THE EFFECT OF THE CONCENTRATION OF ENZYME ON THE RATE OF DIGESTION OF PROTEINS BY PEPSIN.

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The study of the kinetics of enzyme action has led almost invariably to results differing more or less from those predicted by the general laws of chemistry. It would be expected from the general theory of chemical reactions that enzyme reactions should conform to the law expressing the rate of a monomolecular reaction, accelerated by the presence of a catalyst. The rate of reaction, therefore, should be proportional to the concentration of the enzyme and of the substrate and should decrease with time as predicted by the monomolecular formula. This has been found to be true in rare instances[1,2]; but in general the monomolecular formula does not hold for enzyme reactions. It has been found in many cases that the products of reaction interfere with the action of the enzyme. This would account for the divergence of the rate of reaction from that predicted by the monomolecular formula, since, owing to the action of the products, the concentration of the enzyme is changing during the course of the reaction, while the monomolecular formula takes account only of the changes in concentration of the substance decomposed. The rate of reaction of two solutions containing different amounts of enzyme, however, if compared during the same stage of the reaction, should be porportional to the quantity of enzyme, since any effect of the products should be the same in both solutions. It is found in many instances that this is not the case. Enzyme reactions diverge from the expected course of such reactions not only as regards the change in rate with the progress of the reaction, but also in regard to the relation between the rate and the concentration of substrate or enzyme.

[1] Euler, H., *Z. physiol. Chem.*, 1907, li, 213.

[2] Taylor, A. E., *J. Biol. Chem.*, 1906–07, ii, 87. Also Schmitz, H., *J. Gen. Physiol.*, 1919–20, ii, in press.

It was suggested by Brown[3] that these divergences in the case of invertase were due to the fact that the enzyme formed an intermediate compound with the substrate; and several formulas[4] which fit the experimental facts fairly well have been derived on this assumption. They all contain several arbitrary constants, however, and in the lack of any direct evidence in favor of the mechanism which they assume the agreement between calculated and observed values can hardly be considered conclusive. It is assumed in attempting to explain the mechanism of enzyme reactions from the point of view outlined above, that all the enzyme and all the substrate molecules present are equally able to take part in the reaction; in other words, that the active concentration and total concentration of enzyme (or substrate) are the same or directly porportional to each other. It is obvious that, if the active concentration of substrate or enzyme was not equal to the total concentration, the law of mass action would fail to hold if the total concentrations were used in formulas derived from this law, since the law itself states only that the rate of reaction is proportional to the active concentration of the reacting substances. It appears *a priori* quite possible that active enzyme or substrate molecules may exist in solution in equilibrium with other molecules which do not take part in the reaction. The concentration of active enzyme molecules (in the sense of the law of mass action, *i.e.* those which take part in the reaction) would then be some other function of the total concentration and would not be directly proportional to it. The rate of reaction would then also be found to vary as some other function of the total enzyme concentration and not in direct proportion to it. An exactly analogous case is well known in general chemistry; namely, acid hydrolysis.[5] The hydrogen ion is the active part

[3] Brown, A. J., *J. Chem. Soc.*, 1902, lxxxi, 373.

[4] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 146. These authors review the various other formulas proposed. See also Moore, B., in Hill, L., Recent advances in physiology and biochemistry, New York and London, 1906, 43.

[5] For a general discussion of this question see Stieglitz, J., and collaborators, *Am. Chem. J.*, 1908, xxxix, 29, 166, 402, 650. Stieglitz's experiments were made on the hydrolysis of esters. These solutions can hardly be considered heterogeneous and yet show the same divergences from the simple mass action law as do enzyme reactions. This question will be discussed more fully in a subsequent paper.

of the molecule and the rate of reaction therefore varies directly with the hydrogen ion concentration and not with the total acid concentration. In sufficiently dilute solutions the two of course become practically identical since the acid is then completely dissociated. It will be shown in the succeeding part of this paper that pepsin solutions obey the same laws as weak acid solutions in regard to the relation between the total concentration and the rate of hydrolysis; and that the divergence from the law of mass action is not due to any peculiarity of the enzyme reaction itself, but to the fact that the active enzyme concentration is not always directly proportional to the total enzyme concentration.

Experimental Procedure and Results of the Present Investigation.

In a former paper[6] a method was described for determining the rate of pepsin digestion by means of changes in the conductivity of an egg albumin solution to which the pepsin had been added. From these results the time necessary to cause a given change in the conductivity of the solution was determined by graphic interpolation. In the experiments reported in this paper the time in hours necessary to cause the first 10 per cent change was taken as the standard. The reciprocal of this time then $\left(\frac{1}{T \text{ hours}}\right)$ is proportional to the mean rate of digestion for the first 10 per cent of the reaction. For convenience this value will be spoken of as the amount of "active pepsin." The volume noted in the tables is considered in every case as the number of cc. of diluted enzyme solution containing 1 cc. of the original enzyme solution. It is therefore a measure of the dilution of the pepsin before adding to the egg albumin solution. Since 1 cc. of this diluted solution was added to 25 cc. of egg albumin in order to make a determination, the concentration of the pepsin during the actual digestion was $\frac{1}{26}$ of that shown in Tables I, II, III, and V. The conductivity and pH of all solutions were kept equal as nearly as possible. It was pointed out that this change in conductivity did not exactly parallel the change in amino nitrogen of the solution, and so cannot be considered as representing the true course

[6] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 113.

of the reaction. If the amount of egg albumin and all other factors except the amount of pepsin are kept equal, however, the time necessary to cause a given change may be considered as a definite measure of the rate of reaction, which is all that is necessary for the present purpose.

It was stated[6] that the rate of reaction (*i.e.* the reciprocal of the time to cause a given change) was directly proportional to the concentration of enzyme solution, and that any products of reaction present in the enzyme solution did not interfere with the reaction. Both statements were true as regards the pepsin solutions used in the experiments reported. It was found, however, that some pepsin solutions did not obey this law. The rate of digestion, instead of being directly proportional to the enzyme concentration, increased much more slowly. The same phenomena have been observed by Bayliss in the case of trypsin[7] and invertase,[8] and have frequently been observed in enzyme reactions. It has formed one of the arguments for the conception that the enzyme combines with the substrate according to the adsorption formula.[8,9]

Table I is a summary of an experiment illustrating this point. The results are shown graphically in Fig. 1. It is obvious that the value of ET (total pepsin concentration $\times$ the time necessary to cause 10 per cent of the total change in conductivity) is constant for low concentrations but increases in higher concentrations. (If the rate of reaction is directly proportional to the enzyme concentration, the value of ET must of course be constant.) The calculated figures were obtained by a formula considered below. The key to this behavior is given by the results of the experiments shown in Table II. In this experiment 2.5 cc. of an active pepsin preparation were diluted to 10 cc., A, with HCL (pH 2.0) and, B, with a solution of "peptone"[10] prepared by the digestion of egg albumin by a very small amount of pepsin (but containing no active pepsin). Solutions A and B were

[7] Bayliss, W. M., *Arch. Sc. Biol.*, 1904, ii, suppl., 261.

[8] Bayliss, W. M., *Proc. Roy. Soc. London, Series B*, 1911–12, lxxxiv, 90. Duclaux, E., Chimie Biologique, Paris, 1883.

[9] Bayliss, W. M., The nature of enzyme action, Monograph on Biochemistry, London, New York, Bombay, and Calcutta, 3rd edition, 1914.

[10] The word peptone is used in this paper as a general term for substances with which pepsin combines in solution, but does not hydrolyze.

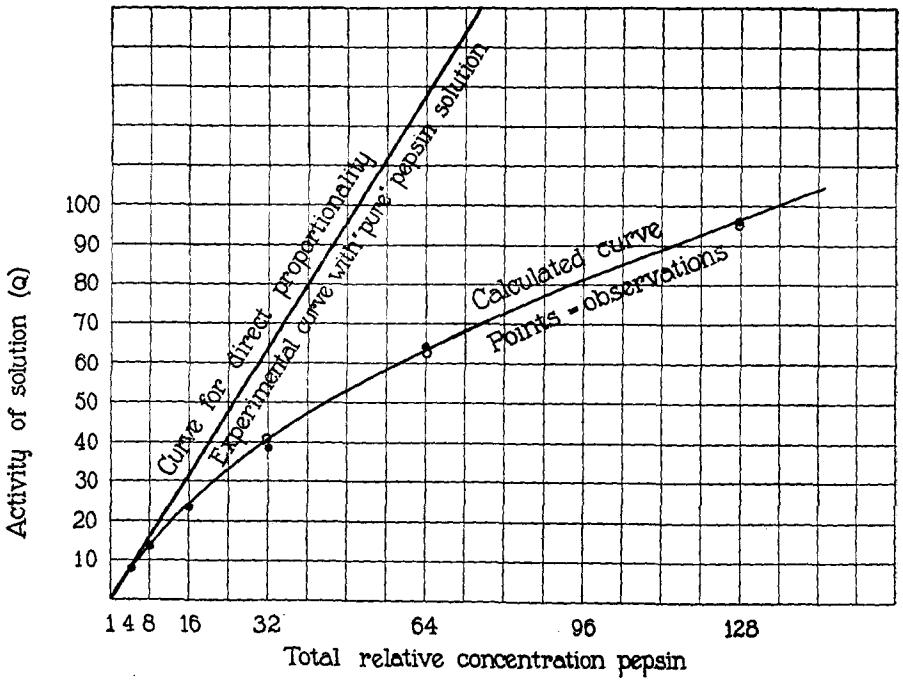

FIG. 1. Curves showing pepsin concentration and rate of digestion (*cf.* Table I).

TABLE I.

Enzyme Concentration and Rate of Digestion.

Pepsin solution. 10 per cent solution of Grübler's pepsin in HCl, pH 2.0.

$$K = 7.2 \qquad d = \frac{30}{v}$$

V = volume containing 1 cc. of original pepsin solution.	E = total pepsin per cc.	$Q = \frac{1}{T}$ = active pepsin per cc.					ET
		Observed.				Calculated.	
		1	2	3	Average.		
1	26.9	9.1	9.7	10.0	9.6	9.7	269
2	13.44	6.25	6.30	6.67	6.39	6.40	206
4	6.72	4.17	3.70	3.57	3.81	4.05	175
8	3.36	2.38	2.50	2.17	2.35	2.42	145
16	1.68	1.39	1.43	1.35	1.39	1.38	120
32	0.84	0.83	0.80	0.78	0.80	0.77	106
64	0.42	0.41	0.40	0.39	0.40	0.40	100
128	[0.21]	0.22	0.20	0.20	0.21	0.20	100

then diluted as shown in the table with HCl while Solution C was diluted with Solution B in which the pepsin had been inactivated by making the solution alkaline for 10 minutes. With Solutions A and C the product of the time into the amount of pepsin present is constant as required by the law of mass action, while in Solution B the value

TABLE II.

Effect of Addition of Peptone to Pepsin Solutions.

Solution A. 2.5 cc. of 2 per cent active pepsin diluted to 10 cc. + HCl, pH 2.0.

Solution B. 2.5 cc. of 2 per cent active pepsin diluted to 10 cc. + 1 per cent peptone solution, pH 2.0.

Solutions A and B then diluted as noted + HCl. Pepsin determined in 1 cc.

Solution C. Same as B except diluted with inactivated B, instead of HCl.

V = volume containing 1 cc. of original pepsin solution.	E = relative concentration of total pepsin taken.	Time for 10 per cent change in conductivity of 25 cc. of egg albumin + 1 cc. of solution. T = hrs. × (10^2).			ET for solution.		
		A	B	C	A	B	C
1.0	100	20	28	21	20.0	28.0	21.0
1.5	66	31	34	29	20.5	22.5	19.1
2.0	50	40	39	41	20.0	19.5	20.5
4.0	25	83	79	81	20.7	19.8	20.3
8.0	12.5	170	162	178	21.2	20.3	22.2

of the product decreases with increasing dilution until it becomes equal to that value obtained from Solutions A and B, and then remains constant.[11] The results are plotted in Fig. 2. The straight line represents direct proportionality.

The solutions were made up to contain the same total concentration of pepsin and in the higher dilutions show the same degree of activity. It seems, therefore, that the divergence of Solution B from the regular law must be due to the fact that the peptone combines with the pep-

[11] This experiment is probably the explanation of the conflicting results obtained by Bayliss[8] and Nelson and Vosburgh (Nelson, J. M., and Vosburgh, W. C., *J. Am. Chem. Soc.*, 1917, xxxix, 790) in connection with the action of invertase. The activity of the solution of invertase used by Bayliss was not proportional to its concentration whereas the activity of that used by Nelson and Vosburgh was directly proportional.

sin to form a rather highly dissociated compound and that the pepsin so combined is inactive. The concentration of active pepsin would therefore be decreased by the peptone and the decrease would be greater in concentrated than in dilute solution. This hypothesis also accounts for the results of Experiment C in which the solution is diluted with an inactivated portion of the same solution. If the inactivated pepsin enters into equilibrium in the same way as the

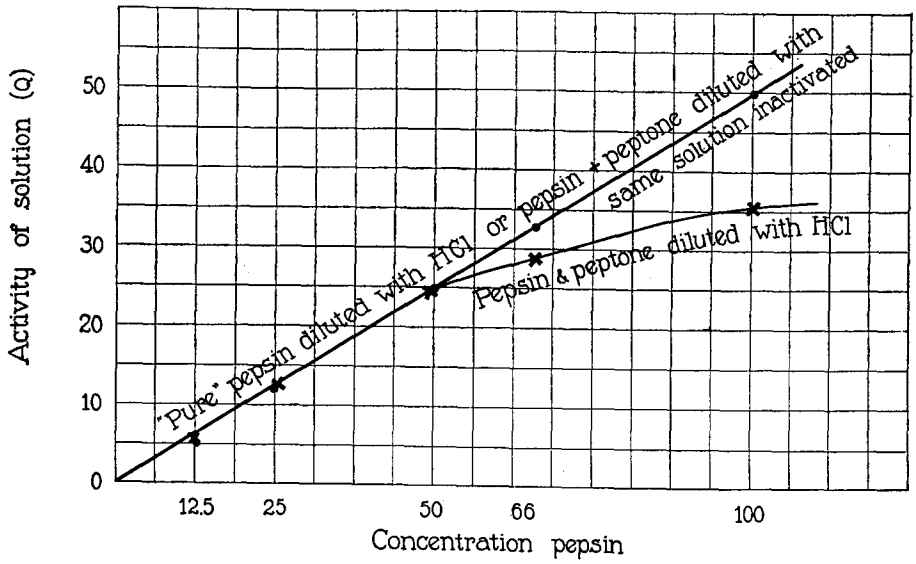

FIG. 2. Curves showing effect of peptone on activity of diluted pepsin solutions (*cf.* Table II).

active pepsin, the concentration of active pepsin in a solution, diluted with an inactivated portion, should decrease in direct proportion to the total concentration. The experiment shows that this is the case. (This question will be taken up more fully later.) The results of this experiment show also that in order to determine the total amount of pepsin present in solution it is necessary to use a dilution such that the rate of digestion is directly proportional to the amount of enzyme solution taken. If this is done the value for the total amount of enzyme, found at dilutions where this value has become constant, is an experimental determination of the total amount of enzyme present, expressed, however, in arbitrary units.

The effect of the peptone in Solution B might be qualitatively explained by the hypothesis that the peptone in the solution combines with the substrate and so reduces the concentration of active substrate molecules, thereby causing the enzyme to become "saturated" with substrate. This explanation, however, fails to explain the results of Experiment C since the same concentration of peptone is present here as in Solution B and yet in this experiment the rate *is* proportional to the amount of enzyme taken.

According to the hypotheses outlined above, the rate of digestion is always directly proportional to the concentration of active pepsin; and the apparent divergence from this relation is due to the fact that the peptone combines with the pepsin and so renders it inactive. The total concentration of enzyme and the active concentration are then no longer equal nor directly proportional; and since the rate is proportional to the active concentration, it is not proportional to the total concentration. It is also assumed that the pepsin and peptone combine according to the law of mass action. This reaction may be considered to take place as follows:

$$\text{Pepsin} + \text{peptone} \rightleftharpoons \text{pepsin}-\text{peptone}$$

and if the reaction obeys the law of mass action the following equation must hold.

$$\frac{\text{Concentration pepsin} \times \text{concentration peptone}}{\text{Concentration pepsin-peptone}} = K \quad (1)$$

or

$$\frac{Q \cdot (d - (E - Q) + x)}{E - Q} = K \quad (2)$$

where E is the total enzyme concentration, Q is the concentration of active (uncombined) pepsin, d is the concentration of peptone present at the beginning of the reaction, and x is the amount of peptone formed during the course of the reaction at the time t. K is the equilibrium constant expressed in arbitrary units since it contains the unit of measurement used. (For the sake of simplicity only the case is considered in which the substance combined with the pepsin at the beginning of the reaction is the same as that formed during the

digestion.) The value of Q then (the active pepsin concentration) at any moment of the reaction would be that defined by equation (2) or

$$Q = -\frac{d - E + x + K}{2} + \sqrt{\left(\frac{d - E + x + K}{2}\right)^2 + KE} \quad (3)$$

The differential equation for the whole process[12] would then be

$$\frac{dx}{dt} = kQ(A - x) \quad (4)$$

in which Q has the value expressed in equation (3), and A is the active concentration of substrate present at the beginning of the reaction. (It seems quite probable that the active substrate concentration is related to the total substrate concentration in the same way as the active and total enzyme concentrations are related. This question will be discussed later. For the present it is assumed that the rate is proportional to the substrate concentration. At low dilutions of substrate this is an experimental fact.) If this value for Q is substituted in equation (4) it becomes too unwieldy in the integral form to use conveniently. The equation may be tested in the differential form, however, by choosing a small constant value for Δx (taken as 10 per cent of the total change in these experiments) and determining Δt experimentally. The reciprocal of the time necessary to cause the change will then be proportional to the mean rate of reaction during the first 10 per cent of the hydrolysis. This rate is of course decreasing constantly due (1) to the decrease in substrate concentration, and (2) to the decrease in the concentration of active pepsin since some pepsin is removed by combination with the products of reaction. The relative decrease in Q due to (1) is the same in every case and cancels out in comparative experiments, such as are considered here, since the total substrate concentration is kept the same in every experiment. The relative decrease in rate (Q) due to (2), however (as may be seen from equation (2)), will not always be the same but will depend to some extent on the relative values of E, d, and x. It will be shown later that a 5 per cent egg albumin solution when completely digested contains about 10 units of peptone (Table III). The

[12] Neglecting any effect of the reverse reaction.

value of x therefore in the first 10 per cent of the reaction will increase from 0 to 1.0. The percentage decrease in Q (the concentration of active pepsin) will depend to some extent on the concentration of peptone (d) present at the beginning of the reaction. That this is actually so is shown by the fact that the relative rates of digestion of two solutions containing the same amount of pepsin but very

TABLE III.

Enzyme Concentration and Rate of Digestion.

Pepsin solution. 1 per cent active pepsin + 10 per cent egg albumin, pH 2.0. Digested 24 hrs. at 37°C. Diluted as below + HCl, pH 2.0.

$$K = 8.5 \qquad d = \frac{19.2}{v}$$

V = volume containing 1 cc. of pepsin solution.	E = total pepsin.	$Q = \frac{1}{T}$ = active pepsin.			
		Observed.			Calculated.
		1	2	Average.	
1.0	10.95	4.35	4.24	4.29	4.40
1.18	9.32	4.17	4.05	4.11	4.08
1.43	7.66	3.84	3.57	3.70	3.71
1.66	6.58	3.45	3.50	3.47	3.39
2.0	5.48	3.03	2.98	3.00	3.06
2.5	4.39	2.94	2.78	2.86	2.67
3.33	3.28	2.17	2.38	2.27	2.17
5.0	2.18	1.75	1.50	1.62	1.61
10.0	1.09	1.0	0.98	0.99	1.05
20.0	0.54	0.57	0.52	0.55	0.52

different amounts of peptone vary, depending on what stage of the reaction is compared. This is due to the fact that the rate of digestion of the solution containing the peptone decreases more slowly than that of the solution containing no peptone. This is in agreement with the formula. The differences in the percentage decrease in the rates of digestion of two solutions during the first 10 per cent hydrolysis, due to variations in the relative values of E and d, were found to be too small to effect the results within the range of values of E and d used in these experiments. The relative mean rate for the first 10 per cent hydrolysis may therefore be considered proportional

to the amount of active pepsin present at the beginning of the reaction; *i.e.*,

$$\text{Rate} = \frac{1}{T} = Q = -\frac{d-E+K}{2} + \sqrt{\left(\frac{d-E+K}{2}\right)^2 + KE}$$

where T is the time in hours necessary to complete the first 10 per cent of digestion. This equation may be tested experimentally by testing the constancy of K for various values of E and d or, better, by comparing calculated and observed values of $\frac{1}{T}$ since small experimental errors cause very large changes in the value of K.

The results of such a series of experiments have been given in Table I. Table III contains the results of a similar experiment in which the pepsin solution was prepared by adding 10 per cent of egg albumin to an active pepsin solution and allowing digestion to be completed at a temperature of 38°C. The solution was then diluted as shown in Table III. As was the case in Experiment 1, the rate of digestion is not directly proportional to the total enzyme concentration. It will be seen that in both Tables I and III the agreement between calculated and observed values is within the experimental error. The figure for E, the total enzyme present, is determined directly from the experiments in high dilution when the value of ET has become constant. It was shown in Experiment 2 that the value for E obtained in this way was really proportional to the total amount of enzyme present. The value for d, the amount of peptone present at the beginning of the reaction, is determined from the figures themselves and therefore must be considered as a second arbitrary constant. This fact, of course, detracts considerably from the significance to be attached to the agreement between the observed and calculated values. It will be shown below, however, that under certain conditions the formula may be still further simplified so as to contain one constant and that it is still found to hold.

Table IV contains a summary of an experiment in which the total concentration of peptone was kept the same and the concentration of pepsin increased. The results are shown graphically in Fig. 3. The values for E, the total pepsin present in the solution of Grübler's pepsin, K, the equilibrium constant, and d, the concentration of pep-

tone originally present, were taken from Table I. The value for E in Solution B was determined by a separate experiment. It will be seen that the total amount of active pepsin found in the solution is not equal to the sum of the amount of active pepsin added plus the amount of active pepsin already present. This shows that the pepsin

TABLE IV.

Addition of Active Pepsin Solution to Solution of Grübler's Pepsin.

Solution A. 10 per cent Grübler's pepsin, pH 2.0.

Solution B. 3 per cent active pepsin, $E = 4.2$.

$K = 7.2$, $d = 3.0$, E (in Solution A) = 2.69 (Table I). 1 cc. of Solution A + noted cc. of B made up to 10 cc. $K = 7.2$.

Volume of Solution B added.	Units of active pepsin added per cc.	Units of active pepsin in Solution A per cc.	$Q = \frac{1}{T}$ = total units of active pepsin.	
			Found.	Calculated.
cc.				
0	0	[2.08]	2.08	2.03
1	0.42	2.08	2.32	2.27
2	0.84	2.08	2.83	2.71
3	1.26	2.08	3.12	3.05
5	2.10	2.08	3.84	3.77
7	2.94	2.08	4.44	4.50
9	3.78	2.08	5.20	5.26

is in equilibrium with the substance that inhibits its action. The fact that the calculated values agree with those found by experiment shows that the equilibrium obeys the law of mass action since the calculated figures are obtained by means of this law.

The Effect of Inactivated Pepsin on the Equilibrium.

The results of Experiment 2 show that if a solution of pepsin (A) containing peptone is diluted with acid, the activity of the resulting solution *is not* directly proportional to the concentration of A. If the same solution is diluted with a portion of itself in which the pepsin has been inactivated with alkali, the activity of the resulting solution *is* directly proportional to the concentration of A. This is the result predicted if it is assumed that the inactive pepsin enters into the equilibrium (*i.e.* combines with the peptone) to the same extent as

the active pepsin. Table V summarizes the result of an experiment similar to Experiment 2 but covering a wider range. In this experiment an impure solution of pepsin (the same as used in Experiment 1) was diluted, A, with acid of the same hydrogen ion concentration, and, B, with the same solution which had been previously inactivated by alkali and then brought back to the same pH as the original. The

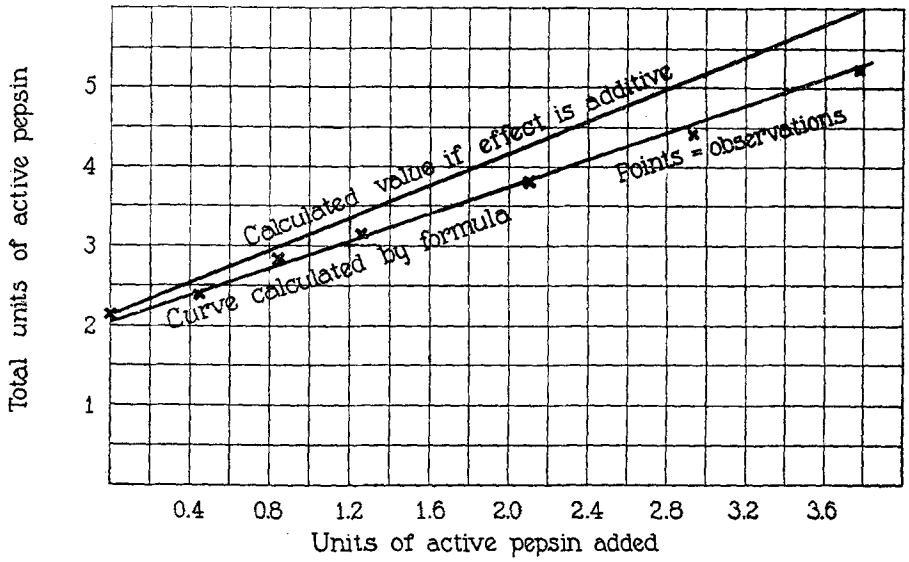

FIG. 3. Curves showing effect of addition of "pure" pepsin to pepsin solution containing peptone (*cf.* Table IV).

activity of the solution diluted with acid is not directly proportional to its concentration. When the same solution is diluted with an inactivated portion of itself, the activity of the resultant solution is directly proportional to its concentration. The figures show again that the result is predicted quantitatively by the hypothesis. Pepsin inactivated by alkali therefore retains the ability to combine with peptone exactly as does the active pepsin. It has, however, lost the power to hydrolyze protein. A very similar phenomenon is known in immunology—the so called toxoids; *i.e.*, toxins which are no longer injurious but are able to bind antibody in the same way as true toxin.

If the pepsin is inactivated by boiling instead of by treatment with alkali the results become irregular and do not agree with the hypothesis that the inactivated pepsin either does or does not enter into the equilibrium.[13] In order to predict them quantitatively it becomes necessary to assume that either the equilibrium constant is changed

TABLE V.

Influence of Inactivated Pepsin on Equilibrium.

10 per cent Grübler's pepsin diluted as noted with, A, HCl, pH 2.0. B, with same solution inactivated by alkali.

V = volume containing 1 cc. of original pepsin solution.	$Q = \frac{1}{T}$ = active pepsin observed in solution.		Calculated.	
	A	B	If inactive pepsin enters equilibrium.	If inactive pepsin does not enter equilibrium.
1	9.6	9.5	9.6	9.6
2	6.39	5.0	4.8	3.6
4	3.81	2.48	2.40	1.4
8	2.35	1.33	1.20	0.8
16	1.39	0.70	0.60	0.4

or that some of the peptone also is destroyed. In any case boiling causes a different change in the properties of a pepsin solution from inactivation with alkali.

Effect of Adding Increasing Amounts of Peptone to Pepsin Solutions.

It is possible to test further the hypothesis outlined in this paper by noting the effect of adding different amounts of peptone to a constant quantity of pepsin and comparing the observed and calculated activity of the resultant solution. If, as assumed in the hypothesis, the pepsin combines with the peptone to form a dissociated compound, the effect of adding successive equal amounts of peptone to a constant quantity of pepsin should not result in a constant decrease in activity of the solution for each unit of peptone added. The first unit of

[13] An apparently similar phenomenon was noticed by Bayliss[7] in his experiments with trypsin.

TABLE VI.

Effect of Adding Increasing Amounts of Peptone to Pepsin Solution.

0.5 cc. of 5 per cent active pepsin solution + noted cc. of peptone solution (from digested egg albumin) and made up to 10 cc. K = 8.5 (Table III), E = 3.16, d = 0.85 per cc. of peptone solution.

Peptone solution.	d = units of peptone added.	$Q = \frac{1}{T}$ = units of active pepsin.			Units of combined pepsin per unit of peptone added. Observed.	Qd
		Observed.	Average.	Calculated.		
cc.						
0	0	3.03 3.14 3.33	3.16			
1	0.85	2.86 2.86 2.94	2.88	2.92	0.33	2.4
2	1.7	2.70 2.70 2.86	2.75	2.75	0.24	4.6
4	3.4	2.38 2.40 2.50	2.42	2.42	0.22	8.2
6	5.1	2.08 2.04 1.96	2.03	2.13	0.22	10.0
8	6.8	1.67 1.85 1.92	1.81	1.91	0.20	12.0

peptone added should have a greater effect than the second, the second a greater than the third, and so on; the relative decrease of the effect depending on the value of the equilibrium constant. Table VI and Fig. 4 give the result of an experiment carried out in this way. It will be seen that the effect of adding increasing units of peptone agrees very well with the calculated values. The compound pepsin-peptone is widely dissociated at this dilution inasmuch as with a total concentration of 0.85 units of peptone and 3.16 units of pepsin only 0.28 units are combined. This fact is shown graphically in

Fig. 4 where the straight line represents the concentration of active pepsin which would be present if the combination was complete. Table VI also shows that the first unit of peptone inactivates more pepsin than the second, etc. This phenomenon is also common in immunology and is known as Ehrlich's phenomenon. As Arrhenius[14] has pointed out it is a general property of any equilibrium system.

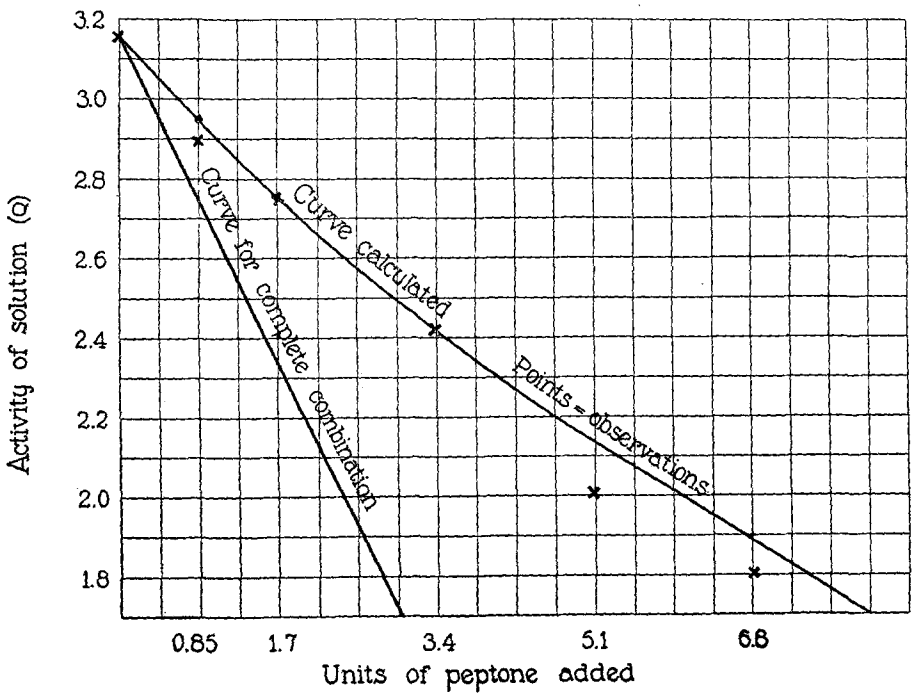

FIG. 4. Curves showing effect of adding increasing amounts of peptone to pepsin solutions (*cf.* Table VI).

In several other respects the action of pepsin on an egg albumin solution is more or less analogous to the action of toxin on an organism. In a sense the pepsin may be said to make the egg albumin solution immune to pepsin. That is, if a small amount of pepsin is allowed to act for a long time on a large quantity of albumin it will at first digest it very rapidly and the rate of digestion will be proportional to the amount of pepsin added. The rate of digestion decreases rapidly,

[14] Arrhenius, S., *Ergebn. Physiol.*, 1908, vii, 480.

however, and finally becomes almost negligible in spite of the fact that there is still a large amount of egg albumin in solution and that the pepsin still retains its activity (as may be demonstrated by diluting the solution, after which digestion will continue). The addition of a further amount of pepsin to the solution will now have little or no effect. The albumin solution is "immune" to the pepsin. This is due to the fact that a small amount of pepsin can cause the production of a very large amount of peptone. Each unit of peptone produced decreases the amount of free pepsin somewhat; but as may be seen from equation (2) it would require an infinite concentration of peptone (d) to reduce the concentration of free pepsin (Q) to 0. Practically, the reaction stops owing to the destruction of the pepsin.[15]

Referring again to Table VI, it will be noted that Qd, the product of the concentration of active enzyme into the concentration of peptone, approaches a constant value as d increases. In other words the concentration of active enzyme becomes nearly inversely proportional to the concentration of peptone, when the latter is present in great excess. This is a well known property of mass action equilibria and follows from the formula, as may be seen below. The formula used in this connection is

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

where Q is the concentration of active (free) enzyme, $[d-(E-Q)]$ the concentration of free peptone, and $E-Q$ the concentration of combined pepsin or peptone. It is obvious that as d increases Q must decrease so that the value of the term $E-Q$ approaches the constant value E. When d becomes very large compared with E the term $d-(E-Q)$ will not differ significantly from d. The equation may then be written

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

d in this equation represents the amount of peptone present at the beginning of the reaction. If the equation is to hold throughout the reaction the concentration of peptone will be represented by $d+x$.

[15] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 465.

If the simplest case is considered in which there is no peptone present at the beginning of the reaction the concentration of peptone at any time is x (since d is 0) and the formula becomes

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

This value for Q may now be substituted in equation (4) which becomes

$$\frac{dx}{dt} = \frac{KE(A - x)}{x} \quad (5)$$

in which A is the concentration of substrate at the beginning of the reaction and x is the amount of substrate decomposed (or of peptone formed) at the time T. For the first part of the reaction the value of $(A-x)$ will not differ much from the value of A and the equation may be still further simplified to

$$\frac{dx}{dt} = \frac{KEA}{x}$$

which states that the rate of digestion at any moment is directly proportional to the enzyme concentration and the substrate concentration, and inversely proportional to the amount of substrate decomposed. K in this equation is a new constant equal to the product of k, the velocity constant, and K (equation (4)), the equilibrium constant. This equation, as has been pointed out by Arrhenius,[16] is the differential form of Schütz's[17] rule, since on integration it becomes

$$TKEA = x^2 \quad \text{or} \quad x = K\sqrt{TEA}$$

That is x, the quantity of peptone formed, is proportional to the square root of the time, the concentration of pepsin, and the con-

[16] Arrhenius, S., *Medd. Kong. vetsakad. Nobelinst.*, 1908, i. An equation similar to this but containing $x\frac{1}{2}$ was found by Bodenstein and Fink (Bodenstein, M., and Fink, C. G., *Z. physik. Chem.*, 1907, lx, 1) to represent the rate of oxidation of SO_2 in the presence of platinum. Dernby, K. G., *Z. physiol. Chem.*, 1914, lxxxix, 425.

[17] Schütz, E., *Z. physiol. Chem.*, 1885, ix, 577.

centration of substrate. It follows from this that if two solutions are compared, each containing the same quantity of substrate, and allowed to digest the same length of time, but with varying concentrations of enzyme, the amount of substrate digested will be proportional to the square root of the enzyme concentration. This is the usual form of Schütz's rule.

It will be remembered that in the derivation of this equation two simplifying assumptions were made: (1) that x, the concentration of peptone, is large compared to Q, the concentration of active pepsin; and (2) that the quantity of substrate present remains relatively constant. The first condition is fulfilled as soon as the digestion has progressed more than a few per cent, provided the original concentration of pepsin is small compared to the concentration of albumin. The second condition, on the other hand, fails to hold after more than 30 or 40 per cent of the substrate is digested. It can be predicted then that Schütz's rule will not hold during the first few minutes of the reaction, or at the end of the reaction, or if the enzyme concentration is too high. As is well known, this is exactly the result obtained by experiment (*cf.* Arrhenius[16]).

The failure of the rule to hold during the first part of the digestion is due to the fact that x at this time is not large compared with Q and hence the relative change in Q is not inversely proportional to the change in x (as assumed in the derivation of the equation) but is much slower as demanded by equation (2). In order to express the fact correctly for the first part of the reaction, then, it would be necessary to substitute for Q in equation (4) the value of Q as defined by equation (3). As has been previously stated, this expression is too unwieldy to handle conveniently. The discrepancy due to changes in the substrate concentration, however, may be corrected very simply if the rate of digestion is directly proportional to the concentration of substrate when the concentration of the latter is low. Experiment shows that this is actually the case. (The effect of the substrate concentration is at present under investigation.) The active concentration of substrate at any moment then will be $A-x$, where A is the original total concentration of substrate and x is the amount transformed. This has already been done in equation (5)

$$\frac{dx}{dt} = \frac{KE(A - x)}{x}$$

which on integration becomes

$$\frac{A \ln \frac{A}{A - x} - x}{ET} = K \quad (6)$$

If the foregoing hypothesis correctly expresses the mechanism of the reaction, the results calculated from Schütz's rule and equation (6) should agree with the experimental results as soon as x has reached a value ten or fifteen times as large as the quantity of active pepsin present. Before x has reached such a value, the results calculated from equation (6) or Schütz's rule, using the values of K at which they are constant, will be higher than those found by experiment. That is, the value of K in Schütz's rule or equation (6) increases for the first 10 or 20 per cent of the total digestion. As was pointed out above, this discrepancy is due to the fact that the formulas are derived on the assumption that the relative change in the pepsin concentration is inversely proportional to the change in the peptone concentration, a condition which does not hold until the peptone is present in large excess. After this point is reached both equations should correctly represent the course of the reaction until the changes in substrate become large. After this change in substrate concentration becomes significant Schütz's rule will no longer hold since there is no term in it that provides for the change in substrate concentration.[18] Equation (6) should hold (*i.e.* give a constant value for K) until the end of the reaction, since this equation takes account of the changes in substrate concentration. Table VII and Fig. 5 give the results of an experiment in which the rate of hydrolysis of an egg

[18] If the substrate concentration is high (*i.e.* more than 1 to 2 per cent) Schütz's rule will be found to hold throughout the greater part of the reaction. This is due to the fact that the rate of digestion in concentrated solutions is nearly independent of the substrate concentration. The falling off in the rate of reaction is therefore almost entirely due to the changes in the pepsin concentration. This change is correctly expressed by Schütz's rule. In high concentration of albumin Schütz's rule therefore fits better than Arrhenius' equation.

albumin solution has been followed by means of the conductivity. The total change was $\frac{1}{170}$ reciprocal ohms. The figures have been calculated to the basis of 1,000. The results show that the equations

TABLE VII.

Rate of Hydrolysis.

Substrate = 2.0 per cent egg albumin solution, pH 2.0, = 10.0 units of peptone per cc. when completely hydrolyzed (from Table III).

Pepsin = 0.02 per cent = 0.2 units per cc.

Temperature 38°C. A = 1,000.

Time. Hrs. × 10^2	Increase in resistance. = X	Values of $K = \frac{X}{\sqrt{ET}}$	Values of $K = \frac{A \ln \frac{A}{A-X} - X}{ET}$
1	1	1	0.06
7	43	16	0.14
11	62	18.7	0.23
18	118	28	0.42
22	143	30	0.51
26	183	36	0.73
31	212	38	0.85
36	240	40	0.94
41	260	40	1.00
51	282	39.5	1.00
61	335	43	1.17
73	360	42	1.17
97	415	42	1.17
125	454	40	1.18
260	582	36	1.12
362	652	34	1.10
462	690	32	1.0
562	740	31	1.1
3,600	1,000		

fit the experimental results almost exactly as was predicted from the derivation. The decline in the value of the constant near the end of the reaction of equation (6) is due to the fact that the changes in conductivity of the solution do not accurately represent the digestion at the end of the experiment.[19] It will be shown later that, when

[19] It has been shown in another paper in this *Journal* (Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 475) that the destruction of pepsin under the conditions of this experiment is so slight as to be negligible. The decrease in the rate of reaction cannot be ascribed to this cause.

the changes in digestion are followed by means of the increase in amino nitrogen, which probably accurately follows the digestion, equation (6) gives a constant value for K.

Equation (6) is identical with that derived by Arrhenius[16] from the action of ammonia on a great excess of ethyl acetate, and applied by him to peptic digestion. Arrhenius, however, considered A as representing the concentration of ammonia (which would correspond to the concentration of pepsin in these experiments). In other words

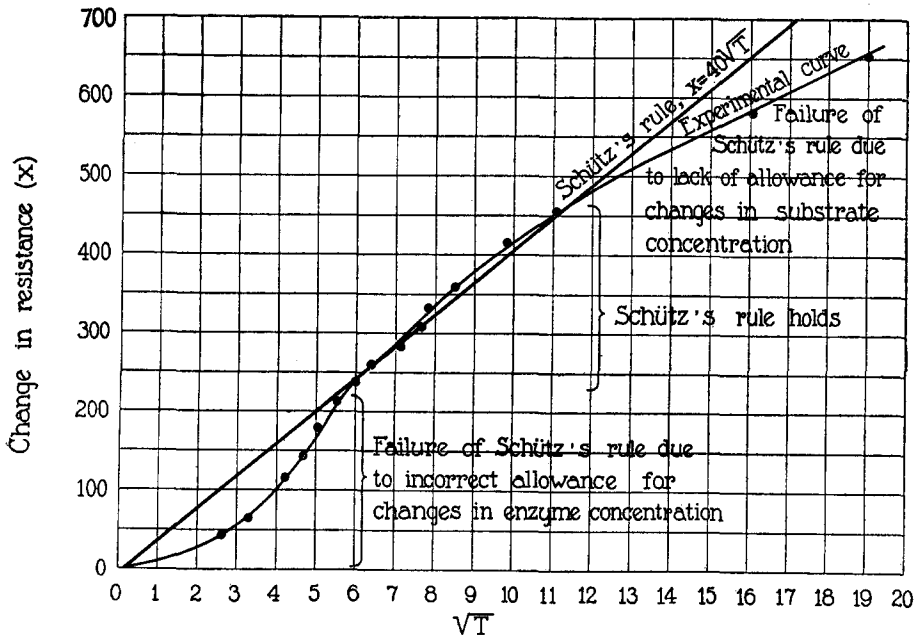

FIG. 5. Curves showing rate of digestion of egg albumin (*cf.* Table VII).

the entire term $\frac{(A-x)}{x}$ in Arrhenius' equation represents the ammonia (or enzyme) concentration while the substrate concentration is considered to remain constant. It is clear from the derivation of the equation presented in this paper, however, that the term $\frac{E}{x}$ represents the enzyme concentration, while $A-x$ represents the substrate concentration. The equation as applied to the hydrolysis of ethyl

acetate by a small amount of ammonia or to the hydrolysis of protein by pepsin is therefore identical in form but differs as to the significance of the term $A-x$.

In all the foregoing experiments the rate of digestion has been followed by means of changes in the conductivity of the solution. Since this value does not accurately represent the course of digestion, the objection might be raised that the agreement between observed and calculated values is due to compensating errors in the derivation of the equation and in the deviation of the conductivity changes from the actual progress of digestion. This could not be the case in the tests of the equation in the differential form since in this case the results are comparative and any deviation of the conductivity changes from the true rate would cancel out. It is possible, however, that the agreement of the equation in the integral form might be due to some such compensation of errors. In order to show that this is not the case a series of experiments was made in which the course of digestion was followed by means of the increase in amino nitrogen. This value was determined by Van Slyke's[20] method as already described,[21] and, as far as is known, accurately represents the progress of digestion. In these experiments the quantity of egg albumin was kept constant (0.5 per cent egg albumin) and the concentration of pepsin varied. The results are summarized in Table VIII. The figures given under X are the increase in amino nitrogen in cc. per 660 cc. of solution. They are the average of three determinations and have an experimental error of about 10 per cent. This is sufficient to account for the variations in the constant of equation (6). The errors in x are greatly magnified in this constant as it depends on the difference between two experimental values.[22] In every case sufficient time had elapsed before the first observation so that x at the time this determination was made was already large compared to the concentration of pepsin. The change in value of the constants for the first minutes of the re-

[20] Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275.

[21] Northrop, J. H., *J. Gen. Physiol.*, 1918–19, i, 607.

[22] The errors in x are reduced in the constant of Schütz's rule. The variations in this constant are therefore outside the limits of experimental error while those of Arrhenius' constant are within the limits of experimental error.

TABLE VIII.

Comparison of Schütz's Rule and Arrhenius' Equation. Rate of Hydrolysis.

Substrate = 0.5 per cent egg albumin solution, pH 2.0.

X = relative increase in amino nitrogen per cc. of solution.

E = relative pepsin concentration.

Time.	X	E	$K = \frac{X}{\sqrt{E\,T}}$	$K = \frac{\left[A \ln \frac{A}{A-X} - X\right]}{E\,T}$
min.				
30	380	32	12.0	0.10
75	500	32	10.0	0.08
135	640	32	10.0	0.08
255	700	32	8.5	0.05
495	806	32	6.6	0.05
840	860	32	5.0	0.04
1,740	975	32	4.0	0.05
4,200	[1,000]	32		
30	224	16	10.0	0.06
75	382	16	12.0	0.07
135	528	16	11.0	0.10
255	608	16	10.0	0.07
495	620	16	7.0	0.04
840	700	16	6.0	0.04
1,740	860	16	5.0	0.04
4,200	[1,000]	16		
30	152	8	10.0	0.06
75	277	8	11.0	0.08
135	390	8	12.0	0.10
255	516	8	11.0	0.10
495	536	8	12.0	0.06
840	656	8	8.0	0.05
1,740	742	8	6.5	0.06
4,200	930	8	2.3	0.07
30	100	4	13.0	0.045
75	174	4	10.0	0.055
135	225	4	11.0	0.055
255	363	4	11.0	0.08
495	415	4	9.4	0.06
840	490	4	8.5	0.055
1,740	636	4	7.6	0.055
4,200	770	4	6.0	0.045

TABLE VIII—*Concluded.*

Time.	X	E	$K = \frac{X}{\sqrt{ET}}$	$K = \frac{\left[A \ln \frac{A}{A-X} - X\right]}{ET}$
min.				
30	90	2	11.5	0.07
75	139	2	11.5	0.07
135	180	2	11.0	0.07
255	265	2	12.0	0.06
495	305	2	10.0	0.06
840	385	2	9.5	0.06
1,740	500	2	8.5	0.06
4,200	630	2	7.0	0.05
30	80	1	14.5	0.10
75	93	1	11.0	0.06
135	112	1	10.0	0.05
255	165	1	10.0	0.06
495	180	1	8.0	0.03
840	230	1	8.0	0.04
1,740	350	1	8.5	0.05
4,200	420	1	6.5	0.03

action, noticed in Table VII, is therefore lacking here. The results show that the equation gives a fairly satisfactory constant when it is considered that the experimental observations are very difficult and that the experiments represent changes in the value of E, x, and T of many hundred per cent. Individual experiments were made which gave much more constant values for K. The present series is given preference, however, since it shows that the equation takes into consideration changes in the enzyme concentration. It is obvious, however, that this equation is merely an approximation formula which will hold only under certain limited conditions and is but little more general than Schütz's rule. The derivation given offers a rational interpretation of both expressions. It may be pointed out also that equation (6) contains only one arbitrary constant K and can therefore hardly be considered as empirical.

DISCUSSION.

It has been shown in the preceding paragraphs that the divergence of the kinetics of pepsin action, from the results predicted from the

law of mass action, may be quantitatively explained by the assumption that the enzyme in solution is in equilibrium with the products of digestion of the protein, or some other substance, and that this equilibrium obeys the ordinary laws of mass action. The results of Peckelharing and Ringer[23] may be taken as experimental proof that the enzyme is so combined. These authors found that very pure solutions of pepsin showed no isoelectric point when tested between two oppositely charged electrodes; but that the addition of peptone caused the pepsin to change the direction of migration at a pH of about 3.0; which corresponds approximately to the isoelectric point of these added substances. It is difficult to explain this experiment otherwise than to conclude that the pepsin combines with the peptone and is carried with it to the electrode. If some of the pepsin combines with peptone, therefore, and so becomes inactive the rate of digestion will evidently not be directly proportional to the total concentration of pepsin but to some other function of the total concentration as defined by the mass action equilibrium. This is exactly analogous to the relation between the hydrogen ion concentration and the total acid concentration. In this case it is only the hydrogen ion which is active in hydrolysis and the activity of the solution is therefore not directly proportional to the total acid concentration. (In the case of acid it is known that the dissociation is electrolytic; *i.e.*, the dissociated parts of the molecule are electrically charged. Whether this is true or not in the case of the pepsin cannot be stated as yet.) The rate of reaction then becomes directly proportional to the active (free) enzyme concentration as demanded by the law of mass action; and the apparent divergence from this law is due to the fact that the total enzyme concentration and the active enzyme concentration are not always directly proportional; just as the total acid concentration and the active acid concentration are not always directly proportional. If this hypothesis is correct, it seems probable that the enzyme does not combine with the substrate for an appreciable length of time, but that the contact of enzyme and substrate molecule results in immediate decomposition of the latter into its products of digestion. There is no doubt that the enzyme actually does combine with the substrate when the latter is not in solution.[6] It is quite possible, however, that this is a case of solution of the en-

[23] Peckelharing, C. A., and Ringer, W. E., *Z. physiol. Chem.*, 1911, lxxv, 282.

zyme in the solid phase and that the kinetics of the reaction are the same there as in the liquid phase. There is some experimental evidence in favor of this point of view. It was found that the rate of digestion of edestin was the same when in solution and when suspended in the enzyme solution.[21] Dauwe[24] has shown that pepsin can diffuse through a membrane of solid protein.

The hypothesis enables us to set an upper limit for the purity of an enzyme preparation. It was found, for instance, in Experiment 1, that the enzyme solution used contained about twenty-seven (arbitrary) units of pepsin and about thirty units of peptone. Assuming that the combining weights of the substances are approximately the same it is obvious that the original preparation could not have been more than 50 per cent pure pepsin. It is, however, quite possible that the enzyme may be combined with some substance and still retain its activity (as found for invertase combined with charcoal by Nelson and Griffin[25]) or that impurities are present which do not combine with the enzyme at all. It is not possible therefore to assume that the active pepsin consists solely of pepsin molecules. For similar reasons it is not possible to draw any definite conclusions from the results of Experiment 2 in which it was found that a 1 per cent egg albumin solution after complete digestion contained about two arbitrary units of peptone while a 1 per cent pepsin solution contained about ten units of pepsin.

It is well known that the kinetics of enzyme reactions differ in another respect from the general laws of chemical reactions in that the rate of reaction in high concentration of substrate does not vary directly with the total substrate concentration. This phenomenon is very similar to the one discussed in the present paper and it would seem that the same explanation applies to both cases; *i.e.*, that the active substrate concentration is not directly proportional to the total substrate concentration.

It may be pointed out that, according to the above mechanism of the reaction, pepsin cannot be considered a catalyst in the sense of the classical definition since it combines with some, at least, of the products of reaction and so enters directly into the equation. Since

[24] Dauwe, F., *Beitr. Chem. Physiol. u. Path.*, 1905, vi, 426.

[25] Nelson, J. M., and Griffin, E. G., *J. Am. Chem. Soc.*, 1916, xxxviii, 1109.

the enzyme combines with one (at least) of the products of reaction its presence must necessarily affect the equilibrium point. The reaction, therefore, would appear to be a special case of bimolecular reaction in which one of the reacting substances (the enzyme) forms a highly dissociated compound with one of the products. The truth of the matter probably is that so called pure catalytic reactions are merely limiting cases in which the combination of the catalyst is so small as to escape detection (Stieglitz).[5]

SUMMARY.

1. In certain cases the rate of digestion of proteins by pepsin is not proportional to the total concentration of pepsin.

2. It is suggested that this is due to the fact that the enzyme in solution is in equilibrium with another substance (called peptone for convenience) and that the equilibrium is quantitatively expressed by the law of mass action, according to the following equation.

$$\frac{\text{Concentration pepsin} \times \text{concentration peptone}}{\text{Concentration pepsin-peptone}} = K$$

It is assumed that only the uncombined pepsin affects the hydrolysis of the protein.

3. The hypothesis has been put in the form of a differential equation and found to agree quantitatively with the experimental results when the concentration of pepsin, peptone, or both is varied.

4. Pepsin inactivated with alkali enters the equilibrium to the same extent as active pepsin.

5. Under certain conditions (concentration of peptone large with respect to pepsin, and concentration of substrate relatively constant) the relative change in the amount of active pepsin is inversely proportional to the concentration of peptone and the equation simplifies to Schütz's rule.

6. An integral equation is obtained which holds for the entire course of the digestion (except for the first few minutes) with varying enzyme concentration. This equation is identical in form with the one derived by Arrhenius[16] for the action of ammonia on ethyl acetate.

7. It is pointed out that there are many analogies between the action of pepsin on albumin solutions and the action of toxins on an organism.