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INTERACTION OF L-ALANOSINE (NSC 153, 353) WITH ENZYMES METABOLIZING L-ASPARTIC ACID, L-GLUTAMIC ACID AND THEIR AMIDES

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Abstract—A comprehensive analysis has been made of the manner in which L-alanosine [L-2-amino-3-(*N*-nitroso, *N*-hydroxy) aminopropionic acid] interacts with the enzymes responsible for the metabolism of the dicarboxylic amino acids and their amides. The drug impedes the transport of L-aspartic acid and, to a lesser degree, of L-glutamic acid, L-asparagine and L-glutamine by lymphoblasts, *in vitro;* in each of these instances, inhibition is apparently competitive in type. Of the enzymes involved in the metabolism of L-aspartic acid, adenylosuccinate synthetase (EC 6.3.4.4), SAICAR synthetase (5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide synthetase) (EC 6.3.2.6), L-aspartyl tRNA synthetase (EC 6.1.1.12), L-aspartate transcarbamylase (EC 2.1.3.2) and L-aspartate aminotransferase (EC 2.6.1.1) were inhibited by L-alanosine; moreover, each of these enzymes except L-aspartyl tRNA synthetase accepted the antibiotic as substrate, although at substantially diminished rates. Of the enzymes involved in the metabolism of L-glutamic acid, L-alanosine inhibited only L-glutamine synthetase (EC 6.3.1.2) and L-glutamate decarboxylase (EC 4.1.1.15) to a prominent degree; this last enzyme was also capable of decarboxylating L-alanosine. Of the enzymes metabolizing L-asparagine and/or L-glutamine, only the L-glutaminase activity of L-asparagine amidohydrolase (EC 3.5.1.1) (with L-glutamine as substrate) and, to a lesser degree, carbamyl phosphate synthetase II (EC 2.7.2.9) were inhibited by the antibiotic. Although L-alanosine provokes a rise in the concentration of inosinic acid (IMP) *in vitro*, pointing to the conclusion that the drug is capable of inhibiting adenylosuccinate synthetase under these circumstances, no such rise was seen *in vivo* either in tumor or liver. However, 1 and 5 hr after administration L-alanosine depressed hepatic ATP and NAD pools, an effect which indicates that the drug is, in fact, restricting the intracellular concentration of adenine nucleotides. Of the metabolites of L-alanosine formed *in vitro*, α-decarboxy alanosine, α-keto alanosine, α-hydroxy alanosine, alanosyl IMP and *N*-carbamyl L-alanosine did not inhibit adenylosuccinate synthetase to any prominent degree, whereas the metabolite generated by SAICAR synthetase powerfully inhibited this enzyme, with a K_i of 0.3μM. Parenteral therapeutic doses of L-alanosine produced striking increases in the concentrations of L-aspartic acid in tumor and liver as well as of L-aspartic and L-glutamic acids in urine. It is concluded that the *N*-hydroxy, *N*-nitroso functionality of L-alanosine is analogous in structure to the β-carboxyls of L-aspartic and L-glutamic acids, respectively; this analogy permits L-alanosine to be anabolized and catabolized via several of the enzymatic routes which ordinarily operate on these dicarboxylic amino acids.

One recurrent theme in the literature on L-alanosine [L-2-amino-3-(*N*-nitroso, *N*-hydroxy) aminopropionic acid], an amino acid antibiotic elaborated by *Streptomyces alanosinicus*, is that the agent interferes with cellular metabolism by virtue of its similarity to L-aspartic or L-glutamic acid [1–4]. This interference involves inhibition of transport [1], anabolism [1–3], and catabolism of these amino acids in one or more systems and in one or more phyla [2, 5, 6]. To characterize this analogy more fully, a comprehensive analysis has been made of the interaction of L-alanosine with the enzymes acting on, or degrading, the dicarboxylic amino acids and their amides.

MATERIALS AND METHODS

L-Alanosine (NSC 153, 353) was obtained from the Drug Research and Development Branch of the National Cancer Institute, Bethesda, MD. DL[1-^{14}C]- or [3-^{14}C]Alanosine (specific radioactivities 7.1 or 9.06 mCi/mmole, respectively) was supplied by Stanford Research Institute, Menlo Park, CA. L-[4-^{14}C]Aspartic acid, L-[U-^{14}C]asparagine, L-[U-^{14}C]aspartic acid, L-[U-^{14}C]glutamic acid, L-[1-^{14}C]glutamic acid and L-[U-^{14}C]glutamine (specific radioactivities 17.4, 103, 231, 100, 23 and 270 mCi/mmole, respectively) were obtained from the Amersham-Searle Corp., Arlington Heights, IL; [^{3}H]-methyl thymidine (specific radioactivity 1 mCi/0.012 mg), and [^{14}C]uridine-5′-triphosphate (specific radioactivity 350 mCi/mmole) were obtained from the New England Corp., Boston, MA. L-Glutamate oxaloacetate transaminase (GOT) (EC 2.6.1.1) from pig heart (sp. act. 180 I.U./mg, 10 mg/ml), L-glutamate pyruvate transaminase (GPT) (EC 2.6.1.2) (sp. act. 80 I.U./mg, 2 mg/ml), and L-glutamate dehydrogenase (GDH) (EC 1.4.1.3) from liver (sp. act. 120 I.U./mg, 10 mg/ml) were products of Boehringer, NY. Purified L-aspartate transcarbamylase from *Escherichia coli* (sp. act. 266.7

I.U./mg) was a gift from Dr. E. R. Kantrowitz of Harvard University, Cambridge, MA. Purified carbamyl phosphate synthetase from *E. coli* (sp. act. 1.1 I.U./mg of protein) was donated by Dr. J. Villafranca of Pennsylvania State University, University Park, PA. Crystalline L-asparaginase (EC 3.5.1.1) from *E. coli* (sp. act. 340 I.U./mg of protein) was a gift of Merck, Sharp and Dohme Research Laboratories, West Point, PA. L-Aspartase (EC 4.3.1.1) from *Bacillus cadaveris* (sp. act. 0.5 to 1 I.U./mg of protein) and ornithine carbamyl transferase from *Streptomyces faecalis* (sp. act. 600 I.U./mg of protein) were purchased from the Sigma Chemical Co., St. Louis, MO. L-Aspartate-β-decarboxylase (EC 4.1.1.12) from *Alcaligenes faecalis* (sp. act. 77 I.U./mg of protein) was a gift of Dr. Suresh Tate of Cornell University School of Medicine. L-Aspartyl kinase (EC 2.7.2.4) was a partially purified preparation from *E. coli*. L-Asparaginase (EC 3.5.1.1) from *Erwinia carotovora* (sp. act. 500 I.U./mg of protein) was procured from the Microbiological Research Establishment, Porton Down, UK. L-Glutaminase (EC 3.5.1.2) from *Aerobacter aerogenes* (sp. act. 5 I.U./mg of protein) was a gift from Dr. J. Wriston, University of Delaware. Adenosine-5′-triphosphate (ATP), argininosuccinate, *N*-acetyl L-aspartate, L-citrulline, nicotinamide adenine dinucleotide (NAD), its reduced form (NADH), guanosine-5′-triphosphate (GTP), inosinic acid (IMP), α-ketoglutaric acid (α-KG), phosphoribosyl pyrophosphate (PRPP), xanthosine-5′-monophosphate (XMP) and oxaloacetic acid were all products of the Sigma Chemical Co. Fructose-6-phosphate (F-6-P), acetyl CoA and carbamyl phosphate were products of Boehringer, NY. Adenylosuccinate and dithiothreitol (DTT) were purchased from the CalBiochem Co., Gaithersburg, MD. Desamido-NAD was donated by Dr. A. Berg of A. D. Little, Inc., Cambridge, MA. Dowex 1 × 8, 200–400 mesh, formate form was a product of Bio-Rad Laboratories, Richmond, CA. DEAE-cellulose was obtained from Whatman, Inc., Maidstone, UK. Anion exchange resin HA-X4 was a product of the Hamilton Co., Reno, NV. Glass fiber filters (Whatman 34-AH) were obtained from the Arthur Thomas Co., Philadelphia, PA. Versilube F-50 silicon oil was purchased from the Harwich Chemical Corp., Cambridge, MA. Most of the microchemical and radiochemical analyses were carried out in Eppendorf 1600 μl polypropylene centrifuge vessels with secure snap-on lids procured from Brinkman Instruments, Inc., Westbury, NY.

Animals

Male BDF_1 mice, on an *ad lib.* diet of Purina mouse chow, were used in the majority of the studies reported here.

Anion exchange column chromatography

Chromatography of urine samples or reaction mixtures was carried out on an 8 × 150 mm column of Hamilton HA-X4 resin using a series of lithium citrate/HCl buffers of increasing ionic strength for elution [7].

Paper electrophoresis was carried out on Whatman 3M paper moistened with sodium phosphate buffer (pH 7.2) at 2000 V for 1 hr. Ascending paper chromatography was carried out on Whatman 3M paper using butanol–acetic acid–water (2:1:1, by vol.) as solvent.

Preparation of 5-amino-4-imidazole carboxylic acid ribonucleotide (AICOR)

AICOR was prepared by alkaline hydrolysis of 5-amino-4-imidazole carboxamide ribonucleotide (AICAR). One mmole (338 mg) of AICAR (prepared essentially by the method of Shaw and Wilson [8] in the presence of triethyl phosphate [9]) in 6 N sodium hydroxide (1 ml of CO_2-free solution) was refluxed for 4 hr, protected by a soda lime tube, then chilled in an ice bath and diluted with 20 ml of absolute ethanol. After vigorous stirring of the resulting mixture, the supernatant fraction was decanted. The syrupy residue became a solid after 6 triturations with ethanol using 10 ml portions. The solid was dried in a vacuum desiccator over P_2O_5 for 1 hr followed by drying for 72 hr at room temperature; the yield was 168 mg (41 per cent); $u.v._{max}$ (extinction × 10^3): in 0.1 N HCl, 245 nm (8.28), 265 nm (10.34); at pH 7.0, 250 nm (9.59); at pH 13.0, 252 nm (10.46). This material when freshly dissolved was chromatographically and electrophoretically homogeneous.

Analysis

Calc. for $C_9H_{11}N_3O_9P\ Na_3\text{-}8H_2O$: C, 19.68; H, 4.96; N, 7.65; P, 5.64. Found: C, 19.55; H, 3.42; N, 7.39; P, 5.46.*

Collection of urine for analysis of the excretory levels of the dicarboxylic amino acids

BDF_1 mice, in groups of twenty, were given a single intraperitoneal injection of varying doses of L-alanosine (500, 200 or 125 mg/kg) or of saline, and were transferred to metabolism cages where urine was collected for various time intervals in receptacles chilled by dry ice. Measurements of the dicarboxylic amino acids and their amides were made by spectrophotometric techniques [10] and checked by amino acid analysis [11].

Transport studies

BDF_1 male mice were injected intraperitoneally with 1×10^6 cells of leukemia 5178Y. Seven days later, tumor cells were aspirated from the peritoneum, collected by centrifugation at 1000 *g*, washed twice with Dulbecco's phosphate-buffered saline containing 2% glucose and 0.007% bovine serum albumin, and then suspended in the same medium to achieve a final concentration of 1×10^6 cells/ml. Transport studies were carried out at 37°. To 2 ml of the above cell suspension, 0.05 ml of the labeled amino acid (0.15 to 10.8 nmoles of L-[U-^{14}C]aspartic acid; 0.37 to 24.3 nmoles of L-[U-^{14}C]asparagine; 1.61 to 10.8 nmoles of L-[U-^{14}C]glutamic acid; or 0.68 to 43.6 nmoles of L-[U-^{14}C]glutamine) was added with constant agitation. At 0.3 min intervals, 900 μl aliquots of the cell suspension were layered

* Discrepancies in the elemental analysis of AICOR may be attributable to the spontaneous decomposition of this compound in solution.

over 700 μl of Versilube F-50 silicon oil in Eppendorf test tubes and immediately centrifuged at 12,000 *g* in an Eppendorf centrifuge for 1 min. The apex of the tube, containing the cell pellet, was cut off; the cell pellet was digested in 1.5 ml of 0.2 N NaOH, and then counted by scintillation spectrometry. To study the effects of L-alanosine on the transport of these amino acids, similar incubation mixtures containing 1 or 10 mM L-alanosine were constituted; 2 ml of cell suspension were preincubated for 1 min with the designated concentration of L-alanosine, before the addition of labeled amino acids.

Preparation of organ homogenates

BDF_1 mice were killed by cervical dislocation, and organs were removed, flash frozen, and homogenized (1:3, w/v) in 0.05 M Tris–HCl buffer (pH 8.0) containing 1 mM dithiothreitol. Homogenates were centrifuged at 108,000 *g* and the supernatant liquid was dialyzed against three changes of the homogenization buffer for 6 hr at 4°.

5-Amino-4-imidazole-*N*-succinocarboxamide ribonucleotide synthetase (SAICAR synthetase) from chicken liver was prepared according to the method of Flaks and Lukens [12]; purification was carried up to the DEAE-cellulose chromatography step.

Preparation of mouse liver tRNA and tRNA synthetases

Mouse liver tRNA was prepared by phenol extraction and further purified by DEAE cellulose column chromatography [13]. The aminoacyl tRNA synthetases were prepared by the procedure of Roe [14].

Analyses of enzymes acting on L-aspartic acid

The assay measures the substrate-dependent enzymatic conversion of L-[4-^{14}C]aspartic acid to a product not susceptible to transamination by L-glutamate oxaloacetate transaminase. The unreacted L-aspartic acid is quantitatively dissipated by enzymatic means. Residual radioactivity in the vessel is, therefore, a measure of enzyme activity. This radioactivity is analyzed by scintillation spectrometry; confirmatory analysis by paper electrophoresis, anion exchange column chromatography, and paper chromatography established that the radioactive products co-migrated with authentic products of the reaction in every case. The compositions of the reaction mixtures are as follows.

L-Aspartate transcarbamylase. In a total volume of 20 μl were admixed 250 nCi of L-[4-^{14}C]aspartic acid, 5 μl of water or L-alanosine (3.0 to 120 mM), 100 nmoles of carbamyl phosphate, and, to initiate the reaction, 5 μl of mouse spleen extract.

Argininosuccinate synthetase. In a volume of 25 μl were admixed. 250 nCi of L-[4-^{14}C]aspartic acid, 50 nmoles of citrulline, 50 nmoles of ATP–$MgCl_2$ in 0.05 M Tris–HCl, (pH 8) or buffer alone, 5 μl of water or L-alanosine (30–150 mM), and, to initiate the reaction, 5 μl of mouse kidney extract.

Adenylosuccinate synthetase. In a total volume of 25 μl were admixed 250 nCi of L-[4-^{14}C]aspartic acid, 100 nmoles of IMP, 100 nmoles of GTP, 100 nmoles of $MgCl_2$, 5μl of water or L-alanosine (15–70 mM) or inhibitor (concentrations bracketing those mentioned in Table 2), and, to initiate the reaction, 5 μl of mouse muscle extract.

L-Aspartate N-acetylase. In a total volume of 25 μl, were admixed 250 nCi of L[4-^{14}C]aspartic acid, 50 nmoles of acetyl CoA, 5 μl of water or L-alanosine (50 mM), and, to initiate the reaction, 5 μl of mouse brain homogenate.

SAICAR synthetase. In a total volume of 30 μl were admixed 250 nCi of L-[4-^{14}C]aspartic acid, 86 nmoles of 5-amino-4-imidazole carboxylic acid ribonucleotide, 250 nmoles of ATP–$MgCl_2$, 500 nmoles of sodium phosphate buffer (pH 7.3), 5 μl of water or L-alanosine (5–100 mM), and, to initiate the reaction, 5 μl of partially purified chicken liver enzyme.

In every case, the reaction mixtures were incubated at 37° for 30 min (during which time a linear rate was verified) and then heated at 95° for 2 min. Decarboxylation of unreacted radioactive L-aspartate was carried out according to the methodology of Cooney *et al.* [15]. The vessels containing radioactive products were then immersed in scintillation fluid, and radioactivity was measured by scintillation spectrometry. To test whether L-alanosine could function as a substrate for these enzymes, appropriate scaled-up reaction mixtures were constituted in which DL-[1-^{14}C]alanosine replaced L-[4-^{14}C]aspartic acid; appropriate controls were included and reaction times were extended to 1 hr. The products were analyzed by paper electrophoresis, paper chromatography, or anion exchange column chromatography [7].

Aminoacyl tRNA synthetases (EC 6.1.1.X)

Aminoacylation was carried out in a reaction mixture containing, in a total volume of 100 μl: 50 mM Tris–HCl (pH 7.2), 2 mM $MgCl_2$, 15 mM KCl, 0.25 mM EDTA, 5 mM ATP–$MgCl_2$, 50 μg tRNA, 0.25 μCi of L-[U-^{14}C]amino acid and 50 μl of the enzyme preparation. After 30 min at 37°, 10% trichloroacetic acid (TCA) was added to arrest the reaction. The precipitated macromolecules were collected on glass fiber filters (Whatman 34-AH), washed three times with cold 5% TCA, once with 95% ethanol, and dried; radioactivity was measured by scintillation spectrometry.

Esterification of L-alanosine to mouse liver tRNA was attempted using a similar procedure, except that DL-[1-^{14}C]alanosine replaced the normal amino acids.

L-Asparagine synthetase (EC 6.3.1.1) assay

This enzyme from mouse pancreas and L5178Y/AR lymphoblasts was measured by a radiometric technique [15, 16].

L-Glutamate oxaloacetate transaminase

In a final volume of 25 μl were admixed: 5 μl of L-[U-^{14}C]glutamic acid (0.25 μCi), 5 μl of 0.05 M Tris–HCl buffer (pH 7.6), 5 μl of 0.03 M oxaloacetic acid, 5 μl of L-alanosine (0.2 to 0.4 M) or water, and 5 μl of L-glutamate oxaloacetate transaminase from pig heart. These reactants were incubated at 37° for 30 min, and then heated at 95° for 2 min. The resultant [^{14}C]α-ketoglutarate was decarboxylated with 50 μl of 1% H_2O_2 in 1.0 N HCl, and any $^{14}CO_2$ so formed was collected in drops of 40% KOH deposited on the underside of the lid.

L-Aspartic acid β-decarboxylase

To measure the ability of L-alanosine to inhibit the β-decarboxylation of L-aspartic acid, 5 μl (0.25 μCi) of L-[U-^{14}C] aspartic acid were incubated with 5 μl (1 I.U.) of L-aspartate β-decarboxylase from *A. faecalis* in 0.66 M sodium acetate buffer (pH 4.8) in the presence or absence of 5 μl of 0.03 M L-alanosine. The generation of $^{14}CO_2$ was assessed as described above. The following reaction mixture was constituted to examine the interaction of L-alanosine with this enzyme: 5 μl (0.25 μCi) of DL[3-^{14}C]alanosine were admixed with 1 I.U. of L-aspartate-β-decarboxylase from *A. faecalis* in 0.66 M sodium acetate (pH 4.8). The reactants were incubated at 37° for 2 hr and then subjected to high voltage paper electrophoresis. The electropherogram channels were cut into 8 mm strips, and radioactivity was measured by scintillation spectrometry.

L-Aspartyl kinase

L-Aspartyl kinase was measured radiometrically by determining the rate of formation of L-[4-^{14}C]aspartyl-β-hydroxamate in the presence of neutral hydroxylamine and ATP–$MgCl_2$. In 1600 μl polypropylene vessels, in a final volume of 20 μl were admixed. 5 μl (0.25 μCi) of L-[4-^{14}C]aspartic acid, 5 μl of freshly neutralized hydroxylamine (0.04 M), 5 μl (0.2 μmole) of ATP–$MgCl_2$, and 5 μl of partially purified enzyme (1 mg/ml). These reactants were incubated at 37° for 1 hr, alkalinized by the addition of 5 μl of 1.0 M Tris base, and then heated in open vessels at 95° for 10 min to destroy hydroxylamine. Residual L-aspartic acid was removed by adding 100 μl of "decarboxylation reagent" described earlier [15]. After 16 hr at 25°, 2 I.U. of L-asparaginase were added to the reaction mixtures; at the same time, a 5 μl droplet of 40% KOH was deposited on the underside of the lid of the vessel. The lids were closed and the assemblies incubated at 37° for 3 hr, when the lids were removed, and any trapped $^{14}CO_2$ was measured by scintillation spectrometry.

L-Aspartase

Crude L-aspartase from *B. cadaveris* was dissolved in 0.05 M Tris–HCl, (pH 8.4) to a final concentration of 1 mg/ml. To determine if L-alanosine was capable of inhibiting L-aspartase, 5 μl of the enzyme were incubated with 5 μl (0.25 μCi) of L-[U-^{14}C]L-aspartic acid, 5 μl of 1 mM $MgCl_2$, and 5 μl of 0.05 M Tris–HCl (pH 8.4) or L-alanosine at final molarities ranging from 1 to 20 mM. Fumaric acid was measured by paper electrophoresis. To determine if L-alanosine could serve as a substrate for L-aspartase, 5 μl of enzyme were incubated at 37° with 5 μl (0.24 μCi) of DL-[1-^{14}C]alanosine and 5 μl of 1 mM $MgCl_2$ for times ranging from 30 to 300 min. Aliquots of the incubation mixture were then either electrophoresed or subjected to high resolution column chromatography on Hamilton HA-X4 resin [7].

Preparation of perchlorate extracts of liver for the measurement of hepatic metabolites

Male BDF_1 mice (normal or bearing subcutaneous L5178Y/AR tumors) were fasted overnight and given single intraperitoneal injections of saline (0.25 ml/mouse) or of L-alanosine (500 mg/kg). One, three and five hr later, the mice were killed by cervical dislocation, and liver and tumor were quickly removed and flash frozen under pressure between flat blocks of dry ice. The frozen tissue was homogenized in 3 vol. (w/v) of 5% perchloric acid, and the resultant homogenates were centrifuged at 12,000 *g* for 3 min. Aliquots of the supernatant fraction were neutralized with 40% KOH, and the precipitated potassium perchlorate was removed by centrifugation. ATP, NAD, L-aspartate and L-glutamate present in the clear yellow supernatant fractions were measured by enzymatic techniques [10,17–20]. An additional concentration step of lyophilization and reconstitution in one-half the starting volume was introduced for the measurement of IMP by anion-exchange chromatography on Hamilton HA-X4 resin [7], by a coupled enzyme assay using double beam spectrophotometry [2], or by high pressure liquid chromatography (as described below).

Measurement of IMP by high pressure liquid chromatography

High pressure liquid chromatography of the PCA extract of livers was carried out on μNH_2 columns (Waters Associates, Milford, MA) developed with 0.01 M ammonium phosphate buffer (pH 2.7); the effluent was monitored at 254 nm. IMP eluted at 23.8 min. A typical profile of liver extract is shown in Fig. 1. The area of the IMP peak was calculated by triangulation and compared with standards to compute the concentration of IMP in the unknown.

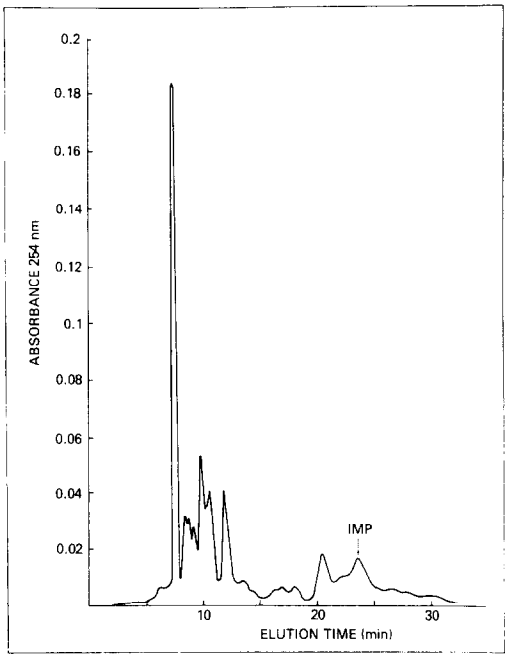


Fig. 1. Measurement of IMP by high pressure liquid chromatography. Details of the preparation of perchlorate extracts and of the chromatography are given in Materials and Methods.

Analysis of enzymes acting on L-*glutamic acid*

L-γ-*Glutamyl-*L-*cysteine synthetase (EC* 6.3.2.2). Flash-frozen kidneys from male BDF_1 mice were homogenized in 3 vol. (w/v) of 0.05 M Tris–HCl (pH 8.4), and the homogenate was centrifuged at 105,000 *g* for 30 min. Five μl aliquots of the resultant supernatant fraction were incubated with 5 μl of neutralized 0.05 M ATP–$MgCl_2$, 5 μl (0.25 μCi) of L-[U-^{14}C]glutamic acid, 5 μl of 0.05 M L-cysteine and 5 μl of 0.05 M L-alanosine or 5 μl of water. After 30 min at 37°, aliquots of the reaction mixtures were subjected to ascending paper chromatography using butanol–acetic acid–water (2:1:1, by vol.). The R_f values of L-glutamic acid and L-γ-glutamyl-L-cysteine in this system were 0.5 and 0.66 respectively.

L-γ-*Glutamyl transpeptidase (EC* 3.4.13.7). A preparation of mouse kidney L-γ-glutamyl transpeptidase was made according to the first three steps of the method of Tate *et al.* [21] and stored frozen at −87°; before use, the enzyme was clarified by centrifugation at 12,000 *g* for 3 min. Five μl aliquots were incubated with 5 μl of 0.01 M neutral reduced glutathione, 5 μl (0.25 μCi) of L-[U-^{14}C]glutamic acid and 5 μl of water or of 0.03 M L-alanosine. After 1 hr, aliquots of the reaction mixture were subjected to high voltage paper electrophoresis at pH 7.2. The electropherogram was cut into strips, and the radioactivity was measured by scintillation spectrometry.

To test if alanosine could be substrate for this enzyme, similar reaction mixtures were constituted except that 0.25 μCi of DL-[3-^{14}C]alanosine replaced radioactive L-glutamine.

L-*Glutamine synthetase (EC* 6.3.1.2). Mouse brain and liver were excised after cervical dislocation, chilled immediately and homogenized (1:4, w/v) in 0.1 M Tris–HCl buffer (pH 7.6) containing 1 mM dithiothreitol. The homogenate was centrifuged at 12,000 *g* for 12 min and the supernatant fraction was used as the source of enzyme. For kinetic analyses, substrate mixtures were prepared containing 0.13 to 4.185 mM L-[U-^{14}C]glutamic acid (specific radioactivity 270 μCi/μmole) along with 0.02 M ATP–$MgCl_2$ and 0.5 M NH_4Cl in 0.1 M imidazole buffer, pH 7.6. In a total volume of 15 μl were admixed: 5 μl of substrate mixture, 5 μl of supernatant fluid, and 5 μl of L-alanosine to achieve a final concentration of 5.3 or 10.6 mM; the vessels were incubated at 37° for 10 min, and then the reaction was stopped by heating at 95° for 10 min. The reaction mixture was centrifuged at 12,000 *g* for 1 min, and 10 μl of supernatant fraction were loaded on to a 1.2 × 1.2 cm column of Dowex 1 × 8 formate, previously washed with distilled water. Newly synthesized L-glutamine was eluted with 5 ml of 0.01 M L-glutamine in 0.05 M Tris–HCl buffer (pH 8.4) and quantitated by scintillation spectrometry.

To study the effect of L-alanosine on the specific activity of L-glutamine synthetase *in vivo*, ten BDF_1 mice were given L-alanosine intraperitoneally at a dose of 500 mg/kg. After 1 hr the animals were killed, and brain and livers were excised; extracts were prepared and L-glutamine synthetase activity was assayed as described earlier.

L-*glutamate pyruvate transaminase.* In a final volume of 25 μl were admixed 5 μl of L-[U-^{14}C]glutamic acid (0.25 μCi), 5 μl of 0.05 M Tris–HCl buffer (pH 7.6), 5 μl of 0.03 M pyruvic acid, 5 μl of L-alanosine (0.2 M to 0.4 M) or water, and 5 μl of L-glutamate pyruvate transaminase from pig heart. These reactants were incubated at 37° for 30 min, and then heated at 95° for 2 min. The α-ketoglutarate that was formed was decarboxylated as described earlier.

L-*Glutamate dehydrogenase (EC* 1.4.1.2). L-Glutamate dehydrogenase was measured spectrophotometrically [10] in the direction:

L-glutamate + NAD → α-ketoglutarate + NH_3 + NADH in the presence and absence of L-alanosine at concentrations ranging from 1 mM to 20 mM. In parallel experiments, the ability of 0.01 M L-alanosine to substitute for an equimolar concentration of L-glutamate in this reaction mixture was examined under similar conditions.

L-*Glutamate decarboxylase (EC* 4.1.1.15). L-Glutamate decarboxylase was measured by the radiometric method described in a companion paper [22]. Inhibition of this enzyme by L-alanosine was measured in the presence of varying concentrations (1.25–125 mM final) of the drug.

Analysis of enzymes acting on L-asparagine

L-*Asparaginase.* L-Asparaginase was measured by a radiometric technique [23].

L-*Asparagine transaminase (EC* 2.6.1.14). The influence of L-alanosine on L-asparagine transaminase was studied by incubating various concentrations of L-alanosine (6.25 to 12.5 mM) with oxaloacetic acid and an extract of mouse liver. A typical reaction mixture, in a total volume of 25 μl, consisted of: 5 μl of L-[U-^{14}C]asparagine, 5 μl of 0.05 M Tris–HCl buffer (pH 8.4), 5 μl of L-alanosine in 0.05 M Tris–HCl, pH 8.4, or buffer alone, 5 μl of 0.03 M oxaloacetate in 0.05 M Tris–HCl, pH 8.4, or 5 μl of buffer alone, and 5 μl of mouse liver extract. The reactants were mixed by centrifugation, incubated at 37° for 30 min, and heated at 95° for 2 min. Decarboxylation of [^{14}C]α-ketocarboxylic acid was conducted as described above.

Analysis of enzymes acting on L-*Glutamine*

Amidotransferases. The following amidotransferases were assayed by the technique given below: desamido NAD: L-glutamine amidoligase (EC 6.3.5.1); XMP L-glutamine amidoligase (EC 6.3.5.2); L-glutamine: D-fructose 6-phosphate aminotransferase (EC 5.3.1.19); ribosylamine-5′-phosphate: pyrophosphate phosphoribosyl transferase (EC 2.4.2.14). This assay measures the substrate-dependent (acceptor-dependent) breakdown of L-[U-^{14}C]glutamine to L-[U-^{14}C]glutamic acid. The following were admixed in Eppendorf vessels in a final volume of 25 μl: 5 μl (0.25 μCi) of L-[U-^{14}C]glutamine (final concentration 0.2 mM), 5 μl of neutralized acceptor (to achieve a final concentration of 0.01 M), 5 μl of neutralized ATP and $MgCl_2$ (each at 0.02 M), and 10 μl of tissue homogenate to initiate the reaction. Blanks without ATP allowed an estimation of any ATP-independent amide transfer or L-glutaminase activity in the extracts. After 30 min at 37°, the reaction mixtures were heated at 95° for 2 min and then cooled. L-[U-^{14}C]Glutamic acid formed in the incubation step was quantitated by

conversion via an exchange transamination at 50 per cent yield to α-ketoglutaric acid, as follows: 20 μl of a solution containing 1 mg of α-ketoglutaric acid and 0.2 mg of L-glutamate oxaloacetate transaminase per ml of 0.5 M Tris–HCl (pH 8.4) were added to the reaction mixtures, and incubated at 37° for 60 min. Subsequently, 50 μl of 1% H_2O_2 in 1 N HCl were added to each vessel; any $^{14}CO_2$ formed was collected in drops of 40% KOH deposited on the lid. At the end of 1 hr, the lids were removed and the radioactivity was measured by liquid scintillation spectrometry.

CTP synthetase (EC 6.3.4.2). This enzyme was assayed by measuring the radiolabeled CTP that is formed from radioactive UTP in the presence of ATP, L-glutamine, and enzyme. UTP and CTP were separated by paper electrophoresis using 0.2 M formic acid (pH 2.6). A typical reaction mixture consisted of the following in a total volume of 30 μl: 5 μl of [U-^{14}C]UTP (50 μCi/ml), 5 μl of 0.05 M ATP–$MgCl_2$, 5 μl of 0.003 M GTP–$MgCl_2$, 5 μl of a mixture containing 0.01 M L-glutamine and 0.01 M KCl in 0.05 M Tris–HCl (pH 7.3), 5 μl of 0.06 M L-alanosine or water and 5 μl of enzyme (dialyzed 105,000 *g* supernatant of rat fetal liver, mouse brain or mouse thymus). After 30 min at 37°, the reaction was terminated by heating at 95° for 2 min; after centrifugation at 12,000 *g* for 3 min, 10 μl aliquots were taken for paper electrophoresis.

Carbamyl phosphate synthetase (EC 2.7.2.9). Carbamyl phosphate synthetase was measured radiometrically. In a final volume of 15 μl were admixed 1 μCi of H[^{14}C]O_3, 0.15 μmole of ATP–$MgCl_2$, 0.15 μmole of L-glutamine, 0.15 μmole of citrulline, 0.1 I.U. of ornithine carbamyl transferase and 0.15 μmole of Tris–HCl (pH 7.4) or L-alanosine. The reaction was initiated by the addition of either crude carbamyl phosphate synthetase from the mutant hamster ovarian cells described by Kempe *et al.* [24] or of purified carbamyl phosphate synthetase from *E. coli*. After 20 min at 37°, during which time the rate was verified to be linear, 50 μl of 1 N HCl were added and the reaction vessels were transferred to ovens maintained at 95°. After 20 min, the radioactivity of the residue was measured by scintillation spectrometry.

L-*Glutaminase*. In a final vol of 15 μl were admixed 5 μl of L-[U-^{14}C]glutamine (specific radioactivity 57.3 μCi/μmole, 5 μl of neutral L-alanosine in 0.05 M Tris–HCl (pH 8.4) or buffer alone, and 5 μl of enzyme. The final concentration of L-alanosine was 0.3 or 0.4 M in the studies with *A. aerogenes* L-glutaminase, 0.002 to 0.005 M in the case of *E. carotovora* L-asparaginase, and 0.002 to 0.005 M in the case of *E. coli* L-asparaginase. In all cases, sufficient enzyme was added to hydrolyze more than 10 per cent but less than 20 per cent of substrate. Vessels were incubated at 37° for 30 min, and then heated at 95° for 5 min. L-[U-^{14}C]Glutamic acid was quantitated by the method used for measuring amidotransferases (see above).

DNA synthesis in P388 cells in vivo

One million P388 cells were implanted intraperitoneally in thirty CDF_1 mice. Six days later, twenty animals received a single intraperitoneal dose of L-alanosine (500 mg/kg). At the designated time interval, groups of three animals were given an intraperitoneal injection of 0.0015 μmole (31 μCi) of [*methyl*-^{3}H]thymidine. After a 30-min interval, they were killed and ascitic tumor cells were collected, washed and resuspended in cold 0.85% NaCl at a concentration of 10^6 cells/ml. Two million cells in triplicate were treated with cold TCA, and the precipitated DNA was filtered on millipore filters, and washed five times with cold TCA followed by 95% ethanol. The filter was dried and the radioactivity was determined by scintillation spectrometry. A group of nine animals constituted the untreated controls.

RESULTS

The interaction of L-alanosine with each of the dicarboxylic amino acids and their amides will be examined first from the standpoint of transport, then of enzymology, and finally of metabolic effects. This last category will encompass changes in the concentrations of these amino acids or of metabolites arising from them. In some cases, consideration will also be given to the net effect of L-alanosine on macromolecular syntheses.

Interaction of L-*alanosine with the* L-*aspartic acid transport system*

Graff and Plageman [1] showed that L-alanosine inhibited the uptake of L-aspartic acid because of its affinity for the system responsible for the transport of this amino acid. We have examined the effect of L-alanosine on the uptake of the dicarboxylic amino acids into murine lymphoblasts (L5178Y/AR). Figure 2, panel A, demonstrates that L-alanosine inhibited the uptake of L-aspartic acid by these cells and that the inhibition is apparently competitive in nature; Eadie-Scatchard plots of these results con-

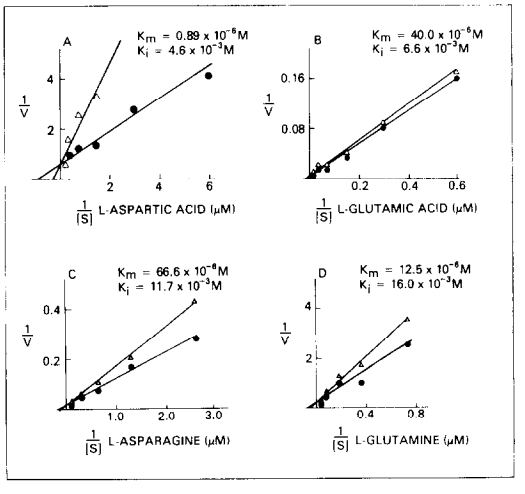


Fig. 2. Influence of L-alanosine on the transport of dicarboxylic amino acids and their amides by L5178Y/AR cells. Studies on the transport of amino acids were carried out by incubating L5178Y/AR cells at 37° with various concentrations of amino acid in the presence (△) or absence (●) of 10 mM L-alanosine, as described in Materials and Methods.

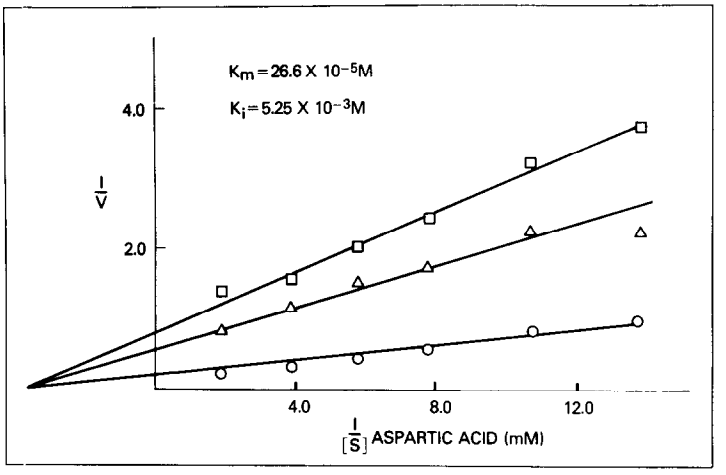


Fig. 3. Inhibition of adenylosuccinate synthetase of mouse muscle by L-alanosine. Adenylosuccinate synthetase activity was measured according to the radiometric method given in Materials and Methods, with L-aspartic acid as the variable substrate in the absence (○) or presence of 3.6 mM (△) or 5.5 mM (□) L-alanosine.

firm that the inhibition was competitive in type (data is not shown). The K_m for L-aspartic acid in this system is 0.89 μM, and the K_i for L-alanosine is 4.6 mM.

More specific examples of the interaction of L-alanosine with the enzymes metabolizing L-aspartic acid are presented in the following section.

Adenylosuccinate synthetase (EC 6.3.4.4). Since L-alanosine inhibits the incorporation of [^{14}C]formate into adenine but not into guanine, Gale and Schmidt [2] suggested that either adenylosuccinate synthetase or adenylosuccinate lyase were likely to be its target enzymes. The finding that Novikoff rat hepatomas, treated with alanosine, accumulated IMP strongly suggested that adenylosuccinate synthetase was the principal site of action of the drug [1]. Although other workers report that L-alanosine, as such, does not inhibit adenylosuccinate synthetase [3, 5], we observed that the drug was capable of inhibiting a crude preparation of this enzyme from mouse muscle. This inhibition was noncompetitive with respect to L-aspartic acid, the K_i being 5.25×10^{-3} M (Fig. 3); dialysis reversed it fully. Intraperitoneal administration of therapeutic doses of L-alanosine to mice bearing subcutaneous L5178Y/AR tumors which are sensitive to L-alanosine therapy inhibited the activity of adenylosuccinate synthetase in muscle and tumor by 40 and 72 per cent, respectively (Table 1); this inhibition was also reversed by dialysis.

Table 1. Effects of L-alanosine on adenylosuccinate synthetase activity *in vivo* in mouse skeletal muscle and L5178/AR*

Tissue	Adenylosuccinate synthetase activity (nmoles/mg protein/hr)		% Inhibition
	Treatment		
	Saline	L-Alanosine	
Muscle	231.75 ± 6.95	136.78 ± 61.14†	40
Tumor	72.35 ± 8.7	19.89 ± 8.92†	72

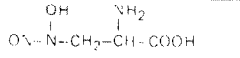
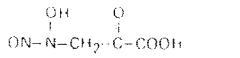
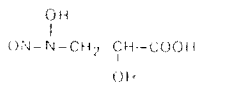
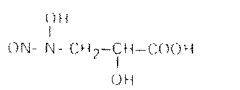
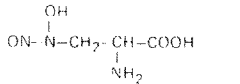
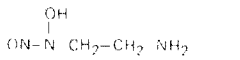
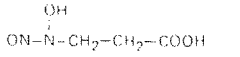
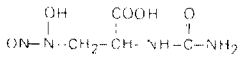
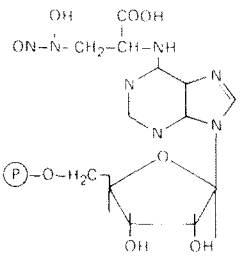
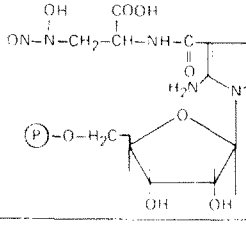
* Male BDF$_1$ mice were injected subcutaneously with 1×10^6 cells/mouse of L5178Y/AR. Ten days later, they were injected intraperitoneally with either saline or 500 mg/kg of L-alanosine. One hr later tumor and skeletal muscle were removed and separately homogenized as described in Materials and Methods; a 12,000 *g* supernatant fraction was used for the assay of the enzyme activity.

† $P > 0.01$.

In an attempt to demonstrate the mode of action of L-alanosine, Gale and Smith [3] incubated the antibiotic with radiolabeled IMP in the presence of adenylosuccinate synthetase from *E. coli.* Chromatographic analysis of such reaction mixtures revealed the presence of a new radioactive species, which these workers postulated to be an adduct of L-alanosine and IMP. However, they neither isolated this adduct nor attempted to demonstrate its inhibitory activity against adenylosuccinate synthetase. Therefore we re-examined the interaction of L-alanosine with this enzyme. Using a partially purified preparation of adenylosuccinate synthetase from rabbit muscle, it was possible to demonstrate that L-alanosine was a true but weak substrate for the enzyme [25]. The resultant product was desalted by absorption onto a column of acid-washed charcoal followed by elution with ammoniacal 60% ethanol. At the concentration used (~ 1μM), the purified adduct failed to inhibit adenylosuccinate synthetase (Table 2).

Argininosuccinate synthetase (EC 6.3.4.5). Argininosuccinate synthetase is considered to be an important regulatory enzyme in the urea cycle of mammals [26]. Adenylosuccinate synthetase and argininosuccinate synthetase catalyze formally analogous reactions, namely the transfer of the carbon skeleton and α-amino nitrogen of L-aspartic acid to the carbonyl group of a suitable receptor, utilizing the energy provided by the hydrolysis of a nucleotide triphosphate. In view of this similarity, it was anticipated that L-alanosine would interfere with the biosynthesis of argininosuccinate. When L-alanosine, at a concentration of 30 mM, was tested as an inhibitor

Table 2. *In vitro* inhibition of adenylosuccinate synthetase by L-alanosine and its metabolites*

Compound	Structure	Concentration required to produce 50% inhibition (mM)
1 L-Alanosine		7
2 α-Keto alanosine		5
3 α-Hydroxy alanosine		2·6
4 Urinary metabolite		2·6
5 D-Alanosine		160
6 α-Decarboxy alanosine		150
7 Desamino alanosine		0·7
8 N-Carbamyl L-alanosine		100
9 Alanosyl IMP		
10 Alanosyl AICOR		0·0003

* Metabolites and anabolites of L-alanosine were prepared as described earlier [25]. The metabolites and anabolites of L-alanosine were tested for inhibition of adenylosuccinate synthetase activity from rabbit muscle, according to the methodology detailed in Materials and Methods. The concentration of alanosyl IMP required to produce 50 per cent inhibition of adenylosuccinate synthetase was not determined.

of the mouse kidney enzyme, the drug did exert substantial (50 per cent) inhibition. Therefore, we made an attempt to determine if L-alanosine could be metabolized by this enzyme. High-resolution anion and cation exchange chromatography [7] of reaction mixtures, wherein L-[1-^{14}C]alanosine replaced L-[4-^{14}C]aspartic acid, failed to reveal the presence of any new radioactive species, a finding which tends to exclude the possibility of metabolism of the drug by this route [25].

L-*Aspartate transcarbamylase (EC* 2.1.3.2). One of the early and critical steps in the *de novo* synthesis of pyrimidines involves the condensation of L-aspartate with carbamyl phosphate to yield carbamyl-L-aspartate. Gale *et al.* [4] found that pyrimidine biosynthesis in *Candida albicans* was repressed by L-alanosine, and that this effect could be antagonized by L-aspartic acid. Analogous antidotal effects, however, could not be observed in rodent tumors [1]. When we examined the interaction of L-alanosine with L-aspartate transcarbamylase from mammalian spleen, we observed that high concentrations of the antibiotic (40 mM) were required to inhibit the enzyme by 50 per cent. This finding suggests that L-alanosine at therapeutic doses is unlikely to affect pyrimidine biosynthesis in mammals. Compared to purified L-aspartate transcarbamylase from *E. coli*, L-alanosine (40 mM) exerted only marginal inhibi-

tion, but, like PALA (*N*-phosphonacetyl-L-aspartic acid) and succinate, was able to stimulate this allosteric enzyme by ~ 200 per cent when it was undersaturated with L-aspartic acid (5×10^{-4} M). Relevant, too, is the observation that L-alanosine was susceptible to enzymic carbamylation catalyzed by purified L-aspartate transcarbamylase from *E. coli* as well as by a crude preparation of the enzyme from PALA-resistant Chinese hamster ovarian cells [25]. The K_m and V_{max} values of the former enzyme for L-alanosine were 12.5 mM and 7.6 nmoles carbamylated/mg of protein/hr; constants with the latter enzyme for the antibiotic were 16.7 mM and 1.2 nmoles carbamylated/mg of protein/hr respectively.

SAICAR synthetase (EC 6.3.2.6). The finding that L-alanosine does not interrupt the biosynthesis of the purine ring points to the fact that the first of the two L-aspartate-utilizing enzymes of the purine biosynthetic pathway are not affected significantly by the drug [1, 2]. Hurlbert *et al.* [27], however, have made the observation that the active metabolite of L-alanosine is an analog of SAICAR; this analog presumably is formed by the condensation of L-alanosine with AICOR. In the interest of completeness and with the goal of establishing the enzymatic basis for the formation of the inhibitory molecule, a more extensive study of this reaction was undertaken. SAICAR synthetase, partially purified from chicken liver, was found to be inhibited by L-alanosine, and the inhibition was formally competitive with respect to L-aspartic acid (Fig. 4), the K_i being 1.61×10^{-3} M. Dialysis reversed it totally.

When L-alanosine was tested as a substrate for SAICAR synthetase, replacing L-aspartic acid, a new product was formed which could be separated by chromatography on high-resolution anion exchange column [7]. A metabolite with identical chromatographic properties was formed irrespective of whether radioactive L-alanosine or radioactive AICOR was used [25]. This metabolite powerfully inhibits adenylosuccinate synthetase (K_i 0.3 μM) (Table 2).

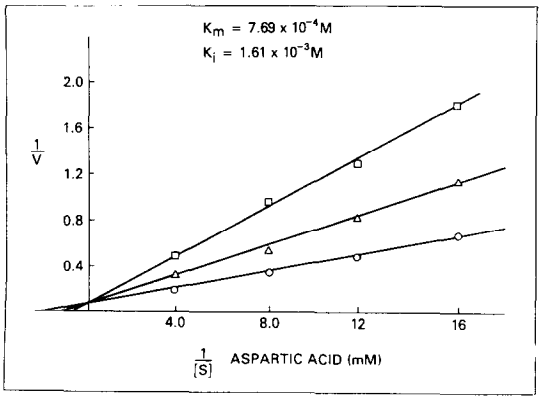


Fig. 4. Interaction of L-alanosine with SAICAR synthetase from chicken liver. SAICAR synthetase activity was measured according to the radiometric technique given in Materials and Methods, and with L-aspartic acid as the variable substrate in the absence (○) or presence of 1.43 mM (△) or 3.6 mM (□) L-alanosine.

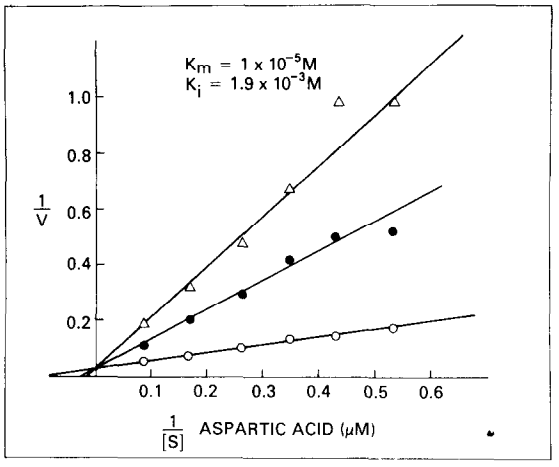


Fig. 5. Inhibition of L-aspartyl tRNA synthetase from mouse liver by L-alanosine. The L-aspartyl tRNA synthetase reaction was carried out as described in Materials and Methods, in the absence (○) or presence of 1.65 mM (●) or 3.3 mM (△) L-alanosine.

L-*Aspartyl tRNA synthetase (EC* 6.1.1.12). Our earlier studies suggested that L-alanosine can be incorporated into protein [25]. For this reason, we chose to explore the interaction of L-alanosine with L-aspartyl tRNA synthetase. In fact, L-alanosine did inhibit the esterification of L-[4-^{14}C]aspartic acid to tRNA in the reaction catalyzed by a partially purified preparation of mouse liver tRNA synthetases: 61 per cent at 5 mM. This inhibition was formally competitive in type, with L-aspartic acid as the variable substrate (Fig. 5); the K_i was 1.9×10^{-3} M. Dialysis fully reversed this effect. Despite this evidence that L-alanosine could occupy the active site of L-aspartyl tRNA synthetase, it has not been possible to demonstrate directly that the antibiotic is esterified to tRNA [25].

L-*Asparagine synthetase.* L-Asparagine competitively inhibits the active uptake of L-alanosine by L5178Y/AR cells *in vitro* [22]. This finding and the steric similarity of L-alanosine to L-aspartic acid justified the expectation that the antibiotic might interact with the enzyme systems responsible for the biosynthesis of L-asparagine. However, even at a concentration of 10 mM, L-alanosine failed to inhibit L-asparagine synthetase from leukemia 5178Y/AR or from normal mouse pancreas, regardless of whether L-glutamine or ammonia was the nitrogen donor. It is noteworthy, though, that HAPA [2-amino-3-(*N*-hydroxy-amino) propionic acid], an analog of L-alanosine, does inhibit these enzymes to a considerable degree (40 per cent at 10 mM). This observation is of importance in light of the fact that HAPA has been suggested to be one of the metabolites of L-alanosine [25].

L-*Aspartyl*-N-*acetylase (EC* 2.3.1.17). In mammalian brain, a particulate enzymatic activity capable of acetylating L-aspartic acid is abundant [28]. This activity was not inhibited significantly by L-alanosine even at 12.5 mM. Moreover, when DL-[1-^{14}C]alanosine was incubated with acetyl CoA and a crude preparation of the enzyme, no new product

Table 3. Activities of L-glutamate oxaloacetate transaminase and glutamate pyruvate transaminase in skeletal and cardiac muscle tissues of the mouse*

Tissue	Enzyme	Substrates	Activity (μmoles/mg protein/hr)
Skeletal muscle	L-Glutamate oxaloacetate transaminase	L-Glutamate and oxaloacetate	0.067
		L-Alanosine and oxaloacetate	0.0125
Cardiac muscle	L-Glutamate oxaloacetate transaminase	L-Glutamate and oxaloacetate	0.096
		L-Alanosine and oxaloacetate	0.017
Skeletal muscle	L-Glutamate pyruvate transaminase	L-Glutamate and pyruvate	0.0496
		L-Alanosine and pyruvate	0.0108
Cardiac muscle	L-Glutamate pyruvate transaminase	L-Glutamate and pyruvate	0.0721
		L-Alanosine and pyruvate	0.0132

* GOT and GPT activities were measured in cardiac and skeletal muscle tissues of the mouse according to the details given in Materials and Methods.

could be detected by anion exchange column chromatography; under the same conditions, L-aspartic acid was acetylated vigorously.

In new born mice, L-alanosine causes severe hypothalamic lesions analogous to those caused by high doses of L-aspartic and L-glutamic acids [6]. On the basis of the negative results presented here, it can be concluded that the enzymatic basis for this lesion does not entail acetylation of the antibiotic.

L-Aspartate aminotransferase. L-Aspartate aminotransferase, a pivotal enzyme in mammalian nitrogen metabolism, exhibits rather restricted substrate specificity [29]. When the effect of L-alanosine on the conversion of L-[4-^{14}C]aspartate (2 mM) to [4-^{14}C]oxaloacetate in the presence of α-ketoglutarate was tested with the mitochondrial enzyme from pig heart, it was observed that the antibiotic inhibited this transaminase in a formally competitive manner with a K_i of 500 mM.

In the second paper of the present series [25], evidence was presented documenting the susceptibility of alanosine to catalytic attack by GOT. The product of that attack was shown to be an α-keto-carboxylic acid, susceptible to decarboxylation by acid hydrogen peroxide. Since cardiac and skeletal muscle, which together constitute ~ 30 per cent of the body mass, are rich in GOT, it was of interest to compare the metabolism of DL-[1-^{14}C]alanosine by extracts of these tissues. The results presented in Table 3 show that the transamination of L-alanosine with oxaloacetic acid catalyzed by crude extracts of cardiac and skeletal muscle proceeds at about one-fifth the rate seen with its physiologic substrate; both cardiac and skeletal muscle tissues are equally abundant in this activity.

L-Aspartate-metabolizing enzymes unique to bacteria: L-aspartase, L-aspartic acid β-decarboxylase and L-aspartyl kinase

Because L-alanosine possesses marked antibiotic properties, we examined its ability to interact with those enzymes of L-aspartate metabolism unique to bacteria. At a final concentration of 0.01 M, L-alanosine neither inhibited the title enzymes nor was a substrate for them.

Table 4 presents a recapitulation of the interaction of L-alanosine with the enzymes metabolizing L-aspartic acid; four of these are inhibited to a marked degree *in vitro*. In order to assess the consequences of such inhibition, we examined the ability of the antibiotic to disturb the concentration of L-aspartic acid in urine, liver and tumor.

Production of aminoaciduria

Previous studies showed that L-alanosine inhibited the active transport of several amino acids [22]. By analogy, it seemed possible that the drug might interrupt tubular resorption in the kidney. In fact, it was found that parenteral L-alanosine provokes a dose-related increase in the excretion of L-aspartic acid in the urine and that this effect lasts for at least 5 hr (table 5).

Alteration of amino acid concentration

Hepatic pools of L-aspartate were measured after drug treatment. A 3 to 5-fold increase was demonstrated (Table 6). No such derangement was observed in nodules of leukemia 5178Y, a neoplasm sensitive to L-alanosine.

Alteration of the concentration of IMP

Inhibition of adenylosuccinate synthetase by L-alanosine or a metabolite thereof should result in the accumulation of IMP *in vivo* [1]. After therapeutic doses of the drug, when hepatic as well as tumoral (L5178Y/AR) IMP pools were measured by spectrophotometric and by chromatographic techniques, no significant increase was noted at 1 or 5 hr after drug treatment (Table 7). It is noteworthy that an apparently larger pool-size of IMP was found when high pressure liquid chromatography was used; this discrepancy might be due to the presence of closely eluting nucleotide species.

Alteration of the concentration of ATP

Since L-alanosine interferes with the biosynthesis of AMP, the concentration of this and related nucleotides should fall as a consequence of drug treatment. This effect has been observed in other systems [1]. One and five hours after the administration of L-alanosine to tumor-bearing mice, a significant decrease in the concentration of ATP was observed in liver and tumor (L5178Y/AR) (Table 7). A modest depression in the concentration of nicotinamide adenine dinucleotide was also seen 5 hr after administration of the drug (Table 7).

Table 4. Summary of the effects of L-alanosine *in vitro* on enzymes of L-aspartate metabolism*

Enzyme	EC No.	Source of enzyme	Maximum conc. tested (mM)	% Inhibition	Nature of Inhibition	K_i	Reversibility	Is alanosine an alternate substrate?
Adenylosuccinate synthetase	6.3.4.4	Mouse muscle	7.1	55	Non-competitive	5.3 mM	Yes	Yes
Argininosuccinate synthetase	6.3.4.5	Mouse kidney	33	56	ND	ND	ND	No
L-Aspartate transcarbamylase	2.1.3.2	Mouse spleen	40	50	ND	ND	ND	Yes
		E. coli	40	5	ND	ND	ND	Yes
5-Amino-4-imidazole-*N*-succinocarboxamide ribonucleotide synthetase	6.3.2.6	Chicken liver	1.5	50	Competitive	1.6 mM	Yes	Yes
L-Aspartyl tRNA synthetase	6.1.1.12	Mouse liver	5	61.6	Competitive	1.9 mM	Yes	No
L-Asparagine synthetase	6.3.1.1	Mouse pancreas	10	0	ND	ND	ND	No
L-Aspartyl *N*-acetylase	2.3.1.17	Mouse brain	12.5	24	ND	ND	ND	No
L-Aspartate aminotransferase (GOT)	2.6.1.1	Pig heart	500	60	Competitive	500.0 mM	Yes	Yes
L-Aspartase	4.3.1.1	*B. cadaveris*	30	0	ND	ND	ND	No
		E. coli	50	0	ND	ND	ND	No
L-Aspartate-β-decarboxylase	4.1.1.12	*A. faecalis*	30	0	ND	ND	ND	No
L-Aspartyl kinase	2.7.2.4	*E. coli*	10	16	ND	ND	ND	No

* Assay techniques for enzyme analysis are given in Materials and Methods. Except in certain cases, detailed kinetic analyses were not carried out unless the $\sim$ ID_{50} was $\sim$ 10 mM or less. The per cent inhibition is that seen at the maximum concentration tested. ND: not determined.

Table 5. Effects of L-Alanosine on the excretion of L-aspartic acid, L-glutamic acid and L-asparagine in urine*

Amino acid	L-Alanosine treatment (mg/kg)	Amount excreted (nmoles ± S.D./ml urine) 3 hr	5 hr
L-Aspartic acid	Saline	348.0 ± 43.5	304.5 ± 43.5
	125	739.0 ± 0.5	848.0 ± 22.0
	250	2326.5 ± 109.0	1010.0 ± 87.0
	500	2869.5 ± 174.0	2043.5 ± 217.5
L-Asparagine	Saline	196.0 ± 65.5	174.0 ± 43.0
	125	130.5 ± 0.5	174.0 ± 43.5
	250	174.0 ± 43.5	130.5 ± 43.5
	500	348.0 ± 87.0	261.0 ± 43.5
L-Glutamic acid	Saline	141.5 ± 13.0	250.0 ± 0.5
	125	930.0 ± 20.5	680.0 ± 49.0
	250	3950.0 ± 64.0	5500.0 ± 1426.0
	500	3850.0 ± 379.5	2640.0 ± 63.5

* Amino acids were measured in clarified urine using techniques described in Materials and Methods. Due to interference from excessive ammonia present in the urine, L-glutamine could not be measured. Drug treatment did not cause any change in the volume of urine excreted; the net excretion of creatinine in the urine of treated mice was not altered significantly.

Table 6. Influence of L-alanosine on the concentration of the dicarboxylic amino acids *in vivo**

Amino acid	Time after treatment (hr)	Concentration (nmoles ± S.D./g tissue) Liver: Saline group	Liver: L-Alanosine group	L5178Y/AR: Saline group	L5178Y/AR: L-Alanosine group
L-Aspartic acid	1	664.6 ± 195.6	3413.6 ± 849.2†	522.4 ± 98.8	599.2 ± 116.4
	5	692.4 ± 138.0	978.6 ± 280.0	334.4 ± 167.2	522.4 ± 89.6
L-Glutamic acid	1	1584.0 ± 758.2	964.4 ± 863.4	759.8 ± 332.3	459.3 ± 328.2
	5	1210.8 ± 747.3	1329.3 ± 1025.4	651.3 ± 362.4	747.3 ± 376.6

* Normal mice or mice bearing 1 cm subcutaneous nodules of leukemia 5178Y/AR were injected intraperitoneally with 500 mg/kg of L-alanosine or with saline. One and five hr later, liver and tumor were removed and processed as detailed in Materials and Methods.

† $P < 0.05$.

Table 7. Influence of L-alanosine on the concentrations of ATP, NAD and IMP in liver and tumor*

Pools	Time after treatment (hr)	Concentration (nmoles ± S.D./g tissue) Liver: Saline group	Liver: L-Alanosine group	L5178Y/AR: Saline group	L5178Y/AR: L-Alanosine group
ATP	1	942.0 ± 53.0	637.0 ± 54.3	194.8 ± 86.0	224.0 ± 96.8
	5	1511.0 ± 64.8	945.5 ± 41.3 ($P < 0.05$)	151.2 ± 86.0	93.0 ± 36.8
NAD	1	882.3 ± 46.9	914.8 ± 46.0		
	5	1176 ± 78.0	889.5 ± 35.0 ($P < 0.05$)		
IMP (spectrophotometric)	1	85.9 ± 24.6	48.0 ± 33.9	19.05 ± 11.7	22.3 ± 2.2
IMP (anion exchange chromatography)	1	114.46 ± 25	124.08 ± 16.08		
IMP (high pressure liquid chromatography)	1	667 ± 148	558 ± 212		
	3	340 ± 84	340 ± 128		
	5	372 ± 88	268 ± 38		

* The pools of ATP, NAD and IMP were measured according to the procedures outlined in Materials and Methods. IMP measurements were made using the three different methods indicated.

DNA synthesis

Since ATP synthesis was affected by L-alanosine both in normal liver and in tumors, it was expected that dATP and consequently DNA synthesis would be affected. P388 cells, which are sensitive to L-alanosine, were treated with the drug *in vivo* and the incorporation of thymidine was studied according to the procedures given under Materials and Methods. Figure 6 illustrates the kinetics of DNA synthesis in these cells following treatment with L-alanosine; this process slows down to about 50 per cent of the control by 2 hr, totally stops by 4 hr, and remains at a negligible level for 24 hr. By 48 hr, DNA synthesis has rebounded.

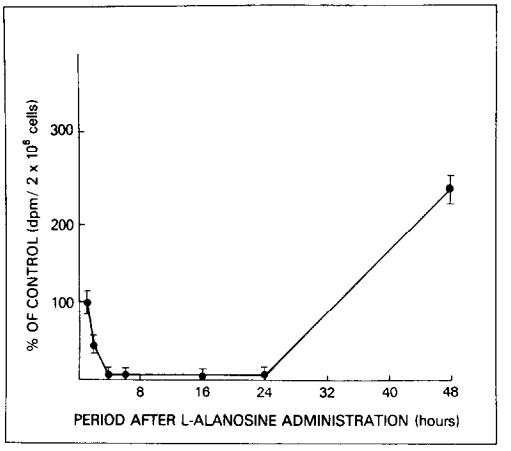


Fig. 6. Inhibition of DNA synthesis by L-alanosine *in vivo*. Incorporation of thymidine into DNA was studied by the procedures detailed in Materials and Methods.

Interaction of L-alanosine with enzymes of L-glutamate metabolism

Earlier studies by Gale and Atkins [30] documented that L-alanosine was a competitive inhibitor of L-glutamate decarboxylase from *E. coli* without at the same time being susceptible to catalytic attack by the enzyme. Our own investigations showed that DL-[1-^{14}C]alanosine could, in fact, be α-decarboxylated by L-glutamate decarboxylase [22]. In the following section we propose to investigate how extensive is the analogy of L-alanosine to L-glutamic acid.

Transport. Figure 2, panel B, shows the effect of L-alanosine on the uptake of L-glutamic acid by leukemia 5178Y/AR cells. The K_m for L-glutamic acid transport is 40 μM; the K_i for L-alanosine is 6.6 mM. It is relevant to point out that L-glutamic acid has been found to impede the uptake of L-alanosine in a competitive manner [22]. Further specific examples of interaction of L-alanosine with enzymes metabolizing L-glutamic acid are presented in the following section.

L-γ-*Glutamyl*-L-*cysteine synthetase.* In the presence of ATP and magnesium ions, L-γ-glutamyl-L-cysteine synthetase catalyzes the formation of a peptide bond between the γ-carboxyl of L-glutamic acid and the α-amino group of L-cysteine. As the first step in glutathione synthesis, this reaction may be of profound importance to the transport of amino acids [21]. *In vitro*, L-alanosine did not inhibit L-γ-glutamyl-L-cysteine synthetase, nor was it metabolized by a crude preparation of this enzyme from rat kidney.

L-γ-*Glutamyl transpeptidase.* Although the title enzyme is not, in the strictest sense, involved in the metabolism of L-glutamic acid, we explored its interaction with L-alanosine for the following reason. Orlowski and Meister [31] have accumulated evidence that L-γ-glutamyl transpeptidase participates in the active transport of L-amino acids. Inasmuch as our earlier studies demonstrated that L-alanosine was transported into murine lymphoblasts by a temperature- and energy-dependent process, it was of interest to determine if the γ-glutamyl cycle might be responsible for this transport [22]. Using high-resolution anion exchange column chromatography in conjunction with paper electrophoresis at pH 7.2, we found no evidence for the conjugation of DL-[1-^{14}C]alanosine to the L-glutamyl moiety of glutathione by a solubilized preparation of the transpeptidase from mouse kidney.

L-*Glutamine synthetase.* L-Glutamine synthetase plays an important role in the nitrogen metabolism of mammals. *In vitro*, L-alanosine inhibits the enzyme from mouse liver (K_i 1.3 mM) and brain (K_i 4.0 mM) in a competitive way (Fig. 7). Dialysis reverses over 90 per cent of this inhibition. *In vivo*, however, therapeutic parenteral doses of L-alanosine

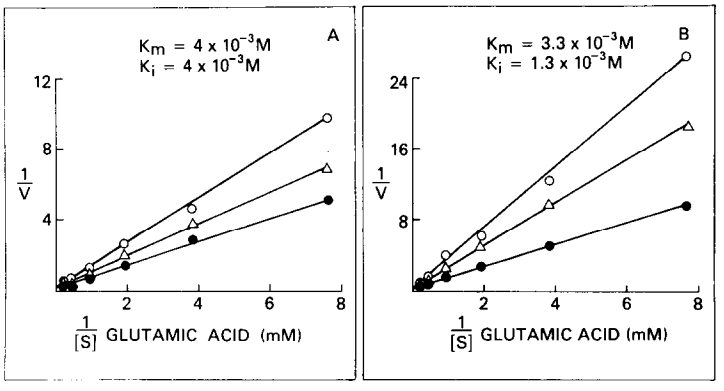


Fig. 7. Interaction of L-alanosine with L-glutamine synthetase from mouse brain and liver. L-Glutamine synthetase was measured in the presence of water (●) or L-alanosine, 5.3 mM (△) or 10.6 mM (○); the assay was conducted as described in Materials and Methods. Panel A represents studies carried out with mouse brain enzyme, and panel B represents the study carried out with the mouse liver enzyme.

Table 8. Summary of the effects of L-alanosine *in vitro* on the enzymes of L-glutamate metabolism*

Enzyme	EC No.	Source of enzyme	Maximum conc. tested (mM)	% Inhibition	Nature of inhibition	K_i	Reversibility	Is alanosine an alternate substrate?
L-γ-Glutamyl L-cysteine synthetase	6.3.2.2	Rat kidney	10	0	ND	ND	ND	No
L-γ-Glutamyl transpeptidase	3.4.13.7	Mouse kidney	10	0	ND	ND	ND	No
L-Glutamine synthetase	6.3.1.2	Mouse liver	8	50	Competitive	1.3 mM	Yes	No
		Mouse brain	8	62	Competitive	4.0 mM	ND	No
L-Glutamyl tRNA synthetase	6.1.1.17	Mouse liver	10	2.4	ND	ND	ND	No
L-Glutamate pyruvate transaminase	2.6.1.2	Pig heart	200	49	Competitive	0.15 M	Yes	Yes
L-Glutamate dehydrogenase	1.4.1.2	Bovine liver	50	0	ND	ND	ND	No
L-Glutamate decarboxylase	4.1.1.15	*E. coli*	125	91	Competitive	1.6 mM	ND	Yes

* Analytical details are given in Materials and Methods. ND: not determined. Percent inhibition is that seen at the maximum concentration tested.

(400 mg/kg) produced negligible effects on the activity of L-glutamine synthetase in brain and liver at time periods up to 4 hr after administration.

L-Glutamyl tRNA synthetase (EC 6.1.1.17). The same considerations which activated our study of the interaction of L-alanosine with L-aspartyl tRNA synthetase prompted us to examine the corresponding enzyme involved in the acylation of L-glutamate. Concentrations of the antibiotic as high as 10 mM produced no inhibition of this enzyme *in vitro*.

L-Glutamate pyruvate transaminase. L-Alanosine, at a final concentration of 0.01 M, failed to inhibit pig heart GPT and produced only 18 per cent inhibition at 0.1 M. Kinetic analysis showed that the antibiotic was an exceedingly weak competitive inhibitor versus L-glutamic acid (K_i 0.299 M). Table 3 documents that GPT is equally abundant in cardiac and skeletal muscle. The rate of the reaction with L-alanosine as a substrate (0.0108 μmoles/mg/hr), although less than that observed with L-glutamic acid as substrate (0.0496 μmole/mg/hr), is nevertheless brisk.

L-Glutamate dehydrogenase. On the basis of the similarity to L-glutamic acid, it was anticipated that L-alanosine might be susceptible to catalytic attack by L-glutamate dehydrogenase. However, even at a concentration of 0.05 M, the antibiotic was neither a substrate nor an inhibitor of this pivotal enzyme.

L-Glutamate decarboxylase. Using a radiometric technique and a partially purified preparation of L-glutamate decarboxylase from *E. coli*, it was observed that 125 mM L-alanosine inhibited this enzyme by 91 per cent. When L-glutamate was replaced by L-alanosine, the antibiotic was α-decarboxylated actively; the K_m for L-alanosine was ~ 20 mM. These results are summarized in Table 8.

Production of aminoaciduria

It was also observed that parenteral L-alanosine was capable of deranging the urinary excretion of L-glutamic acid. Thus, after therapeutic doses of L-alanosine, the concentration of L-glutamic acid in the urine doubled. In contrast to the case with L-aspartic acid, parenteral L-alanosine failed to produce an increase in the hepatic pools of L-glutamic acid (Table 6).

Interaction of L-alanosine with enzymes utilizing L-asparagine

L-Asparagine interferes with the uptake of L-alanosine competitively [22]. Since amino acid transport is most likely an enzymatic process, it was reasonable to determine whether L-alanosine would interfere with the transport of L-asparagine and whether it would inhibit other enzymes acting on L-asparagine.

Transport. The effect of L-alanosine on the transport of L-asparagine is presented in Fig. 2, panel C. The K_m for L-asparagine transport is 67 μM, whereas the K_i for L-alanosine is 12 mM.

The interaction of L-alanosine with individual enzymes of L-asparagine metabolism is presented in the following sections.

L-Asparaginyl tRNA synthetase. For convenience, a partially purified preparation of L-asparaginyl tRNA synthetase from mouse brain was used since that organ, and hence the enzyme prepared from it, is deficient in L-asparaginase. L-Alanosine did not inhibit this enzyme to any important degree at concentrations up to 15 mM.

L-Asparagine transaminase. At a concentration of 12.5 mM, L-alanosine inhibited by 60 per cent the transamination of L-[U-^{14}C]asparagine with oxaloacetate in a reaction system utilizing crude transaminase from adult mouse liver [5].

L-Asparaginase. L-Asparaginase is the mose widely studied enzyme involved in the metabolism of L-asparagine. Its catalytic capabilities are rather broad, encompassing the hydrolysis of amides, nitriles, hydrazides, and other moieties [32]. With L-asparagine as substrate, the L-asparaginases from *E. coli* and *E. carotovora* were not inhibited by L-alanosine, even at a 10 mM concentration (Table 9). Moreover, no degradation of L-alanosine ensued even after protracted incubations with crystalline preparations of L-asparaginase from *E. coli, E. carotovora, Vibrio succinogenes, Dasyprocta aguti*, or with the crude amidohydrolase from mouse liver.

The effect of L-alanosine on the enzymes metabolizing L-asparagine are presented in table 9. The enzymes were not inhibited *in vitro* to any important degree. However, the urinary excretion of L-asparagine was increased after a therapeutic dose of L-alanosine (Table 5). In contrast, drug treatment

Table 9. Summary of the effects of L-alanosine *in vitro* on the enzymes of L-asparagine metabolism*

Enzyme	EC No.	Source of enzyme	Maximum conc. tested (mM)	% Inhibition	Is alanosine an alternate substrate
L-Asparaginyl tRNA synthetase	6.1.1.X	Mouse brain	15	30	ND
					ND
L-Asparagine transaminase	2.6.1.14	Mouse liver	12.5	60	No
L-Asparaginase	3.5.1.1	*E. coli*	10	0	No
		E. carotovora	10	0	No
		D. aguti	10	0	No

* The procedures used for enzyme assays are detailed in Materials and Methods. ND: not determined. The per cent inhibition is that seen at the maximum concentration tested.

caused no increase in the hepatic concentration of L-asparagine (data not shown).

Interaction of L-alanosine with enzymes of L-glutamine metabolism

Originally, L-alanosine was thought to be an antagonist of L-glutamine because of its structural similarity to this amino acid [2]. Our studies on transport of the antibiotic into murine lymphoblasts (L5178Y) showed that this process was inhibited by L-glutamine [22]. For this reason we elected to study the interaction of L-alanosine with select enzymes of L-glutamine metabolism, including those involved in the transport of this amino acid.

Transport. The effect of L-alanosine on the transport of L-glutamine is presented in Fig. 2, panel D. The drug inhibits the transport of L-glutamine in an apparently competitive manner: the K_m for L-glutamine transport is 12.5 μM, whereas the K_i for L-alanosine is 16 mM. The interactions of L-alanosine with individual enzymes of L-glutamine metabolism are presented in the following sections.

L-Glutaminase. Mammalian L-glutaminases provide a portion of the ammonia needed for carbamyl phosphate synthesis in mitochondria [33]. Since L-alanosine produces L-glutamic aciduria, it was relevant to study its effect on a panel of L-glutaminases. Crude hepatic mitochondrial L-glutaminase from the mouse was unaffected by pharmacologically meaningful concentrations of L-alanosine (up to 0.05 M) *in vitro*, in the presence or absence of phosphate (0.01 M). The neutral L-glutaminase from *Aerobacter* was inhibited 50 per cent by 0.3 M L-alanosine. In the case of the L-asparaginases from *E. carotovora* and *E. coli*, 5 mM L-alanosine inhibited their L-glutaminase activity by about 50 per cent; double reciprocal plots of the kinetics of inhibitions of the last two enzymes are presented in Fig. 8. The K_i of L-alanosine for the enzyme from *E. carotovora* is 1.6 mM and that for the *E. coli* enzyme is 5.9 mM. As was mentioned earlier, no evidence was seen for the decomposition of alanosine by any of the above enzymes.

L-Glutamine tRNA synthetase (EC 6.1.1.18). *In vitro*, L-glutaminyl tRNA synthetase from mouse liver was not inhibited by 10 mM L-alanosine. Esterification of L-alanosine to tRNA by L-glutaminyl tRNA synthetase was not examined.

The amidotransferases. L-Glutamine donates its amide nitrogen to any of a dozen or more known acceptors, some of which are involved in the biosynthesis of the purine and pyrimidine ring [34]. Table 10 documents the finding that of the amido transferases tested, only carbamyl phosphate synthetase was inhibited significantly by L-alanosine at 10 mM. The absence of any effect of L-alanosine on 5-phosphoribosylamine synthetase (an early enzyme in purine biosynthesis) or on GMP synthetase is in keeping with our present understanding of the select effect of L-alanosine on purine biosynthesis at the level of AMP in mammals [1, 2, 27].

DISCUSSION

The weight of evidence presented here and elsewhere [25] supports the conclusion that L-alanosine enters cells, and is metabolized, on the basis of its close structural resemblance to the dicarboxylic amino acids or their amides. This metabolism can be viewed in two ways: that which is quantitatively important, and that which is qualitatively important.

In quantitative terms, transamination of L-alanosine and reduction of the resultant α-ketocarboxylic acid appears to be the principal metabolic fate of the antibiotic. Cardiac and skeletal muscles transaminate L-alanosine vigorously; this activity is very likely attributable to GOT and GPT. Although the velocity of transamination of L-alanosine by these enzymes is only one-fifth of that seen with L-glutamic acid, these muscle masses are so rich in GOT and GPT (0.08 I.U./g and 0.06 I.U./g, respectively), and represent such a large fraction of the total mass (30 per cent), that 40 μmoles ($\sim$6 mg) of the antibiotic, in theory could be decomposed by this route per hour, at such times (for example, during the first hour following dosing) as the enzyme is saturated (K_m of GOT for L-alanosine is 0.625 mM). Support for the quantitative importance of transamination comes from the patterns of urinary excretion of the metabolites of L-alanosine: the α-hydroxy derivative appears to be the most abundant excretory product, accounting for a major fraction (60–70 per cent) of the administered dose in several species [25]. The most likely source of this metabolite is via transamination and reduction of the resultant α-ketocarboxylic acid. Inasmuch as this metabolite appears to be metabolically inert (Table 2), its production could be viewed as a kind of detoxification.

In qualitative terms, with therapeutic, toxicologic and enzymologic actions as end points, the most important metabolic fate of L-alanosine is its condensation with 5-amino-4-imidazole carboxylic acid ribonucleotide to yield a fraudulent anabolite capable of powerfully inhibiting adenylosuccinate synthetase ($K_i \sim 0.3$ μM). The synthesis of this key metabolite, which was originally reported by Hurlbert *et al.* [27], may be rather sluggish *in vivo* because DNA synthesis in P388 tumor cells does not begin to be affected until 2 hr after exposure to L-alanosine; (another interpretation of this lag is that, in the absence of *de novo* synthesis, it may take 2 hr to deplete the preformed AMP pool). Similarly, the finding that 24 hr elapse before restitution of DNA synthesis could be a direct reflection of the half-life of this inhibitory molecule. Also, none of the other metabolites or analogs significantly inhibited adenylosuccinate synthetase (Table 2).

IMP dehydrogenase and adenylosuccinate synthetase share IMP as a common substrate, but IMP dehydrogenase has a 10-fold greater affinity for this nucleotide [35, 36]. When, after treatment with L-alanosine, IMP accumulates due to blockage of AMP synthesis, the pool size of XMP or GMP could increase commensurately. In fact, L-alanosine has been shown to produce an increase in the concentration of GMP in L1210 cells *in vivo* [3]. The fact that the present studies show no increase of the hepatic IMP level in mice up to 5 hr after parenteral administration of L-alanosine suggests that the surplus IMP is being utilized for XMP or GMP synthesis.

In summary, the therapeutic and toxicologic implications of the findings reported here are clear.

Table 10. Summary of the effects of L-alanosine *in vitro* on the enzymes of L-glutamine metabolism*

Enzyme	EC No.	Source of enzyme	Maximum concentration tested (mM)	% Inhibition	Nature of inhibition	K_i	Is alanosine an alternate substrate?
L-Glutaminyl tRNA synthetase	6.1.1.18	Mouse liver	10	10	ND	ND	No
L-Glutamine amidohydrolase	3.5.1.2	*E. coli* L-asparaginase	5	20	Competitive	5.9 mM	No
		E. carotovora L-asparaginase	5	59	Competitive	1.69 mM	No
		A. aerogenes L-glutaminase	400	66	Competitive	0.3 M	No
Amidotransferases							
Desamido NAD: L-glutamine amidoligase	6.3.5.1	Mouse liver	10	0	ND	ND	ND
XMP: L-glutamine amidoligase	6.3.5.2	Mouse brain	10	18	ND	ND	ND
UTP: L-glutamine amidoligase	6.3.4.2	Rat fetal liver	10	20	ND	ND	ND
		Mouse thymus	10	20	ND	ND	ND
		Mouse brain	10	20	ND	ND	ND
L-Glutamine: D-fructose 6-phosphate aminotransferase	5.3.1.19	Mouse thymus	10	37	ND	ND	ND
Ribosylamine-5-phosphate: pyrophosphate phosphoribosyl transferase	2.4.2.14	Mouse brain	10	0	ND	ND	ND
ATP: carbamate phosphotransferase	2.7.2.9	Chinese hamster ovary cells	10	49	ND	ND	ND
		E. coli	10	59	ND	ND	ND

* The nature of inhibition was studied in detail only in the case of those enzymes where the ID_{50} was 10 mM or less. ND: not determined. The per cent inhibition is that seen at the maximum concentration tested.

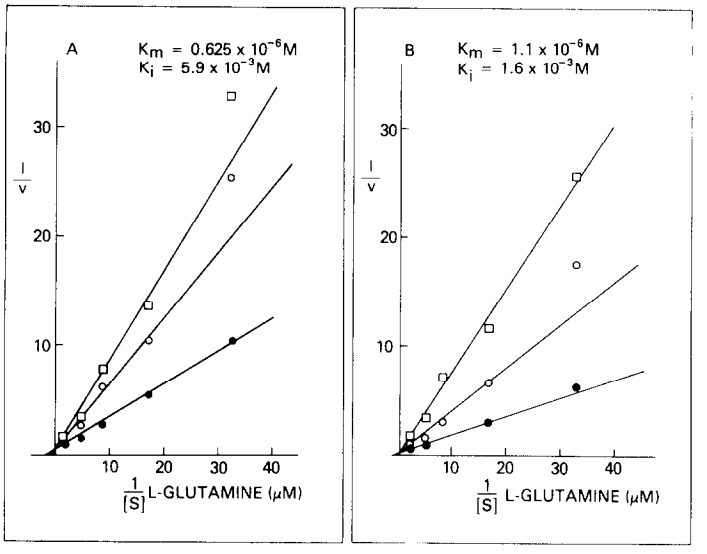


Fig. 8. Inhibition of L-glutaminase activity of L-asparaginases from *E. carotovora* and *E. coli* by L-alanosine. In a final volume of 15 μl were admixed 5μl of L-[U-^{14}C]glutamine at the concentrations shown, 5 μl of neutral L-alanosine [final concentration of 0.005 M (□), 0.002 M (○) or water (●)], and, to initiate the reaction, 5 μl of L-asparaginase from *E. coli* (panel A) or *E. carotovora*, (panel B).

Organs rich in GOT are likely to detoxify L-alanosine before it is anabolized. Tissues and tumors rich in SAICAR synthetase and poor in adenylosuccinate lyase (or other enzymes that would catabolize the inhibitor molecule) are likely to be susceptible to damage by the drug. It is the purpose of studies currently under way to test these inferences directly.

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REFERENCES

1. J. C. Graff and G. W. Plagemann, *Cancer Res.* **36**, 1428 (1976).
2. G. R. Gale and G. B. Schmidt, *Biochem. Pharmac.* **17**, 363 (1968).
3. G. R. Gale and A. B. Smith, *Biochem. Pharmac.* **17**, 2495 (1968).
4. G. R. Gale, W. E. Ostrander and L. M. Atkins, *Biochem. Pharmac.* **17**, 1823 (1968).
5. M. C. Laue, B. P. Yip and F. B. Rudolph, *Biochem. Pharmac.* **26**, 1353 (1977).
6. S. Anandaraj, H. N. Jayaram, J. W. Olney and D. A. Cooney, *Proc. Am. Ass. Cancer Res.* **18**, 219 (1977).
7. A. K. Tyagi, H. N. Jayaram, S. Anandaraj, B. Taylor and D. A. Cooney, *J. Biochem. Biophys. Meth.* **1**, 221 (1979).
8. G. Shaw and D. V. Wilson, *J. chem. Soc.* 2937 (1962).
9. M. Yoshikawa, T. Kato and T. Takenishi, *Bull. chem. Soc. Japan* **42**, 3505 (1969).
10. E. Bernt and H. U. Bergmeyer, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), Vol. 4, p. 1704. Academic Press, New York (1974).
11. J. V. Benson, M. J. Gordon and J. A. Patterson, *Analyt. Biochem.* **18**, 228 (1967).
12. J. G. Flaks and L. N. Lukens, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. 6, p. 52. Academic Press, New York (1963).
13. M. P. J. S. Anandaraj and J. D. Cherayil, *Analyt. Biochem.* **58**, 190 (1974).
14. B. A. Roe, *Nucleic Acids Res.* **2**, 21 (1975).
15. D. A. Cooney, R. L. Capizzi and R. E. Handschumacher, *Cancer Res.* **30**, 929 (1970).
16. H. A. Milman and D. A. Cooney, *Biochem. J.* **142**, 27 (1974).
17. H. U. Bergmeyer, E. Bernt, H. Mollering and G. Pfleiderer, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), Vol. 4, p. 1696. Academic Press, New York (1974).
18. W. Lamprecht and I. Trautschold, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), Vol. 4, p. 2102. Academic Press, New York (1974).
19. J. V. Passonneau and O. H. Lowry, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), Vol. 4, p. 2067. Academic Press, New York (1974).
20. V. Schultz and J. M. Lowenstein, *J. biol. Chem.* **253**, 1938 (1978).
21. S. S. Tate, L. L. Ross and A. Meister, *Proc. natn. Acad. Sci. U.S.A.* **70**, 1447 (1978).
22. J. M. Kelley, R. H. Adamson, D. A. Cooney, H. N. Jayaram and S. Anandaraj, *Cancer Treat. Rep.* **61**, 1471 (1977).
23. D. A. Cooney, H. A. Milman and B. Taylor, *Biochem. Med.* **5**, 190 (1976).
24. T. D. Kempe, E. A. Swyryd, M. Bruist and G. R. Stark, *Cell* **9**, 541 (1976).
25. H. N. Jayaram, A. K. Tyagi, S. Anandaraj, J. A. Montgomery, J. A. Kelley, J. Kelley, R. H. Adamson and D. A. Cooney, *Biochem. Pharmac.* **28**, 3551 (1979).
26. B. Shepartz, in *Regulation of Amino Acid Metabolism in Mammals*, p. 120. W. Saunders, Philadelphia (1973).
27. R. B. Hurlbert, C. J. Zimmerman and D. B. Carrington, *Proc. Am. Ass. Cancer Res.* **18**, 234 (1977).
28. L. Berlinquet and M. Laliberte, *J. Biochem., Tokyo* **38**, 207 (1970).
29. A. Meister, in *Biochemistry of the Amino Acids*, Vol. 1, p. 334. Academic Press, New York (1965).

30. G. R. Gale and L. M. Atkins, *Proc. Soc. exp. Biol. Med.* **129**, 842 (1968).
31. M. Orlowski and A. Meister, *Proc. natn. Acad. Sci. U.S.A.* **67**, 1248 (1970).
32. J. C. Wriston and O. Y. Tobias, in *Advances in Enzymology* (Ed. A. Meister), Vol. 39, p. 185. John Wiley, New York (1973).
33. N. Katunama, T. Katsunama, T. Towatari and I. Tomino, in *Enzymes of Glutamine Metabolism* (Eds. S. Prusiner and E. R. Stadtman), p. 227. Academic Press, New York (1973).
34. S. Prusiner and E. R. Stadtman, in *Enzymes of Glutamine Metabolism* (Eds. S. Prusiner and E. R. Stadtman), p. 605, Academic Press, New York (1973).
35. M. R. Atkinson, R. K. Morton and A. W. Murray, *Biochem. J.* **89**, 167 (1963).
36. T. Spector and R. L. Miller, *Biochim. biophys. Acta* **445**, 509 (1976).