

## THE KINETICS OF TRYPSIN DIGESTION.

### II. CONDITIONS UNDER WHICH THE REACTION IS MONOMOLECULAR.

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A review of the literature on the kinetics of enzyme actions shows that in general the reactions do not obey the simple monomolecular formula. This is particularly true of the proteolytic enzymes. Most of the workers in the field have concluded that the discrepancies are due to some complicating factor, usually considered to be an intermediate compound between enzyme and substrate, and that the reaction is really in agreement with the mass law if these side reactions are taken into account. Henri<sup>1</sup> attempted to formulate an equation that would include these disturbing factors and a number of similar equations have since been proposed. The results in general, however, have been inconclusive owing largely to the fact that the equations inevitably contain several arbitrary constants. The agreement with the experimental results, therefore, loses most of its significance and can always be considered empirical. This was clearly shown by the work of Nelson and Hitchcock<sup>2</sup> on invertase. They found that many of the proposed equations could be reduced to the same form, which, however, did not accurately represent the data even when the constants were derived by the method of least squares. Results such as these have led Bayliss<sup>3</sup> and others to the conclusion that the reactions are not homogeneous at all and that it is useless to attempt to reconcile the results with the mass law. It is possible, however, to attack the problem in another way. Instead of attempting to account for all the complicating factors mathematically, experimental conditions can

<sup>1</sup> Henri, V., *Lois générales de l'action des diastases*, Paris, 1903, 79.

<sup>2</sup> Nelson, J. M., and Hitchcock, D. I., *J. Am. Chem. Soc.*, 1921, xliii, 2632.

<sup>3</sup> Bayliss, W. M., *The nature of enzyme action*, New York, 3rd edition, 1914.

be chosen in such a way as to eliminate these complications. The present paper is an attempt to study trypsin digestion under such conditions and as will be seen the reaction agrees very well with the theory.

The kinetics of trypsin digestion as ordinarily carried out were outlined by Bayliss.<sup>4</sup> 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. The writer has shown<sup>5</sup> 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

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

in which  $S$  is the substrate concentration,  $T$  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 combination 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

<sup>4</sup> Bayliss, W. M., *Arch. sc. biol.*, 1909, xi, suppl., 261.

<sup>5</sup> Northrop, J. H., *J. Gen. Physiol.*, 1921-22, iv, 245, 261.

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. For this reason alone, therefore, the final result would not agree with the monomolecular formula. 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 inactivation of the enzyme is negligible. If a large amount 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 con-

TABLE I.

*Hydrolysis of Casein.*

1 per cent casein.				3.0 per cent casein.		
Time.	$\frac{A-x}{N/50 \text{ protein } N \text{ per } 1 \text{ cc.}}$		$KE = \frac{1}{T} \log \frac{A}{A-x}$	Time.	$\frac{A-x}{N/50 \text{ protein } N \text{ per } 0.5 \text{ cc.}}$	$KE = \frac{1}{T} \log \frac{A}{A-x}$
	Observed.	Calculated.				
<i>hrs.</i>	<i>cc.</i>	<i>cc.</i>		<i>hrs.</i>	<i>cc.</i>	
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	0.255	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			

secutive 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 I. 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 which have been experimentally eliminated in this case.

*Experimental Procedure.*

The casein was prepared by precipitation from acid as described by the writer.<sup>6</sup> The trypsin (Fairchild's) was dissolved in water, titrated to pH 4.5, filtered, and brought back to pH 7.4. This removes most of the accompanying protein. It is necessary to use a powerful buffer solution in order to prevent changes in pH. In these experiments a borate buffer was used. It was prepared by suspending 0.25 mol boric acid in water, titrating to the desired pH, and then making up to 1 liter. The casein is then dissolved in this solution and the pH adjusted again with NaOH. Even under these conditions there is a change in pH which disturbs the reaction when concentrated casein is used. 20 cc. of trypsin solution at 0°C. were added to 130 cc. of 1 per cent casein solution in M/4 borate buffer pH 7.6, also at 0°C. 10 cc. samples were taken at intervals into 10 cc. of cold 10 per cent trichloroacetic acid.<sup>7</sup> The solutions were kept overnight in the ice box, centrifuged, and the precipitate dissolved in alkali and made up to 10 cc. 1 or sometimes 2 cc. of this solution was then analyzed for total nitrogen by Folin and Farmer's<sup>8</sup> micro method. The results are expressed as cc. N/50 alkali. The figures are the average of 4 to 6 determinations. This method determines only the first step in the digestion, *i.e.* the hydrolysis of the casein molecule itself.

*Effect of Varying the Trypsin Concentration.*

If the formula used above is correct, the value of  $KE$  should increase directly as the concentration of trypsin, provided no complicating factors are introduced, *i.e.* the trypsin cannot be too dilute or the effect of the products will become noticeable and the constants will decrease. The trypsin solution itself must be free from such products or the free trypsin will not increase as rapidly as the total trypsin concentration. The result of an experiment in which the trypsin was varied is shown below.

Relative trypsin concentration, $E$ . . . . .	1	2	4	8
Observed velocity constant, $KE$ . . . . .	0.057	0.11	0.24	0.50
Corrected velocity constant, $K$ . . . . .	0.057	0.055	0.060	0.062

The formula evidently correctly expresses the effect of varying the trypsin concentration.

<sup>6</sup> Northrop, J. H., *J. Gen. Physiol.*, 1922-23, v, 749.

<sup>7</sup> Hiller, A., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liii, 253.

<sup>8</sup> Folin, O., and Farmer, C. J., *J. Biol. Chem.*, 1912, xi, 493.

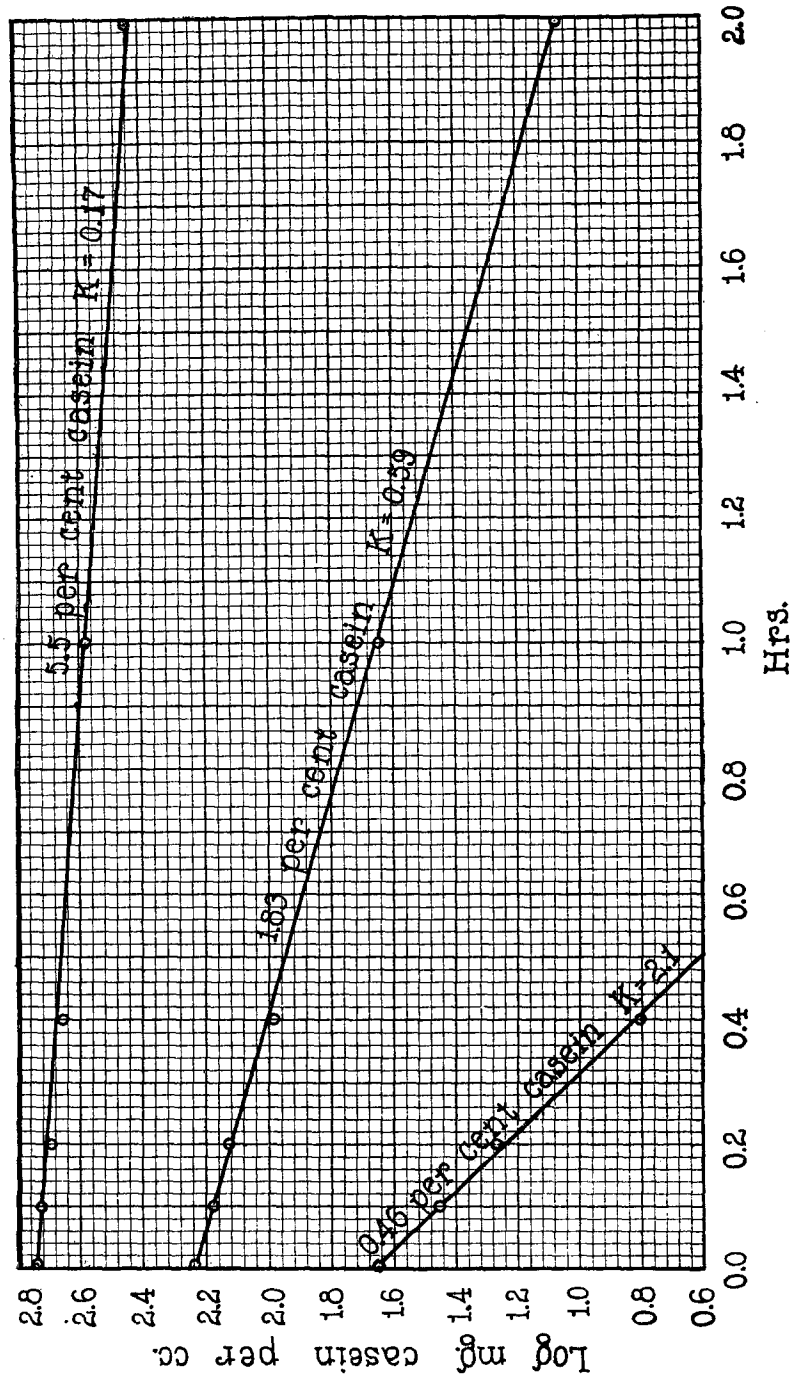


Fig. 1. The rate of hydrolysis of various casein concentrations with constant trypsin.

*Effect of Varying the Casein Concentration.*

If the reaction is really monomolecular with respect to the casein under these conditions the value of the constant  $KE$  should be independent of the casein concentration. The results of experiments in which the casein concentration is varied are shown in Fig. 1 and Table II, and it is obvious that the velocity constant is not independent of the casein concentration, but in high concentrations is nearly inversely proportional to it. Experiments 1 and 2 are not comparable as they were made with different enzyme concentrations. Each reaction by itself, however, was monomolecular. There is an

TABLE II.  
*Casein Concentration and Rate of Hydrolysis.*  
0°C., pH 8.2., M/3 borate buffer.

Experiment 1.				
Concentration of casein, <i>per cent.</i> . . . . .	3.5	1.75	0.87	
$KE$ . . . . .	0.071	0.13	0.29	
$KE \times$ casein concentration . . . . .	0.25	0.23	0.21	
Experiment 2.				
Concentration of casein, <i>per cent.</i> . . . . .	1.36	0.68	0.34	0.17
$KE$ . . . . .	0.51	0.98	1.7	2.3
$KE \times$ casein concentration . . . . .	0.70	0.66	0.58	0.39

indication that in very dilute casein solution the theoretical result would be obtained, but owing to experimental difficulties this cannot be shown conclusively. Similar results had been obtained by Taylor.<sup>9</sup>

The result is exactly analogous to the hydrolysis of cane-sugar by acid in that any one hydrolysis curve is monomolecular but the constants vary with the initial concentration of substrate. In the case of sugar hydrolysis the constants increase with increasing sugar concentration whereas here they decrease. This anomaly in the case of sugar hydrolysis, although the reaction is the classical example of a monomolecular reaction, has never been completely explained.

<sup>9</sup> Taylor, A. E., On fermentation, *Univ. Calif. Pub. Path.*, 1907, i, 239.

The opinion seems to be growing that it is due to an equilibrium between the water and sugar resulting in the formation of hydrated sugar molecules.<sup>10</sup>

The physical meaning of this result is that above about 1 per cent casein the actual amount hydrolyzed instead of the per cent hydrolyzed in a given time is independent of the casein concentration. This is a common result in enzyme reactions and has been usually ascribed to the formation of an intermediate compound. Experimental evidence has already been found, however,<sup>11</sup> which contradicts such an assumption and in any case such an assumption would predict rising velocity constants, during the course of the reaction. This is not the experimental result. We are forced to the conclusion, therefore, that the peculiarity is not due to any reaction between the enzyme and substrate, but between probably the substrate and the water, and also that the products of the reaction must be able to replace the casein in this equilibrium. This conclusion is further indicated by the fact that the effect on the constants depends solely on the relative casein-water concentration and not on the enzyme-casein concentration as might be expected if the equilibrium were between the enzyme and casein, although this evidence is not conclusive in itself. It may be pointed out that the term in the various equations which have been derived for enzyme hydrolysis and which has been supposed to represent an equilibrium between the substrate and enzyme could be interpreted just as well as representing an equilibrium between the substrate and water. The only difference would be as regards the physical significance of the constants. An equation of the same form, assuming a combination of the casein and water will accurately calculate the results shown in Table II, but such an equation contains two arbitrary constants and does not carry conviction in the absence of experimental evidence. It was stated by the writer in a preceding paper<sup>11</sup> that the discrepancy disappeared when the casein remaining in solution was used as the indicator. This result was obtained three times with a certain preparation of casein but has not been found again with any other casein preparations although the experiment

<sup>10</sup> Scatchard, G., *J. Am. Chem. Soc.*, 1921, xliii, 2387.

<sup>11</sup> Northrop, J. H., *J. Gen. Physiol.*, 1921-22, iv, 487; 1923-24, vi, 337.

has been repeated a number of times. The writer is undecided as to whether it was an experimental error or was due to some peculiarity of the casein in question. It is, perhaps, significant that, as will be shown later, the effect is not apparent with edestin, but is still more marked with gelatin.<sup>11</sup> Gelatin and casein have a relatively high viscosity whereas edestin has practically the viscosity of water. The viscosity itself, however, is not the cause as has already been shown. The effect is much too small, as may be seen from Table III, in which the rate of hydrolysis of two solutions is compared, to one of which sugar has been added. There is some effect, but not sufficient to account for the observed figures. Garrett and Lewis<sup>12</sup> have pointed out that if the reaction velocity is affected by the viscosity the reaction is probably between two molecules rather than the decomposition of one, since the latter could hardly be influenced by the viscosity.

TABLE III.  
*Effect of Viscosity.*

	1 per cent casein solution.	1 per cent casein + 30 per cent sucrose.
Relative viscosity, (H <sub>2</sub> O = 1.00) .....	1.2	3.5
<i>K</i> .....	0.24	0.13

It was pointed out above that the constancy of the velocity constants for any one reaction could only be accounted for supposing that the products of the reaction affected the velocity to the same extent as does the casein. An experiment was therefore carried out in which the casein solution was made up (*a*) with water, and (*b*) with a solution of digested casein. The result is shown in Table IV. It is evident that the digested casein has no effect. It must be remembered, however, that this solution of digested casein had been in contact with trypsin much longer than in the experiment itself, where the first products of the splitting of casein are present. It is quite possible, therefore, that the lack of effect is due to this difference. That this is actually the case may be seen from the following experiment.

<sup>12</sup> Garrett, W. H., and Lewis, W. C. M., *J. Am. Chem. Soc.*, 1923, xlv, 1091.



10 cc. of trypsin solution were added to 50 cc. of 1.2 per cent casein and 25 cc. of this solution added to 25 cc. of 1 per cent casein after varying lengths of time. The rate of hydrolysis of these solutions was then followed. All the solutions were at 0°C. and pH 8.4.

TABLE IV.

*Effect of Digested Casein on Reaction Velocity.*

	0.5 per cent casein.	0.5 per cent casein + 0.5 per cent digested casein.	1 per cent casein.
Velocity constant . . . . .	0.98	1.02	0.54

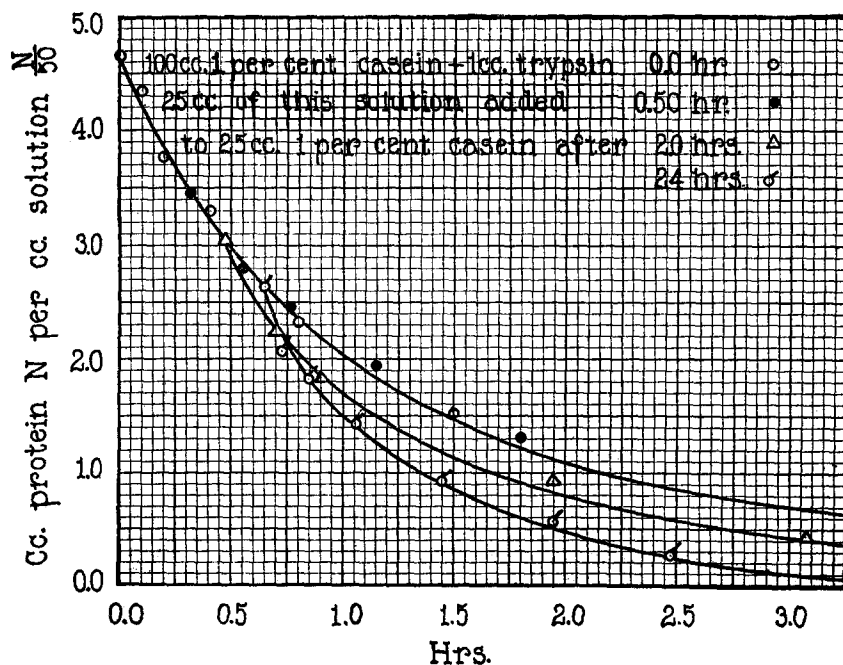


FIG. 2. Hydrolysis of 1 per cent casein solutions containing increasing proportions of digested casein.

Each solution, therefore, contained a total casein concentration of 1 per cent an increasing proportion of which, however, had been hydrolyzed before the experiment was begun. The result is shown in Fig. 2. In this figure the curves have been plotted so as to make

them coincide at the beginning. The second solution, for instance, at the beginning contained an equivalent of 3.45 cc. of casein. This point is therefore plotted on the first curve at 3.45 cc. and the time corresponding to this point considered as the starting time for the

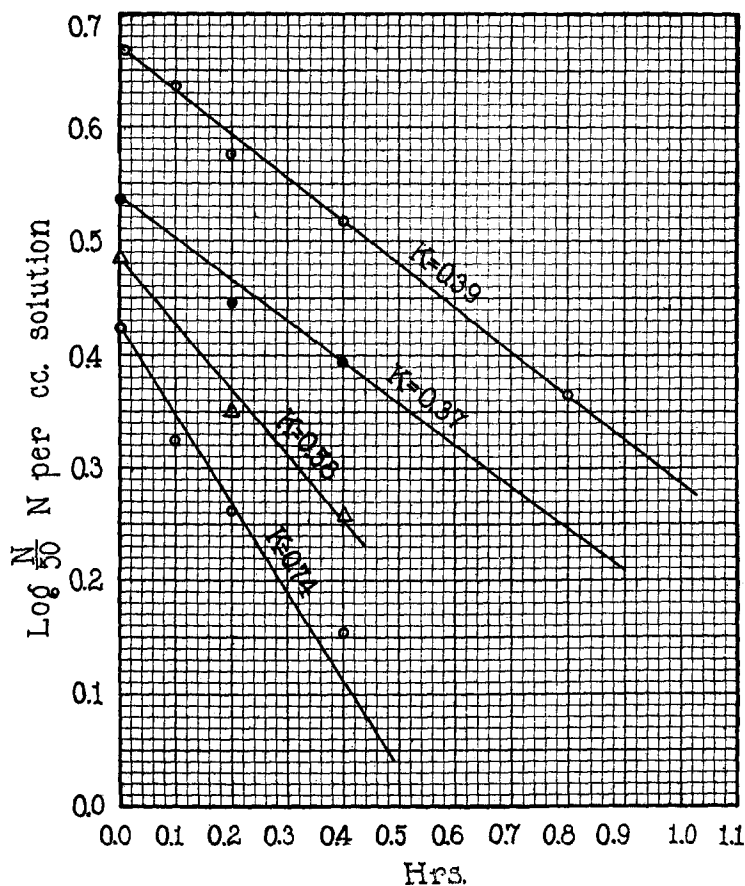


FIG. 3. Rate of hydrolysis of 1 per cent casein solution containing increasing proportions of digested casein.

second solution. It will be seen that all the solutions when plotted in this way are identical as regards casein and trypsin concentration at the first point. It might be expected, therefore, that the curves would all coincide and the figure shows that this is true of the first

two. The next two curves, however, go more rapidly and the last one (in which the casein had been acted on by the trypsin for 24 hours before adding to the fresh casein solution) is progressing at the same rate as though the trypsin had been added in water instead of in digested casein. The experiment shows that the first products formed have the same effect on the reaction as casein itself. That is, if the casein is diluted with a solution of digested casein which has just been formed, the anomalous effect of the substrate concentration disappears and the velocity constant is independent of the casein concentration as it should be. If, however, the digested casein solution has been in contact with trypsin for some time, it does not affect the reaction in the same way as does casein<sup>13</sup> and the reaction proceeds more rapidly the less concentrated the casein.

In Fig. 3 the logarithm of the amount of casein remaining in solution has been plotted against the time. The slope of these curves, therefore, represents the velocity of the reaction. They are somewhat irregular owing to the difficulty of obtaining so many samples accurately and almost simultaneously, but suffice to show the monomolecular character of the separate reactions.

The retarding effects of the products are not noticeable owing to the large amount of trypsin used in the experiment.

#### SUMMARY.

1. The kinetics of enzyme reactions diverge more or less from the simple mass action expression for a monomolecular reaction. There is good reason to believe that these discrepancies are due to other secondary reactions which also agree with the law of mass action. Attempts to incorporate all these reactions in one equation, however, are unsatisfactory owing to the complexity of the relations involved.

2. It is possible, however, to regulate conditions experimentally so that these secondary reactions are reduced to a minimum. This has been done in the case of trypsin digestion by working at a low temperature, which prevents inactivation of the trypsin, by using a

<sup>13</sup> A similar anomalous result, indicating the existence of consecutive reactions, has been observed by Simons in Nelson's laboratory (Simons, H. L., A study of the initial velocity in the hydrolysis of sucrose by invertase, Dissertation, Columbia University, New York, 1921).

large amount of trypsin, which prevents the inhibiting effect of the products from becoming noticeable, and by using the disappearance of the protein as the indicator which obviates the complicating effects of the consecutive reactions.

3. Under these conditions the hydrolysis, for any initial concentration of casein is accurately represented by the monomolecular formula,  $\frac{dx}{dt} = KE (A - x)$ . The effect of variations in the initial trypsin concentration are also correctly predicted.

4. If the initial casein concentration is varied, however, the value of the constant changes for each casein concentration, becoming less as the casein increases and eventually becoming nearly inversely proportional to the casein concentration. It is pointed out that this cannot be due to a compound between enzyme and casein, nor to the viscosity, but is probably owing to an equilibrium between the casein and water, in which the casein can be replaced by the first cleavage products. This is corroborated by the fact that if the casein is dissolved in a freshly prepared solution of digested casein, the anomalous effect of the substrate concentration disappears and the reaction is typically monomolecular in every respect. A solution of digested casein which has been in prolonged contact with trypsin does not have this effect.

5. It is pointed out that the various equations that have been proposed to account for the enzyme reactions on the basis of a compound between the enzyme and substrate could be applied equally well on the basis of a compound between water and the substrate which is attacked by the enzyme.