Biochimica et Biophysica Acta, 484 (1977) 433—442

BBA 68227

FORMAMIDE: THE MINIMUM-STRUCTURE SUBSTRATE FOR UREASE *

WILLIAM N. FISHBEIN

Biochemistry Division, Armed Forces Institute of Pathology, Washington, D.C. 20306 (U.S.A.)

(Received February 7th, 1977)

Summary

Formamide has been found to be a bona fide substrate for jackbean urease (urea amidohydrolase, EC 3.5.1.5) and can be hydrolyzed to completion by the enzyme. The pH maximum for the reaction was 5.2, the Michaelis constant was 0.5 (±0.05) M at pH 5.2 and at pH 6.8, and the maximal velocity was 50-fold lower than that for urea at pH 6.8 its pH maximum, which nevertheless represents the highest V for any of the second-substrates of urease. As a competitive inhibitor of urea at pH 6.8, formamide had a K_i = 0.4 (±0.11) M, indicating that the Michaelis constants for both urea and formamide approximate the true dissociation constants for the respective enzyme-substrate complexes. The ratios of formamide/urea hydrolysis remained constant during purification of the enzyme to maximal specific activity; and formamide hydrolysis was completely inhibited in the presence of 0.8 mM acetohydroxamate, a specific urease inhibitor. Formamide did not produce a progressive inhibition of urease, as had been found to occur with two other substrates possessing the hydroxamate functional group: hydroxyurea and dihydroxyurea. The kinetic constants for urease hydrolysis of formamide compare favorably with those of a representative bacterial aliphatic amidase. Urease did not hydrolyze the closest homologs of formamide: *N*-methyl formamide and acetamide.

Introduction

Although urease (urea amidohydrolase, EC 3.5.1.5) is highly specific towards urea, it does hydrolyze at least three other substrates: hydroxyurea [1], dihydroxyurea [2], and semicarbazide [3]. Recently Sundaram and Laidler [4] reported that urease hydrolzed a large assortment of substituted ureas and car-

* The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

bamate esters, and noted incidently that no hydrolysis of formamide by the enzyme was detectable, although the conditions were not specified. More recently, several of their purported substrates have been contested as artifactual, due to contamination with urea [3]. We have not reinvestigated those substrates, but in this report will present compelling evidence that formamide is a bona fide substrate for urease, and presents the highest reaction velocity of any of the second-substrates, although still far inferior to that of urea. Elements of this work have previously been communicated [5].

Materials and Methods

Materials

Formamide, *N*-methyl formamide, and acetamide were obtained commercially as high-purity reagents, and were used without further modification, Jackbean urease [6] and acetohydroxamate [7] were purified as previously described. Ultra-pure Tris and reagent grade Bis-Tris were used as buffers and made up in charcoal-filtered deionized water with 10 M$\Omega \cdot$ cm resistance at issue.

Methods

Protein determinations were carried out by the method of Lowry et al. using bovine serum albumin as a standard [8].

A number of enzymatic assays were used in this study. Determination of the pH activity curve for urea was carried out in 50 mM Tris and Bis-Tris buffers, with timed dilutions into Nessler reagent for ammonia determination, as previously described [6]. The same procedure was used to determine ammonia liberated during formamide hydrolysis for stoichiometry determinations, the residual formamide being assayed by the method of Bergman [9]. Evaluation of the Michaelis constant for urea, and its competitive inhibition by formamide was carried out by a modified buffer-indicator assay [10], which measured alkaligenic increase in absorbance at pH 6.8 produced by urea hydrolysis, and is insensitive to ammonium formate. Specific activities determined by this method have been normalized to those obtained by the Nessler assay noted above, and are reported in Sumner units for historical comparison. One Sumner unit = 7.14 μmol urea decomposed/min = 14.28 μmol ammonia formed/min = 0.119 μkat, all at 20° C. For calculation of the catalytic constant, the molecular weight of urease was taken as 490 000 [11,12].

All other assays of formamide hydrolysis were carried out by conductimetry at 20° C using a Yellow Springs Instrument Company Model 31 conductivity bridge with electron ray tube and micro-cell with $K = 1$. No acids or bases are used to stop the reaction; and formamide is well-suited to such an assay, since its hydrolytic product, ammonium formate, has an equivalent conductance of 116 $\mu\Omega^{-1}/\mu$mol per cm^3 and a pH-equivalence point near neutral. The usual system contained 16 ml Bis-Tris with concentrations varying from 1.25 mM at pH 5, to 5 mM at pH 8 (to maintain the initial conductance at 200 $\mu\Omega^{-1}$ or less) in a 20-ml vial, to which was added up to 1 ml formamide to provide the appropriate molarity. The reaction was started by adding 0.1 ml urease or less (with negligible increase in conductance), after which the conductance cell was immersed in the solution, and readings taken at minute intervals. In all experi-

ments the conductance increased by 3%/min or more, and was essentially linear in response for 10—20 min. The system was standardized with pure ammonium formate solutions at pH 5.5 and pH 7.7, which gave the same results: the conductance increase was 116 $\mu\Omega^{-1}/\mu$mol ammonium formate per cm^3, ±4% to 10 mM, which encompassed the entire range used for initial rate studies. At 100 mM ammonium formate the conductance dropped to 97 $\mu\Omega^{-1}/\mu$mol per cm^3, and working graphs were used to estimate the fraction hydrolyzed in long-term experiments.

Results

Initial studies were carried out at pH 7.7, where the basal conductance was very low (25—30 $\mu\Omega^{-1}/cm^3$), and increases could be discriminated easily. Upon finding a steady increase in conductance after adding urease, the reaction order in enzyme was evaluated. As shown in Fig. 1, initial velocities increased proportionally with enzyme concentration at both 10 and 100 mM formamide. The marked increased in velocity over this range of substrate concentrations also indicated a high K_m.

Aside from attempts to establish absolute purity of the reactant, the problem of contaminant substrates may be eliminated by demonstrating an exclusive stoichiometry, or by demonstrating conversion of a major fraction of the reactant. In the present case both approaches were used. Table I presents a summary of the fractional hydrolysis of formamide and some congeners, in the presence and absence of urease at several concentrations. 10 mM formamide was quite stable in the reaction mixture for several days in the absence of

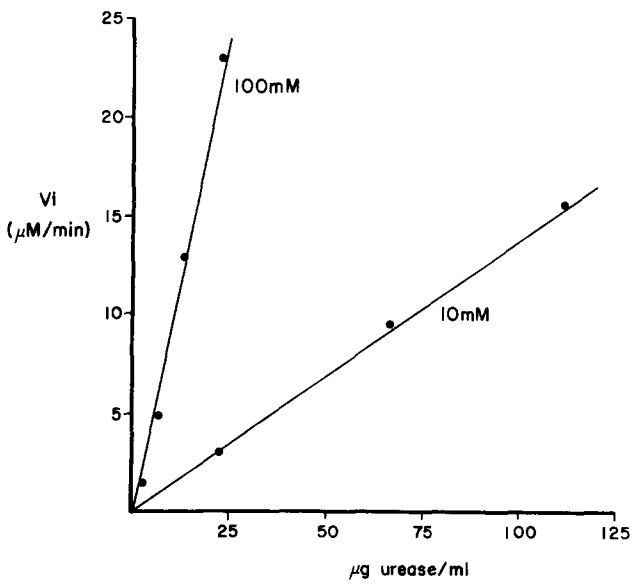

Fig. 1. Reaction order of formamide hydrolysis with urease concentration. Conductimetric assay was used in 5 mM Bis-Tris buffer at pH 7.7. The specific activity of the enzyme was 94 Sumner units/mg based on a standard urea assay.

TABLE I

PERCENT HYDROLYSIS OF FORMAMIDE AND HOMOLOGS IN THE PRESENCE AND ABSENCE OF UREASE

5 mM Bis-Tris, initial pH 7.7, was used in all experiments except *N*-methyl formamide, where initial pH was 5.9. Final pH differed from initial pH by 0.3 units or less. The specific activity of the enzyme was 94 Sumner units/mg based on a standard urea assay. AHA = acetohydroxamic acid.

μg Urease/ml	mM Substrate	% Hydrolysis at 20°C		
		1 Day	2 Days	3 Days
0	10 formamide	0.20	0.28	0.75
23	10 formamide	9.7	10.3	10.6
113	10 formamide	76.5	99.4	97.9
0	100 formamide	0.10	0.14	0.23
7	100 formamide	0.79	0.86	0.91
23	100 formamide	10.7	11.7	11.7
23	100 + 0.8 AHA	0.13	0.28	0.39
0	500 formamide	0.03	0.06	0.08
23	500 formamide	4.7	4.9	4.9
23	500 acetamide	0.0	0.0	0.02
23	500 *N*-methyl formamide	0.07	0.09	0.13
0	500 *N*-methyl formamide	0.04	0.08	0.10

enzyme, but was completely hydrolyzed in 2 days in the presence of 113 μg urease/ml. At 100 mM levels, formamide was again quite stable in the absence of enzyme, but a quite sizeable fraction (12%) was hydrolyzed in the presence of 23 μg urease/ml. The actual amount hydrolyzed in this case was greater than at the lower substrate level. In the presence of the specific urease inhibitor, acetohydroxamate [13,14], at 0.8 mM levels, the hydrolysis was completely blocked, indicating the specific enzymatic nature of the reaction. From initial rate data, 99% inhibition was achieved. At 500 mM levels, formamide was compared with its two nearest homologs, acetamide, and *N*-methyl formamide. Acetamide was very stable and totally unaffected by urease at a level which converted more than 20 mM formamide to ammonium formate in one day. *N*-methyl formamide was tested close to the optimum pH for formamide hydrolysis (as will be seen below), and showed a low rate of spontaneous hydrolysis, which barely increased in the presence of urease. If all of the increase were accepted as catalyzed, it would still represent a rate about 400-fold lower than that for formamide, considering the pH difference of the assays. However, 0.5% of the formamide was hydrolyzed in the first 35 min of the incubation, whereas no detectable hydrolysis of *N*-methyl formamide occurred in that time interval, so we conclude that the latter is not a substrate.

In sum, Table I shows that contaminants cannot be responsible for the hydrolytic reaction, which is at once specific for the enzyme, and specific for the substrate. In this event, the evaluation of substrate purity becomes trivial, and no extra efforts were expended in this direction. If we assume that the conductance measured in solutions of the pure compounds was due entirely to their hydrolytic products, we can estimate the levels of these contaminants, which gave the following values: 0.07% for formamide, 0.2% for acetamide,

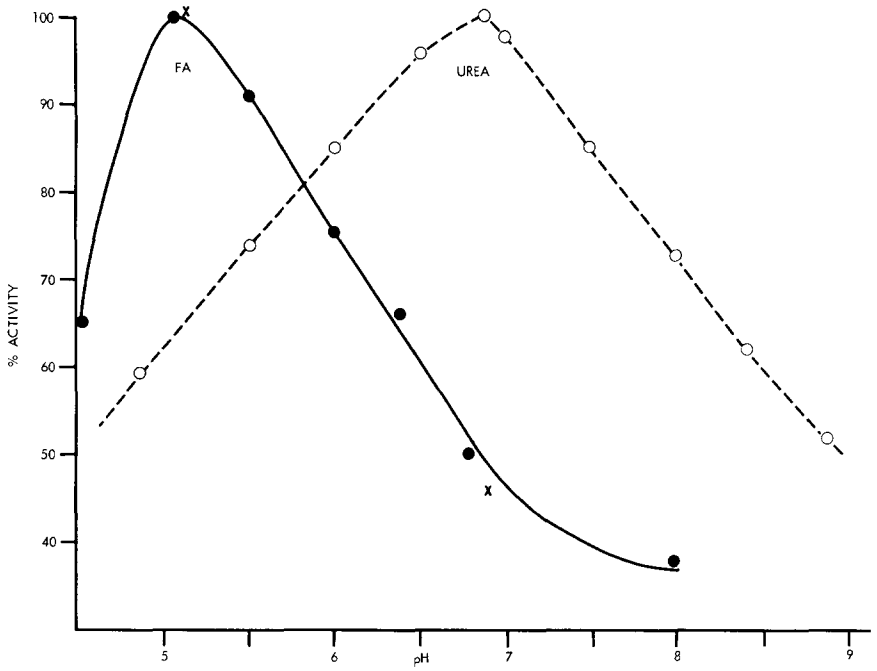

Fig. 2. The pH-activity curves of formamide (FA) and urea hydrolysis by urease (94 Sumner units/mg). Urea was present at 0.1 M in 50 mM Bis-Tris, pH 4.5—6.8 and in 50 mM Tris, pH 7—9, and was assayed by dilution into Nessler reagent. Formamide was present at 0.1 M in 1.25—2.5 mM Bis-Tris, and was assayed conductimetrically (see Materials and Methods). Relative V values for formamide hydrolysis at pH 5.2 and 6.8 denoted x.

and 0.65% for *N*-methyl formamide. After 10 days at 20° C in Bis-Tris buffers, 0.5 M formamide showed 0.06% hydrolysis at pH 6.8, and 1 M formamide showed 0.03% hydrolysis at pH 5.2, in the absence of urease.

Formamide hydrolysis should yield 1 mol of ammonia/mol of formamide decomposed, another distinguishing feature from urea hydrolysis. Evaluation of this stoichiometry by dual dilutions from a solution containing 5 mM formamide and 150 μg urease/ml (94 Sumner units/mg), gave the following values for μM formamide decomposed/μM ammonia formed at pH 7.5: at 5 min 104/101, at 10 min 137/117, at 20 min 170/187, at 40 min 245/297, satisfying the expected relationship.

The pH activity curves were determined for both formamide and urea with the same enzyme preparation, as shown in Fig. 2. Urea hydrolysis was maximal at neutral pH, which was also the case for hydroxyurea and dihydroxyurea [1, 2], whereas formamide hydrolysis was maximal at pH 5.2, and was similar in this respect to semicarbazide [3]. Since both substrates are unionized and the activity curves are quite broad, halfheight perpendiculars provide a satisfactory estimate of the ionization constants of the E · S complex [15]. For urea this procedure is entirely unobjectionable, since the substrate level is at 97% saturation, and gave $pK_a = 4.5$, $pK_b = 9.0$. Previous studies have consistently reported $pK_b = 8.5$—9; but pK_a has varied from 4.5 to 6.6 [4,16—18], very likely due to interacting buffers. In the case of formamide, the substrate level was well below

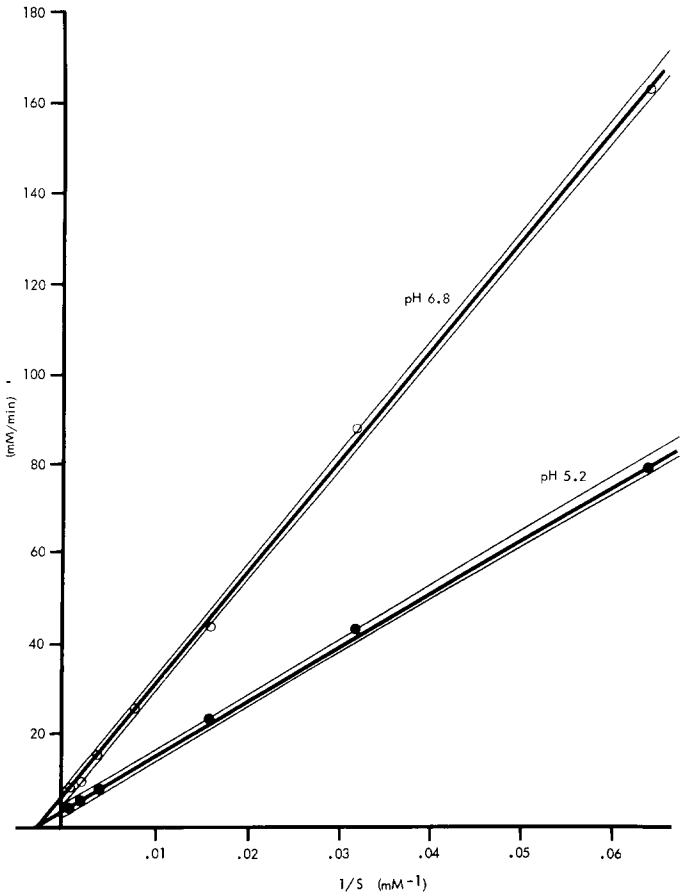

Fig. 3. Double reciprocal plot of formamide hydrolysis at pH 5.2 and 6.8 catalyzed by urease (94 Sumner units/mg). The best-fitting line and its 95% confidence limits are shown, as determined by linear regression analysis. Conditions, kinetic parameters, and correlation coefficients are listed in Table II; the x-axis intercept is $1/K_m$.

saturation, but the maximal velocities determined at pH 5.2 and 6.8 (see below) had the same relationship as that shown in Fig. 2. The values here were $pK_a = 4.5$ and $pK_b = 6.7$, suggesting that the participating acidic group, presumably a carboxyl, is involved in both reactions, whereas the participating basic group differs.

The kinetics of formamide hydrolysis was evaluated at both pH maxima, by double reciprocal plots, and by Hofstee plots [19], as shown in Figs. 3 and 4. An unweighted least-squares regression analysis of y on x was used in each case to determine the best-fitting line and its 95% confidence limits. The results are shown in Table II and indicate a good fit of linearity, but a large variation in the 95% confidence limits. The K_m value does not differ significantly from pH 5.2 to pH 6.8, despite a 2.5-fold fall in maximal velocity, and does not differ significantly between the two plots. The best overall value for K_m from the common confidence limits for both plots at both pH levels was 516 ± 50 mM.

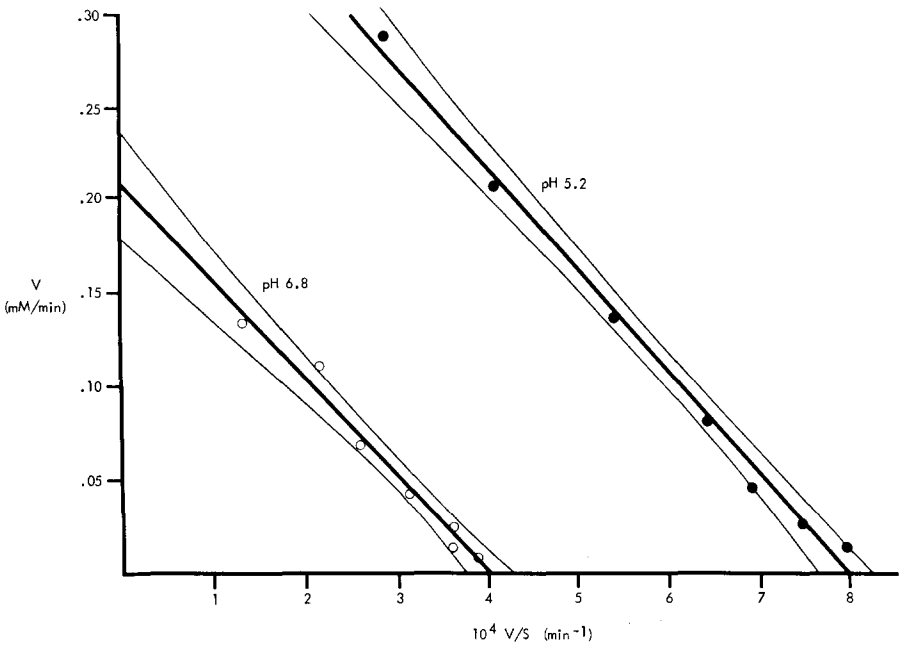

Fig. 4. Hofstee plot of formamide hydrolysis at pH 5.2 and 6.8 catalyzed by urease (94 Sumner units/mg). The best-fitting line and its 95% confidence limits are shown, as determined by linear regression analysis. Conditions, kinetic parameters, and correlation coefficients are listed in Table II; the slope is $-K_m$.

At pH 6.8 urea hydrolysis was found by similar plots to have a K_m of 3 ± 0.5 mM, and formamide was then evaluated as a competitive inhibitor, using an assay system sensitive only to urea hydrolysis. The results of these experiments, shown in Table III, gave a mean K_i = 404 ± 111 mM (95% confidence

TABLE II

KINETIC PARAMETERS FOR UREASE HYDROLYSIS OF FORMAMIDE AT pH 5.2 AND pH 6.8

The conductimetric assay contained 29 μg enzyme/ml 1.25 mM Bis-Tris at pH 5.2 or 2.5 mM Bis-Tris at pH 6.8 (see Materials and Methods). The data are shown in Figs. 2 and 3, and the best-fitting line and its 95% confidence limits (values in parenthesis) were determined by unweighted least-squares regression analysis of y on x. The specific activity of the enzyme was 94 Sumner units/mg based on a standard urea assay.

pH	Plot	V_m (mM/min)	K_m (mM)	r
5.2	$1/V$ vs. $1/S$	0.360	438	0.999
		(0.250 — 0.500)	(303 — 550)	
	V vs. V/S	0.437	516	0.996
		(0.396 — 0.477)	(483 — 621)	
	common limits:	0.436 ± 0.040	516 ± 33	
6.8	$1/V$ vs. $1/S$	0.198	493	0.999
		(0.132 — 0.227)	(312 — 625)	
	V vs. V/S	0.207	551	0.987
		(0.177 — 0.235)	(412 — 623)	
	common limits:	0.202 ± 0.025	517 ± 105	

TABLE III

DETERMINATION OF K_i FOR FORMAMIDE AS COMPETITIVE INHIBITOR OF UREA HYDROLYSIS

A coupled buffer-indicator assay was employed at pH 6.8 with 12 ng urease/ml, and was insensitive to formamide hydrolysis (see Materials and Methods). The specific activity of the enzyme was 115 Sumner units/mg based on a standard urea assay. Values were calculated by the formula for competitive inhibition: $K_i = \alpha K_m I/(S \cdot \alpha S \cdot \alpha K_m)$, using K_m = 3 (±0.5) mM for urea (as determined independently) and V_m taken as the rate observed in the presence of 100 mM urea.

Urea (mM)	Formamide (mM)	Inhibition (%)	$\alpha = v/V_m$	K_i (mM) formamide
5.0	25	11.5	0.611	409
5.0	125	21.0	0.545	319
1.1	125	24.2	0.218	297
1.1	375	60.0	0.172	490

K_i = 404 ± 111 (mean ±95% confidence interval)

interval). Within the error of the procedure then, $K_m = K_i$ for formamide, suggesting that, for both substrates, the Michaelis constants represent true dissociation constants for the respective enzyme-substrate complex. The same conclusion was derived from a similar study of hydroxyurea as a competitive inhibitor of urea hydrolysis [1]. The relative hydrolytic activity towards urea at pH 6.8 versus formamide at pH 5.2 by a series of urease preparations is shown in Table IV. Urease preparations varying from 45 to 100% purity had essentially the same relative activity toward the two substrates, thus eliminating the possibility of a contaminating formamidase in the urease preparation.

Finally, Table V shows the kinetic constants at 20°C for the five unchallenged substrates for urease. Of the five, formamide has the least affinity for

TABLE IV

RELATIVE ACTIVITY OF VARIOUS UREASE PREPARATIONS TOWARD UREA AND FORMAMIDE

Urea hydrolysis was measured at pH 6.8 in a coupled buffer-indicator system containing 0.1 M urea and 5—15 ng urease/ml. Formamide hydrolysis was measured at pH 5.2 in a conductimetric assay containing 0.5 M formamide and 15—45 μg urease/ml (see Materials and Methods). The percent purity was calculated from the rate of urea hydrolysis; the fully purified preparation had a specific activity of 164 Sumner units/mg.

Urease preparation	% purity	Initial Hydrolytic Rate (μmol/min per mg urease)		Urea/formamide
		Urea	Formamide	
U111 SS	30.5	357	5.51	64.8
U113 SS	45.7	536	5.12	104.7
U111 D1S	57.3	671	6.73	99.7
U111 D2S	69.0	807	9.19	87.8
U111 D3S	73.2	857	8.95	95.8
U113 D1S	80.5	942	7.12	132.3
U113 D2S	100.0	1172	11.40	102.7

TABLE V

KINETIC CONSTANTS FOR FIVE UREASE SUBSTRATES AT 20°C

Progressive inhibition denotes the gradual development of noncompetitive inhibition of the enzyme, which is slowly reversible and related to the hydroxamate grouping in these substrates.

Substrate	Reference	pH (max.)	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m $(M \cdot s)^{-1}$	S (mM) tested	Notes
Urea	present	6.8	11 000	0.003	$3667 \cdot 10^3$	1 — 100	
	(25) **	7.0	11 414	0.0033	$3459 \cdot 10^3$	0.5— 50	
Hydroxyurea	(1)	7.0	12	0.0016	7237	0.7— 2.7	$K_i = 0.0025$ * Progressive inhibition
Dihydroxyurea	(2)	7.0	78	0.0125	6197	0.3— 5	Progressive inhibition
Semicarbazide	(3) **	5.0	96	0.060	1602	5 — 40	
Formamide	present	5.2	246	0.516	475	30 —1000	$K_i = 0.404$ *

* Values determined from initial rates as competitive inhibitors of urea hydrolysis at neutral pH.

** Values normalized from 38 to 20°C, assuming the temperature factor determined for urea hydrolysis [25] applied as well to semicarbazide hydrolysis.

the enzyme, but the highest maximal velocity (after urea). Using K_{cat}/K_m as the measure of hydrolytic efficiency, the hydroxyureas are the best of the second-substrates, although 500-fold less efficient than urea. Formamide is yet another order of magnitude less efficient, yet its hydrolysis by urease compares favorably with that catalyzed by a representative bacterial aliphatic amidase, for which k_{cat}/K_m was reported to be 625 $M^{-1} \cdot s^{-1}$ [20]. This gives a quantitative idea as to the remarkable specificity and potency of urease towards its natural substrate.

Discussion

So many false substrates for urease have been reported, that a brief discussion of this problem seems appropriate. The main point to be made is that the kinetic pattern obtained from initial rates gives no information as to the fraction of the reaction completed, and it is this parameter which is crucial in deciding whether a contaminant substrate may be involved. Initial rate studies often involve the decomposition of much less than 1% of the substrate, and this amount of contaminant may be very hard to identify by chromatographic or other means. Where a major fraction of the substrate can be shown to be degraded in long-term studies, as was the case with formamide, the possibility of a contaminant substrate is excluded. Where this is not possible, as was the case with hydroxyurea [1] and dihydroxyurea [2] because of inhibition, the demonstration of an exclusive stoichiometry is necessary to accomplish the same purpose. Specificity for the substrate was shown in the present case by the lack of hydrolysis of the two nearest homologs, acetamide and *N*-methyl formamide, while specificity for the enzyme was shown by inhibition of the hydrolysis by acetohydroxamate, a specific urease inhibitor. Finally, a constant activity ratio of formamide to urea hydrolysis during enzyme purification served to eliminate the possibility of a contaminating formamidase in the urease preparation. Thus formamide itself must now be considered as another

possible contaminant substrate, in compounds which undergo a small amount of hydrolysis in the presence of urease, e.g. formylurea [4].

Although urease hydrolysis of formamide is 7000-fold less efficient than that of urea, it nevertheless compares favorably with hydrolysis of this substrate by a representative aliphatic amidase. Whether this reaction has any physiologic significance is at present unclear, but formamide would seem to be the only second-substrate for urease which is likely to arise metabolically, and the urease content of a number of bacteria and plants is several orders of magnitude greater than that needed for urea hydrolysis. In any event this compound may prove a useful tool in studies of urease structure, since it does not inhibit the enzyme, as do the hydroxyureas, and is hydrolyzed very slowly in comparison with urea. Such studies have assumed increasing importance since the recent discovery that urease is a nickel metalloenzyme [21,22].

Finally we point out that carbamic acid, an intermediate in urea hydrolysis which has generally been assumed to decompose spontaneously [24,25], might also be called hydroxyformamide. By analogy with the hydroxyureas then, this suggests that arease might catalyze carbamate hydrolysis as well, especially at alkaline pH, where its spontaneous hydroytic rate is relatively slow.

Acknowledgements

This investigation was supported in part by United States Public Health Service Research Grant AM-10960 from the National Institute of Arthritis, Metabolic and Digestive Diseases, Department of Health, Education and Welfare, under the auspices of Universities Associated for Research and Education in Pathology, Inc.

References

1 Fishbein, W.N., Winter, T.S. and Davidson, J.D. (1965) J. Biol. Chem. 240, 2402–2406
2 Fishbein, W.N. (1969) J. Biol. Chem. 244, 1188–1193
3 Gazzola, C., Blakeley, R.L. and Zerner, B. (1973) Can. J. Biochem. 51, 1325–1330
4 Sundaram, P.V. and Laidler, K.J. (1970) Can. J. Biochem. 48, 1132–1140
5 Fishbein, W.N. (1975) Fed. Proc. 34, 506
6 Fishbein, W.N. (1969) Ann. N.Y. Acad. Sci. 147, 857–881
7 Fishbein, W.N., Daly, J. and Streeter, C.L. (1969) Anal. Biochem. 28, 13–24
8 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
9 Bergman, F. (1952) Anal. Chem. 24, 1367–1369
10 Ruiz-Herrera, J. and Gonzalez, J. (1969) Anal. Biochem. 31, 366–374
11 Sumner, J.B., Gralen, N. and Eriksson-Quensel, I.-B. (1938) J. Biol. Chem. 125, 37–44
12 Reithel, F.J. and Robbins, J.E. (1967) Arch. Biochem. Biophys. 120, 158–164
13 Kobashi, K., Hase, J. and Uehara, K. (1962) Biochim. Biophys. Acta 65, 380–383
14 Fishbein, W.N. and Carbone, P.P. (1965) J. Biol. Chem. 240, 2407–2414
15 Fromm, H.J. (1975) Initial Rate Enzyme Kinetics, p. 208, Springer-Verlag, New York
16 Myrbäck, K. (1947) Acta Chim. Scand. 1, 142–148
17 Wall, M.C. and Laidler, K.L. (1953) Arch. Biochem. Biophys. 43, 299–311
18 Lynn, K.R. (1967) Biochim. Biophys. Acta 146, 205–218
19 Hofstee, B.H.J. (1956) Enzymologia 17, 273–278
20 Findlater, J.D. and Orsi, B.A. (1973) FEBS Lett. 35, 109–111
21 Dixon, N.E., Gazzola, C., Blakeley, R.L. and Zerner, B. (1975) J. Am. Chem. Soc. 97, 4131–4133
22 Fishbein, W.N., Smith, M.J., Nagarajan, K. and Scurzi, W. (1976) Fed. Proc. 35, 1680
23 Wang, J.H. and Tarr, D.A. (1955) J. Am. Chem. Soc. 77, 6205–6206
24 Blakeley, R.L., Hinds, J.A., Kunze, H.E., Webb, E.C. and Zerner, B., (1969) Biochemistry 8, 1991–2000
25 Blakeley, R.L., Webb, E.C. and Zerner, B. (1969) Biochemistry 8, 1984–1990