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METABOLITES OF ALANOSINE, AN ANTITUMOR ANTIBIOTIC

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Abstract—The metabolism of alanosine, DL-2-amino-3-(*N*-hydroxy, *N*-nitrosamino) propionic acid (NSC-143647), a new antitumor antibiotic, was studied in mice, rats, monkeys and dogs. Urine is the principal excretory vehicle for the drug in these four species. In rats, unchanged alanosine is the principal excretory product. In the other species, a second, more acidic component accounts for the major fraction of the drug-derived radioactivity in urine; this product retains the characteristic u.v. spectrum and both carbons, 1 and 3, of alanosine; it is chromatographically and spectrally indistinguishable from the compound generated by the action of NADH and malate dehydrogenase on the product resulting from the incubation of L-alanosine with L-glutamate oxaloacetate transaminase (GOT) (EC 2.6.1.1) and α-ketoglutarate. On the basis of this evidence, this metabolite is concluded to be the α-hydroxy counterpart of L-alanosine.

The antibiotic was susceptible to transamination *in vitro* by extract of organs rich in GOT; heart was pre-eminent in this regard, and α-ketoglutaric acid was found to be the preferred α-keto partner in the reaction. Crystalline GOT catalyzed an identical reaction *in vitro*, and the product, like oxaloacetic acid, was susceptible to enzymatic condensation with acetyl CoA, in the presence of citrate synthase. Inability to detect the α-keto analogue of alanosine, 2-oxo-3-(*N*-hydroxy, *N*-nitrosamino) propionic acid, in tissues and excreta is attributed to the facile decomposition of this metabolite *in vivo*.

In vitro, alanosine was susceptible to decarboxylation by homogenates of mouse brain and by purified L-glutamate decarboxylase (EC 4.1.1.15) from *Escherichia coli*. No evidence could be adduced for denitrosation of the antibiotic nor for reduction of the nitroso function in a system containing hepatic microsomes and NADPH. However, *L*-amino acid oxidase (EC 1.4.3.2) and high concentrations of pyridoxal phosphate catalyzed the deamination of alanosine at alkaline pH. In confirmation of the observations of Hurlbert *et al.* [Proc. Am. Ass. Cancer Res. **18**, 234, (1977)], alanosine was found to be used by phosphoribosylaminoimidazole-succinocarboxamide synthetase (EC 6.3.2.6) as a fraudulent substrate. Also observed was the condensation of alanosine with IMP, catalyzed by a partially purified preparation of adenylosuccinate synthetase (EC 6.3.4.4) from rabbit muscle; this anabolite exhibited chromatographic properties quite similar to adenylosuccinic acid.

Inasmuch as a substantial percentage of the administered dose of alanosine was found to associate with carcass-macromolecules for protracted periods, attempts were made to determine the basis for this fate. Equivalent labeling was produced irrespective of whether DL-[1—^{14}C] or DL-[3—^{14}C]alanosine was the injectate, so that reutilization of metabolically generated [^{14}C]O_2 is not likely to explain macromolecular retention of the antibiotic. *In vitro*, no esterification of alanosine to tRNA was observed, but the drug did bind to tRNA in an ATP-independent reaction. *In vivo*, ten times more DL-[3—^{14}C]alanosine was incorporated into the hemoglobin of animals recovering from phenylhydrazine anemia than was observed in their saline-treated counterparts. Isolated reticulocytes incorporated only minor amounts of purified DL-[1—^{14}C]alanosine into their molecules; this process was insensitive to inhibition by cycloheximide. *In vitro*, alanosine (used in lieu of L-aspartic acid) neither supported nor inhibited globin synthesis by rabbit reticulocyte lysates. These results leave unsettled the question of whether macromolecular association of alanosine reflects incorporation or affiliation.

L-Alanosine, L-2-amino-3-(*N*-hydroxy,*N*-nitrosamino) propionic acid, is an antibiotic produced by *Streptomyces alanosinicus*; it exhibits antibacterial, antifungal, antiviral and antitumor activity [1, 2].

In the first paper of this series [3], quantitative aspects of the fate and distribution of alanosine in mice, rats, dogs and monkeys were described, and certain metabolic routes suggested. It is the purpose of the present report to document the operation of these routes *in vivo* and *in vitro*, as well as to characterize some of the more prominent and important metabolites of this antibiotic.

MATERIALS AND METHODS

DL-[1— or 3—^{14}C]Alanosine (specific radioactivity 7.1 or 9.06 mCi/mmole, respectively) was supplied by Stanford Research Institute, Menlo Park, CA. The purity of these radiochemicals was greater than 90 percent when determined by thin-layer and column chromatography [3]. In select cases, further purification was accomplished by chromatography of the radiolabeled antibiotic, as described below. L-Alanosine (NSC-153353), DL-alanosine (NSC-143647), 2-amino-3-(*N*-hydroxamino) propionic acid (HAPA,

NSC-159165), 2-(*N*-hydroxy,*N*-nitrosamino) acetic acid (NSC-294817) (compound 3), and α-*N*-carbamyl-L-alanosine (NSC-287091) were obtained from the Drug Research and Development Branch, National Cancer Institute, Bethesda, MD. L-Glutamate decarboxylase (EC 4.1.1.15) from *Escherichia coli* was purified by the method of Shukuya and Schwert [4] to a specific activity of 10 I.U./mg of protein. L-Amino acid oxidase (EC 1.4.3.2) from *Crotalus adamanteus* (sp. act. 0.47 I.U./mg), D-amino acid oxidase (EC 1.4.3.3) from hog kidney (sp. act. 17.6 I.U./mg), acylase I (EC 3.5.1.14) from hog kidney (sp. act. 2000 units/mg), L-leucine aminopeptidase (EC 3.4.11.1, sp. act. 132 units/mg), sulfatase (EC 3.1.6.1) from *Helix pomatia* (sp. act. 5000 units/ml), citrate synthase (EC 4.1.3.7) from pig heart (sp. act. 80 I.U./mg), citrate lyase (EC 4.1.3.6) from *Enterobacter aerogenes* (sp. act. 8 I.U./mg), and β-glucuronidase (EC 3.2.1.31) from bovine liver (sp. act. 10.000 Fishman units/ml) were all products of the Sigma Chemical Co., St. Louis, MO. Catalase (EC 1.11.1.6) from bovine liver (sp. act. 23.000 I.U./mg) and β-glucosidase (EC 3.2.1.21) from almond (sp. act. 2.5 units/mg) were obtained from the Worthington Biochemical Corp., Freehold, NJ. Esterase (EC 3.1.1.1) from hog liver (sp. act. 100 I.U./mg), L-glutamate oxaloacetate transaminase (GOT) (EC 2.6.1.1) from pig heart (sp. act. 180 I.U/mg), L-malate dehydrogenase (MDH) (EC 1.1.1.37) from pig heart (sp. act. 550 I.U./mg), and L-lactate dehydrogenase (LDH) (EC 1.1.1.27) from rabbit muscle (sp. act. 720 I.U./mg) were products of Boehringer, New York, NY. L-Aspartase (EC 4.3.1.1) from *E. coli* (sp. act. 82 I.U./mg) was a gift from Dr. Masanobu Takushige, Kyoto University, Sakyo-Ku, Kyoto 606, Japan. L-Aspartate-β-decarboxylase (EC 4.1.1.12) from *Alcaligenes faecalis* (sp. act. 77 I.U./mg) was a gift from Dr. Suresh Tate, Cornell University School of Medicine, New York, NY. β-Cystathionase from *E. coli* (EC 4.4.1.8) was a gift from Professor J. H. Hong, Department of Biochemistry, Brandeis University, Waltham, MA, and had a specific activity of 8 units/mg of protein. L-Aspartic acid transcarbamylase from *E. coli* (sp. act. approximately 266.7 I.U./mg) was provided by Dr. Evan Kantrowitz, Harvard University, Cambridge, MA. Chinese hamster ovary cells, resistant to *N*-phosphonacetyl-L-aspartic acid and rich in L-aspartic acid transcarbamylase, were donated by Dr. George Stark, Stanford University, Stanford, CA. The sodium salt of 5-aminoimidazole-4-carboxylic acid ribonucleotide [5] (AICOR) was prepared by the saponification in 6 N NaOH of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), which had been synthesized from the corresponding ribonucleoside by treatment with $POCl_3$ in trimethylphosphate [6] at 5°, followed by hydrolysis in cold water.

Chromatographic techniques

Thin-layer chromatography (t.l.c.) was carried out on silica gel, essentially as described earlier [3]. Ascending paper chromatography was conducted on Whatman 3M paper using butanol–acetic acid–water (2:1:1, by vol) with a development time of 16 hr. High voltage electrophoresis was carried out for 1 hr at 3000 V on Whatman 3M paper moistened with 0.1 M sodium phosphate buffer at pH 6.8 or 7.2. Automatic amino acid analysis was performed on the Jeol analyzer, using Jeol AR-50 resin and lithium citrate buffer (pH 2.72) as eluent [7]. Chromatographic analysis of acidic metabolites was carried out on a 0.8 × 15 cm column of Hamilton HA-X4 resin equilibrated with 0.015 M lithium citrate (pH 2.65), and operated at a flow rate of 0.67 ml/min. After loading the sample, this column was developed with the equilibrating buffer for 90 min at 37°, followed by 0.0375, 0.075, 0.15 and 0.225 M lithium citrate buffers (pH 2.72), applied for a period of 60, 60, 75 and 60 min each. Chromatography on DEAE Sephadex was performed under the conditions described in the appropriate figures.

Ultraviolet spectra

Ultraviolet spectra were recorded on a Beckman DGB spectrophotometer with a Beckman 10 inch chart recorder.

Purification of DL-[1-^{14}C]alanosine

Fifty μCi of DL-[1-^{14}C]alanosine were dissolved in 1 ml of 0.015 M lithium citrate buffer (pH 2.67) and loaded on an 8 × 150 mm column of Hamilton HA X4 resin. The column was developed with the same buffer at 37°. DL-[1-^{14}C]Alanosine eluted at 16 min; the active fractions were pooled and lyophilized. The lyophilized fractions were dissolved in 1 ml of 0.15 M lithium citrate buffer (pH 2.72) and loaded on an 8 × 700 mm column of AR-50 resin. Elution was performed at 37° with the same buffer. DL-[1-^{14}C]Alanosine was eluted from this column at 48 min; the active fractions were pooled and lyophilized. To remove salts from the sample, the lyophilized preparation from the previous step was dissolved in a small volume of water and loaded on a 25 × 900 mm column of Bio-Gel, P-2. The column was eluted with distilled water and the active fractions containing DL-[1-^{14}C]alanosine were pooled and lyophilized. Traces of citrate left in the sample were removed by ascending chromatography on Whatman 3M paper with isopropanol–water (85 : 15, v/v) as solvent. The chromatogram was developed for 24 hr. The ultraviolet spots of DL-[1-^{14}C]alanosine were cut from the dried chromatogram, eluted in water, and lyophilized. On rechromatography, this purified sample of DL-[1-^{14}C]alanosine gave a single symmetrical peak on both Hamilton HA X4 and AR-50 (anion and cation exchange) resin columns.

High pressure liquid chromatography

High pressure liquid chromatography was carried out in a micro NH_2 column, (Waters Associates, Milford, MA) developed with a gradient of 0.01–0.2 M ammonium phosphate at pH 7.0, using Curve No. 4 of the model 660 solvent programmer. The gradient was accomplished in 20 min at a flow rate of 2.0 ml/min.

Organ and tumor metabolites of alanosine

Male BDF_1 mice (either normal or where indicated, bearing 10 mm subcutaneous nodules of leukemia 5178Y) were given single intraperitoneal injections of 10 μCi of purified DL-[1-^{14}C]alanosine, and killed 2 hr later. Representative viscera, or tumor nodules, were

removed, frozen on dry ice and homogenized in 0.05 M Tris–HCl (pH 8.4). Aliquots of the homogenates were used as such, or deproteinized with 0.1 M perchloric acid and subjected to t.l.c., paper chromatography, paper electrophoresis and amino acid analysis as described earlier.

Urinary metabolite of alanosine

Collection of urine. Mice, rats, dogs and monkeys were given one intravenous injection of DL-[1-^{14}C]alanosine (10 μCi, 20 mg/kg, in the case of mice and rats, and 10 μCi, 10 mg/kg, in the case of dogs and monkeys), as detailed in our earlier paper [3]. Urine from the larger species was collected in iced vessels; urine from mice was collected in plastic metabolism cages into receptacles chilled externally with dry ice.

Preparation of urinary metabolite (compound 1). Groups of ten BDF_1 mice were injected intraperitoneally with L-alanosine (500 mg/kg) and 10 μCi per mouse of DL-[1- or 3-^{14}C]alanosine. Urine was collected for 24 hr as described earlier, clarified by filtration through Miracloth, lyophilized, and fractionated as follows. The lyophilized urine was reconstituted with a minimum volume of distilled water and applied to a 2.4 × 96 cm column of Dowex 1 × 8 in the formate form. The column was developed with a linear gradient of 0.0–2.0 M formic acid, 2000 ml each; the principal urinary metabolite (compound 1) was eluted at 1.9 M. Fractions containing it were pooled, lyophilized, and chromatographed on a 2.4 × 48 cm column of DEAE Sephadex A-25 in the formate form using a linear gradient of triethylammonium formate, 0.05–1 M, 750 ml each; the principal urinary metabolite was eluted at 0.2 M. Fractions containing it were pooled, lyophilized, and applied to an 0.8 × 15 cm column of Hamilton HA-X4 resin in the citrate form. The urinary metabolite was eluted with 0.015 M lithium citrate, pH 2.65. Fractions containing it were pooled, lyophilized, and chromatographed on a column of DEAE Sephadex identical to that described above. Peak fractions were lyophilized three times, and the resultant yellow powder was stored at −87°.

Decarboxylation and transamination of alanosine

Organs were excised from normal BDF_1 mice, frozen and homogenized (1:4, w/v) in 0.1 M Tris–HCl buffer, pH 7.6 and then dialyzed against the same buffer containing 1 μM pyridoxal phosphate in the cold for 18 hr. Dialyzed organ homogenates were centrifuged at 12,000 *g* for 3 min and the supernatant fluid was used for metabolic studies *in vitro*. Incubation mixtures were constituted in Eppendorf test tubes; in a final volume of 15 μl were admixed: 5 μl (0.25 μCi) of DL-[1-^{14}C]alanosine, 5 μl of a neutral 0.03 M solution of α-ketocarboxylic acid, or 5 μl of 0.05 M Tris–HCl buffer (pH 7.6) and 5 μl of dialyzed organ homogenate. The reactants were mixed by brief centrifugation at 12,000 *g*, and incubated at 37° for 1 hr, at which point the bottoms of the vessels were quickly frozen on a bed of solid CO_2 to arrest the reaction. Droplets (5 μl) of 40% KOH were dispensed onto the underside of the caps of the reaction vessels, and 50 μl of 0.66 M sodium acetate buffer (pH 4.2) containing 1% H_2O_2 were added to the bottom of the vessels. The vessels were capped and incubated at 37° for 2 hr; at the termination of the incubation period, the lids were cut off and immersed in 10 ml of scintillation fluid consisting of ethanol, toluene and Liquiflor (New England Nuclear, Boston, MA) (8:16:1 by vol. respectively), and radioactivity was counted in a Beckman model LS-230 liquid scintillation spectrometer at 87 per cent efficiency. Modifications, if any, of the specified techniques are described in the legends to the appropriate figures and tables. For computation of activity, the total radioactivity liberated as [^{14}C]O_2, when the incubation mixture contained an α-ketocarboxylic acid, was taken to represent both transamination and decarboxylation; [^{14}C]O_2 liberated in the absence of α-ketocarboxylic acid represented decarboxylation alone.

Nonenzymatic transamination of alanosine

In a final volume of 10 μl were admixed: 5 μl (0.25 μCi, 44 nmoles) of DL-[1-^{14}C]alanosine and 5 μl of neutral pyridoxal phosphate at concentrations ranging from 2 to 200 mM. After 1 hr at 37°, 50 μl of 1% H_2O_2 in 1 N HCl were added to the reactants, and at the same time, a 5 μl droplet of 40% KOH was deposited on the underside of the lids of the reaction vessels for the purpose of trapping [^{14}C]O_2. The vessels were closed and further incubated at 37° for 1 hr, at which time any radioactivity on the lids was measured as described earlier.

Oxidation of alanosine

Oxidation of alanosine by L-amino oxidase was studied as follows. In a reaction volume of 15 μl were admixed: 5 μl of DL-[1-^{14}C]alanosine (0.31 μCi, 54.5 nmoles), 5 μl of 0.1 M Tris–HCl buffer, pH 8.0, and 5 μl of L-amino acid oxidase (16.5 I.U./ml). After incubation at 37° for 30 min, 5 μl of 40% KOH were pipetted onto the underside of the lid, and 50 μl of 1% H_2O_2 in 1 N HCl (to α-decarboxylate the product) were added to the bottom of the vessel; the lids were quickly closed and incubation was continued at 37° for 45 min. At this time, the caps were cut off and radioactivity was counted in a scintillation spectrometer.

Attempts to oxidize compound 1 with malate dehydrogenase

Approximately 300 nmoles (5 μl) of compound 1 were incubated at 37° with 5 μl (3.6 I.U.) of malate dehydrogenase, 5 μl of neutral 0.1 M NAD and 5 μl of 0.5 M Tris–HCl buffer, pH 10.2. After 1 hr at 37°, the formation of the product was assessed with paper chromatography and paper electrophoresis.

Analysis of the urinary metabolite (compound 1) using conjugate degrading enzymes

The following enzymes, at the specified concentration, pH and buffer concentrations, were used for degrading the urinary metabolite: acylase-I (5 mg/ml in 0.02 M Tris–HCl, pH 7); β-glucosidase (5 mg/ml in 0.02 M acetate buffer, pH 5); L-leucine aminopeptidase (5 mg/ml in 0.02 M Tris–HCl, pH 8, containing 1 mM $MgCl_2$); esterase (10 mg/ml in 0.02 M Tris–HCl buffer, pH 7.2); sulfatase (5000 units/ml in 0.02 M sodium acetate buffer, pH 5.0); and β-glucuronidase (5 mg/ml in 0.02 M sodium acetate buffer,

pH 5.0). To ≈300 nmoles of urinary metabolite (in a volume of 5 μl) 5 μl of the appropriate enzyme were added; after 16 hr of incubation at 37°, the products were analyzed both by electrophoresis and by paper chromatography.

Preparation of the α-keto analog of alanosine (compound 2)

Three hundred nmoles of DL-[1-^{14}C]alanosine were incubated with 5 μl (1 I.U.) of GOT and 5 μl of neutral 0.1 M α-ketoglutaric acid at pH 8.4. After incubation at 37° for 1–6 hr, the generation of products was analyzed electrophoretically, and chromatographically on Hamilton HA-X4 resin.

Reduction of the α-keto analogue of alanosine (compound 2) with malate dehydrogenase

Three hundred nmoles (1 μCi) of alanosine were incubated at 37° with 5 μl (1 I.U.) of GOT, and 5 μl of neutral 0.1 M α-ketoglutaric acid, pH 8.4, in the presence and absence of 5 μl (3.6 I.U.) of MDH and 5 μl of a neutral 0.1 M solution of NADH. After 16 hr at 37°, the generation of products was assessed electrophoretically, and chromatographically on Hamilton HA-X4 resin.

Examination of denitrosation of alanosine (measurement of nitrite and hydroxylamine)

Male BDF_1 mice were given a single intraperitoneal dose of L-alanosine (500 mg/kg). Urine was collected for the subsequent 24 hr on dry ice. Measurements of nitrite and nitrite plus hydroxylamine were carried out by the method of Csaky [8]. In parallel studies, mouse liver and kidney were homogenized 1:4 (w/v) in 0.05 M Tris–HCl, pH 8.4, and centrifuged at 12,000 *g* for 6 min; 100 μl of the resultant supernatant fluid were incubated with 0.01 M L-alanosine at 37° for 30 min, and then measurements of nitrite and hydroxylamine in a 20 μl aliquot of the incubation mixture were carried out as described above.

Reduction of alanosine

The possibility of reduction of alanosine by microsomal enzymes was tested using rat liver microsomes as well as a supernatant preparation (12,000 *g* for 3 min) from mouse liver prepared by homogenizing freshly excised liver in 3 vol. of 0.05 M Tris–HCl buffer (pH 7.4) containing 0.005 M 2-mercaptoethanol and 0.01 M $MgCl_2$. The supernatant preparation had a protein concentration of 23 mg/ml. Rat liver microsomes were prepared as follows. Liver was homogenized in 0.05 M Tris–HCl (pH 7.4) containing 150 mM KCl (1:3, w/v); the homogenate was centrifuged at 9000 *g* for 30 min and the resultant supernatant fraction was recentrifuged at 105,000 *g* for 60 min. The microsomal pellet was resuspended in the same buffer and diluted to yield a protein concentration of 3 mg/ml.

Under a nitrogen atmosphere, 200 μmoles of L-alanosine containing approximately 0.3 μCi of DL-[1-^{14}C]alanosine were incubated with 0.5 ml of the enzyme (12,000 *g* supernatant fraction from mouse liver or rat liver microsomal preparation) in the presence of 10 μmoles of NADPH or NADH. The incubation was conducted at 37° for 30 min at the end of which time the reaction mixtures were frozen on dry ice and lyophilized. Appropriate controls with or without the donors of reducing equivalents, or with heated enzyme were also included. The generation of the putative reaction product was analyzed by paper chromatography, by paper electrophoresis and by column chromatography on Hamilton HA-X4 resin as described in an earlier section. The chromatograms and electropherograms were analyzed by strip counting, fraction-counting, and autoradiography.

Anabolites of L-alanosine

Via adenylosuccinate synthetase (EC 6.3.4.4). Adenylosuccinate synthetase was partially purified from rabbit muscle by a technique to be reported elsewhere. To generate the adduct of IMP and L-alanosine, 0.1 I.U. of this preparation was incubated in a final volume of 50 μl with 50 nmoles of IMP and GTP–$MgCl_2$, along with 1 μCi of DL-[1-^{14}C]alanosine for 16 hr at 37°. Production of products was monitored on Hamilton HA-X4 resin in the chromatographic system described earlier.

Via phosphoribosylaminoimidazole succinocarboxamide synthetase (EC 6.3.2.6). Enzyme was partially purified from chicken liver by the technique of Flaks and Lukens [9]. In a final volume of 60 μl were admixed: 176 nmoles (1 μCi) of DL-[1-^{14}C]alanosine, 172 nmoles of 5-aminoimidazole 4-carboxylic acid ribonucleotide, 500 nmoles of ATP $MgCl_2$, 500 nmoles of sodium phosphate, pH 7.3, and 36 μg of enzyme protein. After 2 hr at 37°, the reaction mixture was deproteinized with HCl, and analyzed on Hamilton HA-X4 resin as described earlier.

Via argininosuccinate synthetase (EC 6.3.4.5). Fresh mouse kidney and liver were homogenized in 3 vol. of 0.05 M potassium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol and 0.5 mM EDTA; the homogenate was used as the source of enzyme. In a final volume of 50 μl were admixed: 176 nmoles (1 μCi) of DL-[1-^{14}C]alanosine, 250 nmoles of citrulline, 250 nmoles of ATP–$MgCl_2$ and 20 μl of organ extract. After 2 hr at 37°, the reaction mixtures were deproteinized with HCl and analyzed on Hamilton HA-X4 resin as described earlier.

Via L-aspartic acid transcarbamylase (EC 2.1.3.2). To 50 μl of 0.05 M L-alanosine in 0.05 M Tris–HCl, pH 8.4, were added: 1 I.U. of L-aspartic acid transcarbamylase from *E. coli* (sp. act. 266.7 I.U./mg) or from PALA-resistant Chinese hamster ovary cells (sp. act 0.087 I.U./mg), and 200 nmoles of carbamyl phosphate in 0.05 M Tris–HCl, pH 8.4, or buffer alone; additional controls received boiled enzyme. After 1 hr at 37°, the reaction mixtures were chilled; 5 μl aliquots then were taken for ascending paper chromatography (Whatman 3M paper) using butanol–acetic acid–water (1:1:1, by vol.) as solvent, with authentic *N*-carbamyl L-alanosine as standard. Ureido compounds were located by spraying the dried chromatograms with 1% *p*-dimethylaminobenzaldehyde in ethanolic 1 N HCl. Under these conditions, *N*-carbamyl-L-alanosine exhibited an R_f of 0.45 and L-alanosine an R_f of 0.2; both compounds were *p*-dimethylaminobenzaldehyde positive. Confirmatory analyses were carried out using paper electrophoresis at pH 7.0, and column chromatography on Hamilton HA-X4 resin at pH 2.72. In the

former system, *N*-carbamyl-L-alanosine migrated well in advance of L-alanosine; in the latter (cf. the legend to Fig. 5, panel B-2, for the composition of the reaction mixture in this case), the product eluted 63 min after L-alanosine.

Macromolecular metabolism of alanosine

Binding of DL-[1—^{14}C]*alanosine to serum proteins and hemoglobin*, in vivo. Groups of BDF_1 mice were injected intraperitoneally with either 50 mg/kg phenylhydrazine or saline, daily for 5 days. On day 7 the mean hematocrit of the phenylhydrazine group was 26 vs 45 per cent in the saline controls. Groups of 5 phenylhydrazine or saline-treated mice were injected intraperitoneally on day 7 with 10 μCi of DL-[1—^{14}C]alanosine and killed on day 8. The blood was collected, washed with saline, and then the RBC pellet was washed three times with 5% trichloroacetic acid (TCA). The pellet was digested with 40% KOH and counted by scintillation spectrometry.

Binding of DL-[1—^{14}C]*alanosine to reticulocyte RNA*, in vitro. To study the incorporation of DL-[1—^{14}C]alanosine *in vitro* into the RNA of reticulocytes, mice were treated as described earlier with phenylhydrazine (50 mg/kg) or saline, and bled on day 7. One ml of heparinized blood was dispensed into Eppendorf test tubes; 20 μl of 0.05 M Tris–HCl, or 0.05M cycloheximide, were added, and the cells were preincubated at 37° for 5 min. Then 0.3 μCi of purified DL-[1—^{14}C]alanosine (7.0 mCi/mmole) was added and incubated at 37° for 60 min. Cells were centrifuged and washed two times with 1 ml saline. To the washed cell pellet, 1 ml water was added and the hemolysates were centrifuged at 100,000 *g* for 16 hr at 4°. The supernatant fluid was dialyzed, and radioactivity and protein were measured. The pellet was taken up in 1 ml saline, and 500 μl phenol were added. RNA in the aqueous layer was precipitated with ethanol. The pellet was dissolved in 100 μl water and re-precipitated with ethanol. The RNA pellet was dissolved in water and the binding of alanosine was expressed as pCi bound/O.D unit at 260 nm.

Attempts to demonstrate esterification of alanosine to tRNA

Crude aminoacyl tRNA synthetase (EC 6.1.1.X) from mouse liver was prepared as follows. In a Potter–Elvehjem homogenizer, the tissue was homogenized (1:3, w/v) in 0.05 M Tris–HCl buffer, pH 7.2, containing 0.01 M $MgCl_2$, 0.25 M sucrose, 0.01 M 2-mercaptoethanol, 0.1 M KCl, 0.5 mM EDTA and 10% glycerol. The homogenate was spun at 20,000 *g* in an International Centrifuge for 30 min, and the supernatant fluid was brought to 40% saturation with solid ammonium sulfate; the precipitate, collected by centrifugation, was dissolved in a minimum volume of twice-diluted homogenization buffer, and then dialyzed against three changes of the same buffer at 4°. The dialyzed enzyme was clarified by centrifugation before use.

Acylation of alanosine to tRNA was carried out in an incubation mixture (total volume of 200 μl) containing 50 mM Tris–HCl (pH 7.2), 2.0 mM $MgCl_2$, 15 mM KCl, 0.25 mM EDTA, 5 mM ATP, 50 μg of mouse liver total tRNA, 20 μl of the enzyme preparation and 0.15 μCi of DL-[3—^{14}C]alanosine. Incubation was carried out at 37° for 30 min at the end of which time 10% TCA was added to stop the reaction. The precipitated macromolecules were collected on glass fiber filters (Whatman 34AH), washed three times with cold 5% TCA, dried, and counted as described earlier.

Metabolism of alanosine by isolated tumor cells and thymic lymphocytes

Male BDF_1 mice, weighing between 18 and 20 g, were injected by the intraperitoneal route with a suspension of 0.1 million cells of leukemia 5178Y/AR (L5178Y rendered resistant to L-asparaginase by repeated subcurative doses of the enzyme). After 7–10 days, peritoneal fluid containing tumor cells was aspirated aseptically into polypropylene tubes and centrifuged at 600 *g* for 10 min. The tumor cell pellet was washed twice with 10 ml of Hanks' balanced salt solution (HBSS) and resuspended in HBSS to a final concentration of 10 million cells/ml. Thymuses were excised from ten normal BDF_1 mice, pooled, and minced in HBSS. This preparation was passed through four layers of Miracloth and then centrifuged at 600 *g* at 4° for 10 min to separate the lymphocytes; the pellet was washed with HBSS, centrifuged for 10 min at 600 *g*, and finally resuspended in HBSS to a concentration of 10 million cells/ml. Metabolic studies were carried out in scintillation vials modified for the collection of radioactive gases [10]: 100 μl of the cell suspension (1 million cells) and 1 μCi of DL-[1—^{14}C]alanosine (134 nM) were dispensed into the lower compartment and the assemblies were gassed, closed, and incubated at 37° for 1 hr. At the term of the incubation period, the center wells were cut off and counted as described earlier.

Influence of L-*alanosine on mitochondrial respiration*

The influence of L-alanosine on mitochondrial respiration was studied by the technique of Gosalves *et al.* [11], using isolated rat liver mitochondria and concentrations of the antibiotic ranging from 0.2 to 0.4 mM. We are indebted to Dr. M. Gosalves for the performance of these studies.

Preparation of the free acid of L-*alanosine for derivatization*

The sodium salt of L-alanosine (17 mg, 0.1 nmole) was dissolved in 0.2 ml of distilled H_2O, and approximately 0.5 ml of 0.2 N HCl was added drop-wise until a pH of 3.5 was obtained. The solution was cooled to 0° and allowed to stand for 30 min. The resulting precipitate was filtered with Whatman No. 1 paper and successively washed with 1.0 ml EtOH and 1.0 ml ether. The crude free acid was used without further purification.

Trimethylsilylation of L-*alanosine*

To 2.3 mg of L-alanosine free acid suspended in 0.3 ml of redistilled CH_3CN was added 0.15 ml of *N,O*-bis-trimethylsilyltrifluoroacetamide (BSTFA, Aldrich Chemical Co., Milwaukee, WI). The suspension was heated at 100° for 10 min with periodic shaking to give a clear solution. Aliquots (1 μl) of this solution were used for gas chromatography (G.C.) and combined gas chromatography–mass

spectrometry (G.C.–M.S.). The trimethylsilyl-d_0 (TMS-d_0) derivative was prepared in the same manner except that *N,O*-bis-trimethylsilylacetamide d_{18} (BSA d_{18}, Supelco) was used instead of BSTFA, and the derivatization was conducted on a smaller scale because of the high cost of the deuterated reagent.

Gas chromatography and mass spectrometry

Sample mixtures were analyzed on a Varian 2740 gas chromatograph equipped with a flame ionization detector and linear temperature programmer and interfaced to a DuPont 21-492B mass spectrometer via a single stage glass jet separator. A 1.83 m × 2 mm i.d. glass column packed with 3% SE-30 on 100/120 mesh Gas Chrom Q was operated isothermally at 140° for trimethylsilylated alanosine, or temperature-programmed as indicated in the text. Typical G.C. operating conditions employed injector and detector temperatures of 240° and flow rates of approximately 30 ml/min for helium carrier gas, 30 ml/min for hydrogen, and 300 ml/min for air. Isothermal gas chromatographic retention indices were determined graphically. Mass spectra were recorded for all peaks of interest. Standard mass spectrometer operating conditions were: transfer line and jet separator, 255°; ion source, 240°; electron energy, 75 eV; ionizing current, 250 μamps; accelerating voltage, 1.6 kV; and scan speed, 4 sec/decade.

RESULTS

Metabolism of alanosine in vivo

Table 1 documents the finding that a substantial fraction of the dose of DL-[1-^{14}C]alanosine appears in the urine of mice, rats, dogs and monkeys in the form of a metabolite with chromatographic properties different from those of alanosine.

Table 1. Recovery of radioactivity in urine after intravenous administration of DL-[1-^{14}C]alanosine to mice, rats, dogs and monkeys*

Species	Time of collection of urine (hr)	% Radioactivity recovered in the urine as: DL-Alanosine	Urinary metabolite (compound 1)
Mouse	0–4	20	67
	4–24	9	73
Rat	0–4	83	17
	4–24	35	65
Dog	0–4	6	82
	4–24	3	80
Monkey	0–4	26	70
	4–24	20	75

* The percentage of radioactivity is based on analyses of urine using three different systems: t.l.c., paper electrophoresis, and paper chromatography. Total radioactivity in the pools of urine was determined for each collection; radioactivity recovered as the parent (DL-alanosine) and as the principal urinary metabolite (compound 1) during each collection period was expressed as a percentage of this total. Details of the dosage and route of administration of the drug are given in Materials and Methods. The net percentages of the dose of DL-[1-^{14}C]alanosine recovered in the urine over a 24 hr period were 55, 58, 55 and 57 in the case of mice, rats, dogs and monkeys respectively.

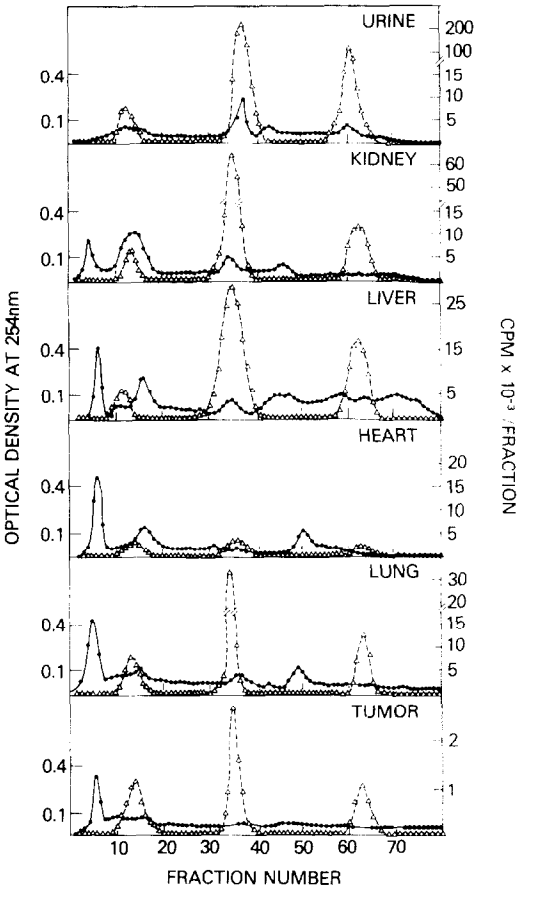

Fig. 1. Chromatography of mouse tissue extracts after parenteral administration of DL-[1-^{14}C]alanosine. DL-[1-^{14}C]Alanosine (10 μCi) was injected, intraperitoneally, into a group of six mice bearing subcutaneous nodules of L5178Y/AR and killed 2 hr later. Representative viscera or tumor were removed, homogenized in 0.05 M Tris·HCl, pH 8.4 (1:4, w/v), deproteinized and chromatographed on DEAE Sephadex A-25 columns (1.5 × 30 cm); elution was accomplished with linear gradients of ammonium formate, 0.01–1.0 M, 200 ml each. Fractions of 2.4 ml were collected, and aliquots were taken for the measurement of radioactivity, and of optical density at 254 nm. Symbols: (○) refers to radioactivity, and (●) refers to absorbance at 254 nm. The first radioactive peak is an impurity present in this preparation; the second radioactive peak corresponds to DL-[1-^{14}C]alanosine; and the third radioactive peak corresponds to the principal urinary metabolite (compound 1).

Pursuant to this observation, and in view of the high and persistent concentration of radioactivity detected in liver, kidney and heart during fate and distribution studies [3], attempts were made to determine whether, and to what extent, the urinary metabolite might be originating in these viscera. To this end, homogenates were prepared of these organs and of tumor tissue of BDF_1 mice bearing the L5178Y lymphoma, 2 hr after a parenteral injection of DL-[1-^{14}C]alanosine; the supernatant fractions were then chromatographed on DEAE

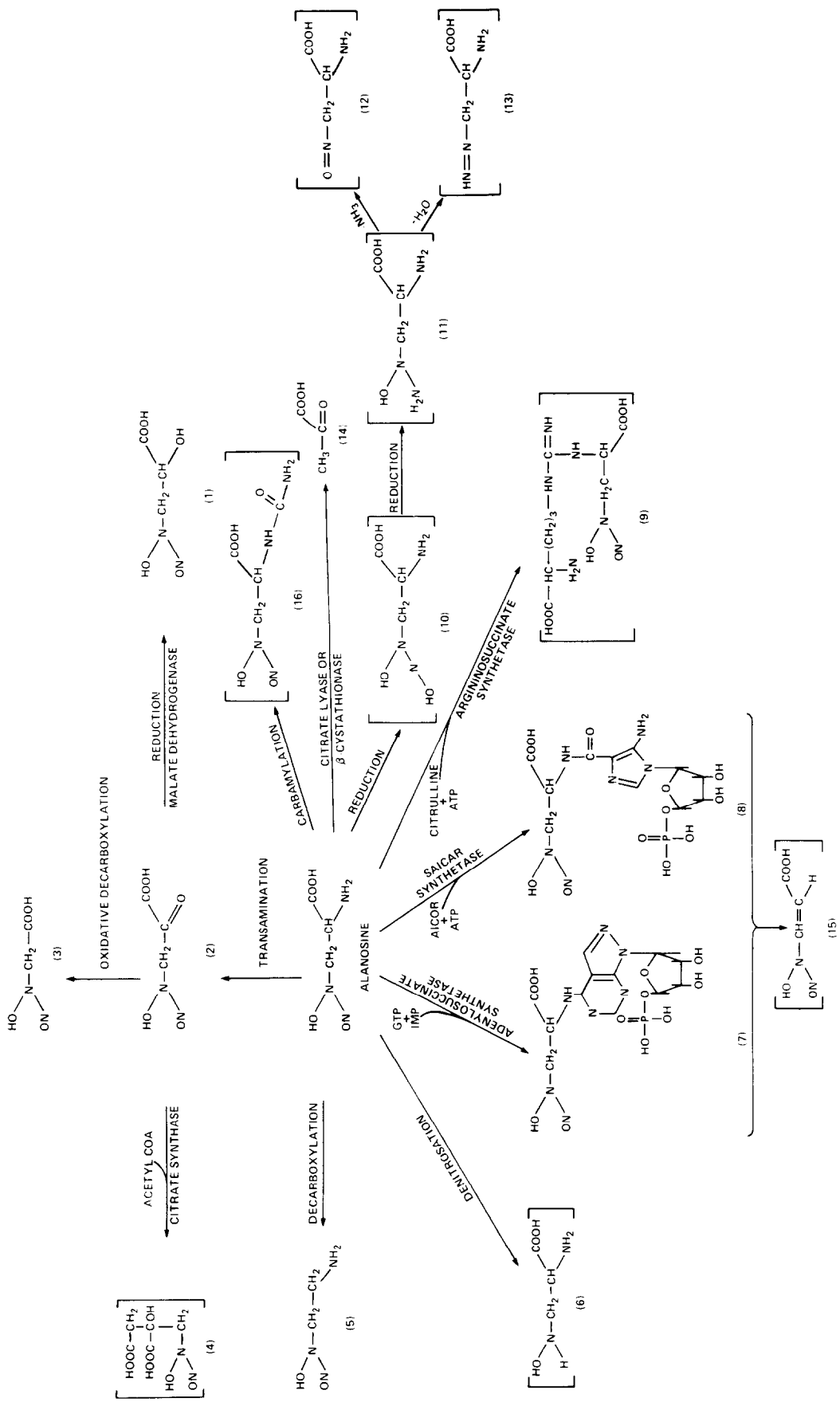

Fig. 2. Routes of metabolism of alanosine.

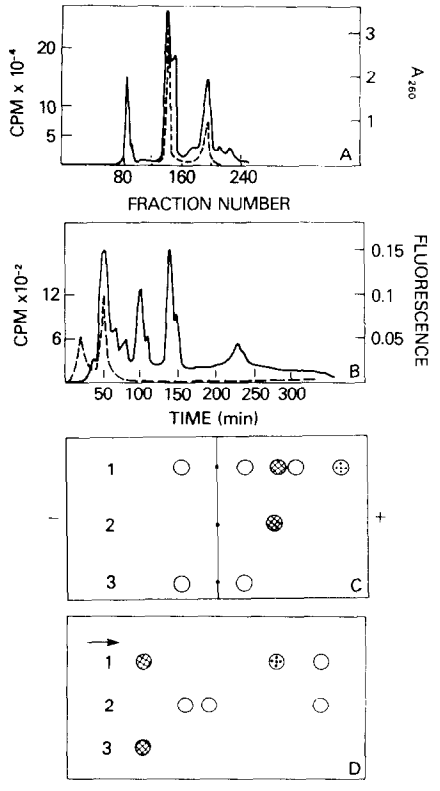

Fig. 3. Analysis of urinary metabolite (compound 1) in four systems. The urinary metabolite was isolated from the urine of a group of ten BDF_1 mice, injected i.p. with L-alanosine (500 mg/kg) and 10 μCi/mouse of DL-[1-^{14}C]alanosine. Urine was collected for 24 hr and then chromatographed as described in Materials and Methods. Panel A: One μCi ($\approx$8.4 μmoles) of the reconstituted, partially purified urinary metabolite was chromatographed on a DEAE Sephadex A-25 column (3 × 45 cm) and developed with a linear gradient of ammonium formate, pH 4.5, 0.01 to 1.0 M. Fractions of 1.6 ml were collected. Symbols: (– – – – –) refers to radioactivity; and (— — —) refers to optical density at 260 nm. Panel B: One μCi ($\approx$8.4 μmoles) of the reconstituted, partially purified urinary metabolite was chromatographed on an AR-50 resin column, 0.8 × 70 cm, using the Jeol analyzer, and 0.15 M lithium citrate buffer, pH 2.72, as described in Materials and Methods. Five min fractions of 3.35 ml were collected. The excitation and emission wavelengths selected for the fluorescence measurements were 250 and 350 nm respectively. Panel C: Paper electrophoresis of the urine from mice treated with DL-[1-^{14}C]alanosine, channel No. 1; NaH[^{14}C]O_3, channel No. 2; or standard DL-[1-^{14}C]alanosine was run in channel No. 3 as detailed in Materials and Methods. Panel D: Paper chromatography of the urine from mice treated with DL-[1-^{14}C]alanosine, channel No. 1, or with NaH[^{14}C]O_3, channel No. 2; standard DL-[1-^{14}C]alanosine was run in channel No. 3; the chromatogram was developed in the ascending mode using butanol–acetic acid–water (2:1:1, by vol.) as detailed in Materials and Methods. In panels C and D, (○) refers to the u.v. absorbing spot; (⊙) refers to the u.v. absorbing, radioactive and ninhydrin-positive spot; and (⊗) refers to the u.v. absorbing, radioactive and ninhydrin-negative spot.

Sephadex A-25 columns (Fig. 1). At this time period, in all three organs, as well as in lung and tumor, a compound was observed whose chromatographic behavior corresponded to that of the principal urinary metabolite (compound 1, Fig. 2). In quantitative terms this metabolite was most abundant in liver, lung and kidney, and least abundant in heart. In view of the antitumor activity of alanosine, the presence of the principal metabolite in tumor was of interest.

On paper electrophoresis at pH 7.2, the principal metabolite from mouse urine (compound 1) migrated toward the anode, well in advance of L-alanosine (Fig. 3C); this finding points to the increased negativity of this species. Additional evidence for this conclusion was provided by the observation that the metabolite was eluted in the void volume (16 min) of the long column of the amino acid analyzer (pH 2.72), whereas DL-alanosine emerged at 48 min (Fig. 3B). In the former system, compound 1 was ninhydrin-negative, and in the latter system it failed to react with basic *O*-phthaldialdehyde. When compound 1 was subjected to paper chromatography (Fig. 3D), it had a faster mobility than DL-alanosine. A chromatographically identical material appeared in the urine of mice given DL-[3-^{14}C]alanosine; in additional studies, the urine of mice treated with NaH[^{14}C]O_3 served as the control for the reutilization of carbon 1 of alanosine (cf. Table 2); no radioactivity corresponding to compound 1 was present in the urine of these mice.

On column chromatography, the principal metabolite was firmly retained on Dowex 1 to × 8 in the formate form, on DEAE Sephadex in the formate form (Fig. 3A), and on Hamilton HA-X4 resin; its u.v. absorption was sufficiently intense at 260 nm to be readily monitored in these systems. Ultraviolet spectrometry revealed that it retained the same absorption maxima as alanosine in 0.1 N HCl (229 nm) and 0.1 N NaOH (248 nm). Taken together these findings suggest that the metabolite retains the carbon skeleton of alanosine, and is devoid of a free α-amino functionality; moreover, the *N*-nitroso-*N*-hydroxyl amino group presumably is intact.

In order that its mass spectrum might be examined, the compound was isolated in apparently pure form by the four chromatographic steps presented in Materials and Methods. The conditions adopted for gas chromatography were those under which the parent drug, L-alanosine, trimethylsilylated as the free acid, showed two peaks of approximately equal area, with isothermal retention indices [12] of 1683 and 1710 (140°, SE-30) respectively (Fig. 4A). The mass spectra of these two peaks were identical (Fig. 4B), suggesting the presence of structural isomers of the *cis* and *trans* types seen with oximes, or the alpha and beta isomers noted with aliphatic azoxy ethers [13–15]. L-Alanosine forms a tetra-trimethylsilyl (TMS) derivative as evidenced by a molecular ion peak of low intensity at *m/e* 437, which shifts to *m/e* 473 when the TMS-d_9 derivative is formed. The legend to Fig. 4 compares the observed fragmentation pattern of the unlabeled and deuterated L-alanosine derivatives, and allows a probable assignment for the corresponding ions. Despite success with alanosine, all attempts to derivatize and analyze the urinary metabolite in the mass spectrometer met with failure. For this reason, efforts were next made to generate the most likely metabolites of alanosine *in vitro*, using pure or well-characterized enzymes in order that the properties of these materials could be compared with those of the principal urinary metabolite.

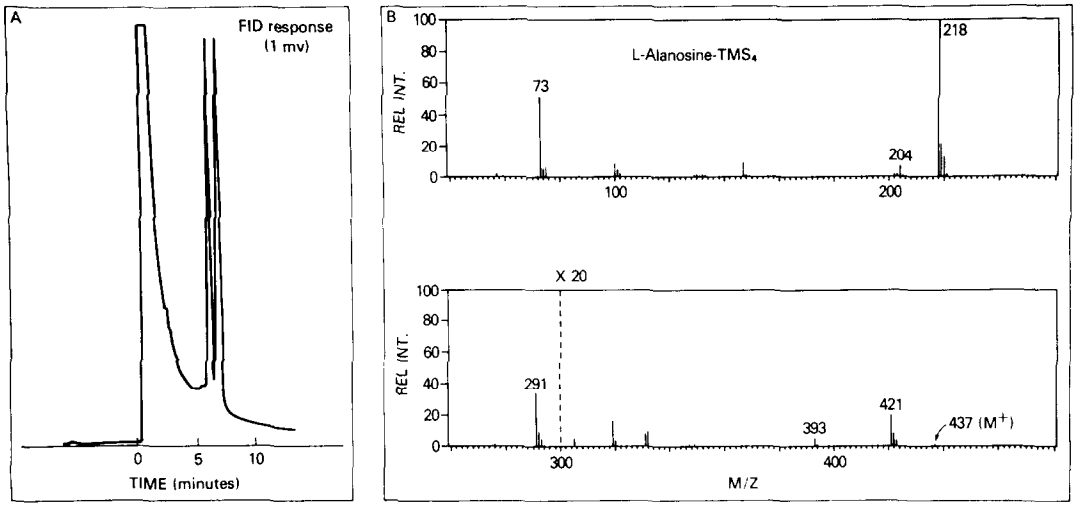

Fig. 4. Panel A. Gas chromatogram of trimethylsilylated L-alanosine, the details of which are presented in Materials and Methods. Panel B. Electron impact (75 eV)mass spectrum of trimethylsilylated L-alanosine. Probable structural assignment of the major fragment ions was based on the mass spectra of alanosine-TMS_4 and compared with the mass spectrum of alanosine $(TMS\text{-}d_9)_4$.

m/e(TMS-d_0)	Relative intensity (%)	m/e(TMS-d_9)	Probable assignment
437	0.03	473	M^+
421	1.0	454	$M-CH_3-H$
393	0.3	426	$M-CH_3-H-CO$
332	0.5	356	$M-CH_3-TMSOH$
331	0.4	355	$M-CH_3-H-TMSOH$
319	0.8	346	$M-CO_2-TMS-H$
291	34.2	318	M–side chain + H
218	100	236	291–TMS
204	7.1	219	
147	9.5	162	$TMS-O{=}Si(CH_3)_2$
100	8.6	109	$TMS-N{=}CH^+$
73	51.7	82	TMS

Conjugates of alanosine

Treatment of a sample of chromatographically pure, radioactive metabolite (compound 1) with acylase I, β-glucosidase, L-leucine aminopeptidase, esterase, sulfatase and β-glucuronidase did not generate a new radioactive electrophoretic species, indicating that the metabolite is not likely to be *N*-acetyl alanosine, alanosine conjugated with glucuronic acid, glucose, sulfate, nor an ester or peptide of the antibiotic.

Transamination of alanosine

Hydrazine is a strong inhibitor of the metabolism of DL-[1-^{14}C]alanosine [3]. This finding made it possible that transaminases might initiate the biotransformation of the antibiotic. A survey using extracts of the principal organs of the mouse revealed that heart was preeminent as a source of this enzymatic activity, followed, in order, by muscle, Harderian glands, liver, brain, seminal vesicles, bone and kidney (Table 2). High voltage paper electrophoresis showed that the product of transamination of L-alanosine with α-ketoglutaric acid catalyzed by myocardium was strongly acidic in character, and migrated toward the positive electrode to a position intermediate between L-aspartic acid and α-ketoglutaric acid; purified GOT plus α-ketoglutaric acid catalyzed the production of an identical product. Presumably this metabolite is the α-keto analog of alanosine, 2-oxo-3-(*N*-hydroxy,*N*-nitrosamino) propionic acid (compound 2).

When reaction mixtures containing DL-[1-^{14}C]alanosine, GOT and α-ketoglutaric acid were chromatographed at pH 2.65 on a high-resolution ion-exchange resin (Hamilton HA-X4), at least four products were demonstrable (Fig. 5, panel A-2). This finding suggests that compound 2, like its analogs, oxaloacetic acid and α-keto-β-sulfinylpropionic acid, is labile in an acidic environment. Interestingly, one of these moieties co-chromatographed with compound 3 (Fig. 5, panel A-3).

In order to determine which keto acid was the best transamination partner for L-alanosine, a survey was undertaken using eleven keto acids and dialyzed extracts of normal liver, kidney, and nodules of leukemia 5178Y/AR, against which the antibiotic exhibits thera-

Table 2. Survey of decarboxylation and transamination of DL-[1-^{14}C]alanosine by extracts of dialyzed mouse tissues*

Organ	Decarboxylation	Transamination
	(pmoles of DL-[1-^{14}C]alanosine converted/hr/g, wet wt)	
Harderian gland	7	252
Brain	189	248
Eye	0	143
Salivary gland	3	59
Thymus	24	57
Lung	21	55
Heart	66	512
Liver	182	249
Spleen	10	60
Pancreas	0	54
Stomach	0	118
Large intestine	0	150
Small intestine	0	81
Kidney	1	205
Adrenal gland	4	98
Pituitary gland	27	31
Muscle	20	325
Bone	9	206
Seminal vesicles	11	237
Residual carcass	16	285

* Tissues from normal BDF_1 mice were excised, extracted and assayed for decarboxylation and transamination activity, as detailed in Materials and Methods.

peutic activity. The results of this study established α-ketoglutarate as the optimal transamination partner for the enzymes from liver and tumor, whereas α-ketovaleric acid was the preferred partner in the case of kidney (Table 3).

When citrate synthase and acetyl CoA were included in reaction mixtures generating compound 2 from DL-[1- or 3-^{14}C]alanosine, in the presence of GOT and α-ketoglutarate, a new radioactive species was formed (Fig. 5, panel A-4). This product (compound 4) is presumed to be a metabolite of the antibiotic analogous to citrate. However, citrate lyase from *Aerobacter aerogenes* failed to decompose it, even after prolonged incubation.*

Since these findings suggested that L-alanosine could be processed through a significant portion of the citric acid cycle, it was relevant to examine its effect on respiration. Using isolated cardiac mitochondria, and isolated Ehrlich ascites cells, Dr. M. Gosalves, at the University of Madrid, could demonstrate no influence of the antibiotic on respiration at concentrations up to 0.4 mM.† Sufficient quantities of compound 4 were not available for analogous tests.

When malate dehydrogenase and NADH were included in reaction mixtures generating compound 2 from alanosine in the presence of GOT and α-ketoglutarate, a product was formed whose optical properties were similar to compound 1 (Fig. 5, panel B-3). In neutral solution, this metabolite was stable at room temperature for 24 hr. It co-electrophoresed with compound 1 at pH 7, co-migrated with compound 1 on paper using butanol–acetic acid and water as solvents, co-chromatographed with compound 1 on DEAE Sephadex and Hamilton HA-X4 resins, and co-eluted with compound 1 in the high pressure liquid chromatograph (Fig. 6). This consonance of behavior in five systems warrants the tentative conclusion that compound 1 is the α-hydroxy analog of L-alanosine, 2-hydroxy-3-(*N*-hydroxy-*N*-nitrosamino) propionic acid (Fig. 2).

Other possible metabolites of alanosine in vitro

Inasmuch as 18 per cent of a dose of DL-[1-^{14}C]alanosine is expired as [^{14}C]O_2 [3], it seemed possible that α-decarboxylation might be responsible for the detachment of carbon 1 of the antibiotic molecule. In the first paper of this series [3], evidence was presented on the abilities of a variety of rodent organs to release [^{14}C]O_2 from DL-[1-^{14}C]alanosine; brain was not included in that series. Inasmuch as alanosine is an acidic amino acid, and because brain is a rich source of the principal mammalian decarboxylase capable of attacking an acidic amino acid, i.e. L-glutamate decarboxylase, the ability of homogenates of cerebrum to release [^{14}C]O_2 from DL-[1-^{14}C]alanosine was examined next. For completeness, various other organs were also studied for that capability. In fact, brain did decarboxylate the drug at a pre-eminently vigorous rate (Table 2).

A parenthetical statement is in order on the ability of the large intestine to decompose alanosine. Previously, we had observed that the metabolism of radiolabeled alanosine to [^{14}C]O_2 was no different in untreated than in bowel-sterilized mice, a finding which warranted the conclusion that gut flora were not important participants in the metabolism of the antibiotic [3]. On enzymatic grounds, though, it is important to note that partially purified L-glutamate decarboxylase from *E. coli* contains the most vigorous 'alanosine-decarboxylase' activity found to date. In fact, this enzymatic reaction has been used as a tool for quantifying unaltered alanosine in plasma [3]. Although Gale and Atkins [17] reported that their purified preparations of squash (*Cucurbita pepo*) and bacterial L-glutamate decarboxylase (presumably from *E. coli*) were inhibited strongly by alanosine but did not accept the antibiotic as a substrate, we have conducted a limited number of additional studies aimed at testing the hypothesis that L-glutamate decarboxylase is, in fact, the enzyme responsible for the decarboxylation of alanosine. L-Glutamate proved to be the strongest inhibitor of this reaction, although many amino acids retarded the rate of decar-

* Commercial citrate lyase, or a contaminant in it, did attack L-alanosine to yield an α-ketocarboxylic acid (Fig. 5, panel A-5) readily reduced by NADH in the presence of lactate dehydrogenase. This treatment also removed the u.v.-absorbing properties of the β-substituent, although no nitrite was released. β-Cystathionase from *E. coli* catalyzed an analogous decomposition (Fig. 5, panel A-6). These findings suggest that L-alanosine is subject to an α,β elimination of the type catalyzed by several pyridoxal enzymes (Fig. 2, compound 14). Confirmation of this suggestion was also provided by studies with tryptophanase, kindly carried out by Dr. E. Snell, University of Texas, Austin, TX. This enzyme also catalyzed the decomposition of L-alanosine to yield pyruvic acid, although further structural identification was not undertaken [16].

† Personal communication from Dr. M. Gosalves, University of Madrid, Spain.

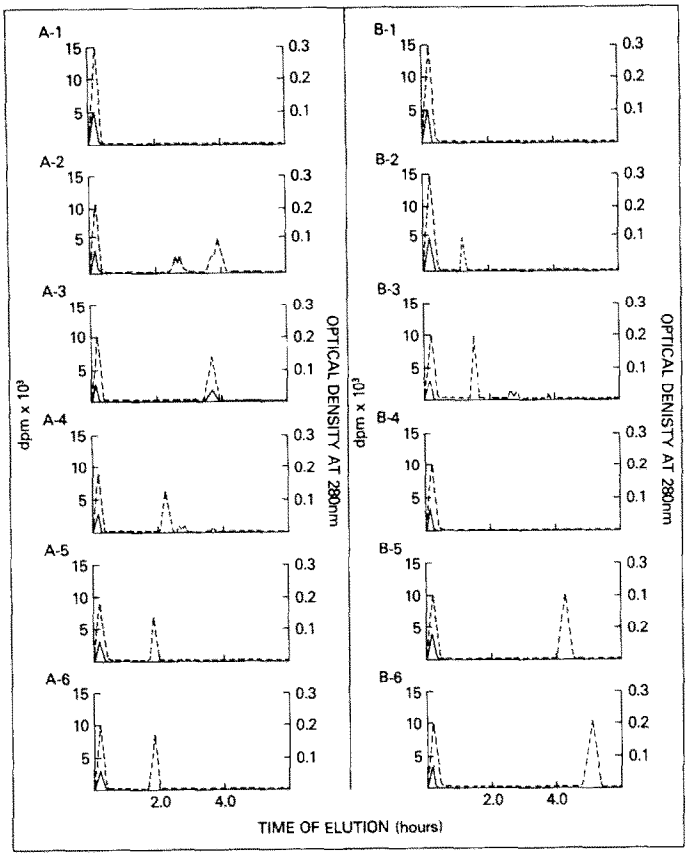

Fig. 5. High resolution chromatography of alanosine and its metabolites. Panels in the figure represent the elution profiles of metabolites of alanosine on the HA-X4 anion exchange column (0.8 × 15 cm) of the Jeol amino acid analyzer. chromatographed according to the technique detailed in Materials and Methods. Symbols: (- - -) refers to radioactivity, and (————) refers to absorbance at 280 nm. Panel A-1: DL-[1—^{14}C]Alanosine (60 nmoles, 0.5 μCi) was chromatographed on the column. Panel A-2: DL-[1—^{14}C]Alanosine (300 nmoles, 2.5 μCi) was incubated with 5 μl (1 I.U.) of GOT and 5 μl of neutral 0.1 M α-ketoglutaric acid in 1 ml of 0.01 M Tris–HCl, pH 8.5, for 16 hr at 37°. The reaction mixture after deproteinization was loaded (0.8 ml) on the column. Panel A-3: Compound 3 (300 nmoles) was mixed with 0.5 μCi of DL-[1—^{14}C]alanosine and then chromatographed on the column. Panel A-4: DL-[1—^{14}C]Alanosine (300 nmoles, 2.5 μCi) was incubated with 5 μl (1 I.U.) of GOT, 5 μl of neutral 0.1 M α-ketoglutaric acid, 20 μl of citrate synthase (3 I.U), and 1 mg of acetyl CoA in 1 ml of 0.01 M Tris–HCl, pH 8.4, for 16 hr at 37°. The reaction mixture after deproteinization was loaded (0.8 ml) on the column. Panel A-5: DL-[1—^{14}C]Alanosine (300 nmoles, 2.5 μCi) was incubated with 20 μl (2 I.U) of citrate lyase in 1 ml of 0.01 M Tris–HCl, pH 8.4, for 16 hr at 37°. The reaction mixture was deproteinized and then chromatographed (0.8 ml) on the column. Panel A-6: DL-[1—^{14}C]Alanosine (300 nmoles, 2.5 μCi) was incubated with 20 μl (0.5 I.U.) of β-cystathionase in 1 ml of 0.01 M Tris–HCl buffer, pH 8.5, for 16 hr at 37°. The reaction mixture after deproteinization was loaded (0.8 ml) on the column. Panel B-1: DL-[1—^{14}C]Alanosine (60 nmoles, 0.5 μCi) was chromatographed on the column. Panel B-2: DL-[1—^{14}C]Alanosine (300 nmoles, 2.5 μCi) was incubated with 20 μl of 0.2 M carbamyl phosphate and 20 μl of aspartate transcarbamylase (40 I.U.) from *E. coli* in 1 ml of 0.01 M Tris–HCl, pH 8.5, for 1 hr at 37°. After deproteinization, an aliquot of 0.8 ml was chromatographed on the column. Panel B-3: DL-[1—^{14}C]Alanosine (300 nmoles, 2.5 μCi) was incubated with 5 μl of GOT (1 I.U.), 5 μl of neutral 0.1 M α-ketoglutaric acid, 5 μl of MDH (3.6 I.U) and 5 μl of neutral 0.1 M NADH in 1 ml of 0.01 M Tris–HCl, pH 8.5, for 16 hr at 37°. After deproteinization, an aliquot of 0.8 ml was loaded on the column. Panel B-4: DL-[1—^{14}C]Alanosine (300 nmoles, 2.5 μCi) was incubated with 10 μl of glutamate decarboxylase (2 I.U) in 1 ml of 0.1 M sodium acetate, pH 4.8, for 16 hr at 37°. After deproteinization, an aliquot of 0.8 ml was loaded on the column. Panel B-5: DL-[1—^{14}C]Alanosine (300 nmoles, 2.5 μCi) was incubated with 200 μl of 0.03 M ATP–$MgCl_2$, 200 μl of 0.1 M $KHCO_3$, 200 μl of 6 mM AICOR (29.5 nmoles) and 200 μl (1 I.U.) of partially purified SAICAR synthetase from chicken liver in 1 ml of 0.01 M Tris–HCl, pH 8.0, for 4 hr at 37°. After deproteinization, an aliquot of 0.8 ml was loaded on the column. Panel B-6: DL-[1—^{14}C]Alanosine (300 nmoles, 2.5 μCi) was incubated with 100 μl of 0.03 M GTP–$MgCl_2$, 100 μl of 0.03 M IMP and 100 μl of adenylosuccinate synthetase from rabbit muscle (1 I.U.) in 1 ml of 0.01 M Tris–HCl, pH 8.4, for 16 hr at 37°. After deproteinization, an aliquot of 0.8 ml was loaded on the column.

Table 3. Rate of transamination of alanosine with a panel of α-keto acids*

	Liver	L5178Y	Kidney
		(μmoles/g tissue/hr)	
α-Ketoglutaric acid	1.30	0.88	0.72
Ketomalonic acid	1.14	0.71	0.27
α-Ketobutyric acid	0.98	0.42	0.29
α-Ketoisocaproic acid	0.95	0.38	0.22
α-Ketovaleric acid	0.85	0.45	0.98
β-Phenylpyruvic acid	0.81	0.62	0.21
Oxaloacetic acid	0.80	0.59	0.28
β-Fluoropyruvic acid	0.74	0.63	0.33
Pyruvic acid	0.71	0.33	0.26
α-Keto-γ-methiol-butyric acid	0.65	0.44	0.17
α-Ketoisovaleric acid	0.59	0.36	0.19

* Transamination of alanosine by extracts of liver, L5178Y tumor and kidney was carried out with a panel of α-keto acids by the technique detailed in Materials and Methods.

boxylation of alanosine.* Lineweaver–Burk plots established that inhibition by L-glutamate was formally competitive in type ($K_i \approx 14.2$ mM). Thus, it is the strong presumption that L-glutamate decarboxylase does, in fact, accept L-alanosine as an alternate substrate.

This presumption was fortified by an examination of the properties of the product resulting from the decomposition of crystalline L-alanosine by L-glutamate decarboxylase, in studies carried out under the following conditions. A 0.005 M solution of L-alanosine in 0.1 M sodium acetate, pH 4.8, was incubated for 2 hr at 37° with a partially purified prepartation of the enzyme. At the end of this incubation, no L-alanosine remained, but a new species was generated. It was ninhydrin-negative, uncharged at pH 7 as adjudged by high-voltage electrophoresis, and eluted in the void volume from a column of Hamilton HA-X4 resin at pH 2.65 (Fig. 5, panel B-4). Like alanosine, this product retained an absorption maximum at 248 nm in basic solution, a finding which points to the integrity of its chromogenic β-substituent. Taken together, these several lines of evidence suggest that the product is 1-amino-2-(*N*-hydroxy,*N*-nitrosamino) ethane, compound 5. The failure of previous workers to observe this decarboxylation may be a consequence of the comparatively slow velocity at which the reaction proceeds.†

It is also relevant to point out that isolated neoplastic lymphoblasts (L5178Y/AR) metabolized DL-[1-^{14}C]alanosine to [^{14}C]O_2 at a rate of 1.7 pmole decarboxylated/million cells/hr; this rate is less than that exhibited by thymic lymphoblasts: 20.9 pmoles decarboxylated/million cells/hr.

In contrast to the behavior of L-glutamic acid decarboxylase, L-aspartate-β-decarboxylase from *Alcaligenes faecalis*, another enteric organism, did not release CO_2 from alanosine at pH 5.0, even after prolonged incubations of 0.01 M solutions of the antibiotic with concentrations of the enzyme as high as 50 I.U./ml. Moreover, such treatments did not diminish the strong ultraviolet absorption of the antibiotic, a finding which suggests that this enzyme does not attack and detach the *N*-nitroso functionality.

Oxidation of L-*alanosine*

The foregoing studies suggest that compound 2 could arise as a consequence of transamination. This product might also be generated *in vivo* by the action of D or L-amino acid oxidases on the racemic mixture used in most of the studies dealt with here. In fact, exposure of DL-[1-^{14}C]alanosine to L-amino acid oxidase from *Crotalus adamanteus* resulted in the generation of a product readily α-decarboxylated by acid hydrogen peroxide. This oxidation proceeded at approximately 5 per cent of the rate observed with L-methionine; presumably the product is compound 2. Under the conditions used, D-amino acid oxidase from hog kidney did not attack alanosine, but the antibiotic was susceptible to nonenzymatic transamination with pyridoxal phosphate; thus at 37° and pH 9.0, 20 per cent of a 4.4 mM solution of DL-alanosine was transaminated/hr in the presence of 0.03 M pyridoxal phosphate.

Denitrosation of alanosine

Enzymatic denitrosation was ruled out by experiments in which homogenates of the principal viscera were incubated with 0.01 M solutions of alanosine for 1- and 2-hr periods, and measurements were made of any nitrite released. None appeared, despite the fact that as little as 10 nmoles of nitrite would have been detected under these conditions.

In this connection, it is relevant to point out that a material with thin-layer chromatographic properties apparently identical to those of HAPA, compound 6 (Fig. 2), the product of denitrosation of alanosine, appears in the urine of rodents given the antibiotic parenterally. However, as mentioned above, attempts to generate this compound *in vitro* met with failure.

* Thus, L-aspartic acid, L-asparagine, L-glutamine, L-cysteine, L-threonine, L-lysine, L-arginine and L-histidine, at a concentration of 0.02 M, inhibited the decarboxylation of DL-[1-^{14}C]alanosine (0.002 M) by *E. coli* L-glutamate decarboxylase (0.16 I.U.), by 25–40 per cent; under these conditions L-glutamate produced 67 per cent inhibition.

† The V_{max} of the decarboxylation of L-alanosine by partially purified L-glutamate decarboxylase from *E. coli* was 20 per cent of V_{max} measured with L-glutamate as substrate; the K_m values, however, were identical (0.02 M).

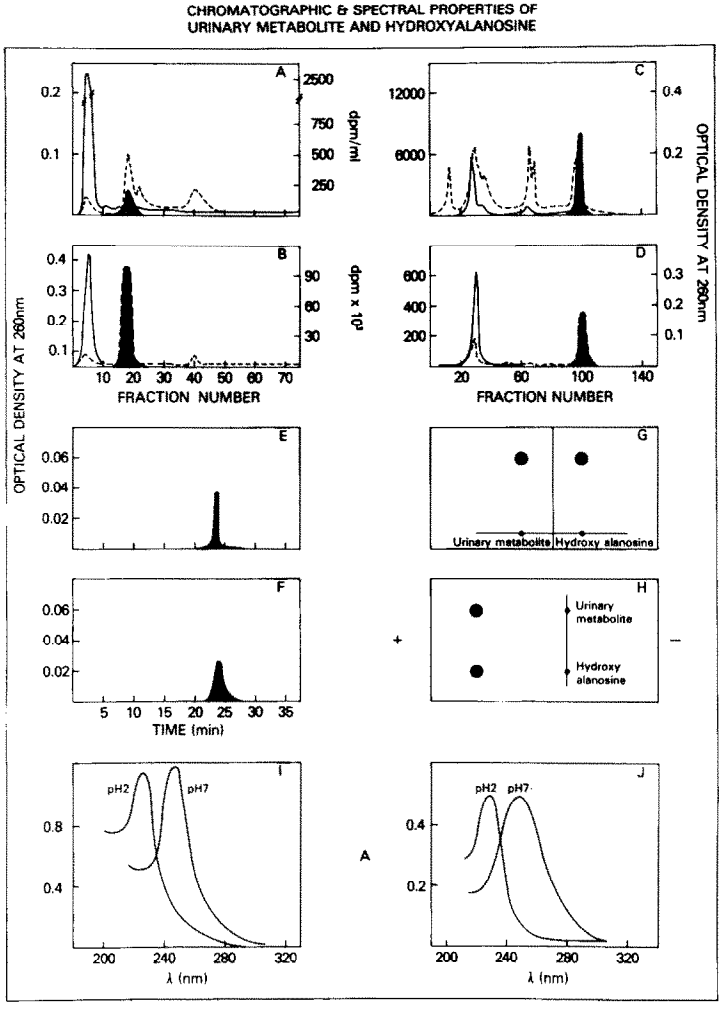

Fig. 6. Chromatographic and spectral profiles of compound 1, and the enzymatically prepared α-hydroxy analog of alanosine. A group of ten BDF_1 mice were injected, i.p., with 500 mg/kg of L-alanosine and 10 μCi/mouse of DL-[1—^{14}C]alanosine. Urine was collected for 24 hr, clarified by filtration through Miracloth, and then partially purified according to the technique detailed in Materials and Methods. α-Hydroxyalanosine was prepared enzymatically by scaling up the reaction mixture specified in the legend to Fig. 5 and then purified by following the same techniques used for partial purification of urinary metabolite. Panels A and B illustrate the chromatographic behavior of the partially purified urinary metabolite and of the α-hydroxy analog of alanosine (1 μCi) respectively, on an anion exchange column, HA-X4, of the Jeol amino acid analyzer, as detailed in Materials and Methods. Panels C and D illustrate the chromatographic behavior of partially purified urinary metabolite and of the α-hydroxy analog of alanosine (2 μCi), respectively, on a DEAE Sephadex A-25 column developed as detailed in Materials and Methods. Panels E and F illustrate the chromatographic behavior of partially purified urinary metabolite and of the α-hydroxy analog of alanosine (1.0 μCi), respectively on μNH_2 columns, as detailed in Materials and Methods. Radioactivity in the effluents was not quantitated. Absorbance was measured at 260 nm. Panels G and H illustrate the paper chromatographic and electrophoretic behavior, respectively, of the partially purified urinary metabolite and of the α-hydroxy analog of alanosine, as detailed in Materials and Methods. Panels I and J represent the u.v. absorption spectra of the α-hydroxy analog of alanosine and partially purified urinary metabolite respectively. The spectra were taken at acid and neutral pHs, in a Beckman DBG recording spectrophotometer, using a quartz cuvette of 1 cm length path. In panels A, B, C and D, (- - -) refers to radioactivity and (——) refers to u.v. absorption at 260 nm. Shaded areas in the elution profiles indicate the position of elution of the principal urinary metabolite or of the α-hydroxy analog of alanosine in these various systems.

Reduction of alanosine

The literature on xenobiotics documents that mammalian microsomes are capable of reducing aromatic nitroso compounds, first to the hydroxamate, and then to the corresponding amine [18,19]. If alanosine had an analogous fate, the resultant intermediate (compound 11) would very likely be unstable; its decomposition could occur through two routes: removal of ammonia or of water. In the former case, the unstable product (compound 12) should spontaneously decompose to yield nitrite. A search for this compound in the urine of mice, given 500 mg/kg of L-alanosine, met with failure. Hepatic microsomes incubated with alanosine and NADPH in an atmosphere of nitrogen also failed to generate either nitrite or hydroxylamine. If water were extracted, diazoalanine (compound 13) would be the product. This amino acid would bear a different net charge than alanosine. Examination of high-voltage electropherograms and ion exchange chromatograms of typical reaction mixtures (alanosine plus microsomes plus NADPH in an atmosphere of O_2 or N_2) showed no evidence for the formation of a new product. Although these results are by no means conclusive, they do suggest that the nitroso moiety of alanosine is not reduced to an appreciable degree in the course of the metabolism of the antibiotic by mammals.

Anabolites of alanosine

Inasmuch as L-alanosine is a substrate for L-glutamic acid oxaloacetic acid transaminase, it is conceivable that the antibiotic could replace L-aspartic acid in other enzymatic situations involving transfer of the α-amino nitrogen. Therefore, experiments were undertaken to determine whether the agent could be condensed, like L-aspartic acid, with IMP in the reaction catalyzed by adenylosuccinate synthetase to yield compound 7 [20], with 5-aminoimidazole 4-carboxylic acid ribonucleotide, in the reaction catalyzed by the phosphoribosylaminoimidazole-succinocarboxamide synthetase to yield compound 8, or with citrulline, in the reaction catalyzed by argininosuccinate synthetase to yield compound 9. Chromatographic evidence for the enzymatic formation of the postulated L-alanosyl-anabolite was found in the first two cases (Fig. 5, panels B-5 and -6), but the conversion was modest (5 per cent) in quantitative terms; no adduct with citrulline could be detected. While the fumaryl analog of L-alanosine (Fig. 2, compound 15) should result when either of the aforementioned adducts is attacked by adenylosuccinate lyase, no unequivocal evidence for the presence of this compound in biologic extracts was uncovered. Purified L-aspartase from *E. coli* also failed to form the "fumaryl analog" of alanosine when DL-[1-^{14}C]alanosine (30 nmoles, 300 nCi) was incubated with the enzyme (20 I.U) for 4 hr at 37°.

Attempts were next made to determine if *N*-carbamyl-L-alanosine might arise through the action on the antibiotic of L-aspartic acid transcarbamylase. This enzyme (from *E. coli* and Chinese hamster ovary cells) did accept L-alanosine as a substrate, but at ≈ 1 per cent of the rate seen with L-aspartic acid (Fig. 5, panel B-2). Moreover, it was observed that carbamyl phosphate, at concentrations greater than 0.01 M, could carbamylate L-alanosine nonenzymatically to yield compound 16 (Fig. 2).

Macromolecular metabolism of alanosine

The previous section authenticated and discussed the principal low molecular weight metabolite of alanosine. However, it should be borne in mind that this antibiotic, in its capacity as an amino acid with structural and chemical similarities to normal amino acids, might be incorporated into protein as a fraudulent building block. Some suggestive evidence for this point of view has been adduced: the macromolecules of animals given DL-[1-^{14}C]alanosine are rendered radioactive, and the half-life of such radioactivity is comparatively long* [3]. These findings are compatible with the incorporation of the antibiotic into structural, or other long-lived macromolecules.

To examine this point, protein synthesis was stimulated in mice by the production of phenylhydrazine anemia; alanosine was administered during the recovery phase, when hemoglobin synthesis was taking place at an exaggerated pace. Twenty-four hr later, the recipients were killed and any radioactivity in their hemoglobin was quantitated by scintillation spectrometry. Under these conditions, ten times more alanosine-derived radioactivity was present per mg of protein in the hemolysate from anemic mice (64 pCi/mg of protein) than in that taken from the saline controls (6.24 pCi/mg of protein).

In order to magnify the extent of this binding, reticulocytes were removed from mice 2 days after the last of five injections of phenylhydrazine (50 mg/kg), and exposed for 1 hr to DL-[1-^{14}C]alanosine, *in vitro*, in the presence or absence of cycloheximide. The nucleic acids were extracted from the ribosomes of these cells, and hemoglobin from the supernatant fraction of the hemolysate. Under these conditions, only small amounts of radioactivity were associated with the nucleic acid fraction (12.4 pCi/O.D. unit at 260 nm); cycloheximide did not alter the extent of this association. The affiliation of DL-[1-^{14}C]alanosine to the protein fraction was also low (64 pCi/mg of protein) and substantially unaltered by exposure to cycloheximide. In order to confirm this point, use was made of a cell free protein-synthesizing system. In the presence of a full complement of normal amino acids (excepting L-aspartic or L-glutamic acids), a rabbit reticulocyte lysate also failed to utilize DL-[1-^{14}C]alanosine for the synthesis of globin; under these conditions, L-[U-^{14}C]glutamic or aspartic acids were incorporated vigorously. Also, the antibiotic (1 mM) did not inhibit protein synthesis by this system in the presence of the normal dicarboxylic amino acids. Indeed, concentrations of alanosine as high as 0.01 M do not interrupt the incorporation of L-leucine by isolated murine leukemic lymphoblasts. At 0.1 M, protein synthesis in L5178Y/AR cells is totally abrogated, but this effect might be attributable to cellular damage from hypertonicity of the medium under these conditions.

Esterification of alanosine to tRNA was also examined using purified tRNA from mouse liver and partially purified hepatic tRNA synthetases. Table 4 documents that the incorporation of DL-[3-^{14}C]alanosine

* Equivalent macromolecular labeling was produced irrespective of whether DL-[1-^{14}C]- or DL-[3-^{14}C]alanosine was injected (data not shown).

into a TCA-insoluble precipitate is independent of ATP and GTP, but dependent on tRNA; total RNA from wheat germ supports the reaction to a minor degree, whereas DNA does not. When the reaction mixture from experiments such as these was analyzed on a Sephadex G-100 column, a minor peak of radioactivity was observed in the tRNA region, indicative of association of alanosine with tRNA. Since this radioactive product was formed by an energy-independent, non-enzymatic process, at alkaline pH, it was presumed not to represent esterification. The quantitatively minor extent of binding of alanosine to tRNA (0.1 μmole/mg) was confirmed by equilibrium dialysis (data not shown). Interestingly, another prototypical macromolecule, glycogen, did bind the antibiotic to a greater extent (0.14 μmole/mg) than either the nucleic acids, lipids, or proteins used (0.09 to 0.104 μmole/mg).

Viewed together, these experiments suggest that isolated systems cannot effect the macromolecular incorporation of alanosine, whereas intact cells can do so. It remains to be determined whether the antibiotic *per se* is used for this purpose, or whether a catabolite of it is responsible.

DISCUSSION

The experiments recapitulated in the present paper warrant the conclusion that alanosine is extensively metabolized in the several species examined. The principal route of this metabolism accounts for 20–60 per cent of a dose of the racemic mixture, and entails conversion of the parent antibiotic to a more acidic material (compound 1), retaining the full carbon skeleton of alanosine and also its *N*-nitroso moiety. Other minor routes involve direct enzymatic detachment of the α-carboxyl group, probably by L-glutamate decarboxylase, to yield compound 5, or begin with transamination to compound 2, which then can be envisioned as undergoing reduction, condensation with acetyl CoA, or oxidative decarboxylation. Alanosine is also capable of being carbamylated and conjugated to IMP as well as to 5-aminoimidazole 4-carboxylic acid ribonucleotide. Hurlbert *et al.* [21] first demonstrated the last of these conjugations, and showed that the resultant anabolite of alanosine is a potent inhibitor of adenylosuccinate synthetase. Thus, although certain of the metabolites of alanosine are formed to a minor extent in quantitative terms, they may be of tremendous qualitative importance.

Unsettled in the present study is the exact basis for the long-term affiliation of alanosine with circulatory and cellular proteins [3]. What is clear, however, is the fact that systems vigorously synthesizing protein *in vivo* do incorporate radioactivity representing or derived from alanosine to a small but measurable degree. It will remain for future studies to assess the importance of this incorporation, and also to evaluate the metabolic and therapeutic properties of the diverse anabolites and catabolites of L-alanosine reported in the present paper.

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Table 4. Interaction of DL-[3–^{14}C]alanosine with mouse liver total tRNA*

Components of the incubation mixture	TCA precipitable radioactivity (pmoles of [3–^{14}C]alanosine)
Complete system	44.0
Minus ATP, plus GTP	42.0
Minus enzyme	21.8
Minus tRNA	15.5
Minus tRNA, plus wheat germ RNA	18.3
Minus tRNA plus DNA	10.5
DL-[3–^{14}C]Alanosine, plus tRNA Plus 33 mM Tris–HCl buffer, pH 7.2	68.9
DL-[3–^{14}C]Alanosine, plus tRNA Plus 33 mM Tris–HCl buffer, pH 9.8	52.8

* DL-[3–^{14}C]Alanosine, used in this experiment, was purified by DEAE Sephadex chromatography, using an ammonium formate gradient at pH 4.5. The details for the complete incubation system and the conditions are given under Materials and Methods. The concentrations of wheat germ RNA and DNA are the same as tRNA; similarly, GTP was substituted for ATP at an equimolar concentration.

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