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The Heat of Hydrolysis of Poly-L-lysine[1]

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The trypsin-catalyzed hydrolysis of poly-L-lysine has been studied calorimetrically. The heat of hydrolysis in tris-(hydroxymethyl)-aminomethane (THAM) buffer at *p*H 7.6 is $\Delta H_{298} = -320 \pm 7$ cal. per residue. Since THAM has a heat of ionization (+ 10,930 cal. per mole) approximately equal to that of the α-amino group of lysine peptides, this heat can be assigned to the hydrolysis reaction which gives fully charged products. Experiments in unbuffered solutions using NaOH to hold the *p*H constant indicate the hydrolysis of 0.26 bond per residue, so that the average heat of hydrolysis is −1240 cal. per bond hydrolyzed.

Introduction

Calorimetric measurements of the heats of hydrolysis of dipeptides have given values[2] ranging from −1550 to −2550 cal. per mole of peptide bond hydrolyzed. The peptide bonds in a synthetic polypeptide might be considered to be more nearly typical of those found in proteins than are the bonds in simple dipeptides. Indeed, evidence[3] has been presented which indicates that in the solid state such polypeptides have in large part a helical structure similar to that proposed by Pauling and Corey[4] for globular proteins, and it appears[5] that this structure may be maintained in solution. If the polypeptide contains only one type of amino acid residue, all the peptide bonds present are identical from a gross chemical point of view, so that a reasonable approximation to the heat of hydrolysis of this type of bond in a protein should be obtainable from experiments performed on the polypeptide.

Waley and Watson[6] have studied in detail the hydrolysis of poly-L-lysine hydriodide catalyzed by trypsin at *p*H 7.6. They found, by using kinetic methods and quantitative paper chromatography, that the reaction can be divided into two phases; in the first phase di-, tri- and tetralysine are rapidly formed, while higher peptides are formed and then decomposed; in the second much slower phase tetralysine is decomposed mainly to dilysine. In view of these results, it appeared that calorimetric experiments on polylysine could be interpreted with sufficient detail to yield a significant average heat of hydrolysis. This paper reports the results of such experiments.

Experimental

Poly-L-lysine hydrobromide, prepared by the N-carboxyanhydride method, was obtained through the kindness of Drs. S. G. Waley and J. Watson.[6] According to their analytical data, this material contained 37.2% bromine and had an average degree of polymerization of 70. These figures indicate 0.96 mole of HBr per residue, and an average residue weight of 207.

Trypsin was prepared from British Drug Houses material by two trichloroacetic acid precipitations. The enzyme was preserved in solution at *p*H 3 in the refrigerator. Its specific activity (benzoyl-L-arginine ethyl ester as substrate) was indicative of a rather pure preparation.

Tris-(hydroxymethyl)-aminomethane (THAM), obtained from Commercial Solvents Co., was recrystallized several times from 95% ethanol and air dried. This material was found by the micro-Kjeldahl method[7] to contain 11.28% N (theory 11.56% N) and lost 0.2% of its weight after being heated to 75° for 24 hours.

The calorimetric procedure has been described[8] previously. All measurements were carried out at 25.00 ± 0.05°. *p*H measurements on the solutions used in the calorimetric experiments were made with a Cambridge Model R meter, taking the *p*H of a 0.10 *M* acetic acid–0.10 *M* sodium acetate solution to be 4.65; in the kinetic experiments on unbuffered solutions, a Beckman Model G meter was employed.

Results

Heat of Ionization of THAM.—Waley and Watson[6] have shown that protons are liberated during the hydrolysis of polylysine at *p*H 7.6. Since these protons are taken up by the buffer with an accompanying heat effect, it is necessary to know the heat of ionization of the buffer acid in order to be able to make appropriate corrections to the observed heats of hydrolysis. The heat of ionization of THAM·H$^+$ was obtained from a series of experiments in which half-neutralized 0.05 *M* THAM was mixed in the calorimeters with an equal volume of dilute HCl (5×10^{-5} to 10^{-3} *M*). The final solutions thus had an ionic strength of 0.013 *M*. Since the ionization reaction

$$(HOCH_2)_3CNH_3^+(aq) + H_2O = (HOCH_2)_3CNH_2(aq) + H_3O^+; \Delta H_1 \quad (1)$$

involves no separation of charges, it may be assumed that the heat of ionization is practically independent of ionic strength.

In these experiments, the observed heat evolution, after appropriate calorimetric corrections, was due to the formation of THAM·H$^+$ from THAM, the dilution of the buffer, and the dilution of the HCl, it being unnecessary with these dilute solutions to apply a vapor pressure correction. The contribution from the dilution of the HCl can be shown to be negligible from the known apparent molal heat content[9] of the acid in water. In experiments of this type the fraction of the HCl which does *not* react with the buffer base is $(\delta/a_0) \approx (4K'_B/b_0)$, where a_0 is the initial molarity of HCl and b_0 is the total molarity of buffer. K_B' is the apparent equilibrium constant for reaction (1) and is approximately 10^{-8} mole per liter. It is thus

(1) Presented before the Division of Biological Chemistry of the American Chemical Society, New York, N. Y., September, 1954.

(2) J. M. Sturtevant, THIS JOURNAL, **75**, 2016 (1954).

(3) See, for example, C. H. Bamford, L. Brown, A. Elliott, W. E. Hanby and I. F. Trotter, *Nature*, **169**, 357 (1953).

(4) L. Pauling and R. B. Corey, THIS JOURNAL, **72**, 5349 (1950); *Proc. Nat. Acad. Sci.*, **37**, 241 (1951).

(5) A. Elliott, *Proc. Roy. Soc.* (*London*), **221A**, 104 (1953); P. Doty, A. M. Holtzer, J. H. Bradbury and E. R. Blout, THIS JOURNAL, **76**, 4493 (1954).

(6) S. G. Waley and J. Watson, *Biochem. J.*, **55**, 328 (1953).

(7) Nitrogen determinations were performed by Mr. Robert Miller, to whom the author is indebted.

(8) A. Buzzell and J. M. Sturtevant, THIS JOURNAL, **73**, 2454 (1951).

(9) J. M. Sturtevant, *ibid.*, **62**, 3265 (1940).

evident that the reaction can be assumed to be complete.

The results of these experiments are summarized in Fig. 1, in which there is plotted the calories liberated per liter of solution *vs.* the moles of THAM neutralized per liter. The data were fitted to a straight line by the method of least squares. From the slope of this line one obtains $\Delta H_1 = +10{,}930 \pm 100$ cal. per mole, where the uncertainty interval includes an allowance for uncertainties in calorimetric calibrations. It may be pointed out that this result is unaffected by any impurities in the THAM which do not react with dilute HCl at *p*H 8. From the intercept of the line the heat of dilution of the half-neutralized buffer is found to be −29.2 cal. per mole of total buffer.

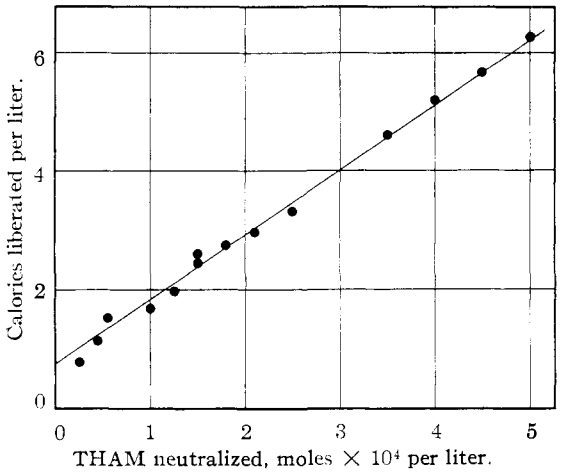


Fig. 1.—The heat of reaction between half-neutralized 0.05*M* THAM and hydrochloric acid.

At 25°, the *p*H of the half-neutralized 0.05 *M* buffer is 8.08. The heat of ionization indicates this *p*H should decrease 0.025 unit per degree.

Calorimetric Experiments.—In these experiments, equal volumes of enzyme and substrate solutions were mixed in the calorimeters after a period of approximately 15 hours had elapsed since filling to allow thermal equilibration. The solutions contained 0.01 *M* $CaCl_2$ to decrease the rate of self-digestion of the trypsin during this period, and were buffered with THAM–THAM·HCl mixtures. Ionic strengths were calculated from the amount of HCl added to give the desired *p*H, and include the contribution from the $CaCl_2$.

The heat evolution in these experiments was found to be linear with time for a considerable period, and then gradually to level off to a slow drift which could not in any way be distinguished from calorimetric drift. The fact that these drifts were in each case of the magnitude usually observed in our apparatus indicates that any long continuing heat evolution or absorption must have been at a rate much lower than observed in the initial stage of the reaction. We have therefore presumably only observed the first phase of the reaction as described by Waley and Watson.[6]

If one assumes the slow thermal drift observed after the first phase of the reaction to be operative during the whole experiment, and deducts this drift from the observed heat evolution, one obtains a time course for the heat evolution illustrated for a typical run in Fig. 2. The extent of reaction plotted in this figure was calculated in the following manner. After subtraction of the drift from the integrator[8] readings, values, q, were obtained which approached a limiting value q_∞. Extrapolation of $(q_\infty - q)$ to $t = 0$, using in this case a linear plot because the heat evolution followed apparent zero-order kinetics, gave $(q_\infty - q_0)$, the total heat evolved not including the heat of mixing the reactants. Then the fraction unreacted was computed as $(q_\infty - q)/(q_\infty - q_0)$. The results of five calorimetric experiments are summarized in Table I, the total heat evolved, $q_\infty - q_0$, being given in calories per residue. The data from the fifth run in the table are plotted in Fig. 2.

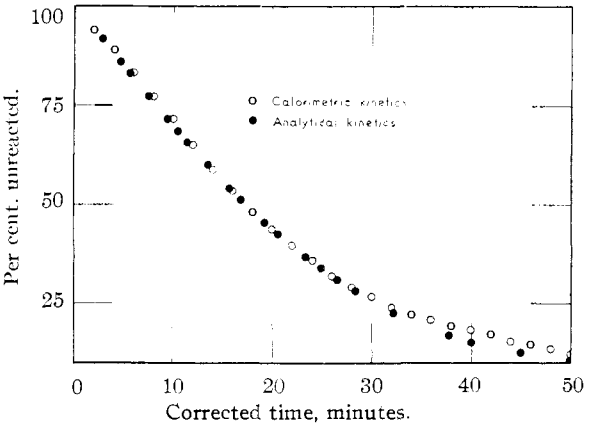


Fig. 2.—The tryptic hydrolysis of poly-L-lysine at *p*H 7.6: calorimetric experiment in buffered solution, ○; kinetic experiment in unbuffered solution, ●. The time scale for the latter experiment has been multiplied by a factor to bring the two experiments into coincidence at 50% completion.

Kinetic Experiments.—In order to estimate the number of bonds hydrolyzed in the calorimetric experiments, the last three runs reported in Table I were performed in unbuffered solutions in which the *p*H was held constant by the addition of 1 *M* NaOH from a microburet, the amount of alkali added being recorded as a function of the time. The solutions contained 0.075 *M* NaCl and 0.01 *M* $CaCl_2$. In these experiments, in which no special precautions to protect the solutions from atmospheric CO_2 were taken, a slow uptake of NaOH was observed after

TABLE I

TRYPTIC HYDROLYSIS OF POLY-L-LYSINE HYDROBROMIDE AT 25°

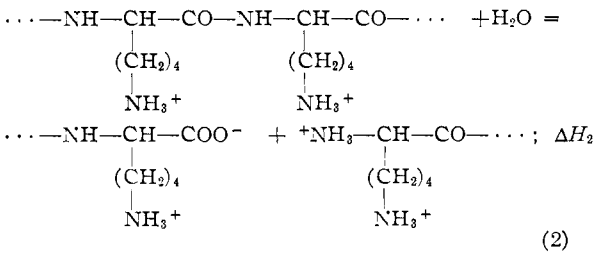
Polymer concn., mg./ml.	Buffer concn., moles/l.	Ionic strength, moles/l.	$-\Delta H_{obs}$, cal. per residue	NaOH to hold *p*H constant, moles/residue
0.99	0.10	0.11	316	...
0.99	.10	.11	324	...
1.99	.10	.11	298	...
1.99	.10	.11	321	...
1.56	.02	.04	323	...
1.08	0	.11	..	0.189
0.74	0	.11	..	.183
2.27	0	.11	..	.193
		Mean	320 ± 7	0.188 ± 0.004

completion of the rapid phase of the reaction which, however, was not significantly more rapid than that observed before addition of the enzyme and which amounted to about 1% of the maximum rate observed immediately after addition of the enzyme. The data in these experiments were therefore corrected for the slow drift in the same manner as the calorimetric data.

It was not feasible to make a direct comparison of the calorimetric and alkali kinetics because of the loss of enzyme activity during the thermal equilibration periods preceding the calorimetric runs. An indirect comparison can be made by multiplying the time scale of an alkali run by a factor to bring it into coincidence with a calorimetric run at 50% apparent completion. When this is done (Fig. 2), it is seen that there is close agreement between the kinetics indicated by the two methods throughout the course of the reaction. The data for the last run in Table I are presented in Fig. 2.

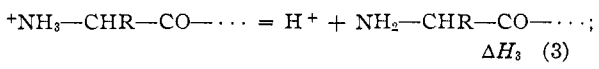
Discussion

In previous work[2] with the dipeptides we have reported the heat of hydrolysis to give *fully charged* products. It is therefore of interest to attempt to evaluate from the present results the average heat of the reaction



(2)

Since protons are liberated in the actual reaction at *p*H 7.6, it is evident that some of the product molecules undergo ionization



(3)

In the buffered calorimetric experiments all protons formed in this way are taken up by reversal of reaction 1. To make a complete correction for these ionization reactions it would be necessary to know in detail the relative amounts of the various lysine peptides formed, and their ionization constants and heats of ionization. These data, in particular the heats of ionization, are not available. Fortunately, the heat of ionization of THAM is probably nearly the same as that of lysine peptides. The apparent heats of ionization,[10] obtained from electromotive force measurements, of the α-amino groups of lysine and lysyllysine are, respectively, 11,600 and 12,700 cal. per mole. The fact that the apparent heat of ionization of the amino group of alanine is reported[10] as 11,600 cal. per mole while calorimetric measurements[11] give 10,960 cal. per mole suggests that the figures for lysine and lysyllysine may be somewhat too large. In any case, it seems best to assume cancellation of the heats due to reaction 3 and those due to reaction 1, and thus to assign the observed heats to reactions of the type (2).

Waley and Watson[6] estimated from their chromatographic experiments the initial rates of formation of di-, tri-, tetra- and pentalysine for a certain enzyme concentration. These totaled 5.0×10^{-6} moles/l. sec. From the known pK' values[12] for these compounds, they then calculated what part of the initial rate of uptake of NaOH in kinetic experiments was due to the formation of each of the products, on the assumption that the pK' of a large polymer molecule is not changed when a small peptide is split off. They concluded that 3.38×10^{-6} mole/l. sec. was due to these products out of a total initial rate of 6.06×10^{-6} mole/l. sec. It may be further noted[13] that the remaining products formed in the early stage of the reaction will be mainly hexa- to octalysine, with degrees of ionization at *p*H 7.6 ranging from about 0.78 to 0.83. If one takes 0.80 as an average, then the initial rate of splitting bonds to form these products will be $(6.06 - 3.38) \times 10^{-6}/0.80 = 3.35 \times 10^{-6}$ mole/l. sec. Thus a total rate of bond splitting of 8.35×10^{-6} mole/l. sec. is accompanied by a proton liberation of 6.06×10^{-6} mole/l. sec., so that at the start of the reaction there is approximately 0.73 mole of alkali taken up per bond split. This figure is not very sensitive to the assumptions made in its evaluation. For example, if it is assumed that all products above pentalysine have the limiting pK' estimated for polylysine from the data of Waley and Watson,[12] the ratio becomes 0.75.

If our procedure of bringing the calorimetric and alkali data into coincidence at the point of apparent half completion is valid, it may be concluded, since both types of experiment give apparent zero-order kinetics, that at the start of the reaction there are 1700 cal. evolved per mole of proton liberated (320/0.188 = 1700; *cf.* Table I). Therefore, as a result of the argument outlined above, the average heat per bond hydrolyzed is $\Delta H_2 = -1240$ cal. This figure is subject to considerable uncertainty, largely because of the assumptions and numerous experimental quantities involved in its derivation. All of Waley's and Watson's experiments were performed in 0.083 M KCl; however it seems unlikely that the various pK' values would be significantly different in 0.11 M ionic strength. In this connection it may be noted that the calorimetric run at 0.04 M ionic strength agreed well with the others.

It cannot be definitely stated that the value -1240 cal. is significantly smaller than the lowest value, -1550, previously observed[2] for the hydrolysis of a dipeptide. The latter figure is for benzoyl-L-tyrosylglycinamide. It would be expected that the separation of positive charges taking place in the hydrolysis of polylysine would introduce a small positive contribution to ΔH_2, provided the temperature coefficient of the effective dielectric constant pertinent to this system is similar to that of pure water. Also, if intramolecular hydrogen bonds are broken in the hydrolysis of the polypeptide, the

(10) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 89.

(11) J. M. Sturtevant, THIS JOURNAL, **64**, 762 (1942).

(12) S. G. Waley and J. Watson, *J. Chem. Soc.*, 475 (1953).

(13) J. Watson, personal communication.

over-all ΔH should be less negative than in the case of a dipeptide. It is unfruitful to attempt to argue these points in any more detail at the present time.

The chief significance of the result reported here is that it constitutes further indication that the heat of hydrolysis of a typical peptide bond in a protein is a smaller negative quantity than has usually been supposed, probably lying between -1000 and -2000 cal. per mole.

Acknowledgments.—The author is indebted to Drs. H. Gutfreund and W. Forrest for helpful discussions. This research was aided by grants from the National Science Foundation and the United States Public Health Service.

NEW HAVEN, CONN.

[CONTRIBUTION FROM THE VIRUS LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY]

The Effect of Pressure on Sedimentation, and Compressibility Measurements in the Ultracentrifuge[1]

BY P. Y. CHENG AND H. K. SCHACHMAN

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For most ultracentrifuge work the large pressures, which may amount to several hundred atmospheres, produced at the bottom of the centrifuge cells cause so little change in the physical properties of the solute or the solvent that the effect of pressure on ultracentrifugal analysis is almost negligible. If, however, the solvent has a density almost equal to that of the sedimenting particles, then the change in density of the solvent throughout the cell can cause marked variations in the effective centrifugal force on the particles in different regions of the cell. In studies on polystyrene latex particles in a solvent of almost equal density, the change in density of the solvent throughout the cell was sufficiently large to cause the particles to float in the high pressure part of the cell while the particles at the top of the cell were sedimenting. Thus sedimenting and floating boundaries could be obtained in a single run and the rate of movement of each boundary could be measured. The flotation rate of the particles in a D_2O–H_2O mixture was, however, much less than that expected from considerations based on the size and density of the particles and the compressibility of the solvent. From the difference between the observed and expected flotation rates, the coefficient of compressibility of the particles was calculated and found to be in good agreement with other compressibility data on solid polystyrene. Other areas of ultracentrifugal analysis in which there is a significant pressure effect are discussed. The application of the ultracentrifuge to the measurement of the compressibility of solids is described.

Introduction

As Svedberg and Pedersen[2] pointed out, the forces developed in modern ultracentrifuges cause sufficient compression of the liquid in ultracentrifuge cells to produce variations in both the density and viscosity of the liquid throughout the cell. These changes in viscosity and density must be considered in the quantitative evaluation of sedimentation data since particles in different regions of the cell are subject to different forces as a result of the pressure gradient. For most systems, and especially those employing aqueous solvents, the density and viscosity variations are almost negligibly small, but increasing use of ultracentrifugal techniques in recent years has encompassed substances for which these compression effects can be very important.

Mosimann and Signer[3] considered the effect of pressure on the viscosity of the organic solvent, acetone, which they employed in a study of the sedimentation of nitrocellulose. They cited data to show that the viscosity of acetone increased by 28% from the meniscus to the bottom of the cell and they made appropriate corrections to the sedimentation velocity observed at different parts of the ultracentrifuge cell. From data on the compressibility of acetone they showed, further, that the buoyancy term in the Svedberg equation[2] varied only 2.4% throughout the cell due to the increase in density of the acetone under the same experimental conditions.

In a recent study on polystyrene latex particles (PSL)[4] in solvents of different densities we have observed that the effect of pressure on the buoyancy term is not negligibly small. In fact, the effect was sufficiently large to justify further study of this problem, and this communication presents the results of this study. As we will show, the sedimentation rate of the PSL is markedly affected by the change in density of the solution due to compression. Furthermore, the magnitude of the differences in sedimentation rate in different parts of the cell can be used, under certain circumstances, to determine the compressibility of the sedimenting solute particles. Though the technique is, at present, limited to certain rather large particles, it is fundamentally a differential method, based on differences in compressibility between solute and solvent, and therefore capable of high accuracy.

Materials and Methods

The suspensions of PSL were the same as those used previously[4] in studies on the validity of Stokes' law of sedimentation and the Einstein viscosity equation. It appears that the PSL are rigid, impermeable spheres of diameter 2640 Å., and density 1.0520 g./cc. Sedimentation coefficients were determined from plots of the logarithm of boundary position, as distance x in cm. from the axis of rotation, *versus* time t in seconds. Boundary positions were determined, as before,[4] by the change in blackening on the photographic plate. This change in blackening on the plate was generally very abrupt and was due to the variation in turbidity in the region between solvent and solution. Because of the large size of the PSL the scattering of light by the

(1) (a) This work was supported by grants from the Corn Industries Research Foundation, Lederle Laboratories and the Rockefeller Foundation. (b) Presented before the Division of Polymer Chemistry at the 126th meeting of the American Chemical Society, September, 1954.

(2) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford University Press, 1940.

(3) H. Mosimann and R. Signer, *Helv. Chim. Acta*, **27**, 1123 (1944).

(4) P. Y. Cheng and H. K. Schachman, *J. Polymer Sci.*, in press.