Inhibition of Glutamic Acid Carboxy-Lyase by Alanosine* (33438)

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Studies of the mode of action of alanosine (L (—) 2-amino-3-nitrosohydroxylamino propionic acid), an antitumor and anti-viral antibiotic characterized by Murthy *et al.* (1), have shown that this compound depresses the rate of *de novo* synthesis of uracil, cytosine, and adenine, with little or no effect on the synthesis of guanine (2, 3). The inhibitory action of the antibiotic on the growth of *Candida albicans* is antagonized by aspartate, and it was suggested that growth inhibition may be mediated through an effect on aspartate transcarbamylase and adenylosuccinate synthetase (3).

The highly reactive character of the nitrosohydroxylamino group of alanosine evoked speculation that this compound may interact with other biochemical systems. The presence of the amino group as well as the hydroxylamino group suggests a possible carbonyl-trapping action; the previously demonstrated (3) antagonism by aspartate of the growth inhibitory action of the antibiotic suggested a possible competition with aspartate for a number of enzymes. As a preliminary test of these two possibilities, the effect of alanosine on aspartate carboxy-lyase (aspartate C-L) was assessed; no inhibition was observed. Subsequently, effects of the antibiotic on other C-L's of microbial origin were determined. All so tested were insensitive with the singular exception of glutamic acid C-L.

The work described in the following report was initiated to compare the effects of alanosine on glutamic acid C-L from three sources and to characterize the nature of the inhibition.

Materials and Methods. All microbial amino acid C-L's were obtained from Nutritional Biochemicals Corporation. Each was used as a finely dispersed suspension except glutamic C-L, which was recovered in the opalescent supernatant solution after centrifugation. Glutamic C-L from squash (yellow crooked neck, *Curcubita pepo*, from the senior author's garden) was prepared as the 38–49% saturated ammonium sulfate fraction described by Melius (4). A 20% homogenate of rat brain, supplemented with pyridoxal phosphate (0.1 mg/ml) (5), served as a source of mammalian glutamic C-L. L-glutamic acid-1-^{14}C was from Calbiochem, and hadacidin (*N*-formyl hydroxyaminoacetic acid) was furnished by Dr. H. T.Shigeura of the Merck Sharp and Dohme Research Laboratories. All other chemicals were of reagent grade from diverse commercial sources.

Activities of the bacterial and squash enzymes were assayed with a Gilson respirometer at 30°C. The buffer was 0.1 *M* Na_2PO_4 adjusted to pH 5.5 with acetic acid. Initial velocities were determined by plotting log $(s - x)$ against time in minutes, where $s =$ initial substrate concentration expressed as μl CO_2 that would be produced upon complete decarboxylation ($=$ μmoles $\times$ 22.4), and $x =$ μl CO_2 produced at any given time. From this, the regression coefficient was calculated which, when multiplied by the factor for conversion to natural logarithms, was k, the velocity constant of the first-order reaction. The initial velocity, v, was then computed by multiplying k by the initial substrate concentration, s; v was expressed as μl CO_2 min^{-1}.

An isotopic method was used to assess the activity of the brain enzyme. Fifty-milliliter Erlenmeyer flasks were fitted with rubber stoppers from each of which was suspended, with a small paper clip, a strip of KOH-impregnated filter paper that served to absorb $^{14}CO_2$ from glutamic acid-1-^{14}C. After incubation at 30° for 30 min, the strips and clips were removed with forceps and placed into vials containing Bray's mixture (6); radioactivity was measured with a liquid scin-

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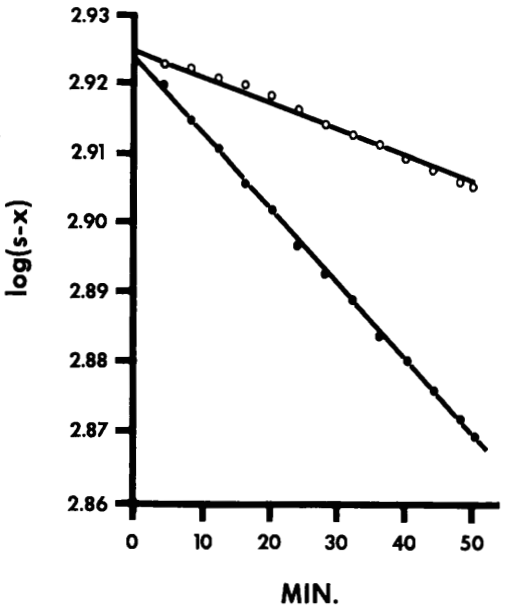

FIG. 1. First-order kinetics of the decarboxylation of glutamic acid by bacterial glutamic acid carboxy-lyase in the absence (●) and presence (○) of alanosine at 6×10^{-4} *M*. Substrate was 1.7×10^{-2} *M*. The enzyme (0.2 ml), added from the side arms of the vessels, was the supernatant solution obtained after centrifugation of a suspension (10 mg/ml) in buffer. Glutamic acid, with and without alanosine, was in the main compartment. Total volume was 2.2 ml.

tillation spectrometer (Mark I, Nuclear Chicago Corp.).

Results. The first-order nature of the decarboxylation of glutamic acid by bacterial glutamic C-L with and without alanosine, is shown in Fig. 1. This linear response was obtained for up to 50 min at all substrate concentrations used in subsequent experiments. In addition, experiments in which v was assessed as a function of enzyme concentration consistently yielded linear responses that intercepted the origin, indicating that no inhibitors or activators were present as contaminants in the enzyme preparation.

A substantially linear relationship was obtained when the initial velocity of the reaction was plotted against the logarithm of the alanosine concentration; 50% inhibition was obtained at a final inhibitor concentration of 4.2×10^{-4} *M* (solid line, Fig. 2). To determine if the alpha-amino group is essential for inhibition, a comparison was made of the action of alanosine with that of hadacidin, a naturally occurring hydroxamate antibiotic devoid of such a group. Under conditions otherwise identical to those described for Fig. 2, hadacidin conferred 50% inhibition at a similar concentration as did alanosine (7.8×10^{-4} *M*), and the regression coefficients of the two dose-response curves were numerically similar (—1.54 and —1.72 for alanosine and hadacidin respectively).

The competitive nature of the inhibition in respect to glutamic acid is shown in Fig. 3, which is typical of 15 such experiments that were performed. The value of K_m, as calculated by least-squares analysis of the experimental points obtained without the inhibitor, was found to be 6.47×10^{-3} *M*, a value that

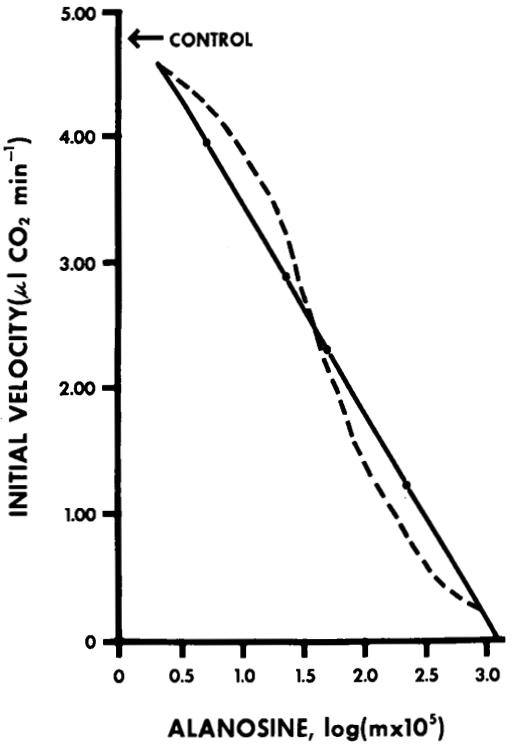

FIG. 2. Initial velocity of bacterial glutamic acid carboxy-lyase as influenced by varying concentrations of alanosine. Substrate was 2.3×10^{-2} *M*. The solid line was computed by the method of least squares to permit an unbiased estimate of the inhibitor concentration conferring 50% inhibition; the dotted line was obtained from the relationship $I/K_i = S/K_m\ (1\text{-}a/a)$.

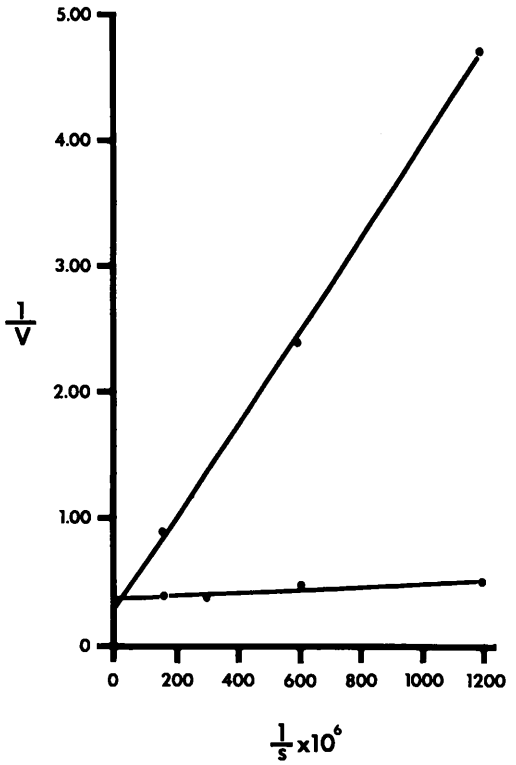

FIG. 3. Competitive inhibition by alanosine, in respect to glutamic acid, of bacterial glutamic acid carboxy-lyase. Alanosine was 6×10^{-4} *M*.

was in fairly good agreement with that found by Roberts ($2.69 \times 10^{-3}M$) (7). Using the alanosine concentration conferring 50% inhibition, as calculated from the experimental points in Fig. 2, the inhibitor constant, K_i, was estimated from the relationship $K_i = K_m I/S$ (7). A value of 1.2×10^{-4} *M* was thus obtained, revealing a K_i/K_m ratio of 0.0185. Substitution of the value of K_i into the equation $I/K_i = S/K_m(1\text{-}a/a)$, the theoretical equation for competitive equilibrium (8), yielded the curve that is shown as the dotted line in Fig. 2, and which displayed reasonably good agreement with the experimental points.

In contrast to the competition of glutamic acid with the antibiotic for the enzyme, the nature of the inhibition in respect to pyridoxal phosphate was strictly noncompetitive; no antagonism whatsoever by the coenzyme was observed (Fig. 4). In the presence of alanosine at 10^{-3} *M* plus pyridoxal phosphate, maximum velocity (V) of the reaction was reduced from 12.7 μl CO_2 min^{-1} to 3.6 μl CO_2 min^{-1}.

The comparative effects of alanosine and hydroxylamine on each of six bacterial amino acid C-L's are shown in Table I. Whereas substantial inhibition was obtained with hydroxylamine in all cases, there was an actual enhancement by alanosine of the rate of decarboxylation of all amino acids tested except glutamic acid. Appropriate control vessels devoid of substrate showed that this was not an apparent elevation of rate due to enzymatic decarboxylation of alanosine; incubation of alanosine alone with each enzyme revealed no CO_2 evolution whatsoever.

Concentrations of alanosine up to 4×10^{-3}

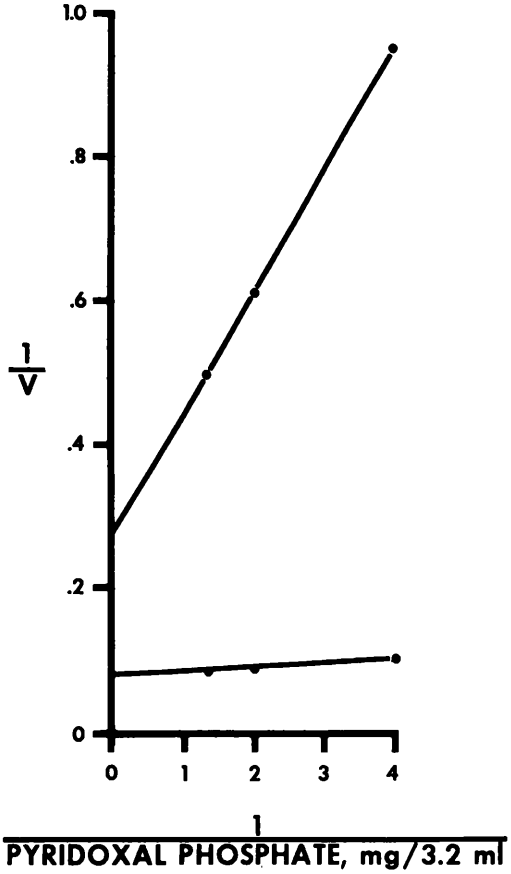

FIG. 4. Noncompetitive inhibition by alanosine, in respect to pyridoxal phosphate, of bacterial glutamic acid carboxy-lyase. Alanosine was 10^{-3} *M*, glutamic acid was 1.6×10^{-2} *M*, and the total volume was 3.2 ml.

TABLE I. Comparison of Effects of Hydroxylamine Hydrochloride and Alanosine on Certain Bacterial Amino Acid Carboxy-lyases.[a]

Carboxy-lyase	Percentage of control velocity in the presence of	
	Hydroxylamine	Alanosine
Arginine	10	112
Lysine	7	126
Histidine	14	220
Tyrosine	24	105
Aspartic acid	47	161
Glutamic acid	27	29

[a] Each enzyme preparation was present at 0.5 mg/ml, substrate was 5×10^{-3} *M*, and hydroxylamine and alanosine were 6×10^{-4} *M*. Velocity was measured at 30° in phosphate-acetate buffer, pH 5.5.

M were totally devoid of any inhibitory action on the glutamic acid C-L from either rat brain or squash.

Discussion. From the foregoing data, two points emerge as worthy of consideration. First, two examples of a high degree of selectivity have been demonstrated: the selectivity of the inhibitor for only one protein species of three that perform the identical catalytic function, and the selectivity for only glutamic C-L of the several C-L's tested from microbial sources. Secondly, while hydroxylamine has been shown to be a competive inhibitor of glutamic C-L (7), a fundamental difference exists between its action and that of alanosine in that other bacterial C-L's are sensitive to hydroxylamine but are not inhibited by alanosine. Further, the inhibitory action of hydroxylamine on glutamic C-L is readily and completely antagonized by pyridoxal phosphate (7), while that of alanosine is not.

The insensitivity of the brain glutamic C-L, if it extends to other mammalian species, may be fortuitous in the sense that an undesirable side effect due to depletion of cerebral γ-aminobutyric acid may be obviated. Aside from other toxic effects that will likely be encountered in subsequent studies, the clinical manifestations of glutamic C-L suppression would certainly be expected to impede therapeutic application of the antibiotic.

Summary. Alanosine is an inhibitor of glutamic acid carboxy-lyase (C-L) of bacterial origin, but not of those from squash and rat brain. Its action is competitive in respect to glutamic acid, but noncompetitive in respect to pyridoxal phosphate. Other bacterial amino acid C-L's tested, while sensitive to hydroxylamine, are not inhibited by alanosine.

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Inactivation of Erythropoietin by Tissue Homogenates* (33439)

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In recent reports Fisher and co-workers (1, 2), Kuratowska (3), and Zanjani *et al.* (4) described a factor in renal extracts capable of inactivation of erythropoietin. *In vitro* studies in our laboratory have confirmed the existence of such a renal factor, but have shown it also to be present in homogenates from livers and spleens. Kinetic measurements

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