THE AUTODESTRUCTION OF PEPSIN IN RELATION TO ITS IONIZATION.

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The irreversible disappearance of the activity of pepsin in alkaline solution was first recorded by Kühne (1). It was confirmed by Langley (2), who observed also "that the destruction by sodium carbonate solutions goes on very much more slowly at 15°C. than at body temperature." Langley's experiments were carried out with neutralized filtrates of extracts of rabbit gastric mucosa made with 0.1 per cent HCl. He found no especial differences in the pepsins from the cat, dog, mole, newt, and snake. He mentions a possibility of frog pepsin being destroyed more rapidly than that of mammals. Biernacki (3) observed that the proteolytic activity of gastric juice was retained at a higher temperature in acid solution than at neutrality. This author found also that the resistance to destruction by heat was increased by the presence of peptones, and that a pure preparation was accordingly less stable than the pepsin in gastric juice. Grober (4) concluded from the average of a number of determinations that urinary pepsin heated with 0.25 per cent HCl was destroyed at 66°C., while pepsin from the same source, dissolved in water, was destroyed at 64°C. This difference he interpreted as indicating a chemical combination between the acid and the pepsin. The combination was thought to retard the hydrolysis of the pepsin. This mechanism was considered by Grober to be the cause of the destruction of pepsin by heat.

Michaelis and Rothstein (5) investigated the kinetics of the destruction of rennin and pepsin. They were unable to distinguish between these two properties of gastric juice preparations by their behaviour towards alkali. They derived an empirical differential equation in which the velocity of autodestruction was proportional to the fourth power of the hydroxyl ion concentration. They found that

at 25°C. and at 35°C., at reactions more acid than pH 6.0, the enzyme suffers no loss in potency, and that on approaching neutrality from the acid side the velocity of destruction suddenly becomes increasingly more rapid, until, in faintly alkaline solutions, it is too great to be measured. Michaelis and Rothstein found that the autodestruction at any given hydrogen ion concentration was not monomolecular. The velocity at constant hydrogen ion concentration in their experiments was proportional to the one and one-half power of the enzyme concentration. The destroyed pepsin was without influence upon the destruction of the remaining active enzyme, from which Michaelis and Rothstein concluded that the reaction is irreversible.

Northrop (6) found that pepsin solutions were most stable at 38°C. at a hydrogen ion concentration of 10^{-5}. "Neither the purity of the enzyme nor the anion of the acid used exerted any marked influence upon the rate of destruction, or on the zone of hydrogen ion concentration in which the enzyme was most stable."

The ionic nature of pepsin was suggested by Loeb in 1909 (7).

These studies of the autodestruction of pepsin are unanimous in their agreement upon the rapid destruction of the enzyme in acidities less than pH 6.0 The purpose of the investigation described below was to discover, if possible, the mechanism of the action of the OH ion. The method differed from that of other workers in that the amount of active enzyme present, at any given hydrogen ion concentration, was determined for zero time. This procedure is facilitated by the fact that the logarithms of the amounts of active enzyme present give a straight line when plotted against the time that the pepsin has been subjected to the hydroxyl ion concentration under consideration.

The zero values at 15°C. so obtained, plotted against pH, fall about a curve which represents the dissociation of a univalent compound with a value for pK of 6.85. The conclusion seems permissible that pepsin is such a univalent compound. This is in accordance with the conclusion of Northrop (8), attained by a totally different method, that pepsin is a univalent compound in the range of hydrogen ion concentrations from pH 1 to pH 7.

When the initial amount of enzyme is taken to be the extrapolated value found for active enzyme at zero time, results at 20°C. and 37°C.,

with those obtained at 15°C., showed that the autodestruction of pepsin is a monomolecular reaction. Michaelis and Rothstein assumed that at all hydrogen ion concentrations 100 per cent of the enzyme was active at zero time. This assumption, however, in the light of the experimental data given below seems to be unsound.

The products of the autodestruction of pepsin exert no inhibiting influence on the rate of subsequent autodestruction. This conclusion is drawn from the absence of any constant diminishing tendency in the values for K, and is similar to that of Michaelis and Rothstein.

The velocity of autodestruction is directly proportional to the hydroxyl ion concentration, when this is greater than pOH 7.7. This linear relationship is shown in Table V and Fig. 3.

It is difficult to interpret the variations in K over the whole range of hydroxyl ion concentrations at which the autodestruction was followed. The curve obtained may represent two different processes, or one process somewhat similar to the neutralization of a strong acid with a strong base.

The relation of autodestruction to ionization and the monomolecular decomposition suggest the following outline of the mechanism involved in the autodestruction of pepsin.

There are two independent processes, both resulting in irreversible loss of enzyme activity. The first is the ionization of the pepsin, with the two obvious possibilities of pepsin as either a weak acid or a weak base. If it is a weak acid, Fig. 2 represents the undissociated residue of the enzyme; and it follows that it is the undissociated form of the enzyme which is active in hydrolysis. If pepsin is a weak base, on the other hand, then the curve represents the degree of dissociation, and it is the pepsin ion which is active. For the purposes of discussion the enzyme will be considered as a weak acid and the curve will be taken to represent its undissociated and hydrolytically active residue. It is the ionized form, therefore, which must be considered to undergo the irreversible change which results in the destruction of the enzyme. This occurs at zero time, *i.e.* as soon as the alkali is mixed with the pepsin. The progressive falling off from 100 per cent, with increasing hydroxyl ion concentration, of the extrapolated values at zero time, makes it necessary to consider this change irreversible. Since ionization is completely reversible, another subsidiary reaction

must be considered to occur, possibly some stereoisomeric transformation, from which the original, undissociated hydrolytically active form, cannot be regained on restoration of the acidity to pH 1.6.

This transformation must permit the newly formed, irreversible compound to function in the ionic equilibrium as well as its precursor. Otherwise the second component of the process of autodestruction about to be discussed could not be defined by the equation for a monomolecular reaction.

The first process can be represented then by the following equations.

$$(1)\quad HA \rightleftarrows H^+ + A^-$$

$$(2)\quad A^- \longrightarrow A_1$$

Equation (1) represents ionization and (2) the instantaneous, irreversible, complete conversion of the anion into an isomer, which, while it may participate in the ionic equilibrium, does not, on recombination with H^+, form active enzyme.

The second mode of irreversible loss of activity of the pepsin can be represented by the equation,

$$(3)\quad HA \longrightarrow B$$

As discussed above, this reaction is defined by the mass law equation for a monomolecular reaction.

The original amount of active enzyme; *i.e.*, a, in the equation $K = \frac{1}{t} \log \frac{a}{a - x}$, is the extrapolated value of active enzyme at zero time.

This quantity is represented by the amount of H A at any given pH at zero time as shown in equation (1). If reaction (3) were dependent upon (1) and (2), then as H A was converted to B, equation (1) would be reversed in order to form H A from H^+ and A^-. If this happened then (3) could not be defined by the monomolecular equation.

The mechanism of this independence of (3) from (1) is probably represented by equation (2) which does not permit the reversion of any of A to H A. This possibility suggested; *viz.*, the formation of an isomer, which, while incapable of taking part in the reconstitution of the hydrolytically active form of the enzyme, may nevertheless func-

tion in ionic equilibrium, does not appear capable of direct experimental verification. A similar conception was advanced by Northrop (9).

The scheme involves two consequences. The first is, that no matter how low the temperature at which a solution of pepsin be maintained, if the C_{H^+} be lowered below that required for minimum ionization, there is an immediate and irreversible destruction of more or less of the pepsin depending on the pH. The second consequence is that the relation of the progressive autodestruction to the C_{OH^-} must be independent of the degree of dissociation of the pepsin, *i.e.* must show no relation to the dissociation curve. Both of the consequences are found in practice.

The first is demonstrated in the fact that an extrapolated value of 100 per cent is not obtained at any pH at which dissociation occurs. The second is indicated by the lack of any obvious relationship between Figs. 2 and 3.

EXPERIMENTAL.

In the experiments at 15°C., egg albumin (Merck) and scale pepsin (Wyeth) were employed. In those at 20°C., and at 37°C., which were performed 2 years previously, egg albumin (Merck) and scale pepsin (Merck) were used.

A 10 per cent solution of pepsin was made in N/10 HCl, giving an acidity approximating pH 3. An electrometric titration was carried out with N/1 NaOH to ascertain the amount of alkali required to bring the pepsin solution to a desired pH. For the autodestruction experiments 10 cc. of the stock 10 per cent pepsin solution was measured into a 50 cc. volumetric flask in a water bath at 15°C. Distilled water employed for dilution, and the N/10 NaOH, were also brought to 15°C. The calculated amount of alkali was added to the volumetric flask followed by distilled water up to the mark. The flask was shaken, and the contents transferred to a 100 cc. Erlenmeyer flask which was stoppered and placed in the water bath at 15°C. At the recorded times 5 cc. of this solution was removed with a pipette and added to 50 cc. of albumin solution at pH 1.6 and at 30°C. The hydrolysis was allowed to proceed for 1 hour when 50 cc. of the digest was removed and pipetted into 12.5 cc. of 10 per cent trichloroacetic

acid. The total nitrogen of the filtrate, after deducting the appropriate blanks, was taken as representing the amount of protein hydrolyzed. After allowing for dilution, the percentage of active enzyme was calculated from the amount of hydrolysis effected by 5 cc. of similar pepsin solution which had been diluted with water only, and to which no alkali had been added. Autodestruction was assumed to have commenced from the moment at which the NaOH was added to the pepsin.

The hydrogen ion concentration was measured both electrometrically and colorimetrically. M/10 phosphate solutions with methyl red, brom-cresol purple, and brom-thymol blue as indicators, were employed as colour standards. The colour of the pepsin solution was not deep enough to interfere with colorimetric estimations. Throughout the range from pH 5.5 to 7.15 the electrometric and colorimetric readings were identical.

On account of the relatively high concentration of albumin employed, 3.2 per cent, it was not necessary to add a buffer to the protein solutions undergoing hydrolysis. With the most active pepsin solution the pH, after 1 hour's hydrolysis at 30°C., rose only from 1.6 to 1.7.

Three controls were used for each hydrogen ion concentration; the estimation of total non-protein nitrogen of albumin and of pepsin, and the determination of the amount of hydrolysis effected by enzyme which had been diluted only with water, *i.e.* had undergone no autodestruction. In the results given in Table I and Fig. 1 these controls have been deducted. The amounts of active enzyme were calculated from the proportion between the amounts of hydrolysis obtained with the enzyme partially destroyed and that effected by enzyme of unimpaired activity.

At each C_{H^+} the extrapolated zero value ((a) in Table I) is therefore stated as a proportion of the original unimpaired activity of the enzyme. Similarly, the values given under $(a - x)$ (Table I) represent the amounts of hydrolysis obtained with partially destroyed enzyme. K (Table I) is calculated by means of the mass law equation for a monomolecular reaction. In Columns (a) and $(a - x)$, 1 per cent represents about 0.15 to 0.3 mg. of N in the macro-Kjeldahl determination.

In Fig. 1 are plotted the logarithms of the amounts of active enzyme

TABLE I.

The Effect of C_{H^+} on Autodestruction of Pepsin at 15°C.

pH	Time	Amount of active enzyme $(a - x)$	Amount of active enzyme at zero time (a)*	$K = \frac{1}{t} \log \frac{a}{a - x}$	Average value for K
	min.	*per cent of original amount*	*per cent of original amount*		
4.35	10	99	99.6	.0002	.0004
	20	96.3		.0007	
	40	96.0		.0004	
	50	94.7		.0004	
4.6	10	(96)	99	—	—
	20	100		—	
	30	98		—	
	40	98		—	
	50	100		—	
5.2	10	95	96	—	—
	20	(91)		—	
	30	96		—	
	50	(99)		—	
	60	96		—	
5.9	10	88.5	88.8	.0002	.0002
	20	(90)		—	
	30	86		(.0007)†	
	40	87.5		.0002	
	50	87.6		.0001	
5.9	10	82	80.9	—	.0005
	20	79		.0005	
	30	78		.0006	
	40	77.6		.0005	
6.22	10	80	80.7	(.0004)	.001
	20	77.5		.001	
	30	75.4		.001	
	40	73.6		.001	
	50	72.0		.001	
6.4	10	71	73.3	.0014	.0014
	20	69		.0013	
	30	66		.0015	

TABLE I—*Concluded.*

pH	Time	Amount of active enzyme $(a - x)$	Amount of active enzyme at zero time (a)*	$K = \frac{1}{t} \log \frac{a}{a - x}$	Average value for K
	min.	*per cent of original amount*	*per cent of original amount*		
6.54	10	68.3	67.9	—	.0007
	20	66.0		.0007	
	40	63.1		.0008	
	50	62.8		.0007	
6.64	15	57.7	58.2	(.0003)	.0014
	30	52.2		.0015	
	45	50.6		.0014	
	60	48.8		.0013	
6.75	10	46	56.2	.009	.011
	20	33.5		.011	
	30	23		.013	
	40	19		.012	
	50	16		.011	
6.85	10	52.2	69.3	.012	.012
	20	37.4		.013	
	30	28.7		.013	
	40	21.3		.013	
	50	18.8		.011	
6.97	4	29.6	44.2	.044	.045
	8	17.9		.049	
	12	12.9		.045	
	16	9.3		.042	
	20	5.3		.046	
7.15	2	18.3	26.3	(.08)	.17
	4	5.5		.17	
	6	2.5		.17	
	8	0.9		.18	
	10	0.6		.16	
7.35	1.25	4	23.2	.61	.66
	2	1		.68	
	3	0.2		.69	

* Obtained by extrapolation (Fig. 1).

† Bracketted values for K were not used in computing the average value for K.

present against the length of time that the pepsin solutions were subjected to the respective hydroxyl ion concentrations. These plots give straight lines, as mentioned above, and are produced to zero time.

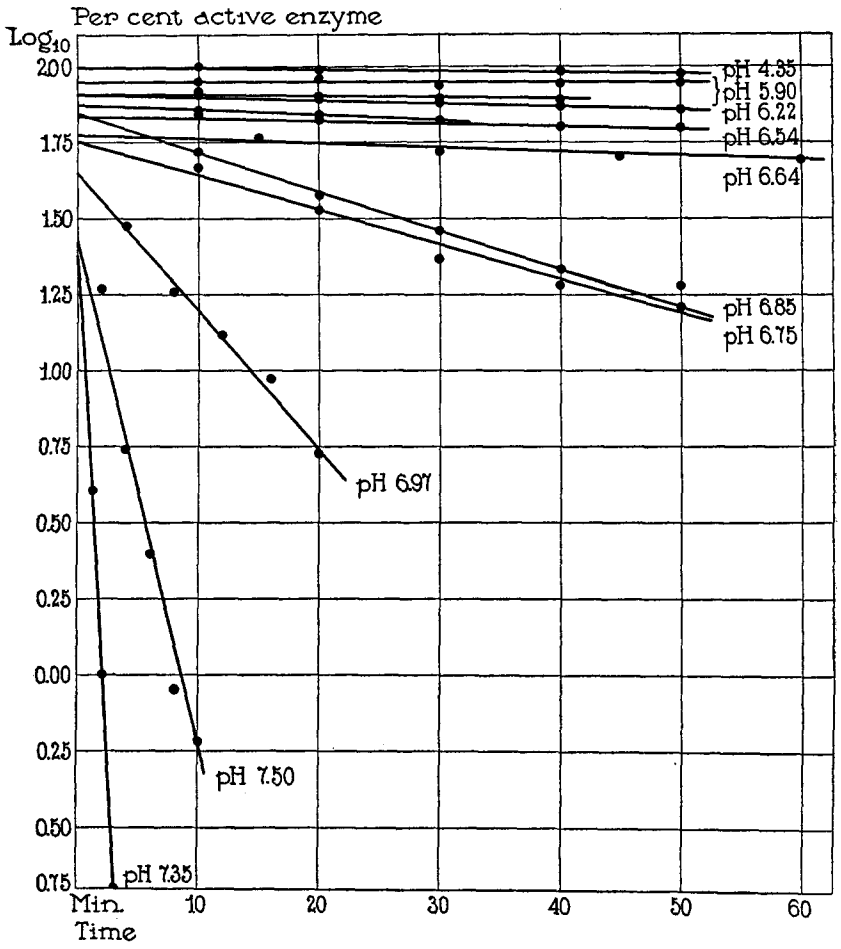

FIG. 1. The autodestruction of pepsin at 15°C. with time, at various reactions. The points are the $\log_{10}$ of amounts of active enzyme (in per cent of original amount). The values for the per cent of active enzyme at zero time are plotted against pH in Fig. 2.

In Tables II and III are given results of autodestruction experiments carried out at 20°C. and at 37°C. The pH values are uncertain and hence these cannot be compared with experiments at 15°C. They

TABLE II.

Autodestruction of Pepsin at Different Hydrogen Ion Concentrations More Alkaline than pH 6.0 and at 20°C.

Time	Amount of active enzyme $(a - x)$	Amount of active enzyme at zero time (a)*	$K = \frac{1}{t} \log \frac{a}{a - x}$	Average K
min.	*per cent of original amount*	*per cent of original amount*		
3	75.9	81.3	.010	.011
6	69.2		.011	
9	64.6		.011	
12	60.3		.011	
3	36.3	44.7	(.030)†	.041
6	25.1		.042	
9	19.1		.041	
12	15.1		.039	
15	10.5		.042	

* Obtained by extrapolation.

†Bracketted values for K were not used in computing the average value for K.

TABLE III.

Autodestruction of Pepsin at Different Hydrogen Ion Concentrations More Alkaline Than pH 6.0 and at 37°C.

Time	Amount of active enzyme $(a - x)$	Amount of active enzyme at zero time (x)*	$K = \frac{1}{t} \log \frac{a}{a - x}$	Average K
min.	*per cent of original amount*	*per cent of original amount*		
15	33	62.4	.018	.017
30	19.6		.017	
105	1.0		.017	
3	36.8	50.1	.045	.045
9	19.2		.046	
12	15.2		.043	
15	10.4		.046	
3	29.6	39.8	.043	.056
6	18.4		.056	
9	12.0		.058	
12	8.8		.055	
15	4.0		.067	

* Obtained by extrapolation.

show, however, that at a given pH the course of the autodestruction of pepsin at these temperatures is defined by the monomolecular equation as at 15°C.

In Table IV are given, for various hydrogen ion concentrations, the values for the undissociated residue of an acid with pK 6.85. These are compared with the extrapolated values of active enzyme at zero time at the same reaction. The data in Table IV are depicted graphically in Fig. 2.

TABLE IV.

Amounts of Active Enzyme at Zero Time Compared with the Dissociation of an Acid, pK 6.85.

pH	Active enzyme at zero time	Undissociated residue of acid
	per cent	*per cent*
4.35	99.6	99.7
4.6	99.0	99.0
5.2	96.0	97.8
5.9	80.9	89.9
5.9	88.8	89.9
6.22	80.7	81.0
6.4	73.3	73.8
6.54	67.9	67.1
6.64	58.2	61.9
6.75	56.2	55.7
6.85	69.3	50.0
6.97	44.2	43.2
7.15	26.3	33.4
7.35	23.2	24.0

In spite of the experimental difficulty of obtaining close agreement in the amounts of active enzyme at zero time, as evidenced by the disagreement in the results for two different experiments at pH 5.9 (Table I) and other discrepancies, the majority of the values obtained fall closely about the ideal dissociation curve (Fig. 2).

These discrepancies do not, in any case, affect the constancy of the value for K at any pH, though they may alter its absolute value, and are insignificant when the whole range of variation of K with pOH is considered.

In Table V are given the average values of K in Table I, the values

for pK, and the corresponding hydroxyl ion concentrations. By extrapolation from the data given by Michaelis (10) the value for pK_w at 15°C. was taken as 14.24. This value minus the pH gives the pOH.

In Fig. 3 are plotted the values of pK against pOH. The curve shows a direct proportionality between the velocity of autodestruction and C_{OH^-} in the range of concentrations greater than pOH 7.7; and possibly another proportionality, also direct, in the range from beyond pOH 9.89 to 7.7.

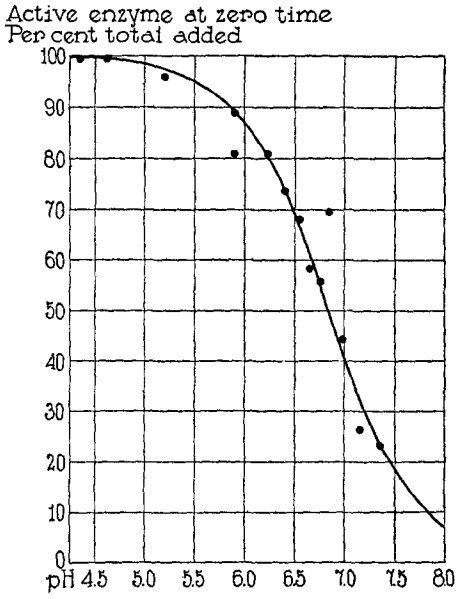

FIG. 2. The ideal dissociation curve of an acid with pK 6.85. The points are obtained from experimental data. They are amounts of active enzyme at zero time for the noted pH values and are obtained by extrapolation (Fig. 1).

Not all the experiments recorded in Table I were carried out with the same albumin solution. In those at pH 4.35, 5.9, 6.22, and 7.35 undenatured albumin was employed, while acid metaprotein served as substrate for the action of the pepsin in the experiments at pH 4.6, 5.2, one at 5.9, 6.4, 6.54, 6.64, 6.75, 6.85, 6.97, and 7.15. In the earlier experiments at pH 6.54, 6.64, 6.85, 6.97, and 7.15 the existence of a strict linear proportionality between the amount of hydrolysis in 1

hour at 30°C. and the concentration of pepsin was assumed. This assumption was made on the basis of results obtained by McFarlane, Dunbar, Borsook, and Wasteneys (11) with acid metaprotein as substrate. They found denatured albumin to give linear proportionality between velocity of hydrolysis and concentration of enzyme. It was found later that this result could not invariably be obtained. This was the case with the denatured albumin employed in the present experiments. An error is therefore introduced in the computations of results in the five experiments mentioned. It is believed, however,

TABLE V.

Relation of the Velocity of Autodestruction, K, to the Hydroxyl Ion Concentration.

pH	pOH	K	pK
4.35	9.89	.0004	3.40
4.6	9.64	—	—
5.2	9.04	—	—
5.9	8.34	.0005	3.30
5.9	8.34	.0002	3.70
6.22	8.02	.0010	3.0
6.4	7.84	.0014	2.85
6.54	7.70	.0007	3.15
6.64	7.60	.0014	2.85
6.75	7.50	.011	1.96
6.85	7.39	.012	1.92
6.97	7.27	.045	1.35
7.15	7.09	.17	0.77
7.35	6.89	.66	0.18

that it does not invalidate the conclusions, because under the experimental conditions the error introduced is not great.

Empirical reference curves were employed in calculating the amount of active enzyme in the majority of the experiments. The data for the reference curve for experiments at pH 4.35, 5.9, 6.22, and 7.35 are given in Table VI and the curve is shown in Fig. 4.

The procedure was as follows: varying amounts of 2 per cent pepsin were added to 50 cc. of 3.2 per cent undenatured albumin at pH 1.6. In order to maintain the nitrogen content the same with each concentration of enzyme the pepsin was diluted with an inactivated solution of the same concentration. The stock pepsin solution was

brought to pH 7.5 at room temperature for 10 minutes, then acidified to pH 1.6 and diluted to a final concentration of 2 per cent. The amount of hydrolysis at 30°C. for 1 hour was measured in the usual manner by precipitation of the unhydrolyzed protein with

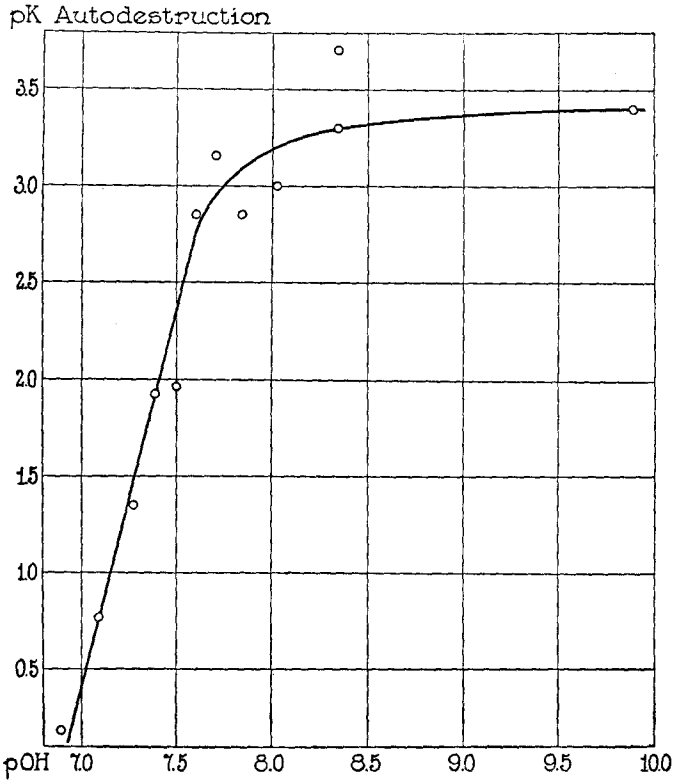

FIG. 3. The relation of the velocity of autodestruction to C_{OH^-} plotted as pK against pOH.

trichloroacetic acid, and estimation of the nitrogen content of the filtrate.

For experiments at pH 4.6, 5.2, one at 5.9, 6.4, and 6.75, acid metaprotein was used, and another reference curve.

The results obtained with the three solutions of albumin are incorporated into one body of data. The values for active enzyme at

zero time, whether obtained with acid metaprotein or with undenatured albumin fall near one dissociation curve as shown in Fig. 2. When

TABLE VI.

Relation between Amount of Hydrolysis in 1 Hour at 30°C. and the Concentration of Pepsin.

2 per cent active pepsin	2 per cent inactive pepsin	3.2 per cent albumin	Albumin N hydrolyzed
cc.	*cc.*	*cc.*	*mg.*
5	0	50	77.6
4	1	50	69.8
3	2	50	59.2
2	3	50	46.8
1	4	50	32.6

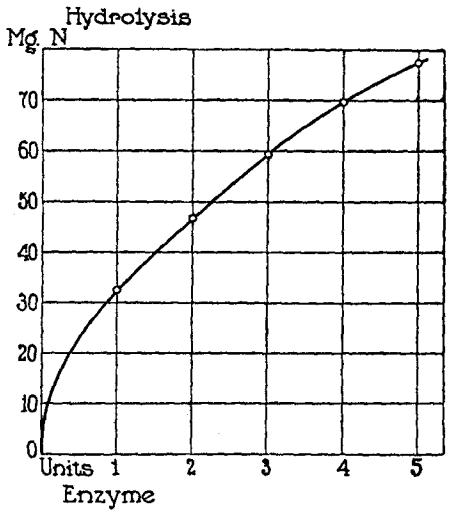

FIG. 4. A reference curve. The relation between the amount of hydrolysis (mg. of N) in 1 hour at 30°C. and the concentration of pepsin (cc. of 10 per cent solution).

the various quantities of active enzyme, calculated in each case according to the nature of the substrate, are inserted into the monomolecular equation, a practically constant value for K is obtained at

each hydrogen ion concentration. These values for K as Fig. 3 shows, vary, in general, with the hydroxyl ion concentration, regardless of the kind of albumin used in the particular experiment. These coincidences give added support for the validity of the methods employed for calculating active enzyme.

Observations made in the course of these experiments demonstrate the need for caution in comparing results obtained with different solutions of presumably the same albumin. The undenatured albumin in the experiments described here was hydrolyzed by a given solution of pepsin nearly three times as quickly as a similar solution of albumin which had been standing at pH 1.6 at room temperature for several weeks. Any relationship found between velocity of hydrolysis and enzyme concentration with a given solution of albumin may not be assumed to obtain with any other similar solution subjected to even slight changes in previous treatment. In order to obtain directly comparable results, the previous history of the albumin used for every determination must be identical.

SUMMARY.

1. Evidence is presented that pepsin is a univalent acid with a value for pK of 6.85 (or a base, with pK 7.39).

2. The autodestruction of the pepsin is shown to be dependent in part upon an instantaneous irreversible change occurring in the ionized form of the enzyme (if it be an acid) or in the unionized form (if it be a base).

3. A further progressive autodestruction of pepsin at any given hydrogen ion concentration and temperature is defined by the mass law equation for a monomolecular reaction

4. The velocity of autodestruction of pepsin is directly proportional to the hydroxyl ion concentration. It is much less in the range of hydroxyl ion concentration from pOH 9.89–7.7, than in the range greater than pOH 7.7. In both of these ranges variations in pK with pOH may be represented by straight lines.

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