THE INFLUENCE OF THE BACKWARD REACTION IN THE PEPTIC HYDROLYSIS OF ALBUMIN.

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In studies of the mechanics of enzyme action unequivocal mathematical relationships based upon theoretical considerations are demonstrated with difficulty. The difficulty is particularly great in the case of protein hydrolysis. These hydrolyses appear to be simple monomolecular reactions; but quantitatively the deviations which are found in the direction of retardation are so significant that different hypotheses have been proposed to account for them. Northrop has suggested, for example, in the case of trypsin (1), and of pepsin (2), that the reaction is inhibited through the combination of the products of hydrolysis with the enzyme, resulting in a reversible compound. In confirmation of this suggestion he derives an equation based upon the mass law. He introduces the conception of an "active mass" of substrate and of enzyme which is not always directly proportional to the total concentration. Michaelis and Menten (3) accounted quantitatively for the action of invertase by considering the affinities of the enzyme for the products of hydrolysis. In these and similar studies the backward reaction has been disregarded.

B. Moore (4) in 1906 clearly pointed out the special significance of the reverse reaction in those cases where hydrolysis of one molecule results in many products. He mentioned proteins, specifically, as examples of this class. He predicted the great facility with which they could be synthesized from concentrated solutions of the products and conversely, their reduced tendency to hydrolysis in concentrated solutions. His predictions were confirmed in a study of the peptic synthesis of protein by Wasteneys and Borsook (5). The conditions found essential for synthesis suggested an important influence of the backward reaction in hydrolysis. The experiments in which synthesis

was effected solely by raising the temperature of a solution in which complete hydrolysis had occurred were particularly suggestive (6). To obtain further evidence for the significance of the backward reaction, investigations into the effect of temperature and of concentration of substrate and of enzyme on hydrolysis were carried out. Coagulated, and also native egg albumin, with pepsin, were employed. It was found that the rate of hydrolysis varied inversely as the concentration of protein, even when the enzyme concentration was maintained throughout in excess and in constant proportion to the substrate. Similarly, as the concentration of albumin increased, the optimum temperature for hydrolysis was approached at a lower and lower level. These effects were obtained with both coagulated and native albumin, with concentrations ranging from 1 per cent to 12 per cent.

By raising the temperature to 65°C. synthesis can be effected in a solution of the products of peptic hydrolysis of albumin corresponding to a 6 per cent solution of protein; but the influence of the backward reaction is evident at a temperature as low as 10°C., and with concentrations of protein lower than 3 per cent. The location of the optimum temperature is influenced not only by the length of time over which the hydrolysis is measured, but also by the concentration of substrate.

The experiments described show clearly that one of the factors present from the outset, retarding the course of hydrolysis, is the backward reaction, whether this be due to concentration of substrate, the products of hydrolysis *per se*, or products introduced as an unavoidable constituent of commercial pepsin.

The effect of the products introduced with the enzyme itself must be considered especially where the relation between enzyme concentration and rate of hydrolysis is under consideration.

The experiments suggest strongly that any mathematical definition of the course of hydrolysis of substances which, like proteins, are hydrolyzed into a number of products, should, to be complete, include the function of the backward reaction.

EXPERIMENTAL.

Egg albumin and scale pepsin were employed throughout. The degree of hydrolysis was measured by estimating the total nitrogen

in the filtrate after precipitation of the unhydrolyzed protein with trichloroacetic acid. To preserve the potency of the pepsin solution, following Morgenroth, and Michaelis and Rothstein (7), sodium chloride was added to a concentration of 10 per cent. The protein and the pepsin solutions were each adjusted electrometrically to pH 1.6 with hydrochloric acid before each experiment. The stock solution of pepsin was made by dissolving a quantity in N/5 HCl. Albumin solutions at acidities less than pH 2.0 are gradually transformed into a jelly on standing at room temperature; the amount of acid required to bring it to pH 1.6 was, therefore, except where otherwise noted, added to the neutral solution of protein immediately before using.

TABLE I.

Effect of Temperature on the Autodestruction of Pepsin.

Temperature at which pepsin solution was incubated.	1.05 per cent pepsin.	2.1 per cent pepsin.	4.2 per cent pepsin.
	Hydrolysis of coagulated albumin (1.3 per cent) with incubated pepsin.		
°C.	*per cent*	*per cent*	*per cent*
10	13.6	19.3	12.2
20	13.6	19.3	12.2
30	13.6	19.3	12.2
40	13.6	19.3	12.2
50	11.8	14.3	10.8
60	6.9	7.6	7.9
70	0.8	0.0	0.0

At the outset it was necessary to ascertain the temperature at which autodestruction of the enzyme begins. Previous work had shown that at acidities greater than pH 5.0, at room temperature, the enzyme may be considered for practical purposes to be stable.

Solutions of pepsin (Merck), of concentrations 1.05 per cent, 2.1 per cent, and 4.2 per cent at pH 1.6, and a suspension of 1.3 per cent heat-coagulated egg albumin at pH 1.6 were employed. The coagulated protein was prepared as follows. Approximately 70 gm. of albumin (Merck) were mixed in a mortar with water, then diluted to 3 litres, and shaken for $\frac{1}{2}$ hour on a mechanical shaker. The solution was centrifuged, and the supernatant liquid, with constant stirring, was heated slowly to boiling and maintained at the boiling point for 2

minutes. A fine easily handled coagulate was obtained, and the supernatant liquid gave no precipitate with trichloroacetic acid.

To determine the effect of temperature on autodestruction of the pepsin, 5 cc. portions of the pepsin solution were immersed in water baths at the temperatures indicated in Table I for 1 hour. 1 cc. aliquots were removed at the end of this time and added to 20 cc. of the suspension of coagulated albumin. The resulting mixture was incubated at 30°C. for 1 hour. The extent of hydrolysis was then measured.

The figures in Table I show that there is no demonstrable inactivation of the enzyme until the temperature is above 40°C.

Comparison of the amounts of hydrolysis due to the three concentrations of enzyme shows the retarding influence of the products. Where the 5 cc. of 4.2 per cent pepsin solution were added to the albumin there was present at the outset of the hydrolysis a 0.8 per cent concentration of products, proteoses and peptone, which are inseparable constituents of commercial pepsin. With the 1.05 per cent pepsin this amounted to 0.2 per cent. The difference, 0.6 per cent of products, was sufficient, therefore, in spite of a fourfold increase in enzyme concentration, to depress the rate of hydrolysis of the former below the latter. This is due, as will be shown, to the inhibiting influence on hydrolysis, of the backward reaction.

The Influence of the Backward Reaction in the Effect of Substrate Concentration on Hydrolysis.

In investigating the effect of concentration of substrate it is important to eliminate any effect referable to enzyme concentration, either the direct effect of introducing variable amounts of products or effects due to different degrees of "saturation" of the substrate. This was attained by maintaining the enzyme in excess over substrate and in constant proportion to the concentration of the latter throughout the range of its variation.

To ascertain the ratio of pepsin to albumin at which the former may be said to be in excess, the experiment recorded in Table II was carried out. To 50 cc. portions of 4.5 per cent native albumin 3 cc. aliquots of pepsin solutions varying from 2.1 per cent to 7.0 per cent were added. The extent of hydrolysis at the end of $\frac{1}{2}$ hour, and 1 hour, at 40°C., was measured by the method described.

The use of concentrations of pepsin higher than 3.5 per cent brought about no increased amount of hydrolysis. From this value upwards there is therefore assumed to be an excess of enzyme over substrate.

Since 3 cc. of 4.2 per cent pepsin were employed with 50 cc. of 4.5 per cent albumin, the proportion of protein to pepsin is 17.9 to 1. A proportion a little higher than this value 16.7 to 1 was maintained throughout in the experiments tabulated in Table III. 12 per cent,

TABLE II.

Effect of Pepsin Concentration on the Rate of Hydrolysis of Albumin.

Enzyme concentration.	Amount of hydrolysis, per 100 cc. of digest in	
	1/2 hr.	1 hr.
per cent	*mg.* N	*mg.* N
2.1	150	194
3.5	170	216
4.2	172	220
5.0	171	205
6.0	173	220
7.0	166	210

TABLE III.

The Effect of Concentration of Albumin on the Rate of Hydrolysis by Pepsin at 35°C.

Concentration of protein.	Amount of hydrolysis in			
	1/2 hr.		1 hr.	
per cent	*mg.* N *in 100 cc.*	*per cent*	*mg.* N *in 100 cc.*	*per cent*
12	230	14.8	309	19.9
7	195	21.5	241	26.6
5	183	28.1	221	34.2
3	139	35.8	163	42.0

7 per cent, 5 per cent, and 3 per cent albumin, and similar concentrations of pepsin were prepared. 3 cc. of 12 per cent pepsin solution were used with 50 cc. of 12 per cent albumin, and this ratio was maintained with the varying concentrations of albumin. The hydrolyses were carried out at 35°C. and samples were removed for analysis at ½ hour and 1 hour.

These results confirm Moore's prediction that the tendency to

hydrolysis diminishes as the concentration of protein increases, and *vice versa*. As the curves (Fig. 1) show, the effect of concentration is present from the beginning of hydrolysis.

When coagulated egg white instead of native albumin is employed

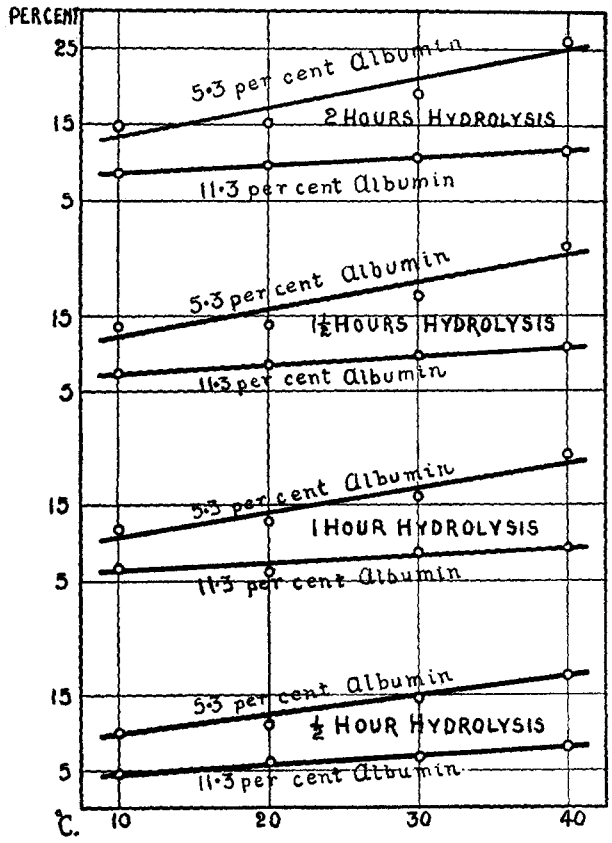

FIG. 1. The effect of temperature on the hydrolysis of 5.3 and 11.3 per cent egg albumin solutions.

as substrate, this effect of concentration manifests itself even more clearly.

Coagulated albumin was prepared as described. 3 cc. of a 2.1 per cent pepsin solution were added to 60 cc. portions of various concentrations of coagulated albumin, and the mixtures were incubated at 40°C. 10 cc. aliquots were removed at ½ hour, 1 hour, 1½ hour,

and 2 hour intervals and analyzed. The concentrations of coagulated albumin were 1.3 per cent, 2.4 per cent, and 2.6 per cent.

The proportion of enzyme to substrate with the lowest concentration of albumin was 12.6 to 1, and with the highest 25.2 to 1. In Table II excess of enzyme over substrate was attained with a proportion of 21.4 to 1. Native albumin in solution, instead of coagulated egg white, was employed in the former instance. Assuming that the same amount of enzyme is required to "saturate" a given amount of coagulated egg white as albumin in solution, the concentration of enzyme even with the most concentrated suspension of albumin is little short of absolute excess. It would be very difficult to account for a nearly fivefold falling off in the rate of hydrolysis of 2.6 per cent albumin by the relatively smaller amount of enzyme employed.

TABLE IV.

Effect of Concentration of Coagulated Albumin on the Rate of Its Hydrolysis by Pepsin at 40°C.

Concentration of protein.	Amount of hydrolysis. (Enzyme N not deducted.)							
	In 1/2 hr.		In 1 hr.		In 1 1/2 hrs.		In 2 hrs.	
per cent	*mg. N in 100 cc.*	*per cent*	*mg. N in 100 cc.*	*per cent*	*mg. N in 100 cc.*	*per cent*	*mg. N in 100 cc.*	*per cent*
2.6	58	4.3	86	6.3	91	6.7	98	7.1
2.4	60	4.5	91	6.9	104	7.9	117	8.8
1.3	—	—	100	28.8	—	—	—	—

This possibility is, moreover, definitely eliminated by the fact that the absolute amount of hydrolysis, expressed as mg. of protein nitrogen, is less with 2.6 per cent albumin than with the 1.3 per cent, though the amounts of enzyme employed in each case were identical. The results in Table IV demonstrate again what was shown in Tables I and III, the diminishing tendency to hydrolysis with increasing concentration of substrate. This influence of substrate concentration is theoretically effective whether the protein in solution is present as such, or in the hydrolyzed form as products. When products are added to a solution of albumin undergoing hydrolysis there is a retardation of hydrolysis to be anticipated as a result of the increase in the concentration of protein corresponding to the albumin equivalent of the products. On

the other hand in a solution of products alone, if the concentration or temperature be adequate, the backward reaction completely overcomes any forward tendency and synthesis takes place.

The Influence of the Backward Reaction in the Effect of Temperature on Hydrolysis.

In a previous publication (6) Borsook and Wasteneys showed that at high temperatures synthesis is possible in solutions where, at a

TABLE V.

Effect of Temperature on Peptic Hydrolysis of Albumin.

Duration of hydrolysis.	Concentration of protein.	Amount of hydrolysis at			
		10°C.	20°C.	30°C.	40°C.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
½	11.3	4.6	6.0	6.6	8.1
½	5.3	10.0	11.0	14.5	17.5
1	11.3	6.7	6.0	8.3	8.7
1	5.3	11.9	12.8	16.1	21.6
1½	11.3	7.3	8.5	9.4	10.7
1½	5.3	13.5	13.8	17.3	24.0
2	11.3	8.2	9.3	10.5	11.8
2	5.3	14.5	15.0	19.1	26.0

lower temperature, hydrolysis was complete. The effect of increase of temperature is to move the equilibrium point more and more to the protein side. From the converse point of view of hydrolysis, the effect of increase of temperature is to produce a relative retardation of the rate. This is difficult to show directly. Indirectly it is shown by the approach of higher concentrations of albumin to their optimum temperature at a lower temperature than more dilute solutions. This manifestation of the influence of the backward reaction again obtains with both coagulated and native albumin.

In the case of the uncoagulated albumin, 3 cc. of a pepsin solution were added to 50 cc. of albumin solution, giving a final concentration of pepsin of 0.12 per cent. Albumin concentrations of 5.3 per cent and 11.3 per cent were employed. Hydrolyses were carried out at 10°C., 20°C., 30°C., and 40°C., and samples were removed for analysis

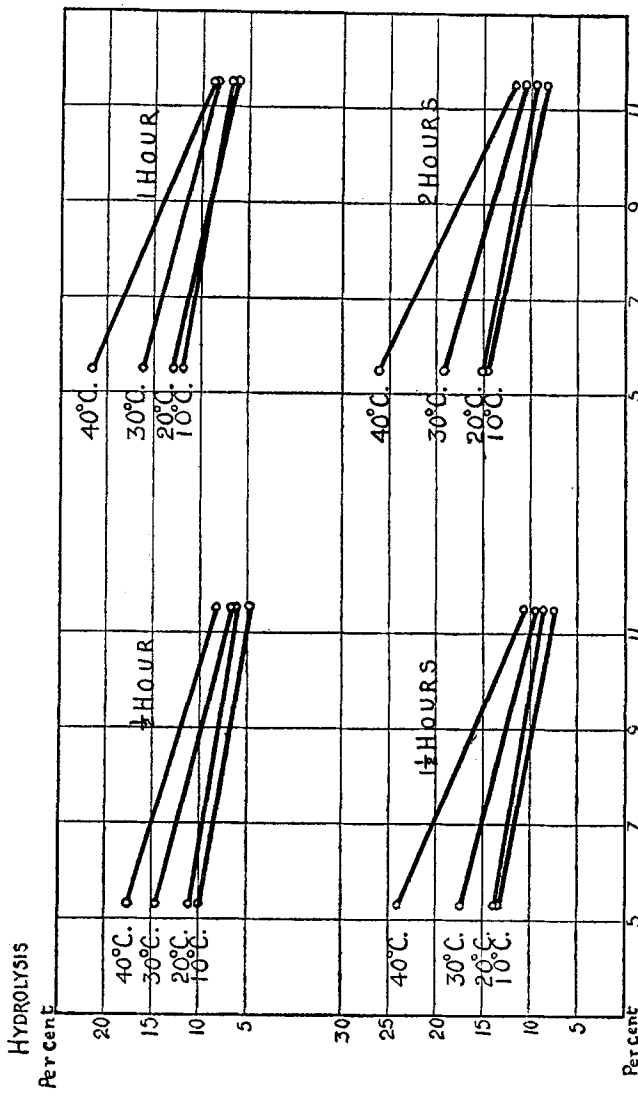

FIG. 2. The effect of concentration of substrate on the hydrolysis of egg albumin solutions at varying temperatures.

at the time intervals indicated in Table V which records the results of this experiment.

The effect of concentration on the temperature optimum is demonstrated very clearly. In the 2 hour period, for example, whereas with 5.3 per cent albumin there is 80 per cent increase in hydrolysis at 40°C. as compared with 10°C. with 11.3 per cent albumin the increase in hydrolysis is only 43 per cent. Stated differently, the 5.3 per cent albumin is hydrolyzed 11.5 per cent more at 40° than at 10°, while the 11.3 per cent albumin is hydrolyzed only 3.6 per cent more at 40° than at 10°.

The accelerating influence of increase of temperature on the backward reaction can be demonstrated by analysis of the same data in another form. In Fig. 2 concentrations of substrate are taken as

TABLE VI.

Influence of Temperature on the Peptic Hydrolysis of Coagulated Egg Albumin.

Duration of hydrolysis.	Concentration of protein.	Amount of hydrolysis at					
		20°C.	30°C.	40°C.	50°C.	60°C.	70°C.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	1.2	5.8	12.5	28.8	37.8	47.0	34.0
	2.3	1.8	2.7	6.9	13.5	17.5	9.7
	2.5	2.1	1.9	6.3	8.7	11.8	7.7

abscissæ and per cent of hydrolysis as ordinates. The curves for 10°, 20°, 30°, and 40° for each time interval are plotted so that their slopes can be compared readily.

The increasing downward slope of all the curves with increase of temperature, becoming more marked as the duration of the hydrolysis becomes greater, shows the progressively increasing inhibition of hydrolysis in the higher concentration, as the temperature increases.

The slope of the curves, Fig. 1, shows that for the 5.3 per cent albumin solution the optimum temperature lies considerably beyond 40°C., even for 2 hours hydrolysis. With 11.3 per cent albumin the optimum temperature is much more nearly attained at 20°C. Temperatures higher than this cause only slight increases in the rate of hydrolysis. There is no influence of the temperature on the stability of the pepsin, to be taken into account here. As Table I shows, heat

destruction of pepsin does not become significant until a temperature higher than 40°C. is reached. Moore pointed out that this retardation of hydrolysis with increased temperature is predictable from thermodynamic considerations of reactions where one molecule breaks down into a number of products and where the heat of reaction is low. This retardation is due essentially to an increased tendency for the occurrence of the backward reaction.

With coagulated egg albumin smaller differences in concentration reveal the influence of temperature, described above, on the equilib-

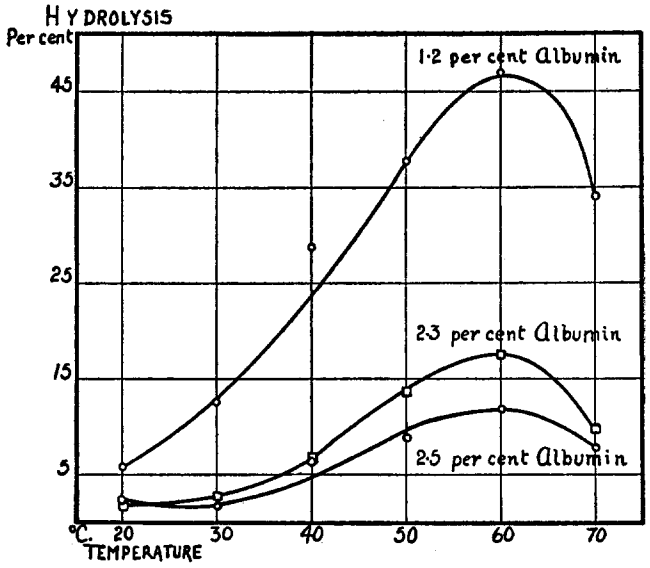

FIG. 3. The effect of temperature on the hydrolysis of 1.2, 2.3, and 2.5 per cent coagulated egg albumin.

rium between the forward and backward reactions. The solutions were the same as those in the previous experiments (Table IV) with coagulated egg albumin. Three concentrations of coagulate were employed, 1.24 per cent, 2.3 per cent, and 2.5 per cent. 3 cc. of a 2.1 per cent pepsin solution were added to 60 cc. of suspension of protein. The results obtained are shown in Table VI.

At 40°C. the 1.2 per cent protein is hydrolyzed in 1 hour to a degree nearly 5 times that of the 2.5 per cent protein. The presence in the

former of relatively twice as much enzyme as in the latter is inadequate in itself to account for the relative difference shown in rate of hydrolysis.

The increased retarding influence of the backward reaction at higher temperatures is shown in Fig. 3 by the diminishing slope of the ascending portions of the curves with increasing concentration of coagulum. This influence of the backward reaction appears to be even greater in the hydrolysis of coagulated egg white than in dissolved albumin. In Fig. 3 the sharp difference between 2.3 per cent suspension of coagulum and 2.5 per cent is striking. This behaviour of an insoluble protein, resembling that of a protein in solution is analogous to the influence of the insoluble protein plastein on the position of the equilibrium point during peptic synthesis (8). Added plastein, though not in solution, retards subsequent synthesis to a degree in direct proportion to the amount added.

The Influence of the Backward Reaction in the Effect of Enzyme Concentration on Hydrolysis.

In the experiments with coagulated albumin it was shown in Table I that an absolute decrease in the amount of hydrolysis could be achieved by increasing the concentration of pepsin. This anomalous result was explained as due to the inhibition of hydrolysis by the products of hydrolysis added with the pepsin.

This phenomenon also is demonstrable with native albumin in solution; but, as in other instances, the effect is not as marked here as with coagulated albumin.

A 3.2 per cent solution of egg albumin (Merck) at pH 1.6 was hydrolyzed for 1 hour at 30°C. in three series with varying concentrations of pepsin (Wyeth). In the one series 5, 4, 3, 2, and 1 cc. of an 0.6 per cent active pepsin solution were added to 50 cc. portions of albumin. To these solutions in the same sequence 0, 1, 2, 3, and 4 cc. of the same pepsin solution, inactivated by neutralization, were added just previous to the addition of the active pepsin. In the two other series 2 per cent and 4 per cent pepsin solutions were similarly employed. In the first series, therefore, there was present at the outset of the reaction with all concentrations of pepsin approximately 0.06 per cent products; in the second series 0.18 per cent; and in the third

series 0.36 per cent. The results are shown in Table VII and Fig. 4.

The effect of varying the initial concentration of products is best shown by the graphic representation of the results in Fig. 4, where 1 cc. of 4 per cent pepsin is plotted as 20 units of enzyme. The three curves are practically parallel; but the greatest velocity of hydrolyses per unit of added active enzyme is obtained where the least amount of products was added. For example to obtain 20 mg. of hydrolysis, the proportions of active enzyme were 3.5:5.5:8, with the lowest value for that solution where the initial concentration of products was least. Similarly for 40 mg. of hydrolysis the proportions were

TABLE VII.

Effect of Initial Concentration of Products Introduced with the Enzyme on the Velocity of Peptic Hydrolysis.

Active enzyme.	Inactive enzyme.	Amount of hydrolysis.					
		0.6 per cent pepsin.		2 per cent pepsin.		4 per cent pepsin.	
cc.	*cc.*	*mg. N in 50 cc.*	*per cent*	*mg. N in 50 cc.*	*per cent*	*mg. N in 50 cc.*	*per cent*
5	0	46.1	19.9	77.6	33.5	98.9	42.7
4	1	40.3	17.4	69.8	30.1	86.9	37.5
3	2	35.1	15.1	59.2	25.5	74.3	32.0
2	3	28.9	12.5	46.8	20.2	65.2	28.1
1	4	18.3	7.9	32.6	14.1	40.8	17.6

11.5:15.5:19.5. Expressing the same result in another form, it is seen from Fig. 4 that where the initial concentration of products was highest a given concentration of added active enzyme, *e.g.* 10 units on the chart, caused 27.5 mg. of hydrolysis; 33.0 mg. was obtained with the intermediate concentration of products; and 37.5 mg. with the lowest concentration.

A noteworthy feature of each of the three curves is that with increasing enzyme concentration the slope diminishes, until, with the latter three or four points on each curve, it approaches a constant; *i.e.*, becomes nearly a straight line. Equal increments with the higher concentrations of enzyme on each curve cause approximately equal increases in velocity. This result fails to confirm Northrop's prediction (2) that equal increments in added enzyme will cause equal

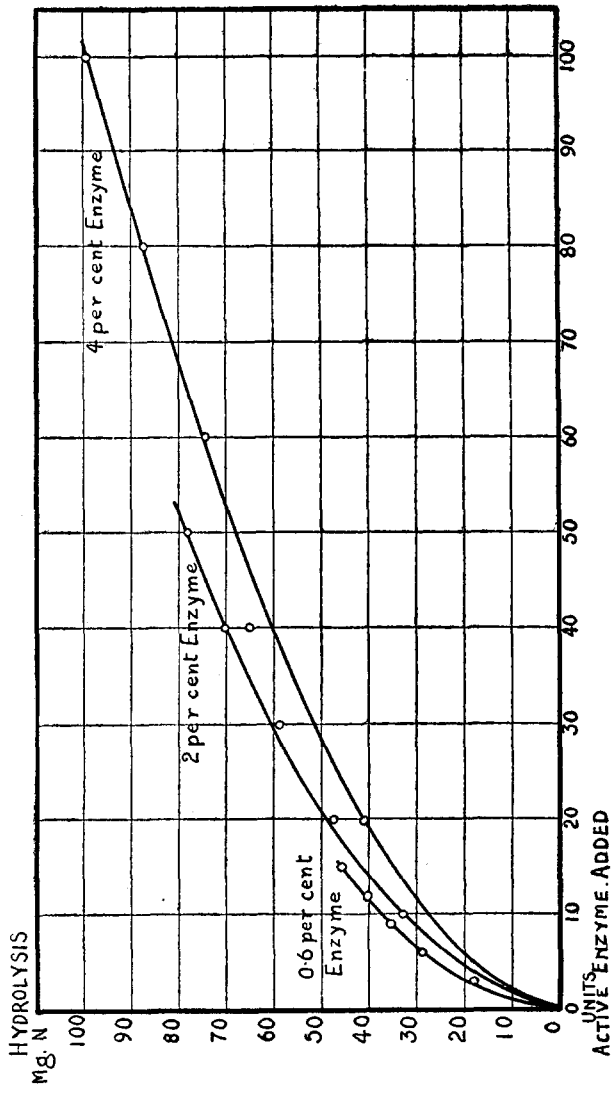

FIG. 4. The effect of the concentration of hydrolytic products contained in commercial pepsin on its activity.

increases in velocity where the enzyme concentration is low, and progressively diminishing increases in velocity with equal increments, where the concentration of enzyme is high. The results in Table VII, and Fig. 4 also differ from Northrop's prediction "that if a solution of pepsin is diluted with a portion of itself in which the pepsin has been inactivated with alkali, the activity of the resulting solution is directly proportional to the concentration of active enzyme solution added."

A possible cause for these differences between our findings and those of Northrop may lie in the difference in our methods for measuring the degree of hydrolysis. Northrop's method consisted essentially in the measurement of changes in the products of hydrolysis. In our experiments the amount of protein hydrolyzed was estimated.

TABLE VIII.

The Effect of Variations in the Initial Amounts of Products on the Rate of Hydrolysis

No.	3.2 per cent albumin.	4 per cent pepsin.	Pepsin digest.	N/40 HCl.	Total non-protein N initially present.	Protein N hydrolyzed.
	cc.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*
1	50	5	5	0	122.4	82.3
2	50	5	4	1	106.9	85.2
3	50	5	3	2	91.3	89.0
4	50	5	2	3	75.7	89.6
5	50	5	1	4	60.1	92.2
6	50	5	0	5	44.5	97.4

Assuming, as Northrop does, a highly dissociated reversible combination of enzyme with products, obeying the mass law, it seems possible to explain qualitatively certain of the results described in Tables III and VII and the changes in slope of any one of the curves in Fig. 4. The effect of temperature, however, Tables V and VI, cannot be so explained unless it be assumed that the dissociation of the pepsin-product combination is less at high than at low temperatures.

The Influence of the Backward Reaction as Exhibited by Variations in the Initial Concentrations of Products.

The retarding influence of the backward reaction on hydrolysis can, of course, also be demonstrated by adding to the protein solution

before the pepsin is allowed to act, varying amounts of hydrolytic products. This is the usual method for exhibiting reversible reactions.

A concentrated solution of the products of the peptic hydrolysis of egg albumin was adjusted to pH 1.6 and its nitrogen content determined. A 4 per cent solution of pepsin (Wyeth) with a similar reaction at pH 1.6 was prepared. A 3.2 per cent solution of egg albumin (Merck), at pH 1.6 was employed as substrate. 5 cc. of the pepsin solution were added to 50 cc. of albumin. The hydrolysis was allowed to proceed at 30°C. for 1 hour, checked by the addition

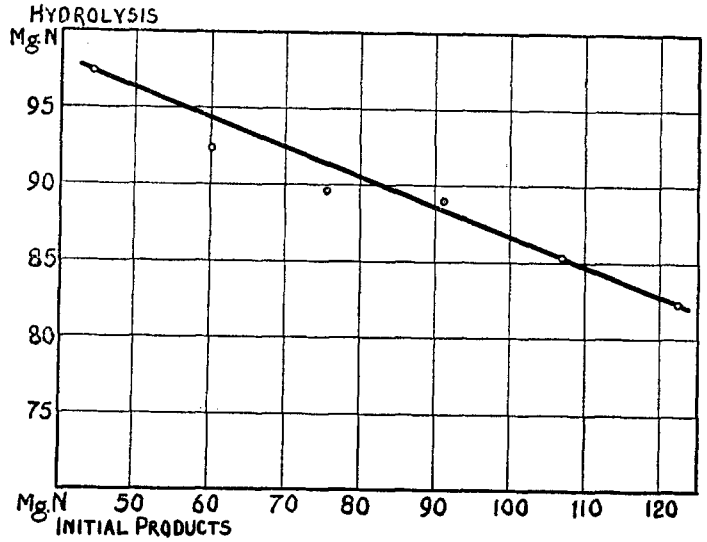

FIG. 5. The effect of addition of varying amounts of hydrolytic products on the peptic hydrolysis of albumin..

of trichloroacetic acid and then estimated in the usual manner. The details and the results of the experiment are described in Table VIII and Fig. 5.

The 44.5 mg. non-protein nitrogen initially present in No. 6, and the other mixtures in Table VIII, were composed of 23.9 mg. N added with the pepsin, and 20.6 mg. present in the albumin solution.

As Fig. 5 shows there is a simple linear relationship here between the inhibition of hydrolysis and the amount of products initially present.

These considerations of the influence of the backward reaction on

peptic hydrolysis are of some practical significance. They show that the optimum temperature for hydrolysis is not only a function of the heat destruction of the enzyme, but also of the concentration of the protein substrate. The greater the concentration of protein the lower is the temperature at which optimum hydrolysis is obtained. Another condition, imposed by the influence of the backward reaction, that must be defined in stating the optimum temperature, is the duration of hydrolysis.

Of more general significance is the necessity which has been demonstrated for taking into account the concentration of substrate, independently of the relation of enzyme to substrate, in any mathematical expression of the course of the enzymatic hydrolysis of protein.

SUMMARY.

1. No destruction of pepsin by heat is demonstrable at pH 1.6 until a temperature of 40°C. is exceeded.

2. The influence of the backward reaction in peptic hydrolysis is shown in the diminishing rate at which increasing concentrations of protein are hydrolyzed.

3. The backward reaction causes the optimum for the hydrolysis of higher concentrations of protein to be attained at a lower temperature than with more dilute solutions.

4. The proteose and peptone associated with commercial pepsin retard hydrolysis in the same sense as the products due to the action of the enzyme.

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