

THE INACTIVATION OF TRYPSIN.

II. THE EQUILIBRIUM BETWEEN TRYPSIN AND THE INHIBITING SUBSTANCE FORMED BY ITS ACTION ON PROTEINS.

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The Equilibrium between Trypsin and the Inhibiting Substance.

The experiments already described¹ show that it is possible to prepare a solution by the action of trypsin on a protein which inhibits the action of trypsin. It has also been shown that the amount of this retardation can be quantitatively measured by comparing the times necessary to cause a given small change in the conductivity of the gelatin solution under the conditions adhered to.

A number of hypotheses may be proposed that will account qualitatively for this retardation. The simplest would be to assume that the inhibiting substance combined with trypsin to form a compound that is inactive and that the activity of the solution is proportional to the concentration of free trypsin remaining in the solution. It has already been shown that if pure trypsin and protein is used the velocity of hydrolysis is proportional to the amount of trypsin taken. This is the experimental fact and is independent of any hypothesis as to the kinetics of the reaction. If it is further assumed that the equilibrium is governed by the law of mass action it is possible to test this hypothesis quantitatively. This has been done in the following experiments.

Influence of the Order of Mixing and of the Time of Standing on the Equilibrium.

Since in most of the experiments the retarding effect of the inhibiting solution has been determined by adding the solution to the gelatin

¹ Northrop, J. H., *J. Gen. Physiol.*, 1921-22, iv, 227.

and then adding the trypsin and determining the rate of hydrolysis at once, it is necessary to know whether or not the order of mixing the solutions or the time during which the trypsin has been allowed to react with the inhibiting substance has any influence on the result. Table I is a summary of experiments planned to answer this question. It shows that the order of mixing and the time during which the trypsin and inhibiting solution are left together has no effect on the final result. That is, the equilibrium between the trypsin and the inhibitor must be reached practically instantaneously and be quantitatively and instantly reversible. (This is only true if the experiment

TABLE I.
Effect of Order of Mixing and Time of Standing.

| SOLUTION. | Time to change 10 points after hours at 6°C. | |
|---|--|------------------------|
| | 0 hours. | 1.0 hours. |
| | hrs. × 10 ² | hrs. × 10 ² |
| (1) 25 cc. gelatin + 1 cc. trypsin..... | 15 | 16 |
| | 16 | |
| (2) 25 cc. gelatin + 1 cc. inhibitor + 1 cc. trypsin added after interval shown..... | 21 | 21 |
| | 25 | |
| (3) 25 cc. gelatin + 2 cc. mixture of 5 cc. inhibitor + 5 cc. trypsin. (Mixture allowed to stand as noted and 2 cc. then added to gelatin)..... | 22 | 20 |

is made under such conditions that the control trypsin solution—without inhibitor—remains unchanged during the course of the experiment. If this condition is not fulfilled, the results depend entirely on the length of time and on the temperature at which the trypsin-inhibitor solution has been allowed to stand.)²

Influence of the Gelatin Concentration.

The retardation could be qualitatively accounted for by assuming that the inhibiting substance combines with the gelatin instead of

² Northrop, J. H., *J. Gen. Physiol.*, 1921-22, iv, 261.

with the enzyme. If this were true the retardation should be less the greater the concentration of gelatin, provided the relative amount of inhibitor to trypsin were kept the same. Table II shows that this is not the case. The amount of retardation is independent of the concentration of gelatin used. The result is confirmed by the experiment discussed below in which the concentration of inhibitor is kept the same and the concentration of trypsin varied. If the inhibitor combined with the gelatin, the resulting solution would act as though

TABLE II.

Influence of Gelatin Concentration on Retardation of Hydrolysis by Inhibiting Solutions.

Control, 25 cc. gelatin of concentration noted + 1 cc. trypsin + 1 cc. 0.01 N NaCl. *Solution*, 25 cc. gelatin of concentration noted + 1 cc. trypsin + 1 cc. inhibiting solution. Temperature 33°C. Specific conductivity of all solutions 1.2×10^{-3} (adjusted with NaCl, and pH of 6.0 adjusted with NaOH).

| Time required for 10 points change with 1 cc. trypsin Time required for 10 points change with 1 cc. trypsin + 1 cc. inhibitor in gelatin concentrations of | | | |
|---|------------------|------------------|------------------|
| 1 per cent. | 2 per cent. | 4 per cent. | 8 per cent. |
| 66 | 65 | 67 | |
| 67 | 65 | | |
| 70 | 70 | | 73 |
| 75 | 75 | 75 | 76 |
| 76 | 70 | 73 | 71 |
| | 75 | 73 | 72 |
| Average 70.8 $\pm 1.8^*$ | 70.0 $\pm 1.4^*$ | 72.0 $\pm 1.2^*$ | 73.0 $\pm 0.7^*$ |

* Average deviation of the mean.

a lower concentration of gelatin had been used and the velocity would still be directly proportional to the amount of trypsin added to the solution. This is not the case. The fact that the inhibiting solution renders trypsin more stable (when no protein is present) also shows that it combines with the trypsin.

Influence of Inactivated Trypsin.

It was found in the case of pepsin³ that pepsin inactivated by alkali took part in the equilibrium just as does the active pepsin

³ Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 471.

(pepsin inactivated by heat does not act in this way). If it should be found that inactive trypsin also took part in the equilibrium it is evident that the experiments are complicated by an additional factor that is very hard to control since there is no independent method for determining the amount of inactive trypsin. In order to determine this point the experiments described in Table III were performed. They show that the inactive trypsin does not take part in the equilibrium. The calculated results are obtained from the law of mass action as described below.

TABLE III.

Addition of Inactive Trypsin.

2 per cent gelatin pH 6.2, specific conductivity 2×10^{-3} . Trypsin, 10 per cent, dialyzed, time to change 10 points = 0.10 hours = 10 units per cc. Inactivated at 65°C. for 2 hours. Active trypsin, 10 per cent, dialyzed, diluted one-third. $P = 10$. $K' = 2.8$.

| Active trypsin. | Inhibitor. | Inactive trypsin. | Time to change 10 points. | Q | | |
|-----------------|------------|-------------------|---------------------------|--------------------|---------------------|------------------------------|
| | | | | Observed. | Calculated. | |
| cc. | cc. | cc. | hrs. $\times 10^2$ | | Inactive no effect. | Inactive enters equilibrium. |
| 1 | 0 | 0 | 36 39 | 2.7 | 2.7 | |
| 1 | 0.5 | 0 | 90 87 | 1.1 | 1.2 | 1.2 |
| 1 | 0.5 | 1 | 90 80 95 | 1.1 1.2 1.05 | 1.2 | 1.9 |

Effect of Adding Increasing Amounts of Inhibitor to a Constant Quantity of Trypsin.

The results of a series of experiments to determine the effect of adding increasing concentrations of inhibitor to the same amount of trypsin are given in Table IV and Fig. I. The experiments were carried out by adding the noted amount of inhibiting solution to 25 cc. of gelatin at 33°C. All the solutions had the same pH, which re-

TABLE IV.

Effect of Increasing Amounts of Inhibitor on the Rate of Hydrolysis.

25 cc. gelatin pH 6.2; specific conductivity 2×10^{-3} . 1 cc. trypsin (10 per cent dialyzed). $V = 28$ cc. $P = 10$. $K' = 2.8$.

| Cc. inhibitor $= \frac{d}{10}$ | Time to change 10 points. <i>hrs. $\times 10^3$</i> | Trypsin. | | | $\frac{Qd}{10}$ | Trypsin combined per 0.125 cc. of inhibitor added. |
|-----------------------------------|--|--|-----------------------------------|--------------------|-----------------|---|
| | | Free $\frac{1}{T} = Q$ observed. | Combined. $E - Q$ observed. | Calculated. Q | | |
| 0 | 44 48 | 2.3 | 0 | 2.3 | | |
| 0.125 | 52 | 1.9 | 0.4 | 1.81 | 0.24 | 1st 0.125 cc. 0.40 |
| 0.25 | 64 | 1.56 | 0.74 | 1.45 | 0.39 | 2nd " 0.34 |
| 0.50 | 90 | 1.10 | 1.20 | 1.00 | 0.55 | 3+4th " 0.18 |
| 1.00 | 155 | 0.65 | 1.65 | 0.58 | 0.65 | 5 to 8th " 0.11 |
| 2.00 | 300 | 0.33 | 1.97 | 0.32 | 0.66 | 9 to 16th " 0.03 |

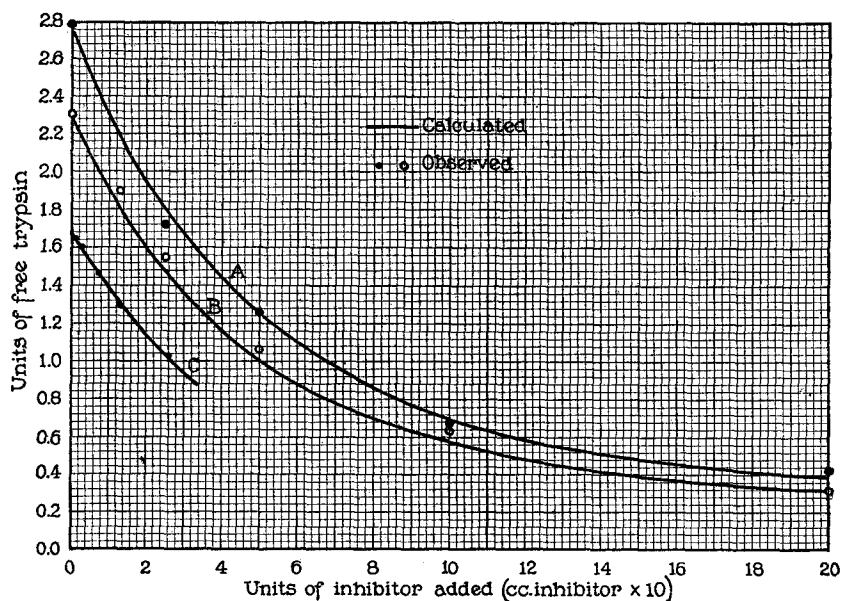
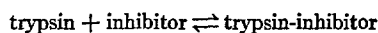


FIG. 1. Effect of adding increasing amounts of inhibitor to trypsin solutions of different strengths. The solid curves are the calculated values, and the points, the observed units of active trypsin present. (This is taken as the reciprocal of the time in hours necessary to cause a change in the bridge reading of 10 points.)

mained constant at 6.3, and the same initial conductivity. 1 cc. of trypsin solution, also of the same pH and conductivity, was then added in each tube and the time required to cause a change of 10 points in the bridge reading determined as already described. The experiments show that the addition of the first cubic centimeter of inhibiting solution has a much greater effect than the subsequent ones and that the effect constantly diminishes and becomes apparently asymptotic. This is evidently the cause of the phenomenon noted earlier that the rate of hydrolysis of a solution already containing some inhibiting substance is less rapidly retarded during the progress of the reaction than one which contains only "pure" trypsin. This is in qualitative agreement with the result predicted by the law of mass action. In order to apply this law quantitatively we may proceed as follows. It has been assumed that the equilibrium was expressed by the equation



and that the rate of hydrolysis was proportional to the concentration of the free trypsin. The law of mass action applied to this equilibrium states that

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

or

$$\frac{\frac{Q}{V} \cdot \left[\frac{d - (E - Q)}{V} \right]}{\frac{E - Q}{V}} = K$$

which may be written

$$\frac{Q [d - (E - Q)]}{(E - Q)} = KV = K' \quad (1)$$

in which Q is the amount of free trypsin in volume, V , of the solution; E , the total amount of trypsin in volume, V , of the solution; d , the total amount of inhibitor in volume, V , of the solution; K , the equilibrium constant in arbitrary units; and K' , new constant equal to the product of the equilibrium into the volume. d will evidently be proportional to the number of cc. of inhibiting solution added and if there are P units of inhibitor per cc. of inhibiting solution, $d = P$ cc.

Solving this equation for Q we find that

$$Q = \pm \sqrt{\left(\frac{d - E + K}{2}\right)^2 + KE} - \frac{d - E + K}{2}$$

and since it is assumed that the velocity of the reaction is proportional to Q

$$\frac{dx}{dt} \propto \frac{\Delta X}{\Delta T} \propto \frac{I}{T} \propto Q \quad \text{and} \quad \frac{T_1}{T_2} \propto \frac{Q_2}{Q_1}$$

All the values in the above equations are known (in arbitrary units) except d and K . If more than one experiment is made it is therefore possible to solve for these two values, and then compare the calculated and observed values for Q . When this is done it is found that below a certain value for d , K is negative while above a certain limit the value of $\frac{K}{d}$ becomes constant owing to the fact that one of the terms of the equation becomes negligible when d is too large. It is also found that the constancy of K is very sensitive to experimental error (as was to be expected since it depends on the difference between two experimental values), so that a comparison of the observed and calculated values of Q is a better test of the formula than the constancy of K . Between the two limits for the values of d there are several values all of which give values of K which permit the calculation of the experimental results. The smallest of these has been taken. This gives a value for K of 0.1 and for P (in the particular inhibiting solution studied) of 10, both expressed in the same arbitrary units (*cf.* Euler and Svanberg⁴ for a discussion of the same equilibrium with invertase, and Northrop³ in the case of pepsin). It will be noted that the expression for the equilibrium as used in this form contains two arbitrary constants; *i.e.*, it is necessary to make two determinations before the others can be calculated. The agreement between the calculated and observed values is close enough to leave little doubt that the formula correctly expresses the facts, but the presence of two constants renders it possible that the agreement is accidental. If this were the case we should expect to find that it was necessary to use different values for K and d in each set of experiments. This is, however, not the case. All the experiments were found to agree

⁴ von Euler, H., and Svanberg, O., *Fermentforschung*, 1920, iii, 330; 1921, iv, 142.

with a value for K of 0.1 as found above, and all done with the same inhibiting solution to agree with the same value of P (or d) as well. Several experiments were made which gave apparently regular results for which, however, it was impossible to find any satisfactory value for K and d . In every case of this kind it was found that the inhibiting solution used contained either active trypsin or some substance which could still be acted on by trypsin. In applying the formula, it is assumed, of course, that the only trypsin present is that in the trypsin solution added and that the substrate concentration is the same in all tubes. These experiments seem to show that the formula is not of such a general character as to fit any regular curve. As would be expected, different inhibiting solutions required different values of P . The results shown in the figure were all calculated from the same values of K' which were obtained from the first part of the experiment in Curve A . All the other results were calculated before the experiment was done, as were those described later in which the conditions were varied in other respects. The figure shows that the calculated and experimental results are identical.

Column 6 in Table IV contains the values for Qd ; *i.e.*, the product of the free trypsin into the total amount of inhibitor. It will be seen that this value increases at first but becomes constant as d becomes large with respect to Q . Q therefore becomes inversely proportional to d . This may also be predicted from the mass action expression, and, as Arrhenius⁵ pointed out, is the condition that leads to Schütz's rule. The steps in the derivation are as follows:

Equation (1) may be written

$$Q = \frac{K(E - Q)}{d - (E - Q)}$$

as d increases the term $(E - Q)$ increases and approaches the constant value E so that $K(E - Q)$ approaches a constant value. If d is large compared to E the term $(E - Q)$ may be neglected in the denominator and the equation written

$$Q = \frac{K''}{d}$$

or Q , the amount of active trypsin is inversely proportional to the amount of inhibitor, which is the experimental result referred to

⁵ Arrhenius, S., *Medd. Kong. Vetsakad. Nobelinst.*, 1908, i.

above (therefore Qd becomes constant). Since the velocity of hydrolysis is proportional to Q and to the concentration of substrate the differential expression for the course of hydrolysis would be $\frac{dx}{dt} = QA$, where A is the amount of substrate. Substituting $\frac{K''}{d}$ for Q we have

$$\frac{dx}{dt} = \frac{K'' A}{d} \quad (2)$$

Since d , the inhibiting substance, is the same as x , the products of hydrolysis, we may substitute x for d . A may be considered a constant for the first few per cents of the hydrolysis. In any case, although A is decreasing, the term $(E - Q)$ (which has also been considered a constant in the numerator) is increasing so that the product of the two will be more nearly constant than either of the two quantities themselves. It is this product that really enters into the equation. Substituting x for d in equation (2) and integrating we obtain

$$\int x dx = \int K'' A dt \text{ or } \frac{1}{2}x^2 = K_3 T \text{ or } x = K_4 \sqrt{T}$$

That is, x , the products formed, is proportional to the square root of the elapsed time. This derivation makes it clear that Schütz's rule will only hold when the concentration of the products formed is large with respect to the amount of enzyme. The author has shown that the same experiments may be performed with pepsin.³ In the case of these two enzymes, at least, therefore, there is direct experimental evidence for Arrhenius' explanation.

Effect of Constant Quantity of Inhibitor on Increasing Amounts of Trypsin.

In all the foregoing experiments the concentration of trypsin has been the same in any one series of experiments and the concentration of inhibitor varied. If the mechanism proposed is correct it should be possible to predict equally well the result of an experiment in which the concentration of inhibitor was kept constant and the amount of trypsin varied. That this is the case is shown in Fig. 2. The calculated results for this experiment were obtained by using the

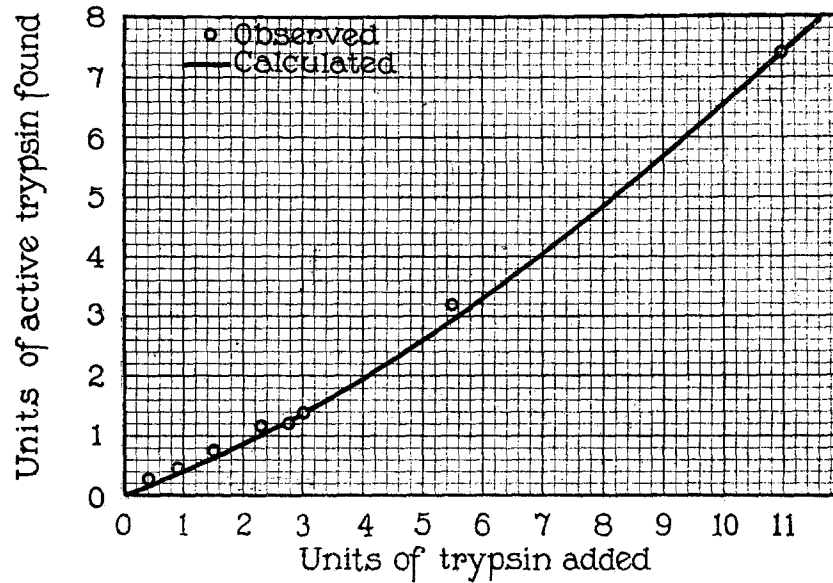


FIG. 2. The influence of the total amount of trypsin on the inactivation caused by 5 units inhibitor. Increased amounts of trypsin were added to series of tubes each containing 25 cc. gelatin solution, and 5 units inhibitor. Duplicate series run at the same time and under the same conditions, but without inhibitor.

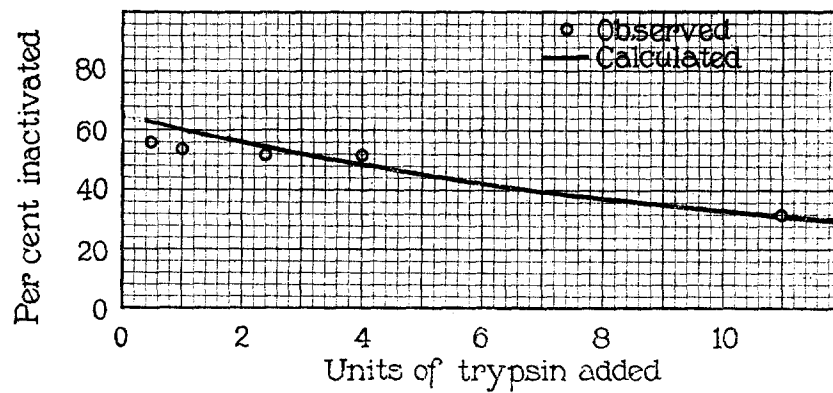


FIG. 3. The influence of the total amount of trypsin present on the percentage retardation caused by 5 units of inhibitor.

values for the constants obtained from the preceding experiments and were worked out before the experiment itself was done. The figure shows that in this case also the experimental and calculated results agree within the limit of experimental error. In Fig. 3 the percentage inactivation of the various amounts of trypsin by the five units of inhibitor have been plotted. As would be expected the smaller the amount of trypsin the greater the percentage inactivation although the absolute amount of trypsin inactivated is less.

It has been shown above that the law of mass action predicts quantitatively the results of the experiments when either the trypsin or the inhibitor concentration is varied. It is possible to vary conditions in another way by keeping the relative amount of trypsin and inhibitor the same and varying the dilution (*i.e.*, the value of v). The calculated and observed results of such an experiment are given in Fig. 4 (Curve *B*). The experiment was performed by mixing the trypsin and inhibitor solution and then adding the noted cubic centimeters of this mixture to 25 cc. of gelatin. It will be seen that in this case also the predicted results are in close agreement with the experiment. In this case the rate of hydrolysis decreases more slowly than the total amount of trypsin taken. This is the result of the fact that as the dilution is increased the trypsin inhibitor compound dissociates and so liberates more active trypsin, so that the concentration of active trypsin does not decrease directly as the total trypsin. Exactly the same curve would be obtained for the rate of hydrolysis by hydrogen ions furnished by a weak acid if the total concentration of acid were plotted against the rate of hydrolysis. In Curve *C* in Fig. 4 the result of an experiment is given in which the concentration of trypsin is varied but the concentration of inhibitor is kept constant. This is a similar experiment to that described in Fig. 2. In this case the rate of hydrolysis decreases more rapidly than the concentration of the trypsin. This is the result of the fact shown in Fig. 3 that the percentage retardation of the action of trypsin with a constant concentration of inhibitor is the greater, the smaller the total amount of trypsin. Curve *A* in Fig. 4 is the dilution-activity curve for "pure" trypsin and gelatin. In this case the velocity is nearly directly proportional to the amount of trypsin taken. It is clear from these curves that unless care is taken to purify the

enzyme and protein solution used, activity-concentration curves may be found to be either convex or concave or a straight line. This probably accounts for the discrepancies in the literature in regard to this point. If the enzyme solution contained products of protein digestion, as is very likely to be the case, the rate of hydrolysis would not increase as rapidly as the enzyme concentration. If the protein solution was already partially hydrolyzed or contained some inhibiting substance, the velocity of hydrolysis would increase more rapidly than the enzyme concentration.



FIG. 4. The influence of the presence of inhibitor on the concentration-activity curve of trypsin. Curve A, "pure" trypsin diluted with water. Curve B, mixture of trypsin and inhibitor diluted with water. The ratio of trypsin to inhibitor is therefore constant. Curve C, mixture of trypsin and inhibitor diluted with a solution of inhibitor of the same concentration as was present in the trypsin solution. The concentration of inhibitor is therefore constant in this experiment.

Effect of the Hydrogen Ion Concentration.

In all the foregoing experiments the hydrogen ion concentration was kept constant at a pH of 6.3. It seemed of interest to determine what effect a variation in this factor would have on the retarding

action of the inhibiting solution. This experiment is difficult to perform since the conductivity method cannot be used and the pH can only be kept constant at ranges other than pH 6.3 by the use of high concentration of buffers which interfere with the formol titration. The results of such an experiment are given in Table V. The pH of the solutions was adjusted by making them all $M/10$ with respect to sodium carbonate and then titrating back to the desired pH with 0.1 N HCl. The course of the hydrolysis was followed by a slight modification of the formol titration already described.⁶ No marked effect of the pH could be noted. Several other experiments were

TABLE V.

Effect of the Hydrogen Ion Concentration on the Inactivation of Trypsin by Inhibitor Solution.

A. Gelatin, 5 per cent, containing 0.2 N Na_2CO_3 . B. Gelatin, 5 per cent, containing 0.2 N Na_2CO_3 and 4 per cent inhibitor solution. 50 cc. samples titrated to pH noted, and noted cc. trypsin added. Samples placed at 33°C. and formal titration run on 5 cc. at intervals.

| | | | | | | | | |
|---|------|------|------|------|------|------|------|------|
| pH at beginning | 6.2 | 6.2 | 8.0 | 8.3 | 8.8 | 8.8 | 10.2 | 10.1 |
| pH at end..... | 6.2 | 6.2 | 7.5 | 7.6 | 7.6 | 8.0 | 9.8 | 9.7 |
| Cc. inhibitor per 50 cc..... | 0 | 2 | 0 | 2 | 0 | 2 | 0 | 2 |
| Time in hours to cause increase of 1.0 cc. in formol titration..... | 0.80 | 1.05 | 0.60 | 0.90 | 0.55 | 0.70 | 0.80 | 1.00 |
| Per cent retardation..... | 20 | | 30 | | 20 | | 20 | |

made all of which gave results approximately the same as those given. No differences in the degree of retardation due to the pH were noted that could be definitely put outside the experimental error. This result was corroborated by the effect of the inhibiting solution on the rate of destruction of trypsin.² Here also no differences in the effect between pH 6 and 10 could be noted. This result seems to show that the "active" concentration of the trypsin and of the inhibiting substance is not markedly effected by the pH (between 6 and 10).

⁶ Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 595.

Does Trypsin Form a Compound with Gelatin?

The hypothesis which has been found to account quantitatively for the experiments described in this paper contains the assumption that the velocity of hydrolysis is proportional to the concentration of *free* enzyme. That is, the enzyme would enter into the formula for the kinetics of the reaction just as would the hydrogen ion concentration in the case of a reaction catalyzed by hydrogen ions or as one of the reactants in any chemical reaction and the time presumably consists of the time required for a molecule of enzyme to come in contact with a molecule of gelatin. The idea was proposed by Brown⁷ and has since been elaborated by various authors that enzymes combine with the substances which are hydrolyzed by them and that the velocity of the reaction depends on the speed of decomposition of this compound, while the time for the compound to form is negligible. If this were true in the case of trypsin it is clear that the mechanism for the equilibrium between trypsin and the inhibiting substance which has been found to agree with the experiments could not be verified since it would be necessary to allow for the amount of trypsin combined with the gelatin. If this mechanism is correct therefore it seems necessary to conclude that the amount of trypsin combined with the gelatin at any one time is negligibly small and that the limiting time element is really the time required for the compound to form as is the case in other chemical reactions. The same reasoning evidently applies to pepsin hydrolysis and to invertase (Euler⁴), since in both these cases it has been found that the equilibrium between the enzyme and an inhibiting substance can be quantitatively accounted for by the assumption that the rate of hydrolysis is proportional to the *free* enzyme. This question will be discussed in another paper.

Can the Results be Accounted for by the Adsorption Formula?

The adsorption formula as given by Freundlich⁸ and as usually used is written

⁷ Brown, A. J., *J. Chem. Soc.*, 1902, lxxxi, 373.

⁸ Freundlich, H., *Kapillarchemie: Eine Darstellung der Chemie der Kolloide und verwandter Gebiete*, Leipsic, 1909.

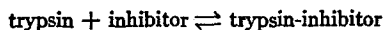
$$\frac{x}{C \frac{1}{n}} = k \cdot m$$

where x is the amount adsorbed, C is the concentration of the substance remaining in the solution, and m is the amount of adsorbent (or the area of the adsorbing surface). k and n are constants. In the present experiments it cannot well be assumed that the enzyme is adsorbed by the inhibiting substance since there is no evidence that this is in other than true solution. It would be necessary to suppose therefore that the inhibiting substance is adsorbed by the enzyme. The amount of enzyme would therefore be represented by m in the above formula and there would be no way to determine how much was combined and how much was free since there is no term in the equation to represent the amount of the adsorbent (in this case the enzyme) which is combined.

Hedin⁹ has found that trypsin is adsorbed by charcoal and that the equilibrium in this case is that demanded by the adsorption formula. Hedin's experiments are, however, not contradictory to those described in this paper since it is quite possible that trypsin may be adsorbed by charcoal and yet react with other substances according to the law of mass action. This is known to be the case with acetic acid and many other substances.

SUMMARY.

1. A study has been made of the equilibrium existing between trypsin and the substances formed in the digestion of proteins which inhibit its action.
2. This substance could not be obtained by the hydrolysis of the proteins by acid or alkali. It is dialyzable.
3. The equilibrium between this substance (inhibitor) and trypsin is found to agree with the equation,



The equilibrium is reached instantaneously and is independent of the substrate concentration. If it be further assumed that the rate of

⁹ Hedin, S., *Biochem. J.*, 1906, i, 484; *Z. physiol. Chem.*, 1906-07, i, 497.

hydrolysis is proportional to the concentration of the free trypsin and that the equilibrium conforms to the law of mass action, it is possible to calculate the experimental results by the application of the law of mass action.

4. The equilibrium has been studied by varying (*a*) the concentration of the inhibiting substance, (*b*) the concentration of trypsin, (*c*) the concentration of gelatin, and (*d*) the concentration of trypsin and inhibitor (the relative concentration of the two remaining the same). In all cases the results agree quantitatively with those predicted by the law of mass action.

5. It was found that the percentage retarding effect of the inhibiting substance on the rate of hydrolysis is independent of the hydrogen ion concentration between pH 6.3 and 10.0.

6. The fact that the experimental results agree with the mechanism outlined under 3, is contrary to the assumption that any appreciable amount of trypsin is combined with the gelatin at any one time; *i.e.*, the velocity of the hydrolysis must depend on the time required for such a compound to form rather than for it to decompose.

7. The experiments may be considered as experimental proof of the validity of Arrhenius' explanation of Schütz's rule as applied to trypsin digestion.

8. Inactivated trypsin does not enter into the equilibrium.

Many of the experiments described in this paper were carried out by Mr. Frank Johnston.