A METHOD FOR THE QUANTITATIVE DETERMINATION OF TRYPSIN AND PEPSIN.

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In the study of proteolytic enzyme reactions an experimental difficulty has always presented itself in the lack of a method permitting the determination of the amount of enzyme present in a given digestion mixture during the course of the reaction. This difficulty has been overcome by a method already described which depends on the change in electrical conductivity during the digestion of egg albumen by pepsin (1), and during the digestion of gelatin by trypsin (2). This method has been employed successfully in a series of experimental studies (2, 3).

While this procedure has been found useful, there are certain studies concerning enzyme reactions in which it cannot be employed. This is particularly true where it is desired to study these reactions when they take place in the presence of "buffer" salts. It occurred to one of the writers that the rate of change in viscosity of gelatin during digestion might be directly related to the amount of enzyme present in the digestion mixture. If this idea is correct, one would expect to find that the time required to cause an equal percentage change in the viscosity of a series of gelatin solutions would be directly proportional to the amount of enzyme added, provided the gelatin solutions are the same but the amount of enzyme in each is varied. That this actually is found to be the case will be shown in this communication.

In order to test the idea stated, it is necessary to consider certain factors which *per se* bring about changes in the viscosity of gelatin solutions; namely, the temperature at which the observations are made, the reaction, and the salt concentration of the solutions. The observations of Loeb (4) regarding the effect of pH and the concen-

tration of salt, and the observation of Loeb and others concerning the effect of temperature, demonstrate the influence of these factors on this property of gelatin. Obviously, then, it is necessary to control these conditions during observations where changes in viscosity are assumed to depend directly upon enzyme action. Further, in the case of experiments on trypsin digestion, the influence of temperature on the stability of the enzyme must be considered.

Fortunately, as far as temperature effects are concerned, we know that a temperature of 34°C. can be used without affecting the stability of trypsin to an extent prohibitive for the conditions of our experiment (2). It so happens that this same temperature is the one at which the minimum thermal effects on viscosity are observed. Regarding the influence of pH, we know, as a consequence of Loeb's observations, that the slope of the curve representing viscosity changes in gelatin solutions as a function of the pH approaches zero and is sensibly constant between pH 7.3 and 7.5. The addition of a "buffer" salt on the alkaline side of the isoelectric point of gelatin easily corrects for any pH effect, but the concentration of the salt used must be one that will not develop a degree of viscosity which will make the time of outflow for the solution too long. On the acid side of the isoelectric point the reaction can be controlled by adding a weak acid, such as H_3PO_4 . We have found it possible by these means to prepare gelatin solutions which meet all the requirements demanded for observations to be made on either side of the isoelectric point.

Preparations for Experimental Procedure.

The gelatin employed was Cooper's powdered preparation which was brought to the isoelectric point and washed as described by Loeb (4), except that larger amounts were made. The isoelectric gelatin was melted and the concentration of gelatin determined by dry weight. A given amount of gelatin was kept at a temperature between 100°C. and 102°C. for 24 hours for this determination. For trypsin digestion the concentrated gelatin preparation was diluted with 0.1 M Na_2HPO_4 so as to contain 3 gm. of gelatin by dry weight per 100 cc., then it was adjusted to pH 7.4 with 0.1 M H_3PO_4 . For pepsin digestion the concentrated gelatin was adjusted to pH 3 with dilute phosphoric acid and diluted to contain 3 gm. of gelatin per 100 cc. The diluted gelatin was in each case distributed in 250 cc. amounts in Erlenmyer flasks and stored in a refrigerator kept at 3°C. A small amount of thymol was added to each flask as a preservative.

The enzyme solutions were prepared as previously described by Northrop (2, 3).

The viscosimeters were made after the pattern of the Ostwald type and were calibrated by regulating the time of outflow of 5 cc. of water at 34°C. to be 60 seconds between two marks. The gelatin-water time ratio was approximately 3. The time of outflow was measured by means of stop-watches. The viscosimeters were held in a stand which was kept constantly in a deep water bath. The viscosity obtained in this way is the mean viscosity over the period of time elapsed during the measurement. For convenience in plotting, however, the value is assumed to represent the viscosity at the center of this time interval. The error introduced by this method is negligible.

EXPERIMENTAL RESULTS.

For trypsin digestion 50 cc. of 3 per cent gelatin, pH 7.4, were pipetted from a flask that had been in the water bath at 34°C. over night and put into an empty flask also immersed in the water bath. 0.5 cc. of trypsin dilution was then added. After thorough mixing, 10 cc. of this gelatin-trypsin mixture were pipetted into each of 5 viscosimeters and the time of outflow was observed at frequent intervals. The time intervals of observations were based on hundredths of the hour for convenience and were measured from the time of addition of the trypsin. This procedure was carried out with four different dilutions of trypsin, the relative dilutions were of the order 1-2-4-8. The actual times in seconds required for the digestion mixture to pass between the two chosen points on the viscosimeter were plotted as ordinates against the time intervals of observation as abscissæ; then smooth curves were drawn through these points. The time required to cause the same percentage change in the viscosity of the different solutions in each viscosimeter was then interpolated from these curves. Some difficulty was experienced in determining the zero point for drawing these curves. At first we attempted to use for the zero the number of seconds required for the outflow of gelatin when diluted with a volume of water equal to that of the trypsin dilution used. We found, however, that this point frequently did not fall on the same curve as subsequent readings. In such cases the zero was determined by extrapolating back. Later we found that it was much better to follow this procedure entirely.

The time values obtained by interpolation for each group of dilutions were averaged and the mean of this figure was taken as the time

required to cause the change. A group of these curves for different dilutions in the same viscosimeter is shown in Fig. 1. It was found that the values so obtained varied nearly directly as the reciprocal of the relative amount of trypsin taken; that is, double the amount of enzyme requires half the time to cause the same change (Arrhenius' "QT" rule). In Table I the data obtained from such an experiment are shown. The figures are the average of four determinations. Several similar experiments were made. It will be noted that the method permits the calculation of the quantity of enzyme present

FIG. 1. Effect of increasing quantities of trypsin on the changes in viscosity of gelatin solutions.

with approximately 5 per cent error which we believe to be a satisfactory degree of precision.

One experiment was made to observe whether the method would serve with an equal degree of precision in the case of gelatin-pepsin digestion. Different dilutions of pepsin were mixed with properly adjusted gelatin solutions and these mixtures were run in duplicate. The results obtained, as in the previous experiments, show that the method can be applied satisfactorily in this case.

Another experiment was made to determine whether there was any relation between conductivity changes and viscosity changes during digestion with trypsin. No parallelism was observed between

TABLE I.
Change Caused in Viscosity of 3 Per Cent Gelatin Solution pH 7.4 by Various Amounts of Trypsin Q.
Temperature 34°C. $\pm 0.02^\circ\text{C}$.

Change in viscosity.	Mean time to cause per cent change noted. (Time interpolated from curve.)				Value of K.				Mean for all values of QT.	Q calculated. Mean of all values of QT Mean time	Q taken.
	When Q =				When Q =						
	1	2	4	8	1	2	4	8			
per cent	hrs. $\times 10^2$	hrs. $\times 10^2$	hrs. $\times 10^2$	hrs. $\times 10^2$							
5	26.8 $\pm$ 0.3	12.3 $\pm$ 0.4	6.5 $\pm$ 0.1	3.3 $\pm$ 0.1	26.8 $\pm$ 0.3	24.6 $\pm$ 0.8	26.0 $\pm$ 0.4	26.4 $\pm$ 0.8	25.9 $\pm$ 0.6	0.97 $\pm$ 0.05 0.98 $\pm$ 0.05 0.99 $\pm$ 0.06 0.94 $\pm$ 0.04	1
10	57.8 $\pm$ 0.5	27.0 $\pm$ 0.9	14.3 $\pm$ 0.3	7.1 $\pm$ 0.2	57.8 $\pm$ 0.5	54.0 $\pm$ 1.8	57.2 $\pm$ 1.2	56.8 $\pm$ 1.6	56.5 $\pm$ 2.7	2.10 $\pm$ 0.12 2.00 $\pm$ 0.12 2.10 $\pm$ 0.13 2.05 $\pm$ 0.11	2
15	97.0 $\pm$ 0.9	45.6 $\pm$ 1.5	24.2 $\pm$ 0.4	12.2 $\pm$ 0.4	97.0 $\pm$ 0.9	91.2 $\pm$ 3.0	96.8 $\pm$ 1.6	97.6 $\pm$ 3.2	95.7 $\pm$ 4.6	4.00 $\pm$ 0.10 3.95 $\pm$ 0.24 3.95 $\pm$ 0.21 4.01 $\pm$ 0.18	4
20	154.0 $\pm$ 2.1	74.8 $\pm$ 1.9	35.5 $\pm$ 0.2	17.0 $\pm$ 0.4	154.0 $\pm$ 2.1	149.6 $\pm$ 3.8	142.0 $\pm$ 0.8	136.0 $\pm$ 3.2	145.5 $\pm$ 5.3	7.89 $\pm$ 0.38 7.35 $\pm$ 0.45 7.82 $\pm$ 0.35 8.55 $\pm$ 0.42	8

these. Since it is probable that the change in viscosity represents the hydrolysis of the gelatin molecule itself while we know the conductivity change to be a function, under the proper conditions of experiment, of the complete hydrolysis, no direct connection between viscosity and conductivity changes would be expected.

The course of the reaction as followed by the viscosity curve is not monomolecular, so it differs from the changes in viscosity taking place during spontaneous hydrolysis as studied by von Schroeder (5).

SUMMARY.

A quantitative method is described which permits a determination of the relative amount of trypsin or pepsin present in a gelatin-enzyme digestion mixture, provided the gelatin and trypsin solutions are purified.

This method is dependent upon the change in viscosity of such solutions. It is found that the time required to cause a given percentage change in the viscosity is nearly inversely proportional to the amount of enzyme present.

It is pointed out that the particular value of the method lies in the fact that enzyme reactions which take place in the presence of "buffer" salts may be studied.

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