THE DEPENDENCE OF THE SPECIFIC ACTIVITY OF UREASE UPON THE APPARENT ABSOLUTE ENZYME CONCENTRATION

By JACK PETERSON,* KENT M. HARMON,† and CARL NIEMANN

(From the Gates and Crellin Laboratories of Chemistry,‡ California Institute of Technology, Pasadena)

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If the activity of an enzyme preparation is determined under conditions in which a further increase in substrate concentration is without demonstrable effect, all other factors being held constant, it is ordinarily assumed that the specific activity of the enzyme, expressed in terms of arbitrary units per unit weight of the enzyme, is independent of the absolute enzyme concentration (1, 2). However, with urease solutions stabilized with hydrogen sulfide or cysteine (1) we have observed that the specific activity of a given urease preparation, when determined under the above conditions, increases with decreasing apparent enzyme concentration over a wide range of concentrations and that this increase in specific activity proceeds with a measurable velocity at temperatures above 15°. This phenomenon was observed with crude urease preparations, such as jack bean meal, and with two, three, and seven times recrystallized urease. Since little or no difference was observed in the behavior of three and seven times recrystallized urease, the data presented in this paper are limited to those obtained with thrice recrystallized preparations. Urease activity was determined by a modification of the procedure described by Van Slyke and Cullen (3). The precision of the modified procedure was ±2 to 3 per cent.

EXPERIMENTAL

Determination of Urease Activity

Reagents—The buffer solution, 0.1 M in phosphate adjusted to pH 7.0, used in all experiments was prepared from dipotassium hydrogen phosphate and potassium dihydrogen phosphate. The 1.0 M solution of urea was prepared daily in order to minimize the effects of bacterial contamination. The crystalline urease was prepared from jack bean meal by the method of Dounce (4), all operations subsequent to the initial extraction being carried out at 5°. Thrice recrystallized urease from 200 gm. of meal was

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† National Research Council Predoctoral Fellow.

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dissolved in 3 to 5 ml. of water, 1 per cent saturated with hydrogen sulfide, and this stock solution kept at 5° prior to its use. Hydrogen sulfide solutions were prepared daily by appropriate dilution of a solution saturated at 0°. Redistilled water was used in all cases.

Procedure—Clean 18 × 150 mm. reaction tubes were charged with 2.0 ml. of buffer solution and 1.0 ml. of 1.0 M urea solution (or standard ammonium sulfate solution containing 100 γ of ammonia nitrogen per ml.) and placed in a constant temperature bath at either 25° or 15°. When thermal equilibrium had been attained, the enzyme solution was added (usually 1.00 or 0.79 ml.) and the time noted. After the desired time interval had elapsed (usually 2 minutes), 0.5 ml. of 1.0 M sulfuric acid was added to each of the tubes; the latter were shaken and placed in an ice bath. Exactly 10 ml. of 0.01 M sulfuric acid were placed in each 18 × 150 mm. absorption tube and the tubes fitted with rubber stoppers, each bearing a 4 mm. glass inlet tube and a capillary type critical orifice designed to permit an air flow of 300 to 400 ml. per minute. To each of the reaction tubes in the ice bath were added 3 to 4 gm. of anhydrous potassium carbonate, and the tubes were fitted with rubber stoppers, each bearing a 4 mm. inlet tube as well as a 4 mm. U-tube serving to connect the reaction tube with the absorption tube. Each reaction tube was connected to an absorption tube with a short length of rubber tubing and placed in a 55° bath, the inlet and outlet tubes connected to manifolds, and the aeration started. After 20 minutes each reaction tube was disconnected and shaken so as to wash down the sides of the tube with carbonate solution. After this process, the tubes were reconnected and allowed to aerate for a second period of 20 minutes, when the washing process was repeated. After a final 20 minutes aeration the absorption tubes were disconnected and placed in an ice bath. To the chilled contents of each of the absorption tubes was added 1.0 ml. of Nessler's reagent (5), the tubes were removed from the ice bath, and after 10 minutes the intensity of the color measured in a Klett colorimeter. With the apparatus at our disposal it was possible to run eleven determinations simultaneously and in every such series two or more determinations were blanks in which either the urea solution or the urease solution was replaced by an equal volume of water or standard ammonium sulfate solution. For precise results such blank determinations were found to be necessary. The conversion of colorimeter readings to micromoles of ammonia was based upon a series of determinations of the ammonia recoverable in the range of 10 to 200 γ of ammonia nitrogen from a standard solution of ammonium sulfate. The specific activity was calculated from the relation specific activity $= 10x/ta$, where x = the number of micromoles of ammonia liberated, t = the time in minutes during which hydrolysis occurred, and a = the micrograms of protein nitrogen present in the solution.

Dilution Experiments

Procedure—Relatively concentrated solutions of thrice recrystallized urease in water 1 per cent saturated with hydrogen sulfide, *i.e.*, containing 300 to 1500 γ of protein nitrogen per ml., were diluted with solutions of hydrogen sulfide or cysteine previously adjusted to pH 7.2 to 7.5 by the addition of anhydrous potassium carbonate.[1] The specific activity of urease in these solutions was then determined as a function of time, zero time being taken as the time of mixing. In those experiments in which a stepwise dilution technique was employed the solutions were allowed to stand at 25° for 2 hours after the initial dilution. A 1:10:6000 stepwise dilution is defined as an initial 1:10 dilution which has subsequently been diluted to effect a final over-all dilution of 1:6000. The data obtained in these experiments are given in Tables I to III.

Determination of Michaelis Constants

Procedure—The kinetics of the hydrolysis of urea by thrice recrystallized urease were studied at 25° and pH 7.0. The activity of the urease solutions was determined as described previously and the rate of determinations were made at nine different urea concentrations; *i.e.*, 0.004, 0.005, 0.006, 0.007, 0.008, 0.010, 0.015, 0.025, and 0.250 M urea. The data so obtained were found to obey the Michaelis-Menten equation, $v = VS/(Km + S)$, where v = the rate of hydrolysis at a urea concentration S and V = the maximum or limiting rate of hydrolysis over the concentration range of substrate studied. In practice it was found convenient to follow the suggestion of Lineweaver and Burke (6) and to transform the original Michaelis equation into its linear form $1/v = Km/VS + 1/V$ for evaluation of the data. It should be noted that the concentration of urea corresponding to a given rate was always taken as the average urea concentration obtaining during the determination of that rate.

DISCUSSION

The dependence of the specific activity of urease upon the apparent enzyme concentration in systems containing hydrogen sulfide was first observed when relatively concentrated solutions of urease in water containing hydrogen sulfide were diluted approximately a thousand fold with the same solvent.[2] It was further observed[2] that in the absence of hydrogen sulfide rapid inactivation of the urease occurred and that the effect observed with solutions containing hydrogen sulfide could be obviated by the presence of silver ion. In earlier experiments it was not appreciated that urease is

[1] The pH of water 1 to 10 per cent saturated with hydrogen sulfide was found to vary between 5.5 and 4.5.

[2] Unpublished experiments of the authors.

slowly inactivated at pH 4.5 to 5.5 in solutions containing hydrogen sulfide. However, it was found that this inactivation could be minimized or suppressed by maintaining the systems at approximately pH 7 at all times.

The data presented in Columns 2 and 3 of Table I are typical of those observed when relatively concentrated solutions of urease are diluted at 25° and pH 7 with water containing hydrogen sulfide and the specific activity of the urease in these solutions determined as a function of time, with zero time as the time of mixing. The initial values are not particularly accurate, since the actual determination of urease activity requires a minimum of 2 minutes if precise results are to be obtained. Nevertheless the data

TABLE I

*Dilution of Urease Solutions with Water 1 Per Cent Saturated with Hydrogen Sulfide at 25° and pH 7.2 to 7.5**

Time after dilution	Specific activity at 25°, pH 7.0†					
	1:6000 dilution		1:4:6000 dilution		1:10:6000 dilution	
	Experiment I	Experiment II	Experiment I	Experiment II	Experiment I	Experiment II
(1)	(2)	(3)	(4)	(5)	(6)	(7)
min.						
2	136	131	138‡	146‡	156	148
10	141	134‡	145	151	161	153
30	149	139	156‡	154	168	154
70	169	163	186	170‡	181	163
120	186	175	207	186	196	184
180	198	191‡	218		203‡	
240	210	207	228	204	210‡	204
300	220		228		217	

* The original urease solution contained 1365 γ of protein nitrogen per ml.

† Average of duplicate determinations, agreeing within ±2 per cent.

‡ Obtained from a smooth curve through other points of the series.

in Columns 2 and 3 of Table I clearly illustrate that the specific activity of the urease in these solutions increases with time.

The increase in the specific activity of urease upon dilution noted above was observed when urease solutions containing 1365 γ of protein nitrogen per ml. were diluted to give solutions containing approximately 0.23 γ of protein nitrogen per ml. In order to determine whether this effect could be observed with dilutions of lower order dilutions of 1:4 and 1:10 were employed; these solutions were allowed to stand sufficiently long to attain equilibrium, and then diluted uniformly to the point at which it was possible to determine the specific activity of the urease contained therein rea-

sonably accurately. As before, the specific activity of the urease in the final solutions was determined as a function of time (Table I, Columns 4 to 7). It will be noted that in every case the specific activity increased with time and reached, within experimental error, the same maximum limiting value. Further, it is clear from these data that the initial 1:4 and 1:10 dilutions were effective in diminishing the percentage change in specific activity observable upon the final 1:6000 dilution, and one may conclude not only that dilutions of low order were attended by changes in specific activity, but also that at equilibrium and with relatively high concentrations of urease the specific activity of urease is a function of the enzyme concentration if the latter is taken as being equivalent to the amount of protein nitrogen present in solution.

TABLE II

*Dilution of Urease Solutions with Water 1 Per Cent Saturated with Hydrogen Sulfide at 15° and pH 7.3**

Time after dilution	Specific activity at 15°, pH 7.0, after 1:4000 dilution	
	Experiment I	Experiment II
min.		
2	88	83
15	88	
45	86	88
60	91	93

* The original urease solution contained 1365 γ of protein nitrogen per ml.

An attempt was made to estimate the magnitude of the temperature coefficient of the above reaction by measuring the change in the specific activity of urease brought about by a 1:4000 dilution of a relatively concentrated urease solution at 15° and 25° and at pH 7.3. Unfortunately the data obtained in these experiments (Table II) did not justify the calculation of a value for the temperature coefficient. However, they do provide evidence that the reaction is dependent on temperature.

The above observations on the behavior of urease in solutions containing hydrogen sulfide suggested the desirability of investigating the effects observable with another so called stabilizing agent. In Table III are presented data which were obtained in preliminary experiments in which cysteine was used instead of hydrogen sulfide. While these data are not as extensive as those available for hydrogen sulfide, it is clear that in both systems similar, but not necessarily identical, reactions are operative.

The Michaelis constant (1, 2, 7) of an enzyme is often taken as a char-

acteristic property of the enzyme, though it is recognized (7) that the constant may be dependent upon temperature and pH.[3] Using crude preparations, Van Slyke and Cullen (3) and Ambros and Münch (8) obtained data which give values of 0.011 M urea at 20° and pH 7 and 0.0082 M urea at 50° and pH 7.6 for the Michaelis constant of urease. With urease solutions containing 0.065 to 0.095 γ of protein nitrogen per ml., which had been prepared by dilution with water 1 per cent saturated with hydrogen sulfide and allowed to stand at 25° for 3 to 4 hours, values for the Michaelis constant of five different urease preparations, obtained from two different lots of jack bean meal, were found to be 0.0098, 0.0116, 0.0098, 0.0112, and 0.0103 M urea at 25° and pH 7.0 respectively. The variation in the Mi-

TABLE III

*Dilution of Urease Solutions with Solutions of Cysteine at 25° and pH 7.0**

Time after dilution	Specific activity at 25°, pH 7.0†			
	0.01 M cysteine	0.002 M cysteine		
	1:5000 dilution	1:5000 dilution	1:4:5000 dilution	1:10:5000 dilution
min.				
2	119	137	123	98
10	123	141	129	103
30	134	150	136	109
70	154	169	152	121
120	179	183	166	130
180	188	190	171	132‡
240	198	193	175	132
360	198	176	189	150

* The original urease solution contained 1365 γ of protein nitrogen per ml.

† Average of duplicate determinations, agreeing within ±2 per cent.

‡ Obtained from a smooth curve through other points of the series.

chaelis constant noted above, *i.e.* 0.0107 ± 0.0009, was somewhat greater than that observed, *i.e.* 0.0106 ± 0.0003, when the constant of a given urease preparation was redetermined at intervals over a period of several months.

The fact that a substantial increase in the specific activity of urease is observed when a relatively concentrated solution of urease in water containing hydrogen sulfide or cysteine is diluted with the same solvent appears to preclude the possibility that the effect observed is simply an activation of urease by hydrogen sulfide or cysteine (9). An alternative explanation may be that the urease molecule dissociates into smaller units upon dilution and that this process is accompanied by an increase in the number of re-

[3] Ionic strength and the nature of the buffer may also be important variables.

active sites, the hydrogen sulfide or cysteine merely serving to prevent inactivation. A second explanation may be that the crystalline urease preparations are contaminated with a naturally occurring inhibitor, which is not removed by the repeated recrystallization of urease from relatively concentrated solutions, and that the urease-inhibitor complex dissociates in dilute solutions, the degree of dissociation being a function, within limits, of the degree of dilution. Although there are insufficient data to determine whether all of the above hypotheses are operative or whether any one should be completely excluded, it is clear that the observed effect must be taken into account if studies on urease action are to be properly evaluated.

SUMMARY

It has been observed that the specific activity of urease in solutions containing hydrogen sulfide, or cysteine, and expressed in terms of arbitrary units of urease activity per unit weight of enzyme taken as protein nitrogen is dependent, within limits, upon the apparent enzyme concentration. The Michaelis constants of several urease preparations have been determined at 25° and pH 7.0 under conditions minimizing the above phenomenon.

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