Effects of Certain Hydroxamic Acids on L-Glutamate 1-Carboxy-lyase* (33955)

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A recent report from this laboratory (1) described the nature of the inhibition of bacterial L-glutamate 1-carboxy-lyase (GCL) by alanosine [L(—)-2-amino-3-nitrosohydroxylaminopropionic acid], an antibiotic with antiviral and antitumor properties (2). The corresponding enzyme from rat brain or squash was not inhibited, nor were five other bacterial amino acid CL suppressed. Inhibition was competitive in respect to glutamic acid, similar to that induced by hydroxylamine (3), but was noncompetitive in respect to pyridoxal phosphate. Hadacidin (*N*-formylhydroxyamino acetic acid), which is devoid of an amino and a nitroso group, was also inhibitory to bacterial GCL (1).

A structural feature common to both antibiotics is the (=N-OH) group. Consequently, the likelihood was considered that certain synthetic hydroxamic acids may prove to be inhibitory to GCL. A limited number of hydroxylamine derivatives were indeed tested by Roukema *et al.* (4) and active congeners were found. Inhibition conferred by certain aliphatic hydroxamates conceivably could be mediated via competition with substrate, as was shown with alanosine (1), or through binding with pyridoxal phosphate; this latter reaction has been demonstrated adequately in nonenzymatic systems (5–7). A group of synthetic hydroxamates was consequently tested for inhibitory action on GCL from two sources; a report follows.

Materials and Methods. Bacterial (*Escherichia coli*) GCL was from Nutritional Biochemicals Corporation. The squash (*Cucurbita pepo*) enzyme was the 38–49% saturated ammonium sulfate fraction described by Melius (8). Since the activity of this preparation was not enhanced by the addition of pyridoxal phosphate, the apoenzyme was assumed to be saturated with respect to coenzyme. The hydroxamic acids (HA) were synthesized by Hynes Chemical Research Corporation, Durham, N. C., and glutamic acid-1-^{14}C was from Calbiochem. All other chemicals were of reagent grade from various commercial sources.

The rate of evolution of $^{14}CO_2$ from glutamic acid-1-^{14}C in potassium phosphate buffer (0.05 *M*, pH 5.5, 37°) was used as a measure of the reaction velocity. Erlenmeyer flasks (25 ml) served as reaction vessels, and each contained a total fluid volume of 4.1 ml. The gas evolved was trapped on a KOH-impregnated filter paper strip suspended by a no. 3 paper clip embedded in the rubber stopper. All materials except the hydroxamic acids were prepared in buffer. Glutamic acid-1-^{12}C was prepared as a stock solution at 0.05 *M* in buffer; isotopic glutamate was added to this at 0.125 μCi/ml.

The bacterial enzyme was suspended in buffer (1.0 mg/ml). After gentle homogenization, the insoluble material was sedimented (1000*g*, 5 min) and 2 ml of the opalescent supernatant solution was added to each reaction vessel. The ammonium sulfate fraction containing the squash enzyme was diluted to the desired level of activity with buffer. Addition of either enzyme to the reaction flask was followed by the addition of 0.1 ml of dimethyl sulfoxide (DMSO), with or without the appropriate hydroxamate. After incubation at 37° for 20 min in a Dubnoff metabolic shaker, the reaction was initiated by adding 2.0 ml of substrate solution. The rubber stoppers with the appended KOH-saturated papers were immediately inserted, and the vessels were further incubated for 5–30 min, the exact interval being determined

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TABLE I. Hydroxamic Acids (HA) which were Devoid of Appreciable Inhibitory Action at 10^{-3} *M* against Bacterial and Squash L-Glutamate 1-Carboxy-lyase.

Hydroxyurea	2-Amino-6-hydroxypyrimidino-HA
Glycyl HA	3-Hydroxynaphtho-2- HA
L-Lysyl HA	2-Hydroxynaphtho-1 HA
Oxalyl diHA	Isonicotinyl HA
Methoxyoxamide	4-Nitrobenzoyl HA
3-Aminobutyryl HA	4-Fluorobenzoyl HA
Succinyl monoHA (59%)[a]	Trimesyl triHA
Pimelyl monoHA	3-Hydroxybiuret
L-Alanyl HA	Benzoyl HA
D-Alanyl HA	3-Aminobenzoyl HA
β-Alanyl HA	4-Aminobenzoyl HA
Sorbyl HA	3,5-Diaminobenzoyl HA (73%)[a]
Glutaryl monoHA (39%)[a]	Salicyl HA
Acetoxyoxamide	4-Hydroxybenzoyl HA
3-Nitrosalicyl HA	2,3-Dihydroxybenzoyl HA
3,4,5-Trimethoxybenzoyl HA	3-Methylsalicyl HA
4-Chlorobenzoyl HA	5-Bromosalicyl HA
4-Iodobenzoyl HA	Terephthalyl monoHA (19%)[a]
Naphtho-1 HA	Terephthalyl diHA
Phenylcarbamoyloxyoxamide	4-Ethoxysalicyl HA
4-Bromobenzoyl HA	Acetylsalicyl HA
4-Acetamidobenzenesulfonhydroxamide	

[a] Values in parentheses denote extent of stimulation of reaction velocity by that hydroxamate.

by the nature of the experiment being done. The reaction was terminated either by removal of the stoppers, or by addition of H_2SO_4 with a syringe and needle through the stopper. No substantial difference was noted between experiments terminated in either fashion. The clips with the strips were removed with forceps and placed into liquid scintillation vials containing 15 ml of Bray's (9) phosphor solution. After allowing 24 hr for complete elution of the $K_2{}^{14}CO_3$, radioactivity was measured with a liquid scintillation spectrometer (Mark I, Nuclear Chicago Corporation). Ultraviolet absorption spectra were recorded with a Cary model 15 spectrophotometer.

Results and Discussion. Table I lists compounds which were found to be virtually devoid of any inhibitory action; Table II shows those which were active against each enzyme.

The squash enzyme was chosen for most kinetic studies because of its higher degree of purity than that of bacterial origin. From double reciprocal plots of the data of the preliminary studies, it was found that this enzyme was moderately inhibited by relatively low substrate concentrations ($>2 \times 10^{-3}$ *M*). However, least squares treatment of the linear portion of the reciprocal plots of several experiments employing quite low substrate concentrations ($< 2 \times 10^{-3}$ *M*) yielded a value of $K_m = 2.32 \times 10^{-3}$ *M* (Fig. 1). This value was only slightly lower than that reported by Roberts (3) for bacterial GCL (2.69×10^{-3} *M*).

Satisfactory double reciprocal plots could not be obtained with the data derived from enzyme-inhibitor systems. In the presence of inhibitor, the plots were invariably nonlinear and frequently intercepted the ordinate at a value of $V_I = \infty$. However, in no case did the plots obtained in the presence of inhibitor intercept the ordinate at a value of $V_I < V$; *i.e.*, no evidence of noncompetitive inhibition was observed. These bizarre plots were considered likely to be due to the complex effects of inhibitor in the presence of a possible inhibiting concentration of substrate; an additional factor may well have been that most

TABLE II. Inhibition of Bacterial and Squash L-Glutamate 1-Carboxy-lyase (GCL) by Certain Hydroxamic Acids.[a]

	Inhibition (%) at 10^{-3} *M*	
Hydroxamic acid (HA)	Bacterial GCL	Squash GCL
Oxamyl HA	98	90
L-Aspartyl-β-HA	90	82
2-Aminobenzoyl HA	0	60
2,3,4-Trihydroxybenzoyl HA	60	71
2-Aminoterephthalyl diHA	5	99
3,5-Diisopropylsalicyl HA	89	99
2-Fluorobenzoyl HA	85	72
3-Aminopyrazino-2-HA	99	99
3,4,5-Trimethoxyphenylacetyl HA	89	71

[a] Substrate was present at a final concentration of 2.5×10^{-2} *M*.

TABLE III. Antagonism by Substrate of the Inhibitory Action of Certain Hydroxamic Acids on L-Glutamate 1-Carboxy-lyase from Squash.

Inhibitor and Concentration (M)	Substrate (M)	Percentage of control velocity
3,5-Diisopropylsalicyl HA (6×10^{-4})	2×10^{-4}	21
	2×10^{-3}	47
Oxamyl HA (2×10^{-5})	2×10^{-4}	15
	2×10^{-3}	47
3,4,5-Trimethoxyphenylacetyl HA (2×10^{-4})	2×10^{-4}	30
	2×10^{-3}	66
L-Aspartyl-β-HA (6×10^{-4})	2×10^{-4}	4
	2×10^{-3}	21
3-Aminopyrazino-2-HA (10^{-5})	2×10^{-4}	20
	2×10^{-3}	63

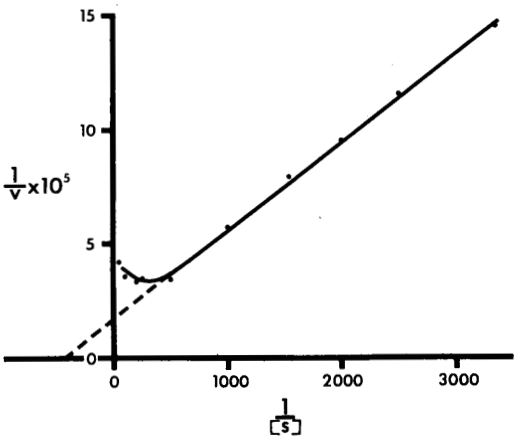

FIG. 1. Double reciprocal plot of the relationship of substrate concentration, $[S]$, to reaction velocity, V, for L-glutamate 1-carboxy-lyase from squash.

of the more active inhibitors were also antagonized by pyridoxal phosphate (see below). However, in all cases the course of the reaction was linear within the period of measurement and was considered to be a valid estimation of reaction velocity.

Consequently, the velocity of the reaction was assessed using each of the 5 most active inhibitors at a single concentration, and using 2 concentrations of substrate. Table III shows the antagonism by substrate of the action of each inhibitor, clearly suggestive of a competitive relationship between substrate and inhibitor. Following this, the dose–response relationships of each of the 9 most active compounds were determined. From the experimental points of Fig. 2, the concentration of each compound yielding 50% inhibition was determined. From the relationship (10)

$$K_I = \frac{K_m[I]}{[S]},$$

the inhibitor constant of each compound was computed (Table IV), and the values so obtained were substituted into the expression for competitive equilibrium (10)

$$\frac{[I]}{K_I} = \frac{[S]}{K_m} \cdot \frac{1-a}{a},$$

where a = fractional activity at each value

TABLE IV. Kinetic Parameters of the Inhibitory Action of Certain Hydroxamates on L-Glutamate 1-Carboxy-lyase (GCL).[a]

Compound	50% inhibitory concentration (M)			
	Bacterial GCL	Squash GCL	Squash GCL K_I	K_m/K_I
3,4,5-Trimethoxyphenylacetyl HA	1.86×10^{-4}	1.32×10^{-4}	1.22×10^{-5}	190
2,3,4-Trihydroxybenzoyl HA	4.27×10^{-4}	1.66×10^{-5}	1.54×10^{-6}	1506
3-Aminopyrazino-2-HA	2.24×10^{-5}	8.71×10^{-6}	8.08×10^{-7}	2871
Oxamyl HA	2.09×10^{-5}	1.55×10^{-5}	1.44×10^{-6}	1611
2-Fluorobenzoyl HA	3.02×10^{-4}	4.07×10^{-4}	3.78×10^{-5}	61
L-Aspartyl-β-HA	1.62×10^{-5}	3.98×10^{-5}	3.69×10^{-6}	629
3,5-Diisopropylsalicyl HA	4.57×10^{-5}	3.98×10^{-5}	3.69×10^{-6}	629
2-Aminobenzoyl HA	(inactive)	2.69×10^{-4}	2.50×10^{-5}	93
2-Aminoterephthalyl diHA	(inactive)	3.99×10^{-5}	3.70×10^{-6}	627

[a] Substrate was present at a final concentration of 2.5×10^{-2} M.

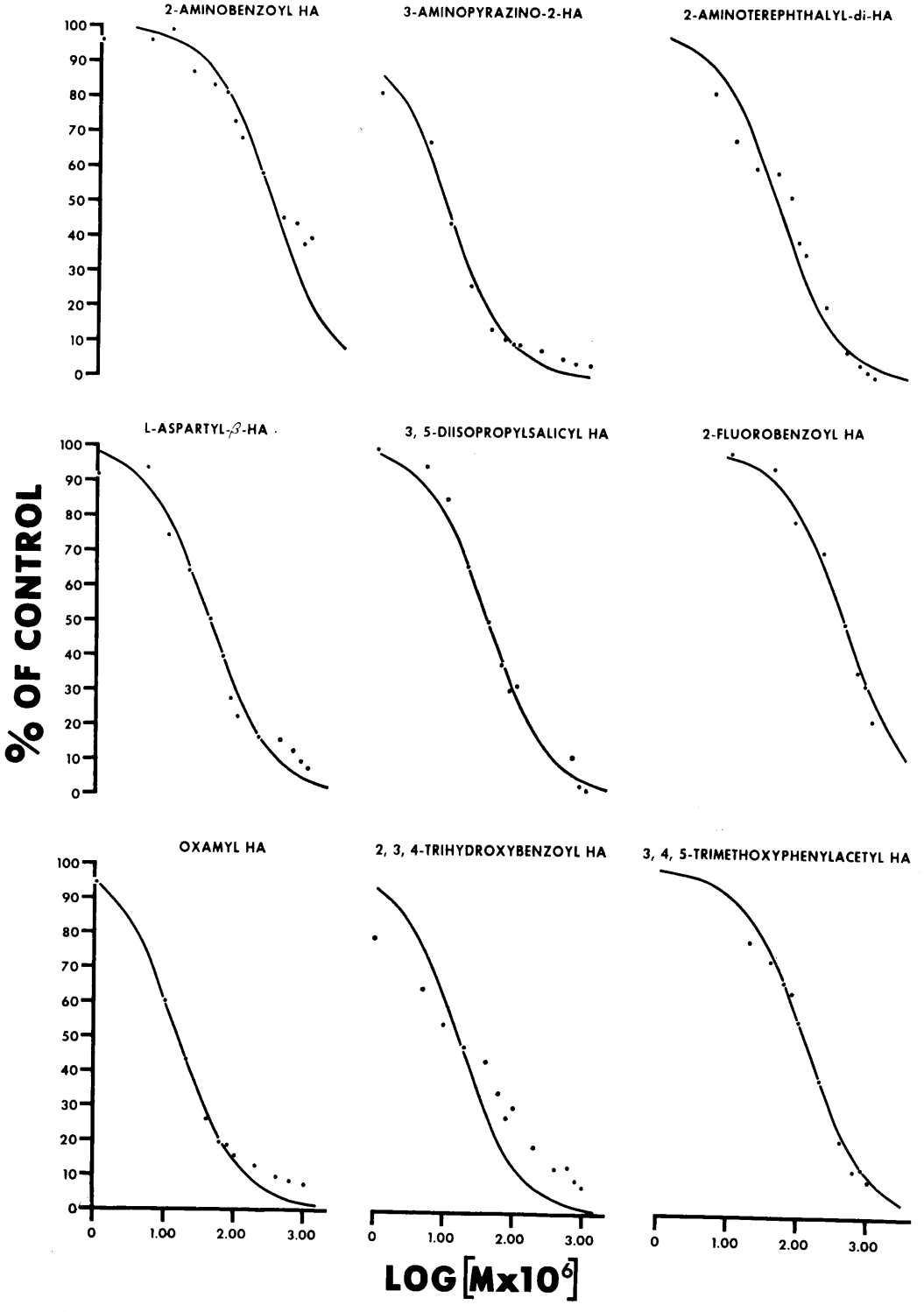

FIG. 2. Dose–response relationships of squash L-glutamate 1-carboxy-lyase to the actions of certain inhibitory hydroxamates: the points were measured experimentally; the lines were calculated from the expression of competitive equilibrium as described in the text.

TABLE V. Effects of Excess Pyridoxal Phosphate (PyP) on the Inhibition by Certain Hydroxamates of L-Glutamate 1-Carboxy-lyase from Squash.[a]

Compound and concentration (M)	PyP (4×10^{-4} M)	Percentage of control velocity
3,5-Diisopropylsalicyl HA	−	63
(6×10^{-4})	+	100
2,3,4-Trihydroxybenzoyl HA	−	36
(6×10^{-4})	+	88
3,4,5-Trimethoxyphenylacetyl	−	35
HA (6×10^{-4})	+	100
L-Aspartyl-β-HA (6×10^{-4})	−	23
	+	99
2-Aminoterephthalyl diHA	−	53
(10^{-4})	+	37
Oxamyl HA (10^{-4})	−	16
	+	100
3-Aminopyrazino-2-HA	−	20
(8×10^{-5})	+	99

[a] Substrate was present at a final concentration of 2×10^{-3} M.

of $[I]$. Results are shown as solid lines in Fig. 2. With only 1 of the 9 inhibitors used was there a substantial departure from a generally good fit to the experimental points, implying a competitive nature of the observed inhibition in respect to substrate.

The effects of an excess of pyridoxal phosphate (PyP) on the pattern of inhibition conferred by 7 of the most active compounds are shown in Table V. With one quite striking exception, there was virtually complete antagonism by the coenzyme. The singular exception, the actual potentiation of inhibition in the case of 2-aminoterephthalyl diHA, was reproducible. Even though the mechanism of this enhanced inhibition was not investigated, a likely explanation may be that a product of interaction of the hydroxamate with PyP was an inhibitor which was not antagonized by free coenzyme.

Nonenzymatic interaction of PyP with certain hydroxamates as manifest by changes in the PyP ultraviolet absorption spectrum is well documented (5–7). The aliphatic hydroxamates which inhibited the squash GCL in the present study did not absorb appreciably in the ultraviolet (200–400 mμ) region. Figure 3 shows that oxamyl HA and L-aspartyl-β-HA at 10-fold the concentration of PyP induced quite similar alterations, indicative of interaction with the coenzyme in a common fashion. The inhibitory aryl hydroxamates, however, absorbed quite strongly in the region scanned; consequently, they were used at only 2-fold the concentration of PyP. 2-Aminoterephthalyl HA conferred only a subtle change consisting of a shift to the right of the major 221 mμ peak. 3-Aminopyrazino-2-HA abolished the minor 388 mμ peak, similar to the effects of oxamyl HA and L-aspartyl-β-HA. 3, 4, 5-Trimethoxyphenylacetyl HA and 2, 3, 4-trihydroxybenzoyl HA were without effect. 2-Aminobenzoyl HA, 3, 5-diisopropylsalicyl HA, and 2-fluorobenzoyl HA induced marked but dissimilar spectral aberrations; these are shown in Fig. 4. Thus, capability of inducing spectral changes is not a requisite for inhibitory action on GCL; conversely, certain HA which strikingly modify the spectrum of the coenzyme (6) are devoid of notable GCL inhibitor action.

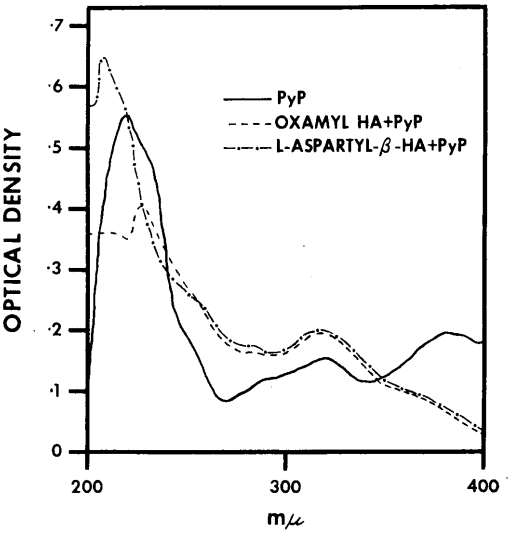

FIG. 3. Changes in the ultraviolet absorption spectrum of pyridoxal phosphate (PyP) induced by the presence of certain aliphatic hydroxamic acids (HA): HA, 5×10^{-4} M; PyP, 5×10^{-5} M; phosphate buffer, pH 5.5; the reference cell contained the appropriate HA alone.

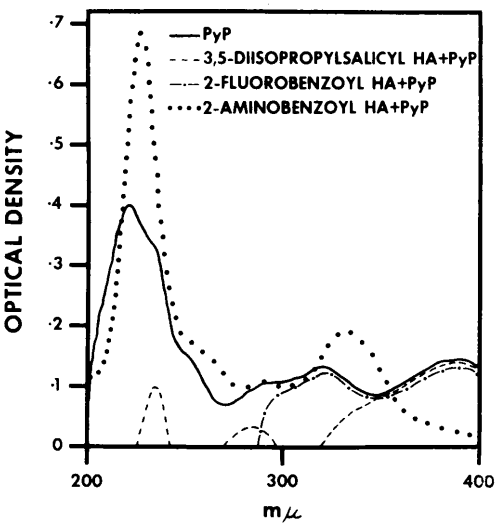

FIG. 4. Changes in the ultraviolet absorption spectrum of pyridoxal phosphate (PyP) induced by the presence of certain aryl hydroxamic acids (HA): HA, 10^{-4} *M*; PyP, 5×10^{-5} *M*; phosphate buffer, pH 5.5; the reference cell contained the appropriate HA alone.

Summary. Over 50 hydroxamic acids (HA) were surveyed for inhibitory action against bacterial and vegetable glutamic acid carboxy-lyase (GCL). Appreciable activity against the bacterial enzyme was shown by 7 compounds. These 7, in addition to 2 others, also inhibited the vegetable enzyme. Four HA stimulated the activity of the vegetable enzyme, but none had a similar effect on bacterial GCL. Kinetic studies indicated that inhibition was predominantly competitive with substrate, but with the exception of 1 compound, was also antagonized by pyridoxal phosphate (PyP). Certain of the active inhibitors interacted with PyP in a nonenzymatic system as shown by changes in the ultraviolet absorption spectrum of the coenzyme, but this property was not a requisite for GCL inhibitory action.

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