the value 12.4×10^{-6} cm., and also $\langle \bar{L}_0^2 \rangle_z / \bar{M}_w = 1.52 \times 10^{-16}$ cm.^{2} mole g.$^{-1}$; this last value cannot be directly compared with analogous figures reported in the literature[14] without a heterogeneity correction. The slope, α, of the upper line in the Fig. 4 is 3.5×10^{-8} g.^{2} mole^{-1} cm.$^{-1}$.

The lower plot in Fig. 3, that of intrinsic viscosity ratio V *versus* A_2, is also essentially linear; an analogous linearity was observed by Krigbaum and Carpenter[14] on plotting $[\eta]$ *versus* A_2 for a polystyrene in cyclohexane over a range of temperatures.

Finally, it is possible to calculate from the data in Table V the values of the quantity $\langle \bar{R}^2 \rangle_z^{1/2} / V^{1/3}$, which should be constant if the intrinsic viscosity is to be proportional to the cube of a linear dimension of the polymer coil; the eleven values obtained from the data in Table V (omitting that for phloroglucinol) are indeed closely similar and give a mean value for $\langle \bar{R}^2 \rangle_z^{1/2} / V^{1/3}$ of 596 ± 13 Å. (one s.d.).

[CONTRIBUTION NO. 1653 FROM STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY, NEW HAVEN, CONN.]

Heats of Hydrolysis of Peptide Bonds[1]

BY MARY RAWITSCHER, INGEMAR WADSÖ[2] AND JULIAN M. STURTEVANT

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The heats of hydrolysis, to form fully charged products, of the tyrosine-glycine bond in benzoyl-L-tyrosylglycine and L-tyrosylglycinamide have been determined by direct calorimetry. The results obtained, $\Delta H_{298} = -1330 \pm 90$ and -1300 ± 150 cal. per mole, respectively, are slightly less negative than the value previously reported for the related peptide benzoyl-L-tyrosylglycinamide, the small differences finding a reasonable interpretation in terms of electrostatic effects. The heat of hydrolysis of benzoyl-L-tyrosine, $\Delta H_{298} = -1980 \pm 100$ cal. per mole, has also been determined. From this result and other data obtained in this Laboratory, the heat of hydrolysis of the benzoic acid—tyrosine bond in benzoyl-L-tyrosylglycinamide is calculated to be $\Delta H_{298} = -2230 \pm 210$ cal. per mole. Accurate calorimetric values are now available for the heats of hydrolysis of eight different peptide bonds in synthetic peptides. These values range from -1240 to -2250 cal. per mole, with a mean value of approximately -1800 cal. per mole.

It was reported in 1952[3] that the heat of hydrolysis of the tyrosine–glycine bond in benzoyl-L-tyrosylglycinamide (BTGA) to give fully charged products is $\Delta H_{298} = -1550 \pm 100$ cal. per mole

$$\mathrm{RCO\text{-}NHR' + H_2O = RCOO^- + {}^+NH_3R'} \quad (1)$$

Since BTGA carries no charge, it might be supposed that this small heat of hydrolysis would be more or less typical for interior peptide bonds in a protein. It was of interest to investigate the effect of having a charged or partially charged group close to the peptide bond under attack, as is the case in the hydrolysis of a terminal bond in a peptide. We have accordingly determined the heats of hydrolysis of the tyrosine-glycine bond in benzoyl-L-tyrosylglycine (BTG) and L-tyrosylglycinamide (TGA), using enzymic catalysis so that the reactions could be carried out at 25° at approximately neutral *p*H. Since the enzyme used with BTG, carboxypeptidase, also attacks the product, benzoyl-L-tyrosine (BT), it was necessary to determine the heat of hydrolysis of this compound.

Experimental

The calorimetric equipment and method have been described in detail elsewhere.[4] In each experiment, 14.0 ml. of substrate solution was charged to the top compartment and 14.0 ml. of enzyme solution to the bottom compartment of one calorimeter and 28.0 ml. of water to the other calorimeter. Because of the necessity to remove solutions for analysis, it was impossible to make duplicate runs on each filling of the calorimeters. All measurements were made at 25.00 ± 0.05°. The enzymes employed, carboxypeptidase and leucineaminopeptidase, were purchased from Worthington Biochemical Company, Freehold, N. J., and were used without further purification. The enzymic hydrolyses were buffered by 0.03–0.05 *M* tris-(hydroxymethyl)-aminomethane (tris) plus enough HCl to give the desired *p*H.

Preparation of Substrates.—BT was prepared from benzoyl chloride and L-tyrosine; m.p. 166–168° after recrystallization several times from H_2O. Titration equivalent weight 293; calcd. 285.3.

BTG.—Freshly distilled glycine ethyl ester was coupled to BT with N,N'-dicyclohexylcarbodiimide,[5] and the product was recrystallized from ethyl acetate and acetone; m.p. 158–165°; yield 62%. The benzoyltyrosylglycine ethyl ester was dissolved in methanol and saponified at room temperature by addition of two equivalents of aqueous NaOH. After 20 min., the reaction mixture was brought to *p*H 5 by addition of HCl, and the methanol was removed by distillation at room temperature. The product was recrystallized from H_2O; m.p. 196–202°, with softening at 160°; yield 95%. Titration equivalent weight 341; calcd. 342.3.

Anal. Calcd. for $C_{18}H_{18}O_5N_2$: C, 63.15; H, 5.27; N, 8.18. Found: C, 63.09; H, 5.39; N, 8.01.

TGA.—Carbobenzoxytyrosine was prepared according to Martin, *et al.*[6]; m.p. 98° after recrystallization from H_2O. It was treated with glycine ethyl ester and N,N'-dicyclohexylcarbodiimide,[5] and the product was recrystallized from ethyl acetate; m.p. 168–170°. Treatment of the ester with NH_3 in methanol[7] gave carbobenzoxytyrosylglycinamide; m.p. approximately 113° (melt not clear until 135°). The carbobenzoxy group was removed by hydrogenation over palladinized charcoal in methanol containing an excess of acetic acid.[8] The resulting acetate of tyrosylglycinamide was recrystallized from methanol–ethanol (2:5). The product did not show a sharp melting point; there appeared to be a transition at 163°, followed

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(2) Fellow of the Sweden America Foundation. Thermochemical Laboratory, University of Lund, Lund, Sweden.

(3) A. Dobry, J. S. Fruton and J. M. Sturtevant, *J. Biol. Chem.*, **195**, 149 (1952).

(4) A. Buzzell and J. M. Sturtevant, *J. Am. Chem. Soc.*, **73**, 2454 (1951); A. Dobry and J. M. Sturtevant, *J. Biol. Chem.*, **196**, 141 (1952).

(5) See, for example, J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

(6) C. J. Martin, J. Golubow and A. E. Axelrod, *J. Biol. Chem.*, **234**, 294 (1959).

(7) M. Bergmann and J. S. Fruton, *ibid.*, **118**, 255 (1937).

(8) J. S. Fruton and M. Bergmann, *ibid.*, **145**, 260 (1942).

by decomposition at about 200°. Titration equivalent weight 301; calcd. 297.3.

Anal. Calcd. for $C_{13}H_{19}O_5N_3$: N, 14.1. Found: N, 13.7.

Results

Hydrolysis of BT.—Carboxypeptidase, in a concentration of approximately 3.3 mg. per ml., was used to catalyze this hydrolysis. Initial substrate concentrations were 4–5 × 10^{-3} *M*.[9] Li^+ was added at 0.75 *M* to make the enzyme sufficiently soluble. Even with this high enzyme concentration, the reaction was so slow as to make it inadvisable to permit the reaction to go to completion in the calorimeter. After about 100 minutes, the reaction solution was removed from the calorimeter, and an aliquot was heated rapidly to 100° and held there for 5 minutes to destroy the enzyme. After filtration and dilution the amount of free tyrosine was estimated spectrophotometrically. The calorimetric record was extrapolated forward to the time of enzyme denaturation. Two additional samples, taken at later times, were treated similarly.

The results of 7 experiments in the *p*H range 7.05 to 8.05 are given in Table I. For each experiment, the figure in the fifth column is the mean heat evolution, in cal. per mole, calculated from the figures in the third and fourth columns. The last column gives the heat of hydrolysis corrected for ionization effects as described below. The estimated uncertainty in the mean heat of hydrolysis, $\Delta H_h = -1980$, includes allowances for uncertainties in the analyses, the calorimetric extrapolations and calibrations and the ionization corrections.

TABLE I

THE CARBOXYPEPTIDASE-CATALYZED HYDROLYSIS OF BENZOYL-L-TYROSINE AT 25°

*p*H	Reaction time, min.	Amount reacted, μmole	Obsd. heat evolution, cal. × 10^2	Mean $-\Delta H_{obsd.}$, cal./mole	Mean $-\Delta H_h$, cal./mole
7.05	87	32.5	6.88		
	88	32.6	6.94		
	91	34.0	7.13	2115	2105
7.15	87	36.7	6.67		
	92	34.7	7.00		
	97	39.2	7.32	1900	1885
7.50	78	34.9	6.16		
	81	33.1	6.36		
	89	38.5	6.92	1825	1795
7.50	94	39.8	8.09		
	99	43.4	8.44		
	105	45.5	8.86	1975	1945
7.50	91	34.2	7.07		
	96	37.3	7.38		
	102	38.5	7.78	2020	1990
7.95	91	28.0	6.41		
	96	31.6	6.73		
	101	34.2	7.05	2160	2075
8.05	125	31.0	6.80		
	126	31.3	6.85		
	127	32.5	6.91	2170	2065
				Mean	1980 ± 100

Following our previous procedure, we wish to evaluate the heat of hydrolysis to fully charged products. The actual process observed in the calorimeter may be written as

$$RCONHR' + \alpha TNH_2 + H_2O \longrightarrow RCOO^- + \alpha NH_2R' + \alpha TNH_3^+ + (1-\alpha)^+NH_3R'; \quad \Delta H_{obsd} \quad (2)$$

where TNH_2 represents the uncharged form of tris, and α is the degree of ionization of $R'NH_3^+$ at the *p*H of the experiment. If we add to this equation the equations for the two ionization processes

$$\alpha TNH_3^+ \longrightarrow \alpha H^+ + \alpha TNH_2; \quad \alpha\Delta H_B \quad (3)$$

$$\alpha NH_2R' + \alpha H^+ \longrightarrow \alpha \overset{+}{N}H_3R'; \quad -\alpha\Delta H_i \quad (4)$$

where ΔH_B and ΔH_i are, respectively, the heats of ionization of tris and of tyrosine, we obtain reaction 1 for which the heat change is ΔH_h. Therefore

$$\Delta H_h = \Delta H_{obsd} - \alpha(\Delta H_i - \Delta H_B) \quad (5)$$

The quantity α is readily estimated if the pK' of $R'NH_3^+$ is known. We have used the value 9.11.[11] On the basis of comparison with other amino acids, we estimate ΔH_i = 10,000 cal. per mole. According to Sturtevant,[12] ΔH_B = 10,900 cal. per mole, while Podolsky and Morales[13] report ΔH_B = 11,600. We have used the mean of these two values in calculating the corrected heats of hydrolysis given in the last column of Table I.

Hydrolysis of BTG.—This reaction was catalyzed by carboxypeptidase at a concentration of approximately 0.5 mg. per ml. The solutions contained 0.10 *M* Li^+, and the total ionic strength was 0.15 *M*. Each calorimetric experiment was interrupted after 40–200 min., the enzyme inactivated by rapid heating and the solution filtered. As in the experiments with BT, the calorimetric curves were extrapolated forward to the time of denaturation of the enzyme. An aliquot was analyzed for glycine and tyrosine in a Specialized Instruments Co. Model 120 Amino Acid Analyzer. Correction was made for the contribution to the observed heat evolution arising from the small extent of hydrolysis of the BT formed in the reaction, the assumption being made that no hydrolysis of the benzoic acid–tyrosine bond took place in the original substrate. Correction to the reaction yielding fully charged products was made using ΔH_i = 10,600 cal. per mole for the heat of ionization of glycine,[14] and taking pK' = 9.60.[11] The data for 8 experiments are summarized in Table II.

The hydrolysis of BTG by carboxypeptidase is unusual from a kinetic point of view. Consider, for example, the second and seventh experiments

(9) Two experiments, in which the initial rate of hydrolysis of BT by carboxypeptidase at *p*H 7.5 was measured spectrophotometrically at several substrate concentrations, gave for the Michaelis–Menten[10] constant for this system K_m = (2.7 ± 0.5) × 10^{-3} *M*. Thus the calorimetric experiments were run under conditions of enzyme saturation.

(10) See, for example, R. A. Alberty, in "The Enzymes," P. D. Boyer, H. Lardy and K. Myrbäck, eds., Academic Press, Inc., New York, N. Y., 1959, p. 150.

(11) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, pp. 84–85.

(12) J. M. Sturtevant, *J. Am. Chem. Soc.*, **77**, 1495 (1955).

(13) R. J. Podolsky and M. F. Morales, *J. Biol. Chem.*, **218**, 945 (1956).

(14) J. M. Sturtevant, *J. Am. Chem. Soc.*, **63**, 88 (1941).

TABLE II

THE CARBOXYPEPTIDASE CATALYZED HYDROLYSIS OF BENZOYL-L-TYROSYLGLYCINE AT 25°

*p*H	Reaction time, min.	Initial substrate concn., $M \times 10^3$	Amount produced, μmole Glycine	Amount produced, μmole Tyrosine	Obsd. heat evolution, cal. × 10^2	Heat evolution due to BT, cal. × 10^2	$-\Delta H_{obsd.}$, cal./mole	$-\Delta H_h$, cal./mole
7.0	87	3.28	30.1	2.5	4.26	0.51	1245	1245
7.0	47	3.71	25.6	1.5	3.12	.30	1100	1100
7.0	56	4.18	33.0	1.6	4.30	.32	1205	1205
7.5	58	3.66	34.4	3.1	5.05	.63	1285	1280
7.5	72	3.66	32.8	3.1	6.12	.63	1675	1670
7.5	43	3.28	26.3	2.6	3.78	.53	1235	1230
8.0	199	3.71	40.6	5.6	7.66	1.13	1610	1590
8.0	66	4.18	33.3	1.6	4.79	0.32	1340	1320

Mean $\Delta H_h = -1330 \pm 90$ cal. per mole

in Table II. A four-fold increase in reaction time increased the yield of tyrosine by a factor of 3.7, while the splitting of the tyrosine-glycine bond was increased by a factor of only 1.6. It is unlikely that the difference in the *p*H of these two experiments had much influence on the product distribution.

It is possible, by making observations at two different wave lengths, to determine roughly the product distribution as a function of time, and several experiments of this type were performed. Optical densities were determined for BTG, a mixture of BT and G, and a mixture of B, T and G, all at 10^{-4} *M*, at 250 and 264 mμ, in solutions containing 0.05 *M* tris buffer, *p*H 7.5, with the results given in Table III. A typical kinetic experiment is illustrated in Fig. 1; in this experiment the initial concentration of BTG was 1.37×10^{-4} *M* and the enzyme concentration was approximately 4×10^{-6} *M*. The spectrophotometric blank contained the same concentration of enzyme. Interpolation of optical densities at various time intervals and computation of the yields of the products gave the results shown in Fig. 2. It was necessary to add

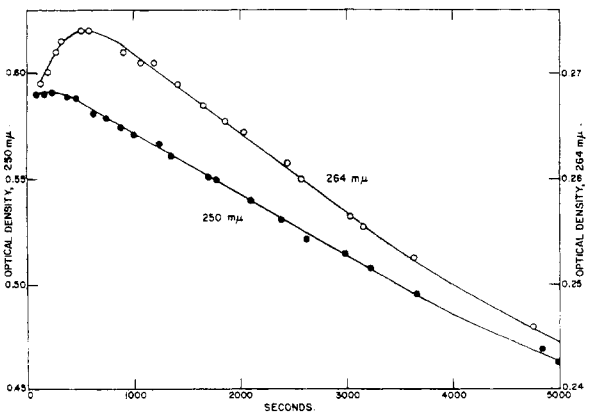

Fig. 1.—The variation in absorption at 250 and 264 mμ during the hydrolysis of benzoyl-L-tyrosylglycine by carboxypeptidase at 25° in 0.05 *M* tris buffer, *p*H 7.5; initial substrate concentration, 1.37×10^{-4} *M*; enzyme concentration, approximately 4×10^{-6} *M*.

0.003 to the readings at 264 mμ to avoid negative values for the yield of benzoic acid in the initial stage of the reaction; this small correction has very little effect on the general form of the curves in Fig. 2. Although the results of this experiment cannot be considered to be very accurate, primarily because of the relatively small differences in absorption caused by hydrolysis of the tyrosine-glycine bond in BTG, they do not permit the drawing of qualitative conclusions. It appears that after the initial splitting of the tyrosine-glycine bond has proceeded to the extent of about 30%, there is very little further attack on this bond, and further reaction consists primarily in splitting the benzoic acid-tyrosine bond in the intermediate BT. A separate experiment with 0.88×10^{-4} *M* BTG in the presence of 3.4×10^{-4} *M* benzoate, tyrosine and glycine showed that these products have no significant effect on the time course of the change of absorption at 250 mμ. We are unable to propose an explanation for the observed kinetics. It may be noted that a situation such as this could conceivably lead to an erroneous conclusion in the utilization of carboxypeptidase for end group determination.

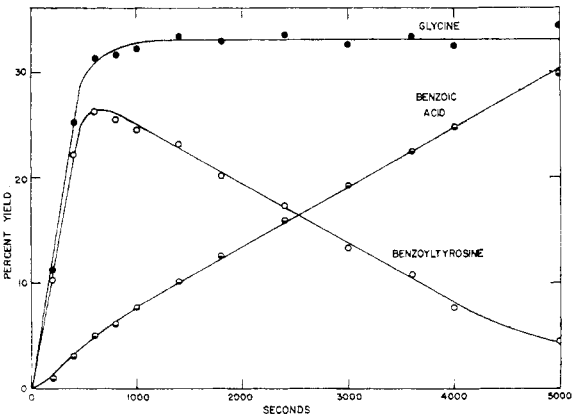

Fig. 2.—Product distribution during the hydrolysis of benzoyl-L-tyrosylglycine by carboxypeptidase, calculated from the data of Table III and Fig. 1.

TABLE III

OPTICAL DENSITIES OF 10^{-4} *M* SOLUTIONS AT *p*H 7.5

Solute	Optical density 250 mμ	Optical density 264 mμ
BTG	0.425	0.194
BT + G	.468	.228
B + T + G	.117	.137

Hydrolysis of TGA.—Leucine aminopeptidase was employed as catalyst for the hydrolysis of TGA. The enzyme (about 0.07 mg. per ml.) was

activated with 0.004 M Mg^{++}. The ionic strength was made up to 0.3 M by addition of KCl. Substrate concentrations in the range 1.3 to 2.3 $\times$ 10^{-3} M were used. Heat evolution appeared to be complete within about 1 hr., but each reaction mixture was checked with the Amino Acid Analyzer to prove completion of the reaction and absence of hydrolysis of the amide bond. The enzyme was destroyed before analysis by adding glacial acetic acid to pH 2. The data for 6 experiments are summarized in Table IV. The corrections for ionization in these experiments cannot be estimated directly. It can be shown that

$$\Delta H_h = \Delta H_{obsd.} + \alpha'(\Delta H_{TGA} - \Delta H_B) - \alpha''(\Delta H_{Tyr} - \Delta H_B) - \beta(\Delta H_{GA} - \Delta H_B) \quad (6)$$

where ΔH_h is the heat of hydrolysis of fully charged TGA to give fully charged tyrosine (Tyr) and glycinamide (GA), α', α'' and β are, respectively, the degrees of ionization of the substituted ammonium groups of TGA, Tyr and GA, and ΔH_{TGA}, ΔH_{Tyr} and ΔH_{GA} are the corresponding heats of ionization. The pK' of TGA is unknown, but it is probably approximately equal to that of GA, 7.93,[11] so that both α' and β are greater than 0.5 in these experiments. In addition, the heats of ionization of TGA and GA are unknown.[15] However, since the observed heats of hydrolysis are independent of pH, although α' and β would be expected to vary from 0.57 to 0.84, and α'' from 0.07 to 0.24, it seems reasonable to assume that $\Delta H_h = \Delta H_{obsd.}$ The uncertainty assigned to this quantity has been increased to include an estimated allowance of $\pm$120 cal. per mole for the ionization corrections.

TABLE IV

THE LEUCINE AMINOPEPTIDASE-CATALYZED HYDROLYSIS OF L-TYROSYLGLYCINAMIDE AT 25°

pH	Amount of substrate, μmole	Tyrosine formed, μmole	Heat evolution, cal. $\times$ 10^2	$-\Delta H_{obsd.}$, cal./mole
8.00	54.3	54.4	7.06	1300
8.20	31.9	32.0	4.59	1440
8.20	31.9	31.9	3.96	1240
8.20	41.7	41.8	4.76	1140
8.60	41.7	41.8	5.24	1255
8.60	41.7	41.6	5.76	1380

$\Delta H_h = -1300 \pm 150$ cal. per mole

(15) It has been found[3] that the value ΔH_{GA} = 9800 cal. per mole gives corrected values for the heat of hydrolysis of BTGA in phosphate buffer which are independent of pH with correction terms amounting to 18–85% of the observed heats.

Discussion

The substrate concentrations used are sufficiently low so that the heats of hydrolysis reported here can be equated with the standard heats of hydrolysis. In the experiments with BT, relatively large enzyme concentrations were employed, but even here the molar ratio of substrate reacted to enzyme present was always greater than 25. It is thus probable that the observed heats are not appreciably affected by the presence of the enzyme.

From the heats of hydrolysis of BT and TGA determined in this work, and the heat of hydrolysis of BTGA previously reported,[3] we may calculate the heat of hydrolysis of the benzoic acid–tyrosine bond in BTGA to be -2230 ± 210 cal. per mole. As mentioned above, there is some uncertainty as to the charge state of the TGA formed in this reaction.

Table V summarizes the peptide and amide hydrolysis heats which have been evaluated in this Laboratory. It appears that the hydrolysis of peptide bonds involves smaller enthalpy changes than have usually been assumed on the basis of earlier work.[16] The large difference in enthalpy values between peptides and amides is only in part attributable to the greater enthalpy of ionization of the ammonium ion as compared to substituted ammonium ions. Presumably the remaining difference in heats of hydrolysis of peptides and amides to form uncharged products is largely due to the greater interaction of ammonia with the solvent.

Very little can be said concerning the variation, in some cases substantial, of heats of hydrolysis with structure. It may, however, be noted that comparison of the data for BTG, TGA and BTGA, and for BT and the benzoic acid–tyrosine bond in BTGA, suggests that the presence, within a few ångström units, of a positive charge on the carboxyl side or a negative charge on the amino side of a peptide bond decreases the magnitude of the heat of hydrolysis by approximately 250 cal. per mole. This effect is in the direction expected on

TABLE V

HEATS OF HYDROLYSIS OF SYNTHETIC PEPTIDES AT 25°

Substrate	Bond hydrolyzed	$-\Delta H_h$, cal./mole	Reference
Poly-L-lysine	Lysine–lysine	1240 (average)	12
L-Tyrosylglycinamide	Tyrosine–glycine	1300 $\pm$ 150	This work
Benzoyl-L-tyrosylglycine	Tyrosine–glycine	1330 $\pm$ 90	This work
Benzoyl-L-tyrosylglycinamide	Tyrosine–glycine	1550 $\pm$ 150	3
Benzoyl-L-tyrosine	Benzoic acid–tyrosine	1980 $\pm$ 100	This work
Carbobenzoxyglycyl-L-leucine	Glycine–leucine	2110 $\pm$ 50	17
Benzoyl-L-tyrosylglycinamide	Benzoic acid–tyrosine	2230 $\pm$ 210	This work
Carbobenzoxyglycyl-L-phenylalanine	Glycine–phenylalanine	2550 $\pm$ 50	18
Benzoyl-L-tyrosinamide	Amide	5840 $\pm$ 220	18
Glycyl-L-phenylalaninamide	Amide	6220 $\pm$ 150	17
Benzoyl-L-argininamide	Amide	6650 $\pm$ 200	19

(16) See the summary by H. Borsook, *Advances in Protein Chemistry*, **8**, 127 (1953).

(17) J. M. Sturtevant, *J. Am. Chem. Soc.*, **75**, 2016 (1953).

(18) A. Dobry and J. M. Sturtevant, *J. Biol. Chem.*, **195**, 141 (1952).

(19) W. W. Forrest, H. Gutfreund and J. M. Sturtevant, *J. Am. Chem. Soc.*, **78**, 1349 (1956).

the basis of a simple consideration of electrostatic interactions. According to the Bjerrum formulation,[20] the electrostatic contribution to the free energy of hydrolysis of BTG should differ from that of BTGA by the quantity

$$\Delta(\Delta F_{\text{elect}}) = \frac{-N\epsilon^2}{rD} \qquad (7)$$

where N is Avogadro's number, ϵ is the protonic charge, D is the dielectric constant of the medium and r is the distance between the charges in the glycine zwitterion. Differentiation with respect to temperature gives

$$\Delta(\Delta H_{\text{elect}}) = \frac{-N\epsilon^2}{rD}\left(1 + \frac{\mathrm{d}\ln D}{\mathrm{d}\ln T}\right) \qquad (8)$$

Since d ln D/d ln T for water at 25° is −1.37, the enthalpy difference should be a positive quantity. With $r = 3 \times 10^{-8}$ cm., $\Delta(\Delta H_{\text{elect}}) = 520$ cal. per mole, which is about twice as large as the observed effect.[21]

(20) N. Bjerrum, *Z. physik. Chem.*, **106**, 219 (1923).

(21) It is well known that use of the bulk dielectric constant of the solvent in calculations involving dipolar ions is at best a rough approximation. See for, example, C. Tanford and J. G. Kirkwood, *J. Am. Chem. Soc.*, **79**, 5333 (1957).

[CONTRIBUTION FROM THE CLAYTON FOUNDATION BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS, AUSTIN, TEXAS]

The Inhibition of Urease by Metal Ions at *p*H 8.9

BY WILLIAM H. R. SHAW[1] AND DILIP N. RAVAL

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Urease is inhibited by these various ions arranged in order of decreasing toxicity: Cu(II), Zn(II), Ni(II), Co(II), Fe(II) Mn(II). Metal ion inhibition is non-competitive, and the inhibition index is found to depend on the square of inhibitor concentration. Reaction of the metal ions with sulfhydryl groups removed from the catalytically active sites is postulated. Correlation of toxicity with metal sulfide insolubility and with the sum of the first and second ionization potentials of the metals is reported. Comparison also shows that the ranking of metal ions in the "natural order" coördination compound stability sequence is identical with a ranking according to relative toxicity. The effect of ionic strength on the inhibition is discussed.

Introduction

Results obtained by other investigators on the inhibition of urease by metal ions have been collected from the literature and summarized in a previous paper.[2] On the basis of these data, it was found possible to arrange the common metal ions in a tentative sequence of relative inhibitory effectiveness. From a consideration of this earlier work it became apparent that several points required clarification. The dependence of metal ion inhibition on substrate concentration, for example, had not been studied; consequently, the nature of the inhibition could not be elucidated. The problem of finding a buffer that does not interact strongly with metal ions or with the enzyme was also a difficult one to solve. It had been established,[3] however, that the products of ureolytic activity bring water solutions to a constant *p*H of 8.95 ± 0.1. Thus it is possible to perform experiments at this *p*H without added buffer salts. The present work utilizes this principle.

Experimental

The preparation of urease and general experimental techniques have been described previously.[2-4] Hydrogen sulfide was not employed to stabilize the enzyme containing solutions. Best results were achieved with urease stock preparations that had aged at least one month. The metal ion solutions were made from metal sulfates of the best available grade. J. T. Baker analyzed urea was employed, and both urea and metal salts were used without further purification. All solutions were initially brought to approximately *p*H 8.5 by addition of a dilute NaOH solution. During reaction, the products of ureolytic activity buffered the solutions to a constant *p*H[5] of 8.95 ± 0.1.

In preparing the experimental solutions for one part of a typical run, two 10 ml. volumetric flasks were employed. To each of these flasks 2 ml. of enzyme stock was added. A suitable aliquot of a solution containing the metal ion under investigation was next introduced into one flask and distilled ion-exchanged water was added to bring the level in both flasks to the fiducial mark. After gentle mixing, the two solutions were allowed to stand for 2 hr. in the refrigerator[5] and 1 hr. in the water bath at a constant temperature of 25.0°. For the kinetic runs, 1 ml. of the metal containing enzyme solution was added rapidly with mixing to a test tube holding 25 ml. of a urea solution. Also 1 ml. of the metal-free enzyme solution was similarly introduced into a second test tube containing an identical urea solution. The concentrations of metal ion and substrate in these reaction mixtures were calculated and employed in reporting results. After a measured time, reaction was stopped by addition of acid, and the concentration of ammonium ion produced was determined by the usual spectrophotometric technique.[4] The rate of urea hydrolysis in the metal containing reaction mixture was recorded as the inhibited rate, V_i. The uninhibited rate, V_u, was calculated from the urea hydrolyzed in the metal-free reaction mixture. All experiments were performed at 25°

Results

The metal ions here listed, arranged in order of decreasing toxicity, were found to inhibit urease: Cu(II), Zn(II), Ni(II), Co(II), Fe(II), Mn(II). Results obtained with Fe(II) are least reliable since some oxidation to Fe(III) during the course of the experiments probably took place. For each ion, the ratio of inhibited rate, V_i, to uninhibited rate, V_n, at a fixed inhibitor concentration was measured at various substrate concentrations (Table I). These results indicate that the inhibi-

(1) Department of Chemistry The University of Georgia, Athens, Georgia.

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(4) (a) W. H. R. Shaw and D. N. Raval, *ibid.*, **83**, 2866 (1961); (b) G. B. Kistiakowsky, P. C. Mangelsdorf, Jr., A. J. Rosenberg and W. H. R. Shaw, *ibid.*, **74**, 5015 (1952).

(5) Experiment established that the degree of inhibition reached a constant value after the enzyme had been in contact with the metal ion solution for approximately ten minutes.