

THE INACTIVATION OF TRYPSIN.

III. SPONTANEOUS INACTIVATION.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 15, 1921.)

In addition to the inactivation of trypsin caused by its combination with some of the products of the hydrolysis, which has been discussed in the first part of this series, trypsin undergoes a second or spontaneous inactivation. This inactivation is independent of the action of the enzyme, irreversible and distinct from the reversible retardation of the action of the enzyme by the products formed during the reaction. Tammann¹ clearly recognized this complicating factor and attempted to correct for it, and there has since been considerable discussion as to the nature and course of this reaction. The rate of destruction of a number of enzymes has been studied, especially by Madsen and Walbum² who found in general that the reaction was monomolecular.

The inactivation of trypsin was studied by Vernon³ who found that the reaction was not monomolecular but became progressively slower than the rate predicted by the monomolecular formula. He concluded therefore that the solution must contain a number of different forms of the enzyme some of which were more stable than others. He also found that, as is generally the case, the purity of the solution had a marked influence on the rate of decomposition. As will be seen from the experiments in this paper it is this factor which causes the divergence from the monomolecular formula so that it is unnecessary to assume the existence of a series of enzymes differing in their degree of stability.

¹ Tammann, G., *Z. physik. Chem.*, 1889, iii, 25.

² See Arrhenius, S., *Immunochemie*, Leipsic, 1907.

³ Vernon, H. M., *J. Physiol.*, 1904, xxx, 330.

Methods.

The methods used in the present experiments were the same as those described above. The amount of active trypsin present was determined by measuring the time required for 1 cc. of the solution to cause a small amount of hydrolysis of a gelatin solution at 33° and a pH of 6.2 (in one experiment the pH was 10). The hydrolysis was followed either by the formol titration or the change in conductivity.

Influence of the containing vessels.—Since the formula for a mono-molecular reaction is the same as that for diffusion it is necessary to know whether the enzyme is really being destroyed or simply diffus-

TABLE I.

Influence of Containing Vessel on Decomposition.

10 cc. dialyzed trypsin placed in vessel noted, and left at 20°C., pH 6.2. trypsin determined in 1 cc. after interval noted.

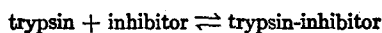
Time at 22°.	Trypsin per cc. after time noted at 22°C.			
	Containing vessel.			
	Platinum 5 cc.	Quartz 10 cc.	Paraffin 10 cc.	Glass 50 cc.
<i>hrs.</i>				
0	1.16	1.19	1.10	1.20
17	0.64	0.60	0.68	0.66

ing to the walls of the containing vessel. In the latter case the rate of disappearance of the enzyme will depend on the size and character of the containing vessel, while if the process is chemical it will probably be independent of these factors. In order to test this point trypsin solutions (prepared as described in the preceding paper) were placed in various vessels and kept at 22° for 17 hours. The activity of the solution was tested before and after this interval. The results of this experiment are shown in Table I. It is evident that the destruction of the enzyme is independent of the container and is therefore probably not a diffusion process. The same conclusion is indicated by the temperature coefficient.

Influence of the Purity of the Solution on the Course of the Reaction.

Fig. 1 contains the results of a series of experiments on the rate of inactivation of various trypsin solutions at 38° and pH 6.2. The amount of active trypsin remaining in solution at any time has been plotted as the logarithm so that if the reaction were monomolecular the resulting curve would be a straight line. As the figure shows this is true in the case of the dialyzed trypsin. This particular solution had been dialyzed under pressure for 18 hours at 6°C., filtered and redialysed. The constant found is 0.005 (time in minutes and common logs). This experiment could not be repeated with certainty but in general, the more carefully the solution was purified the more nearly the reaction was found to be monomolecular. The figure also shows that undialyzed trypsin solutions and those to which gelatin had been added are apparently inactivated at first more rapidly than the pure solutions and then much more slowly. On the other hand, solutions containing inactivated trypsin or substances which had been found to interfere with the action of the enzyme, are much more stable, and if a large amount of these substances are present amount of decomposition is too small to determine in the interval of time chosen. The addition of glycine is without effect.

It has been shown in the previous paper that the products formed by the action of trypsin on proteins form a compound with the trypsin that is inactive. The simplest explanation for the present experiments would be to assume that exactly the same mechanism is at work here and that the compound, trypsin-inhibitor, is stable as well as inactive. It was found that the experiments referred to were in quantitative agreement with the hypothesis that the trypsin and inhibiting substance unite to form a compound according to the equation



and further that this equilibrium conformed to the law of mass action; *i.e.*

$$\frac{\text{Concentration of free trypsin} \times \text{Concentration of free inhibitor}}{\text{Concentration of trypsin-inhibitor}} = \text{a constant}$$

It follows from this equation that the amount of free trypsin present in a solution containing a given amount of inhibitor is a function of the dilution. The more concentrated the solution the more trypsin will be combined. Since the determination of the amount of free trypsin was made by adding 1 cc. of the trypsin to 25 cc. of gelatin it is possible for most of the trypsin to be active (uncombined) under these conditions but nearly all combined (inactive and stable) in the undiluted trypsin solution.

If the trypsin solution at the beginning of the experiment contains undigested protein it is evident that the concentration of the inhibiting substance, which is found by the action of trypsin on the protein, will increase during the experiment, and that the observed result will be the combination of two effects: first, reversible inactivation of the trypsin due to the presence of the inhibiting substance, and second, irreversible destruction of the free trypsin. The combination of these two factors will give a curve which, compared to the curve for the pure trypsin solution, will drop too rapidly at first, due to the increasing concentration of inhibiting substance and will then decrease too slowly owing to the fact that the trypsin is nearly all combined and therefore stable. This is the result shown in Fig. 1. This explanation may be verified by diluting the solution sufficiently before determining its activity. Since the inactivation due to the inhibiting substance is reversible and depends on the concentration of the solution whereas the spontaneous inactivation is irreversible, the two effects may be separated in this way and the resulting value alone will be a measure of the irreversible spontaneous decompositions. The result of an experiment performed in this way is shown in Fig. 2. The curve for the formol titration of the trypsin solution alone is also given. The figure shows that when the trypsin solution is sufficiently diluted before the determination is made, the initial rapid drop disappears and also that the time during which this drop is noticeable in the less diluted solution corresponds to the time during which the formol titration is increasing.

There remains to be explained the subsequent retardation of the inactivation in solutions containing protective substances. It follows from the law of mass action which, as has been shown, correctly expresses the equilibrium, that the smaller the amount of trypsin

present the greater the percentage of combined and therefore stable trypsin. In a solution, therefore, originally containing trypsin and inhibiting (protective) substances, the percentage of the trypsin that

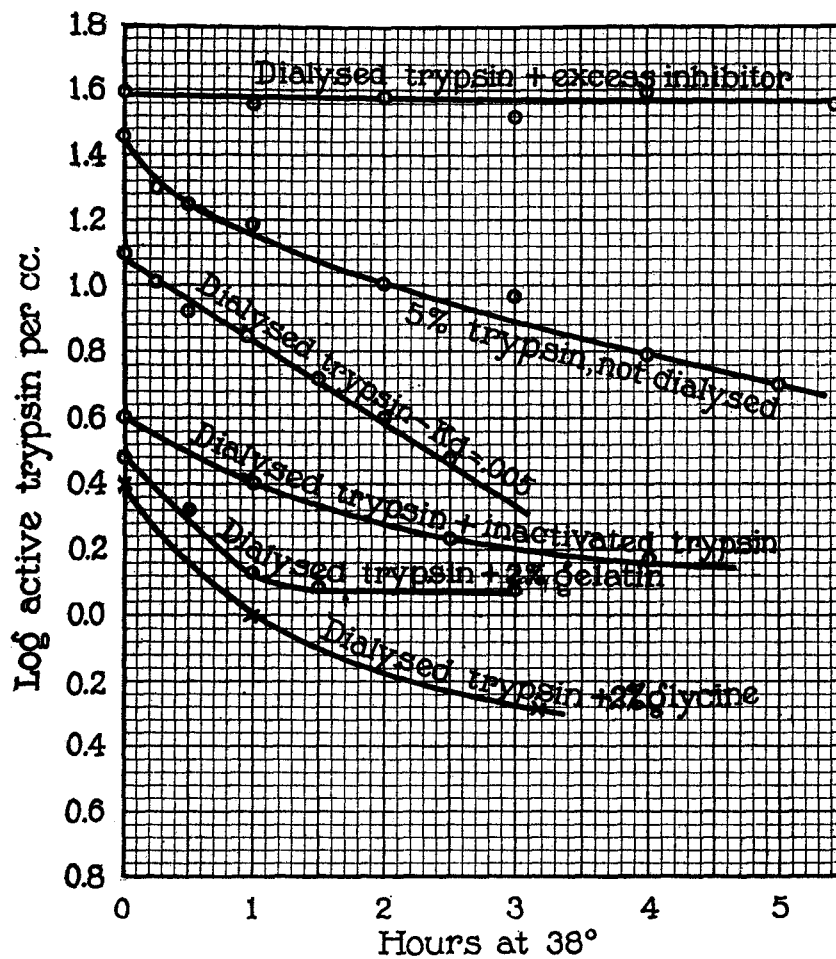


FIG. 1. Inactivation of various trypsin solutions at 38°C.

is free is constantly decreasing. Since it is this quantity that determines the rate of inactivation, the rate of inactivation will also constantly decrease and the resulting decomposition curve will fall more

slowly than demanded by the monomolecular formula. As has been stated this is the experimental result.

The above explanation may be tested quantitatively as follows. If the total concentration of trypsin and inhibitor be known the concentration of free trypsin at any dilution may be calculated by the law of mass action, since the equilibrium constant is known from the experiments described in the preceding paper. The constant

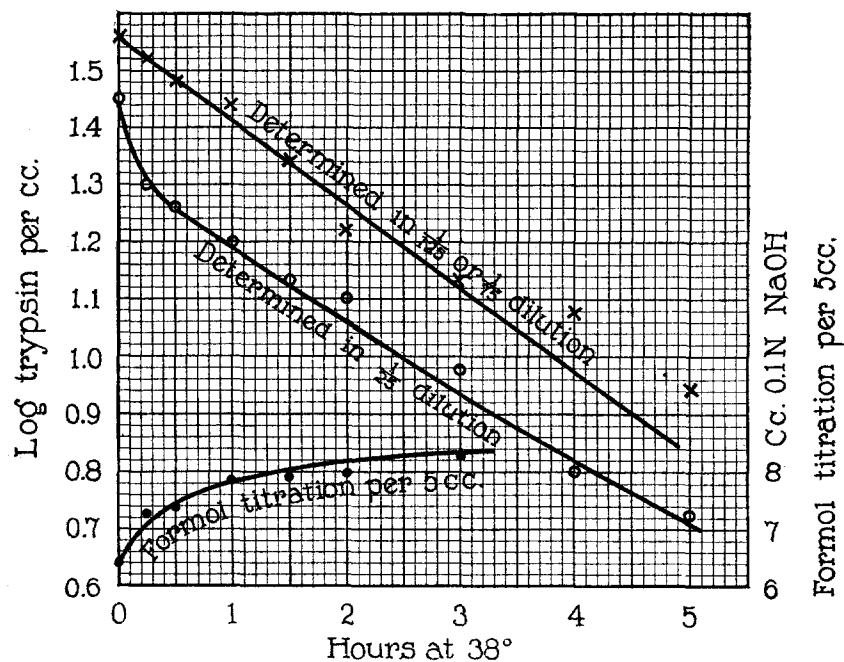


FIG. 2. Inactivation of trypsin in solution containing protein at 38°C.

for the destruction of the free trypsin is known from the decomposition curve of the purified trypsin. It was found to be about 0.005, using common logs and expressing the time in minutes. That is, very nearly half the amount of trypsin present is destroyed in 1 hour at 38° and a pH of 6.2. If a known amount of inhibitor is added to a known amount of trypsin, therefore, it is possible to calculate concentration of free trypsin in this solution. Since the value of K_d (the rate of decomposition) is known, the percentage of this free

trypsin that will be inactivated in any given interval of time can be approximated. The total amount of trypsin remaining in solution can then be found by difference and the amount of this which will be free and active at the concentration used in the determination, and in the presence of the known concentration of inhibitor can be calculated. This value should agree with that found by experiment. It must be remembered that the above calculation is only a first approximation since it contains two assumptions that are not strictly correct. (1) That the free trypsin is inactivated at the same rate as the same concentration of pure trypsin. As a matter of fact the amount of trypsin destroyed under the conditions of the experiment will be slightly greater than the quantity which would be destroyed if there were no combined stable trypsin present, since some of this will be dissociated as the free trypsin is destroyed and the amount of free trypsin and therefore the amount destroyed increased in this way. If the experiment is limited to the first part of the reaction (as was done) this difference is within the experimental error. (2) It was assumed that the only inhibiting (protective) substance present was added as the inhibiting solution. The decomposition curve of the trypsin solutions alone, however, show that in general there is always some protective substance present in the trypsin solution. The fact that the addition of inactivated trypsin renders the enzyme more stable indicates also that the solution contains some protective substances. This effect can hardly be ascribed to the inactive trypsin itself since it was shown in the first part of this paper that inactivated trypsin took no part in the reaction. The neglect of this quantity tends to make the calculated amount of trypsin destroyed too low. This is the result that is obtained. (If this quantity of inhibitor present in the trypsin solution is taken into account, the calculated and observed values may be made identical, but since there is no independent method of determining the value to be used, the process really consists in adding another arbitrary constant to the formula and so does not add much to the validity of the proof.)

The result of an experiment calculated and carried out as described above is shown in Table II. The agreement is as good as could be expected in view of the many sources of experimental error and of the fact that the calculation involves the extrapolation of the equilib-

rium equation over a range of 2,500 per cent dilution, since the values for the constants were determined in a solution which had been diluted 25 times and which in addition contained gelatin, whereas in the calculation the same formula was applied to a solution which had not been diluted at all and which contained no gelatin. This experiment appears to furnish strong confirmation of the validity

TABLE II,

Concentration of Inhibitor and Rate of Inactivation of Trypsin at 38°C.

10 cc. dialyzed trypsin and noted units inhibitor placed at 38°C. 1 cc. removed after intervals noted and added to 25 cc. of 2 per cent gelatin pH 6.2, specific conductivity 2.2×10^{-3} , at 33°C. and rate of hydrolysis followed; this gives the units of free trypsin per cc. solution when diluted 1:26. $K = 0.1$. Kd (decomposition trypsin) = 0.005.

Experiment No.	Inhibitor per cc. trypsin solution.	Free trypsin per cc. solution diluted 1:26 after hours at 38°C.					
		0 hours.		0.6 hours.		1.0 hours.	
		Observed.	Calculated.	Observed.	Calculated.	Observed.	Calculated.
	<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>
1	0	3.45	(3.45)	2.6	2.5		
1	0.17	3.40	3.35	3.0	2.3		
1	0.50	3.0	3.2	2.9	2.2		
1	1.7	2.3	2.6	2.2	2.15		
		2.5					
2	0	2.5	(2.5)			1.1	1.25
2	1.7	1.9	1.8			1.7	1.5
		2.0					
3	0	3.3	(3.3)			1.65	1.65
3	1.8	2.7	2.75			2.2	1.8

of the mechanism proposed for the reaction between the trypsin and inhibitor. It also shows that the equilibrium is not effected to any extent by the gelatin. The conclusion seems unavoidable that little or no trypsin is combined with the gelatin. The fact that gelatin has no protective influence on the trypsin also points to the same conclusion.

The Influence of the Manner in which the Inhibiting Solution is Added.

It was found in the study of the effect of the inhibiting solution on the rate of digestion by trypsin that the order in which the solutions were mixed and the time of standing was without effect on the result provided the control solution of pure trypsin did not alter during the experiment. If, however, the experiment is made at 38° where the control solution is rapidly destroyed the results are very different. The results of such an experiment are shown in Fig. 3. It will be seen that the solution to which all of the inhibitor had been added at the beginning of the experiment is much more active at the end than the one to which the inhibitor was added at intervals. This is evidently very similar to the Danysz phenomenon in immunology. In the present experiment the result is more marked if a relatively small quantity of the inhibitor (\approx antitoxin) is added at the beginning of the experiment whereas in the Danysz phenomenon it is necessary to add an excess. This is due to the fact that in the present experiment it is the free trypsin (\approx toxin) that is irreversibly changed during the experiment while in the Danysz experiment it is apparently the free antitoxin (\approx inhibitor) which is altered.²

The Decomposition of the Trypsin-Inhibitor Compound.

The rate of destruction of this compound at 38° is so slow in comparison to that of the free trypsin that it may be neglected. At 62°, however, it decomposes quite rapidly and follows the course of a monomolecular reaction (as was to be expected) provided an excess of inhibitor has been added so that practically all the trypsin is combined in the undiluted solution. The result of an experiment is given in Fig. 4. Irregular results were obtained in some cases owing to the formation of a precipitate presumably of coagulated protein. In such cases the amount of active trypsin remaining in solution shows a sudden drop at the time of formation of the precipitate.

Influence of the pH on the Rate of Decomposition.

Fig. 5 contains the result of an experiment in which the trypsin solutions were adjusted to various pH by the addition of HCl or NaOH before being placed at 38° for 1 hour. The activity of the

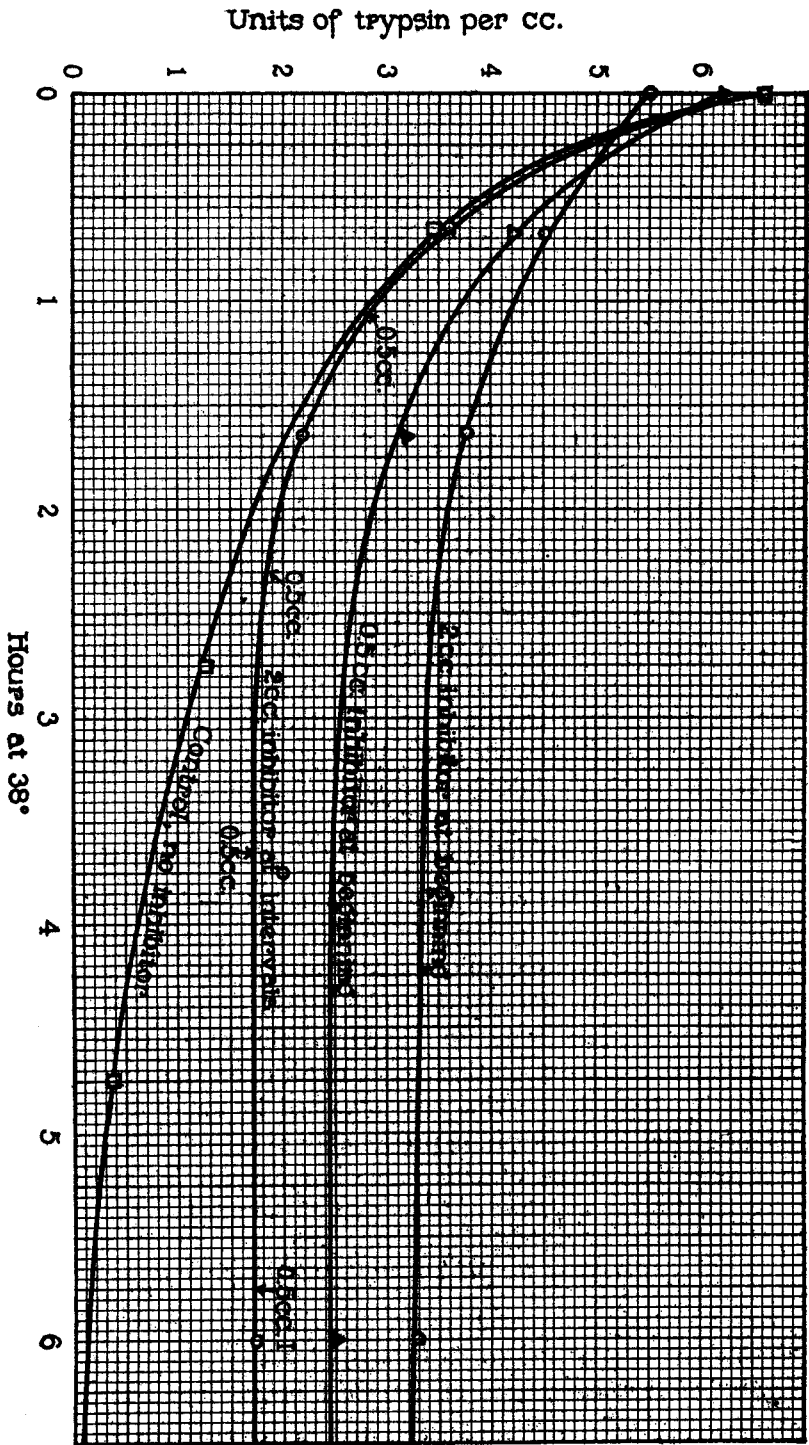


FIG. 3. Effect of time of adding inhibitor on decomposition of trypsin at 38°C.

solutions was determined by adding 1 cc. to 50 cc. of 5 per cent gelatin containing 0.2 M Na_2CO_3 (pH = 10) and the rate of hydrolysis followed by the formol titration. Trypsin is evidently most stable at a pH of 5. The rate of decomposition increases quite rapidly if the solution is either more or less acid.⁴ It differs in this respect from pepsin which is stable over quite a wide range and resembles the invertase studied by Hudson.⁵ The rapid increase in the rate of destruction

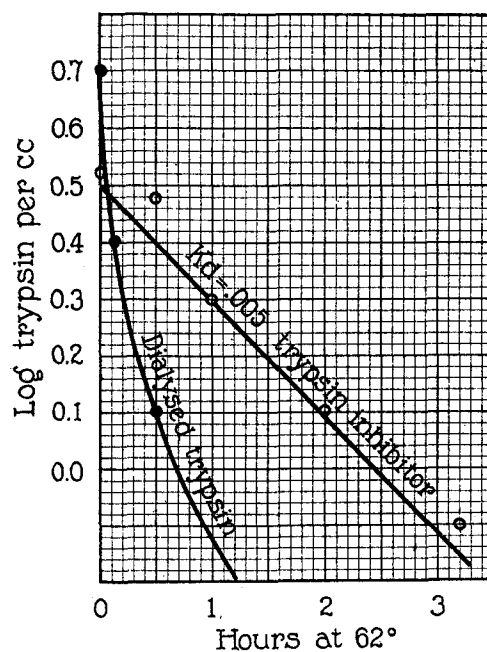


FIG. 4. Inactivation of trypsin and trypsin-inhibitor compound at 62°C.

with increasing alkalinity makes it evident that this behavior must enter to a large extent in deciding the optimum pH for the action of the enzyme. It was not found possible to determine quantitatively the effect of the pH owing to the fact that the reactions are rarely strictly monomolecular and it is therefore difficult to find a value

⁴ This agrees with the experiments of Ringer (Ringer, W. E., *Z. Physiol. Chem.*, 1921, cvi, 107).

⁵ Hudson, C. S., and Paine, H. S., *J. Am. Chem. Soc.*, 1910, xxxii, 774.

which correctly expresses the rate of decomposition. The time required for a certain amount to be destroyed could not be determined owing to experimental difficulties.

Influence of the pH on the Protective Effect of the Inhibiting Substance.

A comparison of the curves (Fig. 5) for the dialyzed trypsin solution and the solution to which inhibiting substance had been added shows that the protective action of the latter is also a function of the pH. There is little or no protective action on the acid side of pH 5.

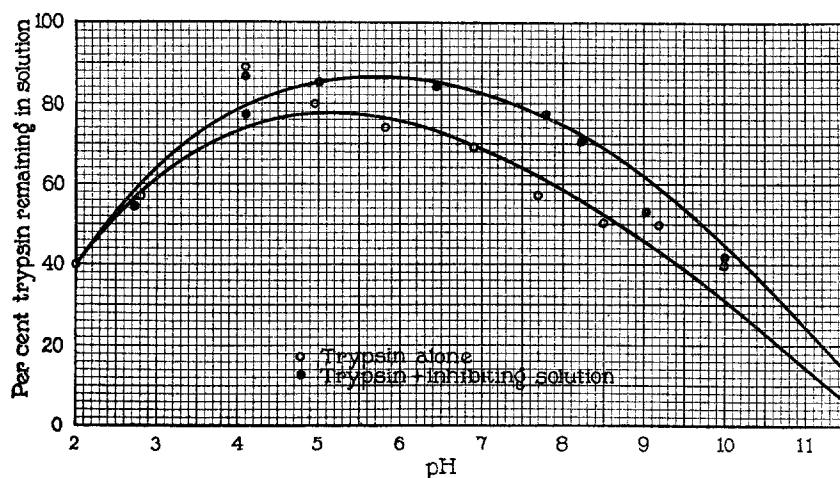


FIG. 5. Decomposition of trypsin solutions at 38°C. and different hydrogen ion concentrations. Per cent of trypsin remaining active after 0.5 hours at 38°C.

As the solution becomes more alkaline the protective action increases and then decreases slightly although the experiments are hardly accurate enough to be certain of this second decrease. If the hypothesis which has been used to account for experiments so far is correct this behavior evidently means that on the acid side of pH 5, trypsin does not combine with the inhibiting compound and that the combination has a maximum somewhere near a pH of 8 to 9.

This experiment confirms those described in the preceding paper in which it was found that the retarding influence of the inhibiting solu-

tion was not markedly effected by variations in the pH of the solution in the range of pH 6 to 10.

The mechanism which has been found to agree with the experiment described in this paper will also account for a peculiar fact which has been frequently observed in the study of the destruction of enzyme; namely, that the rate of decomposition at any one concentration will be strictly monomolecular, but that the rate becomes increasingly greater the more dilute the solution, instead of being independent of the concentration as is demanded by the monomolecular formula. If, as has been shown to be true for trypsin, the rate of decomposition depends on the amount of uncombined enzyme, it follows that the more dilute the solution the more rapidly the enzyme will become inactivated since the enzyme-inhibitor compound dissociates with increasing dilution. If, further, the inactivated enzyme reacts with the inhibitor to the same extent as does active enzyme (which was found to be the case with pepsin) then the rate of decomposition will be strictly monomolecular at any one concentration but will be the greater the more dilute the solution. This is the experimental result.

SUMMARY.

1. The rate of inactivation of purified trypsin solutions approximates closely that demanded by the monomolecular formula. The more carefully the solution is purified the closer the agreement with the formula.

2. The products formed by the action of trypsin on proteins renders the trypsin more stable. Gelatin and glycine have no effect.

3. The rate of inactivation of trypsin solutions containing these products does not follow the course of a monomolecular reaction but becomes progressively slower than the predicted rate.

4. The protective action of these substances is much greater if they are added all at once at the beginning of the experiment than if they are added at intervals.

These observations may be quantitatively accounted for by the hypothesis that a compound is formed between trypsin and the inhibiting substance which is stable as well as inactive, and that the rate of decomposition depends on the amount of uncombined trypsin present.

5. Trypsin is most stable at a pH of 5 and is rapidly destroyed in strongly acid or alkaline solution.

6. The protective effect of the inhibiting substances is small on the acid side of pH 5, increases from pH 5 to 7, and then remains approximately constant.