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THE DYNAMICS OF PEPSIN AND TRYPSIN[1]

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ENZYMES appear in many respects to be the connecting link between living and inanimate matter, since many of the reactions occurring in the living organism are controlled and directed by these unstable and illusive substances, while the enzymes themselves do not have the attributes associated with what are called living organisms. Enzymes themselves, however, have certain peculiarities which have always made them a source of interest and also of controversy. They are usually classed as catalysts which in itself is sufficient to surround them with an air of mystery. This distinction between catalytic and other reactions, although it has undoubtedly been of value, is, in the writer's opinion, to a very large extent artificial and it would be much better to define catalytic reactions with Falk as a reaction in which "the chemical composition of one of the initial substances is the same as that of one of the products of the reaction." A second peculiarity of enzymes which has attracted attention is the so-called specificity, i.e., they have more effect on certain reactions than on others. This peculiarity, although of the greatest practical importance, is also to a large extent artificial, since the specificity is quantitative and not qualitative, and from a quantitative standpoint all reactions are specific. The question of specificity is therefore one of chemical reactions in general and is not at all peculiar to enzyme reactions. This is well illustrated by the recent work of Levene and Simms, who found that the rate of hydrolysis of dipeptides either by acid or erepsin was a function of the acid and basic dissociation constants of the peptide. In regard to the "catalytic" or "specific" nature of the reaction, therefore, enzyme reactions do not appear to differ qualitatively from other chemical

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reactions although the peculiarities are certainly more marked. There remain, however, certain other characteristics which have been supposed to distinguish them from the usual chemical reactions.

The kinetics of enzyme reactions were early found to differ from those of simple inorganic reactions and they were considered by many workers to be therefore exempt from the usual chemical laws. The work of Bredig and Arrhenius among others showed that complete analogies could be found for all the peculiarities of enzymes in inorganic reactions and it is now generally admitted that no sharp line can be drawn between enzymes and other chemical compounds. There is still considerable uncertainty, however, as to the laws expressing the kinetics of the reactions. A number of workers consider that enzymes are colloidal substances and that the law of mass action cannot be expected to hold, but that the reactions are more nearly analogous to gas reactions occurring at the surface of a metal. In confirmation of this idea it is possible to find very close analogies to enzyme reactions with such heterogeneous or colloidal systems. On the other hand, it is possible to find equally good analogies in reactions, such as hydrogen ion catalysis, which are undoubtedly homogeneous. The problem seems to resolve itself therefore into which point of view will furnish the simplest and most accurate method of calculating the kinetics of the reaction in question. Now since the kinetics of homogeneous reactions are much better understood both theoretically and practically than heterogeneous reactions, it seems more sensible, on account of the convenience if for no other reason, to use the law of mass action as long as the experiments are found to conform to it, and this has been the point of view in the present experiments.

The mechanism of enzyme reactions has usually been attacked by a study of the kinetics of the reactions themselves. Kinetics of reactions in general fail to show agreement between theory and experiment, and even the classical hydrolysis of sugar presents certain anomalies which have never been satisfactorily accounted for. This method of attack also leads invariably to equations containing a number of arbitrary constants whose physical

meaning is a matter of doubt and which detract very considerably from the significance to be attached to the agreement between calculated and observed values. Also the question as to whether or not the reactions are monomolecular, about which so much discussion has been centered, is not decisive as regards the nature of the reaction since the same formula is predicted for a heterogeneous reaction regulated by the rate of diffusion and a homogeneous, catalyzed reaction. It has seemed more advisable therefore to study the reaction between the enzyme and some substance which it does not decompose.

Since the only quantitative method for the determination of enzymes consists in determining the rate at which they react, it is necessary first of all to establish the relation between this rate and the concentration of enzyme. In the case of the proteolytic enzymes there are a number of methods by which the reaction can be followed, the choice depending largely on the nature of the experiment. In these experiments, the increase of amino nitrogen determined by Van Slyke's method or by the formol titration, the change in conductivity of the solution, and the change in viscosity, as well as the amount of protein left in solution have all been used. For the present purpose the conductivity and viscosity changes are the most convenient since comparative results only are needed and it is not necessary to interpret the observed changes in terms of the actual chemical reactions occurring, all that is necessary being the relation between this change and the concentration of enzyme.

The results of such experiments in which the concentration of a purified enzyme solution was varied are shown in tables 1, 2 and 3. Table 1 shows the relation between the time required to cause a small change in the viscosity of a standard gelatin solution with various concentrations of trypsin. Table 2 gives the change in the conductivity of a solution of egg albumin with increasing pepsin concentration, and table 3 the same experiment with trypsin. In every case it will be seen that the time required to cause a small, equal change in the substrate is inversely proportional to the concentration of the enzyme. It will be assumed therefore *under the conditions of these experiments* that

TABLE 1

Change caused in viscosity of 3 per cent gelatin solution pH 7.4 by various amounts of trypsin Q. Temperature 34°C. ± 0.02°C.

CHANGE IN VISCOSITY	MEAN TIME TO CAUSE PER CENT CHANGE NOTED (TIME INTERPOLATED FROM CURVE) When Q =				VALUE OF K When Q =				MEAN FOR ALL VALUES OF QT'	Q CALCULATED. MEAN OF ALL VALUES OF $\frac{QT'}{\text{MEAN TIME}}$	Q TAKEN
	1	2	4	8	1	2	4	8			
per cent	*hours* × 10^2	*hours* × 10^2	*hours* × 10^2	*hours* × 10^2							
5	26.8±0.3	12.3±0.4	6.5±0.1	3.3±0.1	26.8±0.3	24.6±0.8	26.0±0.4	26.4±0.8	25.9±0.6	0.97±0.05 0.98±0.05 0.99±0.06 0.94±0.04	1
10	57.8±0.5	27.0±0.9	14.3±0.3	7.1±0.2	57.8±0.5	54.0±1.8	57.2±1.2	56.8±1.6	56.5±2.7	2.10±0.12 2.00±0.12 2.10±0.13 2.05±0.11	2
15	97.0±0.9	45.6±1.5	24.2±0.4	12.2±0.4	97.0±0.9	91.2±3.0	96.8±1.6	97.6±3.2	95.7±4.6	4.00±0.10 3.95±0.24 3.95±0.21 4.01±0.18	4
20	154.0±2.1	74.8±1.9	35.5±0.2	17.0±0.4	154.0±2.1	149.6±3.8	142.0±0.8	136.0±3.2	145.5±5.3	7.89±0.38 7.35±0.45 7.82±0.35 8.55±0.42	8

TABLE 2

Concentration of pepsin and change in conductivity

Solution, 2.5 cc. of 2 per cent active pepsin diluted to 10 cc. + HCl, pH 2.0, and then diluted as noted + HCl. Pepsin determined in 1 cc.

Q = RELATIVE CONCENTRATION OF PEPSIN TAKEN	TIME FOR 10 PER CENT CHANGE IN CONDUCTIVITY OF 25 CC. OF EGG ALBUMIN + 1 CC. OF SOLUTION— T = hours × (10^2)	QT
100	20	20.0
66	31	20.5
50	40	20.0
25	83	20.7
12.5	170	21.2

TABLE 3

Concentration of trypsin and change in conductivity of gelatin solution

RELATIVE CONCENTRATION OF TRYPSIN	TIME REQUIRED TO CHANGE 10 POINTS OBSERVED	(QT=550) CALCULATED	QT
	hours × 10^2		
64	7.5 7.3	8.5	480 467
32	17.5 16.0	17.0	560 510
16	34 35	34.0	540 560
8	80 82	68	640 656
4	155 160	136	620 640
2	300 250	272	600 500
1	660 680	542	660 680

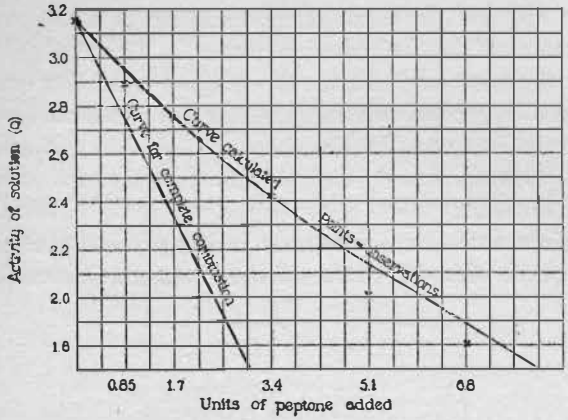

FIG. 1. CURVES SHOWING EFFECT OF ADDING INCREASING AMOUNTS OF PEPTONE TO PEPSIN SOLUTIONS

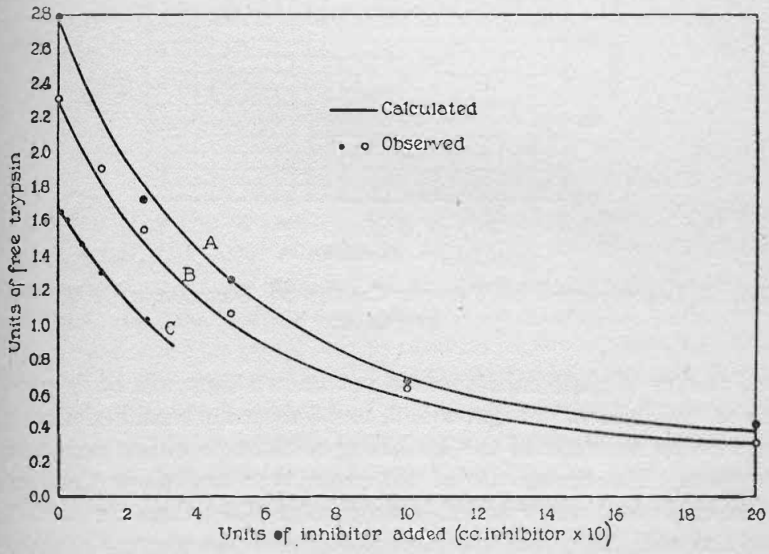

FIG. 2. EFFECT OF ADDING INCREASING AMOUNTS OF INHIBITOR TO TRYPSIN SOLUTIONS OF DIFFERENT STRENGTHS

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.)

the time required to cause such a change is a measure of the concentration of (active) enzyme present. This makes it possible to study the reaction between the enzyme and other compounds.

Enzymes in general are inhibited by the products formed during the reaction, and Bayliss showed that this was also true of trypsin. It is also known that plasma contains substances having a marked inhibitory effect on trypsin digestion. These substances are therefore convenient for study in the present case since they are connected with the course of the reaction. Since the chemi-

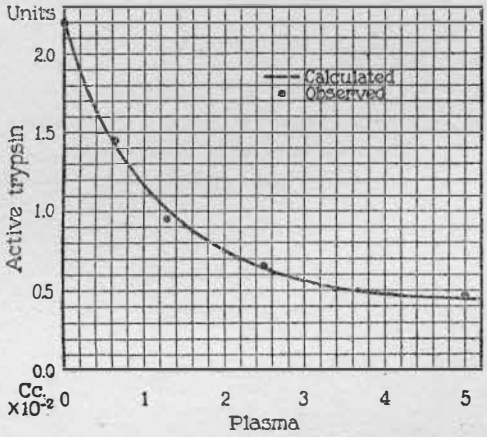

FIG. 3. INACTIVATION OF TRYPSIN CAUSED BY INCREASING QUANTITIES OF PLASMA

cal nature of these substances is not known they will be referred to as "inhibitors." Figures 1, 2, and 3 show the results of adding increasing amounts of the inhibiting solution to either pepsin or trypsin. The curves are of the same type and show that the first cubic centimeter added has a greater effect than the second, and so on. They are also asymptotic and the enzyme activity will evidently not be completely depressed until an infinite concentration has been added. 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.

The simplest assumption would be that the equilibrium was expressed by the equation

$$\text{trypsin} + \text{inhibitor} \rightleftarrows \text{trypsin-inhibitor}$$

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

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

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', a new constant equal to the product of the equilibrium constant into the volume. d will evidently be proportional to the number of cubic centimeter of inhibiting solution added and if there are P units of inhibitor per cubic centimeter of inhibiting solution, $d = P$ cc.

Solving this equation for Q we find that

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

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. 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 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. 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 figures 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 figures show that the calculated and experimental results are identical.

EFFECT OF CONSTANT QUANTITY OF INHIBITOR ON INCREASING AMOUNTS OF TRYPSIN

In the foregoing experiments the concentration of enzyme 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 figure 4. The calculated results for this experiment were obtained by using the 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.

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 figure 5 (Curve B). The experiment

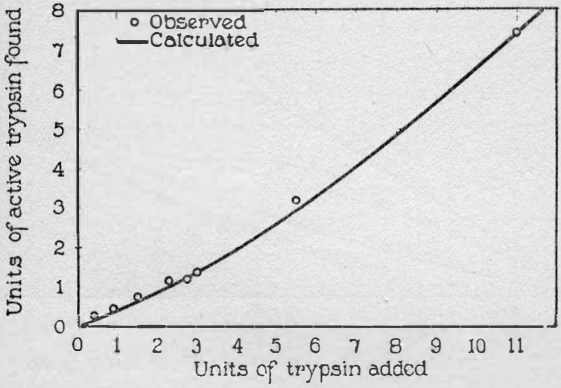

FIG. 4. THE INFLUENCE OF THE TOTAL AMOUNT OF TRYPSIN ON THE INACTIVATION CAUSED BY 5 UNITS INHIBITOR

Increasing 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.

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 figure 5 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

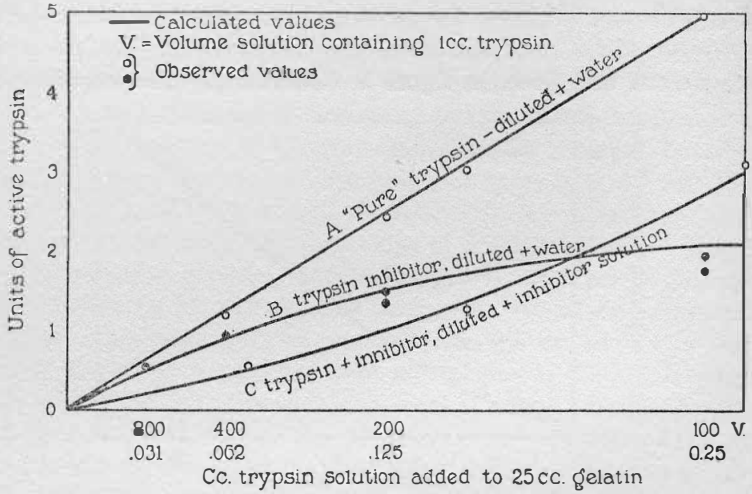

FIG. 5. THE INFLUENCE OF THE PRESENCE OF INHIBITOR ON THE CONCENTRATION-ACTIVITY CURVE OF TRYPSIN

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.

described in figure 4. In this case the rate of hydrolysis decreases more rapidly than the concentration of the trypsin. This is the result of the fact 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 figure 5 is the dilution-activity curve for "pure" trypsin. 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 taked 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 total enzyme concentration. If the protein solution was already partially hydrolyzed or contained some inhibiting sub-

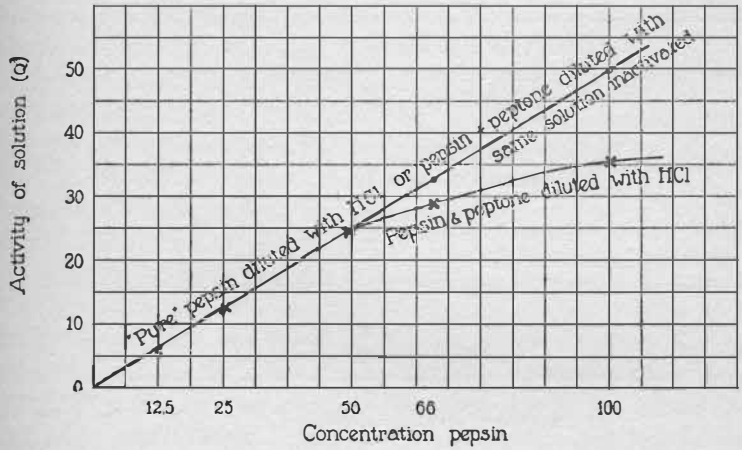

FIG. 6. CURVES SHOWING EFFECT OF PEPTONE ON ACTIVITY OF DILUTED PEPSIN SOLUTIONS

stance, the velocity of hydrolysis would increase more rapidly than the total enzyme concentration.

Figure 6 gives the result of a similar experiment with pepsin solutions. It will be noted in this case that pepsin inactivated by alkali still reacts with the inhibitor, i.e. it is analogous to what, in Ehrlich's terminology is called a "toxoid."

The preceding experiments show that pepsin and trypsin react with inhibiting substances in a way which may be accurately calculated by means of the law of mass action. They may be also calculated by means of Freundlich's adsorption formula and

it is possible to obtain results with charcoal that give a very similar curve. There is, however, one important difference between the two sets of experiments. In the case of the solutions referred to above, the effect is instantly and completely reversible and is independent of the order of mixing provided the

TABLE 4

Influence of order of mixing

	T HOURS PER 20 PER CENT CHANGE IN VISCOSITY	$Q = \frac{1}{T}$ HOURS
50 cc. gelatin + {trypsin 2:25 dilution, 0.5 cc. / water, 0.5 cc.} (control)	0.87	1.15
50 cc. gelatin + {trypsin 2:25 dilution, 0.5 cc. / plasma 1:20 dilution, 0.5 cc.}	1.30	0.77
(50 cc. gelatin—plasma 1:20 dilution, 0.5 cc.) + trypsin dilution 2:25, 0.5 cc.	1.36	0.75

TABLE 5

Effect of order of mixing

METHOD OF PREPARING MIXTURE	TIME REQUIRED FOR 10 PER CENT CHANGE IN VISCOSITY
	hours
1. 1 cc. of H_2O + 0.2 cc. of trypsin, kept 5 minutes at 20°C., 10 cc. of gelatin added	0.41
2. 1 cc. of charcoal suspension (= 0.1 gram of charcoal) + 0.2 cc. of trypsin kept 5 minutes at 20°C., 10 cc. of gelatin added.	>3.00
3. 10 cc. of gelatin + 1 cc. of charcoal suspension kept 5 minutes at 20°C., 0.2 cc. of trypsin added	0.42

experiment is done under conditions which prevent the "spontaneous" inactivation of the enzyme, whereas in the case of charcoal the result is markedly dependent on the order of mixing and is not reversible. This is shown in tables 4 and 5. This criterion allows a choice in favor of the view that the reaction

is homogeneous and is actually in accord with the law of mass action.

The foregoing experiments furnish good evidence that pepsin and trypsin may be considered as in true solution and justify the use of the law of mass action in respect to them. Euler and his co-workers have found that the retardation of invertase by certain ions may also be calculated by the mass law.

"SPONTANEOUS" INACTIVATION OF PEPSIN AND TRYPSIN

One of the characteristics of enzymes is the fact that they are relatively very unstable and quickly lose their activity even at moderate temperatures. Here again the course of the reaction is usually anomalous. It is predicted by the law of mass action that the rate of decomposition of a substance should be proportional to its concentration. The percentage inactivation should, therefore, be independent of the original concentration and the course of the reaction should follow the monomolecular formula. Experimentally, however, this is usually not the case. If a series of enzyme solutions is prepared of varying concentrations, it is usually found that the more dilute one is the most rapidly inactivated and, on the other hand, that the larger the percentage inactivation of any one solution the more slowly the remaining enzyme is inactivated. This latter experiment has led to the view that there are a series of enzymes present which differ in their stability to heat. This assumption, however, is unnecessary since, as will be seen, the formation of a compound between inhibiting substances and the enzyme which has just been discussed is sufficient to account for the experimental observations. The results of some experiments with trypsin are shown in figure 7. In this figure the logarithm of the amount of active trypsin remaining in solution at any time has been plotted against the time 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 eighteen hours at 6°C., filtered and redialyzed. 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

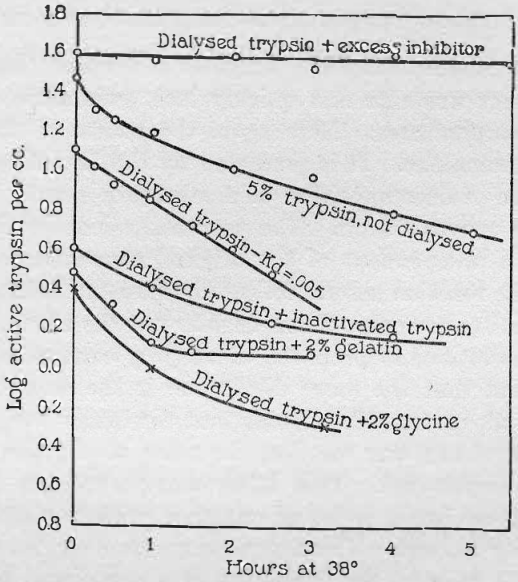

FIG. 7. INACTIVATION OF VARIOUS TRYPSIN SOLUTIONS AT 38°C.

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 is present, the 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 above that the products formed by the action of trypsin on protein form a compound with the trypsin that is inactive. The simplest explanation for the present ex-

periments 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 follows from this mechanism 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.

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, with a constant concentration of inhibiting substances, 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 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 EFFECT OF BOTH "SPONTANEOUS" INACTIVATION AND INHIBITING SUBSTANCES

The foregoing experiments show that the reaction follows quite closely the simple theoretical course when the "pure" enzyme is inactivated by heat alone or by the addition of inhibiting substances alone. If, however, the experiment is made at a higher temperature, 38°C., so that both reactions occur simultaneously, the result is very different. The results of such an experiment are shown in figure 8. It will be seen that the solution to which all of the inhibitor had been added at the beginning of the ex-

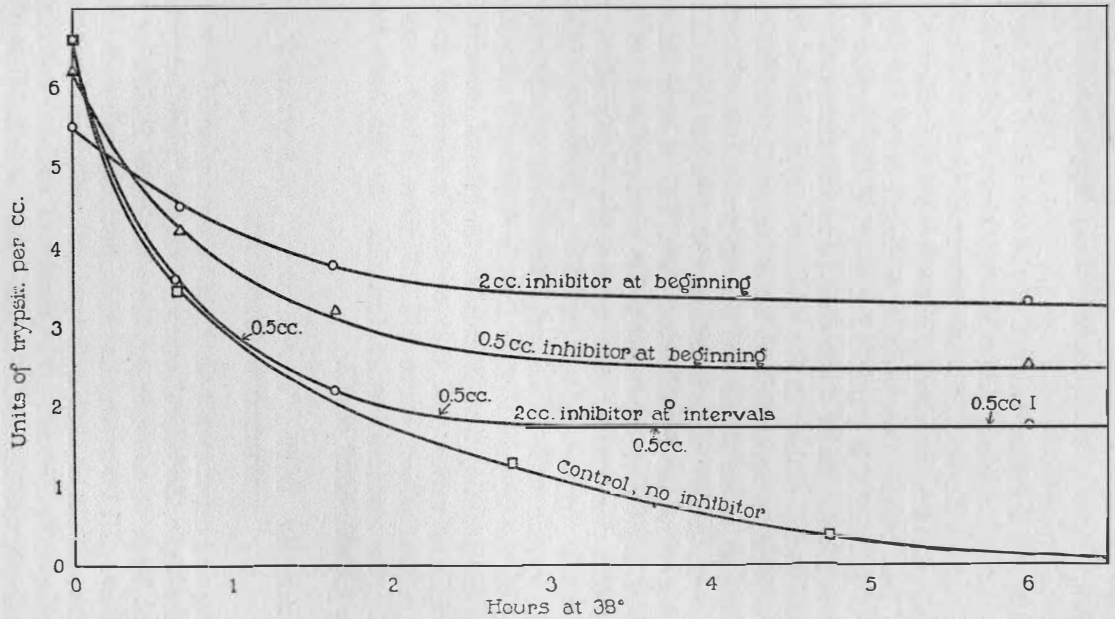

FIG. 8. EFFECT OF TIME OF ADDING INHIBITOR ON DECOMPOSITION OF TRYPSIN AT 38°C.

periment 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.

The experiment at first sight is not at all simple and does not agree with any simple theoretical curve. It is merely the result, however, of the fact noticed above, that the compound of trypsin-inhibitor is much more stable than the free enzyme and that the solution therefore, to which the largest amount of inhibitor has been added, retains its activity longest.

INFLUENCE OF pH ON THE RATE OF INACTIVATION

As might be expected the inactivation of the enzyme depends to a large extent on the pH of the solution. Trypsin is most stable at a pH of about 5.0, while pepsin becomes very unstable as soon as the pH is raised above 6.0. In this connection Michaelis has reported the interesting and unexpected result that if the change of pH with temperature is taken into account the inactivation of pepsin has no temperature coefficient.

INACTIVATION BY RADIUM EMANATION AND X-RAY

The inactivation of pepsin, trypsin, and invertase on exposure to radiation from radium emanation have been studied by Hussey and Thompson. These workers also found that with purified enzymes in dilute solutions the course of the reaction was monomolecular and its velocity was proportional to the radiant energy received by the solution. The inactivation of trypsin by x-ray was studied by Clark and the writer with the same result. The rate of inactivation of the enzyme from x-ray could be accurately calculated from the results of Hussey and Thompson with radium emanation, if it were assumed that the rate of inactivation was simply in proportion to the relative ionizing power of the two radiations. This is in accord with physical theory which attributes all reactions due to radiation to ionization, irrespective of the type of ray, but differs from the opinion usually expressed in biological work, that the effect of the radiation depends on the type of ray as well as upon the energy.

These experiments also brought out the point that the relative stability to heat of all the enzymes studied so far is qualitatively the same as their stability to radiation.

KINETICS OF THE REACTION

The kinetics of trypsin digestion as ordinarily carried out were outlined by Bayliss. Bayliss found that the reaction was not monomolecular, and that the amount of substrate hydrolyzed did not increase in proportion as the enzyme concentration increased nor as the substrate concentration increased. In other words, the reaction disagreed with the simple mass action theory in almost every respect and Bayliss concluded that the reaction could not be considered homogeneous, but that the results were due to an intermediate adsorption compound. Bayliss also showed, however, that the enzyme was inactivated during the reaction and was inhibited by the products formed. It was shown above that both of these reactions by themselves agree with the law of mass action. It is impossible, however, to introduce the necessary corrections into the formula owing to the complexity of the relations involved.

There is no doubt that the reaction takes place between the water, enzyme, and protein, so that, assuming one molecule to react, the equation governing the reaction must have the general form

$$\frac{dx}{dT} = K \text{ (substrate) (enzyme) (water)} \qquad (1)$$

in which x is the amount of substrate decomposed, T the time, and the terms in parentheses concentrations. By using dilute solutions, the water concentration may be considered constant and so disappears from the equation. If the enzyme is also considered constant, the ordinary monomolecular formula is obtained. It is easy to show experimentally, however, that the enzyme is not constant, due to two effects; first, it is continually undergoing spontaneous inactivation, the more rapidly the higher the temperature; and second, it is continually being inhibited by com-

bination with the products of hydrolysis. This effect will be less the greater the relative amount of enzyme compared to the products. There is another complication due to the fact that the digestion of protein is not one reaction but a series of consecutive reactions and there is every reason to suppose that these reactions occur at different rates. These complications cannot be corrected for mathematically but it is possible to choose experimental conditions under which they are reduced to a minimum. If the reaction is carried out at low temperatures, the

TABLE 6

Hydrolysis of casein

1 PER CENT CASEIN				3.0 PER CENT CASEIN		
TIME	$A - x$ N/50 protein N per 1 cc. Observed	$A - x$ N/50 protein N per 1 cc. Calculated	$KE = \frac{1}{T} \log \frac{A}{A - x}$	Time	$A - x$ N/50 protein N per 0.5 cc.	$KE = \frac{1}{T} \log \frac{A}{A - x}$
hours	*cc.*	*cc.*		*hours*	*cc.*	
0.01	5.68	5.68		0	8.60	
0.11	5.38	5.376	0.235	0.20	7.20	0.39
0.215	5.08	5.075	0.236	0.40	6.02	0.39
0.53	4.25	4.27	0.242	0.80	4.37	0.36
1.0	3.16	3.29	0.225	1.60	2.20	0.37
2.51	1.44	1.444	0.238	3.40	0.57	0.35
4.00	0.62	0.637	0.238			

inactivation of the enzyme is negligible. If a high concentration of enzyme is used, the inhibiting effect of the products is also very small, and if the first step in the reaction alone is followed, the effect of the consecutive reactions disappears. These conditions can be fulfilled by digesting casein at 0° with a large amount of trypsin and following the reaction by the disappearance of the protein as indicated by its precipitation with trichloroacetic acid.

The result of an experiment carried out under these conditions is shown in table 6. The figures show that the reaction follows

the monomolecular time curve with a considerable degree of accuracy. It furnishes very strong proof of the fact that the discrepancies ordinarily observed are due to the complications enumerated above and these have been experimentally eliminated in this case.

THE COURSE OF THE REACTION WITH CONSTANT SUBSTRATE UNDER CONDITIONS CAUSING INACTIVATION OF THE ENZYME

It was shown in the preceding section that if experimental conditions are so chosen as to avoid a decrease in the amount of active trypsin either by spontaneous inactivation or inhibition by the products of digestion, the reaction followed accurately the monomolecular formula. The present experiments were planned to show the course of the reaction when a single disturbing factor, the spontaneous inactivation of the enzyme, is at work. These conditions can be approximated by using a very concentrated solution of gelatin as substrate and carrying on the hydrolysis at 60°C., with a high concentration of trypsin. At this temperature, the enzyme is rapidly inactivated so that the reaction soon stops. The inhibiting effect of the products is reduced to a minimum since only a small amount of hydrolysis occurs and since a relatively large amount of trypsin is used. The percentage of the gelatin hydrolyzed is so small that the substrate concentration may be considered approximately constant.

The basic formula for the reaction is again

$$\frac{dx}{dT} = K \text{ (substrate) (enzyme) (water)}$$

Since under these conditions the substrate does not change significantly the substrate concentration may be included with the water concentration in the constant so that the formula can be written

$$\frac{dx}{dT} = K \text{ (enzyme)} \qquad (2)$$

Further, since the effect of the products on the enzyme is small, the enzyme concentration is decreasing as a function of time only. In order to integrate the formula it is necessary to know what this function is, but as a first approximation it is possible to compare the average reaction rate over a short time interval with the average amount of enzyme present during this interval, and which is determined in a separate sample. This procedure is, of course, not strictly correct, since it assumes that the reac-

TABLE 7

Relation between rate of reaction and trypsin concentration

ΔT = TIME INTERVAL $T_2 - T_1$.	Δx $x_2 - x_1$	$\frac{\Delta x}{\Delta T}$	$\frac{E_{T_1} + E_{T_2}}{2}$, AVERAGE TRYPSIN FOR CORRESPONDING INTERVAL	$\frac{\frac{\Delta x}{\Delta T}}{\text{TRYPSIN CONCENTRATION}}$
0 -0.05	0.60	12	2.2	5.5
0.05-0.10	0.35	7	1.47	4.7
0.10-0.15	0.25	5	1.15	4.3
0.15-0.20	0.20	4	0.90	4.5
0.20-0.30	0.27	2.7	0.65	4.2
0.30-0.40	0.18	1.8	0.45	4.0

tion velocity is constant over the interval. The formula for this purpose is

$$\frac{\Delta x}{\Delta T} = \frac{x_2 - x_1}{T_2 - T_1} = \frac{C\,(E_{T_1} + E_{T_2})}{2}$$

in which x_1 is the amount of hydrolysis at time, T_1; x_2 the amount at time, T_2; E_{T_1} the amount of the enzyme at time, T_1; and E_{T_2} the amount of enzyme at time, T_2. The amount of enzyme must be determined by an independent experiment. The value of

$$C = \frac{\frac{\Delta x}{\Delta T}}{\frac{(E_{T_1} + E_{T_2})}{2}}$$ should then be constant.

Table 7 shows that this is approximately true.

It was shown above that the inactivation of trypsin by heat

approximates a monomolecular reaction and that the discrepancies are due to the fact that more or less of the enzyme is combined with one of the hydrolysis products to form a compound which is more stable than the free enzyme. If it is assumed that the enzyme does decompose monomolecularly, the enzyme concentration at any time can be expressed as a function of time and equation (2) can be integrated. If E_o is the enzyme concentration at the beginning, the enzyme concentration at any time, T, is $E_o e^{-KT}$ in which e is the base of the natural logarithms. Equation (2) may therefore be written

$$\frac{dx}{dT} = C\,E_o e^{-KT}$$

which on integration becomes

$$x = \frac{C\,E_o}{K}\left(1 - e^{-KT}\right)$$

in which K is the constant for the decomposition of trypsin. Further,

when $T = \infty$

$$x = \frac{C\,E_o}{K} \tag{3}$$

That is, the total amount of hydrolysis will be directly proportional to the amount of enzyme—a result which is at first sight directly contradictory to that expected in any catalytic reaction.

Let the value of x at the end of the reaction $= A = \frac{CE_o}{K}$, then $x = A - Ae^{-KT}$ which is the ordinary monomolecular formula and may be written $K = \frac{1}{T}\,ln\,\frac{A}{A - x}$. K, however, is not the velocity constant for the hydrolysis of the gelatin but is the constant for the rate of inactivation of the enzyme, while A is not the total substrate present but the amount decomposed at the end of the reaction. The curves for the inactivation of the

trypsin and for the hydrolysis of the gelatin should, therefore, both be approximately monomolecular and should both give the same value for the constant, K. That this is true is shown in Table 8. The figures are as good as can be expected with the very short time intervals which must necessarily be employed. The gradual decrease is due to the protective action of the products of the reaction on the trypsin as explained above. The experiment shows that the decrease in rate of the reaction is simply due to the inactivation of the enzyme as was predicted from the theory and that if the enzyme could be kept constant also the time curve would be a straight line.

TABLE 8

Monomolecular constants for digestion and for inactivation of enzyme

TIME	K ENZYME	K DIGESTION
hours		
0.02	5.4	3.0
0.04	3.5	2.9
0.08	2.5	2.9
0.15	2.0	2.4
0.30	2.0	2.3
0.60	2.5	2.5

EFFECT OF VARYING THE INITIAL ENZYME CONCENTRATION

It was noted above that the velocity of the reaction depended solely on the amount of enzyme present at any time and that the constant for the reaction, therefore, represents the constant for the decomposition of the enzyme. If this is true the value of the constant should be independent of the concentration of enzyme, a result which is just the opposite of the usual catalytic reaction. In order to test this point the experiment was repeated with a series of different enzyme concentrations. The result is shown graphically in figures 9 and 10. In figure 10 the log of the amount of hydrolysis is plotted against the time so that if the reaction is monomolecular the resulting curves should be straight lines. If the velocity constants are the same, the lines

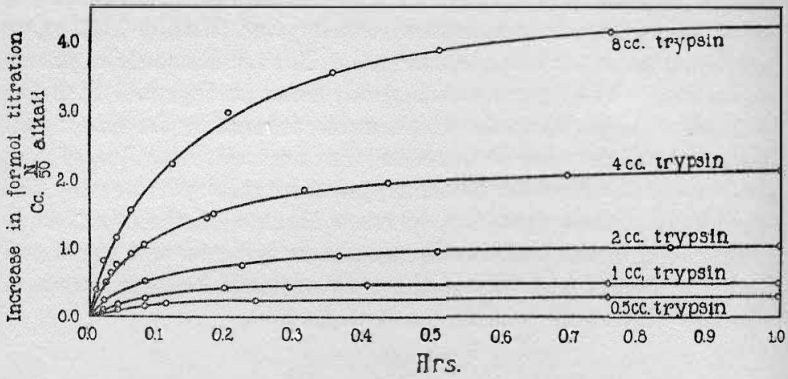

FIG. 9. HYDROLYSIS OF GELATIN WITH INCREASING CONCENTRATIONS OF TRYPSIN AT 60°C.

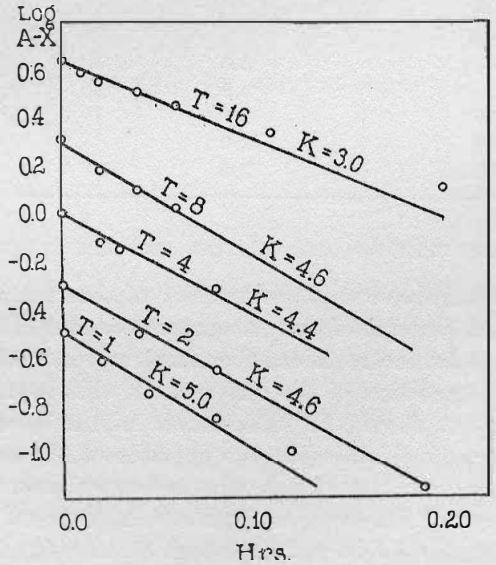

FIG. 10. HYDROLYSIS OF GELATIN WITH INCREASING CONCENTRATIONS OF TRYPSIN

should be parallel and the figure shows that this is approximately the case. In figure 9 the actual increases in the formol titration are plotted against the time. The experiment shows that at

TABLE 9

Varying concentrations of catalase

$H_2O_2 \doteq 112$ cc. O_2. (Morgulis, p. 368)

CATALASE	t	$A - x$	K	CATALASE	t	$A - x$	K
cc.	*minutes*	cc.		cc.	*minutes*	cc.	
6.75	0	113		3.75	0	71	
	5	56.4	0.060				
	10	33.0	0.053		10	23.9	0.047
	15	19.0	0.052				
	20	10.9	0.051		20	7.5	0.048
	25	6.7	0.050				
	30	4.2	0.047		30	1.8	0.053
	40	1.2	0.050		40	1.0	0.046
5.63	0	105		3.0	0	59	
	5	56.0	0.054				
	10	35.7	0.047		10	24.2	0.0387
	15	22.9	0.045				
	20	12.5	0.046		20	12.6	0.034
	25	7.5	0.045				
	30	4.0	0.047		30	5.8	0.033
	40	1.3	0.053		40	2.6	0.034
					45	1.8	0.034
4.5	0	89					
	5	51.6	0.046				
	10	33.9	0.042				
	15	21.6	0.041				
	20	12.9	0.042				
	25	8.3	0.041				
	30	5.3	0.041				
	35	3.2	0.040				
	45	1.0	0.043				

any time the amount of hydrolysis is proportional to the original enzyme concentration, while the time required to cause a constant amount of hydrolysis is not proportional to the enzyme

concentration, but decreases more rapidly than the enzyme increases. This result has been obtained in other instances and has been used as evidence of the heterogeneous nature of the reaction. These experiments show, however, that it is simply due to the fact that the enzyme is decreasing as a function of time, instead of as a function of x. The final value reached is also proportional to the original enzyme concentration. This is the result predicted by equation (3) and is exactly the opposite of the result ordinarily obtained in enzyme or catalytic reactions; namely, that the velocity constant is proportional to the concentration of catalyst while the total amount of hydrolysis is independent of the concentration of catalyst. In these experiments the velocity constant is independent of the amount of trypsin and the total amount of hydrolysis directly proportional to it. If this experiment had been performed alone without any further knowledge of the action of trypsin it would undoubtedly be cited as conclusive evidence that trypsin was not a catalyst at all but that the reaction was simply stoichiometric. Results similar to these in the case of catalase have been described by Morgulis. Morgulis' results can be satisfactorily calculated on the basis of the same mechanism as has been employed here.

The result of a recalculation of Morgulis' data on this basis are shown in table 9.

THE COURSE OF THE REACTION WHEN BOTH SUBSTRATE AND ENZYME ARE DECREASING

The preceding section showed that the reaction rate could be satisfactorily calculated when the enzyme concentration was decreasing due to spontaneous inactivation while the substrate concentration remained nearly constant. It is of interest to follow the course of the reaction with a lower substrate concentration so that this also is variable. These conditions can be fulfilled by using edestin as substrate at 40°C. This case had been discussed by Tammann.

Under these conditions the equation becomes

$$\frac{dx}{dt} = K\,(A - x)\,E_t$$

in which $A - x$ is the substrate concentration at the time, t, and E_t is the enzyme concentration at the time, t, instead of a constant as would be the case for a simple catalytic reaction. If it is assumed further as a first approximation that the enzyme is decomposing monomolecularly, then the enzyme concentration at any time will be $E_t = E_o e^{-Kt}$, E_o being the enzyme concentration at 0 time, K, the constant of inactivation of the enzyme, and e, the base of the natural logarithm. The equation for the rate of hydrolysis of the edestin, therefore, is

$$\frac{dx}{dt} = k_r (A - x) E_o e^{-Kt}$$

which on integration becomes (using logs to base 10)

$$k_r = \frac{K \log \left(\frac{A - x}{A} \right)}{E_o (10^{-Kt} - 1)} \qquad (4)$$

K in the equation is the constant of inactivation of the enzyme shown in table 10. It is therefore determined by an independent experiment and is not simply an arbitrary constant. The values for the reaction constant k_r are given in table 11. They are as satisfactory as could be expected when the approximate nature of the assumptions on which the equation is based are taken into account. The disturbing factor is the decomposition of the enzyme which does not follow the simple monomolecular reaction rate as assumed in deriving this equation. These irregularities can be accounted for as has been shown but cannot be taken into account mathematically in any simple way. If the substrate concentration is kept constant but the initial enzyme varied, there is no simple relation between either the amount of hydrolysis at a given time or the time required to form a given amount of hydrolysis. This is due to the fact that the rate of the reaction is a function of both time and of the value of x. It has been frequently pointed out that in comparing the two reactions it is necessary to make the comparison over corresponding parts of the curve. It is usually stated that the time required

to cause a certain effect is a better measure than the effect produced after a certain time. This is probably usually true but there is no reason to predict *a priori* that one method will be better

TABLE 10

TIME	$E = 1.2$ $K = \frac{1}{T} \log \frac{E_o}{E_t}$
hours	
0.5	0.158
1.0	0.165
2.0	0.150
3.0	0.130
4.0	[0.114]
6.0	[0.085]
Average K	$= 0.15$

TABLE 11

Value of $k_r = \frac{K}{E_o} \log \frac{\frac{A-x}{A}}{(10^{-Kt} - 1)}$

$K = 0.15$

$E = 1.2$

$A = 1.19$

$\frac{K}{E_o} = 0.125$

TIME	k_r
hours	
0.5	0.187
1.0	0.172
2.0	0.17
3.0	0.175
4.0	0.173
6.0	0.182

than the other. If the rate of the reaction is decreasing as a function of time then the comparison should be made at equal times. If it is decreasing as a function of the extent of the reaction then the comparison should be made at corresponding

stages of the reaction. Failure to give a simple ratio by either method, however, does not prove that the reaction is contrary to any simple mass action formula.

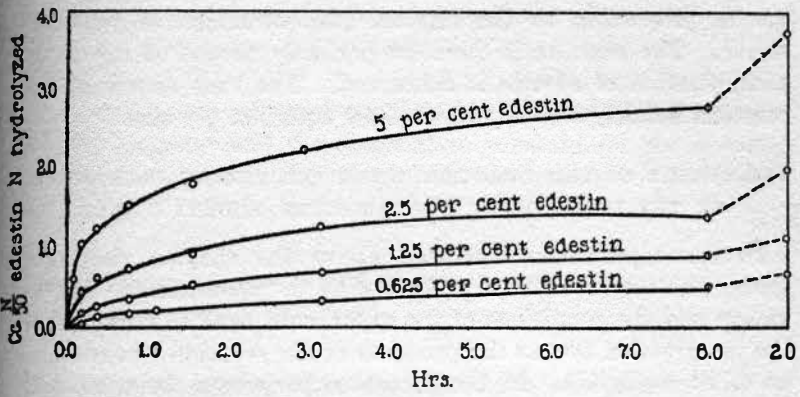

FIG. 11. RATE OF DIGESTION OF DIFFERENT CONCENTRATIONS OF EDESTIN WITH CONSTANT TRYPSIN CONCENTRATION

TABLE 12

Amount of edestin hydrolyzed at different times with varying initial concentrations of edestin and constant trypsin. Edestin hydrolyzed as cubic centimeter N/50N

TIME	INITIAL EDESTIN CONCENTRATION IN PER CENT			
	0.62	1.25	2.5	5.0
hours				
0.5	0.15	0.30	0.60	1.30
1.0	0.22	0.43	0.80	1.60
2.0	0.28	0.59	1.05	2.00
4.0	0.37	0.75	1.35	2.40
8.0	0.50	0.95	1.45	2.80
20.0	0.70	1.15	2.00	3.75

EFFECT OF VARYING THE INITIAL SUBSTRATE CONCENTRATION

Equation (4) predicts that if the substrate concentration is varied the amount decomposed at any time will be in proportion to the original concentration. There will be no simple relation

between the times required to cause any given amount of hydrolysis and the initial concentration.

The result of an experiment with different edestin concentrations is shown in figure 11 and table 12. The amounts hydrolyzed are in proportion to the original concentrations as predicted above. The reaction is therefore perfectly normal as far as the concentration of edestin is concerned. The time course of this reaction is fairly well represented by equation (4) also.

THE COURSE OF THE REACTION WHEN THE ENZYME IS INHIBITED BY THE PRODUCTS OF THE REACTION; SCHÜTZ'S RULE

In the experiments discussed above the enzyme decreased during the course of the reaction owing to "spontaneous" inactivation and the conditions of the experiment were so chosen that the inactivation due to the products of the reaction was so small as to be negligible. In the present experiments the conditions are such that the spontaneous inactivation of the enzyme is negligible but the inactivation by the products of the reaction is marked. These conditions are obtained by digesting casein at 0°C. with a small concentration of enzyme. Under these conditions the substrate concentration is decreasing owing to hydrolysis, and the active enzyme is decreasing owing to the combination with the products of hydrolysis. The mass action equation for the reaction is, therefore, again

$$\frac{dx}{dt} = KQ\,(A - x) \tag{5}$$

in which Q is also a function of x. It has been found above that the reaction between the enzyme and the products of hydrolysis is accurately expressed by the equation

$$\text{enzyme} + \text{inhibitor} \rightleftarrows \text{enzyme} - \text{inhibitor} \text{ or } \frac{Q \cdot (x - (E - Q))}{E - Q} = K$$

in which $E - Q$ is the combined, inactive enzyme. It was pointed out by Arrhenius that this equation could be written

$$Q = \frac{KE}{x}$$

as soon as x becomes large with respect to E. This is a property of any similar mass action system and was verified experimentally. If this value is substituted for Q, in equation (5), we obtain

$$\frac{dx}{dt} = K_A E \frac{A - x}{x} \tag{6}$$

in which K_A is a constant equal to the product of the reaction velocity constant and the dissociation constant for the enzyme-product compound. The integral of this expression (Arrhenius) is

$$K_A = \frac{A \ ln \frac{A}{A - x} - x}{ET}$$

This equation assumes that the reaction rate is proportional to the concentration of substrate and inversely proportional to the concentration of the products of the reaction. It evidently will not hold at the beginning of the reaction since the amount of enzyme is not inversely proportional to the products of the reaction until this value is large with respect to E.

If the substrate concentration is high there will be a period in the reaction during which it will not change markedly. If this also is considered constant, equation (6) becomes

$$\frac{dx}{dt} = K'_S \frac{E}{x}$$

or on integration

$$K'_S = \frac{x}{\sqrt{ET}}$$

which is Schütz's rule.

These equations were found by Arrhenius to agree quite well with Sjöqvist's results and the experiments were repeated and confirmed by the writer. In both cases the methods used to follow the digestion (conductivity or amino acid increase) are

open to the objection that the value A, the total substrate concentration, is rather uncertain, and also to the objection that the whole series of consecutive hydrolyses are followed. In the present experiments these points have been eliminated by following directly the disappearance of the protein.

According to Schütz's rule, the amount of protein hydrolyzed, plotted against the product of the square root of the enzyme

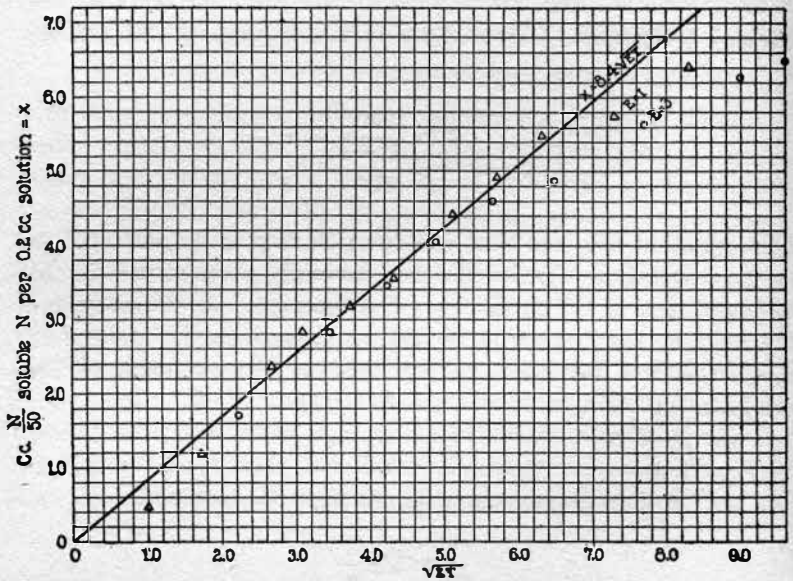

FIG. 12. RATE OF DIGESTION OF CASEIN SOLUTION PLOTTED AGAINST THE SQUARE ROOT OF THE ENZYME CONCENTRATION × THE TIME IN DAYS

concentration into the elapsed time, should be a straight line and the curves for various enzyme concentrations should be superimposable. Figure 12 shows that this is true over a part of the curve but that the observed points are below the line at the beginning and end of the experiment. The discrepancy at the beginning is due to the fact mentioned above that the square root relation does not hold until the products of the reaction are present in excess. The discrepancy at the end is due to the decrease in the substrate concentration which, in the derivation

of Schütz's rule, is assumed constant. It will be noted, however, that the relation holds over a much wider range than could be expected on the basis of constant substrate, since the equation holds fairly well over a range in which the substrate concentration decreases from 5 to 2. This result is due to the fact that the rate of hydrolysis is not proportional to the total substrate concentration but nearly independent of it when the casein is

TABLE 13

Comparison of Arrhenius' equation (K_A) and Schütz's Rule (K_S) applied to the digestion of casein by trypsin

$E = 3$				$E = 1$			
TIME	$A - x$	$K_S = \frac{x}{\sqrt{ET}}$	$K_A = \frac{A \ln \frac{A}{A-x} - x}{ET} \times 10$	TIME	$A - x$	$K_S = \frac{x}{\sqrt{ET}}$	$K_A = \frac{A \ln \frac{A}{A-x} - x}{ET} \times 10$
days				*days*			
0	7.60			0	7.60		
1	6.38	0.74	0.33	3	6.40	0.70	0.3
4	4.68	0.85	0.62	5	5.86	0.78	0.5
6	4.15	0.84	0.63	14	4.40	0.85	0.67
8	3.52	0.82	0.74	19	3.85	0.85	0.74
11	3.00	0.80	0.74	26	3.17	0.87	0.85
20	1.95	0.73	0.78	33	2.65	0.87	0.82
27	1.27	0.70	0.89	40	2.08	0.87	1.00
				54	1.65	0.80	1.00

over 1 per cent. The assumption used to derive Schütz's rule, that the rate of digestion is independent of the substrate concentration, is therefore experimentally correct over a rather wide range.

Arrhenius' equation, on the other hand, assumes that the rate is directly proportional to the substrate concentration. This is not experimentally the case, so that Arrhenius' correction is too

large and the constants for his equation shown in table 13 rise toward the end of the reaction. The experimental curve is therefore between the two theoretical curves, since the rate of digestion is not quite independent of the substrate concentration

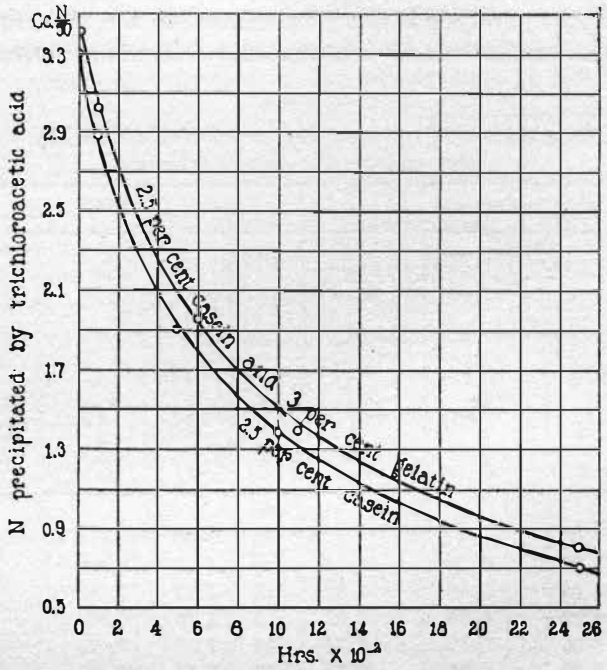

FIG. 13. THE RATE OF DIGESTION OF CASEIN ALONE AND IN THE PRESENCE OF 3 PER CENT GELATIN

Nitrogen precipitated by trichloroacetic acid (ordinates) is plotted against the time of reaction (abscissae).

nor is it directly proportional to it. Neither equation expresses accurately the effect of the substrate concentration on the rate of hydrolysis. Schütz's rule is better with high concentrations of substrate and Arrhenius' is better with low concentrations. A similar result was obtained by Dernby.

EVIDENCE CONCERNING THE EXISTENCE OF A COMPOUND BETWEEN ENZYME AND SUBSTRATE

It was mentioned that the rate of hydrolysis of casein by trypsin was not proportional to the casein concentration as it should be on the simple mass law assumption but that in concentrations of over 2 per cent the rate was nearly independent of the original casein concentration. This is a common result in enzyme reactions. Following the original suggestion of Brown and of Henri, it has been frequently assumed that this peculiarity was due to the existence of a compound between the enzyme and substrate. Michaelis and Menten, and Van Slyke have shown indeed that certain experiments can be calculated on this basis. The writer has found, however, that if both substrate and inhibitor concentrations are varied, results are obtained which cannot be calculated in this way. It has also been found that casein is digested just a rapidly in the presence of gelatin as when no gelatin is present. The result of such an experiment is shown in figure 13. It is evident that the enzyme cannot be combined with both the casein and gelatin at the same time and hence this experiment shows conclusively that the enzyme is not combined to any appreciable extent with the substrate, unless it be further assumed that there is a separate enzyme for each protein. No evidence could be found for this assumption and in addition it can be shown that the distribution of the enzyme between solid particles of protein and the surrounding solution is such as to preclude the existence of a compound between the enzyme and the protein. It may also be pointed out that the formulae which have been used, based on the assumption of a compound between enzyme and substrate, can be derived just as well by assuming an equilibrium between substrate and the water.

INFLUENCE OF THE pH ON THE RATE OF DIGESTION

The activity of all enzymes is markedly affected by the pH of the solution. It was pointed out by Michaelis that these curves were of the type of dissociation curves and he suggested that

enzymes were electrolytes and that the pH effect was due to the formation of enzyme ions. In the case of pepsin, however, the experiments did not bear out this assumption very well and it was further known that the pH optimum was different with different proteins. It was of interest, therefore, to compare the rate of digestion of the proteins with the titration curves of the

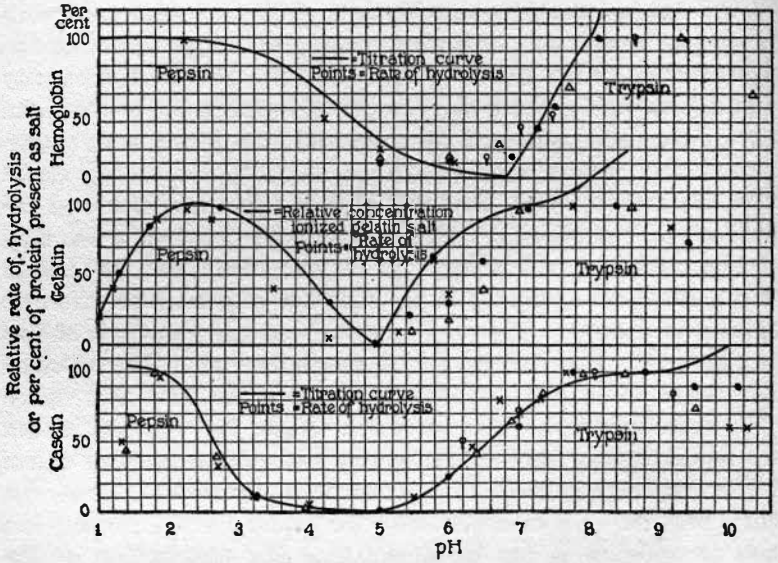

FIG. 14. COMPARISON OF TITRATION CURVES TO RATE OF DIGESTION OF VARIOUS PROTEINS

proteins themselves. The results of a series of experiments of this kind are shown in figure 14. There is evidently a close relationship between the concentration of ionized protein and the rate of digestion of the protein. The assumption that only the negative protein ions are attacked by trypsin and only the positive ions are attacked by pepsin will serve quite well, at least as a first approximation.

THE IONIC NATURE OF PEPSIN AND TRYPSIN

The ionization of a substance which may be prepared in any reasonable degree of purity may be easily shown by the conductivity method. In the case of substances, however, which cannot be isolated, the proof of the existence of ions is difficult. The method of migration in an electric field is uncertain, owing to the fact that particles of practically any material show movement under these conditions and that such movement does not necessarily indicate the existence of ions.

The theory of membrane equilibrium advanced by Donnan, however, predicts conditions which offer a criterion for the ionic nature of a substance. Donnan showed by thermodynamic reasoning that, if a solution containing a mixture of diffusible and non-diffusible ions was separated by a membrane from another solution containing only diffusible ions, the concentration of diffusible ions would be different on the two sides of the membrane. At equilibrium the product of the concentration for any one pair of oppositely charged diffusible ions of the same valence on one side of the membrane must be equal to the product of the concentrations of the same pair on the opposite side of the membrane. It follows that the ratio of the concentration of any pair of negative ions inside to that outside must be equal to the ratio of the concentrations of any pair of positive ions of the same valence outside to that inside the membrane.

Expressed mathematically, the equation is

$$(A_o^{-n})^{1/n}(B_o^{+m})^{1/m} = (A_i^{-n})^{1/n}(B_i^{+m})^{1/m}$$

or

$$\frac{(A_o^{-n})^{1/n}}{(A_i^{-n})^{1/n}} = \frac{(B_i^{+m})^{1/m}}{(B_o^{+m})^{1/m}} = \frac{(C_i^{+l})^{1/l}}{(C_o^{+l})^{1/l}} = \cdots\cdots$$

in which (A_i) is the concentration of an n valent negative ion inside the membrane, (A_o) is the concentration of the same ion outside. B, C, etc., are any other diffusible ions present, having the valence m, l, etc. In order to test the ionic nature of a substance therefore it is only necessary to set up such an equilib-

TABLE 14

Effect of pH on distribution of chloride ions and trypsin, inside and outside of gelatin particles

pH	2.0	2.5	3.0	3.5	4.0	4.7	5.5	6.0	6.5
Ratio: $\frac{Cl^-\text{ conc. liquid}}{Cl^-\text{ conc. gelatin}}$	0.60	0.40	0.24	0.13	0.38	1.0	1.3	1.6	2.0
Ratio: $\frac{\text{Trypsin conc. gelatin}}{\text{Trypsin conc. liquid}}$	0.50	0.30	0.23	0.17	0.35	1.5 11.0 1.6 20.0	1.5 4.0 6.0 1.2	—	1.4	2.5
pH	7.0	8.0	8.5	9.0	9.2	9.4	9.6	9.8	10.0
Ratio: $\frac{Cl^-\text{ conc. liquid}}{Cl^-\text{ conc. gelatin}}$	1.9	1.9	1.8	2.0	2.4	2.6	2.5	2.2	2.3
Ratio: $\frac{\text{Trypsin conc. gelatin}}{\text{Trypsin conc. liquid}}$	1.9	1.7	2.1	2.0	2.6	2.5	2.6	2.0	1.9
pH	10.2	10.4	10.6	10.8	11.0				
Ratio: $\frac{Cl^-\text{ gelatin}}{Cl^-\text{ liquid}}$	0.45	0.55	0.50	0.50	0.45				
Ratio: $\frac{\text{Trypsin gelatin}}{\text{Trypsin liquid}}$	1.0	0.38	0.43	0.43	0.30				

rium system, measure the ratio of the concentrations—on the two sides of the membrane—of some ion, such as hydrogen or chloride, and compare this ratio with the concentration ratio of the substance under investigation. The only difficulty lies in the fact that the equation predicts the concentration of the ions and not the total concentration, so that if the substance is not completely ionized or is combined in non-ionic form in the solution, the determinaton of the total concentration will not lead

TABLE 15

Comparison of Br or Cl and Pepsin Ratios at Various pH with H_2SO_4 and HCl

ACID	CONCENTRATION OF ACID	pH	RATIO: Br IN EGG ALBUMIN / Br IN FILTRATE	RATIO: PEPSIN IN EGG ALBUMIN / PEPSIN IN FILTRATE
	M			
	—	6.0	0.70	0.71
HCl	0.004	4.5	4.6	4.1
	0.008	4.2	10.0	7.2
	0.020	3.8	23.0	15.0
	0.04	3.3	36.0	22.0
H_2SO_4 + 0.02 KBr	0.002	5.0	2.5	2.0
	0.005	4.7	3.6	2.9
	0.010	4.4	4.3	4.6
	0.020	4.0	5.8	5.3
	0.040	2.8	7.8	7.7
	0.060	1.6	5.6	5.9
	0.100	1.0	4.2	4.6

to the correct ratio. In other words, if the experimental results do not agree with the ratio, the discrepancy may be due to complicating factors and no definite conclusion can be drawn, whereas if they do agree, the conclusion seems justified that the substance is ionic. Pepsin and trypsin have been studied under the above conditions, using small particles of gelatin or egg albumin to set up the equilibrium, and the relative concentration of the enzyme compared to those of the Cl or Br ion. The results of some of these experiments are shown in tables 14 and 15. They show

that trypsin behaves like a monovalent positive ion from pH 2.0 to 10.2. At this point it behaves as though it were un-ionized and on the alkaline side of 10.2 behaves like a monovalent negative ion. Pepsin behaves like a monovalent negative ion on the acid side of pH 6.0.

These results have a bearing on the kinetics of the reaction. They show that there is no change in the ionization of either enzyme over the range of pH in which they are active and also that there is no compound formed between these enzymes and either gelatin or egg albumin since the entire experiment rests on the assumption that the total amount of enzyme present in the protein is uncombined and active.

In reviewing these experiments the attempt has been made to show that pepsin and trypsin do not differ in their reactions from other better-known chemical substances. The complex and anomalous nature of the reactions as usually carried out are due not to any divergence from the classical laws of reactions but merely to the fact that there are several simultaneous reactions, each simple and each by itself following the predicted course. If experimental conditions are so chosen as to eliminate these side reactions the anomalies disappear. In respect to one other property at least it has also been shown that these enzymes do not differ from more simple compounds, since they distribute themselves in a system in which a Donnan equilibrium is set up in the same quantitative manner as do ordinary diffusible ions such as chloride or bromide.

LITERATURE

The present paper is a summary of investigations which have been presented in detail in a series of papers in the *Journal of General Physiology*, vols. i to vi. These papers contain the experimental details and references to the literature.