THE INFLUENCE OF THE SUBSTRATE CONCENTRATION ON THE RATE OF HYDROLYSIS OF PROTEINS BY PEPSTIN.

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In contrast to the numerous papers on the influence of changes in the pepsin concentration, the influence of varying the protein concentration on the rate of digestion of protein has been but little studied. Weis found that the rate was nearly directly proportional to the protein concentration in low concentration but increased more slowly than the latter in concentrations of more than 2 to 3 per cent. The experiments were made with a crude enzyme preparation which contained several proteolytic enzymes, and were made in such a way as to compare the changes in different solutions after the same time interval, instead of comparing the times required to cause an equal change. They are therefore difficult to interpret.

Preliminary experiments made with a purified pepsin and purified egg albumin showed in general the same results as those found by Weis. In concentrations of more than 2 to 3 per cent the rate of digestion increases more slowly than the protein concentration and finally becomes nearly independent of it. This phenomenon is a very general one in enzyme reactions and many explanations have been offered to account for it. Brown suggested that the relative decrease in the rate of digestion with increasing substrate concentration was due to the fact that the enzyme remained combined with the substrate for a period of time large compared with the time necessary for combination to take place. The enzyme therefore becomes more and more "saturated" with substrate as the relative concentration of substrate to enzyme increases. Van Slyke and Cullen

1 Weis, Med. Carlsberg Lab., 1903, v, 127.
showed that an equation might be derived on the assumption of the above mechanism, based on the law of mass action. The validity of this derivation has, however, been questioned by Falk. Bayliss has advocated the view that the combination is due to adsorption and cannot be considered a mass action phenomenon. He states, as do Armstrong and Armstrong, that the fact that the rate of hydrolysis in many cases is nearly independent of the total substrate concentration cannot be explained on the law of mass action, and must be due to some saturation effect of the enzyme with the substrate.

The fact is frequently overlooked that purely chemical catalysis in strictly homogeneous solutions also shows apparent divergences from the mass law. This point is, however, discussed by Bredig, Mellor, Lewis, and especially by Falk. In the hydrolysis of cane sugar by acids, for instance, the rate of hydrolysis is not proportional to the total concentration of acid used but to the hydrogen ion concentration. As is well known, the hydrogen ion concentration in heavily "buffered" solutions is almost independent of the total acid concentration in certain ranges so that the hydrolysis of cane sugar by such a solution would show an analogous behavior to enzyme reactions in that the rate of hydrolysis of the sugar would be nearly independent of the total acid concentration. The apparent discrepancy of the mass law is therefore due to the fact that the "active concentration" (on which the mass law is based) is in many cases not identical with the total concentration. There is a further discrepancy in these cases due to the fact that the rate of hydrolysis in certain concentrations is not proportional to the \( C_H^+ \) as determined by the conductivity ratio. This is the so called salt effect and is probably

due to increased activity of the hydrogen ions by the salt. In any case, it is not due to any peculiarity of the catalytic reaction since the same discrepancy is found in comparing the hydrogen ion concentration as determined by the conductivity and E.M.F. methods. The apparent discrepancy between the mass action law and the kinetics of acid catalysis as outlined above is analogous to the case in enzyme reactions where the rate is not proportional to the enzyme concentration.

Acid hydrolysis, moreover, also shows the same peculiarity in regard to the sugar concentration, i.e. the rate does not increase directly as the sugar concentration, as expressed in grams or molecules per liter. In the case of acid hydrolysis the rate increases more rapidly than the concentration. Arrhenius\textsuperscript{11} has suggested that this behavior is due to the fact that the active concentration of sugar is not correctly expressed by the molecular concentration and has shown that very much better results are obtained if the osmotic pressure of the sugar solution is used as a measure of the active concentration. He further assumes that the acid affects the equilibrium between active and inactive sugar molecules and so accounts for the "salt effect." The same mechanism is assumed to account for the effect of temperature, which is much greater than that predicted by the kinetic theory. This hypothesis, of course, fits the facts, but in the absence of independent evidence is really an assumption of the law of mass action rather than a proof of the law. Several authors have proposed explanations for catalytic reactions on the same basis; i.e., that the catalyst changes the concentration of certain molecules and so increases the speed of the reaction. Stieglitz\textsuperscript{12} and his coworkers have been able to verify this hypothesis experimentally in the case of the acid hydrolysis of imido esters. This reaction shows the same peculiarities as that found in many enzyme reactions; namely, the rate is not proportional to the total ester concentration. Stieglitz was able to show, however, that the rate was directly proportional to the concentration of ester ions. He considers that the acid causes the formation of imido ester salts and

\textsuperscript{11} Arrhenius, S., \textit{Z. physik. Chem.}, 1899, xxviii, 317.
thereby increases the concentration of active ions. He was able to confirm this by independent measurement of the ion concentration (by means of the conductivities). It follows, as emphasized by Stieglitz, that if the above mechanism is correct so called catalytic reactions are merely limiting cases of ordinary reactions in which the combination of the "catalyst" with the substrate or with the products of hydrolysis is too small to be measured. It seems probable that enzyme reactions are of the same type. There is no doubt, at least, that the enzyme often combines with the products of the reaction and so shifts the equilibrium. Bodenstein and Dietz\textsuperscript{13} have shown experimentally that this is true in certain cases. It would seem better, therefore, to consider enzyme reactions as cases of bimolecular reaction in which one of the products dissociates more or less completely with the liberation of active enzyme; if the dissociation is complete the result would be a monomolecular reaction and, if no dissociation whatever takes place, a bimolecular reaction. Most enzyme reactions are apparently intermediate. The specificity of enzyme reaction thus becomes neither more nor less remarkable than the specificity of any other chemical reaction. (The author has had the privilege of discussing the above points with Dr. K. G. Falk who has reached independently similar conclusions.) It was shown in a previous paper\textsuperscript{14} that the above conception of enzyme reactions as applied to pepsin gives a quantitative explanation for the kinetics of the reaction and explains the fact that the rate is not always proportional to the total concentration of pepsin. Arrhenius\textsuperscript{15} has pointed out that it also gives the explanation of Schütz's rule and the divergence from the monomolecular law.

It is clear from the brief account of catalytic reactions given above that the same apparent divergences from the law of mass action are to be found in these reactions as in enzyme reactions and that the divergences in many cases at least are caused by the fact that the active concentration is not the same as the total concentration of substance. It seems quite probable that the same explanation applies to both. There is no doubt that the saturation theory is sufficient

\textsuperscript{13} Bodenstein and Dietz, Z. Elektrochem., 1906, xii, 605.
\textsuperscript{14} Northrop, J. H., J. Gen. Physiol., 1919-20, ii, 471.
\textsuperscript{15} Arrhenius, S., Med. Nobelinst., 1908, i.
to explain many of the facts but in the absence of direct evidence it can hardly be considered proved.

According to this theory a certain amount of enzyme can act only on a limited amount of substrate; after this quantity is reached any excess of substrate has no effect on the reaction. It is clear that according to this mechanism it is the ratio of the concentration of substrate to that of the enzyme which causes the relative decrease in the rate of digestion of the substrate as the concentration of substrate increases, and not the actual concentration of substrate present in the solution. If the effect, however, is due to the fact that the active concentration of substrate is not directly proportional to the total concentration then the falling off of the rate of reaction with increasing substrate concentration is independent of the ratio of substrate to enzyme and depends only on the actual concentration of substrate.

It occurred to the writer that this question might be tested experimentally by comparing the rate of digestion of different substrate concentrations when hydrolyzed with different enzyme concentrations. Assume, for instance, that the substrate at concentration 10S is found to hydrolyze five times as rapidly as the substrate at concentration S, when enzyme concentration E is used. According to the monomolecular formula the substrate at concentration 10S should digest ten times as rapidly as the substrate at concentration S. The saturation hypothesis would explain this divergence by the assumption that the enzyme becomes saturated with substrate at a concentration of the latter of less than 10S. In concentration 10S, therefore, much of the substrate takes no part in the reaction and the rate of reaction is less than the expected. It would be predicted further that increasing the substrate concentration from 10S to 20S would have relatively less effect on the rate of reaction than increasing the substrate concentration from S to 2S. This is true. It follows also on the saturation hypothesis that increasing the enzyme concentration from E to 10E should have a relatively greater effect on the rate of digestion of substrate 10S than on the rate of digestion of substrate at concentration S; since it was assumed in accounting for the effect of increasing the substrate concentration that the enzyme (at concentration E) was more saturated with substrate at (substrate) concent-
tration 10 \( S \) than at (substrate) concentration \( S \). According to the saturation theory, the rate of digestion in concentration 10 \( S \) is limited only by the concentration of enzyme while the rate at concentration \( S \) is limited both by the concentration of enzyme and by the concentration of substrate; hence changing the enzyme concentration should have a greater effect at substrate concentration 10 \( S \) than at substrate concentration \( S \). The experiments show that this prediction is not fulfilled. The relative increase in the rate of digestion of substrate at concentration \( S \), caused by increasing the concentration of enzyme from \( E \) to 10 \( E \), is identical with the relative increase in rate of digestion of the substrate at concentration 10 \( S \), caused by the same increase in enzyme concentration.

If, on the other hand, the relative decrease in rate with increase in concentration of substrate is due to an equilibrium in the substrate solution which causes the concentration of active molecules to differ from the total concentration, the rate of hydrolysis of the substrate at concentration 10 \( S \) should be always five times the rate of digestion at concentration \( S \) (in the example just discussed), irrespective of the enzyme concentration. Experiments show that this is actually the case. It is necessary, of course, in making such experiments to be sure that the range covered is such that the enzyme cannot be considered saturated in both substrate concentrations. That is, the range of substrate concentrations must be such as to show nearly direct proportionality between the rate of digestion and the substrate concentration in the lower, but not in the higher concentrations of substrate. It is also necessary to measure the time required to cause a constant change in the substrate and not a constant percentage change or the change made in a given time. The failure to recognize this has led to much confusion in discussion of the kinetics of enzyme reactions (cf. Bredig).7

This is due to the fact that in most enzyme reactions the products retard the action of the enzyme. It will be clear therefore (irrespective of the mechanism by which this retardation takes place), that comparative results can be obtained only when a constant amount of products is formed. The actual amount of products formed for example by 10 per cent hydrolysis of varying substrate concentrations will be very different. The larger the concentration of substrate the greater the amount of products formed by 10 per cent hydrolysis and the greater
the consequent slowing up of the enzyme due to the inhibiting effect of the products. It is also clear that the retardation will be proportionally greater if a small amount of enzyme is present than if a large amount is present (irrespective of the mechanism by which the retardation is affected). The same reasoning holds for the case when the amount of products formed in a given time is taken as the measure of the rate of reaction. This question was discussed fully in a previous paper.14

In all the experiments given in this paper, therefore, the rate of digestion is measured as the reciprocal of the time necessary to cause a small absolute change in the substrate concentration. According to the law of mass action as applied to monomolecular reactions the time necessary to cause this change should be nearly inversely proportional to the substrate concentration, provided the change is small compared to the total change in the lowest concentration. If wider variations than this are used it is necessary to calculate the predicted time according to the monomolecular formula. It may appear that the above method of testing the reaction is a very indirect one and that a simpler and more exact method would be to express the course of a single reaction, according to the mechanism proposed, in a single equation. This equation could then be tested experimentally. Such a procedure, however, leads inevitably to an equation with two or more constants, the value of which must be determined from the experiments themselves, so that but little weight can be attached to the agreement of such an equation with the experimental facts. It seems better, therefore, to limit the experimental conditions in such a way as to leave but one variable.

In all the experiments reported in this paper, the changes are within the above limits and the time required to cause a constant change should, therefore, (according to the mass law) be nearly inversely proportional to the substrate concentration at the beginning of the reaction. As will be seen this is not the case if the total concentration of protein is considered as the active concentration but is approximately true if the concentration of ionized protein is considered as the reacting mass.

In these experiments the rate of hydrolysis was followed by means of changes in the conductivity of the solution. It is, therefore, necessary to be sure that the production of the same amount of peptone in each of the solutions used causes the same change in conductivity. This was tested experimentally in each experiment by adding 1 cc. of peptone solution (prepared from egg albumin by the action of pepsin) to 25 cc. of the protein solution and determining the change in conductivity. It was found that the addition of an equal amount of peptone to protein solutions of varying concentrations (from 20 to 1 per cent) does cause an equal change in conductivity provided the hydrogen ion concentration of the solution is greater than pH 1.8. If the solution is less acid than this the change in conductivity of the solution on the addition of a constant quantity of peptone in the presence of a large amount of protein is less than that caused in the presence of a small amount of protein. This is obviously due to the buffer action of the protein in high concentration and can be foreseen from the titration curve of the protein.
Fig. 1 gives the results of two experiments on the effect of the pepsin concentration on the relative rate of digestion of protein solutions of different concentration. In Experiment 1, (Curve I, Fig. 1) 25 cc. of protein solution containing 8, 4, 2, 1, and 0.5 per cent protein, were hydrolyzed at 25° with the addition of (a) 1 cc. of 2 per cent pepsin, and (b) 1 cc. of 0.2 per cent pepsin. All solutions were brought to a pH of 1.8 with hydrochloric acid. The time necessary to cause a given change (about \(1.4 \times 10^{-4}\) reciprocal ohms) in the specific conductivity was determined. The reciprocal of this time, therefore, gives the mean rate of digestion of the various solutions for the first \(1.4 \times 10^{-4}\) reciprocal ohm change. In order to compare the two series, the rate of digestion of the concentrated egg albumin (in each series) was considered as 100 and the rate of digestion of the other concentrations calculated on this basis. The curve shows that the relative rate of digestion of the 8 per cent egg albumin compared to the rate of digestion of 4, 2, 1, or 0.5 per cent egg albumin is the same irrespective of whether 2 or 0.2 per cent pepsin was used. The curve also shows that in low concentrations, 0.5 to 2 per cent, the increase in rate is nearly proportional to the increase

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in substrate concentration but increases much more slowly in high concentrations. Experiment 2, (Curve II) shows the same result. It was made the same way but at a pH of 1.6. The points in this case are the average of two determinations. They are therefore more reliable and as the figure shows also more nearly equal. In both experiments the relative activities of the two pepsin solutions were as 4.7: 1. It follows from the experiments that this ratio is also inde-

TABLE I.

<table>
<thead>
<tr>
<th>Ratio: Rate of hydrolysis of 15 per cent albumin with Rate of hydrolysis of 1 per cent albumin</th>
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<tr>
<td>0.08 per cent pepsin.</td>
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<tr>
<td>8.9</td>
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<tr>
<td>9.4</td>
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<td>10.0</td>
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<td>9.8</td>
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<td><strong>Average</strong></td>
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<td>9.52</td>
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**Ratio:** Rate of hydrolysis with 0.08 per cent pepsin in Rate of hydrolysis with 0.008 per cent pepsin

<table>
<thead>
<tr>
<th>15 per cent protein.</th>
<th>1 per cent protein.</th>
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<tr>
<td>5.3</td>
<td>5.2</td>
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<tr>
<td>5.0</td>
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<td>4.7</td>
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<td>5.3</td>
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<td><strong>Average</strong></td>
<td><strong>Average</strong></td>
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<tr>
<td>5.06</td>
<td>5.34</td>
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pendent of the substrate concentration in which the tests were made. (It was shown in a previous paper that the discrepancy in the rate of digestion as compared with the enzyme concentration can be quantitatively explained on the basis of a mass action equilibrium between the pepsin and peptone.) Table I shows a similar experiment in which several duplicate determinations were made at two protein concentrations with two enzyme concentrations. The results are more accurate and also in closer agreement than those shown in Fig. 1.
It seems necessary to conclude from these experiments that the relative decrease in the rate of digestion of protein solutions of increasing concentration is independent (within the limits of error of these experiments) of the enzyme concentration used.

There does not appear to be any direct experimental evidence on the above point in connection with other enzymes. It is frequently stated, however, (Nelson and Vosburgh,\textsuperscript{17} Van Slyke and Cullen\textsuperscript{3}) that the velocity of reaction is directly proportional to the enzyme concentration under all conditions and irrespective of the substrate concentration. If this is true it follows necessarily that the relative rate of digestion of various substrate concentrations, when hydrolyzed with any given enzyme concentration, is independent of the enzyme concentration used.

It appears to the writer that this is contrary to the result predicted by the saturation theory. According to this theory it would be predicted that the falling off in the increase in the rate of digestion as compared to the increase in concentration of a protein solution (above a certain low concentration) is due to the fact that at this concentration the enzyme begins to become saturated with substrate; \textit{i.e.}, the time necessary for the enzyme to combine with the substrate becomes small compared with the time during which it remains combined. If this saturation effect becomes noticeable at a concentration of protein of 2 per cent with 0.08 per cent pepsin it should become noticeable at a lower protein concentration with 0.008 per cent pepsin. The experiment shows this is not the case. If anything, the figures show that the rate of digestion of the substrate falls off more rapidly (as compared to the concentration) with the higher pepsin concentration than with the lower. In Table I, which is more reliable owing to the larger number of determinations there is less than 1 per cent difference.\textsuperscript{18}

It seems necessary to conclude therefore that the relative decrease in the rate of digestion as compared with the increase in protein concentration is independent of the enzyme concentration used.


\textsuperscript{18} It probably cannot be assumed that, according to the saturation theory, the rate of digestion (caused by increasing the enzyme concentration ten times) should be increased ten times as much in the concentrated as in the dilute substrate concentration. The increase should be large enough to detect, however.
centration is due to some equilibrium in the protein solution itself and is independent of the enzyme concentration.

It is well known that in acid solution protein exists in an ionized condition. The concentration of ionized protein is not directly proportional to the total concentration but will increase more slowly than the total concentration. It is obvious, therefore, that the rate of digestion will be more nearly proportional to the concentration of ionized protein than to the total concentration of protein. The hypothesis, then, that the ionized protein is the form which takes part in the reaction, will allow a nearer approach to the predicted rate of reaction. Pauli\(^{19}\) has suggested that the enzyme attacks the ionized protein; there seems, however, to be no direct evidence for this view. It can be tested experimentally by comparing the rate of digestion with the degree of ionization of the protein.

The concentration of ionized protein can be determined approximately from the pH and conductivity measurements. If the total conductivity and the hydrogen ion concentration of a solution are accurately known, the conductivity due to the protein-salt ions can be determined by subtracting the conductivity of the free HCl from that of the solution. The validity of this method rests on three conditions: (1) the conductivity of the free HCl in the solution is the same as that of the same concentration of acid in water solution; (2) the C\(_{H^+}\) is equal to or greater than the C\(_{Cl^-}\); and (3) the hydrogen ion concentration as determined by the e.m.f. method must agree with that found by the conductivity method.\(^{20}\) The first assumption cannot be tested directly but it has been shown by Hardy\(^{21}\) and by Loeb\(^{22}\) that the viscosity of the solution has no significant effect on the conductivity since the viscosity may increase till the solution is nearly solid without an appreciable change in the conductivity. This experiment was repeated and confirmed. The second condition can be shown to hold also by direct measurements of the chlorine ion concentration by means of concentration cells as was done by Manabe and Matula.\(^{23}\) Many measurements of this kind were made and confirmed those of the above mentioned authors; namely, the chlorine ion concentration is always equal to or greater than the hydrogen ion concentration. It was

\(^{19}\) Pauli, W., Arch. ges. Physiol., 1910, cxxxvi, 483.

\(^{20}\) For the purpose of these experiments it is only necessary that the conductivity and e.m.f. methods should agree. The absolute value for the C\(_{H^+}\) is immaterial.

\(^{21}\) Hardy, W. B., J. Physiol., 1905, xxxiii, 251.

\(^{22}\) Loeb, J., J. Gen. Physiol., 1918-19, i, 559.

\(^{23}\) Manabe, K., and Matula, J., Biochem. Z., 1913, lii, 369.
found, however, that if the conductivity of the H⁺ and the total Cl⁻ were subtracted from that of the solution the remaining values were within the limits of error of the measurements. In other words, the conductivity due to the protein ion itself is very small compared to that due to the excess chlorine ion (by excess Cl⁻ is meant the difference between the total CCl⁻ and the CCl⁻ = C⁺). Since, however, the protein ion must equal in concentration the excess chlorine ion, the value for the conductivity obtained by subtracting the conductivity of the free HCl from that of the solution may be considered as proportional to the amount of ionized protein. (Recent work, by Noyes, Milner, and others, has rendered questionable the exact interpretation to be put upon conductivity ratios; they very probably do not represent the actual ion concentration in all cases.)

The third condition may be experimentally fulfilled by standardizing the apparatus used for the C⁺ determinations against HCl solutions of known conductivity and taking the C⁺ as that determined by the conductivity ratios. This method was used in the present experiments. The final values for the conductivity due to the protein-salt ions are the difference between two large figures so that the error is very large and becomes larger as the solution becomes more dilute. Below 1 per cent protein solution (at pH 1.7) the value is meaningless as it usually lies within the limit of error.

The egg albumin was crystallized three times and then dialyzed under pressure at the isoelectric point until the specific conductivity was lower than 1 × 10⁻⁴ reciprocal ohm. The solutions were then brought to a pH of 1.6 to 1.8 with HCl and then diluted with HCl of exactly the same pH. The solutions varied from 16 to 1 per cent egg albumin. The time necessary to cause a constant small change in the conductivity of the resulting solution by the same amount of pepsin was then determined as described previously. The reciprocal of this time is plotted in the curves as the rate. The conductivity of the solution was measured on an aliquot part of the solution to which the equivalent amount of inactivated pepsin had been added. The C⁺ was determined by the E.M.F. method on this solution. The value given for the specific conductivity of the protein is obtained by subtracting the specific conductivity of the free HCl from that of the solution. The experimental error of the value is 5 to 10 per cent in the high concentrations and 20 to 30 per cent in the lower. The figures given are the averages of three determinations. All measurements were made at 25° ± 0.01.

The conductivity and rate of digestion of the egg albumin was measured in this way. It was found in general that the conductivity of the protein solution was, within the rather large limits of error, directly proportional to the rate of digestion of the solution. In other words, the rate of digestion is that predicted by the mass law if the ionized protein is considered as the reacting form. The results of three such experiments are given graphically in Fig. 2 in which...
the rate of digestion is plotted against the conductivity of the protein. This figure shows that the two values are approximately directly proportional.

![Graph showing rate of digestion and conductivity of egg albumin solutions.]

**Fig. 2.** Rate of digestion and conductivity of egg albumin solutions.

**Viscosity of the Solution.**

It is impossible to use egg albumin in more concentrated solutions than 16 per cent as the higher concentrations set to a jelly very rapidly. It seemed quite possible that the increasing viscosity of the solution might affect the rate of digestion (as found by Colin and Chaudun\(^\text{24}\) for invertase). This question can be tested experimentally by taking advantage of the well known hysteresis of albumin solutions.

500 cc. of a 25 per cent egg albumin solution were titrated to pH 1.6 with HCl, placed at 25°, and the viscosity and rate of digestion of a sample determined at intervals for about 10 hours. The amount of pepsin used was such that the viscosity of the digesting solution did not change appreciably during the determination. This was due to the fact that the decrease in viscosity by the pepsin was equalized by the increase of the viscosity with time. The viscosity of the solution at the beginning of the experiment was about three times that of water and at the end too large to measure by the viscosimeter. At the beginning of the last

Substrate concentration and hydrolysis
digestion test, the solution could hardly be pipetted with a wide-mouth pipette
and was semisolid. The figures for viscosity are doubtless all too low since the
viscosimeter was not known to obey Poiseuille's law and almost certainly did not
obey it since the time of outflow for 20 cc. of water was only 10 seconds.

The result of the experiment is shown graphically in Fig. 3. The
rate of digestion is not affected appreciably, until the viscosity has
increased four to five times that of water. This is far greater than
the viscosity of any solution used in the other experiments referred
to. There is no doubt, however, that when the solution becomes
nearly solid the rate of digestion is greatly decreased. It was found
that the same decrease was noticed if the viscosity of the solution
was increased by the addition of agar. The presence of the agar
alone is not the cause of the decrease in the rate as was shown by the
fact that the rate of digestion was unaffected by the agar if the experi-
ment was conducted at 40° (when the agar caused no increase in
viscosity). It would seem, therefore, that the effect of viscosity
must be a mechanical one due to interference with the diffusion of

Fig. 3. Influence of viscosity on rate of digestion.

For a discussion of this question see Washburn, E. W., and Williams, G. Y.,
the enzyme rather than to a change in the resistance of the protein. This is borne out by the fact that pepsin diffuses only very slowly through coagulated protein (cf. for instance Dauwe). Reformatsky has shown that the rate of hydrolysis of methyl acetate by acids is identical in water solution and in a solid agar gel. In this case the rate of diffusion of the H⁺ is also independent of the viscosity (Voigtländer).

Ringer has pointed out that the optimum pH for the digestion of protein coincides approximately with the maximum viscosity and has suggested that the rate of digestion is dependent on the degree of hydration of the protein; the viscosity of the solution is also assumed to be a measure of the degree of hydration. It would seem from the experiment just described that an increase in viscosity decreases the rate of digestion instead of increasing it, as supposed by Ringer. If the protein ion is the active form of the protein the optimum pH should depend on the maximum degree of ionization. According to Pauli the maximum viscosity also depends on the ionization. Loeb has shown, however, that this is not true. The hypothesis outlined above requires that the rate of digestion of a protein solution at different pH should be directly proportional to the amount of protein ionized. Preliminary experiments show that this is true, qualitatively at least. Unfortunately the change in conductivity (as pointed out above) cannot be used to follow the rate of digestion at lower C⁺⁺ so that the experimental difficulties are much greater.

The results of the present paper may be considered in qualitative agreement at least with the mechanism of pepsin digestion as outlined in the preceding paper. The hypothesis advanced considers that there is an equilibrium in the pepsin solution between pepsin and peptone (substances combining with pepsin and so rendering it inactive). There is also an equilibrium between ionized and unionized protein in the protein solution. The reaction takes place according

23 Ringer, W. E., *Arch. Neerl. Phys.*, 1918, ii, 571; *Z. physiol. Chem.*, 1915, xcv, 195. Ringer considers that the charge on the protein is also of importance. This agrees with the present experiments.
to the law of mass action between the uncombined pepsin and the protein ion. The mechanism may be formulated as below.

\[ \text{Pepsin} + \text{peptone} \rightleftharpoons \text{pepsin-peptone} \]

or

\[ C_{\text{pepsin}} = K \frac{C_{\text{pepsin-peptone}}}{C_{\text{peptone}}} \]  
(1)\textsuperscript{a}

and

\[ \text{Protein-chloride} \rightleftharpoons \text{protein}^+ + \text{Cl}^- \]

or

\[ C_{\text{protein ion}} = K \frac{C_{\text{protein-chloride}}}{C_{\text{Cl}^-}} \]  
(2)\textsuperscript{a}

The reaction would be expressed by

\[ \text{Protein ion} + \text{pepsin} \rightleftharpoons [\text{protein ion-pepsin}] \rightleftharpoons \text{peptone-pepsin} \rightleftharpoons \text{pepsin} + \text{peptone} \]

The rate of hydrolysis of the protein at any instant of time would therefore be proportional to the concentration of protein ions and of free pepsin present in the solution at that instant and the differential expression for the rate of reaction would be

\[ -\frac{dC_{\text{protein ion}}}{dt} = K C_{\text{protein ion}} \cdot C_{\text{pepsin}} \]

where \( C_{\text{protein ion}} \) and \( C_{\text{pepsin}} \) are determined by equations (1) and (2). There is probably little doubt that the enzyme and substrate unite to form an addition product, but according to the experimental evidence found in this paper the time during which they are combined is negligible in the consideration of the kinetics of the reaction.

The mechanism outlined above will explain, at least qualitatively, the peculiarities in the kinetics of other enzyme reactions. It seems very unlikely, however, that the equilibrium in the substrate solution should always be ionic. It may be any isomeric equilibrium. Since,

\textsuperscript{a} The equilibrium expressed in (2) is certainly, and that expressed in (1) is probably, influenced by the hydrogen ion concentration.
in such cases, it is extremely difficult to obtain any independent measurement of the equilibrium, there seems to be no way to test the proposed mechanism.

SUMMARY.

1. It is pointed out that the apparent exceptions to the law of mass action found in enzyme reactions may be found in catalytic reactions in strictly homogeneous solutions.

2. These deviations in the rate of reaction from the law of mass action may be explained by the hypothesis that the active mass of the reacting substances is not directly proportional to the total concentration of substance taken.

3. In support of this suggestion it is shown that for any given concentration of pepsin the relative rate of digestion of concentrated and of dilute protein solutions is always the same. If the rate of digestion depended on the saturation of the surface of the enzyme by substrate the relative rate of digestion of concentrated protein solutions should increase more rapidly with the concentration of enzyme than that of dilute solutions. This was found not to be true, even when the enzyme could not be considered saturated in the dilute protein solutions.

4. The rate of digestion and the conductivity of egg albumin solutions of different concentration were found to be approximately proportional at the same pH. This agrees with the hypothesis first expressed by Pauli that the ionized protein is largely or entirely the form which is attacked by the enzyme.

5. The rate of digestion is diminished by a very large increase in the viscosity of the protein solution. This effect is probably a mechanical one due to the retardation of the diffusion of the enzyme.