Urease Inhibitors for Hepatic Coma. II. Comparative Efficacy of Four Lower Hydroxamate Homologs *in Vitro* and *in Vivo*[1] (34949)

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Although it is now well established that the hydroxamic acids are specific and effective inhibitors of plant and bacterial urease (1–3) with potential application in the fields of veterinary nutrition (4) and clinical medicine (5), only one member of this class of compounds, acetohydroxamic acid, is currently being investigated, with some promise, in the treatment of hepatic insufficiency (6). A previous report from this laboratory documented in some detail the antiureolytic effect of this compound in mice, and presented the rationale for the application of such compounds to the treatment of hepatic coma and other diseases (7). In this report the kinetics of urease inhibition *in vitro* and in mice by acetohydroxamic acid is compared with that produced by three of its nearest chemical neighbors to evaluate structure–function relationships. Although the sum of the evidence indicates that acetohydroxamic acid is the most effective inhibitor, the other compounds are sufficiently active to suggest that consideration of toxicity would be the major factor in selection of a therapeutic agent.

Methods and Materials. Formohydroxamic acid (FHA), acetohydroxamic acid (AHA), propionohydroxamic acid (PHA), and isobutyrohydroxamic acid (IBHA) were synthesized and purified as previously described (8). Hydroxyurea (HU) was obtained from Mann Research Lab. Lyophilized jackbean urease was obtained from Sigma Chemical Company with a specific activity of 0.7 Sumner units/mg (Type II), and 7 Sumner units/mg (Type VI). The type VI enzyme was used for all experiments except where otherwise noted. Urease was assayed at pH 7 in 0.1 *M* Tris–0.1 *M* maleate buffer containing 0.1 *M* urea (unless otherwise noted), by nesslerization of five aliquots removed at 1-min intervals (9). Preincubation of 0.1–0.2 mg of enzyme with the inhibitor for given time intervals followed by 20- to 50-fold dilution for enzyme assay was used as before, to evaluate the rate of development of inhibition (2).

For *in vivo* studies the compounds were dissolved in sterile water and at once injected ip into male CAF_1 mice followed, after 10 min, by sc injection of 0.4 ml sterile saline containing 1.88 μCi of ^{14}C-urea, SA 0.173 mCi/mg. The animals were placed in an all-glass metabolic chamber, and the expired carbon dioxide was collected in normal NaOH, precipitated as barium carbonate, washed, filtered, dried, and counted at infinite thickness in a gas-flow proportional counter. The details of the experimental procedure have been described (7). The fraction of administered ^{14}C-urea expired as $^{14}CO_2$ in the treated mice was compared to the value obtained from a series of control mice to determine the fractional inhibition of ureolysis.

Results and Discussion. The pH-inhibition curve for three of the hydroxamates with type VI urease is shown in Fig. 1. It is clear that the compounds are active over a broad pH range, with maximum inhibition at pH values near neutral. In fact, the curves resemble the pH-activity curve for urease,

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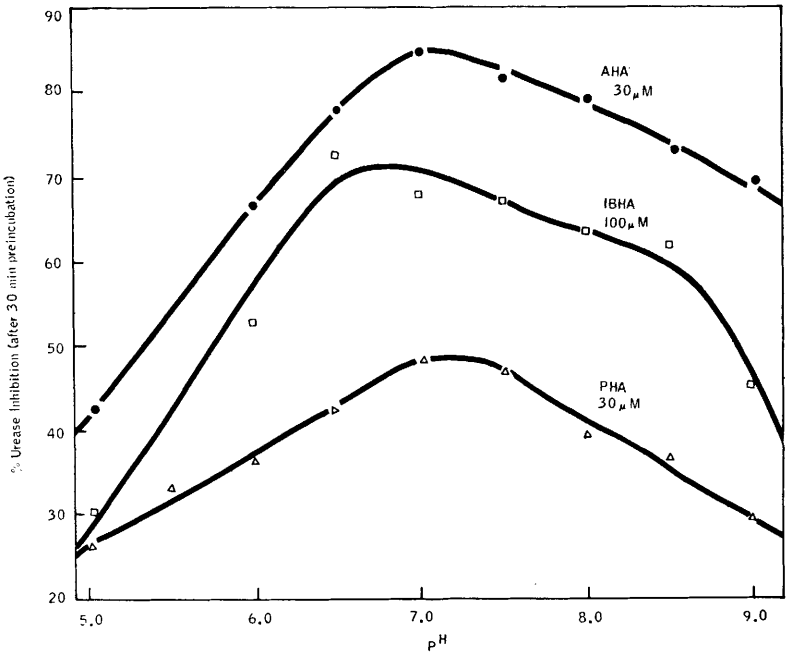

FIG. 1. Inhibition-pH curves for three aliphatic hydroxamates with urease, 7 Sumner units activity/mg. Enzyme and inhibitor were incubated for the same time interval at each pH in 0.1 *M* Tris–0.1 *M* maleate buffers, and the activity remaining was compared to that of enzyme alone at the same pH.

which also had a broad pH range. FHA had a pH-inhibition curve similar to those shown in Fig. 1. Although Kobashi *et al.*, initially reported the hydroxamates to be ineffective at pH 6 (1), they are active at this pH as shown here, and in previous studies by ourselves (2), Gale and Atkins (3), and Waid and Pugh (10). Further studies of inhibition kinetics were carried out at pH 7.0.

The development of urease inhibition in the presence of hydroxamates is slow enough to provide for ready measurement by preincubation of the enzyme and inhibitor for periods of 5–180 min, followed by dilution into urea solution for assay. By adjusting the time intervals and the inhibitor concentration, conditions can be selected so that no further inhibition develops during the time of the urease assay, and the activity is not altered whether or not the inhibitor concentration is maintained when the enzyme is diluted (9). The bimolecular rate constants for association of the inhibitor and enzyme may thus be obtained by decay plots such as those shown in Fig. 2. The data shown are for IBHA and for AHA, each at two concentrations, an order of magnitude apart. In addition, AHA inhibition is shown with urease type II as well as the type VI enzyme used for all other experiments. The agreement between the two experiments with AHA is sufficient to indicate that the purity of the enzyme is not an important factor in determining the association rate constants.

All experimental values for inhibition half-times and rate constants are summarized in Table I. The only other value in the literature is our own previous study of AHA in which the k_a was 1060 M^{-1} min^{-1}, which was obtained with a different substrate concentration and a different assay (2). AHA had the most rapid inhibitory effect of the four congeners, with the rate constant declining for PHA and IBHA as the alkyl chain becomes larger, but also for FHA which has no alkyl group. The measurement of k_a for FHA had the greatest error, because only short time intervals could be used for prein-

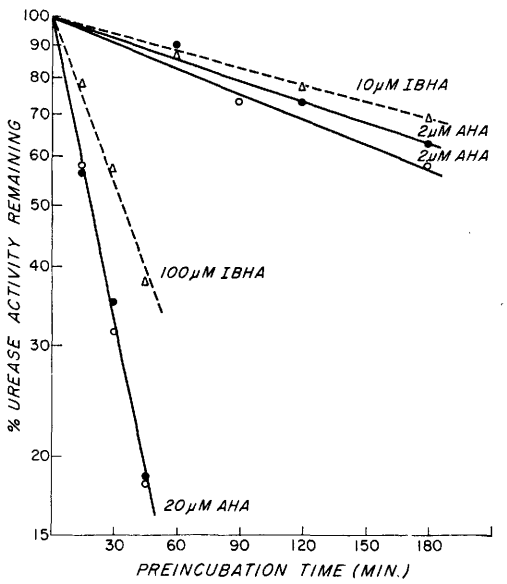

FIG. 2. Urease activity decay plots in the presence of AHA and IBHA. The enzyme, at a concentration of 0.7 Sumner units/ml, was preincubated with inhibitor for the time intervals noted, then diluted 20- to 50-fold into 0.1 *M* urea for assay. Tris–maleate buffer, 0.1 *M*, pH 7.0 was used throughout. IBHA with urease of 7 Sumner units activity/mg (△); AHA with urease of 7 Sumner units/mg (●); AHA with urease of 0.7 Sumner units activity/mg (○).

cubation, since it alone, of the four compounds studied, reached equilibrium state. The other three showed kinetically irreversible inhibition patterns as we had noted before for AHA (2), and Kobashi *et al.*, found for a large number of hydroxamates (1). In other words, the inhibition proceeded to 100% with continued preincubation, and K_I values could not legitimately be derived by the Michaelis formulation, which applies only to equilibrium state. However, Gale and Atkins found no preincubation effect in the case of a number of other hydroxamic acids (3); and it appeared that FHA might provide a clue to the solution of this disparity, since it was the only member for which we could obtain both the association rate constant and the equilibrium constant. The latter was, therefore, evaluated in a combination experiment involving four concentrations of inhibitor over a 10-fold range, varied against four concentrations of substrate over a 100-fold range.

Lineweaver-Burk plots of the substrate-velocity data in the absence of FHA yielded straight lines with extrapolated K_m values for urea of 3.5–4.5 m*M* in agreement with most previously reported values (3, 9, 11). The pattern of FHA inhibition is shown in the double-reciprocal inhibitor plot of Fig. 3, in which all values should lie on a single line for a noncompetitive inhibitor. Although point scatter is evident, inspection reveals no tendency for higher substrate concentrations to produce lines with greater slopes, as would be expected for a competitive inhibitor; and the best straight-line fit for all points (excluding the one obviously errant value) was, therefore, determined by regression analysis. The solid line shows this result, which gives a K_I value for FHA of 3.8 μM, representing about a 1000-fold greater affinity for the enzyme than has its substrate. The discrimination level of the experiment is shown by the dashed lines, which indicate the patterns that should have been obtained at 1, 10, and 100 m*M* urea for the same inhibitor and substrate constants (and assuming a unit intercept), if the inhibition had been competitive. FHA inhibition is thus clearly noncompetitive in pattern, as has been the case with all

TABLE I. Inhibition Half-times ($t_{½}$) and Bimolecular Rate Constants (k_a) for the Formation of Urease:Hydroxamate Complex.

Urease	Hydroxamate		$t_{½}$ (min)	k_a (M^{-1} min^{-1})	
VI	AHA	20 μM	18.7	1848	1555
		2 μM	274.0	1263	
II	AHA	20 μM	18.0	1923	1733
		2 μM	224.0	1543	
VI	PHA	40 μM	20.0	866	944
		4 μM	170.0	1020	
VI	IBHA	100 μM	35.0	198	204
		10 μM	330.0	210	
VI	FHA	20 μM	31.5	1100	1200
		2 μM	266.0	1300	

Calculations were made from activity-decay plots as shown in Fig. 2. Lyophilized jackbean urease type VI had SA of 7 Sumner units/mg (100 IUB units/mg); type II had 10-fold lower SA.

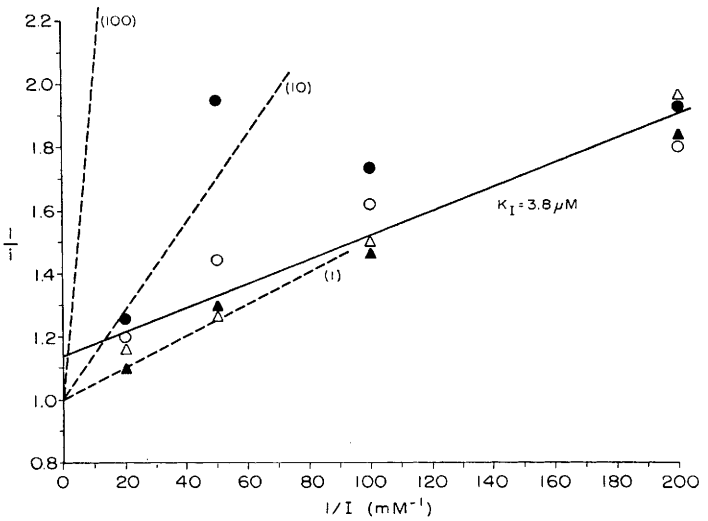

FIG. 3. Double-reciprocal inhibitor plot of FHA inhibition of urease (7 Sumner units activity/mg) at pH 7.0 in 0.1 *M* Tris–maleate buffer. All assays were carried out by 50-fold dilution into urea solutions after 60-min preincubation, by which time equilibrium had been attained. The solid line was determined by computer-programmed regression analysis for ungrouped data, employing all points save the one grossly errant measurement, assuming noncompetitive inhibition, and gives $K_I = 3.8$ μM. The interrupted lines show the slopes that should have been obtained for 1, 10, and 100 m*M* urea if the inhibition were competitive, assuming the same K_m and K_I values, and a unit intercept. Urea concentrations were: 100 m*M* (●) 10 m*M* (○) 5 m*M* (▲); 1 m*M* (△).

the aliphatic hydroxamates in which such data could be obtained (2, 3, 9). This implies that structurally the inhibitor site is spatially distinct from the active site, and pharmacologically that no level of urea would be sufficient to prevent the hydroxamate from inhibiting the enzyme.

Since $K_I = K_d/K_a$, the first-order rate constant for the dissociation of EI complex could be calculated in the case of FHA, which is of some relevance in the interpretation of the kinetic irreversibility of urease inhibition by its congeners. The calculated k_d for FHA of 4.56×10^{-3} min^{-1} is better understood in terms of its corresponding halftime for dissociation which is 2.53 hr. Thus it would take 2.5 hr for any urease–FHA complex formed to dissociate 50% and thus regenerate half the enzymatic activity, and about 10 hr to recover 93% of the initial activity after all free FHA was removed.

If, as seems plausible, the other hydroxamates complex similarly with urease, then their dissociation halftimes must be considerably longer than that for FHA, or they would also have reached equilibrium during the period of observation. It follows then, that in the case of AHA and, to a lesser extent in the case of PHA, the dissociation constant must be about an order of magnitude lower than that of FHA, since their k_a values are similar to, or greater than, that of FHA, while IBHA might have a K_I about the same as that of FHA. The kinetically irreversible pattern of inhibition by the other three compounds would then be a consequence of tight binding, plus sufficiently low rate constants for enzyme-inhibitor complex formation and dissociation, so that it was not feasible at the usual enzyme concentrations to follow the reaction over sufficiently long time periods for equilibrium to be reached. This type of reaction is analogous to pseudoirreversible inhibition (12), and represents, we believe, the correct interpretation of the reaction of the hydroxamates with urease. The pertinence of these considerations to the *in vivo* effects of the hydroxamates will be pointed out below.

Since our laboratory has recently demonstrated that urease exists in a multiplicity of active molecular forms, and that lyophilized preparations represent a polydisperse collection of these isozymes (13, 14), the inhibition constants cited above can hardly be considered definitive, but it is likely that the relative effects of the four congeneric hydroxamates would be similar for any isozyme selected. In any event, the pharmacologic utilization of these compounds depends upon their reacton *in vivo*, which involves not plant urease, but a wide assortment of bacterial ureases with perhaps different kinetic characteristics; and this was, therefore, explored directly by evaluating the efficacy of these compounds in CAF_1 mice.

Inhibition of ureolysis in male CAF_1 mice. Ten control mice injected with ^{14}C-urea, expired as $^{14}CO_2$ the following percentage (mean $\pm$ SE) of the injected radioactivity: 6.55 $\pm$ 0.41 during the first 2 hr, 3.20 $\pm$ 0.28 during the next 2 hr, and 1.52 $\pm$ 0.20 during the third 2 hr period. The cumulative 6-hr figures were 11.27 $\pm$ 0.62% of the administered dose of radioactive urea. These values were about 10% greater than those obtained in previous studies (7), and provided the basis for calculating the inhibition after administering hydroxamates. No dosage of any of the hydroxamates produced a significant change in the total weight of barium carbonate collected for individual time periods; and there was thus no evidence of gross disturbance in body metabolism that might interfere with the evaluation of urease inhibition.

A log dose-response curve was estimated for at least two concentrations of each of the hydroxamates for the first 2-hr interval during which the accuracy of measurement is highest. The data plotted in Fig. 4 show, that except for the highest dose of FHA, parallel lines may be fitted for all of the hydroxamates, indicating a 15% increase in inhibition for each doubling of the dose. For a given equimolar dose, it is apparent that the efficacy in mice decreases as the size of the aliphatic sidechain increases. A single dose study of HU is also included, and demonstrates the marked inferiority of this compound as a bacterial urease inhibitor in comparison to the aliphatic hydroxamates. The dose producing 50% inhibition of ureolysis (ED_{50}), in millimoles per kilogram body weight, was 1.45 for FHA, 2.10 for AHA, 2.20 for PHA, and 5.35 for IBHA. If a similar dose-response pattern is assumed for HU, the corresponding ED_{50} for this compound would be 16.0.

The decline of inhibition *in vivo* was evaluated by delaying the sc injection of ^{14}C-urea for 16 hr after the administration of a large dose of each hydroxamate, and comparing the residual inhibition with that obtained during the first 6 hr. The data are shown in Table II, using equimolar doses of each hydroxamate, except for FHA, where only half the dose was administered because of its greater toxicity (15). The value for the 6-hr interval is not a simple average of the three successive 2-hr intervals, since it is based on data cumulated for each mouse, and therefore has greater accuracy as interval fluctuations tend to cancel out. Inspection of the SE indicates that the accuracy of determination declined considerably for each sequential 2-hr interval as the amount of available ^{14}C-urea declined.

All four compounds produced maximal inhibition during the first 2 hr, with relatively sharp decline during the immediately following two intervals. All four were thus rapidly distributed to the gastrointestinal tract, and exerted their inhibition without delay. This agrees with direct studies of the physiologic disposition of these compounds in mice, in which the absorption and excretion rates approximated that of urea (16). Table II also demonstrates that three of the compounds still exerted significant inhibition of ureolytic activity 16–22 hr after administration. Only PHA was inactive in this time period (the mean values indicating activation are not significantly different than 0 effect), and the 2-hr collection periods now show a fairly steady level of inhibition. In the case of AHA, where the biologic half-life in CAF_1 mice has been determined as 3 hr (17), less than 3% of the administered dose is still in the body 16 hr

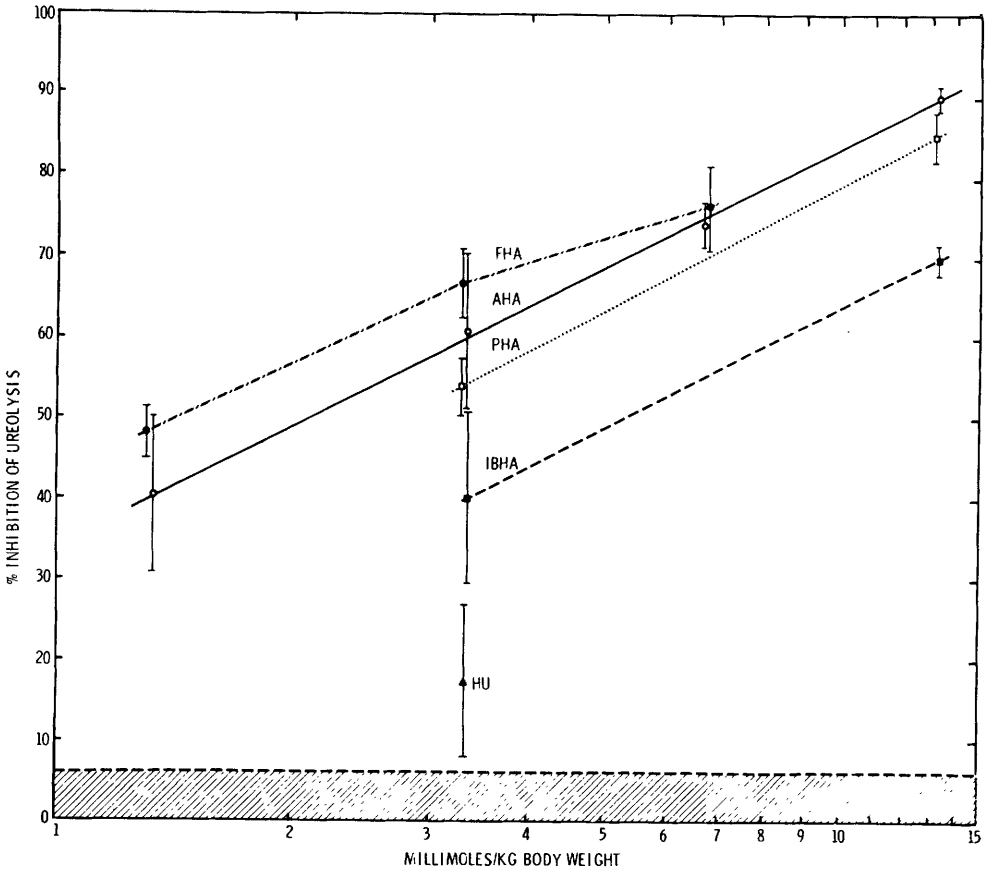

FIG. 4. Log-dose response curves for the inhibition of ^{14}C-urea hydrolysis in male CAF_1 mice by equimolar doses of four aliphatic hydroxamates during the first 2 hr after ip injection. The mean ± SE values are shown for five mice in each case, at doses of 1.33, 3.33, 6.67, or 13.33 mmoles/kg body weight; and a single-dose trial of the aminohydroxamate, hydroxyurea (HU), is also included for comparson. The shaded area represents the SE (in terms of percentage of inhibition) for a group of 10 control mice for the same 2-hr interval. The parallel lines indicate a 15% increase in inhibition for each doubling of the dose.

later, and the persistence of effect, therefore, suggests a component of irreversible inhibition.

We believe that this phenomenon is due to the very slow dissociation of EI complex, as was shown to be the case *in vitro*, so that even after removal of the inhibitor from the body, a delay period is required before enzyme reactivation is manifest. However PHA does not show this delayed inhibition, although its EI complex must also dissociate quite slowly. Although this can be explained by assuming that PHA has much less affinity for bacterial than for plant urease, a more plausible explanation might be that PHA is rapidly metabolized, so that its effective biologic life is much shorter than that of the other hydroxamates.

On balance, AHA would appear to be the most suitable of the four compounds for choice as a urease inhibitor, considering the ED_{50} and the rapidity and duration of action. However FHA and PHA are quite close to AHA in efficacy, and IBHA is not a great deal weaker. Their antiurease activity might be expected to differ relatively little in different animal species, since the enzyme being inhibited is not contributed by the host, but

TABLE II. Inhibition of ^{14}C-Urea Hydrolysis in CAF_1 Mice at Various Time Intervals After Intraperitoneal Injection of Four Short-Chain Aliphatic Hydroxamic Acids.

Hours after injection	% Inhibition (mean ± SE)[a]			
	FHA 6.72 mm/kg	AHA 13.33 mm/kg	PHA 13.33 mm/kg	IBHA 13.33 mm/kg
0–2	76.3 ± 5.3	89.8 ± 1.5	85.4 ± 3.2	69.8 ± 2.0
2–4	51.4 ± 3.4	68.5 ± 3.6	62.7 ± 7.8	63.7 ± 5.6
4–6	48.6 ± 9.5	65.4 ± 6.4	44.4 ± 11.5	42.8 ± 10.6
0–6	65.5 ± 2.6	80.5 ± 2.1	73.5 ± 3.9	64.6 ± 3.4
16–18	22.1 ± 3.9	27.6 ± 3.5[b]	(0.0 ± 8.5)	16.8 ± 4.5
18–20	27.1 ± 7.1	35.4 ± 5.5[b]	(−20.2 ± 13.8)[c]	17.1 ± 9.2
20–22	25.6 ± 8.7	37.6 ± 7.9[b]	(−12.5 ± 16.5)[c]	(25.5 ± 15.5)
16–22	24.0 ± 1.6	31.1 ± 3.1[b]	(− 7.5 ± 10.1)[c]	18.0 ± 6.3

[a] The percentage inhibition was determined by comparison with a group of 10 control mice whose mean percentage of ^{14}C-urea hydrolysis in each corresponding time interval was taken to represent 0% inhibition. The SE as % I for any group is then obtained as 100 × SE as % $^{14}CO_2$ divided by the mean value for the control group. Data for the control group are given in the text. For the 6-hr intervals, the data were cumulated for individual mice.

[b] Eight animals used in this group; five in all other groups.

[c] A minus sign indicates activation; all values except those in parentheses differ significantly ($p < .10$) from the control mean.

by resident bacteria. Toxicity considerations may, therefore, be the most important determinants in the selection of a therapeutic agent. In this regard, FHA was considerably more toxic than the other hydroxamates in mice, while IBHA was the least toxic (15). Moreover teratogenesis has been reported to be elicited by FHA and AHA, but not by PHA (18). The potential application of one or more of these compounds in veterinary and clinical medicine, therefore, will probably depend more on their toxicologic behavior than on differences in their pharmacologic efficacy.

Summary. Formo- (FHA), aceto- (AHA), propiono- (PHA), and isobutyro- (IBHA) hydroxamic acids have been evaluated as inhibitors of jackbean urease *in vitro* and of bacterial urease in male CAF_1 mice. All four were active throughout the pH range 5-9, bound tightly to the enzyme as manifested by K_I values of 4 μM or less, and exhibited low association rate constants for the formation of EI complex. The development of inhibition was kinetically irreversible for all of the compounds except FHA, but this is believed to represent simply the operational effect of low rate and equilibrium dissociation constants, rather than a true organochemical alteration of the enzyme. In mice the ED_{50} for the inhibition of ureolysis during the first 2 hr after administration, in millimoles/per kilogram body weight, was 1.45 for FHA, 2.10 for AHA, 2.20 for PHA, and 5.35 for IBHA. Hydroxyurea was much inferior to any of these compounds as a urease inhibitor. The inhibition was maximum during the first 2 hr after administration in all cases; yet all, except PHA, demonstrated significant residual inhibition 16–22 hr later.

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