

Det Kgl. Danske Videnskabernes Selskab.

Mathematisk-fysiske Meddelelser. **XIV**, 11.

---

# THE STABILITY OF CRYSTALLINE PEPSIN

BY

JACINTO STEINHARDT



KØBENHAVN

LEVIN & MUNKSGAARD

EJNAR MUNKSGAARD

1937

A preliminary account of these measurements was presented to the Kemisk Forening of Copenhagen on 19 May, 1936, and appeared in abbreviated form in *Nature*, 11 July, 1936.

# THE STABILITY OF CRYSTALLINE PEPSIN

BY

JACINTO STEINHARDT\*

(from the Institute of Physical Chemistry of the University of Copenhagen)

---

## I. Alkaline Inactivation.

The availability of crystalline enzyme preparations which are well-defined and homogeneous to degree offers obvious incentives to renew physico-chemical investigations of enzyme behaviour. The results of such investigations should be relatively free of the uncertainties which necessarily attended the many ingenious experiments with grossly impure and undefinable solutions, in the last decade. Examination of the variation of activity, stability, and certain convenient physical properties over a wide range of conditions may not only elucidate the mechanism of enzyme action, but also furnish indications of their chemical structure. Further incentive lies in the great advances in solution theory during the last fifteen years. These have a special importance for the crystallizable enzymes which, as with other proteins, must be regarded as highly polyvalent acids entering into involved electrolytic equilibria; their departure from ideal behaviour will be as great, or greater, than any the physical-chemist has already studied. Finally, the accumulation, in recent years, of accurate and essential physico-chemical protein data facilitates and compels unambiguous interpretation of enzyme experiments.

\* Fellow of the Nation Research Council, and of the General Educational Board, U. S. A. during the course of this research.

Although purification of an enzyme makes possible its analytical and thermodynamic investigation, its kinetic properties are still most characteristic and most significant. Unfortunately the substrates of the most easily available crystalline enzymes are themselves proteins and undergo reaction in many successive steps. Study of such systems is extraordinarily difficult, practically and theoretically; one must not only succeed in distinguishing the effects of the experimental variables on each of two complex substances, but must also isolate experimentally a single link in the chain of successive reactions — for which at present there is no certain procedure — and must find conditions under which the accumulating diversity of reaction products will not alter the initial state of the system beyond definition. The instability of both enzyme and substrate create other difficulties (NORTHROP has shown that some kinetic experiments merely measure the rate of destruction of the enzyme, and give little information about its reaction with the substrate). Knowledge of the stability of both components is a necessary prerequisite for kinetic studies on the reaction between them. Such knowledge can only result from other, simpler kinetic experiments.

Study of the kinetics of enzyme inactivation, because it avoids the difficulties just cited and furnishes necessary information for later work, is thus a logical first step in a general investigation of enzyme kinetics. Here there is no other substance under examination than the enzyme itself.

Pepsin was selected for the present research because it is easily and cheaply prepared, and because it has been subjected to more thorough tests of homogeneity (including ultracentrifugal analysis: PHILPOT and ERIKSSON-QUENSEL, 1933; PHILPOT, 1935) than other crystalline preparations.



Unlike trypsin, its destruction is almost completely irreversible under ordinary conditions (NORTHROP, 1930—31). Methods of measurement therefore involve fewer assumptions or complications of procedure.

Earlier inactivation experiments with impure pepsin have led to a number of unusual conclusions. It has been reported that a definite fraction of the total enzyme (the size of the fraction varying with the hydrogen-ion concentration) is instantly inactivated on bringing a solution to a neutral or alkaline pH; the remainder then becomes inactive unimolecularly at a measurable rate (GOULDING, WASTENEYS, and BORSOOK, 1926—27). The inactivation rate has been shown to depend greatly on the acidity, as in protein denaturation, but different investigators have reported different degrees of dependence. MICHAELIS and ROTHSTEIN (1920) who believed the kinetics to follow a three-halves order, found the rate to vary inversely with the fourth power of the hydrogen-ion concentration over a wide range. EGE (1925) found a third power dependence; the less extensive data of GOULDING, et al., indicate an exponent between these two, although these authors describe the inverse relation to hydrogen ions as linear. It is shown in the present paper that all these results are partly fortuitous, but a large exponent relating the rate to hydrogen-ions is common to all and requires explanation. Interest also attaches to the large thermal increments, also characteristic of protein denaturation, reported for pepsin by ARRHENIUS (75,000 calories) and by MICHAELIS and ROTHSTEIN (58,000 calories) since, as in many cases of denaturation, these large values require much slower rates of reaction at ordinary temperatures than are actually observed if the thermal increment is to be given its usual theoretical significance.

In the present research the course of inactivation has been followed over the widest possible range of acidity and salt concentration with four different buffers. In addition, the effect of temperature has been studied by measuring the dependence of velocity on acidity at a second temperature as well.

## II. Experimental.

**Pepsin.** — The enzyme was crystallized from PARKE, DAVIS pepsin, 1:10,000, by PHILPOT's modification (1935) of NORTHROP's method.<sup>1</sup> In a few experiments pepsin crystallized once only, by NORTHROP's unmodified method (1930), was used, but most experiments were made with pepsin crystallized three times according to PHILPOT's directions, except for the occasional omission of stirring in the second and third crystallizations. Some 25 stock-solutions were prepared at various times, as described below, from 8 different preparations; the non-protein nitrogen impurity, estimated by precipitation of the protein with 10 volumes of 0.15 M trichloroacetic acid at 80° and KJELDAHL analysis of the filtrate, varied from 4 to 25 per cent. of the total nitrogen. In spite of these differences the kinetic data obtained with all the stock solutions are a homogeneous body of data, and it is impossible to correlate minor individual variations with particular batches of crystals, or with the amount of impurity.

The specific activity, measured by the hemoglobin method, was always somewhat higher than the figure given by ANSON and MIRSKY (1932—33). This is partly due to the high dilution in which the activity was tested, since the hemoglobin method is not entirely independent of concentration (see Measurement of Pepsin Concentration, below). When calculated by the method described below, different solutions prepared from the same or different batches of crystals showed the same range of variation in specific activity, about 10 per cent.

After thorough washing with 0.001 M HCl, stock solutions were prepared by stirring the crystals in small volumes of 0.005 M HCl until no more would dissolve. After filtering off the residual crystals this procedure was repeated with fresh solvent, in some cases

<sup>1</sup> I am indebted to Mr. PHILPOT for kindly making available to me before publication his convenient method for crystallizing pepsin.



as often as 30 times. The apparent solubility falls off rapidly with successive extractions of the same crystals, and appears to approach a limiting value; the exact determination of this limit is difficult since the small quantity of crystals which remain for the last extractions do not permit the rapid attainment of equilibrium. The apparent change in solubility is not accompanied by any significant change in specific activity, if the latter is calculated on the basis of pepsin nitrogen. Non-protein nitrogen drops to about 0.04 mg. per ml. after three or four extractions. This is a satisfactorily small fraction of the 'soluble' extract (about 5 per cent.), but since the later extracts are very much less concentrated, this constant level of non-protein nitrogen finally represents a quarter or more of the total nitrogen. Analyses in solutions are thus a poor indication of the amount of impurity in the crystals, for non-protein nitrogen is a larger fraction of the total nitrogen when the latter is small than when it is great, as can also be shown by using buffers in which pepsin is very soluble, — here non-protein nitrogen may be reduced to a negligible fraction. Conversely, non-protein nitrogen rises to some 50 per cent., in more concentrated HCl (at the isoelectric point) where the enzyme is less soluble.

The apparent fractionation with respect to solubility is very similar to the protein fractionations reviewed by SØRENSEN (1930) but appears to have a simple explanation, — gradual removal of electrolyte from the crystals by successive portions of solvent. This may be complicated to some extent by removal of acid from the solvent by the crystals in the first few exposures to solvent. When the solvent contains somewhat higher HCl concentrations and a sufficient amount of salt this drift in solubility is never observed after one or two washings. The constant amount of non-protein N always present may be due to admixture of slightly soluble solid nitrogenous material; it is noteworthy that tyrosine (in which pepsin is rich, and which is often seen in crystalline form in old denatured pepsin suspensions) would contribute the amount of non-protein nitrogen always found (WINNEK and SCHMIDT, 1935). If the first hypothesis suggested here is accepted, the solubility of pepsin in dilute HCl is lower than previously reported (NORTROP, 1929—1930 b), at this concentration about 0.07 mg. N per ml. In 0.1 MKCl the solubility is approximately three times as great.

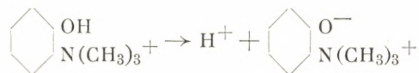
The first, more concentrated extracts sometimes but not always contained a quantity of inactive protein (from 15 to 35 per cent. in the first extract) which, like pepsin, is precipitated by 0.15 M

trichloroacetic acid, but, unlike pepsin, is not rendered insoluble at the isoelectric point in the presence of half-saturated sodium sulfate by previous treatment with strong base. This material is usually largely removed in the first few extractions (25 ml. portions of solvent to an initial 6 gr. filter cake). Its occasional presence in kinetics experiments was without effect.

The stock solutions were kept at 3°–4° until needed. They underwent only slight deterioration in periods of 2–3 weeks before use.

Choice of buffers. — Pepsin is inactivated at measurable rates at pH between 5.7 and 7.1. Since the reaction velocity depends on the buffer, it was desirable to cover as much of the range as possible with single buffers. Phosphate excepted, none of the buffers conventionally used are available in this range, and phosphate was eliminated by the choice of monobasic acids to facilitate ease of calculation and control of ionic strength, and to avoid other buffered regions which might interfere with adjustment of pH for subsequent activity measurements. With the exception of a few orienting experiments with citrate, the buffers were made from *p*-nitrophenol (most frequently), trimethylacetic acid, and trimethyl-*o*-aminophenol halide. The classical dissociation constant of these acids (on the conventional acidity scale defined below) at  $\mu = 0.1$  and 25°, are given in Table 1<sup>1</sup>. All four buffers were purified by

<sup>1</sup> I am grateful to Professor J. N. BRØNSTED for a highly purified sample of trimethylacetic acid, and for suggesting the use of trimethylaminophenols. These monobasic acids carry a positive charge, owing to the methylated nitrogen; their conjugate bases, the form in which they occur uncombined, are zwitterions. The charged group causes the phenolic group to dissociate at much higher hydrogen-ion concentrations than in phenol, and the methylation avoids the complications of polybasicity:



The meta- and para- acids are approximately equally strong (ca.  $1 \times 10^{-8}$ ). All three forms have the useful property of crystallizing, on half neutralization of the base, as "basic salts" which can be dissolved to give solutions containing equal concentrations of acid and its conjugate base. The hydrogen-ion concentration of these solutions is thus equal to the dissociation constant. The dissociation constants and heats of dissociation (5165 calories for the ortho-compound) were measured by Mr. THOMAS ROSENBERG and by Fru cand. polyt. AGNES DELBANCO.



Table I.

Classical dissociation constants of acids used in preparing the buffers. Values refer to 25° and ionic strength 0.1.

Acid .....	K	pK
<i>p</i> -nitrophenol.....	$1.12 \times 10^{-7}$	6.95
trimethylacetic .....	$1.0 \times 10^{-5}$	5.00
trimethyl- <i>o</i> - aminophenol halide .....	$3.81 \times 10^{-8}$	7.42

repeated recrystallization. The total concentration of acid plus base in the reaction mixture was kept at 0.02 M, except in the experiments with the aminophenol, in which the concentration was varied. In every case sufficient KCl (in a few experiments KNO<sub>3</sub>, or KCl + NaCl) was added to bring the ionic strength up to the desired value (in the experiments at  $\mu = 0.5$ , in Table III only, the same amount of salt was used at every pH,  $\mu$  rising in the more alkaline solutions to 0.51). Since *p*-nitrophenol is less than half dissociated at the more alkaline pH used, ionic-strengths much below 0.02 could be used with this buffer.

pH measurement. — Acidity measurements were made in triplicate at the conclusion of each experiment, with a quinhydrone electrode connected through saturated KCl to a 1 M KCl calomel half-cell. These were mounted in the thermostat used for the kinetic experiments; this precaution was required because two of the buffers used have a high temperature sensibility. The reaction velocity is so greatly dependent on pH and salt concentration that the comparative accuracy of the data is limited by the pH measurement rather than by the velocity-constant. Thus, it was also necessary to correct the observed potentials for the small differences of liquid junction potential in solutions of different ionic strength. The procedure used was similar to that of GUGGENHEIM and SCHINDLER (1934), but the potential of the calomel half-cell was assigned by measuring it against the quinhydrone electrode immersed in a solution, 0.005 M in HCl and 0.095 M in NaCl. The pH of this solution, at both temperatures, was arbitrarily defined as 2.339, in accordance with the older SØRENSEN scale; it is recognized that the true  $p_{\text{aH}}$  scale probably lies .065 units higher. The correction for liquid-junction potential, based on recent determinations of the transport numbers of K<sup>+</sup> and Cl<sup>-</sup>

(McINNES and DOLE, 1931) becomes uncertain to the extent of one millivolt in the most alkaline solutions of ionic strength below 0.03, owing to the relatively large content of buffer anion of unknown mobility. In all calculations of pH, allowance was made for the effect of the ion concentration on the normal potential of the quinhydrone electrode (SØRENSEN, SØRENSEN, and LINDERSTRØM-LANG, 1921).

The quinhydrone electrode gives slightly drifting potentials in nitrophenol buffers. The presence of this drift has offered no difficulty in practise; the potentials obtained in the interval between 3 and 8 minutes are reproducible to 0.3 millivolts.

The solutions with  $\mu = 0.5$  included in Table III were not measured at 25° but at room temperature. The pH has been corrected to 25° from the known heat of dissociation.

**Kinetic procedures.** — 40 ml. of 0.04 M buffer were placed in a 150 ml. glass-stoppered flask in the thermostat. Another flask contained the enzyme solution (diluted with 0.0005 M HCl to contain from 0.005 to 0.028 mg. pepsin N per ml.). Both solutions had been adjusted to the same ionic-strength with salt, and the buffer solution contained sufficient extra base to neutralize the acid in the enzyme solution. After half an hour, 40 ml. of enzyme solution were run into the buffer, a stop-watch being started at the time of half-delivery (usually 7–8 seconds). The vessel was whirled for a few seconds and allowed to stand in thermostat without further shaking except in certain experiments described later. At the desired intervals 3 ml. samples were pipetted off and discharged into small test-tubes containing sufficient HCl to neutralize all the buffer base, and to bring the free acid concentration to 0.0005 M. These samples keep in the cold for many hours without noticeable change; activity tests were usually made in from one to three hours, but in very slow experiments the solutions were tested a few minutes after sampling. A zero-time sample was also taken, in the same way, from enzyme that had been run into an equal volume of dilute HCl, or, as a further check, into the buffer acid. Comparison of these controls with one another and with the calculated effect of dilution is described in the discussion of the data.

This procedure was varied slightly in a number of experiments. Those involving very low ionic-strengths required that all the added salt should be in the enzyme solution and none in the buffer. In one series (Table III) the vessels were shaken regularly by a horizontal movement of 5 cms. 170–185 times per minute.



Measurement of pepsin concentration. — Pepsin was estimated by measurement of its catalytic activity, using two different test-proteins. In most experiments the enzyme in 1 ml. of the diluted sample was measured in duplicate by the hemoglobin method of ANSON and MIRSKY (1932—33), using commercial denatured hemoglobin (MERCCK'S). This change from the purified native hemoglobin solution of these authors necessitated redetermination of blanks, and calibration of the relation between colorimeter readings and enzyme concentration. Plotting the logarithm of the tyrosine equivalent determined colorimetrically, after subtraction of the reading with solutions containing no enzyme, against the enzyme concentration results in a straight line; but the slope of this line is not 1, as it should be for a linear relation. The 'proportionality blank' of ANSON and MIRSKY was not used, since tests with actual dilutions of a tyrosine standard added to the filtrates showed that it was not required. The relation between enzyme concentration and tyrosine equivalents in the digest is accurately given by the equation:

$$E = kTy^{1.265} \quad (1)$$

with enzyme concentrations between 0.0003 and 0.01 mg. pepsin N per ml. If the concentration of the tyrosine standard is doubled, and higher enzyme concentrations are used, the relation is more nearly linear, and holds over a correspondingly higher range of concentrations with the exponent 1.150 instead of 1.265. Both concentration ranges have been used with completely consistent and comparable results.

Eq. 1 shows that, if the reaction kinetics are of the first order, the logarithm of the tyrosine reading is interchangeable with the logarithm of the enzyme concentration in giving a straight line against time. If the velocity constant is calculated from the slope this must be divided by the appropriate exponent. This procedure has been followed in calculating the velocity constants given in the tables.

Calculation of specific activities of pepsin preparations on the assumption that tyrosine readings obtained with this modification of the hemoglobin method are linearly related to enzyme concentration will give a result depending on the dilution of the enzyme tested. If, however, the activity is defined as the reciprocal of  $k$  in Eq. 1, and  $E$  and  $Ty$  are expressed in the same units as in ANSON and MIRSKY'S definition, the figure obtained, 0.045 — 0.049,

is independent of concentration. The residual random variation is not greater than the limits of error in pepsin N measurements in the dilute stock solutions employed.

In order to get results of the highest reproducibility it was necessary to time all operations, i. e., the precipitated digestion mixture was filtered rapidly after standing for six minutes, the colour developed with the Folin reagent was measured after waiting one hour, etc. A deep red filter, inserted in the eyepiece of the colorimeter eliminated the interference of the yellow colour of nitrophenol.

In a few experiments KAHLBAUM's casein was used as test-protein. Here the amount of digestion after one hour was estimated by KJELDAHL analysis of the filtrate after precipitation with trichloroacetic acid, as described by NORTHROP (1932—33), but the protein solution was prepared according to HOLTER (1930). Blanks were deducted for the quantity of nitrogen introduced by the buffer (with *p*-nitrophenol this blank is uncertain, owing to partial loss of nitrogen from the buffer during destructive digestion). Velocity constants determined in this way were entirely consistent with those obtained with the hemoglobin method (See Fig. 3, in which the points obtained with casein are marked by a thin vertical line).

Temperature control. — The experiments were carried out at 25° and 15°. A large mercury-toluol regulator and efficient stirring kept the temperature constant to better than 0.01°.

### III. The Data.

The results of 85 experiments at 25° are given in Tables II and III, the division corresponding to whether or not the reaction-vessels were shaken during the experiment. Owing to the large number, it is impractical to present detailed data for each experiment; only the logarithm of the unimolecular velocity-constant, and the initial enzyme concentration are given for each set of experimental conditions. Each velocity constant represents duplicate analyses after at least ten time-intervals spaced over at least 80 per cent. completion of the reaction in over half the experiments (in many,



Table II.

Velocity of inactivation at 25° when the reaction-vessel was not shaken.

Ionic Strength	pH	$\log_{10} k_{10}^{\text{min.}}$	Initial Enzyme (mg. N/ml.)
(a) <i>p</i> -nitrophenol buffers			
0.50	6.012	-3.708	0.00364
	6.250	-2.489	.00368
	6.663	-0.838	.00349
0.30	5.994	-3.642	0.00338
	6.147	-2.854	.00376
0.20	3.81	-5.614	0.00579
	5.706	-5.188	.00254
	5.830	-4.322	.00604
	5.894	-4.207	.00254
	6.290	-2.273	.00577
	6.442	-1.484	.00222
0.10	5.861	-4.598	0.00388
	6.178	-2.968	.00378
	6.502	-1.470	.00391
0.05	5.964	-4.390	0.00359
	6.095	-3.752	.00378
	6.205	-3.101	.00415
	6.382	-2.307	.00435
	6.529	-1.777	.00411
	6.668	-1.628	.00437
0.0317	6.097	-3.920	0.00307
	6.290	-3.126	.00381
	6.325	-2.907	.00370
	6.526	-2.155	.00405
	6.895	-0.945	.00376
0.0248	5.994	-4.742	0.00418

(continued on next page)

Table II.

(continued)

Ionic Strength	pH	$\log_{10} k_{10}^{\text{min.}}$	Initial Enzyme (mg. N/ml.)
0.02	6.043	— 4.745	0.00290
	6.099	— 4.439	.00326
	6.356	— 3.152	.00311
	6.70	— 2.070	.00296
	6.94	— 1.303	.00319
0.012	6.044	— 5.117	0.00460
	6.374	— 3.452	.00456
	6.69	— 2.502	.00452
	7.03	— 1.534	.00458
0.0317 (KNO <sub>3</sub> as salt)	6.013	— 4.189	0.00341
	6.475	— 2.232	.00340
0.20 (with 1% HCHO)	5.838	— 4.305	0.00376
	6.279	— 2.108	.00376
(b) Trimethylacetic acid buffers			
0.20	5.994	— 4.344	0.00363
	6.079	— 3.844	.00328
	6.476	— 2.041	.00359
0.05	6.176	— 3.919	0.00346
(c) Trimethyl- <i>o</i> -aminophenol buffers			
0.20	6.297	— 2.620	0.00337
	6.571	— 1.517	.00372
0.042	6.438	— 2.791	0.00322

over 95 per cent.). As indication of the adequacy of the data, and as justification of the employment of unimolecular constants as a description of the individual experiments, detailed results for three experiments from Tables II and III are shown in Fig. 1. These three representative runs have been selected only because their rates (at the same ionic-

Table III.

Velocity of inactivation at 25° with the reaction-vessel shaken.  
Data marked (') were obtained with casein as test-protein:  
all others with hemoglobin.

Ionic Strength	pH	$\log_{10} k_{10}^{\text{min.}}$	Initial Enzyme (mg. N/ml.)	
(a) <i>p</i> -nitrophenol buffers				
0.50—0.51	5.91	— 3.187	0.00800	
		5.97	— 3.333	.0131
		5.99	— 3.211	.0130
		6.04	— 3.325	.0131
		6.08	— 3.003	.00939
		6.16	— 2.614	.0126
		(') 6.28	— 2.326	.025
		6.33	— 2.142	.0119
		6.35	— 1.888	.0131
		6.38	— 1.860	.00735
		(') 6.38	— 1.880	.025
		6.46	— 1.542	.00980
		6.49	— 1.604	.00936
		6.56	— 1.175	.0130
		6.64	— 0.882	.0111
	0.30	6.044	— 3.051	0.0125
6.145		— 2.767	.00806	
6.234		— 2.643	.00806	
6.316		— 2.076	.0139	
6.408		— 1.772	.00799	
6.452		— 1.483	.0127	
6.587		— 1.047	.0127	
0.20	3.789	— 3.494	0.00578	
	5.780	— 3.388	.00678	
	5.851	— 3.454	.00596	
	5.910	— 3.366	.0122	
	6.034	— 3.305	.0126	
	6.284	— 2.327	.0129	
	6.631	— 0.931	.0139	

(continued on next page)

Table III.

(continued)

Ionic Strength	pH	$\log_{10} k_{10}^{\text{min.}}$	Initial Enzyme (mg. N/ml.)
0.10	6.071	— 3.364	0.0102
	6.225	— 2.618	.00950
	6.436	— 1.642	.0104
0.05	5.958	— 3.676	0.00360
0.0248	5.980	— 3.582	0.00395
0.02	6.040	— 3.671	0.00320
0.012	6.028	— 3.420	0.00295

(b) Trimethyl-*o*-aminophenol buffers. Concentration 0.025 M at pH 6.73 and 0.05 for the others.

0.50	( <sup>1</sup> ) 6.410	— 2.072	0.025
	( <sup>1</sup> ) 6.73	— 0.77	0.025
	( <sup>1</sup> ) 6.79	— 0.67	0.025

(<sup>1</sup>) Test protein was casein

strengths and in the same buffer) are not too far apart to be shown on a single graph; even so, there is an approximately fifty-fold variation in the half-periods of these three experiments, in the narrow pH interval between 6.290 and 6.631. The ordinates represent a quantity proportional to the logarithm of the pepsin concentration (as described under Methods), and the half-periods are shown by broken vertical lines.

It is clear that the data are well represented by kinetic equations of the first order, at least until the reaction is almost complete. The slight slowing at over 80 per cent completion is insufficient to permit application of three-halves order kinetic equations, as in the analysis of MICHAELIS and ROTHSTEIN. When the 52 experiments of Tables II and V are tested with both equations, 27 are satisfactorily fitted by



first order to over 80 per cent. completion, and only 2 appear to be better fitted by the fractional order; the remainder are indeterminate, either because they were not followed far enough, or in a few cases because the points scatter too

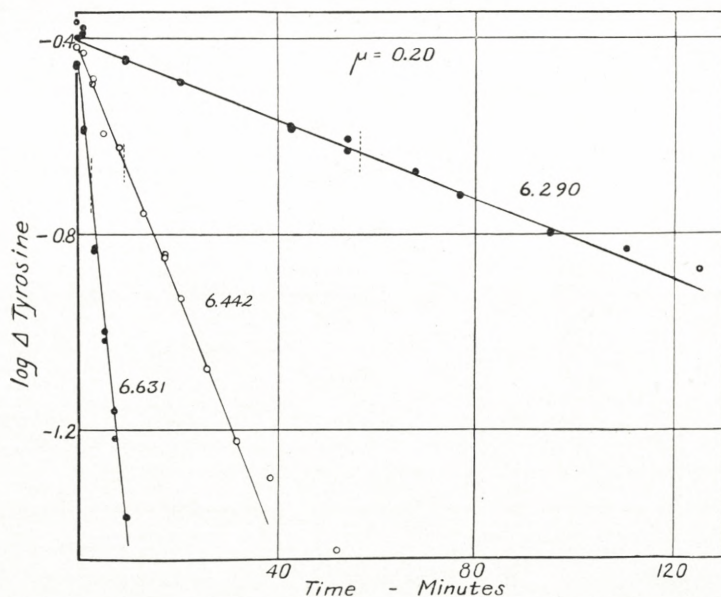


Fig. 1. The individual kinetic experiments at an ionic-strength of 0.2 at 25°. The buffer was *p*-nitrophenol. The experiment at pH 6.442 is from Table I, the others from Table II (reaction-vessels shaken). Broken vertical lines show the half-periods.

widely. An even more conclusive reason for rejecting the fractional order is that a fivefold variation in initial enzyme concentration fails to change the time for half completion. Thus, the slowest reaction shown in Fig. 1 (plotted as though it had the same ordinates as the other two sets of data) had an initial enzyme concentration only one-quarter that of the others; however, its rate stands in the same relation to pH as the others, as later described (see Fig. 2). In general, ex-

periments at this salt concentration fall into two groups of widely different initial concentrations, with no corresponding differences in velocity.

The deviations from first-order kinetics at the end cannot be due to approaching equilibrium, for the position of these deviations varies from one experiment to another, apparently at random, with no relation to pH. Similar absence of correlation with particular enzyme preparations likewise excludes the possibility of a small admixture of a more stable active substance. The progressive absorption of very small amounts of carbon dioxide during sampling would, in view of the great pH dependence, produce such deviations, but it is also possible that these effects indicate insufficient standardization in the complicated analytical procedure. When an almost completely inactivated pepsin solution is allowed to stand at pH 5 there is a slight recovery of activity for some days (NORTHROP, 1932—33, and unpublished experiments of the author). The amount recovered is small, but when 90 per cent. or more of the pepsin initially present in these very dilute solutions has been destroyed this amount is a considerable fraction of the remaining activity. The apparent falling off in inactivation rate when nearly all the pepsin has been destroyed may be due to such recovery of activity in the interval between acidifying the sample and testing its activity. The necessary addition of acid before each test introduces an unavoidable ambiguity<sup>1</sup>.

Whatever their cause, these deviations are too small to

<sup>1</sup> PHILPOT has suggested (1935) that base does not denature pepsin but changes it to something more susceptible to subsequent acid denaturation. Should this be true, the present measurements nevertheless refer to the reaction initiated by base, but there might well be competing reactions on addition of acid before the tests. One of these may slightly reverse the effect of base.

have an appreciable effect on the velocity-constants, which are fixed by the data to within 5 per cent. (in a few bad cases, 10 per cent.). This accuracy is ample for estimation of the effects of acidity, ionic-strength, and temperature, since the range of effects produced by variation in these conditions is very wide: there is a factor of about 88,000 between the fastest and slowest rates listed in Tables II and III.

The three experiments shown in Fig. 1 exhibit no instant inactivation of the kind described by GOULDING, BORSOOK, and WASTENEYS (1926). No such effects were found in any of the experiments. Effects of this kind can be detected only by comparing the enzyme concentration of the stock solution (corrected by the factor of dilution), or the enzyme concentration found in a blank dilution (with dilute HCl, or the buffer acid) against the enzyme concentration extrapolated to the beginning of the experiment from the straight line fitted to the experimental points. No better agreement can be expected than the closeness with which the ordinary experimental points fit the best straight line. In practise discrepancies of as much as 10 per cent. are occasionally found between extrapolated and calculated zeros; usually the agreement is better. The extrapolated figure is often higher than that calculated from the stock solution rather than lower the discrepancies seem therefore to be without special significance. It is reasonable to find slightly greater scattering in the zero time measurement since the procedure in sampling and estimating this solution is not exactly the same as the procedure for all the others. There is also reason for believing, in view of the discussion of the experiments of Table III in a later section, that some slight inactivation may occur at the start of an experiment from the act of mixing the solutions; a few per cent. of inactivation has been demon-



strated by comparison of the calculated zero (from measurement of the stock solution) and control dilutions with the acid of the buffer. Such inactivation, due to a heterogeneous reaction at air interfaces, is naturally not reproducible.

Although NORTHROP (1930 *a*) and HERRIOTT and NORTHROP (1934) have published data on crystalline pepsin which appear to confirm the existence of instantaneously inactivated fractions varying with pH, the procedure described — analysis after a single time-interval a few minutes after mixing — could not distinguish between such effects and continuous inactivation at a rate increasing with pH. For crystalline pepsin, at least, it seems safe to conclude that this instantaneous inactivation does not exist, and need not be allowed for or explained.<sup>1</sup>

This description of the data of Table II applies equally to Table III, with a single reservation. The latter data, obtained in experiments during which the reaction-vessel was shaken, are of two kinds, as will be shown in the following pages: experiments pH 6.1 and above are in no way different from those already described, — unimolecular, and with no instant inactivation; below this pH, the velocity observed is predominantly that of a heterogeneous reaction, and is independent of pH. This reaction is likewise of the first order, but its velocity can be altered by the speed of shaking. This sometimes increased at night when the laboratory voltage rose; the precise value of the constants in the more acid solutions is therefore sometimes uncertain, and the results of different experiments are not all strictly comparable.

<sup>1</sup> The explanation proposed by GOULDING and his collaborators requires the assumption that both active and inactive pepsin can act as a single chemical species in determining an equilibrium.



Since NORTHROP has shown that the inactivation of pepsin can be reversed, to a very small extent, at pH between 5.0 and 5.6, indications of an equilibrium point were sought in the more acid experiments. No marked deviations from first-

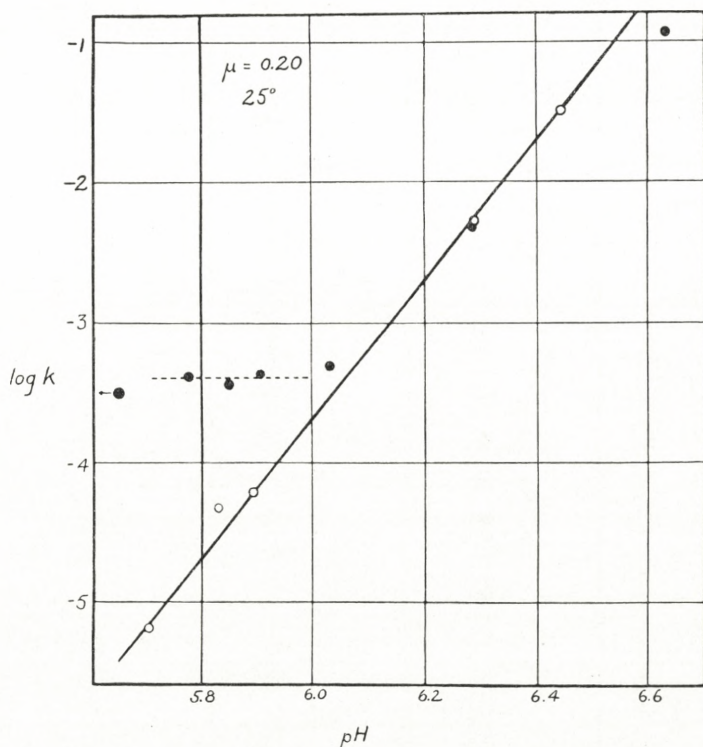


Fig. 2. The relation of the logarithm of the velocity-constant to pH at an ionic-strength of 0.2 at  $25^\circ$  with *p*-nitrophenol buffers. The open circles represent experiments from Table I (no shaking), the filled circles data from Table II (vessels shaken). The point marked with an arrow represents an experiment at pH 3.79.

order kinetics were shown at over 80 per cent. completion even in the slowest experiment in Table II (pH 5.706). Thus, even at acidities only slightly above those used by NORTHROP, the equilibrium point lies far over on the side of total

destruction at this temperature; other experiments at slightly higher pH show that this is also true at 3°—4°.

Dependence of velocity on pH. — These experiments not only confirm the existence of an enormous effect of acidity on the velocity of inactivation, but show that this effect is even greater than has been reported. Since the series with *p*-nitrophenol buffers at  $\mu = 0.2$  covers a wider range than the others, data for this ionic-strength from both tables are combined in Fig. 2. Because the points represent half-periods differing by a factor of 20,000 (2.5 minutes to 36 days) the logarithms of the decadic velocity constants in reciprocal minutes are plotted instead of the constants themselves. This method of plotting shows strikingly the great regularity of the pH relationship. All the points from Table II (open circles) lie on a straight line, the slope of which is exactly five. The points from Table III (filled circles) representing experiments in which the vessels were shaken, lie on the same line, except at pH below 6.1, where the reaction rate becomes entirely independent of pH and is presumably that of a different reaction path, involving phase interfaces<sup>1</sup>. The first of the filled circles, marked with an arrow, represents an experiment at pH 3.79 — it is included here to show how far this independence of pH extends. At the intersection of the horizontal and slanting

<sup>1</sup> The product of the heterogeneous reaction is much less soluble than that of the homogeneous reaction in dilute salt solutions at pH below 6.3. The lower pH limit for the solubility of the latter is not known, since it is formed so slowly at pH below 6.1 that there may be time for it to be hydrolysed by the remaining pepsin; but it is soluble in more acid solutions than the product of the heterogeneous reaction, which appears as a finely divided suspension almost as soon as shaking commences, in the more acid solutions. The conclusion that the heterogeneous reaction occurs at air interfaces depends on failure to effect its velocity by coating the inside of the reaction-vessel with various waxes.

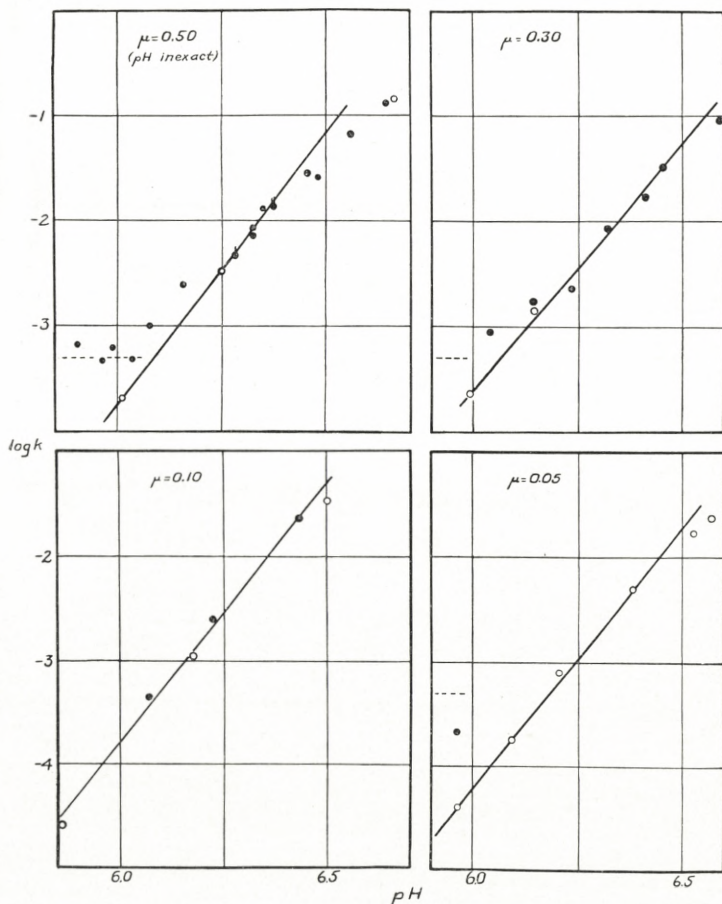


Fig. 3. The relation of the logarithm of the velocity-constant to pH at ionic-strengths of 0.5, 0.3, 0.1, and 0.05 at 25° with *p*-nitrophenol buffers. Open circles are data obtained without shaking (Table I), filled circles data with the vessels shaken (Table II). In the set for  $\mu = 0.5$ , two experiments with casein as test-protein are marked by thin vertical lines.

lines the velocity of both homogeneous and heterogeneous reactions is comparable; at higher and lower pH one or the other reaction is so much the faster that the contribution of the second to the total velocity can be neglected.

It will be observed that the highest point lies a little



below the line; this is shown later to be true of every series of experiments. At the highest velocities, the proportionality of velocity to the inverse fifth power of the hydrogen-ion concentration no longer prevails; further increase in pH produce a relatively smaller increase in velocity. At this temperature and salt concentration this limit is reached only at the highest measurable velocities, but a lower temperatures and and in more dilute solutions, these deviations can be followed further; just noticeable here, they are shown later to be important for the theory of the fifth-power dependence which covers a velocity-interval of about 1 to 5000 in these data.

Data for all other ion-concentrations show the same relationship to pH. In Fig. 3 the results, with and without shaking, for four different ionic-strengths (*p*-nitrophenol buffers) are shown separately. In all four sets the fifth-power dependence holds over a wide range; as with the data of Fig. 2 the velocity becomes independent of pH in the more acid solutions — when the vessels are shaken. In each case, again, the points for highest velocities always lie a little below the straight line — which should evidently curve off in this region. Three of the four sets of data have practically the same coordinates, but in the fourth set ( $\mu = 0.05$ ) the velocities at the same pH are considerably lower. These effects of salt concentration are described more fully in a later section, in which data for still lower ionic-strengths are also presented.

The data for  $\mu = 0.5$  scatter more than the others because temperature was not controlled during measurement of pH in part of this series (see Methods). These data show how slight errors in pH measurement, combined with the normal deviations from the straight line at high pH, and the results of shaking at low pH, can appear to give a line of

lower slope than five. MICHAELIS and ROTHSTEIN reported a slope of four; possibly, failure to control ionic-strength also contributed to this fortuitous result in their experiments, as well as in those of EGE, and of GOULDING, et al.

It has been shown by EGE, and confirmed by unpublished experiments of the author, that inactivation of pepsin by acids is directly proportional to the hydrogen-ion concentration. By extrapolating the line of slope 5 in Fig. 2, and drawing a line of slope  $-1$  through the point for pH 3.81 (Table II), the pH of maximum stability is found at the intersection of the two lines at pH 5.28, close to where NORTHROP found maximum reversal of inactivation. At this pH the extrapolated value for the half-period of each inactivation reaction is approximately eight years. In more dilute salt solutions and at lower temperatures the enzyme is even more stable.

Significance of the fifth-power. — A dependence on acidity so great as to change the velocity of inactivation 10,000 times in one pH unit suggests how proteins may sometimes appear to possess sharp, almost discontinuous pH-stability regions (SVEDBERG, 1930). It also demands that secondary effects on acidity of such other experimental variables as salt concentration and temperature must be estimated and allowed for before interpretation of their primary effects on protein denaturation is undertaken. For this it is necessary to postulate a mechanism of this unusual pH dependence.

The view that denaturation of proteins is catalyzed by hydrogen and hydroxyl ions (in the sense of P. S. LEWIS, 1926, 1927; W. C. M. LEWIS, 1931) must be rejected as a primary mechanism in this case because of the large ex-

ponent; the salt effect, discussed later, helps to exclude it also. Catalysis by the basic ions of the buffer (BRØNSTED, 1928) is inapplicable for the same reasons, and can be further excluded in other ways. The high exponent admits only explanations involving equilibria, and the fact that hydrogen-ions are involved in the proportionality suggests that the equilibria concern dissociation of acid groups in the enzyme molecule.

Pepsin, a highly polyvalent amphoteric electrolyte, undergoes many stages of acid dissociation; consequently, many different kinds of pepsin ion coexist in the solutions in which the reaction was studied. It is necessary to assume that of the many groups which dissociate a hydrogen-ion in this range of acidity, the dissociation of a certain 5 groups greatly increases the susceptibility of the enzyme to inactivation. It is an essential part of this assumption that the dissociation of five groups and not fewer is required for the great increase in instability. Nothing can be postulated about the stability of intermediate ions in which one, two, three, or four of the five groups have given off a hydrogen-ion except that they are much more stable. At the level of stability observed, it is the dissociation of the fifth hydrogen-ion which causes the significant change.

The five groups involved are probably not the only groups which dissociate in this range of acidity; it is only assumed that the dissociation of the fifth of these groups has a much greater effect on the stability than the dissociation of any other group in this range. Since it is known that pepsin bears both positive and negative charges, and that its net charge is already strongly negative in the lowest pH interval of these experiments, the effect of these 5 dissociations is not likely to be unspecific and due solely to a change in



net charge<sup>1</sup>. It is more likely that this effect on the stability is produced by 5 specific chemical groups, probably of one kind: however, this last supposition is not an essential part of the explanation of the fifth-power dependence proposed here.

If all other stages of dissociation than the 5 in question are ignored, and the dissociation constants  $K_1$ ,  $K_2$ ,  $K_3$ ,  $K_4$ , and  $K_5$ , are assigned to the successive stages, then the fraction of the total pepsin (in all ionic forms) present as ions of the fifth kind can be expressed in the following mass-law identity, in which  $P_1$ ,  $P_2 \dots P_5$  represent pepsin ions formed by the 5 stages of dissociation, and  $P_0$  represents all the other possible species of pepsin ions, on the more acid side of these dissociations:

$$\left. \begin{aligned} & \frac{P_5}{P_0 + P_1 + P_2 + P_3 + P_4 + P_5} = \\ & = \frac{1}{\frac{a_H^5}{K_1 K_2 K_3 K_4 K_5} + \frac{a_H^4}{K_2 K_3 K_4 K_5} + \frac{a_H^3}{K_3 K_4 K_5} + \frac{a_H^2}{K_4 K_5} + \frac{a_H}{K_5} + 1} \end{aligned} \right\} (2)$$

The left-hand member of this equation is proportional to the reaction-rate only when the respective ratios of activities to concentrations for the several ionic species remain fixed, as in the case of measurements made at constant ionic-strength in dilute solutions. The right-hand member is multiplied by a complex but constant factor in changing the Ps from activities to concentrations, under these conditions, to get an expression proportional to velocity.

<sup>1</sup> The related assumption that dissociation of any five groups out of a larger available number produces the decisive effect on the stability suffices to give the slope of five, and cannot be excluded. It leads to much more complicated equations for the exact pH-velocity function. For this reason, and because it is inherently less probable, its possibilities are not further developed.

When  $K_1 \ll a_H$  it follows that  $K_2, \dots, K_5 \ll a_H$ , and only the first term of the series in the right-hand denominator need be considered; this is equivalent to the condition that the strongest of the 5 groups is only slightly dissociated. The equation then becomes:

$$\frac{P_5}{\sum_0^5 P_i} \approx \frac{K_1 K_2 K_3 K_4 K_5}{a_H^5} \quad (2')$$

omitting the additional constant factor mentioned above. The region over which the straight line adequately represents the data in the logarithmic plots (Figs. 2 and 3) is the region over which the condition for this approximation prevails.

Further conclusions are more easily drawn by considering the relations that the  $K$ s may bear to one another (theoretically, given a wide enough range of data, it is deducible from the curvature at the alkaline end). Accepting the presumption that all 5 groups are chemically identical, the fact that there are at least three carbon atoms, and usually a nitrogen atom also, between the nearest neighboring protein acid groups, will cause these groups, unless placed under special steric influences, to have apparent dissociation constants which differ only by limiting statistical factors (ADAMS, 1916). When these factors are assigned in the usual way, Eq. 2 can be rewritten:

$$\frac{P_5}{\sum_0^5 P_i} = \frac{1}{\alpha^5 + \frac{5}{\alpha^4} + \frac{10}{\alpha^3} + \frac{10}{\alpha^2} + \frac{5}{\alpha} + 1} \quad (3)$$

when the constant factor is omitted as before. In this equation,  $\alpha = \frac{K_0}{a_H}$  where  $K_0$  is a constant which is the same for each acid group (its apparent dissociation constant

separated from the purely statistical effect due to the presence of the others in the same molecule). This equation is a special case of (2) and will therefore give the approximate Eq. 2', but greater interest attaches to the exact form, which should

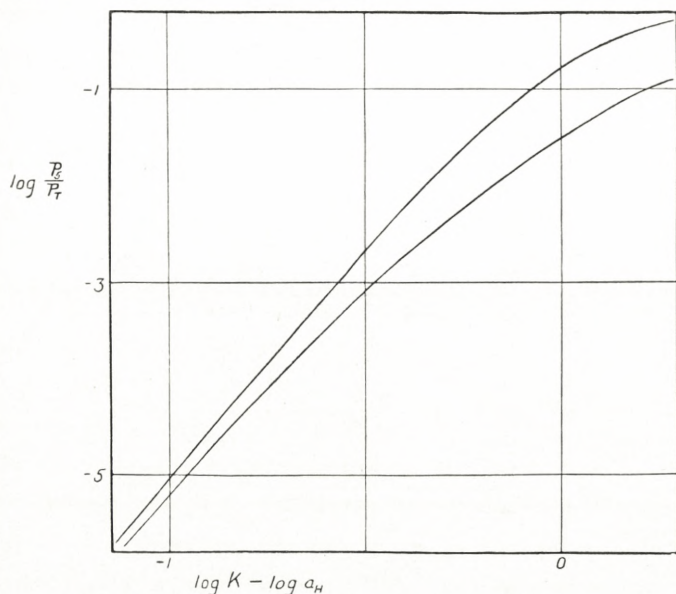


Fig. 4. Graphical representation of Eqs. 3 (lower) and 3' (upper) on the coordinate system of Figs. 2 and 3.

describe the curved as well as the linear portions of Figs. 2 and 3, if this interpretation of the fifth-power effect is correct.

If no allowance is made for statistical factors, the numerical factors drop out of Eq. 3:

$$\frac{P_5}{\sum_0^5 P_i} = \frac{1}{\frac{1}{\alpha_5} + \frac{1}{\alpha_4} + \frac{1}{\alpha_3} + \frac{1}{\alpha_2} + \frac{1}{\alpha} + 1} \quad (3')$$

Both (3) and (3') are shown plotted, on the same coordinates as the data, in Fig. 4 — the abscissa has been made



independent of particular values of pH or  $K_0$ . Both equations have been shown, because the overlapping of other dissociating groups in pepsin in the same pH range makes the assignment of statistical factors uncertain. Overlapping tends to make the apparent  $K$ s more nearly alike. Since the factors may even tend to approach unity, their omission may be preferable to their inclusion, which would be strictly necessary only if the 5 groups were the only acid radicals in the molecule. As the figure shows, the result is not greatly affected; when factors are included the curve turns off from a straight line more gradually. It is interesting to observe to what extent dissociation of the first group may proceed before marked deviations from a straight line appear: when one-sixth of the total enzyme has dissociated at least one hydrogen from one or more of the five groups, the velocity predicted by Eq. 3' is only 20 per cent. lower than the corresponding value on the straight line, a just appreciable difference on this scale.

Figs. 5 and 8, which follow (further discussed later) illustrate the satisfactory way in which one of these two equations (3') fits the data — the fit with (3) is definitely less satisfactory. The two lowest ionic-strengths (0.02 and 0.012) are less well fitted, but it is probably without significance that (3) describes them better since there is reason for believing that data for very dilute salt solutions should depart from the simple theoretical relationship (this is given in discussion of the salt effect). The decision between the alternative forms therefore rests on the more concentrated solutions. None of the data extend as far into the region of curvature as would be desirable for the most critical test of fit. Consequently, the coordinates assigned to the theoretical curve are, within narrow limits, somewhat arbitrary; cal-

culations which follow, based on these fitted coordinates, are uncertain to the extent of this arbitrariness. It should be possible to avoid this in future work by extending the data into the region of curvature, by working at lower temperatures; but the present measurements are sufficiently unambiguous to permit some conclusions.

The antilogarithm of the pH (with sign changed) which corresponds, for each set of data, with the abscissa zero of the curve in Fig. 4, is equal to  $K_0$  which enters into  $\alpha$  in Eq. 3'. At all ionic-strengths between 0.1 and 0.5 almost identical values are found; the maximum uncertainty in fitting any one set of data is 0.1 pH unit (usually it is half of this); the maximum difference between the most probable value of each ionic strengths is under 0.4 unit. The mean value of the pH at the abscissa zero is 6.76, corresponding to  $K_0 = 1.74 \times 10^{-7}$ .

This constant falls in a range occupied by very few types of acids. Of the limited number of acid radicals in proteins (carboxyl, imino, ammonium ( $R-NH_3^+$ ), phenylhydroxy, thiol, imido, and guanido) only imino groups, as in histidine, and ammonium groups, are near enough in strength to this value to be considered. In most amino-acids and polypeptides, the charged ammonium groups have much smaller acid constants than are required here, but the constant increases 40—60 times with increasing length of the polypeptide chain to a limiting value of pK at approximately 7.8 (COHN, 1931). This limit agrees very well the figure 7.5 calculated by LINDERSTRØM-LANG (1935) for the protamine clupein — a polypeptide of 28 amino-acids residues. Since the clupein chain is studded with 20 well-spaced positive charges from its guanido groups, LANG's finding probably represents the greatest acidity which protein amino groups can attain,

when they are derived from aliphatic monoamino-acids. They are thus 5 or 6 times too weak to fit the requirements of this analysis.

The imino group of histidine has  $pK = 6.0$  or  $6.1$  (LEVY, 1935, and other investigators). Its strength has been measured in the polypeptides histidyl-histidine, in which the mean value for the two imino groups is hardly affected and histidylglycine, in which there is a slight increase (GREENSTEIN, 1931; 1933). It therefore appears unlikely that it could be weakened sufficiently in any combination; the group is fairly remote from the amide linkage.

The strength of amino groups in the tetravalent sulfur-containing amino-acid, cystine, is 15—40 times greater than that of amino groups from other aliphatic amino acids. No measurements are available in polypeptides, but in the amino-acid  $pK$  is about 7.65 (CANNAN and KNIGHT, 1927). By analogy with the behavior of the same group in other amino-acids, its strength in polypeptides and proteins may easily equal or go below the value, 6.76, required by the present interpretation of the kinetic data. This tentative identification of the 5 groups with amino groups of cystine is consistent with NORTHROP's chemical analysis of pepsin which shows that the enzyme contains ten atoms of sulfur, which could correspond to five cystine residues. The analytical data of HERRIOTT and NORTHROP (1934) also show that there are from 3 to 5 primary amino groups per molecule — a remarkably small number, doing much to explain the very acid isoelectric point of the enzyme. Recently, GORTER, VAN ORMONDT, and MEIJER (1935) have shown, by analysis of the pH dependence of surface spreading of pepsin, that the number of amino groups is very small. There is no difficulty in assigning these amino groups to



cystine rather than to lysine, which contributes many of the amino groups of other proteins.

These comparisons neglect the possibility that the constants derived from the kinetic data may be compound, and include a constant for a secondary tautomeric equilibrium. The fact that the analysis has suggested dissociations localized in cystine, already known to be involved in changes accompanying protein denaturation (HOPKINS, 1930; ANSON and MIRSKY, 1935—36) encourages credence in its conclusions. To test them further, experiments were made in buffer solutions containing 1 per cent. formaldehyde (this concentration is without effect on the hemoglobin method). In the simplest case, combination of one HCHO molecule per amino group, and assuming that only the uncombined amino groups govern the stability, the reaction rate should be hardly changed in acid solutions, but should be increasingly diminished with rising pH. The effect actually found was very small — but was greater rather than smaller in more acid solutions. Since it is known that as many as three HCHO molecules can combine with a single amino group, since 5 groups are involved, and since no confident prediction can be made about the reactivity of the various complexes, these tests must be considered to have resulted inconclusively.

The salt effect. — The effect of changes in ionic strength, shown in Fig. 5, is extraordinarily great. Between ionic-strengths of 0.012 and 0.10 the velocity at a given pH increases about 40 times; above this the effect is much smaller and finally reverses. If the explanation proposed for the pH dependence is correct, the ionic-strength can affect the velocity in two ways, corresponding to primary and

secondary salt effects (BRØNSTED, 1928). The latter is not a direct effect on the velocity, but only displaces the dissociation equilibrium which determines the concentration of the

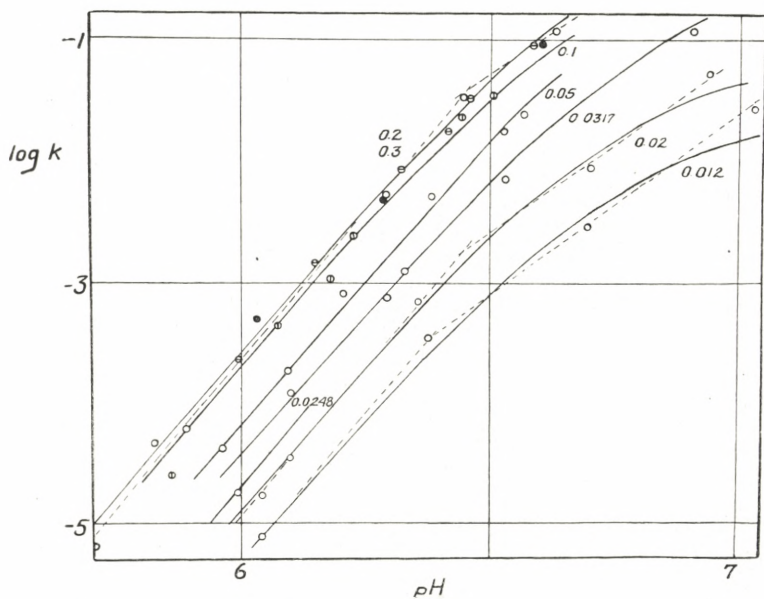


Fig. 5. The assembled data for *p*-nitrophenol buffers at all ionic-strength except 0.5, omitted to avoid crowding. Data from Table II (filled circles) are omitted at pH at which the heterogeneous reaction predominated. The fitted curves are from Eq. 3'; the broken straight lines are an alternative method of determining the relative positions of each set of data, — the upper branch has a slope of three.

reactive pepsin ion. A first-order calculation of the size and sign of this displacement in terms of the foregoing views, may be tested against the displacement along the abscissa of the theoretical curves fitted to the data.

The 5 stages of dissociation may be represented as a single process:



for which the following mass-law equation is valid:

$$\frac{a_{\text{H}^+}^5 f_{\text{P}_5^-} C_{\text{P}_5}}{f_{\text{PH}_5} C_{\text{PH}_5}} = K'. \quad (4)$$

The same argument used for justifying the assignment of identical values to the 5 dissociation constants also requires that the activity-coefficients will be governed by the charge on each group (here +1 on the acid, and zero on the conjugate base), rather than by the net charge of the entire molecule. The subscripts in the equation are misleading in this connection, since the species written  $\text{PH}_5$  actually bears a negative charge, and  $\text{P}_5^-$  differs from it by having 5 positive charges the fewer, so that the ion becomes 5 charges more negative. That the localized charge on a group rather than the net charge on the protein determines the dissociation behaviour of that group as a function of changes in the medium is effectively shown by the successful use of alcohol-water mixtures as a solvent in which to titrate the amino groups of proteins with base, as introduced by FOREMAN (1920) and WILLSTATTER and WALDSCHMIDT-LEITZ (1921); there is a different effect of the medium on the relative strengths of amino groups and indicator, — directly referable to the positive charge which changes to zero on dissociation of a hydrogen ion from the amino group in the course of the titration. The fact that the net charge of the protein is negative is without effect.

Eq. 4 may be written (subscripts now indicate postulated charges;  $\text{P}_0$  is the former  $\text{P}_5$ )

$$\frac{C_{\text{P}_0}}{C_{\text{PH}_5^+}} = K' \frac{f_{\text{PH}_5^+}}{f_{\text{P}_0}} \frac{1}{a_{\text{H}^+}^5} \quad (4')$$



Over the range in which the 5th-power relation between velocity and pH prevails, the left-hand member differs inappreciably from the fraction which enters into the apparent velocity-constant,  $\frac{C_{P_0}}{\sum_0^5 C_{P_i}}$ , and in a small pH-interval this difference is nearly a constant factor, close to unity. With only slight approximation, then:

$$\log \frac{C_{P_0}}{\sum C_{P_i}} + \log k + \log F \cong \log K' + 5 \text{ pH} + \log f_{\text{PH}_5^+} - \log f_{P_0} \quad (4'')$$

where  $k$  is the constant factor, and  $F$  combines all other factors entering into the velocity-constant, including dimensional factors, the primary effect of temperature, and the primary salt effect. The final term is practically independent of ionic-strength in dilute solutions and its variation may be neglected. By writing (4'') for two different ion concentrations, and choosing values of pH at which the same ion ratio is found in the two sets of data, the ion ratio and certain other terms may be eliminated by subtraction, with the result:

$$\text{pH}_{\mu_2} - \text{pH}_{\mu_1} \cong \frac{1}{5} \left[ \log \frac{(f_{\text{PH}_5^+})_{\mu_1}}{(f_{\text{PH}_5^+})_{\mu_2}} + \log \frac{F_{\mu_2}}{F_{\mu_1}} \right] \quad (5)$$

in which the numerical subscripts refer to the two ionic-strengths.

If, on the contrary, a zero charge on the acid groups were assumed the final result, arrived at in an analogous manner, would be:

$$\text{pH}_{\mu_1} - \text{pH}_{\mu_2} \cong \frac{1}{5} \left[ \log \frac{(f_{P^-})_{\mu_1}}{(f_{P^-})_{\mu_2}} + \log \frac{F_{\mu_1}}{F_{\mu_2}} \right]. \quad (5')$$

At constant temperature the second term in the bracket

represents, by definition, the primary salt effect, and may be eliminated by determining  $\text{pH}_{\mu_2} - \text{pH}_{\mu_1}$  from the horizontal component of the displacement in the fitted theoretical curve (Eq. 3') instead of from the pH-interval between the two sets of data for the same velocity (the vertical component of the shift corresponds to the primary salt effect). The remaining term may then be approximately evaluated by analogy with the familiar variation of activity coefficients for the same charge-types as these in simpler ions. On increasing the salt concentration from high dilution to  $\mu = 0.1$ , the activity coefficients of such ions most often fall from values near unity down to about 0.7. Using these figures in (5) and (5') the calculated value for  $\Delta \text{pH}$  in the horizontal component of the displacement over this interval of ionic-strengths is  $-0.031$  or  $+0.031$  respectively, when  $\mu_1$  is taken as the higher concentration. This is an extremely small displacement, at the limit of certainty with which the theoretical curves can be fitted to the data.

Table IV.

Relation of apparent  $\text{pK}_0$  to ionic-strength, determined by superposition of the theoretical curve (upper curve, Fig. 4) to the data.

Ionic Strength	Apparent $\text{pK}_0$ (middle value of possible range)	Displacement (from $\mu_1 = 0.3$ )
0.50	6.800	.044
0.30	6.756	...
0.20	6.765	.009
0.10	6.755	— .001
0.05	6.838	.082
0.0317	6.779	.023
0.02	6.771	.015
0.012	(6.755)	(— .001)

The horizontal displacements represented by the curves in Fig. 5 are given in Table IV.<sup>1</sup> The figures are hardly consistent enough to warrant certainty as to sign (they favour slightly Eq. 5, consistent with amino or imidazole groups), but they agree very well with the requirement, arrived at by the use of the fraction  $\frac{1}{5}$ , that the displacement should be very small.<sup>2</sup> The result also justifies the approximation made when the factor  $k$  was introduced as a constant; over so small a pH interval it cannot vary appreciably.

The greater part of the large effect of ionic-strength shown in Fig. 5 is thus a primary one. In BRØNSTED'S theory of the primary salt effect the formation of an intermediate complex preceding reaction is postulated. When this complex and the ions that form it (the reacting species of the stoichiometric equation) are of unlike charge type, their respective activity-coefficients vary in different ways with the ionic-strength, and the concentration of the complex, on which depends the velocity, varies with the resulting ratio. When the reacting species have other than zero charge this leads to prediction of an exponential salt effect over the range in which the DEBYE-HÜCKEL limiting law is valid, according to the following equation (20°):

$$\log \frac{k}{k_0} = Z_A Z_B \sqrt{\mu} \quad (6)$$

where  $k_0$  is the velocity-constant at infinite dilution, and  $Z_A$  and  $Z_B$  are the charges on the ions forming the critical

<sup>1</sup> The broken straight lines of slopes 5 and 3 in Figs. 5 and 8 show an alternative approximate method for determining these displacements. In every case the displacements of their intersections have closely agreed with the displacements of the coordinates of the theoretical curve.

<sup>2</sup> Were the velocity dependent on the concentration of hydroxyl-ions as MICHAELIS and ROTHSTEIN thought possible, this displacement should be much greater.



complex. This equation shows that a straight line of integral slope should result when the logarithms of the velocity-constants are plotted against the square root of the ionic-strength, and that the slope of this line should be positive if the ions

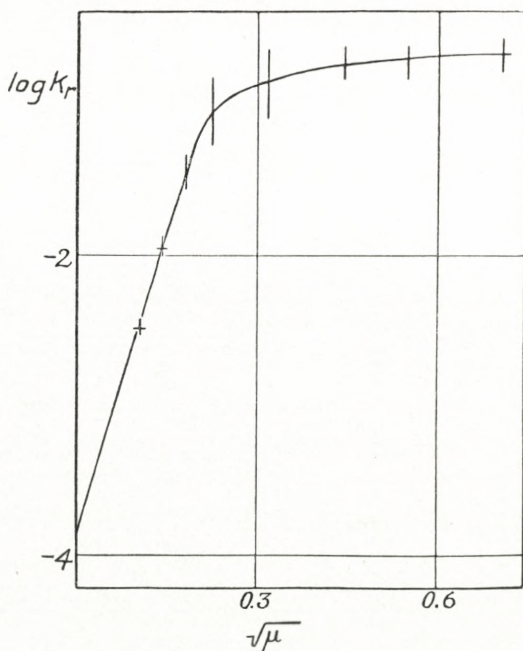


Fig. 6. The primary salt effect with *p*-nitrophenol buffers at 25°. The vertical lines show the range of uncertainty resulting from freedom in fitting Eq. 3' to the data for each ionic-strength.

are of like sign, and negative otherwise. The equation has been applied successfully to a large number of reactions with values of  $Z_A Z_B$  between  $-4$  and  $+4$ . Outside this range it has been less successful, probably because the limiting law itself fails for ions of large charges.

Application of Eq. 6 to the present data must take account only of the change in ordinates between each of the fitted theoretical curves in Fig. 5. In Fig. 6 these ordinates are

plotted against the square root of the ionic-strength, with the length of each vertical line indicating the limits of uncertainty due to curve-fitting. The result is consistent with a linear relationship in the range of dilution in which agreement with Eq. 6 may be expected. The slope, however, is very great, approximately +13. This figure need not imply charges of more than 5 or 6 since ions of high charges sometimes produce limiting slopes of two or even three times the theoretical value. This distinctively large effect, reminiscent of the large salt effects that have been demonstrated in other protein phenomena, notably solubility, is apparently inconsistent with the preceding assignment of activity coefficients on the basis of single group charges. It remains obscure at the present time.

In one respect it requires further consideration: an unusually high sensitivity to ionic-strength entails a high sensitivity to specific ion effects at lower concentrations than those at which they are usually manifested, and the exact application of the principle of the ionic-strength is then limited to even lower salt concentrations than those to which it is generally applied. A practical consequence for these experiments is that mixtures of buffer-ions and neutral salt in different proportion to give the same "ionic-strength" will not give equivalent ionic effects. This is particularly likely at low concentrations, where the ratio of neutral salt to (for example) sodium nitrophenolate, varies over wide extremes. To estimate the magnitude of such specific effects, two experiments were made at  $\mu = 0.0317$ , substituting  $\text{KNO}_3$  for  $\text{KCl}$ ; the nitrate was chosen as most likely among common ions to manifest specific difference from the chloride. The velocities obtained at pH 6.013 and 6.475 were about 50 per cent. and 35 per cent. greater than

when KCl was used. Substitution of small amount of NaCl for KCl was without effect at  $\mu = 0.5$ , where sensitivity to changes in concentration is also low. These experiments show that no appreciable distortion of the pH-velocity relationship is to be expected from specific ion effects except at the lowest salt concentrations, where a very small distortion may result at the least acid end of the data. Higher velocities will be found, owing to the specific effect of the large concentration of the larger nitrophenol ion. This is the direction in which the experimental data deviate from the theoretical curve at  $\mu = 0.02$  and  $0.012$ .

The specific effects of buffers. — Any explanation of the fifth-power relation which depends on a catalytic mechanism is very unlikely; the small secondary salt effect more specifically excludes hydroxyl-ion catalysis. General basic catalysis (by the buffer anion) can only be excluded rigidly by showing that the velocity does not depend on the concentration of the buffer, or on the strength of the buffer acid. The pH relationship itself is incompatible with such a dependence on the concentration of the anion, provided the dissociation constant of the buffer is not the same as the dissociation constant used in calculating the theoretical curve. Nevertheless, in order to obtain good buffering, the buffer selected will tend to have a constant close to this.

In most of the experiments the buffer was *p*-nitrophenol; the *pK* of this acid in 0.1 M KCl is 6.95 while that of the postulated pepsin groups is 6.76. The difference, though small, is much outside the limits of error, but to exclude the possibility of basic catalysis beyond a doubt experiments were made with other buffers of widely differing strengths,



If basic catalysis is involved, the pH range over which an inverse fifth-power relation to acidity is found should differ with each buffer.

Fig. 7 shows data obtained with trimethyl-*o*-aminophenol

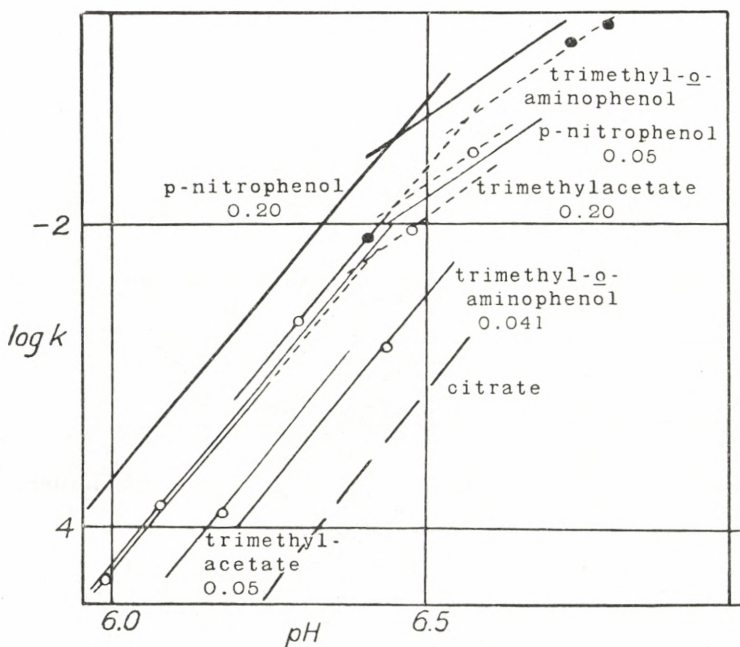


Fig. 7. Data for other buffers compared with the results with *p*-nitrophenol at 25°. The latter are represented by broken lines of slope 5 and 3, taken from Fig. 5. The position of the citrate data, indicated by a line, is only approximate.

halide ( $pK \cong 7.4$ ) at  $\mu = \text{ca. } 0.20$  and  $0.041$ , and with trimethylacetic acid ( $pK \cong 5.0$ ) at  $\mu = 0.20$  and  $0.05$ . For reference, curves representing the data for nitrophenol at  $\mu = 0.20$  and  $0.05$  are included. The general position of very much rougher results with poorly buffered citrate solutions is also shown by a broken line. Although there are few points, and they do not all represent strictly com-

comparable experimental conditions, it is clear that the relation of velocity to pH is not changed, although the three acids differ in strength by about 200 times. It is also evident that comparable salt effects are found with all the buffers.

The absolute rate of reaction differs with each buffer; even disregarding the results with citrate there is a factor of three between two of the others, though with no relation to the acid strength. *p*-Nitrophenol, which gives the fastest rate, has a strength between that of the other two. The latter differ in strength by about 200 times and give almost identical rates.

The experiments of MICHAELIS and ROTHSTEIN, with phosphate buffers, add further detail to the situation shown in Fig. 7, if the effect of impurities in their solutions may be neglected. The velocities reported are much lower than any in Tables II and III, and are comparable with the results obtained with citrate. This specific influence of the buffer cannot be explained at present.

The effect of temperature. — The large temperature coefficients hitherto reported for protein denaturation have been obtained by comparing the velocity at two different temperatures at the same pH. When values so obtained are transformed into "energies of activation" through the ARRHENIUS equation, they invariably lead to such large figures (with two enzymes, trypsin and ptyalin, the values are about 150,000 and 120,000 calories respectively; trypsin, at least, is a protein) that the observed rate of reaction cannot be accounted for by any known process for the redistribution of energy between molecules in gases or in solution. This method of calculating activation energies is incorrect if the view of the pH-velocity dependence set forth in this

paper is accepted, for the concentration of the reactive ionic species also increases at higher temperatures, and this increase contributes to the total kinetic temperature effect. This is made plain by Fig. 8, which shows the reaction rates as a

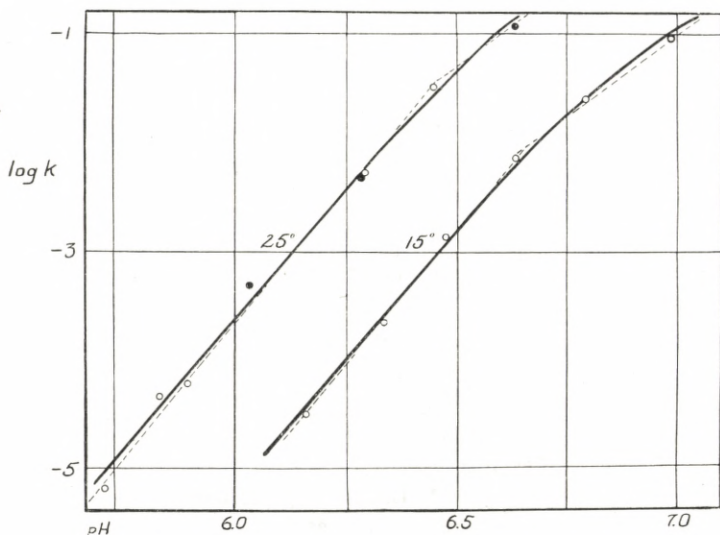


Fig. 8. A comparison of the pH-dependence of the velocity-constant at two temperatures. The curves are from Eq. 3'. The broken straight lines are included as an additional method of appraising the shift in the coordinates of the sets of data, as in Fig. 5.

function of pH at 15° and 25° at the same ionic strength, and with the same buffer. The data for the lower temperature are given in Table V. Even casual inspection shows that the greater part of the temperature effect consists of a horizontal shift in the curves, and that only a small part of the increase in velocity at the same pH is due to a vertical change in coordinates, — i. e. an increase in the rate at which the unstable ion decomposes. Precise appraisal of the two components of the total effect is handicapped by failure of the data to extend to more alkaline solutions.



Table V.

Velocity of inactivation at 15° and ionic strength 0.20; *p*-nitrophenol buffers. No shaking.

pH	$\log_{10} k_{10}^{\min}$	Initial Enzyme (mg. N/ml.)
6.161	-4.483	0.00288
6.333	-3.638	.00290
6.473	-2.851	.00309
6.630	-2.120	.00328
6.788	-1.593	.00266
6.982	-1.021	.00341

Within the limits of uncertainty imposed by this circumstance, the most probable value for the horizontal shift can be set as 0.23 pH units. This is equivalent to a heat of dissociation for each of the five groups (again assumed to be alike) of 9040 calories.<sup>1</sup> While this figure is only approxi-

<sup>1</sup>  $\Delta H = 4.571 \Delta \text{pH} \cdot \frac{T_2 T_1}{T_2 - T_1}$  by assuming  $\Delta H$  to be constant over the 10° temperature interval. This equation is not affected by the simultaneous functioning of 5 groups. This is easily shown over the range in which the logarithmic graph is linear:

$$\log \frac{P_5}{\sum_0^5 P_i} \cong 5 \log K_0 + 5 \text{pH}.$$

Combining this with the familiar equation for the dependence of  $K$  on  $T$ , it becomes:

$$\log \frac{P_5}{\sum_0^5 P_i} \cong 5 \left[ \frac{\Delta H}{4.571 T} + C \right] + 5 \text{pH}.$$

For equal values of the left-hand member, at two temperatures:

$$5 \left[ \frac{\Delta H}{4.571 T_1} + C \right] + 5 \text{pH}_1 = 5 \left[ \frac{\Delta H}{4.571 T_2} + C \right] + 5 \text{pH}_2.$$

The factor 5 and the constant  $C$  thus cancel out. Use of the exact equation 3' does not affect the result. The only assumption required is identity of the dissociation constants and their heat effects.

mate, it clearly favours the previous identification of the acid constants with ammonium-like groups. In amino-acids these groups have heats varying from about 9000 calories (in histidine and tyrosine) to 12,800 calories. The highest value characteristic of other groups is 7000 calories for the imino group of histidine.

In regions where both curves are approximately straight lines,  $Q_{10} = 40$ , corresponding to an apparent energy of activation of 63,500 calories. This is fairly near to the figure, 58,000, given by the data of MICHAELIS and ROTHSTEIN. Of this quantity, 45,200 ( $5 \times 9040$ ) is the heat of dissociation of the stages determining the concentration of the unstable ion. Thus, the true value of  $E$  (activation energy) for decomposition of the reacting ion is the remainder, 18,300 calories. The same figure is given by direct estimation of the vertical displacement between the two curves. The relative uncertainty in  $E$  is necessarily larger than in  $\Delta H$ ; this does not affect the main point of interest here which is the great difference between the apparent and true energies of activation, and the fact that the true value, 18,300, is a magnitude commonly met with in ordinary chemical processes.<sup>1</sup>

This difference has manifest significance for kinetic calculations since it removes the paradox associated with the larger value. When the ARRHENIUS equation is rewritten in the form:

$$k = Ze^{\frac{-E}{RT}}$$

<sup>1</sup> MICHAELIS and ROTHSTEIN suggested from their temperature data that practically the entire effect of temperature could be accounted for by its influence on  $C_{OH}$  at fixed pH, if the reaction-mechanism was catalysis by hydroxyl ions. This suggestion involves the assumption that the inverse kinetic relation to  $C_H$  signifies direct dependence on  $C_{OH}$ . Were this true, in light of their data,  $E$  should be zero, and the reaction should be immeasurably fast.

in which  $k$  is the natural velocity-constant expressed in reciprocal seconds, and  $Z$  is a factor which may be regarded, to a first approximation, as the number of molecular collisions per second per unit volume, — its most common value in simple bimolecular gas-reactions is approximately  $10^{11}$  in close agreement with the predictions of the kinetic theory of gases. Recent surveys of reactions in solutions (MOELWYN-HUGHES, 1933 *b*; CHRISTIANSEN, 1924) have shown that in many cases comparable values of  $Z$  are found, and that in most cases  $Z$ , regarded as an empirical factor seldom departs from  $10^{11}$  [by a factor of more than 100. Values of  $\log Z$  over 14 or 15 are paradoxical because the number of collisions between reactant molecules and other molecules is not likely to exceed  $10^{14}$ ; even this value can be attained only by very large molecules with large persistence of velocity after collision (MOELWYN-HUGHES, 1933 *a*). No mechanism is then provided for the initiation of individual molecular reactions, nor even for the maintenance of a number of molecules with sufficient energy to react.

$Z$  has been calculated for the present data, using alternatively the apparent and corrected values of  $E$ . The velocity-constant has been taken from the *p*-nitrophenol data extrapolated to infinite dilution (Fig. 6) at a pH corresponding to  $\frac{K}{a_H} = 0.446$ . Use of data for the other buffers would give lower values, but a factor of 3 is of little importance in these calculations.

$\log k$  (extrapolated) is  $-3.9$ , which, after correction to reciprocal seconds and Napierian logarithms, becomes  $\ln k_{\text{sec.}} = -13.1$ . This number must still be transformed from a decadic to a natural base (in the first order kinetic equation), on which it becomes  $-12.26$ . The reaction-rate is still



expressed in terms of the total pepsin; on changing to the amount present as the reactive ion ( $P_5$ ),  $\ln k'_{\text{sec.}}^{\text{Nap.}} = -9.74$ . Since  $E$  is 18,300  $\frac{E}{RT}$  is 31.2. Combining these numbers,  $\ln Z = 21.46$ , or  $\log Z = 9.3$ . This result is well within the range of values commonly found. Interpreted literally, it signifies that approximately one out of one hundred collisions between unstable ions possessing the activation energy and solvent molecules, result in reaction. Even the use of the fastest data (nitrophenol buffers) and correction for the very small amount of reactive ions, has given a result that is low rather than high.

If a straightforward calculation is made with the uncorrected value, 63,500 calories,  $\log Z$  is approximately 41, and the observed rate is thus at least  $10^{26}$  times too fast to be accounted for by ordinary kinetic considerations. This anomaly is present in all earlier kinetic data on protein denaturation or enzyme destruction and various proposals (among others, the existence of reaction chains and contributions to the activation energy from many degrees of freedom) have been advanced to account for it (see, for example, MOELWYN-HUGHES, 1933 *a*, and LEWIS, 1931). Other authors have tried to account for the high energy without recognizing the paradox associated with it (MIRSKY and PAULING, 1936, among others). Should the present calculations prove applicable to other proteins, the difficulties arising from the large temperature effect in denaturation may prove to be illusory.<sup>1</sup> Data on ptalin (EGE, 1932) and hemoglobin (P. S. LEWIS, 1926, 1927) show that the occurrence of a large exponent in the kinetic depen-

<sup>1</sup> Theories of the mechanism of denaturation which partly rest on large values for  $E$  (such as MIRSKY and PAULING's) may then require some revision.

dence on acidity, on which these calculations are based, is common.

The foregoing computations, though limited in exactness, support the interpretation of the pH effect which underlies them. They can also be extended to explain the frequently observed variation of the apparent  $Q_{10}$  or  $E$  with pH in protein denaturation (it is readily seen in Fig. 8, at pH above 6.5). The observation that  $E$  appears to become smaller and approaches a limiting value at high temperatures (LEWIS'S recalculation of the data of CHICK and MARTIN) when measured at a single pH is also understandable from the shift of the curves left-ward with increasing temperature, — regions of smaller slope, therefore smaller vertical separation, coming to lie at the pH of the experiment (most of the protein becoming transformed to the unstable ion). The limits found, with serum proteins about 23,000 calories, are only slightly higher than the 18,300 computed here. It is noteworthy that the temperature effect in acid denaturation is frequently much smaller than in alkaline solutions (see, for example, EGE, 1925, 1934). In acid solutions only carboxyl groups can be dissociating, and the effect of temperature on their dissociation is vanishingly small.

The author wishes to record his indebtedness to Professor J. N. BRØNSTED for generous provision of laboratory facilities and assistance, and for affording frequent opportunities to discuss the ideas developed in this paper. He is likewise grateful to Dr. KAI LINDERSTRØM-LANG and Dr. HEINZ HOLTER of the Carlsberg Laboratories, to Professor J. A. CHRISTIANSEN of the Chemical Laboratory, Danmarks tekniske Højskole, and to Mr. EINAR GÜNTEMBERG of the Institute of Physical Chemistry, for much fruitful advice information. The kind interest of Dr. J. N. NORTHROP of the

Rockefeller Institute for Medical Research at Princeton was an important factor in encouraging the author to undertake this work.

Experimental assistance was rendered by Miss TOVE PONTOPPIDAN THOMSEN.

## VI. Summary.

Crystalline pepsin, in solutions of constant ionic-strength on the alkaline side of its pH stability maximum inactivates unimolecularly at a rate which is inversely proportional to the fifth power of the hydrogen-ion concentration. This relation has been found to apply over a range of velocity of 1 to 5000 in the results of nearly 100 kinetic experiments at two temperatures with four different buffers, and at various ionic-strengths. The rate varies with the buffer but the fifth-power dependence does not. Over a still wider range the results are in quantitative agreement with the view, supported by other evidence, that the rate depends on the concentration of a single pepsin ion in which all 5 of the primary amino groups of the enzyme have lost their positive charges. If all five groups are of equal strength, their acid dissociation constant is  $1.74 \times 10^{-7}$ . Evidence is submitted for localizing these groups in cystine residues.

On shaking the reaction-vessels, a heterogeneous reaction, independent of pH and  $\mu$ , obscures the results at pH below 6.1. Above this, the homogeneous reaction predominates, and shaking cannot modify the results otherwise obtained. Experiments without shaking show that at the pH stability maximum (5.28 at  $\mu = 0.2$ ) the half-period of inactivation is approximately 8 years.

With increasing salt concentration ( $\mu = 0.012$  to  $\mu = 0.1$ ) the velocity at constant pH increases nearly ex-



ponentially about 40 times. At higher ion-concentrations the change is smaller, and finally reverses. The virtual absence of a secondary salt effect is consistent with the theory of the pH dependence.

If account is taken of the effect of temperature on the size of pepsin fraction present as the unstable ion, the temperature effect on the rate of the actual reaction (decomposition of this ion) is much smaller than the observed change in velocity. When the ratio  $\frac{k_{25^\circ}}{k_{15^\circ}}$  (about 40) is used to calculate the critical increment, the result is 63,500 calories, — a high figure, characteristic of protein denaturation. When the shift in the pH dependence is allowed for, assuming all five groups alike, an ordinary value, 18,300, is obtained. The exponent produces a large disparity, though the heat of dissociation of each group is only 9040 calories. The latter value favours the view that primary amino groups are involved.

The observed reaction rates stand in reasonable relation to the number of molecular collisions with activated unstable pepsin ions, when the corrected figure, 18,300, is used for the energy of activation. They are too high by a factor of  $10^{26}$  when the uncorrected figure, 63,500, is used. Should this result apply to the proteins generally, the theoretical difficulties arising from the great temperature effect in denaturation may prove to be illusory. The frequently observed variation of critical increment with pH, and its decline to a limiting value at high temperatures, can also be explained.

## Citations.

- ADAMS, E. Q., *Journ. Am. Chem. Soc.*, 1916, **38**, 1503.
- ANSON, M. L., and MIRSKY, A. E., *Journ. Gen. Physiol.* 1932—33, **16**, 59; *Journ. Gen. Physiol.* 1935—36, **19**, 427, 439, 451, 559, 571.
- ARRHENIUS, S., *Quantitative Laws in Biological Chemistry*, New York, 1915.
- BRØNSTED, J. N., *Chem. Rev.*, 1928, **5**, 231.
- CANNAN, R. K., and KNIGHT, B. C. J. G., *Biochem. Journ.* 1927, **21**, 1384.
- CHRISTIANSEN, J. A., *Zts. physik. chem.*, 1924, **113**, 35.
- COHN, E. J., *Ergebn. d. physiol.*, 1931, **33**, 781.
- EGE, R., *Zts. f. physiol. chem.* 1925, **143**, 159; *Biochem. Zts.* 1932, **244**, 243; *Zts. f. physiol. chem.*, 1934, **224**, 129.
- FOREMAN, F. W., *Biochem. Journ.*, 1920, **14**, 451.
- GORTER, E., VAN ORMONDT, H., and MEIJER, T. M., *Biochem. Journ.*, 1935, **29**, 38.
- GOULDING, A. M., WASTENEYS, H., and BORSOOK, H., *Journ. Gen. Physiol.*, 1926—27, **10**, 451.
- GREENSTEIN, J. P., *Journ. Biol. Chem.*, 1931, **93**, 479; *Journ. Biol. Chem.*, 1933, **101**, 603.
- GUGGENHEIM, E. A., and SCHINDLER, T., *Journ. Phys. Chem.*, 1934, **38**, 533.
- HERRIOTT, R. M., and NORTHROP, J. H., *Journ. Gen. Physiol.*, 1934—35, **18**, 35.
- HOLTER, H., *Zts. f. physiol. chem.*, 1931, **196**, 1.
- HOPKINS, F. G., *Nature*, 1930, **126**, 328.
- LEVY, M., *Journ. Biol. Chem.*, 1935, **109**, 361.
- LEWIS, P. S., *Biochem. Journ.*, 1926, **20**, 965, 978, 984; 1927, **21**, 46.
- LINDERSTRØM-LANG, K., *Trans. Farad. Soc.*, 1935, **31**, 324.
- McINNES, D. A., and DOLE, M., *Journ. Am. Chem. Soc.*, 1931, **53**, 1357.
- MICHAELIS, L., and ROTHSTEIN, M., *Biochem. Zts.*, 1920, **105**, 60.
- MIRSKY, A. E., and PAULING, L., *Proc. Nat. Ac. Sci.*, 1936, **22**, 439.

- MOELWYN-HUGHES, E. A., *Erg. d. Enzymforsch.*, 1933 *a*, **2**, 1; *Kinetics of Reactions in Solution*. Oxford, 1933 *b*.
- NORTHROP, J. H., *Journ. Gen. Physiol.*, 1929—30, **13**, 739; *Journ. Gen. Physiol.*, 1929—30, **13**, 767; *Journ. Gen. Physiol.*, 1930—31, **14**, 713; *Journ. Gen. Physiol.*, 1932—33, **16**, 33.
- PHILPOT, J. St. L., and ERIKSON-QUENSEL, I.-B., *Nature*, 1933, **132**, 82.
- PHILPOT, J. St. L., *Biochem. Journ.*, 1935, **29**, 2458.
- SVEDBERG, T., *Trans. Farad. Soc.*, 1930, **26**, 740.
- SØRENSEN, S. P. L., *Cont. rend. d. trav. d. Lab. Carlsberg*, 1930, **18**, 5.
- SØRENSEN, S. P. L., SØRENSEN, M., and LINDERSTRØM-LANG, K., *Cont. rend. d. trav. d. Lab. Carlsberg*, 121, **14**.
- WILLSTATTER, R., and WALDSCHMIDT-LEITZ, E., *Ber. d. deutsch. chem. Gesell.*, 1921, **54**, 2988.
- WINNEK, P. S., and SCHMIDT, C. L. A., *Journ. Gen. Physiol.*, 1934—35, **18**, 889.
-



