STUDIES ON:

THE SPECIFIC ACTIVITY AND INHIBITION OF UREASE;

TRYPSIN AND CHYMOTRYPSIN - CATALYZED HYDROLYSIS OF SIMPLE PEPTIDES;

ISOMERIC TRANSITIONS OF RADIOACTIVE TELLURIUM.

Thesis by

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I wish to express to Professors Niemann and Dodson my deepest appreciation for their guidance and instruction, and to my wife my gratitude, not only for typing this manuscript, but also for her constant love and encouragement during my career as a graduate student.

Abstract

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 noted phenomenon.

It has been observed that urease is competitively inhibited by phosphate, but not by maleate or glycine buffers. The effects of several substituted ureas upon the kinetics of the urease-catalyzed hydrolysis of urea have been compared.

It has been observed that although the trypsin-catalyzed hydrolysis of benzoyl-l-arginineamide is apparently first order with respect to substrate, the first order rate constant is dependent upon the initial substrate concentration. Similar results have been obtained in a study of the hydrolysis, by chymotrypsin, of N-acetyl-l-tyrosylglycineamide, and possible explanations for the two cases have been advanced. Inhibition of chymotrypsin by N-acetyl-d-tyrosylglycineamide has been observed.

Unsuccessful attempts have been made to obtain proof of a proposed mechanism for the chemical changes which accompany the isomeric transition of $Te^{127-129}$. Further information regarding the nature of these changes has been obtained.

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Part I

The Dependence of the Specific Activity of Urease Upon the Apparent Absolute Enzyme Concentration

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If the activity of an enzyme preparation is determined under conditions where 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 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 $\pm$ 2-3 per cent.

Experimental

A. Determination of Urease Activity

Reagents.- The buffer solution, 0.1 molar in phosphate adjusted to pH 7.0, used in all experiments was prepared from dipotassium hydrogen phosphate and potassium dihydrogen phosphate. The 1.0 molar 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) with all operations subsequent to the initial extraction being carried out at 5°. Thrice recrystallized urease from 200 gm. of meal was dissolved in 3-5 ml. of water 1% 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 x 150 mm. reaction tubes were charged with 2.0 ml. of buffer solution and 1.0 ml. of 1.0 molar urea solution (or standard ammonium sulfate solution containing 100 μg. of ammonia-nitrogen per ml.) and placed in either a 25° or 15° constant temperature bath. When thermal equilibrium had been attained the enzyme solution was added (usally 1.00 or 0.79 ml.) and the time noted. After the desired time interval had elapsed (usally two minutes) 0.5 ml. of 1.0 molar sulfuric acid was added to each of the

tubes, the latter shaken, and placed in an ice bath. Exactly 10 ml. of 0.01 molar sulfuric acid was placed in each 18 x 150 mm. absorption tube and the tubes fitted with rubber stoppers each bearing a 4 mm. glass inlet tube and a capillary type orifice designed to permit an air flow of 300-400 ml./min. To each reaction tube in the ice bath was added 3-4 g. of anhydrous potassium carbonate and the tubes 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. With each reaction tube connected to an absorption tube with a short length of rubber tubing the reaction tubes were placed in a 55° bath, the inlet and outlet tubes connected to manifolds and the aeration started. After twenty 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 twenty minutes, when the washing process was repeated. After a final twenty 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 removed from the ice bath and after ten 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 blank determinations 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 10-200 μg. of ammonia-nitrogen from a standard of ammonium sulfate. The specific activity was calculated from the relation: specific activity = $10\chi/ta$, where χ = number of micromoles of ammonia liberated, t = time in minutes during which hydrolysis occurred, and a = micrograms of protein-nitrogen present in the solution.

B. Dilution Experiments

Procedure.- Relatively concentrated solutions of thrice recrystallized urease in water 1 per cent saturated with hydrogen sulfide, i.e., ones containing 300-1500 μg. of protein-nitrogen/ml., were diluted with the solutions of hydrogen sulfide or cysteine previously adjusted to pH 7.2-7.5 by the addition of anhydrous potassium carbonate.*

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

The specific activity of urease in these solutions was then determined as a function of time taking zero time as the time of mixing. In those experiments where a stepwise dilution technique was employed the solutions were allowed to stand at 25° for two 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 - III inclusive.

C. 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 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 molar urea. The data so obtained were found to obey the Michaelis-Menton equation, $v = VS/(K_m + S)$, 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 $1/v = \frac{K_m}{V} \cdot \frac{1}{s} + \frac{1}{V}$ 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 the 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.* It was further observed* 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 slowly inactivated at pH 4.5-5.5 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

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* Unpublished experiments of the authors.

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 taking 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 two minutes if precise results are to be obtained. Nevertheless the data 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 g. protein-nitrogen/ml. were diluted to give ones containing approximately 0.23 γ g. protein-nitrogen/ml. In order to determine whether this effect could be observed with lower order dilutions the device was adopted of carrying out dilutions of 1-4 to 1-10, allowing these solutions to stand sufficiently long to attain equilibrium, and then diluting these solutions uniformly to the point where it was possible to determine the specific activity of the urease contained therein reasonably accurately. As before the specific activity of the urease in the final solutions was determined as a function of time (Table I, columns 4-7 inclusive). It will be noted that in every case the

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 the low order dilutions 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 be taken as being equivalent to the amount of protein nitrogen present in solution.

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 temperature dependent.

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 using

cysteine 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 characteristic property of the enzyme though it is recognized (7) that the constant may be dependent upon temperature and pH.* Using crude preparations Van Slyke and Cullen (3) and Ambros and Münch (8) obtained data which give values of 0.011 molar urea at 20° and pH 7 and 0.0082 molar urea at 50° and pH 7.6 for the Michaelis constant of urease. With urease solutions containing 0.065-0.095 μg. of protein-nitrogen/ml., which had been prepared by dilution with water 1% saturated with hydrogen sulfide and allowed to stand at 25° for three to four 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 molar urea at 25° and pH 7.0 respectively. The variation in the Michaelis 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 extending over a period of

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* Ionic strength may also be an important variable.

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 reactive sites, the hydrogen sulfide or cysteine merely serving to prevent inactivation. A second alternative 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.

Table I

Dilution of Urease Solutions With Water 1% Saturated With Hydrogen Sulfide at 25° and pH 7.2-7.5***

Time After Dilution in Minutes	Specific Activity at 25° and pH 7.0**					
	1-6000 Dilution		1-4-6000 Dilution		1-10-6000 Dilution	
	I	II	I	II	I	II
(1)	(2)	(3)	(4)	(5)	(6)	(7)
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	___

Table II

Dilution of Urease Solutions With Water 1% Saturated With Hydrogen Sulfide at 15° and pH 7.3***

Time After Dilution in Minutes	Specific Activity at 15° and pH 7.0 After a 1-4000 Dilution**	
	I	II
(1)	(2)	(3)
2	88	83
15	88	—
45	86	88
60	91	93

Table III

Dilution of Urease Solution with Solution of Cysteine At 25° and pH 7.0***

Time After Dilution in Minutes	Specific Activity at 25° and pH 7.0** 0.01M Cysteine	0.002M Cysteine		
	1-5000 Dilution	1-5000 Dilution	1-4-5000 Dilution	1-10-5000 Dilution
(1)	(2)	(3)	(4)	(5)
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

Notes to Tables

* Obtained from a smooth curve through other points of series.

** Average of duplicate determinations agreeing within ± 2 per cent.

*** Original urease solution contained 1365 μg. protein-nitrogen/ml.

Part II

Nature of the Inhibition of Urease

by

Phosphate and Substituted Ureas

One of the best approaches to an understanding of the mechanism of enzyme-catalyzed reactions is through an investigation of the effect of inhibiting substances upon the kinetics of such reactions. In view of the simplicity of its substrate and probable inhibitors, the ease of determination of its activity, and the high degree of its specificity, crystalline urease is especially suitable for a study of this type.

An early investigation of the inhibition of urease by substituted ureas, carried out by Ambros and Münch (8), was inconclusive because of the crudity of their analytical methods. Recent work by Dr. Jack Peterson, in these laboratories, resulted in a precise method for the measurement of urease activity (described in Section I) and gave a good indication of the direction in which further research would be fruitful. The research here reported is an extension and confirmation of Peterson's results.

Theory

A theory which has received wide application in explanation of the observed kinetics of enzyme-catalyzed reactions is that of Michaelis and Menten (10). They postulated equilibrium intermediate compound formation between enzyme and substrate. Simple modifications of their theory lead to equations which represent the action of various types of inhibitors. The following considerations are based on the

treatment given by Lineweaver and Burk (6) and by Ebersole, Guttentag, and Wilson (11).

Consider a system containing enzyme, substrate, and an inhibitor. If E represents an enzyme molecule, S a substrate molecule, I an inhibitor molecule, and S' the reaction products, one may assume reactions 1-5 to occur.

(1) $E + S \rightleftharpoons ES$

(2) $ES \longrightarrow E + S'$

(3) $E + I \rightleftharpoons EI$

(4) $ES + I \rightleftharpoons ESI$

(5) $EI + S \rightleftharpoons ESI$

Let
e = total enzyme concentration
s = substrate concentration
i = inhibitor concentration
p = concentration of ES, the enzyme-substrate complex
q = concentration of EI, the enzyme-inhibitor complex
g = concentration of ESI, the enzyme-substrate-inhibitor complex
e-p-q-g = concentration of free enzyme
k = rate constant for reaction 2

Then

(6) $K_m = \frac{s(e-p-q-g)}{p}$ = dissociation constant of ES

(7) $K_c = \frac{i(e-p-q-g)}{q}$ = dissociation constant of EI

(8) $K_g = \frac{ip}{g}$ = equilibrium constant for reaction 4

By the use of equations 7 and 8, q and g may be

eliminated from equation 6. The latter may then be solved for p, to give

$$p = \frac{esK_cK_g}{K_mK_cK_g + iK_mK_g + sK_cK_g + isK_c} \quad (9)$$

If the reaction velocity v is proportional to the concentration of the enzyme-substrate complex, $v = -\frac{ds}{dt} = kp$.

$$v = \frac{kesK_cK_g}{K_mK_cK_g + iK_mK_g + sK_cK_g + isK_c} \quad (10)$$

Let V represent the velocity, in the absence of inhibitor, when s is large enough that all enzyme is combined with substrate (p = e). Then V = ke. Making this substitution and taking the reciprocal of both sides, one gets equation 11:

$$\frac{1}{v} = \left(1 + \frac{i}{K_c}\right)\left(\frac{K_m}{V}\right)\left(\frac{1}{s}\right) + \left(1 + \frac{i}{K_g}\right)\left(\frac{1}{V}\right) \quad (11)$$

According to equation 11, a straight line should result when $\frac{1}{v}$ is plotted (usually as ordinate) against $\frac{1}{s}$. As shown below, an inhibitor may affect the slope of the line, the ordinate intercept, or both. The type of inhibition which occurs is defined on the basis of the effect on slope and intercept.

A- Reaction in the absence of inhibitor

If i = 0, only reactions 1 and 2 occur, and equation 11 reduces to the Michaelis-Menten equation,

$$\frac{1}{v} = \frac{K_m}{V} \cdot \frac{1}{s} + \frac{1}{V} \quad (12)$$

As the ordinate intercept is $\frac{1}{V}$ and the slope is $\frac{K_m}{V}$, V and K_m may be readily evaluated.

B- "Competitive" inhibition

Competitive inhibition is assumed to result from the equilibrium compound formation of the inhibitor with the enzyme at the same point as that with which the substrate unites. This situation is represented by equations 1-3. Equation 11 may be adapted to represent only equations 1-3 by setting K_g equal to ∞(reaction 4, then, does not occur). If this is done, equation 13 results.

$$(13) \qquad \frac{1}{v} = (1 + \frac{i}{K_c})\ (\frac{K_m}{V})\ \cdot \frac{1}{s} + \frac{1}{V}$$

A competitive inhibitor thus affects only the slope; the addition of inhibitor causes the slope to increase by the factor $(1 + \frac{i}{K_c})$.

C- "Non-competitive" inhibition

Non-competitive inhibition is usually regarded simply as a condition in which some of the enzyme molecules are rendered inactive. The net effect of adding a non-competitive inhibitor should be merely that of lowering the total enzyme concentration, and should show up as a greater intercept (a lower maximum velocity in the presence of

inhibitor) and a proportionally greater slope. This result is predicted by equation 11 if $K_c = K_g$.

D- "Un-competitive" inhibition

It is occasionally observed that addition of an inhibitor causes an increase in intercept with no change in slope. If $K_c = \infty$ (reaction 3 does not occur), equation 11 reduces to equation 14, which expresses this effect.

$$(14) \qquad \frac{1}{v} = \frac{K_m}{V} \cdot \frac{1}{s} + \left(1 + \frac{i}{K_g}\right) \cdot \frac{1}{V}$$

E.

Apparently unrelated changes in intercept and slope may result upon addition of an inhibitor if K_c and K_g are unequal.

In general, any change in slope is an indication that reaction 3 is occurring; any change in intercept is an indication of reaction 4 or 5 or both 4 and 5.

Thus the results of an investigation of enzyme inhibition are best summarized and compared in the form of the slopes and ordinate intercepts of the straight lines obtained by plotting $\frac{1}{v}$ against $\frac{1}{s}$.

Experimental

Urease activities were measured as described in Section I-A. The kinetics of the hydrolysis of urea were investigated as described in Section I-C.

Thrice recrystallized urease was diluted to a concentration of about one μg. protein N/ml. with a solution which was 0.01 formal in buffer and which was stabilized with H_2S. The dilute solution, pH 6.5, was maintained at 25° for five hours before use.

The maleate buffers were prepared by addition of solid reagent grade sodium hydroxide to a solution of recrystallized maleic acid; the glycine buffers, by addition of recrystallized sodium carbonate to a solution of recrystallized glycine. At pH 7.0, the glycine buffer had a glycine: bicarbonate ratio of 100:6.

The reaction mixtures were prepared as described previously. Reaction times of three minutes were used for all activity measurements whose results are reported. Early experiments with phosphate buffer gave the erroneous impression that the activity at the low urea concentration, and consequently the slope: intercept ratio, was dependent upon the reaction time. This effect was found to be caused by insufficient mixing of the reaction mixture after addition of enzyme. Similar experiments with maleate buffer, in which the reaction time was varied, and in which the solutions were thoroughly mixed, showed only the usual experimental

differences.

A least sqares treatment, in which the data were weighted proportionally to the reaction velocities, was used in computing the slopes and intercepts of the $\frac{1}{v}$ vs $\frac{1}{s}$ lines.

Discussion

A- Effect of Buffers

That urease activity is affected by the type of buffer, and its concentration, was reported by Van Slyke and Zacharias (12), who observed that urease activity is depressed by phosphate, and by Howell and Sumner (13). The latter authors noted the dependence of the activity--pH curve upon the type of buffer, using phosphate, citrate, and acetate buffers.

In the course of this investigation of the kinetics of the urease--urea system, it has been found that phosphate exerts a marked inhibiting influence of the competitive type. The data pertaining to the effect of phosphate are summarized in Table IV, Exp'ts I and II, and presented graphically in Fig. 1. It is evident that the intercept of the $\frac{1}{v}$ vs $\frac{1}{s}$ line remains essentially unchanged upon increasing the phosphate concentration, while the slope increases markedly. The increase in slope is approximately linear for the lower phosphate concentrations. Extrapolation to zero phosphate gives a value for the slope of 4.5. The slope: intercept ratio for

zero phosphate concentration, or Michaelis constant, is about 3 μmole/ml. or 0.003 formal urea.

From equation 13, for a case of competitive inhibition the slope is $$m = (1 + \frac{i}{K_c})\ (\frac{K_m}{V})\ .$$

Using the extrapolated value of $m = \frac{K_m}{V} = 4.5$, K_c may be calculated from the relation

$$K_c = \frac{4.5\ i}{m-4.5}\ ,\ \text{where } i = \mu\text{mole inhibitor/ml.}$$

For i = 30 to 161 μmoles phosphate per ml., the average value of K_c is 0.035 formal phosphate (Table IV, Column 7).

That the inhibition by phosphate is not simply an ionic strength effect is shown by the markedly different results obtained with maleate and glycine buffers (Table IV, Exp't. III; Figs. 2 and 3). Increasing the sodium maleate concentration from 0.16 to 0.53 formal causes no change in slope, and only a relatively small increase in intercept. The increase in slope upon increasing the glycine concentration, when buffering with glycine, may or may not be significant, since the variation is within experimental error, as judged by runs with the same concentration of phosphate buffer. Glycine also has a small effect upon the intercept.

It is interesting to note that with the maleate and glycine buffers, a slope: intercept ratio is obtained which is not greatly different from the ratio obtained from the phosphate data upon extrapolation to zero phosphate concentration.

The discovery of competitive inhibition of urease by phosphate is an indication that other biological systems, which are often studied in phosphate buffer, may be more affected by the phosphate than is now supposed.

B- Effect of substituted ureas

The results of the investigation of the inhibition of urease by various compounds related to urea are summarized in Table V (Figs. 4 and 5).

It is clear that n-butyl urea has a marked effect upon the kinetics of the system, whether buffered with maleate or phosphate. Unfortunately, the slopes for the $\frac{1}{v}$ vs $\frac{1}{s}$ lines are too erratic to permit calculation of a reliable value for K_c. The intercepts, however, increase linearly with the concentration of inhibitor. K_g may be calculated from the expression for the intercept in equation 11,

$$\text{Intercept} = (1 + \frac{i}{K_g}) \frac{1}{V} \quad .$$

The values for K_g, as calculated for n-butyl urea inhibition in both a maleate and a phosphate buffer, are tabulated in Table V, column 8. They are all reasonably close to the average of 0.032 formal n-butyl urea.

In phosphate buffer, neither t-butyl urea nor methyl urea has much effect at low inhibitor concentration (0.05 formal or less).

In maleate buffer, on the other hand, both compounds exert a definite influence upon the kinetics of the reaction. Values of the constants K_c and K_g may be calculated for both inhibitors, although such values should not be considered as highly reliable, since only a single experiment was carried out with each compound. The constants obtained from the data are:

methyl urea --	$K_c = 0.132$ formal
	$K_g = 7.9$ formal
t-butyl urea --	$K_c = 0.057$ formal
	$K_g = 0.425$ formal

Thus, of the inhibitors investigated, n-butyl urea has much the greatest effect.

Table IV

Effect of Buffer Concentration

Upon Kinetics of Urease-Urea System

Exp't. No. (1)	Buffer (2)	Buffer Conc. (3)	Slope "m" (4)	Intercept "b" (5)	m/b (6)	K_c (7)
I	Phosphate	.030 f.	8.12	1.27	6.4	.038
		.056	11.3	1.28	8.8	.037
		.109	22.1	1.26	17.5	
II	"	.056	11.9	1.49	8.0	.034
		.109	19.4	1.53	12.7	.033
		.161	25.8	1.62	16.0	.034
		.267	52.0	1.39	37.6	
		.38	69.1	1.71	40.5	
III	Maleate	.16	7.84	1.70	4.6	
		.32	7.80	1.80	4.3	
		.53	7.82	2.30	3.4	
	Glycine	.16	6.76	1.45	4.65	
		.32	7.00	1.54	4.6	
		.53	7.21	1.68	4.3	

Table V

Effect of Substituted Ureas

Upon Kinetics of Urease-Urea System

Exp't.	Buffer	Inhibitor	Inhibitor Conc.	Slope "m"	Intercept "b"	m/b	K_g
(1)	(2)	(2)	(4)	(5)	(6)	(7)	(8)
IV	.134 f. maleate	n-butyl urea	0	5.20	1.40	3.71	
			.0053 f.	4.96	1.73	2.87	.027
			.021	6.95	2.36	2.94	.034
			.037	6.36	3.21	1.98	.030
			.053	11.9	3.72	3.20	.034
V	.053 f. phosphate	"	0	10.8	1.48	7.33	
			.0053	15.6	1.64	9.50	.040
			.016	14.9	2.14	5.17	.033
			.032	18.2	3.13	5.82	.028
			.053	18.0	3.90	4.62	.031
VI	.134 f. Maleate	t-butyl urea	0	5.1	1.58	3.2	
			.035	7.6	1.71	4.4	
VII	.056 f. phosphate	"	0	11.7	1.36	8.6	
			.012 f.	11.7	1.38	8.4	
			.025 f.	12.7	1.34	9.5	
			.037 f.	12.4	1.39	8.9	
			.049 f.	12.8	1.41	9.1	
VIII	.134 f. maleate	methyl urea	0	5.1	1.58	3.2	
			.050 f.	6.5	1.63	4.0	
IX	.056 f. phosphate	"	0	11.3	1.28	8.8	
			.053 f.	11.1	1.28	8.7	
			.263 f.	14.1	1.39	10.1	

Demonstration of Competitive Inhibition

By Phosphate

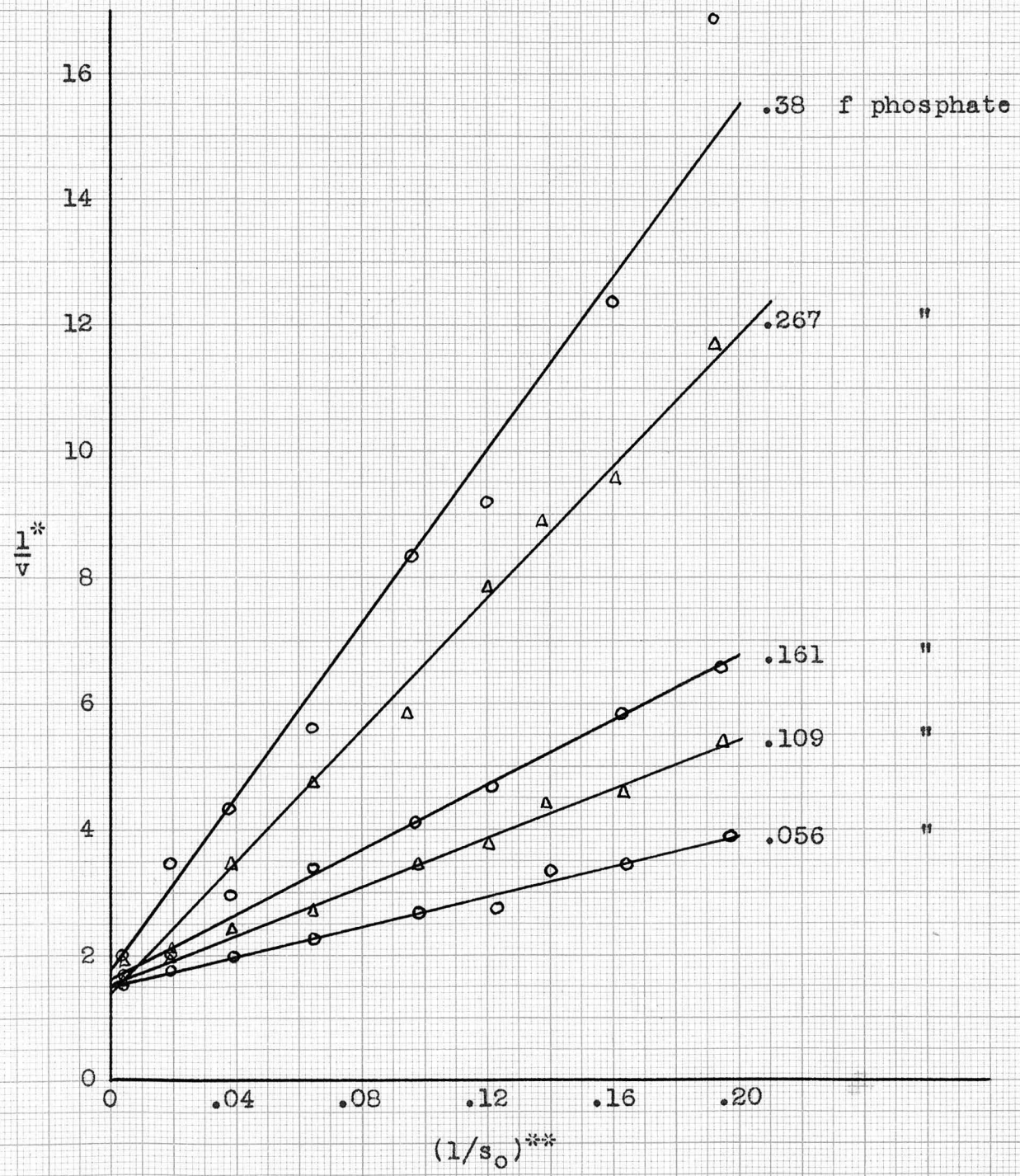

Fig. 1

Effect of Glycine Buffer

Upon Kinetics

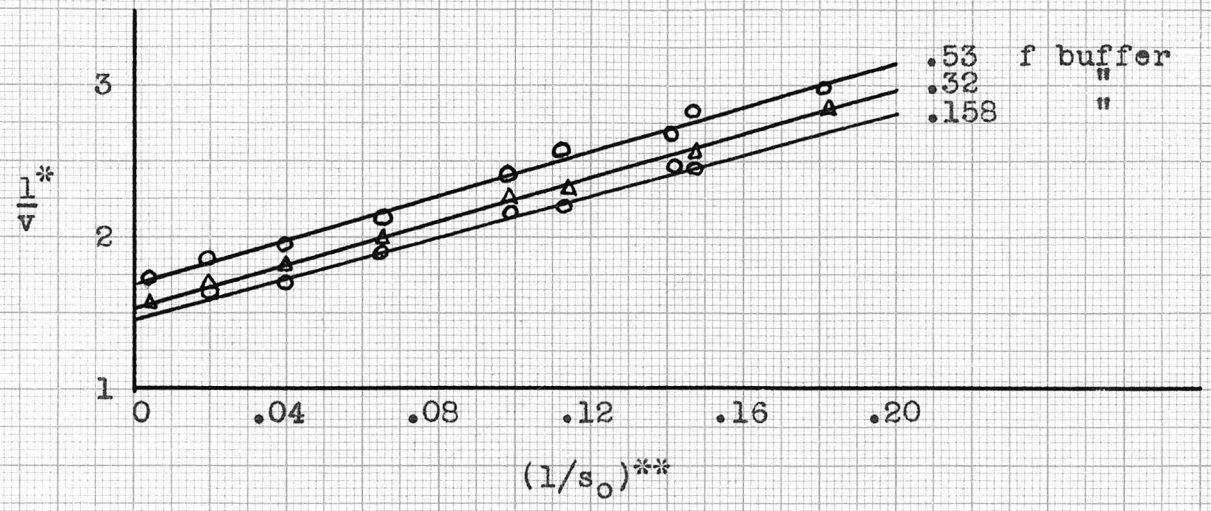

Fig. 2

Effect of Maleate Buffer

Upon Kinetics

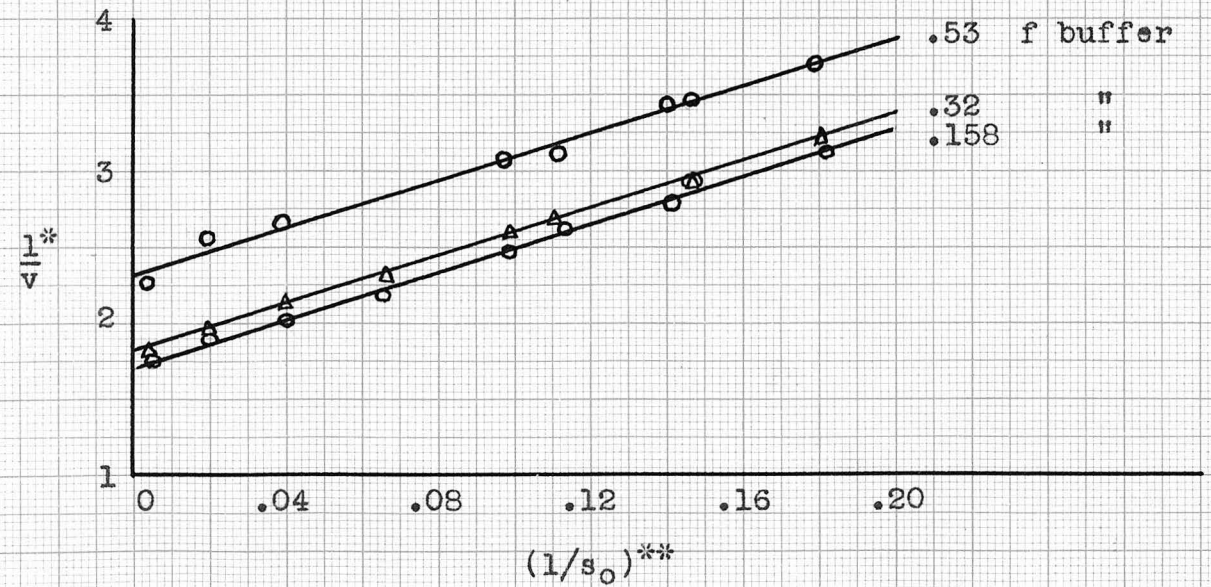

Fig. 3

Effect of n-Butyl Urea

In 0.134 f. Maleate Buffer

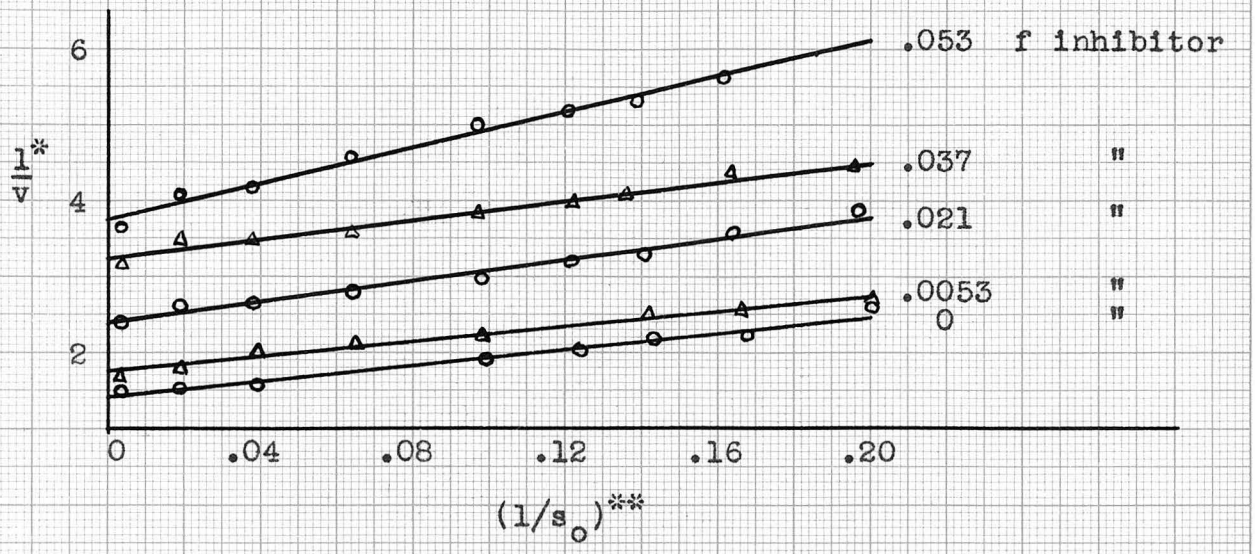

Fig. 4

Effect of n-Butyl Urea

In 0.053 f. Phosphate Buffer

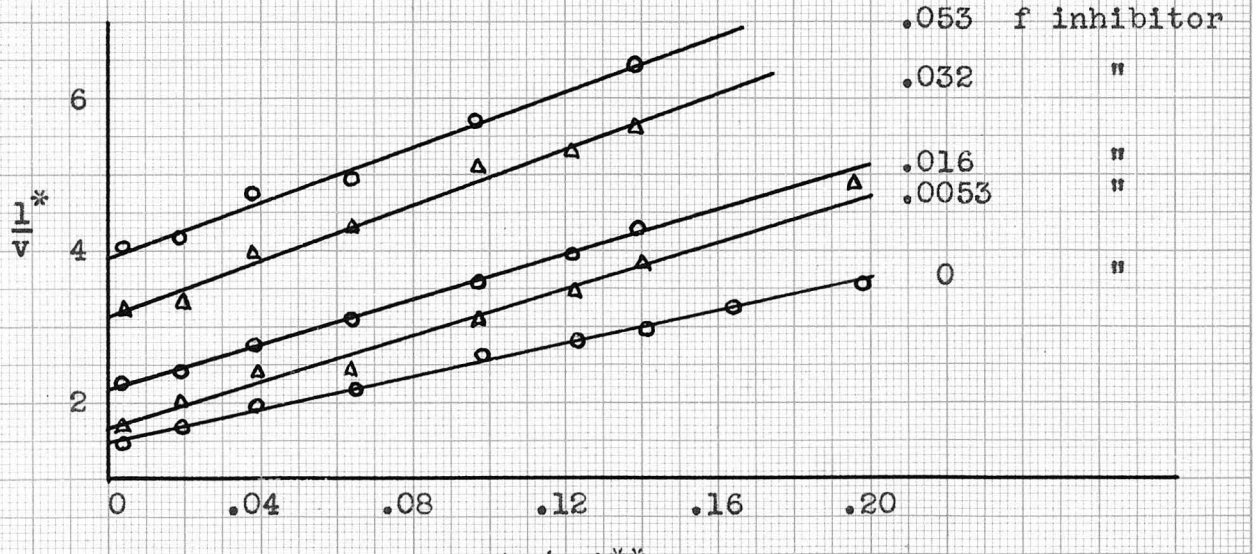

Fig. 5

Notes to Figures 1-5

* (μmole NH_3)$^{-1}$ ml.min.

** (μmole urea)$^{-1}$ ml.

Part III

Experiments with Trypsin and Chymotrypsin

A- Kinetics of the Trypsin-Catalyzed Hydrolysis of Benzoyl-l-Arginineamide

Crystalline trypsin has been shown (14-19) to catalyze the hydrolysis at the amide link, of α-benzoyl-l-arginine-amide (BAA), α-hippuryl-l-arginineamide, α-toluenesulfonyl-l-arginineamide, α-benzoyl-l-lysineamide, and α-hippuryl-l-lysineamide; and, at the ester link, of the methyl esters of benzoyl-l-arginine (BAME) and toluenesulfonyl-l-arginine. Previous investigations of the kinetics of the amide hydrolyses were carried out only with initial substrate concentrations of 0.043 to 0.05 formal. They led to the conclusion that the reaction rate is first order with respect to the concentration of both the enzyme and the substrate. This conclusion was drawn because for any single experiment, with a given enzyme concentration E_o and an initial substrate concentration of about 0.05 formal, the rate of hydrolysis was found to decrease as predicted by equation 15. Since

$$-\frac{ds}{dt} = k'E_o s \qquad (15)$$

s = substrate concentration
k' = proteolytic coefficient

these results were obtained with initial 0.05 formal substrate concentrations, the Michaelis constants were assumed to be relatively large.

On the other hand, Neurath's studies (18) of the hydrolysis of BAME show this reaction to be zero order with

respect to the substrate up to 95% hydrolysis. As the initial substrate concentration was 0.0063 formal, a very low Michaelis constant is indicated for this reaction.

Hydrolysis of benezoyl-l-arginineamide (BAA)

A further investigation of the kinetics of the trypsin-catalyzed hydrolysis of BAA has shown that the initial reaction velocity varies little with inital BAA concentration, over the range of 0.01 to 0.07 formal BAA (see Table VI columns 2 and 4). The initial reaction rates obey the Michaelis-Menten equation, $v = -\frac{ds}{dt} = \frac{V\,s}{K_m+s}$, within experimental error. At 25° C. K_m was found to be ~0.0021 formal BAA. It was also observed that for any value of s_o, the reaction apparently follows a first order kinetics, although at high initial substrate concentrations, the reaction rate decreases somewhat more rapidly with time than expected for a reaction which is truly first order with respect to the substrate concentration. The first order rate constant k', however, increases as s_o decreases. Further, the reaction products have a marked inhibitory effect upon the reaction rate, although ammonium ion alone is without effect.

These results parallel those recently reported by Neurath et al (20) for the hydrolysis of both the ester and amide of benzoyltyrosine by crystalline chymotrypsin. These investigators observed no inhibition by the products

of the reaction, and concluded that their results agreed with the integrated Michaelis-Menten equation,

$$(16) \qquad ket = 2.3\ K_m \log \frac{s_o}{s} + (s_o - s) \quad .$$

However, if the rate of an enzymatic reaction is consistent with the latter equation, it should not decrease as rapidly with time as it should if the rate were truly first order with respect to the substrate concentration. Neurath doesn't give suffienct data to tell whether or not this is true.

In view of the observed inhibition of the trypsin - BAA hydrolysis by the split products, a different explanation seems in order. One possible theory is outlined below (21).

Consider the equilibria:

$$\underset{(e-p-q)}{E} + \underset{(s)}{BAA} \overset{K_m}{\rightleftharpoons} \underset{(p)}{E \cdot BAA} \xrightarrow{k} E + BA + A$$

$$\underset{(e-p-q)}{E} + \underset{(s_o-s+I)}{BA} \overset{K_i}{\rightleftharpoons} \underset{(q)}{E \cdot BA}$$

e= total enzyme concentration
s= substrate concentration
s_o= initial substrate concentration
p= concentration of the enzyme-substrate complex
q= concentration of the enzyme-split product complex
I= initial concentration of the split product
e-p-q= free enzyme concentration

$$(17) \qquad K_m = \frac{s\ (e-p-q)}{p} \qquad pK_m = s\ (e-p-q)$$

(18) $$K_i = \frac{(s_o-s+I)(e-p-q)}{q} \qquad qK_i = (s_o-s+I)(e-p-q)$$

From 18, $$q = \frac{(s_o-s+I)\ (e-p)}{K_i+s_o-s+I}$$

Substituting into 17,

$$p = \frac{esK_i}{K_m(K_i+s_o - s+I)+sK_i}$$

If $K_i = rK_m$, $$p = \frac{es}{K_m+ \frac{s_o}{r} + \frac{I}{r} + \frac{(r-1)\ s}{r}}$$

(19) $$\frac{-ds}{dt} = kp = \frac{kes}{K_m+ \frac{(s_o+I)}{r} - \frac{(1-r)s}{r}}$$

(20) Integrating, $$ket = 2.3\left[K_m+\frac{1}{r}(s_o+I)\right] \log \frac{s_o}{s} - \frac{(1-r)}{r}(s_o-s)$$

(21) If $I = 0$, $$\frac{-ds}{dt} = \frac{kes}{K_m+\frac{s_o}{r} - \frac{(1-r)s}{r}}$$

(22) And at $t = 0$, $$-\frac{ds}{dt} = \frac{kes_o}{K_m+s_o}$$

(23) If $r = 1$ $$-\frac{ds}{dt} = \frac{kes}{K_m+s_o+I}$$

In equations (19-23), r represents the relation between K_i and K_q. If r = 1, the reaction should appear to be exactly first order with respect to the substrate.

An equation similar to equation 23 is obtained by application of Klotz' linear equation (22) for the binding of ions by a protein with n equivalent binding sites per molecule.

$$(24) \qquad \frac{e}{A_e} = \frac{K}{n} \cdot \frac{1}{A} + \frac{1}{n}$$

where e = protein (enzyme) concentration

A = ion concentration (in this case, concentration of substrate plus split product)

A_e = no. of moles of ions bound to protein per unit volume of solution

Taking the reciprocal,

$$A_e = \frac{enA}{K+A}$$

Assuming that only the substrate is bound to the enzyme,

$$s_e = \frac{ens}{K+s}$$

where s = substrate concentration

s_e = bound substrate concentration.

If the rate of reaction is proportional to s_e,

$$(25) \qquad v = \frac{kens}{K+s}$$

Equation 25 has the same form as the Michaelis-Menten equation, with a different significance applied to the constants.

If one assumes, on the other hand, that the combining

site of the enzyme may be occupied equally well by either the substrate or one of the split products,

$$s_e = \frac{s}{A} A_e = \frac{s}{A} \cdot \frac{enA}{K+A}$$

Then

(26)
$$v = \frac{kens}{K+A}$$

If in equation 20 r is less than one, the split product must be more firmly bound to the enzyme than is the substrate. In such a case, as the reaction proceeds the rate should decrease more rapidly than would be predicted from a first order kinetics. (see equation).

Such a deviation from a first order reaction has been observed in all experiments in which s_o was high. A value of r of about 0.5 has been found to fit such cases fairly well. However, with low s_o values, the deviation was not observed--perhaps because of the experimental difficulties involved.

The rate constants k, calculated from equation 22 are listed as observed for various initial substrate concentrations, enzyme concentrations, and temperatures, in columns 3 and 5-8 of Table VI . At 25° C, consistent results--as indicated by the uniformity of the k values - were obtained. They gave values of k = 0.0022*, K_m = 0.0021, and r $\cong$ 0.5.

---

* k = millimoles $min.^{-1}$ (mg. enzyme nitrogen)$^{-1}$. This enzyme preparation was about one-half as active as those of Bergmann.

At 40°, however, the k values varied considerably from one experiment to another.

It is evident that the Michaelis constant, K_m, for the reaction is temperature dependent, ranging from 0.0021 formal BAA at 25°C to 0.0082 at 40°C.

It seems of interest to compare experimental values for the extent of hydrolysis (s_o-s) with those calculated from equation 20, using different values of r. These results are tabulated in Table VII, for r = 0.43 and r = 1.0. Generally speaking, the agreement is better with the lower value of r, although r = 1 fits more closely at the very low initial substrate concentrations.

The effect of the reaction products upon the rate is shown in Table VIII. 0.05 formal NH_4^+ causes no demonstrable change. Since benzoylarginine is only slightly soluble,*

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* In view of the low solubility of benzoylarginine, it is surprising that no crystallization of split products from the reaction solution was observed, even after standing for 24 hours. If the inhibiting split product crystallized out upon reaching the concentration u, the rate from that time on would be expressed by the equation

$$v = \frac{kes}{(K_m + \frac{u}{r}) + s}$$

Consequently, the plot of log s (as ordinate) against time would give a curve which was concave upward from zero time to the time when the split product concentration became equal to u, and which was concave downward from that time to the end of the reaction. Such an effect was not observed in any of the experiments.

it could not be tested alone. A solution of 0.05 formal BAA was completely hydrolyzed, the enzyme destroyed by heating, fresh BAA added, and fresh trypsin. The rate was then followed in this solution, which was initially 0.042 formal in benzoylarginine and NH_4^+, and was found to be markedly decreased. The results obtained in this experiment are compared with those calculated from equation 20 (using the values of k = 0.0027 and r = 0.53), in Table VIII. The agreement is good.

The data presented fit well with the theory, although Neurath's observations upon the rate of hydrolysis of BAME argue against the postulated inhibition by benzoylarginine. Perhaps both NH_4^+ and benzoylarginine are necessary for inhibition.

Experimental

Reagents: The data presented were obtained with a preparation of crystalline trypsin procured from Armour. Similar results were given by crystalline trypsin from Lehn and Fink. The trypsin solutions were prepared by dissolving a weighed quantity of the dry enzyme - $MgSO_4$ mixture (7.5% nitrogen) in water and centrifuging to remove the small amount of insoluble material. Such a solution preserved its activity essentially unchanged for several hours.

The substrate solutions were prepared by dissolving a weighed amount of benzoyl-l-argininеamide hydrochloride in

water. A 0.05 formal solution had a pH of 3 to 4. In the early experiments, the pH was adjusted to 8 by addition of a minute amount of K_2CO_3. This practice was discontinued because it proved to be unnecessary and because it was found that carbonate in large amounts (0.04 formal) acted to decrease the activity of the trypsin.

A 0.1 formal phosphate buffer, pH 7.9, was prepared from recrystallized potassium dihydrogen phosphate and disodium hydrogen phosphate. Glass-redistilled water was used for enzyme, substrate, and buffer solutions.

Reagent grade formaldehyde was found to have too low a pH for use in formol titrations (23). This situation was remedied by shaking the formaldehyde with basic magnesium carbonate, allowing to stand two or three days, and filtering. The pH of the resulting solution was about 8.

Procedure: In the usual procedure, 5.0 ml. of substrate solution and 1.0 ml. of buffer were pipetted into a 1" x 6" test tube, which was placed in a constant temperature bath. 1.0 ml. of trypsin solution was added from a pipet, the mixture shaken, and a 1.0 ml. sample withdrawn for the blank determination. The blank was corrected for the one-half to one minute time interval required for this operation. 1.0 ml. samples were withdrawn thereafter at the times desired.

Each sample was added immediately to an equal volume of formaldehyde in either a 10 ml. beaker or a beaker of the same diameter but $2\frac{1}{2}$ to 3 inches high. The beaker was placed in a rubber stopper which in turn was mounted on the shaft of

an inverted air-driven stirrer in such a way as to permit rotation of the beaker during the titration. The pH meter electrodes in the solution acted as stationary stirrers.

Titration of the sample was carried out by the addition of 0.01 f. sodium hydroxide from an automatic buret, calibrated in hundredths of a milliliter. A small diameter glass tube, its lower end drawn to a fine opening and immersed in the solution, delivered the base directly into the sample.

The end-point of the titration was taken at pH 8.1, as measured by a Beckman pH meter using electrodes especially shaped for use in a limited space. It was found necessary to thoroughly rinse the electrodes after each titration, and check them against a standard buffer, as their calibration changed somewhat during the course of a titration. It was also found advisable to titrate a sample as soon as possible after it was added to the formaldehyde, as the titer changed upon standing.

Blank runs were made with solutions containing no enzyme. No appreciable hydrolysis of the substrate was observed under these conditions.

Table VI

Reaction Rates and Rate Constants
pH = 7.7

s_o*	25° C. E_o=.055** K_m=.0021 formal		25° C. E_o=.034**		30° C. E_o=.043** K_m=.0033 formal	40° C. E_o=.064** K_m=.0082 formal	40° C. E_o=.042**
	s_o-s* (in 20")	k	s_o-s* (in 60")	k	k	k	k
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
70							.0057
50			3.7	.0022			
43.4					.0065	.0074	
37							.0059
30			3.7	.0024	.0067	.0076	
10	1.7	.0023			.0060	.0076	
8.7					.0066	.0061	
5.8					.0061	.0073	
5.0	1.5	.0023					
4.6			2.0	.0018			
4.0	1.2	.0020					
3.0	1.0	.0021	1.5	.0021	.0066	.0074	
2.0	0.9	.0023					
1.5					.0054		

* μ moles BAA per ml.
** mg. protein N/ml.

Table VII

Comparison of Experimental and Calculated Results 25° C.

K_m= 0.0021 formal BAA; k= 0.0022; E_o= 0.055 mg. N/ml.

s_o moles/ml. (1)	Time (min.) (2)	Percent hydrolysis (3)	s_o-s (obs.) μ mole/ml. (4)	s_o-s (calc.) μ mole/ml. r= 0.43 (5)	s_o-s (calc.) μ mole/ml. r=1 (6)
10	11.5	11.2	1.12	1.11	1.11
	20	17.0	1.70	1.70	1.83
	40	28.5	2.85	2.90	3.31
5.0	10	16.2	0.81	0.76	0.80
	20	29.8	1.49	1.30	1.47
	30	37.0	1.85	1.78	2.02
	40	45.8	2.29	2.14	2.49
	50	51.0	2.55	2.48	2.88
	60	56.8	2.84	2.76	3.20
4.0	10	16.7	0.67	0.68	0.73
	20	30.0	1.20	1.19	1.31
	30	41.8	1.67	1.58	1.79
	40	48.3	1.93	1.92	2.18
	50	59.0	2.36	2.06	2.51
	60	62.8	2.51	2.42	2.77
3.0	10	20.0	0.60	0.58	0.57
	20	32.0	0.96	1.03	1.14
	30	44.7	1.34	1.35	1.53
	40	59.0	1.77	1.61	1.84
	50	65.3	1.96	1.73	2.08
	60	71.0	2.13	2.00	2.27
2.0	10	26.5	0.53	0.48	0.52
	20	46.0	0.92	0.72	0.90
	30	61.5	1.23	1.05	1.18
	40	69.5	1.39	1.24	1.39
	50	70.5	1.41	1.38	1.54
	60	80.5	1.71	1.49	1.66

Table VIII

Effect of Reaction Products upon the Rate

25° C. E_o= .062 mg. N/ml. s_o= 50 μ moles/ml.

Reaction Product added	Time (min.) (1)	Percent hydrolysis (2)	s_o-s (obs.) μ moles/ml. (3)	s_o-s (calc.)* μ moles/ml. (4)
None	60	16.8	8.42	8.90
	120	29.0	14.51	14.5
	270	50.0	25.0	25.3
.05 formal NH_4^+	60	16.9	8.47	8.90**
	120	29.4	14.68	14.5 **
.042 formal benzoyl-arginine; .042 formal NH_4^+	60	8.27	4.13	3.8 ≠
	120	15.50	7.75	7.0 ≠
	240	27.3	13.62	12.8 ≠

* using k= .0027 , r= .53
** Assuming no inhibition by ammonium ion.
≠ Assuming inhibition by benzoylarginine.

B- Chymotrypsin-Catalyzed Hydrolysis of l- and dl- N-Benzoyltyrosylglycineamide and N-Acetyltyrosylglycineamide

Contrary to the report of Bergmann and Fruton (24), benzoyl-dl-tyrosylglycineamide is rapidly hydrolyzed by crystalline chymotrypsin. At 40° C. and pH 7.8, in a 0.00344 formal solution of the dl- mixture, the l- component is split as rapidly as in a 0.00172 formal solution of benzoyl-l-tyrosylglycineamide. Experiments at markedly higher substrate concentrations, while desirable, could not be done because of the low solubility of the substrate. The data are presented in Table IX.

A more complete picture is presented by experiments which were done with (l-) and (dl-) acetyltyrosylglycineamide. The solubility of this compound was large enough to permit work with solutions 0.05 formal with respect to the (l-) component. At pH 7.8 and at either 25 or 40° C., the rate of hydrolysis of the (l-) component in a 0.1 formal solution of the (dl-) mixture is about one-half the rate in a 0.05 formal solution of the (l-) compound. At 40°, in solutions 0.00172 formal with respect to the (l-) component, the rates are identical within experimental error for the (l-) and (dl-) compounds. These data, compiled in Table X, thus indicate that in 0.10 formal (dl-) solutions the (d-)

form acts as an inhibitor. In solutions of low (dl-) concentration, the enzyme is unsaturated and no inhibition occurs.

In the course of these experiments, as shown by the tables, the extent of hydrolysis of each reaction mixture was measured at suitable successive time intervals. A plot of log s* against time gives an approximately straight line in each case, indicating a reaction which is first order with respect to the substrate. Rate constants and proteolytic coefficients, calculated by the commonly used first order rate expression, are included in Tables IX and X. Comparison of the proteolytic coefficients show that the benzoyl peptide is hydrolyzed much more rapidly than the acetyl peptide under the same conditions. These constants, however, should be taken only as a rough approximation, since they represent the sum of two reactions--the hydrolysis of the tyrosylglycine peptide bond and the comparatively slow splitting of the amide link (25).

Based upon the same approximation--omission of consideration of the amide splitting--was an attempt to investigate the kinetics of the hydrolysis, catalyzed by crystalline chymotrypsin, of N-acetyl-l-tyrosylglycineamide at 25° C. and pH 7.8. The rate of this reaction was

---

* s = substrate concentration at time t; s_o = initial substrate concentration.

measured for several different initial substrate concentrations. As in the case of the trypsin-catalyzed hydrolysis of benzoyl-l-arginineamide (BAA) and the chymotrypsin-catalyzed hydrolysis of both the ester and the amide of benzoyl-tyrosine (20), an apparent first order kinetics is observed for the reaction at all initial substrate concentrations; but the first order rate constant k' increases as s_o decreases. In many of the experiments, the reaction rate falls off less rapidly with time than would be expected of a truly first order reaction.

Either of two possible explanations for this behavior may be advanced:

1) If inhibition by the split products occurs, as in the trypsin--BAA reaction, the course of the reaction would agree with equation (20).
2) If no inhibition by split products occurs, the rate law may be expressed by the integrated Michaelis-Menten equation, equation 16.

Either equation would fit the data equally well. Since the data are meager, and nothing is known of possible reaction product inhibition, the simpler explanation embodied in equation (16) was adopted for purposes of comparison with experimental data. In Table XI are tabulated the experimental values of s_o-s and those calculated from equation (16), using the best experimental values of K_m = 0.03 formal and

$k = 0.0089^{*}$. The agreement is within experimental error.

Experimental

Crystalline chymotrypsin preparations obtained from Lehn and Fink and from Armour were compared and gave about the same results. The solutions were buffered with 0.015 formal phosphate, pH 7.8.

The procedure was the same as that described in Part III-A. The extent of hydrolysis was determined by formol titration to pH 8.1, using a pH meter for end-point determinations. One-half ml. samples of the 0.05 formal substrate solutions were used; 2 ml. samples of the 0.0017 formal solutions were titrated.

---

* k = millimoles min^{-1} (mg. enzyme nitrogen)$^{-1}$.

Table IX

Hydrolysis of N-Benzoyltyrosylglycineamide*
40° C.; pH = 7.8

E_o (mg. prot. N/ml.) (1)	Time of Reaction (min.) (2)	$s_o - s$ μmoles/ml. dl (3)	l (4)	k'# dl (5)	l (6)	C/ dl (7)	l (8)
.0154**	10	.66	.70	.049	.052	3.2	3.4
	20	1.18	1.22	.058	.062	3.7	4.0
.0154***	10	.71	.65	.053	.047	3.5	3.1
	20	1.24	1.15	.063	.055	4.1	3.6
	30	1.60	1.38				

* Concentration of dl substrate = 3.44 μmole/ml.
" " l " = 1.72 "
**Crystalline chymotrypsin preparation from Armour.
*** " " " " Lehn and Fink.
# k' is the first order rate constant, $min.^{-1}$.
/ C is the proteolytic coefficient.

Table X

Hydrolysis of N-Acetyltyrosylglycineamide
pH = 7.8

Temp. °C	E_o*	s_o**	Time (min.)	s_o-s μ mole/ml. dl	l	k'*** (min.$^{-1}$) dl	l	C† dl	l
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
40	.0154	1.72	30	.29	.36	.0061	.0079	.39	.51
			60	.60	.64	.0071	.0077	.46	.50
40	.031	1.72	20	.45	.42	.015	.014	.45	.45
			40	.63	.70	.011	.013	.37	.42
			60	.90	.85	.012	.011	.40	.36
40	.060	50	20	7.36	12.76	.0079	.015	.13	.24
			40	13.46	22.0	.0077	.015	.13	.24
25	.075	50	20	3.92	7.52	.0041	.0081	.054	.11
			40	8.06	14.20	.0044	.0083	.058	.11
			60		19.96		.0085		.11
			80	13.74		.0040		.054	
25	.30	50	10	7.64	14.1	.0165	.0329	.055	.11
			20	12.6	24.7	.0146	.0342	.049	.11
			30		32.2		.0342		.11
			40	24.4		.0167		.056	

* Mg. protein nitrogen /ml. of Lehn and Fink chymotrypsin.
**Concentration of l- form of substrate, μ mole/ml.
***k' is the first order rate constant.
† C is the proteolytic coefficient.

Table XI

Comparison of Experimental and Calculated Results
25°C; pH = 7.8; E_o = 0.15 mg. prot. N/ml.
(K_m = 0.03; k = 0.0089)

s_o mole/ml. (1)	Time of Reaction (min.) (2)	Percent Hydrolysis (3)	s_o-s (obs.) μmole/ml. (4)	s_o-s (calc.) μmole/ml. (5)
50	10	14.9	7.44	8.1
	20	27.6	13.8	15.7
	40	48.6	24.3	27.3
30	10	16.9	5.08	6.3
	20	37.1	11.1	11.6
	40	65.2	19.6	20.0
20	10	26.1	5.22	4.9
	20	45.2	9.04	8.9
	30	59.4	11.9	12.4
8.3	10	31.1	2.6	2.5
	20	53.2	4.4	4.3
	30	68.7	5.7	5.7
5.0	10	31.0	1.55	1.6
	20	53.3	2.67	2.7
	30	70.6	3.53	3.5

Part IV

Chemical Effects Accompanying the Isomeric Transitions of Radioactive Tellurium

The isomeric transitions undergone by $Te^{127,129}$,

$$Te^{127} \xrightarrow[\text{I. T.}]{\text{90 day}} Te^{127} \xrightarrow[\beta-]{\text{9.3 hr.}} I^{127}$$

and

$$Te^{129} \xrightarrow[\text{I. T.}]{\text{32 day}} Te^{129} \xrightarrow[\beta-]{\text{72 min.}} I^{129}$$ (26),

are known to bring about the reduction of telluric acid. The molecular species resulting from this reduction is commonly assumed to be tellurous acid, since it may be separated from the solution, along with added inactive tellurous acid, as elemental tellurium following fractional reduction with SO_2 in 3N HCl. This procedure has been used as a method of separating the nuclear isomers. Similar effects are found to accompany the isomeric transitions of Br^{80} (27-32), Se^{79}, and Se^{81} (33).

In an early paper on the subject, Segrè and his coworkers remarked (28) that the recoil energy, imparted to the atom either by the transition gamma or by an electron resulting from internal conversion, might be enough to break the necessary bonds. However, it was later shown that the recoil energies are much smaller than the bond energies involved (27).

It is further known that the $Te^{127,129}$ and Br^{80} transition gammas are largely internally converted. The conversion electrons play a part in the phenomena as has been shown in an experiment (27) in which the daughter activities of $Te^{127,129}$ were collected from the walls of a vessel in

which gaseous di-ethyl tellurium had been allowed to decay. In a similar experiment with Zn^{69}, which undergoes an isomeric transition which would be expected to impart a much higher energy to the recoiling zinc atoms, but whose gammas are largely unconverted, no daughter isomer was found to have separated.

An attractive theory (31) lays the cause of molecular disruption of this type to an Auger process, initiated by the conversion electron. The Auger process should result in a partial stripping of the valence electrons from the decaying atom, leaving the molecule with a net positive charge. The molecule would thereby become unstable and fly apart.

If this theory is correct, the fragments from the disrupted molecules should have a positive charge, and it should be possible to collect them upon a negatively charged plate in an electric field. De Vault and Libby (31) performed such an experiment, placing oppositely charged plates in gaseous ethyl bromide containing Br^{80}. At the conclusion of the experiment there was no difference between the activities on the plates. This result was attributed to a neutralization of the charge on the fragments before they came in contact with the plates.

In this laboratory, charged plate experiments, similar to that of De Vault and Libby, but using gaseous hydrogen telluride and dimethyl-tellurium, were also carried out without success. These attempts are described below. Also

described in the following pages are a number of solution experiments which show that tellurous acid is indeed produced from telluric acid as a result of the isomeric transition.

Experimental

Reagents

Radioactive tellurium: Tellurium containing the radioactive isotopes Te^{127} and Te^{129} was obtained from the Manhattan District. All subsequent references to compounds containing tellurium refer to this material, unless otherwise specified.

Hydrogen telluride: H_2Te was prepared by electrolysis, as described by Ernyei (34). A platinum anode and a tellurium cathode, in compartments separated by a fritted disc, were immersed in a 50% H_2SO_4 solution. A four volt potential was applied; with the passage of current through the cell, H_2Te was formed. A stream of N_2 through the cathode compartment swept the H_2Te into a cold trap. The cell was cooled in an ice-salt bath, to diminish thermal decomposition of the H_2Te.

Dimethyl Tellurium: $(CH_3)_2Te$ (35) was prepared by reactions represented by the equations:

$$Te + 2\ CH_3I \longleftrightarrow Te(CH_3)_2I_2$$

$$Te(CH_3)_2I_2 + H_2O + Na_2SO_3 = Te(CH_3)_2 + Na_2SO_4 + 2HI$$

1 gm. Te (powdered) and 2.23 gm. CH_3I were mixed, sealed into a glass tube, and heated at 80°C. for 24 hours. After breaking open the tube, the contents were extracted with hot $CHCl_3$. Red crystals of $Te(CH_3)_2I_2$ formed upon cooling the $CHCl_3$ solution. One gm. of the iodide compound was added to a frozen solution of 0.7 gm. Na_2SO_3 in several cc. H_2O in a small flask, the flask was connected to a vacuum line, evacuated, and the mixture warmed. With care, it was possible to distil the $(CH_3)_2Te$ and only part of the water into an adjoining cold trap. Repeated fractional distillation under vacuum finally gave a bright yellow solid, which was substantially water-free and which melted to give a light yellow liquid upon warming. Low yields were obtained.

Tellurous acid: Te was dissolved in HNO_3 with a small amount of HCl added, to give tellurous acid. The solution was neutralized with NaOH, precipitating the tellurium. This was then dissolved in 3N HCl, to give a solution of tellurous acid in approximately 3N HCl.

Telluric acid: Tellurium oxide, precipitated as in the preparation of tellurous acid, was mixed with conc. H_2SO_4, and H_2O_2 was added. When the solid had dissolved, the solution was evaporated until formation of crystals was observed. The solution was cooled, and conc. HNO_3 was added, causing crystallization of telluric acid. The crystals were heated almost to the melting point to drive off the nitric acid, then recrystallized twice from water. The crystals were

dissolved in water to give a telluric acid solution.

Gas Phase Experiments

The first charged plate experiments were made with gaseous H_2Te, in a small cell with iron plates as electrodes. H_2Te proved unsatisfactory, however, due to its instability; activity with the half lives of the parent isomers was found on both plates. Decompostion of the H_2Te seemed to occur preferentially on the positive electrode, as it was often found to pick up markedly more activity than did the negative plate.

Because of the difficulties in preventing decomposition of H_2Te, all following gas phase work was done with $Te(CH_3)_2$. Several charged plate experiments were carried out using stainless steel plates, each about 3 cm^2 in area, and 1 to 2 mm. apart, and with gas pressures from 20 to 30 mm. A potential drop as high as 340 volts was applied across the gap for five hours. Essentially zero activity was found on both plates, although an activity of 1850 c/min. was recovered from the gas phase.

Believing that with larger plates, there would be a greater chance of success, Exp't. II (Table XII) was carried out using the cell shown in Fig. 6 . This consisted of 2 concentric glass cylinders, with the annular space enclosed and sealed at the ends. The inner surface of the outer

cylinder and the outer surface of the inner cylinder were rendered conducting with a thin, continuous layer of colloidal graphite (the graphite was applied by painting onto the glass a suspension of alcohol-dag in acetone). The cell was filled with $(CH_3)_2Te$ to a pressure of 10 mm. and 60 volts applied for 15 minutes. It was then discovered that a small current was flowing. The voltage was reduced to 37½ volts and was continued for five hours. At that time, the gas in the cell was frozen out into the side arm and removed; the cylinders were separated, and the graphite surfaces swabbed down with conc. HNO_3 to remove any deposited Te. The HNO_3 solutions were concentrated and finally evaporated to dryness on glass microscope slides, on which they were counted. The gas removed with the bulb was also treated with conc. HNO_3 and mounted for counting in the same way. The results, given in Table XII show that the activities collected on the two plates were nearly the same.

As a check on the work of Seaborg (33), Exp't. III was performed. $Te(CH_3)_2$ was allowed to stand at low pressure in a sealed glass bulb. After 10 hours, the gas was frozen out and the walls were washed down with conc. HNO_3. The decay curve of the activity obtained from the walls was analyzed into curves which showed the 9 hr. and 72 min. half-lives of the daughter isomers.

Solution Experiments

If the theory that an Auger type process, with the resultant loss of electrons from the molecule, is responsible for the effects observed, one would expect an oxidation to occur during the isomeric transition.* It seemed well to investigate the statement that the isomeric transitions of $Te^{127,129}$ bring about reduction of telluric acid to tellurous acid, in view of the scarcity of experimental evidence that it is actually tellurous acid which is formed.

1) In-active telluric acid carrier was added to a solution of labelled sodium tellurite (2800 c/min.), then separated by precipitation of zinc tellurate. The precipitated tellurate was found to have an activity of 30 c/ min. Apparently the isomeric transition does not result in oxidation of tellurous to telluric acid, under these conditions.
2) Several mg. of freshly precipitated, inactive tellurium was added to 5 cc. of labelled tellurous acid (350 c/min.). After shaking for ten minutes, the tellurium was filtered

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* In a paper which appeared since this investigation was carried out, Williams (36) reported that in acid solutions, 55% of the isomeric transitions of Te^{129} cause reduction of telluric to tellurous acid. In alkaline solution, however, only 40% of the transitions bring about this reduction, while 15% cause the oxidation of tellurous to telluric acid.

off and counted. Its negligible activity of 10 c/ min. indicated that tellurous acid was not reduced either to tellurium or to a form which exchanged rapidly with tellurium. In another experiment, the mixture was allowed to stand over night. Similar results were obtained.

3) The common experiment of mixing tellurous acid carrier with labelled telluric acid was carried out. The tellurous acid was selectively reduced with SO_2, in a 3N HCl solution, and was found to have picked up activity, with the half life of the 9 hr. daughter isomer. (By the time this experiment was performed, most of the 32 day parent isomer had decayed).

4) An Experiment similar to the above was performed. In this case, however, the tellurous acid was precipitated in three fractions, the first two as the oxide, by neutralizing with NaOH, and the third as the element, by reduction. Fractions 1 and 2 were dissolved in HCl and reduced to elemental tellurium for counting. The specific activities were measured and compared. As may be seen from Table (XIII), the specific activities of the three fractions are practically the same. If the product finally resulting from the reduction induced by the isomeric transition were anything but tellurous acid, one would not expect nearly as close an agreement between the specific activities as was obtained.

Except where otherwise specified, all samples were reduced to elemental tellurium and mounted on a circle of filter paper for counting. A bell jar type counter tube, with a thin mica window , was used.

Table XII

Gas-Phase Experiments with $(CH_3)_2Te$

at Room Tenperature

Exp't No.	Pressure (mm.)	Voltage between Plates	Distance between Plates (mm.)	Area of Each Plate ($cm.^2$)	Time (Hrs.)
I	25	340	1	3	4¾
II	10	37	½ - 2	1200	5
III	42	—	—	—	10

Exp't. No.	Act. on Negative Plate (c/min.)	Act. on Positive Plate (c/min.)	Activity Recovered From Gas (c/min.)	$T_{\frac{1}{2}}$ of Act. on Plates
I	6	7	1865	—
II	440	385	535	9 Hr. 72 min.
III	Activity on walls- 2120		5942	9 Hr. 72 min.

Table XIII

Fractional Separation of Daughter Isomer

From Telluric Acid Solution

Fraction	Ppt's as	Wt. of Te. Counted	Total Activity	Specific Activity (9 hr. half life)
1	+ 4 oxide	4.2 mg.	400 c/min.	95 c/mg/min.
2	+ 4 oxide	8.5 mg.	697 c/min.	82 c/mg/min.
3	Tellurium	11.8 mg.	955 c/min.	81 c/mg/min.

Cell Used in Experiment II

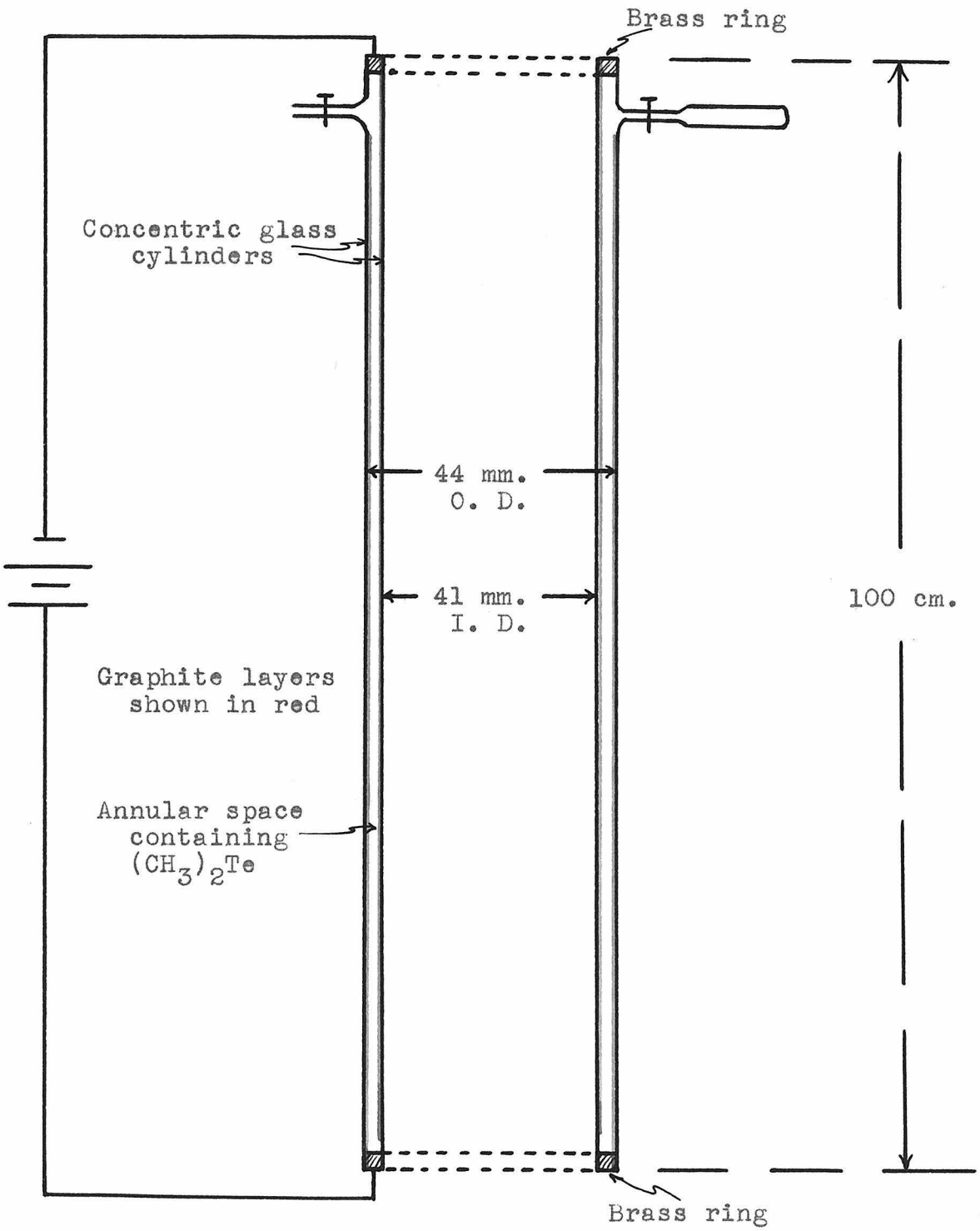

Fig. 6

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Propositions

1) The general methods used in the investigation of urease inhibition (see Thesis, Part II) should be applied to the following systems:
   a) The inhibition of trypsin by acylarginine compounds;
   b) The inhibition of trypsin and chymotrypsin by the optical antipodes of their substrates;
   c) The inhibition of urease by p-chloromercuribenzoate (Hellerman et al, J. Biol. Chem. 147, 443 (1943).

2) The reversible inactivation of trypsin has been insufficiently investigated and should be further studied using simple substrates for which the specificity of trypsin has been demonstrated.

3) a) It is proposed that the irreversible inactivation of trypsin at pH 8 would be inhibited by benzoyl-l-arginine.
   b) The claim that the basic ϵ-amino group of lysine is necessary for trypsin activity (Hofmann and Bergmann, J. Biol. Chem. 130, 81) is based on inconclusive evidence.

4) The effect of an inhibitor upon an enzymatic reaction may be that it causes changes in K_m and k. (see Thesis, p. 18).

5) The study of an enzyme reaction under only a limited number of different conditions may be very misleading.

6) It is proposed that the effect of phosgene upon animal life is not caused simply by the release of HCl in the cells, and that the true effect may be discovered by the use of tracer methods, using radio carbon of the high specific activity now available.

7) Information as to the mechanism of the oxidation of organic compounds could be obtained by tracer studies with O^{18}.

8) The exchange of SO_2 with SO_3, and of ferro- and ferricyanide should be reinvestigated.

9) The attempted verifications of the postulated Auger-effect mechanism for the chemical effects which accompany the isomeric transitions in tellurium (see Thesis, Part IV) probably failed because of the high ratio of inactive to active tellurium. It is proposed that the experiment be repeated, using TeF_6 containing tellurium of a much higher specific activity than has heretofore been available.

10) The words "sublimed" or "resublimed" upon bottles of chemicals are not good indications of the purity of the contents.