glutamate-aspartate transaminase by β-methylene-DL-aspartate leads to a marked change in the spectrum of the enzyme [2]. Thus, a solution of 2.4 mg of enzyme in 1 ml of 100 mM potassium phosphate buffer (pH 6.2) showed absorbance at 435 nm of 0.175 and absorbance at 335 nm of 0.070. Following incubation with 2.0 mM β-methylene-DL-aspartate for 5 hr at 25°, the absorbance at 435 nm against a blank lacking enzyme decreased to 0.051, and a new peak appeared with an absorbance of 0.240 at 335 nm. Similar spectral changes were noted after inactivation of glutamate-aspartate transaminase by DL-vinylglycine [18].

Inability to detect α-keto acid formation in rat tissue homogenates containing β-methylene-DL-aspartate. Tissue extracts were prepared and γ-cystathionase activity was assayed in these extracts with L-homoserine as substrate according to the method of Greenberg [26]. Thus, brain, liver and kidney were separately homogenized in 10 vol. of ice-cold 100 mM potassium phosphate buffer (pH 7.2). Following low speed centrifugation (10,000 *g*), the supernatant fluids were assayed for their ability to convert 10 mM L-homoserine to α-ketobutyrate [26]. In order to determine whether β-methylene-DL-aspartate is a substrate of γ-cystathionase (or of other enzymes, such as threonine deaminase, which are capable of catalyzing double bond migration), the following experiment was carried out. Reaction mixtures (0.1 ml) containing supernatant fluid (10 μl) and 20 mM β-methylene-DL-aspartate in 100 mM potassium phosphate buffer (pH 7.2) were incubated at 37° for 6 hr; 0.1% 2,4-dinitrophenylhydrazine in 2 M HCl (0.05 ml) was then added and, after a further 20 min at 37°, 0.85 ml of 1.2 M KOH was added and the absorbance at 430 nm was determined against a blank lacking β-methylene-DL-aspartate. No increase in absorbance relative to the blank was noted for any of the tissues. ($E_{430\,nm}$ for a typical α-keto acid is $\sim 1.5 \times 10^4$.) Conditions were such that a rate of α-keto acid formation from β-methylene-DL-aspartate as low as 0.001% of the rate of α-ketobutyrate formation from L-homoserine would have been detected in liver homogenates. It should be noted that failure to detect α-keto acid is not proof of non-binding of β-methylene-DL-aspartate to γ-cystathionase or to threonine deaminase. It is possible that the resulting enamine is more stable than that derived from homoserine and hydrolysis or attack at the α-carbon by 2,4-dinitrophenylhydrazine is not favored.

Effect of thiol nucleophiles on the rate of inactivation of glutamate-aspartate transaminase by β-methylene-DL-aspartate. Thiols add readily to activated double bonds and, therefore, have the potential of affecting the rate of enzyme inactivation by a β,γ-unsaturated substrate. Examples of acceleration, deceleration and no change in rate of inactivation are all known [27, 28]. The $T_{\frac{1}{2}}$ for inactivation of glutamate-aspartate transaminase in the presence of 40 mM β-methylene-DL-aspartate was 2.6 min (pH 7.2; 25°). When 60 mM 2-mercaptoethanol, dithiothreitol or glutathione was included separately, the $T_{\frac{1}{2}}$ values were 90, 45 and 3.6 min respectively (Fig. 4). The very slight protection found with glutathione suggests that inactivation did not

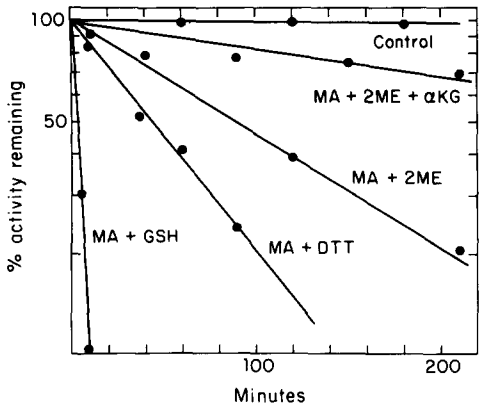

Fig. 4. Effect of sulfhydryls on the rate of inactivation by β-methylene-DL-aspartate. The reaction mixtures contained glutamate-aspartate transaminase (5 μg), 40 mM β-methylene-DL-aspartate (MA) and 60 mM sulfhydryl-containing compound in 0.1 ml of 100 mM potassium phosphate buffer (pH 7.2), 25°. At intervals 2 μl aliquots were withdrawn and assayed as described in the legend to Fig. 1. Abbreviations: 2ME, 2-mercaptoethanol; DTT, dithiothreitol; GSH, glutathione; and αKG, α-ketoglutarate (4 mM). The $T_{\frac{1}{2}}$ for inactivation of enzyme in a reaction mixture containing β-methylene-DL-aspartate, but lacking sulfhydryl-containing compounds, was 2.6 min (curve not shown).

occur following release of a reactive species from the active site. The data suggest that Michael addition of 2-mercaptoethanol to structure I (Fig. 2) competed effectively with addition of enzyme-X. Presumably, dithiothreitol was less efficient than 2-mercaptoethanol because it was bulkier; glutathione was too large to interact effectively at the active site. It is interesting to note that β-mercaptoethylamine protects D-amino acid transaminase against inactivation by β-chloro-D-alanine, presumably by reacting at the enzyme active site with the unsaturated aminoacrylate intermediate; glutathione is much less effective [28]. It is notable that protection by α-ketoglutarate and 2-mercaptoethanol was additive (compare Figs. 3 and 4).

It has been reported recently that, at pH 8.3, cysteine and cysteamine readily undergo Michael addition with γ-methyleneglutamate [29]. We carried out similar experiments with 2-mercaptoethanol; 10 mM DL-γ-methylene-glutamate was incubated for 24 hr with 100 mM 2-mercaptoethanol under nitrogen in 100 mM potassium phosphate buffer (pH 7.0) or in 100 mM sodium borate buffer (pH 8.6), 25°. An intense new ninhydrin-positive spot (R_f = 0.35, solvent 1, Table 1) was detected following paper chromatographic analysis of the pH 8.6 incubation mixture; no new amino acid was detected in the pH 7.0 incubation mixture. When a similar experiment

Table 1. Paper chromatography of β-methylene-DL-aspartate and reference amino acids*

Amino acid	$R_f \times 100$ Solvent 1	Solvent 2
β-Methylene-DL-aspartate	42	36
DL-β-Methyl-DL-aspartate	45	40
L-Aspartate	37†	35†
L-α-Aminobutyrate	69	62
L-Alanine	57†	49†
L-Glutamate	45†	42†
γ-Methylene-DL-glutamate	54	42

* Solvent 1: methyl ethyl ketone–*tert*-butyl alcohol–formic acid–water (40:30:15:15). Solvent 2: *n*-butyl alcohol–acetic acid–water (50:25:25).
† In agreement with Ref. 4.

was carried out with 10 mM β-methylene-DL-aspartate and 100 mM 2-mercaptoethanol, traces of two new amino acids (R_f = 0.26 and 0.56) were detected in the pH 8.6 incubation mixture and no new amino acid was detected in the pH 7.0 incubation mixture. These findings suggest that the double bond of β-methylene-DL-aspartate did not undergo facile Michael addition but became more reactive when the β-methylene-DL-aspartate was transformed to a ketimine structure during processing at the active site of glutamate-aspartate transaminase (Fig. 2).

Interaction of β-methylene-DL-aspartate with other pyridoxal-P-containing enzymes. β-Methylene-DL-aspartate did not inactivate pig heart glutamate-alanine transaminase even after prolonged incubation at a concentration of 20 mM (with or without 20 mM pyruvate). L-Aspartate is a poor inhibitor of pig heart glutamate-alanine transaminase and has a large dissociation constant [30]. However, it is a substrate, albeit a poor one (0.13% relative to alanine; 0.16% relative to glutamate; Ref. 30). β-Methylene-DL-aspartate is not a substrate of pig heart glutamate-alanine transaminase; no alanine was detected by paper chromatographic analysis of a reaction mixture containing 20 mM β-methylene-DL-aspartate, 20 mM sodium pyruvate, 100 mM potassium phosphate buffer (pH 7.1), and 50 μg of enzyme in a final volume of 0.1 ml incubated for 10 hr at 23° (data not shown). A rate of 0.001% relative to glutamate would have been detected. In contrast, DL-vinylglycine has been found to be a substrate of pig heart glutamate-alanine transaminase [17] at about 1% the rate exhibited with L-alanine. This rate is similar to the relative rate (1.4%) exhibited with L-α-aminobutyrate, the fully saturated analog of vinylglycine [30].

Other pyridoxal-P-containing enzymes which are not inactivated by β-methylene-DL-aspartate include soluble glutamine transaminase K, *E. coli* glutamate decarboxylase, and *P. fluorescens* GABA transaminase.* In addition, when rat organ homogenates were incubated at 25° for 1 hr in the presence of 10 mM potassium phosphate (pH 7.2) and 10 mM β-methylene-DL-aspartate, no significant decrease relative to a control lacking β-methylene-DL-aspartate was noted for the following enzyme activities: glutamine transaminase L (liver), asparagine trans-

* β-Methylene-DL-aspartate is neither a substrate nor an irreversible inhibitor of bacterial D-amino acid transaminase (T. E. Soper and J. M. Manning, personal communication) or of L-aspartase from *Bacillus cadaveris* (A. J. L. C., unpublished data).

Table 2. Inactivation of mouse kidney glutamate-aspartate transaminase following intraperitoneal injection of β-methylene-DL-aspartate (2 mmoles/kg)*

Time after injection (hr)	Activity [μmoles · min^{-1} · (g wet wt)$^{-1}$]	N	% Control
Control	55.5 ± 2.4	9	100
6	33.6 ± 2.2†	9	61
24	49.1 ± 3.7	6	88

* β-Methylene-DL-aspartate was injected intraperitoneally. At the intervals shown, animals were killed and enzyme activities were determined as described in the text: N = the number of animals killed in each group.
† Different from control values with $P < 0.001$.

Table 3. Effect on glutamate-aspartate transaminase *in vivo* in mice injected intraperitoneally with β-methylene-DL-aspartate (5 mmoles/kg)*

Tissue	Activity [μmoles · min^{-1} · (g wet wt)$^{-1}$]	N	P
Brain (control)	52.5 ± 2.2	6	
Brain + βMA	55.5 ± 4.0	6	NS†
Liver (control)	87.8 ± 3.7	6	
Liver + βMA	67.3 ± 5.6	5	<0.01
Kidney (control)	85.2 ± 4.5	6	
Kidney + βMA	51.0 ± 4.8	6	<0.0005
Heart (control)	171.6 ± 5.3	6	
Heart + βMA	173.0 ± 4.5	6	NS
Skeletal muscle (control)	68.5 ± 2.5	6	
Skeletal muscle + βMA	63.8 ± 3.8	6	NS

* Mice were killed after 6 hr, and glutamate-aspartate transaminase activity was determined as described in Experimental Procedures; N = number of separate tissues analyzed. βMA, β-methylene-DL-aspartate.
† Not significant.

Table 4. Effect of β-methylene-DL-aspartate on several transaminase activities *in vivo**

Tissue	Transaminase	Activity [μmoles · min^{-1} · (g wet wt)$^{-1}$]	N	P
Liver	Glutamate-alanine (control)	16.7 ± 1.0	6	
	Glutamate-alanine + βMA	16.8 ± 1.3	5	NS†
	Tyrosine (control)	2.00 ± 0.41	6	
	Tyrosine + βMA	7.69 ± 0.36	5	<0.0001
	Asparagine (control)	0.44 ± 0.05	6	
	Asparagine + βMA	0.72 ± 0.08	5	<0.01
Kidney	Glutamine (control)	2.76 ± 0.10	6	
	Glutamine + βMA	2.57 ± 0.19	6	NS

* Mice were injected intraperitoneally with β-methylene-DL-aspartate (5 mmoles/kg), and 6 hr later the animals were killed. The enzyme activities were determined as described in Experimental Procedures; N = number of animals in each group. βMA, β-methylene-DL-aspartate-treated group.
† Not significant.

aminase (liver), ornithine transaminase (liver), branch-chain (i.e. valine, leucine and isoleucine) transaminase (kidney) and GABA transaminase (brain) (data not shown).

Although β-methylene-DL-aspartate has no effect on purified pig heart glutamate-alanine transaminase, it does appear to inhibit rat liver glutamate-alanine transaminase in homogenates (but not *in vivo*, see below). Rat liver homogenates (50 μl) were incubated at 25° in 100 μl of potassium phosphate buffer (pH 7.2) containing 5 mM β-methylene-DL-aspartate. At intervals, portions (1–5 μl) were withdrawn and assayed for glutamate-alanine and glutamate-aspartate transaminase activities; the times to reach 50% inactivation were 16 and 4 min respectively. Addition of pyruvate, α-ketoglutarate or glutamate to the mixture resulted in a decrease in the rate of inactivation of glutamate-alanine transaminase; however, addition of L-alanine enhanced the rate of inactivation (data not shown). β-Methylene-DL-aspartate also appeared to inactivate rat brain glutamate decarboxylase. Portions (20 μl)

Table 5. Interaction of β-methylene-DL-aspartate and related amino acids with L-amino acid oxidase*

Amino acid	Concn (mM)	Initial velocity (μmoles·hr^{-1}·mg^{-1})	K_m (mM)	Ref.
DL-Vinylglycine	40	1090		38
DL-Vinylglycine†		660‡	8	37
L-Aspartate		0.85‡	330	Present work
β-Methylene-DL-aspartate	80	<0.01		Present work
DL-β-Methyl-DL-aspartate	40	<0.02		Present work

* Except where indicated the reaction was carried out at 23° and the rate of semicarbazone formation was measured [8].

† O_2 consumption was measured at 25°.

‡ V_{max} values.

of rat brain homogenates were incubated in 100 μl of 100 mM potassium phosphate buffer (pH 7.2) containing 5 mM β-methylene-DL-aspartate at 37° and, at intervals, were assayed for glutamate decarboxylase. Activity was lost, with a $T_{\frac{1}{2}}$ of ~60 min compared to a control lacking inhibitor. (Under the same conditions, 50% loss of glutamate-aspartate transaminase activity occurred after 5 min.)

Inactivation of mouse kidney and liver glutamate-aspartate transaminase following intraperitoneal injection of β-methylene-DL-aspartate. Mice were injected with β-methylene-DL-aspartate (2 mmoles/kg); separate groups were killed at 6 and 24 hr, and total (i.e. soluble plus mitochondrial) glutamate-aspartate transaminase activities in brain, liver and kidney were determined.* A highly significant decrease in kidney glutamate-aspartate transaminase was found 6 hr after intraperitoneal administration of β-methylene-DL-aspartate, but the activity approached the control value by 24 hr (Table 2). No decrease of brain glutamate-aspartate transaminase activity was observed at 6 and 24 hr. Liver activity was consistently decreased 10% at 6 hr but not at 24 hr.

In a separate experiment, mice were injected with β-methylene-DL-aspartate (5 mmoles/kg) and killed after 6 hr.* The activity of total glutamate-aspartate transaminase decreased by 40% in kidney and 23% in liver (Table 3). No significant decrease in brain, skeletal muscle or heart glutamate-aspartate transaminase activity was found. The greater inhibition of kidney enzyme compared to the liver enzyme may have been due to more facile transport of β-methylene-DL-aspartate into the kidney cell. For example, it is known that kidney slices take up L-[U-^{14}C]glutamate more readily than do liver slices [31]. The lack of inhibition of mouse brain glutamate-aspartate transaminase may have been due to exclusion of β-methylene-DL-aspartate at the blood–brain barrier. (L-Aspartate does not readily cross the blood–brain barrier [32].) However, β-methylene-DL-aspartate *can* effectively inactivate rat brain glutamate-aspartate transaminase if the blood–brain barrier is circumvented.

* No obvious behavioral differences between mice injected with β-methylene-DL-aspartate and controls were discernible.

It was important to ascertain whether or not intraperitoneal injection of β-methylene-DL-aspartate affects protein synthesis directly or indirectly. Therefore, several transaminase activities were determined in tissues from the same group of mice in which a decrease in kidney and liver glutamate-aspartate transaminase activity had been found. In no other case was a decrease in activity noted (Table 4), suggesting that β-methylene-DL-aspartate was not a direct protein synthesis inhibitor *in vivo*. No significant change in activity of kidney glutamine transaminase K or of liver glutamate-alanine transaminase was noted; both tyrosine and asparagine transaminase activities were elevated (Table 4). The very large increase in tyrosine transamine activity was not anticipated. However, it is well known that tyrosine transaminase activity can be greatly increased by a variety of metabolic manipulations (see Refs. 33 and 34). The change in tyrosine and asparagine transaminase activities suggests that β-methylene-DL-aspartate caused profound metabolic changes *in vivo* which indirectly affected synthesis or degradation (or both) of certain enzymes.

Studies with D- and L-amino acid oxidases. L-Aspartate is a poor substrate of L-amino acid oxidase [35, 36] whereas DL-vinylglycine is a good substrate [37, 38] with a high V_{max} (Table 5). DL-Vinylglycine also irreversibly inactivates L-amino acid oxidase [37, 38]. Reported $T_{\frac{1}{2}}$ values for inactivation are 4 min (borate buffer, pH 8.0, 23°; semicarbazone assay) [38] and 3.4 min (Tris buffer, pH 8.0, 25°; oxygen uptake assay) [37] at DL-vinylglycine concentrations of 40 and 6.25 mM respectively. β-Methylene-DL-aspartate may be regarded as a substituted aspartate and also as a substituted vinylglycine. It was of interest, therefore, to determine the substrate characteristics of β-methylene-DL-aspartate (Table 5). Based upon the considerations discussed above, one might have expected β-methylene-DL-aspartate to be a moderately good substrate and to inactivate L-amino acid oxidase more slowly than vinylglycine. However, no activity toward 80 mM β-methylene-DL-aspartate or toward 40 mM DL-β-methyl-DL-aspartate was detected (Table 5). Furthermore, no inactivation of L-amino acid oxidase was noted after incubation with 80 mM β-methylene-DL-aspartate for 1 hr at pH 8.1, 25° (data not shown).

Relative to longer-chain aliphatic amino acids, D-aspartate [39] and DL-vinylglycine were moder-

ately good substrates of D-amino acid oxidase and, unlike L-amino acid oxidase, D-amino acid oxidase is not inactivated by DL-vinylglycine [37]. With 2 mM β-methylene-DL-aspartate, we were unable to detect any product formation or inactivation of enzyme over a period of 24 hr at 25°. Thus, in a reaction mixture (0.1 ml) containing 50 mM 2-mercaptoethanol, 2 mM β-methylene-DL-aspartate, 25 μg D-amino acid oxidase, 5 μg catalase and 100 mM Tris–HCl buffer (pH 8.2), no α-keto acid was detected (as the 2,4-dinitrophenylhydrazone; see Ref. 38). (Apparently, vinylglyoxylate, or its imino analogue, rapidly polymerizes but can be trapped with mercaptoethanol [38]. Therefore, 2-mercaptoethanol was included to trap any product as a precaution against polymerization and loss of carbonyl function.) Parallel experiments with DL-β-methyl-DL-aspartate also failed to yield a discernible product.

DISCUSSION

Glutamate-aspartate transaminase is not completely inactivated by β-methylene-DL-aspartate. The 3–6% activity remaining accounts for the slight deviation from monophasic kinetics seen in Fig. 1. Gehring *et al.* [40] have noted that inactivation of mitochondrial and soluble glutamate-aspartate transaminases by DL-vinylglycine is not complete; ~10% residual activity remains. However, in the presence of α-ketoglutarate, pseudo first-order kinetics are observed over the entire inactivation process, suggesting that residual activity in the absence of α-ketoglutarate is due to the pyridoxamine-P form of the enzyme [40]. In the present experiments, the residual activity could not have been due to traces of pyridoxamine-P enzyme; residual activity was still apparent in the presence of 2 mM α-ketoglutarate. We should point out that the commercial enzyme used in these studies was prepared by a method that involves a step requiring a maleate buffer. Martinez-Carrion *et al.* [41] have shown that this treatment may result in some alkylation of enzyme. While it is possible that the residual activity resulted from some modification with maleate buffer, the virtually complete inactivation (≥99.5%) in the presence of 20 mM GSH makes this idea seem unlikely. Birchmeier *et al.* [42] have shown that pig heart glutamate-aspartate transaminase possesses five sulfhydryls per monomer. Three sulfhydryls (I–III) are external, while two (IV and V) are buried. Some of these sulfhydryls have subtle but not essential roles in the catalytic processes [42]. Under certain conditions (NEM treatment, followed by low concentrations of thiols such as mercaptoethanol or GSH), sulfhydryl III can be induced to form disulfide linkages [42]. Residual activity (5–17%) of sulfhydryl III-modified enzyme was noted [42]. The present findings of (a) residual activity with β-methylene-DL-aspartate-treated enzyme and (b) decreased residual activity in the presence of excess GSH also suggest a subtle interaction of sulfhydryls during the inactivation process with β-methylene-DL-aspartate. We note that, although strict pseudo first-order kinetics are often stated as a criterion for inactivation of an enzyme by a suicide inhibitor, there are many apparent examples in the literature where biphasic loss of activity or residual activity is observed. In fact, highly purified non-maleate-treated mitochondrial and soluble forms of rat brain glutamate-aspartate transaminase are inactivated by vinylglycine in a non-monoexponential fashion [43]. The rat brain soluble and mitochondrial preparations contain ~20% and ~8%, respectively, of a component resistant to inactivation by vinylglycine. Similarly, a 1% residual activity has been reported for DL-vinylglycine-inactivated bacterial D-amino acid transaminase [17]. The residual activity of this enzyme may possibly be due to alkylated enzyme in which the enzyme contains pyridoxamine-P bound in modified Schiff's base linkage which is still catalytically active [17]. Johnston *et al.* [44] noted biphasic inactivation of bacterial cystathione γ-synthase and bacterial methionine γ-lyase by propargylglycine. Deviation from monophasic kinetics has also been noted for inactivation of D-amino acid transaminase by gabaculine [45] and inactivation of glutamate decarboxylase (*E. coli*) by (RS)-(fluoromethyl)glutamate [46]. Burnett *et al.* [47] have noted 1–3% residual activity of propargylglycine-inactivated glutamate-alanine transaminase. It appears that 97% inactivation is associated with incorporation of one mole of propargylglycine per enzyme dimer. Modification of one subunit apparently alters binding characteristics of the other subunit so that the non-alkylated monomer processes alanine at a much slower rate and is then no longer susceptible to inactivation by propargylglycine [47]. Johnston *et al.* speculate that "the kinetics of inactivation reactions induced either by affinity labels or by suicide substrates may be rather more complicated (and revealing?) than hitherto suspected" [44]. In any case, the explanation for the residual activity in the various preparations of glutamate-aspartate transaminase must await further study.

The present findings, that (a) no turnover of glutamate-aspartate transaminase in the presence of β-methylene-DL-aspartate and α-ketoglutarate could be detected and that (b) α-ketoglutarate slowed the rate of inactivation, are similar to the findings of Soper *et al.* [17] and of Gehring *et al.* [40]. Soper *et al.* [17] showed that α-ketoglutarate does not increase the rate of inactivation of soluble glutamate-aspartate transaminase by DL-vinylglycine; Gehring *et al.* [40] showed that α-ketoglutarate actually slows the rate of inactivation of both soluble and mitochondrial glutamate-aspartate transaminase by DL-vinylglycine. Moreover, inactivation is accompanied by <0.2 turnover events per mole of enzyme inactivated [17, 40]. The findings with vinylglycine- and β-methyleneaspartate-inactivated glutamate-aspartate transaminase are in marked contrast to the findings obtained with bacterial D-amino acid transaminase. Thus, in the presence of DL-vinylglycine, bacterial D-amino acid transaminase turns over hundreds of times for each inactivation event and the presence of α-keto acid substrate markedly increases the rate of inactivation [17].

It should be noted that the original proposal for the mechanism of inactivation of soluble glutamate-aspartate transaminase by vinylglycine involved Michael addition to the γ-carbon of a conjugated ketimine [18]. [We favor such a mechanism

for the inactivation of glutamate-aspartate transaminase by β-methylene-DL-aspartate (Fig. 2).] However, it was subsequently found, following denaturation of vinylglycine-inactivated enzyme, that pyridoxal-P, and not the expected pyridoxamine-P, was released [40]. Moreover, when enzyme was inactivated with [1-^{14}C]vinylglycine and then chemically and enzymatically degraded, a peptide that contained radioactivity in an ε-nitrogen adduct of lysine was obtained; electrophoretic data suggested that the adduct also contained the α-amino group of vinylglycine [40]. Therefore, Gehring *et al.* [40] suggested that inactivation occurs following a 1,3-prototropic shift after formation of the initial aldimine intermediate and an α-CH bond cleavage; the resulting conjugated enamine is an electrophilic Michael acceptor which alkylates the ε-amino group of an active site lysine. (However, Morino *et al.* [48] found that glutamate-aspartate transaminase catalyzes elimination of HCl from α-amino-β-chlorobutyrate without concomitant inactivation; presumably, chloride elimination gives rise to an enamine structure idential to that proposed by Gehring *et al.* [40].) Clearly, an enamine intermediate as Michael acceptor cannot account for the present findings that pyridoxamine-P was isolated from β-methylene-DL-aspartate-inactivated enzyme. Moreover, in model systems, Michael addition has been shown to occur on the methylene carbon of amino acids containing a CH_2=C(CO_2H)—linkage [29, and present work].

There appear to be subtle differences between the modes of inactivation of glutamate-aspartate transaminase by DL-vinylglycine and by β-methylene-DL-glutamate. The rate of inactivation by β-methylene-DL-aspartate was slowed in the presence of 2-mercaptoethanol; on the other hand, 2-mercaptoethanol has been reported to have no effect on the rate of inactivation with DL-vinylglycine [18]. It is conceivable that the methylene carbon of the conjugated ketimine (Fig. 2) is more accessible to nucleophilic attack by 2-mercaptoethanol than is the conjugated enamine derived from vinylglycine.

A conjugated ketimine as an intermediate in the inactivation of glutamate-aspartate transaminase by L-2-amino-4-methoxy-*trans*-3-butenoic acid appears to be well established [25, 40]. Gehring *et al.* speculated that the different pathways of inactivation of glutamate-aspartate transaminase by vinylglycine and by L-2-amino-4-methoxy-*trans*-3-butenoic acid may be due to the stabilizing effect of the methoxy group on the β,γ-double bond, decreasing the probability of isomerization of the conjugated ketimine [40]; indeed, studies with model compounds support this conclusion [25, 40]. Similarly, the differences noted above between DL-vinylglycine and β-methylene-DL-vinylglycine inactivation of glutamate-aspartate transaminase may be due to the stabilizing effect of the β-carboxyl on the β,γ-double bond of β-methylene-DL-aspartate. Clearly, further work is needed to elucidate the exact mechanism(s) of inactivation of glutamate-aspartate transaminase by both vinylglycine and β-methylene-DL-aspartate.

Several approaches have been used for the design of irreversible inhibitors of pyridoxal-P enzymes. For example, Severin and colleagues synthesized a series of cyclic internal hydroxamic esters (substituted cycloserines, cycloglutamates) [49, 50] and amino-hydroxamic esters [50] and tested them as irreversible inhibitors of three glutamate (i.e. -aspartate, -alanine, -tyrosine) transaminases and glutamate decarboxylase. Binding strengths and differential inactivation with some of these compounds were pronounced, but none was totally specific for glutamate-aspartate transaminase.* Propargylglycine was shown to inactivate pig heart glutamate-alanine transaminase, but not glutamate-aspartate transaminase [51]. DL-Vinylglycine has been shown to inactivate pig heart glutamate-aspartate transaminase, but not glutamate-alanine transaminase [17]. However, the high binding constant (~100 mM; Ref. 18) and susceptibility to enzymatic conversion to α-keto acid (either by direct oxidation [37] or by double bond migration [37]) preclude vinylglycine as a metabolic inhibitor.

β-Methylene-DL-aspartate was initially developed as an analog of β-methylaspartate in the course of study of the vitamin B-12-dependent β-methylaspartate mutase reaction in bacteria [3]. Since β-methylene-DL-aspartate is structurally related to both vinylglycine and aspartate, it occurred to us that β-methylene-DL-aspartate might be a better inhibitor of glutamate-aspartate transaminase than is DL-vinylglycine. The present work confirms this hypothesis and demonstrates that β-methylene-DL-aspartate also inhibits glutamate-aspartate transaminase *in vivo*. No evidence was found for enzymatic conversion of β-methylene-DL-aspartate to an α-keto acid. Of special importance is the finding that β-methylene-DL-aspartate had no effect on soluble pig heart glutamate-alanine transaminase *in vitro*. The selectivity of β-methylene-DL-aspartate also occurred *in vivo*. Intraperitoneal administration of β-methylene-DL-aspartate into mice resulted in highly significant decreases of liver and kidney glutamate-aspartate transaminase activity (Table 3) but had no effect on liver glutamate-alanine transaminase activity (Table 4). (Glutamate-alanine transaminase activity in kidney was not measured; its activity is very low [52] and difficult to measure accurately.) We have found one case in which glutamate-alanine transaminase is apparently inhibited by β-methylene-DL-aspartate; that is in rat liver homogenates. However, even in this case, glutamate-aspartate transaminase activity decreased several-fold faster than glutamate-alanine transaminase activity.

We were surprised to find that glutamate-alanine transaminase activity was susceptible to inactivation in rat liver homogenates containing β-methylene-DL-aspartate. Purified glutamate-alanine transaminase from rat liver has been reported to exhibit a high degree of specificity toward glutamate, alanine and their respective α-keto acids [53]. Moreover, as noted above, β-methylene-DL-aspartate has no effect on purified pig heart glutamate-alanine transamin-

* One table was presented showing that 10 mM valine-hydroxamic carboxyethyl ester inactivated glutamate-aspartate transaminase by 40% but had no effect on three other pyridoxal-P enzymes [50]. No further details were given.

ase. The observed inhibition of this enzyme in homogenates may reflect a marked difference in structure of the active site between rat liver and pig heart glutamate-alanine transaminases. (It is interesting to note that there are differences in heat stability and immunological properties between rat liver and pig liver glutamate-alanine transaminases [52].) The unexpected increase in the rate of β-methylene-DL-aspartate inactivation of glutamate-alanine transaminase by L-alanine in crude tissue homogenates may be due to non-specific secondary reactions. Whether or not β-methylene-DL-aspartate inactivates rat liver glutamate-alanine transaminase as a true suicide inhibitor is not yet clear. β-Methylene-DL-aspartate also inactivates glutamate decarboxylase in rat brain homogenates but not in the commercial *E. coli* preparation.* This finding is consistent with what is known about the purified mouse brain enzyme [54]. L-Aspartate is a substrate (about 3–5% the rate exhibited with L-glutamate), and both D-glutamate and L-aspartate are strong competitive inhibitors ($K_i = 0.9$ and 3 mM respectively; K_m, glutamate = 0.8 mM) [54]. Inspection of Dreiding models suggests that L-glutamate binds to the active site in a non-extended conformation and that the β-methylene group of β-methylene-DL-aspartate can occupy a region close to that occupied by the 3-carbon (methylene) of glutamate. The present findings suggest that binding studies with β-methylene-DL-aspartate and purified glutamate decarboxylase may yield information concerning the active site of brain glutamate decarboxylase.

Aminooxyacetate is often used to inhibit glutamate-aspartate transaminase *in vitro* (e.g. Ref. 55) but this compound is a non-selective pyridoxal-P enzyme inhibitor, its inhibition is sometimes reversible [56, 57], it can form oximes with α-keto acid substrates [56], and under certain conditions it can be metabolized to glycolate [58]. Smith *et al.* [56] have suggested the use of the suicide inhibitor, 2-amino-4-methoxy-*trans*-3-butenoic acid [cf. Ref. 25] for inhibition of glutamate-aspartate transaminase. Ferré and Williamson [59] later used 2-amino-4-methoxy-*trans*-3-butenoic acid to study the role of glutamate-aspartate transaminase in hepatic gluconeogenesis in suckling newborn rat. More recently, Smith and Freedland [60] have utilized this compound to inhibit glutamate-aspartate transaminase in isolated rat hepatocytes. However, 2-amino-4-methoxy-*trans*-3-butenoic acid is converted to a saturated α-keto acid (presumably in a manner analogous to the conversion of vinylglycine to α-ketobutyrate [37]) by liver threonine (serine) dehydratase (deaminase) [61]. Indeed, Kapke and Davis [61] speculated that one of the functions of the amino acid dehydratases may be to detoxify certain naturally occurring β,γ-unsaturated amino acids. Furthermore, 2-amino-4-methoxy-*trans*-3-butenoic acid is a potent methionine antagonist and competes with methionine carrier systems *in vivo* [62]; the compound is also an inhibitor of ATP:L-methionine *S*-adenosyltransferase (although the *cis*-isomer is more effective) [63]. We believe that, β-methylene-DL-aspartate is probably the most promising inhibitor now available for studying the metabolic consequences of selective inhibition of glutamate-aspartate transaminase.† This is especially true for organ preparations such as rat brain, where glutamate-alanine transaminase activity is very low and where its concomitant inhibition should not be a major problem. Indeed, we have shown recently that, in rat cerebral slices respiring on glucose, β-methylene-DL-aspartate strongly inhibits both glutamate-aspartate transaminase and oxygen consumption [64]. The inhibition of enzyme activity is time dependent and is linearly correlated with the degree of inhibition of oxygen consumption [64].

In summary, β-methylene-DL-aspartate appears to be a reasonably selective inhibitor of glutamate-aspartate transaminase, does not appear to be metabolized to an α-keto acid, and is active against rat liver and kidney glutamate-aspartate transaminase *in vivo*.

Acknowledgements—We wish to thank Dr. Alton Meister for helpful suggestions and Martin Haber for expert technical assistance.

* β-Methylene-DL-aspartate also inactivates rat liver cysteine sulfinate decarboxylase (O. W. Griffith, personal communication).

† Attempts to improve the selectivity of a glutamate-aspartate transaminase inhibitor still further, by synthesizing analogues in which a methylene-H of β-methylene-aspartate is replaced with an alkyl or benzyl group, are currently under way. Aspartates in which the α- or β-carbon contains a bulky substituent bind effectively to glutamate-aspartate transaminase (e.g. α-methyl-, *erythro*-β-hydroxy- and α-hydroxymethylaspartates [21, 65–67]). Therefore, compounds such as β-ethylideneaspartate may still bind effectively to glutamate-aspartate transaminase but not to rat liver glutamate decarboxylase or to rat liver glutamate-alanine transaminase.

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