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EFFECT OF ACYL RESIDUES OF HYDROXAMIC ACIDS ON UREASE INHIBITION

KYOICHI KOBASHI, KENJI KUMAKI AND JUN'ICHI HASE
Faculty of Pharmaceutical Science, University of Toyama, Toyama (Japan)

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SUMMARY

1. As regards the relationship between carbon number of acyl- or *p*-alkoxybenzohydroxamic acids and their inhibitory powers on urease (urea amidohydrolase, EC 3.5.1.5) activity, heptylo- and caprylohydroxamic acids in the series of the former and *p*-methoxybenzohydroxamic acid in the series of the latter showed the maximum inhibitory power. An increase in the number of carbon atoms of the acyl- or alkoxy-moieties led to a marked decrease in inhibitory power, which might be attributed to the decrease of their hydrophilic properties.

2. Substitution with various groups at the *meta*- or *para*-position of benzohydroxamic acid did not affect the inhibitory power. *Ortho*-substituted derivatives, however, were markedly less inhibitory.

These observations cannot be explained as being due to the effect of electronic polarization, but can be accounted for as being brought about by the "*ortho* effect", in the sense that a steric hinderance was caused by *ortho*-substitution in the benzohydroxamic acid at the active site of urease.

3. *o*-Aminobenzohydroxamic acid, a unique example among *ortho*-substituted derivatives, was found to be one of the most powerful inhibitors. Therefore a certain electronegatively charged group might possibly be located close to the active site of urease. However, methylation of the *o*-amino group of the compound reduced markedly its inhibitory power, this observation probably being attributable to the increase of steric size in the *ortho*-position.

4. Among hydroxamic acids derived from pyridine carboxylic acid, the position of the hydroxamic acid moiety influenced significantly the inhibitory power on the urease activity.

The α-amino group of hydroxamic acid derived from some α-amino acids did not affect the inhibitory power.

5. Compared with various related compounds of hydroxamic acid and urea on their effect on urease activity, it is very probable that –CONHOH is the group which is absolutely necessary in the chemical structure for the inhibition of urease activity. Both the properties of hydroxamic acids to form a coloured complex with Fe^{3+} and their ionization constants had no correlation with their inhibitory powers on urease activity.

INTRODUCTION

Hydroxamic acids have been reported to be potent and specific inhibitors of urease activity (urea amidohydrolase, EC 3.5.1.5)[1-3]. In our previous papers[1,4,5], their inhibition was found to be specific for the urease activity of plant and bacterial origin: the inhibitory power of hydroxamic acid was almost independent of the purity of the urease preparation used and also was not influenced by the addition of a large amount of egg albumin to the crystalline urease prior to the addition of the inhibitor. Furthermore, hydroxamic acid did not show any inhibitory action at a final concentration of $1.0 \cdot 10^{-3}$ M on a variety of other enzymes, in which hydrolases and sulfhydryl enzymes were included.

Urease reacted with aliphatic hydroxamic acid to form a stable enzyme–inhibitor complex, which could be isolated either by acetone precipitation as octahedral crystals or by Sephadex gel filtration. The molar ratio of hydroxamic acid incorporated to the crystalline enzyme was measured, using ^{3}H-labeled caprylohydroxamic acid, and found to be 2 moles per mole of enzyme[4], based on molecular weight of urease being 480 000 (ref. 6). Using a urease preparation of low specific activity, the ratio of hydroxamic acid to inactive complex was determined, based on ureolytic activity, and found to be the same as in the case of crystalline urease. These results show that urease reacts with the same number of moles of hydroxamic acid to form an inactive complex, regardless of the purity of the urease activity.

From a comparison of the effect of hydroxamic acid and its various related compounds on urease activity, we have already indicated that –CONHOH is the main group necessary for inhibition[1]. In the present report, we have synthesized more hydroxamic acids and its analogues, and investigated their effects on urease activity of sword bean extract. We used an enzyme preparation of low specific activity for the measurement of the inhibitory power of hydroxamic acids and their related compounds. The results obtained in this report, however, can be related to the pure state of the enzyme, because hydroxamic acid is a highly specific inhibitor of urease activity, as already mentioned above. Through these investigations we have made an attempt to clarify the correlation between the chemical structure or physico-chemical properties of hydroxamic acids and their inhibitory powers.

MATERIALS AND METHODS

Preparation of enzyme

Sword bean powder was stirred for 1 h with 5 vol. of 0.1 M phosphate buffer (pH 7.7). The suspension was centrifuged at 10 000 $\times$ *g* for 15 min and the supernatant fluid (5.7 units/mg of protein) was used for the measurement of inhibitory powers of hydroxamic acids and related compounds.

Synthesis of hydroxamic acids

Hydroxamic acids synthesized in our laboratory include eleven aliphatic-, nine araliphatic- and thirty-seven aromatic hydroxamic acids. Some of the hydroxamic acids were unknown compounds, of which melting points, analytical values and other properties were listed in Table I.

TABLE I

A LIST OF UNKNOWN HYDROXAMIC ACIDS

Compound	*m.p.*	*Formula*	*Analysis (%)*						*Appearance*	*Recrystallisation solvent*
			Calculated			*Found*				
			C	*H*	*N*	*C*	*H*	*N*		
(1) Aliphatic hydroxamic acids										
Heptylo	71.5–72°	$C_7H_{15}O_2N$	—	—	9.66	—	—	9.73	White plates	Benzene
Nonylo	81.5–82.5°	$C_9H_{19}O_2N$	—	—	8.09	—	—	8.31	White plates	Benzene
Undecano	89–90.1°	$C_{11}H_{23}O_2N$	—	—	6.66	—	—	7.03	White plates	Benzene
(2) Araliphtic hydroxamic acids										
α-Phenylbutyro	124–125°	$C_{10}H_{13}O_2N$	67.02	7.31	7.82	67.46	7.28	7.58	White needles	Chlorobenzene
β-Phenylbutyro	121–122°	$C_{10}H_{13}O_2N$	67.02	7.31	7.82	66.92	7.24	8.02	White needles	Chlorobenzene
γ-Phenylbutyro	85°	$C_{10}H_{13}O_2N$	67.02	7.31	7.82	67.12	7.29	7.98	White needles	Chlorobenzene
α-Phenylhexyro	74–75°	$C_{12}H_{17}O_2N$	69.54	8.27	6.76	69.55	8.13	6.50	White plates	Benzene
β-Phenylhexyro	113–114°	$C_{12}H_{17}O_2N$	69.54	8.27	6.76	69.72	8.29	6.63	White plates	Benzene
(3) Aromatic hydroxamic acids										
p-Hydroxybenzo	168°	$C_7H_7O_3N$	54.90	4.61	9.15	54.75	4.71	8.96	White needles	Methanol
m-Aminobenzo	137–138°	$C_7H_8O_2N_2$	55.25	5.30	18.41	54.97	5.11	18.09	Slightly pink needles	Chlorobenzene
o-Monomethlyaminobenzo	119°	$C_8H_{10}O_2N_2$	57.82	6.07	16.86	57.79	5.98	16.59	Slightly pink needles	Isopropyl ether
o-Chlorobenzo	158°	$C_7H_6O_2NCl$	48.98	3.51	8.16	48.91	3.36	8.14	Slightly red powder	Benzene
m-Chlorobenzo	164°	$C_7H_6O_2NCl$	48.98	3.51	8.16	49.06	3.40	8.40	Slightly red needles	Water
o-Methylbenzo	128°	$C_8H_9O_2N$	63.56	6.00	9.27	63.19	6.00	9.70	White needles	Chlorobenzene
m-Methoxybenzo	77–80°	$C_8H_9O_3N$	57.48	5.43	8.38	56.24	5.36	8.34	White plates	Water
p-Methoxybenzo	162°	$C_8H_9O_3N$	57.48	5.43	8.38	57.66	5.47	8.36	Slightly red needles	Water
m-Butoxybenzo	107–110°	$C_{11}H_{15}O_3N$	63.14	7.23	6.69	62.91	6.96	6.85	White plates	Benzene
p-Butoxybenzo	160–162°	$C_{11}H_{15}O_3N$	63.14	7.23	6.69	63.40	7.27	6.92	White plates	Acetone
m-Hexyloxybenzo	115°	$C_{13}H_{19}O_3N$	65.80	8.07	5.90	65.96	8.09	6.02	White plates	Water
p-Hexyloxybenzo	155–158°	$C_{13}H_{19}O_3N$	65.80	8.07	5.90	65.57	8.00	5.88	White plates	Acetone
m-Octyloxybenzo	83–86°	$C_{15}H_{23}O_3N$	67.89	8.74	5.28	67.61	8.41	5.20	White plates	Light petroleum
p-Octyloxybenzo	152–153.5°	$C_{15}H_{23}O_3N$	67.89	8.74	5.28	67.99	8.86	5.40	White powder	Acetone
o-Dodecyloxybenzo	82–83°	$C_{19}H_{31}O_3N$	70.99	9.72	4.36	70.95	9.75	4.74	White powder	Methanol
m-Dodecyloxybenzo	100–102°	$C_{19}H_{31}O_3N$	70.99	9.72	4.36	72.35	9.56	3.39	White powder	Benzene
p-Dodecyloxybenzo	130–132°	$C_{19}H_{31}O_3N$	70.99	9.72	4.36	72.00	9.80	4.73	White powder	Methanol

Measurement of approximate I_{50} value of hydroxamic acid

The percentage of inhibition was measured under the following conditions. A mixture of 140 units of crude urease in 0.3 ml of 0.1 M phosphate buffer (pH 7.7) and of 0.30–3000 nmoles of hydroxamic acid to be tested in 0.3 ml of the same buffer was pre-incubated at 35° for 30 min. After pre-incubation, 5.0 ml of 3.0% urea solution in 0.1 M phosphate buffer (pH 6.7) and 0.05 ml of alcoholic solution of 0.1% phenol red were added and urease activity was measured colorimetrically[7]. Fig. 1

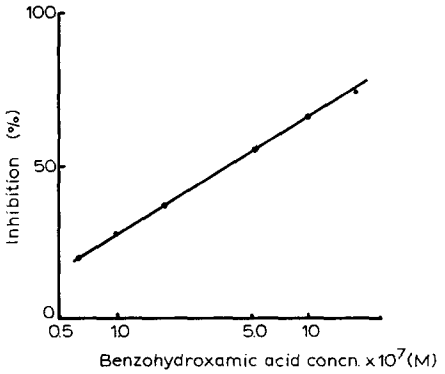


Fig. 1. Measurement of approximate I_{50} value of hydroxamic acid.

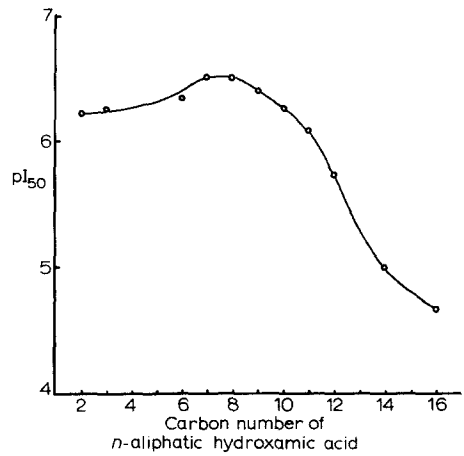


Fig. 2. Relation between carbon number of *n*-aliphatic hydroxamic acids and their inhibitory power on urease activity.

shows the inhibition of urease activity by benzohydroxamic acid, the approximate I_{50} value of which was measured to be $3.7 \cdot 10^{-7}$ M in a final concentration.

RESULTS

Relation between carbon number of n-aliphatic or p-(n-alkyloxy)benzohydroxamic acids and their inhibitory powers on urease activity

The pI_{50} values of fatty acyl monohydroxamic acids were dependent upon the carbon number of their acyl moieties as shown in Fig. 2. Among the eleven derivatives tested, heptylo- and caprylohydroxamic acids were the most potent inhibitors. As shown in this figure, an increase in the carbon number of the hydroxamic acid to a value greater than nine led to a marked decrease in inhibitory power. The increase in carbon number of alkyl residues leads to a decrease in the solubility of hydroxamic acid in water. In this case, a small amount of ethyl alcohol was used as an aid in dissolution in order to obtain an original solution of 10 μmoles/ml of fatty acyl hydroxamic acids. Ethyl alcohol had no effect on either urease activity or on urease inhibition by hydroxamic acids under the conditions described in MATERIALS AND METHODS. The marked decrease in inhibitory power of hydroxamic acids of higher chain length might be attributable to the decrease of hydrophilic properties of the compounds.

Similar results were obtained in the series of *p*-alkyloxybenzohydroxamic acid as shown in Fig. 3. The methylation of *p*-hydroxybenzohydroxamic acid which had a pI_{50} of 6.45, increased the inhibitory power to the maximum pI_{50}, 6.65. A further increase in carbon number of the series led to a sharp decrease in the inhibitory power on urease activity.

Benzene nucleus, the C–C bond and C–O bond have 2.78, 1.54 and 1.47 Å

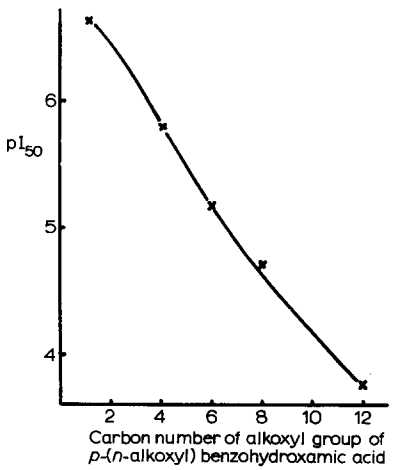


Fig. 3. Effect of carbon number of alkoxyl groups of *p*-(*n*-alkoxyl)benzohydroxamic acid.

lengths, respectively. Using these three values, both the length of the molecule and the inhibitory power of *p*-methoxybenzohydroxamic acid corresponded to those of heptylohydroxamic acid. When the abscissa of Figs. 2 and 3 are graduated in units corresponding to the chain length of the molecule, the curves in the two figures almost coincide.

Effect of substitutions of benzohydroxamic acid

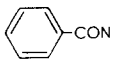
Effect of various substituents of aromatic hydroxamic acids on their I_{50} values is shown in Table II. The *meta*- and *para*-substituted derivatives tested, except the *p*-carboxyl group, gave almost the same I_{50} values as benzohydroxamic acid. These observations could not be explained according to Hammett's rule in electronic theory. On the contrary, *ortho*-substituted benzohydroxamic acids, except the *o*-amino derivative, were markedly less inhibitory. For example, as can be seen in this table, chloro-, methoxy- and nitroderivatives had I_{50} values of $3.0 \cdot 10^{-7}$, $2.3 \cdot 10^{-7}$ and $3.7 \cdot 10^{-7}$ M, respectively, in the *para*-position and $4.3 \cdot 10^{-7}$, $5.4 \cdot 10^{-7}$ and $3.7 \cdot 10^{-7}$ M in the *meta*-position. However, a marked decrease was observed in I_{50} values of $7.5 \cdot 10^{-5}$, $4.5 \cdot 10^{-4}$ and $1.2 \cdot 10^{-2}$ M, respectively, when these substituents were in the *ortho*-position.

o-Carboxybenzohydroxamic acid was non-inhibitory, and it is possible that the *o*-carboxyl group has an intramolecular interaction with the hydroxamic acid group, which is the main group necessary for the inhibition. In order to exclude this possibility, *o*-carbomethoxy- and *o*-carboamide derivatives, which had substantially no interaction with the hydroxamic acid group, were synthesized. As shown in Table II,

TABLE II

EFFECT OF SUBSTITUTION OF BENZOHYDROXAMIC ACID

Experimental conditions were described in detail in MATERIALS AND METHODS and Fig. 1.



; $I_{50} = 3.7 \cdot 10^{-7}$ M.

Group	I_{50} *values (M)*		
	ortho	*meta*	*para*
$-NH_2$	$2.7 \cdot 10^{-7}$	$3.0 \cdot 10^{-7}$	$4.1 \cdot 10^{-7}$
$-NHCH_3$	$5.5 \cdot 10^{-6}$	—	—
$-N(CH_3)_2$	No inhibition	—	—
$-OH$	$2.5 \cdot 10^{-6}$	—	$4.3 \cdot 10^{-7}$
$-CH_3$	$1.6 \cdot 10^{-5}$	$6.3 \cdot 10^{-7}$	$1.1 \cdot 10^{-6}$
$-Cl$	$7.5 \cdot 10^{-5}$	$4.3 \cdot 10^{-7}$	$3.0 \cdot 10^{-7}$
$-OCH_3$	$4.5 \cdot 10^{-4}$	$5.4 \cdot 10^{-7}$	$2.3 \cdot 10^{-7}$
$-NO_2$	$1.2 \cdot 10^{-2}$	$3.7 \cdot 10^{-7}$	$3.7 \cdot 10^{-7}$
$-OC_4H_9$	No inhibition	$3.6 \cdot 10^{-7}$	$1.8 \cdot 10^{-6}$
$-OC_6H_{13}$	No inhibition	$7.0 \cdot 10^{-7}$	$2.3 \cdot 10^{-6}$
$-OC_8H_{17}$	No inhibition	$3.6 \cdot 10^{-6}$	$2.1 \cdot 10^{-5}$
$-OC_{12}H_{25}$	No inhibition	No inhibition	No inhibition
$-COOH$	No inhibition	—	No inhibition
$-CONH_2$	No inhibition	—	—
$-COOCH_3$	No inhibition	—	—

they did not inhibit urease activity at all. Therefore the effect of the *o*-carboxyl group may be not due to its acidity but to its steric structure.

Substituents in the *ortho*-position could be arranged in descending order of inhibitory power as follows: amino-, hydroxyl-, methyl-, chloro-, methoxy-, nitro- and carboxyl group. This order might be explained on the basis of steric structure rather than of electronic polarization. In this regard, however, the *o*-amino group did not influence the inhibitory power at all, which strongly suggests the possibility of the presence of an electronegatively charged group close to the active site of urease. Methylation of the *o*-amino derivative markedly reduced its inhibitory power to one-twentieth of the original value in the monomethyl- and brought about non-inhibition in the dimethylamino derivative. This effect of methylation, which reduces the

TABLE III

I_{50} VALUES OF ARALIPHATIC HYDROXAMIC ACIDS

Hydroxamic acid	I_{50} *(M)*
Phenylaceto	$1.2 \cdot 10^{-6}$
β-Phenylpropio	$2.0 \cdot 10^{-7}$
α-Phenylbutyro	$1.1 \cdot 10^{-5}$
β-Phenylbutyro	$4.6 \cdot 10^{-7}$
γ-Phenylbutyro	$1.0 \cdot 10^{-6}$
α-Phenylhexyro	$8.9 \cdot 10^{-6}$
β-Phenylhexyro	$1.2 \cdot 10^{-6}$
1-Naphthylaceto	$8.6 \cdot 10^{-6}$
α-Benzoylaminocinnamo	$1.0 \cdot 10^{-7}$

inhibitory power, is probably attributable to the increase of steric size in the *ortho*-position.

The I_{50} values of araliphatic hydroxamic acids are shown in Table III. β-Phenylpropio- and α-benzoylaminocinnamohydroxamic acid equalled heptylo- and caprylohydroxamic acids in I_{50} values which were the most potent inhibitors in the series of aliphatic hydroxamic acids. Three β-phenyl fatty acyl hydroxamic acids and γ-phenylbutyrohydroxamic acid showed strong inhibitory powers, but their I_{50} values decreased almost proportionally to their molecular weights, possibly owing to the decrease in their hydrophilic properties. However, α-phenylbutyro- and α-phenylhexylohydroxamic acid were markedly less inhibitory, showing one-twentieth and one-seventh the I_{50} values, respectively, of those of the corresponding β-phenyl derivatives. 1-Naphthylacetohydroxamic acid also was less inhibitory than α-phenylacetohydroxamic acid. This remarkable effect of the α-phenyl group and α-naphthyl group in araliphatic hydroxamic acid is probably due to steric hinderance, in the same fashion as that of the *ortho*-substituted benzohydroxamic acid.

With regard to the "*ortho* effect" in aromatic and the "α-effect" in araliphatic hydroxamic acids, it seems probable that a certain electronegatively charged group is located close to the active site of urease, and possibly the same group sterically makes *o*- and α-substituted hydroxamic acids inaccessible to the active site of enzyme.

In addition, all the carboxylic acids and esters corresponding to the hydroxamic acids shown in Figs. 2 and 3, Tables II and III had no inhibitory action on urease activity, and had neither an interfering effect nor a reactivating action on urease inhibition by the corresponding hydroxamic acid.

Effect of basic group

The I_{50} values of heterocyclic hydroxamic acids are shown in Table IV. Among three hydroxamic acids derived from pyridine carboxylic acids, nicotinohydroxamic acid was equal in I_{50} value to caprylohydroxamic acid. Isonicotinohydroxamic acid

TABLE IV

I_{50} VALUES OF HETEROCYCLIC HYDROXAMIC ACIDS

Hydroxamic acid	I_{50} *(M)*
Picolino	$1.4 \cdot 10^{-3}$
Nicotino	$3.2 \cdot 10^{-7}$
Isonicotino	$5.7 \cdot 10^{-6}$
Thiophene-2-carbo	$4.8 \cdot 10^{-7}$
Furan-2-carbo	$9.1 \cdot 10^{-7}$

was less inhibitory and picolinohydroxamic acid was almost non-inhibitory. This observation suggests that the position of the basic group influences significantly the inhibitory power on urease activity. Thiophene-2-carbo, furan-2-carbo- and nicotinohydroxamic acid were powerful inhibitors with almost the same I_{50} value as benzohydroxamic acid.

The I_{50} values of derivatives of amino acids were shown in Table V. Glycyl- and DL-alanylhydroxamic acid were equal to the corresponding aceto- and propio-

TABLE V

I_{50} VALUES OF α-AMINOACYLHYDROXAMIC ACIDS

Hydroxamic acid	I_{50} *(M)*
Glycyl	$2.3 \cdot 10^{-6}$
DL-Alanyl	$6.4 \cdot 10^{-7}$
L-Leucyl	$4.6 \cdot 10^{-6}$
L-Tyrosyl	$3.8 \cdot 10^{-5}$

hydroxamic acid, respectively, in I_{50} values and thus the α-amino group did not affect the inhibitory power. L-Leucyl- and L-tyrosylhydroxamic acid were less inhibitory than the corresponding caprylo- and β-phenylpropiohydroxamic acids, respectively. The lesser inhibitory power of these two α-aminoacylhydroxamic acids might be due to their low solubility.

I_{50} values of various other hydroxamic acids

Table VI shows I_{50} values of monohydroxamic acids derived from di- and tricarboxylic acids and of arabinohydroxamic acid. Maleino-, succino- and adipinomonohydroxamic acid were markedly less inhibitory than the corresponding aliphatic hydroxamic acid, and γ-D-isocitrinomonohydroxamic acid showed no inhibition on urease activity. In general, monohydroxamic derivatives of di- and tricarboxylic acids were markedly less inhibitory, probably due to steric hindrance of a neighboring

TABLE VI

I_{50} VALUES OF DI- OR TRICARBOHYDROXAMIC ACIDS AND SOME OTHER HYDROXAMIC ACIDS

Hydroxamic acid	I_{50} *(M)*
Terephthalomono	$1.8 \cdot 10^{-3}$
Terephthalodi	$5.9 \cdot 10^{-6}$
Monomethylesteroxalo	No inhibition
Maleinomono	$3.9 \cdot 10^{-5}$
Succinomono	$1.8 \cdot 10^{-5}$
Adipinomono	$1.8 \cdot 10^{-5}$
γ-D-Isocitrino	No inhibition
Monochloroaceto	$1.8 \cdot 10^{-6}$
D-Arabino	$2.7 \cdot 10^{-6}$

carboxylic group or the effect of its anionic property. Terephthalomonohydroxamic acid also did not inhibit urease activity. Therefore, it seems that a certain electronegative group is situated near the active site of the enzyme and that this group makes these anionic derivatives inaccessible to the active site. On the other hand, terephthalodihydroxamic acid showed considerably powerful inhibitory action, but was less inhibitory than benzohydroxamic acid. D-Arabinohydroxamic acid, as a unique example of a sugar derivative, was found to be a potent inhibitor.

Necessary chemical structure for the inhibition of urease activity

It seems likely, as reported previously[1], that the main group which is necessary for the inhibition of urease activity is –CONHOH. To obtain a definite conclusion with

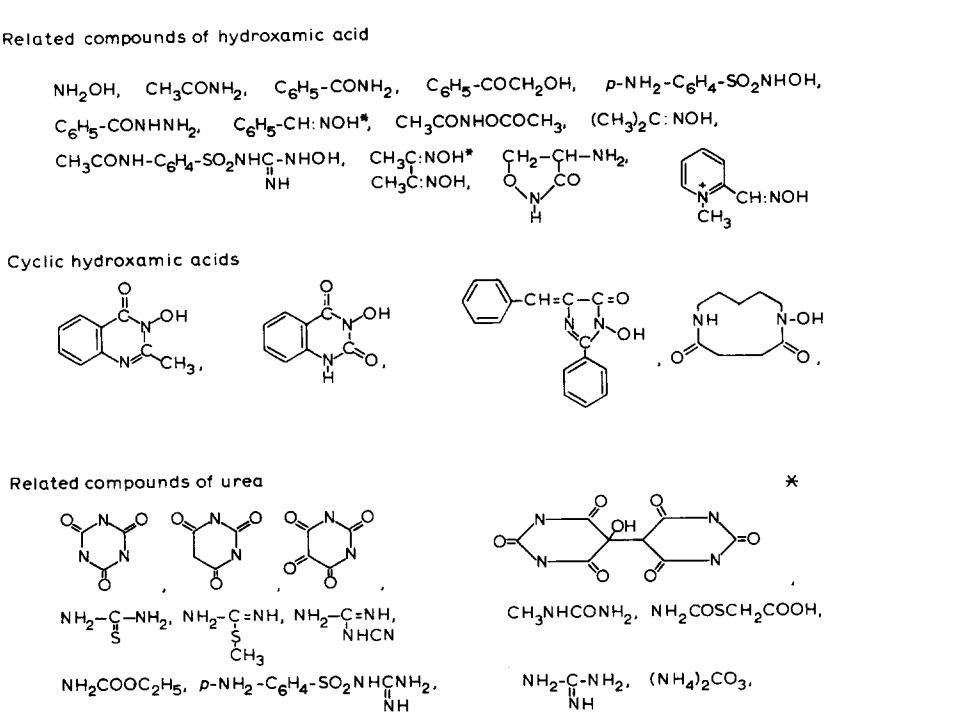


Fig. 4. Non-inhibitory related compounds and analogues of hydroxamic acid. Some compounds which inhibit urease activity less than 10% in the final concentration of $1 \cdot 10^{-3}$ M were shown in this figure as non-inhibitory ones.

* These samples were measured in the saturated solution.

more evidence, thirty samples of related compounds of hydroxamic acid and urea, as shown in Fig. 4, were measured for their inhibitory powers. All of the compounds have a modified structure in which a certain atom of the –CONHOH group was replaced by various other groups in their molecule. However, none of them inhibited urease activity and in addition they had neither an interfering nor a reactivating effect on the urease inhibition by hydroxamic acids. From these results, it appeared that the –CO– function of the hydroxamic acid group could not be replaced by any group such as $-SO_2-$, $-(C=NH)-$, or $-CH=$, since I, II or oximes were not inhibitory. The hydrogen atom of the –NH– of the group also could not be modified, as is shown in the structure of cyclic compounds. The –OH of the group was necessary for inhibition, because amides, *O*-acetyl derivatives and cycloserine were inactive. It is concluded that –CONHOH is the absolutely necessary structure for the inhibition of urease activity.

Hydroxamic acid, in general, forms a violet or blue complex with Fe^{3+}, which is the principle of the determination method according to the method of LIPMANN AND TUTTLE[8]. All the hydroxamic acids, which were non- or markedly less inhibitory, formed almost the same highly coloured complexes, in which were involved the long-chain aliphatic- (Fig. 2), long-chain *p*-alkoxybenzo- (Fig. 3), *o*-substituted benzo- (Table II), picolino- (Table IV), di- or tricarboxylic mono- (Table VI) and the cyclic hydroxamic acids (Fig. 4). Therefore, the ability of hydroxamic acid to form a

TABLE VII

COMPARISON OF I_{50} VALUES OF CAPRYLOHYDROXAMIC ACID WITH THOSE OF OTHER INHIBITORS

Crude urease preparation (5.7 units/mg) was used for the measurement of I_{50} values of these compounds.

Inhibitor	I_{50} *(M)*
Caprylohydroxamic acid	$9.3 \cdot 10^{-8}$
Hydroxylamine	$8.8 \cdot 10^{-3}$
Phenylhydrazine	$2.6 \cdot 10^{-2}$
Thiosemicarbazide	$2.9 \cdot 10^{-2}$
Semicarbazide	$5.4 \cdot 10^{-2}$
NaF	$1.3 \cdot 10^{-3}$
H_2O_2	$1.8 \cdot 10^{-2}$
Cu^{2+}	$3.0 \cdot 10^{-4}$
Hg^{2+}	$2.0 \cdot 10^{-5}$
Ag^{+}	$1.0 \cdot 10^{-5}$
p-Chloromercuribenzoate	$4.9 \cdot 10^{-5}$

coloured complex with Fe^{3+} in the acid pH range has no correlation with the inhibitory power on urease activity.

Comparison of I_{50} values of hydroxamic acids with those of other inhibitors

Table VII shows the comparison of I_{50} value of caprylohydroxamic acid with those of carbonyl reagents such as hydroxylamine[2], heavy metal ions, oxidizing reagents and F^- (unpublished observation). From these results, hydroxamic acid was found to be the most powerful inhibitor which was equal in its inhibitory power to suramin[9], the most powerful inhibitor known in the literature.

These I_{50} values were measured using a water extract of sword bean powder under the same conditions as described in MATERIALS AND METHODS. As previously reported[1,4], I_{50} values of hydroxamic acids were almost independent of the purity of urease preparations: the I_{50} value of caprylohydroxamic acid was measured to be $4.8 \cdot 10^{-8}$ M using crystalline enzyme (3500 units/mg) and to be $9.3 \cdot 10^{-8}$ M using sword bean extract (5.7 units/mg). Inhibition by other inhibitors shown in Table VII, however is markedly dependent upon the purity of enzyme preparations.

The inhibition by hydroxamic acids was neither protected nor reversed by 0.03% egg albumin or by the following biochemical compounds in the final concentration of $1.8 \cdot 10^{-3}$ M: L-cysteine, glutathione, L-arginine, L-aspartic acid, L-asparagine, L-glutamic acid, L-serine, DL-threonine, L-tryptophan ($9.0 \cdot 10^{-4}$ M), L-tyrosine, creatine, taurine, betaine and pantothenic acid (saturated solution).

These results support hydroxamic acid to be a strictly specific inhibitor on urease activity.

Effect of organophosphoric compounds

Organophosphorus compounds are well-known inhibitors of hydrolases such as acetylcholinesterase, chymotrypsin, trypsin, thrombin and some lipases. These inhibitions, particularly on acetylcholinesterase, have been shown to be reactivated by treatment with hydroxylamine, hydroxamic acid or oxime[10] as reported by WILSON[11,12] and WILSON *et al.*[13], who postulated that nucleophilic hydroxamic acids or al-

doximes might attack the phosphoryl group bound to active sites and split it from the enzyme, and so restore the activity. Moreover, hydroxamic acid was found to be an accelerant of the hydrolysis of phosphoric ester[14]. Based on these observations, investigations were made upon the effect of organophosphoric compounds, diisopropylfluorophosphate (DFP), dimethyldichlorovinylphosphate (DDVP) and *O,O*-dimethyl-2,2,2-trichloro-1-hydroxyethyl phosphate (dipterex), on urease activity and on inhibition of urease activity by hydroxamic acids. All of these organophosphorus compounds did not inhibit urease activity at all in a final concentration of $1.7 \cdot 10^{-3}$ M. In addition, they did neither protect nor reactivate the inhibition of urease activity by both aliphatic and aromatic hydroxamic acids, even when they were added to a vessel in 1000 times molar excess of the hydroxamic acid. As shown in Fig. 4, oximes which have a reactivating effect on organophosphoric inhibition did neither inhibit urease activity nor affect the inhibition by hydroxamic acids. These facts show that the properties of the active site of urease are quite different from those of other hydrolases.

DISCUSSION

Ortho-substituted derivatives of benzohydroxamic acids showed markedly less inhibition of urease activity than *meta*- and *para*-substituted derivatives. The order of the substituted groups at the *ortho*-position in their inhibitory power could be arranged in proportion to their steric size. In a series of araliphatic hydroxamic acids, substitution of the phenyl group at the α-position showed a distinct decreasing effect upon the urease inhibitory power. Thus a possible conclusion from these observations is the presence of a sterically hindered group close to the active site of the enzyme. This group makes the –CONHOH group of the hydroxamic acid derivative substituted by a sterically bulky group at the *ortho*- or α-position inaccessible to the active site. Among *ortho*-substituted benzohydroxamic acids, substitution of the amino group, with a single exception, did not influence the inhibitory power at all, a fact which suggests the possibility of the presence of a certain electronegative group near the active site. This group could be assumed to be the same one having both "*ortho*- and α-phenyl effect" without any contradiction.

Monohydroxamic derivatives of dicarboxylic acids, in general, were markedly less or non-inhibitory on urease activity. Maleino- or succinomonohydroxamic acids have a carboxylic anion at the same distance from the hydroxamic acid group as the *o*-carboxybenzoderivative, and their weak inhibitory power also would be due to "*ortho* effect" as described above. However, the effect of the δ- or *p*-carboxylic anion of adipino- or terephthalomonohydroxamic acid cannot be explained by the "*ortho* effect", but could be attributable to the presence of another electronegative repelling group in the enzyme. Therefore, it seems likely that another electronegative area is located comparatively apart from the active site and shows a "repelling effect" against adipino- and terephthalomonohydroxamic acids.

For the purpose of examining whether or not the ionization constant of hydroxamic acids affects their inhibitory power, pK_a values of some hydroxamic acids were measured at 25° as shown in Table VIII. Although *ortho*- and *para*-substituted benzohydroxamic acids have almost the same pK_a in the cases, for example, of nitro- and methoxyderivatives, *ortho*-substituted benzohydroxamic acids were

TABLE VIII

pK_a AND pI_{50} VALUES OF SOME HYDROXAMIC ACIDS

Hydroxamic acid	$pK_a(25)$	pI_{50}
Aceto	9.4	6.21
Benzo	8.9	6.42
o-Nitrobenzo	8.2	2.92
p-Nitrobenzo	8.0	6.42
o-Methoxybenzo	8.9	3.35
p-Methoxybenzo	9.0	6.64
o-Hydroxybenzo	7.4	5.60
Picolino	8.7	2.85
Nicotino	8.3	6.49
Isonicotino	7.8	5.74

markedly less inhibitory than the *para*-substituted ones. Among the pyridine derivatives, we could not observed any correlation between pK_a and pI_{50}. Moreover, the pK_a values of the powerful inhibitors, of which the pI_{50} is greater than 6.0, are distributed at random from 8.0 to 9.4. From these results, we could not correlate the inhibitory power of the hydroxamic acids with their ionization constant. In regard to this fact, it is difficult to explain the effect of acyl moieties of hydroxamic acids on inhibition of urease based on Hammett's rule in electronic theory, we observed that the *meta*- and *para*-substituted derivatives of benzohydroxamic acid had the same I_{50} values as benzohydroxamic acid.

In the present study, we used a crude extract of sword bean powder for the measurement of the inhibitory power of hydroxamic acid. We have already reported that hydroxamic acid inhibits urease activity with high and strict specificity and that the I_{50} value was almost the same, regardless of the purity of the enzyme preparations used: the I_{50} value of caprylohydroxamic acid was measured to be $4.8 \cdot 10^{-8}$ M when crystalline urease (3500 units/mg) was used and to be $9.3 \cdot 10^{-8}$ M when crude extract (5.7 units/mg) was used. The specific activity of the enzyme was 600 times higher than that of the crude enzyme, but the I_{50} value in the pure state was only half that of the I_{50} value in the crude state. Therefore, the inhibitory powers of the hydroxamic acids measured in this paper are probably of the same magnitude with the pure state of enzyme and the conclusion obtained through these investigations is to be interpreted with respect to pure enzyme without any objection.

As previously reported[1], the inhibition by acetohydroxamic acid was progressive with time. FISHBEIN AND CARBONE[2] demonstrated acetohydroxamic acid to be an irreversible inhibitor of urease with $K = 1 \cdot 10^3\ M^{-1}$ at 25° and suggested that our data[1] should be expressed in terms of rate constant. However, we found aromatic hydroxamic acid to be a reversible and instantaneous inhibitor and *n*-aliphatic hydroxamic acid to be a progressive inhibitor with time. The inhibition by the latter also was found to be very slowly reversed. All the data in this paper are expressed in terms of the I_{50} values, which are more appropriate for completely reversible inhibitors. Therefore the I_{50} values of *n*-aliphatic hydroxamic acids may not represent the exact inhibitory power in kinetic terms. Studies on the difference of the mechanism of inhibition between aliphatic and aromatic hydroxamic acid are now in progress.

REFERENCES

1 K. KOBASHI, J. HASE AND K. UEHARA, *Biochim. Biophys. Acta*, 65 (1962) 380.
2 W. N. FISHBEIN AND P. P. CARBONE, *J. Biol. Chem.*, 240 (1965) 2407.
3 R. L. BLAKELEY, J. A. HINDS, H. E. KUNZE, E. C. WEBB AND B. ZERNER, *Biochemistry*, 8 (1969) 1991.
4 K. KOBASHI, J. HASE AND T. KOMAI, *Biochem. Biophys. Res. Commun.*, 23 (1966) 34.
5 J. HASE AND K. KOBASHI, *J. Biochem. Tokyo*, 62 (1967) 293.
6 J. B. SUMMER, N. GRALÉN AND I.-B. ERIKSSON-QUENSEL, *J. Biol. Chem.*, 125 (1938) 37.
7 D. D. VAN SLYKE AND R. M. ARCHIBALD, *J. Biol. Chem.*, 154 (1944) 623.
8 F. LIPMANN AND L. C. TUTTLE, *J. Biol. Chem.*, 159 (1945) 21.
9 E. D. WILLS AND A. WORMALL, *Biochem. J.*, 47 (1950) 158.
10 D. R. DAVIES AND A. L. GREEN, *Biochem. J.*, 63 (1956) 529.
11 I. B. WILSON, in J. L. WEBB, *Enzyme and Metabolic Inhibitors*, Vol. 1, Academic Press, New York, 1963, p. 630.
12 I. B. WILSON, *J. Am. Chem. Soc.*, 77 (1955) 2383.
13 I. B. WILSON, S. GINSBERG AND E. K. MEISLICH, *J. Am. Chem. Soc.*, 77 (1955) 4286.
14 B. E. HACKLEY, Jr., R. PLAPINGER, M. STOLGERG AND T. W. JAUREGG, *J. Am. Chem. Soc.*, 77 (1955) 3651.