intensity is decreased tremendously. Throughout the rest of the spectra there are no great differences between the spectra of the two states of morpholine other than the usual vapor shifts and general broadening of bands in the vapor spectrum.

The greatest differences between morpholine and dioxane spectra exist in the same two regions discussed above. Dioxane liquid has no band at 3300 but does produce a band at 3500 cm.$^{-1}$ which morpholine does not show. However, Errera[12] has pointed out that vapor or pure dry liquid dioxane do not produce this band. The determinations by McKinney, *et al.*, were made with open cells so that absorption of water by hygroscopic dioxane was probable. Errera's statements have been checked briefly by determining the dioxane vapor spectrum in this region (Fig. 2); the 3500 cm.$^{-1}$ band is not found. The morpholine, which is also hygroscopic, may possibly have taken up water but pains were taken to prevent this occurrence by keeping the sample desiccated and filling the sealable cells in a stream of dry nitrogen. Other differences in the two spectra occur at: (a) the long wave length region in which dioxane vapor exhibits no band comparable to the 809 and 768 cm.$^{-1}$ morpholine bands, (b) the 1200–1300 cm.$^{-1}$ region in which four strong bands occur in morpholine to only two in dioxane, and (c) the band at 1649 cm.$^{-1}$ in morpholine which is attributable to NH bending.

The Raman spectra values for morpholine in the table show close comparison with the infrared values, particularly at longer wave lengths.

(12) Errera, *Physica*, **4**, 1097 (1937).

Summary

The infrared absorption of liquid and gaseous morpholine has been investigated between 1.4 and 15.0 μ with a rock salt prism spectrometer. Several important differences exist between the two spectra and indicate that intermolecular association probably of the van der Waals type, is quite strong in the liquid state. Comparison with liquid and gaseous dioxane enables detection of bands produced in morpholine by the substitution of the NH group for one of the ether linkages in dioxane, and indicates the weaker intermolecular association present in the latter. Frequencies found for morpholine compare closely with those of the Raman spectrum.

PITTSBURGH, PA. RECEIVED OCTOBER 17, 1946

[CONTRIBUTION FROM STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY]

The Heat of the Inactivation of Pepsin[1]

BY MARGARET BENDER[2] AND JULIAN M. STURTEVANT

The general problem of the structure of proteins is of the greatest importance in biology, as evidenced by the tremendous literature on this subject. Relatively little information is available concerning the energetics of reactions involving these important substances. It is with the hope of gaining new insight into the protein problem that we have undertaken calorimetric measurements of the heats of protein and allied reactions. The present paper reports data obtained three years ago on the inactivities of pepsin by alkali, using a calorimeter described in previous papers.[3] We have chosen this reaction as a first point of attack chiefly because the very careful work of Steinhardt[4] has demonstrated that it is characterized by relatively clean-cut first order kinetics. The present work is to be considered as preliminary in character; we feel that the results, though not as extensive as might be desired, are nevertheless of considerable interest. Certain weaknesses in our apparatus which appeared in the course of this work have led us to propose an extensive redesign before further work in this field is undertaken.

Experimental

Preparation of Pepsin.—Parke, Davis 1:10,000 pepsin was purified and crystallized essentially as described by Northrop.[5] After four crystallizations the pepsin was dialized against distilled water until no further sulfate or chloride was removed, and the pepsin suspension was then lyophilized. Dried pepsin was weighed out for each run.

Analytical Methods. Nitrogen Determinations—All nitrogen determinations were made by the Kjeldahl method, in some cases macro[6] and in some semimicro. Non-protein nitrogen (NPN) was estimated in the filtrate from the treatment of a sample with 25 ml. of 0.3 *M* trichloroacetic acid after heating to boiling. Protein nitrogen (PN) was either taken by difference or determined in the trichloroacetic acid precipitate with the application of a suitable blank.

Determination of Peptic Activity.—The enzymatic activity of the pepsin solutions was determined essentially as described by Anson.[7] The tyrosine[8] formed by digestion of hemoglobin by the pepsin under carefully standardized conditions was estimated colorimetrically. Our

(1) Calorimetric Investigations of Organic Reactions. V. Previous paper in this series. THIS JOURNAL, **64**, 762 (1942).

(2) Present address: Madison, Wisconsin.

(3) J. M. Sturtevant, *J. Phys. Chem.*, **45**, 127 (1941); THIS JOURNAL, **63**, 88 (1941); **64**, 762 (1942).

(4) J. Steinhardt, *Kgl. Danske Videnskabernes Selskab. Math. fys. Medd.*, **XIV**, 11 (1937).

(5) J. H. Northrop, "Crystalline Enzymes," Columbia University Press, New York, N. Y., 1939, pp. 128 ff.

(6) The macro Kjeldahl determinations were performed at the Connecticut Agricultural Experiment Station through the courtesy of E. M. Bailey, to whom the authors wish to express their indebtedness.

(7) M. L. Anson, *J. Gen. Physiol.*, **22**, 79 (1938).

(8) Strictly speaking, one estimates unspecified products formed in the digestion which are not precipitated by trichloroacetic acid and which give the same color as tyrosine solutions.

use of an Evelyn photoelectric colorimeter (with a 660 filter) in place of a visual colorimeter necessitated some changes in the tyrosine determinations. Frequent calibration of the tyrosine method with a series of dilutions of a standard solution of tyrosine showed the method to be accurately logarithmic, if care was taken to maintain the trichloroacetic acid concentration at the value met with in the hemoglobin digestions. Colorimeter readings were always made ten minutes after the dropwise addition of the Folin–Ciocalteu[9] phenol reagent was half completed.

Peptic digestions were run as described by Anson,[7] except that no tyrosine was added to the blank filtrates. Each stock solution of pepsin was analyzed in two main dilutions, and two to five sub-dilutions of each of these were used in the individual digestions. Most of the digestions were carried out at 25°.

The data for each set of dilutions of a stock solution were found to follow, within experimental error, the quadratic equation

$$PU/T = 0.584 + 164T \qquad (1)$$

where PU and T are, respectively, the pepsin units present in the diluted sample and the millimoles of tyrosine found in the digestion filtrate. The calibration curve given by Anson corresponds approximately to values of the constants in this equation of 0.52 and 200.

Duplicate analyses of pepsin stock solutions indicated a precision of ±3 to 4% in the peptic activity determinations. Eighteen different stock solutions gave a mean value of 0.175 for the PU per mg. PN, with an average deviation of ±6.2%. Northrop[10] gives the value 0.20 for his earlier preparations, and 0.22–0.26 for his later preparations, and states that the difference is probably due in part to differences in the hemoglobin method.

Determination of *p*H.—All *p*H's were measured with a Beckmann glass electrode *p*H meter at the same temperature as that used in the rate and heat measurements. In order to make our data comparable with those of Steinhardt[4] we have employed the same *p*H scale as he did, defined by assigning the *p*H 2.34 to a solution 0.005 *M* in hydrochloric acid and 0.095 *M* in sodium chloride, at the temperatures at which we made measurements. *p*H determinations were reproducible to ±0.02 *p*H unit.

Calorimetric Procedure.—The procedure for the calorimetric experiments can be illustrated by a typical run (Run 6, Table II). A concentrated pepsin solution was prepared by suspending dry pepsin in water and adding $N/5$ potassium hydroxide slowly with stirring[11] until the pepsin dissolved. The ionic strength was adjusted by adding potassium chloride to approximately 0.15, and a weighed amount of the solution introduced into the dilution cup. The *p*H of this solution was 4.6, at which value the inactivation is immeasurably slow.

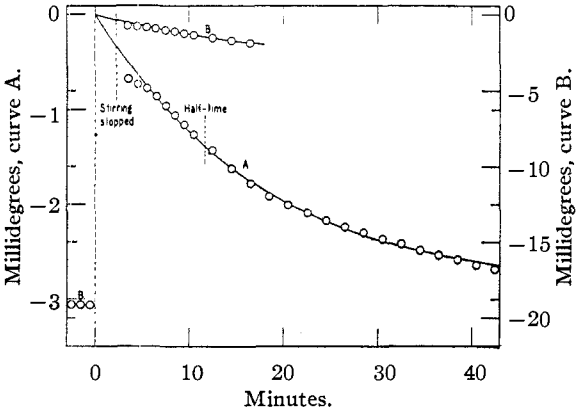

Fig. 1.—Observed (circles) and calculated (curves) temperatures for Run 6, Table II.

The calorimeter can contained 0.05 *M* *p*-nitrophenol[12] buffer of ionic strength 0.15. Sufficient buffer was present in all runs to give a minimum value of 20 to the ratio (moles of *p*-nitrophenolate ion)/(moles of protein). The reasonably good first order kinetics observed (see below) can be taken as indication that the solutions were well buffered in all the experiments.

The method of the calorimetric observations has been described in previous publications.[3] The temperature readings (after correction for deviation from strictly adiabatic conditions) are plotted on two different scales in Fig. 1; the set A (left-hand ordinates) show the *decrease* in temperature during the reaction, and the break in the set B (right-hand ordinates) shows the relatively large *increase* in temperature accompanying the mixing of the protein at *p*H 4.6 with the buffer at *p*H 6.56.

Temperatures at times $(t - 12)$, t and $(t + 12)$ minutes after the initiation of the reaction were interpolated by means of a smooth curve through the experimental values. Six sets of interpolated temperatures gave the rate constants,[3] k, and total temperature changes given in Table I. The constancy[13] of these quantities is good evidence that the reaction is first order. The total temperature change during the reaction was only 0.00297°; an error of half a minute in the time of initiation of the reaction would produce an error of 3% in this figure.

The curve A in Fig. 1 was calculated from the average rate constant and initial and final temperatures derived from the interpolated temperatures. The observed temperatures, between 25 and 90% completion of the reaction, deviate from the curve on the average ±20 microdegrees (±0.8% of the total temperature change).

The heat capacity of the calorimeter and its contents was determined at the end of the experiment. The value obtained, 0.4105 ± 0.1% joule per microvolt, gives 1.448 joules for the energy *absorbed* during the reaction.

TABLE I

THE INACTIVATION OF PEPSIN AT *p*H 6.25 AND 25° (RUN 6, TABLE II)

t, min.	18.5	19.5	20.5	21.5	22.5	23.5
k, com. logs., min.	0.0266	0.0263	0.0258	0.0256	0.0250	0.0251
Total temp. change, microvolts	3.49	3.53	3.53	3.54	3.55	3.53

The *p*H of the solution after removal from the calorimeter was found to be 6.25 (25°). This was taken to be the *p*H at which the reaction occurred. Peptic activity and protein and non-protein nitrogen determinations were carried out on the original pepsin solution by the methods described above.

Experimental Results

In Table II are listed the pertinent data for eleven experiments, three at 15° and eight at 25°. The headings in the table are self-explanatory. The *p*H's of the buffer and final solutions were measured at the temperature of the reaction, and

(9) The reagent described by O. Folin and V. Ciocalteu [*J. Biol. Chem.*, **73**, 627 (1927)] was diluted with 2 volumes of water.

(10) Ref. 5, pp. 18 ff.

(11) During this process 22% of the activity of the dry pepsin was lost. This figure varied between 0 and 40% in the other runs. However, in spite of the loss of total activity, the activity in terms of protein nitrogen was essentially constant in all solutions prepared.

(12) Classical dissociation constants were calculated from the known compositions and observed *p*H's of the various buffer solutions. The values obtained in this way for the *p*K are 7.19 ± 0.02 at 15°, 7.06 ± 0.01 at 25°, and 6.92 ± 0.02 at 35°. These values give for the heat of ionization at 25° + 23,000 joules per mole. Steinhardt found the *p*K at 25° and 0.1 ionic strength to be 6.95.

(13) The small downward trend in k probably is due to the slight deviations in adiabatic conditions between the experiment and the rating period, since it was in the opposite direction in some runs and negligible in others.

TABLE II

THE RATE AND HEAT OF INACTIVATION OF PEPSIN

Heat values in international joules; rate constants calculated using common logarithms, time in min.

	Pepsin solution in cup											Inactivation						
Run	Initial temp., °C.	Pepsin prepn.	g.	PU/g.	Mg. NPN/g.	Mg. PN/g.	pH (room temp.)	pH Buffer	pH Final	Heat change, mixing, j.	Rate constant, 100k	Av. dev. in k (%)	$10^3 \Delta t$, °C.	Av. dev. %	Heat change, j.	Heat of inactivation j./PU	Heat of inactivation j./g. PN	% Reaction used in calculations
1	15.03	F	8.791	2.13	7.5	12.7	4.7	6.90	6.62	−8.65	8.40	±3.6	−0.92	±2.2	0.451	0.0241	4.04	58–92
2	15.01	F	8.840	2.51	9.1	12.8	4.7	6.90	6.66	−9.24	9.73	±3.2	−1.35	±1.5	.665	.0300	5.89	64–96
3	15.01	F	8.240	1.87	6.8	10.8	4.8	6.97	6.77	−6.41	10.44	±2.4	−1.75	±2.5	.856	.0555	9.60	69–97
4	24.72	C	8.512	1.45	7.1	9.0	4.6	6.47	6.20	−6.41	2.88[a]	±5.2	−1.80	±0.2	.879	.0715	11.5	29–80
5	25.02	C	8.945	2.64	7.2	14.5	5.0	6.46	6.23	−5.12	2.57	±3.8	−3.71	±0.1	1.827	.0774	14.1	13–82
6	25.01	E	8.171	2.21	7.0	11.8	4.6	6.56	6.25	−9.33	2.57	±3.3	−2.97	±1.0	1.448	.0800	15.0	32–88
7	24.71	E	8.500	1.96	...	..	4.8	6.51	6.26	−6.88	2.12[a]	±7.0	−2.71	±0.5	1.320	.0790	...	22–84
8	25.00	C	5.751	2.40	6.8	13.8	5.0	6.43	6.29	−3.98	3.88	±0.2	−2.85	±0.2	1.365	.0989	17.2	27–86
9	25.01	E	8.351	1.93	7.3	11.6	4.8	6.60	6.36	−6.36	4.13	±5.4	−4.51	±1.6	2.201	.1367	22.6	36–87
10	25.01	C	7.651	1.61	4.6	9.4	5.1	6.51	6.385	−2.85	5.15	±0.6	−3.46	±0.4	1.688	.1372	23.5	26–88
11	25.02	E	8.645	1.98	...	..	4.8	6.64	6.43	−5.25	5.46	±0.9	−4.66	±0.2	2.280	.1333	...	43–91

[a] Corrected to 25° using the rough temperature coefficient at constant pH observed in this work.

the velocity constants (except for runs 4 and 7 as indicated) and heat values refer to this temperature. Six additional runs at 25° were discarded, 4 because of calorimetric failure, and 2 because of excessive drifts in k's and calculated temperature changes. Seven experiments were performed at 35°. The data obtained in these experiments are not given in this paper; in some of these runs there was evidence of mold formation in the calorimeter and we therefore feel it advisable to check these results before publishing them.

Analytical Reaction Rate Determination.—As pointed out below, our rate constants differ widely from those reported by Steinhardt.[4] It therefore seemed advisable to run some experiments in which the reaction was followed by measuring the loss of peptic activity. In these experiments pepsin solution and 0.05 M p-nitrophenol buffer were mixed, and at desired intervals 2-ml. samples were removed and run into about 75 ml. of ice-cold dilute acetic acid buffer (pH 4.75) in a 100-ml. volumetric flask. Buffer was added to the mark and a 1-ml. sample used for determination of the peptic activity. In some cases duplicate activity determinations were made. In all cases at least 10 samples of the reaction mixture were taken.

The results of these experiments are summarized in Table III. The first column gives the final pH of the reaction mixture. The next two columns give the initial pepsin concentrations; the values of PU per ml. were obtained by the same extrapolation method as used with the calorimetric data, and the values of mg. PN per ml. were calculated from these using our average value of 0.175 per mg. PN. The fourth and fifth columns list the final enzyme concentrations obtained by extrapolation and by direct observation. The first set of rate constants (column six) was calculated from data interpolated on a curve through the experimental points (giving zero weight to the infinity reading), and the second set from the slopes of the log PU *vs.* time plots. This second method of calculation is that employed by Steinhardt.

TABLE III

ANALYTICAL DETERMINATION OF THE RATE OF INACTIVATION AT 25°

Final pH	Initial enzyme concentration PU per ml.	Initial enzyme concentration Mg. PN per ml.	Final enzyme concentration, PU per ml. Calcd.	Final enzyme concentration, PU per ml. Obs.	Rate constant, k, mins., common logs. Increment method	Rate constant, k, mins., common logs. Graphical method	Frac. of reacn. used in calculations, %
6.14	0.0115	0.066	0.0025	0.0003	0.0241	0.0154	78
6.15	.0081	.046	.0023	.0003	.0248	.0107	71
6.26	.0059	.034	.0004	.0003	.0374	.0318	75
6.38	.0078	.045	.0007	.0005	.0911	.0561	84
6.385	.0067	.038	.0003	.0003	.0819	.0620	65

The individual points in the first run listed in Table III showed the worst scattering of any of these runs. These points, sixteen in number, deviate on the average ±7.0% from the values calculated from the velocity constant, initial concentration and final concentration deduced by the method of calculation used in the calorimetric runs.

The calculated final enzyme concentrations in the first two runs in Table III are about one-fourth the initial concentrations, and larger than the observed final concentrations. No explanation for this behavior can be given.

Discussion of Results

Criticism of Results.—It should be emphasized that the several measurements and manipulations involved in each calorimetric run are difficult and characterized by an unfortunate lack of precision or reproducibility, so that the overall results are of a rather low order of accuracy. The nitrogen determinations indicate that all our pepsin solutions were contaminated by large amounts of non-protein nitrogenous impurities, the PN constituting only 56% (Run 4) to 67% (Runs 8 and 10) of the total N. The fact that the measured peptic activity per unit of PN was constant to within experimental error indicates that the protein present in each case was mainly pepsin. It is known from past experience that reaction rates are in general markedly more affected by impurities because of catalysis than are

reaction heats; correspondingly, our heat values show much better correlation with *p*H than do our reaction rates. A comparison of the relatively satisfactory kinetics observed in each run with the scattering of the rate constants when plotted as a function of *p*H indicates that some impurity or impurities have exerted an erratic catalytic effect.

The heat effect accompanying the denaturation is very small, so that the sensitivity of the calorimeter was severely taxed in making the measurements. It was therefore necessary to use relatively concentrated protein solutions, making comparison with previous rate measurements uncertain, and giving a somewhat high protein-buffer ratio.

The present results cover a very restricted *p*H range. Attempts will be made in future work to extend this range, though one is limited, particularly at 15°, by too small a heat effect at low *p*H's and by too fast a reaction at higher *p*H's.

Rate Constants.—The common logarithms of the rate constants at 25° are plotted against *p*H in Fig. 2, the filled circles representing the calorimetric values and the open circles the analytical values. In the latter case the values calculated

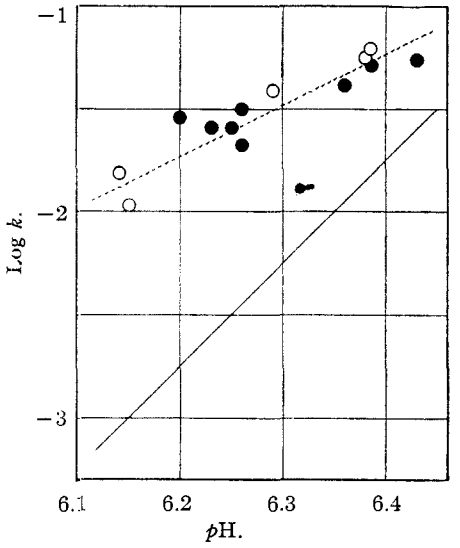

Fig. 2.—The rate of the inactivation of pepsin at 25°: filled circles, calorimetric method; open circles, analytical method; solid line, Steinhardt's data.

by the graphical method have been used. The rates obtained by the two methods are in fair agreement, *indicating that the rate-controlling process is the same in each case.* The calorimetric and analytical values both deviate from the line of slope d log *k*/d *p*H = 2.5 on the average ± 0.03 *p*H unit, which is only a little above the expected uncertainty in the *p*H measurements. If the deviations are thrown entirely into the velocity determinations, the average deviation is about ±17%. In view of the short *p*H range covered, the slope is to be considered as only roughly established.

The rate constants at 15° also show an increase with increasing *p*H, though the apparent slope is smaller than at 25°. Very little significance can be attached to this result because of the small number of experiments and the narrow *p*H range.

Thermal Data. Heats of Inactivation.—Considerably more interest attaches to the heat data obtained in these experiments, since, as pointed out above, the impurities present can be expected to have less effect on the apparent heat of reaction than on the rate. The heat effect accompanying the inactivation process is termed for convenience the heat of inactivation or denaturation, although it is recognized that secondary processes may contribute to the over-all heat effect. Such secondary processes must be rapid compared to the inactivation proper, at least at 25°, because of the agreement between the analytical and calorimetric rate constants.

The heats of inactivation are plotted against the *p*H in Fig. 3; filled circles refer to heats expressed as joules per PU present at the start of the reaction, and open circles to heats expressed as joules per g. PN at the start. The adherence

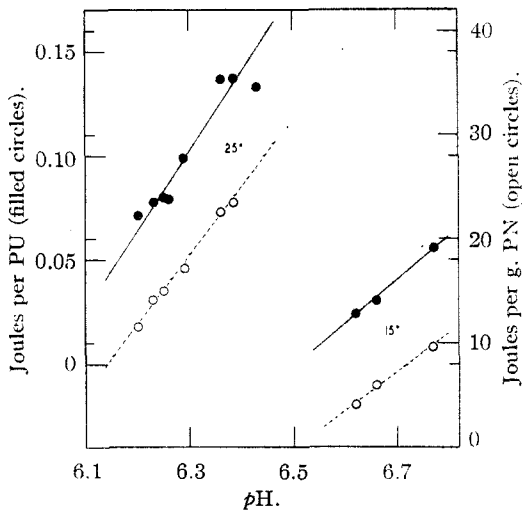

Fig. 3.—The heat of inactivation of pepsin at 15 and 25°.

of the experimental points to a linear dependence on *p*H is remarkably good, particularly when the protein concentration is measured by the PN present. The average deviation of the points from their respective straight lines is, in this case, less than ±0.01 *p*H unit at both temperatures. The two different measures of the amount of protein reacting give entirely consistent results; thus the predicted *p*H's at which the heat effect at 15° should vanish differ by only 0.002 *p*H unit. The slopes of the lines in Fig. 3 have the values given in Table IV. The ratio of the slope in terms of PN to that in terms of PU is seen to be essentially constant.

TABLE IV

APPARENT CHANGE OF HEAT OF INACTIVATION WITH *p*H

Temp., °C.	Joules per PU per *p*H unit	Joules per g. PN per *p*H unit	Ratio column 3 to column 2	Cal. per mole per *p*H unit
15	+0.21	+37	180	+49.000
25	+0.38	+66	170	+88.000

An estimate of the heat effect in *calories*[14] *per mole* of pepsin can be obtained by assuming that all the protein present was pepsin, and that pepsin contains 15% nitrogen and has a molecular weight[15] of 37,000. On this basis (cal./mole) = 1330 (joules/g. PN), and there are approximately 10^6 pepsin units per mole. Thus the quantity here termed the heat of inactivation has values between approximately 5,000 and 50,000 cal. per mole, depending on temperature and *p*H.

Heats of Mixing.—In the eleventh column of Table II are listed the observed heat effects accompanying the mixing of the protein and buffer solutions. In view of the non-protein impurities present, it has not seemed worthwhile to attempt any detailed analysis of these data. It might be pointed out that the approximate heat of ionization of *p*-nitrophenol[12] indicates that the part of the mixing heat due to the protein is a positive heat effect (heat absorption), which is what one would expect from the fact that the groups in amino acids and proteins undergoing dissociation of a proton in the *p*H range 5–7 have positive heats of ionization.

Change of Heat of Inactivation with *p*H.—The most surprising result of the present work is the strong dependence of the heat of inactivation on *p*H. We consider this behavior to be reasonably well established at 25° where the heat of reaction at *p*H 6.4 is approximately double that at *p*H 6.2. The slope of the heat *vs.* *p*H curve at 15°, though not well established, appears to be approximately half that at 25°.

Previous results obtained with this calorimeter[3,16] exclude the possibility that these large slopes can be attributed in any significant degree to calorimetric lags.

It is, of course, impossible to separate heat effects due to rapid secondary processes such as ionization from that due to the inactivation itself. The hypothesis therefore suggests itself that the variation in the total heat effect with *p*H is to be attributed to changes, resulting from the denaturation, in the pK values of groups ionizing in the *p*H region of interest here, without any accompanying change in the total number of such groups. The following discussion indicates that no reasonable assumptions concerning the number and pK's of such groups can account for more than a small fraction of the observed change of heat of reaction with *p*H.

It is obvious that only those ionizable groups which have pK's within one or two units of the *p*H values of interest can contribute significantly to the heat effect. Suppose that there are m such groups per molecule of protein, all of which have the ionization constant K_N in the native protein and K_D in the denatured protein, and that there is no interaction between these groups.[17] If ΔH_i, the heat effect accompanying the loss of 1 mole of H^+ from the protein and the gain of 1 mole of H^+ by the buffer anion, is assumed to be the same regardless of which ionizable group loses the proton and whether the ionizable group is in the native or denatured state, then it can be shown that the change of the total heat effect, ΔH, with *p*H should be given by

$$-\frac{1}{2.3m\Delta H_i}\frac{d\Delta H}{dpH} = \frac{d}{d\ln x}\left(\frac{\alpha}{\alpha+x} - \frac{1/\alpha}{1/\alpha+x}\right) \quad (2)$$

where $\alpha \equiv (K_D/K_N)^{1/2}$, $x \equiv a_H(K_DK_N)^{1/2}$. Here a_H is the hydrogen ion activity. The maximum value of the term on the right side of equation 2 increases as α increases, approaching the limiting value of 0.25 for large α and $x \approx \alpha$. This value is practically reached for $\alpha = 10$, or $pK_N - pK_D = 2$.

If we take +9000 cal.[18] for the heat of dissociation of 1 mole of H^+ from the protein and −5,500 cal.[12] for the heat of association of 1 mole of H^+ with the *p*-nitrophenolate ion, ΔH_i has the value +3,500 cal. per mole. In order to account for the observed slope at 25°, m would have to be given the *minimum* value 44; this is far greater than any reasonable number which can be assigned to the groups ionizing in this restricted *p*H region.

It is obvious that the above discussion also excludes the possibility that the observed change of ΔH with *p*H is due to the ionization in the denatured state of a *constant* number of groups which were un-ionized in the native state.

Another possible explanation of the large observed slopes is that a considerable fraction of the heat effect is actually due to digestion of the denatured pepsin by the remaining native pepsin. In future work it will be important to determine the PN at the end as well as the beginning of each experiment. It seems unlikely that any appreciable error could have been introduced by self-digestion, since the optimum *p*H for digestion by pepsin is about 1.5.[19] Furthermore, a simple calculation, which we will not reproduce here, indicates that if self-digestion were responsible to any significant degree for the observed slopes, the latter should have been found to vary markedly with *p*H even within the restricted *p*H range of our experiments. Any digestion into relatively large fragments, such as has been observed to occur with various proteins[20] at *p*H's far removed

(14) By definition, 1 calorie = 4.1833 int. joules.

(15) J. St. L. Philipot and I. B. Eriksson-Quensel, *Nature*, **132**, 932 (1933).

(16) J. M. Sturtevant, *Physics*, **7**, 232 (1936).

(17) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, p. 452.

(18) Reference 4, p. 45.

(19) G. Haugaard and R. M. Roberts, THIS JOURNAL, **64**, 2664 (1942).

(20) See, for example, M. L. Petermann, *J. Phys. Chem.*, **46**, 183 (1942).

from the optimum *p*H, would presumably be accompanied by a relatively small heat effect, and would thus probably not be of great importance.

The calorimetric observations do not in themselves indicate whether or not the reaction is reversible. However, Steinhardt[4] has shown that at these *p*H's the equilibrium point, as judged by the loss of proteolytic activity, is far on the side of inactive protein, and this observation is confirmed by our analytical rate determinations at 25°.

It would seem that one can conclude from our observations that the nature of the inactivation process varies significantly even with small changes in *p*H. The various inactive products obtained, which may or may not be readily interconvertible as a result of *p*H changes, have in common the property of being unable to digest hemoglobin. The variation of ΔH with *pH* could be the result, for example, of a change with *p*H in the average number of hydrogen bonds broken per molecule or in the average number of ionizable groups liberated per molecule.

Comparison with Previous Results.—Steinhardt,[4] in much the most important work on the kinetics of this reaction, studied the rate in *p*-nitrophenol buffers as a function of the *p*H, the ionic strength and the temperature. He followed the reaction by the loss of peptic activity, and found it to follow the first order law. The most interesting result of his work is the finding that the specific reaction rate is inversely proportional to the *fifth* power of the hydrogen ion activity, as measured by the *p*H, over a range of *p*H (about 0.7 unit) sufficient to cause a 5000-fold change in reaction rate.

Our results at 25°, over a much narrower *p*H range, indicate a slope of log *k vs.* *p*H of about 2.5 as compared with Steinhardt's value 5.0. At the lower end of the *p*H range our rates are about 10 times those interpolated in Steinhardt's data for ionic strength 0.15 (lower line in Fig. 2). Steinhardt also made measurements at 15° and ionic strength 0.2; if we assume that the change from this ionic strength to 0.15 has the same effect at 15 as at 25°, we find our rate constants at this temperature are 6 to 9 times as large as his, the discrepancy again being smaller at the higher end of the *p*H range.

Measurements, by a method entirely different from that employed here, of the heat of denaturation of pepsin have been reported by Conn, Gregg, Kistiakowsky and Roberts,[21] working at 30.6°. In one series of experiments these authors measured the heat evolved on adding sufficient alkali to take native pepsin from *p*H 5.0 to 9.1, where denaturation is practically instantaneous, and that evolved on adding the same amount of alkali to the denatured protein after it had been returned by addition of acid to the original *p*H. The difference in these quantities may be called the heat of denaturation *if it is assumed that the denaturation process varies only with respect to velocity as the* *p*H *is varied.* The value obtained was 16.2 cal. per g. PN, which corresponds to 90,000 cal. per mole, if we take the molecular weight as 37,000.

Another series of measurements performed by these authors was similar, except that different initial *p*H's were used in order to compare the apparent heat of denaturation with the amount of peptic activity remaining at the start of the reaction. The results of these experiments led the authors to conclude that the heat of denaturation was not proportional to the amount of active protein present as measured by the peptic activity. This conclusion is at variance with our observation that the analytically and calorimetrically determined kinetics are essentially the same.

The total heat of denaturation found by Conn Gregg, Kistiakowsky and Roberts is of the order of magnitude found in our work. A more detailed comparison of results seems impossible in view of the great difference in the methods used. It seems probable that the two methods are not concerned with the same process or set of processes. An additional indication of this is afforded by some titration curves given by Conn, Gregg, Kistiakowsky and Roberts. It was found, for example, that about 6 millimoles of potassium hydroxide per g. of nitrogen was required to take "native" protein from *p*H 4.5 to about *p*H 7.6, and that after *two hours* the *p*H of the solution dropped to 7.4; the same amount of potassium hydroxide took denatured protein from *p*H 4.5 to 7.1. There can be no doubt that the inactivation process considered in Steinhardt's and our work would be practically instantaneous at *p*H 7.6 and 31°. It thus appears that the slow drift in *p*H is due to a *different* denaturation process which is very rapid at *p*H 9.5; the heat data of Conn, Gregg, Kistiakowsky and Roberts presumably include the heat of this high-*p*H denaturation with the heat of varying amounts of the low-*p*H inactivation.

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Summary

The heat effect accompanying the loss of proteolytic activity of pepsin in *p*-nitrophenol buffers has been measured at 15 and 25°, by a calorimetric method which permits separation of the relatively large heat of mixing at the initiation of the reaction from the true heat of the reaction. The reaction is accompanied by absorption of heat, the amount absorbed being a strong function of *p*H in the restricted *p*H range investigated. This observation is interpreted to indicate that the nature of the inactivation process undergoes significant change as the result of small changes in *p*H.

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(21) J. B. Conn, D. C. Gregg, G. B. Kistiakowsky and R. M. Roberts, THIS JOURNAL, **63**, 2080 (1941).