# THE COMBINATION OF ENZYME AND SUBSTRATE.

## I. A Method for the Quantitative Determination of Pepsin.

## II. The Effect of the Hydrogen Ion Concentration.

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### *I. A Method for the Quantitative Determination of Pepsin.*

Considerable experimental evidence has been obtained by various authors to show that enzymes are removed from their solutions by insoluble substances.[1,2] Pepsin has been especially studied from this view-point. Dauwe[3] showed that this property of removing pepsin from its solution was not common to all substances and was connected in some way with the digestive action of the enzyme. He was also able to show that the size of the particles, at least in the case of egg albumin, was without any marked effect on the amount of pepsin removed.

Abderhalden[4] and his coworkers state that pepsin is removed almost completely from its solution by insoluble proteins on which it acts and they consider that this plays an important rôle in the kinetics of the reaction. These results were partially confirmed by Leary and Shieb.[5]

[1] For the literature see Bayliss, W. M., The nature of enzyme action, London, 3rd edition, 1914.

[2] Nelson, J. M., and Griffin, E. G., *J. Am. chem. Soc.*, 1916, xxxviii, 1111.

[3] Dauwe, F., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 426. This paper reviews the earlier work.

[4] Abderhalden, E., and Steinbeck, E., *Z. physiol. Chem.*, 1910, lxviii, 293. Abderhalden, E., and Strauch, F. W., *ibid.*, 1911, lxxi, 315. Abderhalden, E., and Wachsmuth, F., *ibid.*, 339. Abderhalden, E., and Friedel, F., *ibid.*, 449. Abderhalden. E., and Kramm, F., *ibid.*, 1912, lxxvii, 462.

[5] Leary, J. T., and Shieb, S. H., *J. Biol. Chem.*, 1916–17, xxviii, 393.

Bayliss[1] attaches considerable importance to the combination of enzyme and substrate and considers it an essential point in the theory of enzyme action. Van Slyke and Cullen[6] were able to formulate the kinetics of enzyme action on the basis of the law of mass action by assuming the existence of a compound between enzyme and substrate.

The study of this combination, however, has not furnished any quantitative experimental data as to the nature of the reaction or the influence of various factors on it—due largely to the difficulty of determining quantitatively the amount of enzyme.

It seemed important, therefore, to obtain quantitative experimental data on this subject. In order to do this it was necessary to have a convenient and accurate method for the determination of pepsin. It was found that the change in the conductivity during the digestion of egg albumin by pepsin afforded such a method. Sjöqvist[7] found that there were marked changes in the conductivity during pepsin digestion. His results were confirmed with the exception that the change was found not to follow the actual rate of digestion. It can be used therefore only as an empirical method for the determination of pepsin and not for the study of the kinetics of the reaction, as was done by Sjöqvist. The reason for this divergence is probably due to the fact that the change in conductivity is due to two causes; (1) the liberation of free acid (carboxyl) groups which increase the conductivity, and (2) the liberation of free amino groups which bind some of the free acid and so decrease the conductivity. This explanation is borne out by the following facts.

With dilute solutions of egg albumin containing strong acetic acid (pH 2.3) there is a regular small increase in the conductivity which nearly parallels the increase in free amino groups as followed by the increase in amino nitrogen. This is due to the fact that, owing to the very large excess of free undissociated acid present, the ions which are removed from solution by combination with the free amino groups are replaced by the dissociation of more acid, and so kept nearly constant. The slight increase in conductivity is therefore due to the liberation of free carboxyl groups. In strong acid solu-

[6] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 141.

[7] Sjöqvist, J., *Skandin. Arch. Physiol.*, 1893–95, v, 277.

tions, however, as hydrochloric, there is a rapid decrease in the conductivity—due to the removal of acid ions by combination with the liberated amino groups. This change is so much larger than the increase due to the acid groups set free by the protein that the increase in conductivity due to the latter is more than compensated. It has already been shown[8] that the actual rate of digestion is approximately the same in all acids at the same reaction so that the differences in the changes in the conductivity cannot be ascribed to differences in the rate of digestion.

It was found that the maximum change occurs in strong solutions of egg albumin titrated to pH 2.6 with hydrochloric acid. This solution was therefore used. The conductivity was followed by means of the apparatus described by Taylor and Acree.[9] The electrodes were of the dipping type and were immersed in the solution in order to make a reading. It was found that the percentage change in conductivity was constant for a given quantity of pepsin, irrespective of the absolute value of the original conductivity. The readings and figures are therefore given in terms of the increase in the scale readings on the bridge, which for small readings are equivalent to the percentage change. The measurements were carried out as follows.

25 cc. of a 3 per cent egg albumin solution were pipetted into a series of large "Non-sol" test-tubes and suspended in a water bath at 37° ± 0.02°. The electrodes were immersed in the solution and 1 cc. of the pepsin solution was added. The external resistance was then set so as to give a bridge reading of 500; *i.e.*, the middle of the bridge. The change in conductivity was now followed by the bridge readings. These figures are related to the actual change in resistance of the solution by means of the formula $\frac{X}{R} = \frac{A}{1{,}000 - A}$ where $X$ = resistance of the solution, $R$ is the external resistance, and $A$ is the bridge reading.

The figures given in Table I are the increase in the value of $A$ and are very nearly proportional to the percentage increase in the resistance.

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

[9] Taylor, W. A., and Acree, S. F., *J. Am. Chem. Soc.*, 1916, xxxviii, 2396.

Readings were taken at intervals so as to give points corresponding to changes of 2 to 4 units of the bridge reading (which could be easily read to 0.25 units). These points were then plotted on a large scale by means of a flexible "spline" and weights, so that the curves were

TABLE I.

*Change in Conductivity of Solution of Egg Albumin with Varying Amounts of Pepsin.*

25 cc. of egg albumin solution, pH 2.6. *R* 320.
Temperature 37° ± 0.02°C. *A* at beginning 500.

Relative amount of pepsin added in volumes of 1 cc.

| 100 | | 50 | | 25 | | 12.5 | |
|---|---|---|---|---|---|---|---|
| Time. | Increase in scale reading. | Time. | Increase in scale reading. | Time. | Increase in scale reading. | Time. | Increase in scale reading. |
| *min.* | | *min.* | | *min.* | | *min.* | |
| 3 | 4.0 | 4 | 3.0 | 3 | 1.5 | 7 | 2.2 |
| 8 | 8.5 | 13 | 7.8 | 17 | 6.0 | 9 | 2.7 |
| 14 | 12.0 | 23 | 11.0 | 28 | 8.0 | 26 | 5.2 |
| 18 | 14.5 | 39 | 16.0 | 46 | 11.5 | 34 | 6.7 |
| 31 | 21.0 | 51 | 19.5 | 60 | 13.5 | 67 | 9.2 |
| 43 | 26.0 | 88 | 26.5 | 93 | 18.5 | 102 | 13.2 |
| 83 | 38.0 | | | | | | |

| Change. | | Time necessary to cause change. | | |
|---|---|---|---|---|
| Increase in scale reading. | Time. | | | |
| | *min.* | *min.* | *min.* | *min.* |
| 5 to 10 | 6.0 | 11.8 | 22.4 | 44.0 |
| 10 to 15 | 8.1 | 14.4 | 32.0 | |

| Interval. | Relative amount of pepsin calculated. | | | |
|---|---|---|---|---|
| 5 to 10 | (100) | 51 | 27 | 13.6 |
| 10 to 15 | (100) | 56 | 25 | |
| Average..... | (100) | 53 | 26 | 13.6 |

30 to 50 cm. long. The time necessary to cause a given change was then interpolated from these curves. In this way errors in individual readings were smoothed out and, as the curves are regular, accurate values for the time necessary to cause a given change could be ob-

tained. Since there is always some uncertainty about the zero reading the interval from 0 to 5 was omitted and the time to cause a change of from 5 to 10 and from 10 to 15 units was taken. The values obtained in this way were then averaged and the result taken as proportional to the reciprocal of the amount of pepsin present. By comparing this figure with the corresponding one from a control containing a standard amount of pepsin, the relative quantity of pepsin present could be determined, since it was found that the time

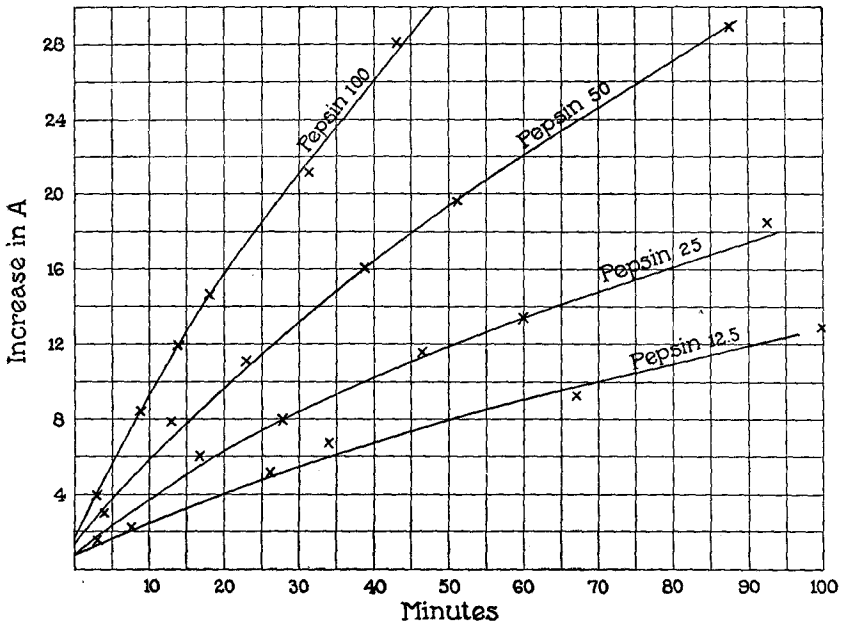


FIG. 1.

necessary to cause any given change was inversely proportional to the amount of pepsin present. That is, double the quantity of pepsin requires half the time, etc. (Arrhenius' "QT" rule).[10]

Table I and Fig. 1 give the results with a series of solutions containing different amounts of pepsin. It will be seen that the amount of pepsin can be determined with an accuracy of ± 2 to 3 per cent,

[10] Arrenhius, S., Quantitative laws in biological chemistry, London, 1915.

and further that ten to fifteen determinations can be made in the course of 1 to 2 hours depending on the amount of pepsin present.

It was now possible to determine quantitatively the amount of pepsin removed from solution by various substances.

Table II shows the results of such a series with coagulated and dried egg albumin. The control experiments show that the decrease in the amount of pepsin is not due to the destruction of the pepsin on standing or to the retarding effect of the products of reaction.

TABLE II.

*Change in Concentration of Pepsin in Various Solutions.*

Temperature 25°C.
pH of all solutions, 2.5.
1 cc. of solution pipetted off and pepsin estimated at time noted.

| Time. | Relative amount of pepsin per cc. | | |
|---|---|---|---|
| | 20 cc. of pepsin solution. | 20 cc. of pepsin solution + 1 gm. of egg albumin in solution. | 20 cc. of pepsin solution + 1 gm. of coagulated egg albumin. |
| *min.* | | | |
| 1 | (100) | 101 | 95 |
| 10 | 101 | 101 | 84 |
| 20 | 94 | 96 | 82 |
| 40 | 95 | 100 | 86 |
| 80 | 100 | 128* | 80 |
| 160 | 97 | 98 | 86 |

* This is an experimental error. The curve was irregular and gave widely divergent results for different intervals.

A series of experiments was now made with various substances. The results are summarized in Table III. It is obvious that the removal of the pepsin is not purely a matter of surface but that it is dependent in some way on the substance itself.[11]

This fact is brought out more strongly in the experiments summarized in Table IV and Fig. 2, where coagulated egg albumin of different sized particles was used. It is evident that the equilibrium

[11] The author does not doubt the existence of adsorption or concentration in the surface layer in the sense of Willard Gibbs. This phenomenon, however, is evidently of subordinate importance here.

reached is independent of the size of the particles and therefore of their surface. These experiments were repeated under slightly different conditions several times—always with the same result. This would indicate that the process is either one of solution, in which

TABLE III.

*Removal of Pepsin from Solution by Different Substances.*

10 cc. of pepsin solution, pH 2.5, + 0.5 gm. of substances noted. Allowed to stand 10 min. at 25°C. and pepsin estimated in 1 cc. of solution.

| Substance. | Relative quantity of pepsin per cc. |
|---|---|
| Control; pepsin solution alone | 100 |
| Starch | 98 |
| $CaSO_4$ | 86 |
| Agar | 103 |
| Kaolin | 100 |
| Blood charcoal | 15 |
| Casein | 70 |
| " (coagulated, dried, and ground to 40 mesh) | 74 |
| " (extracted with boiling alcohol for 24 hrs.) | 60 |
| " C (charred at 150°C.) | 90 |
| Egg albumin (coagulated, dried with acetone, and finely powdered) | 10 |

TABLE IV.

*Effect of Size of Particles of Egg Albumin, Coagulated, Dried, and Ground.*

2.0 gm. in 20 cc. of pepsin solution titrated to pH 2.5 + HCl.
1.0 cc. pipetted off and analyzed for pepsin at time noted.

| Time. | Size of particles. | |
|---|---|---|
| | Through 10 mesh but not through 20 mesh. | Through 80 mesh. |
| | Relative amount of pepsin per cc. | |
| *min.* | | |
| 1 | | 46 |
| 2 | 98 | 21 |
| 4 | 93 | 30 |
| 8 | 72 | 12 |
| 30 | 32 | 10 |
| 60 | 12 | 14 |
| 120 | 10 | 11 |

case the law of partition coefficients should be found to hold, or of chemical combination, in which case the law of mass action should apply. Preliminary experiments indicate that the process follows the law of partition coefficients.

It may be objected that the effective surface is not the actual surface of the particles but some fine interior structure which is the same in all. The particles, however, swell in acid and appear translucent and homogeneous. It would seem that any interior surfaces must be of nearly intermolecular dimensions. In this case, of course, all chemical phenomena may be considered "surface" phenomena.

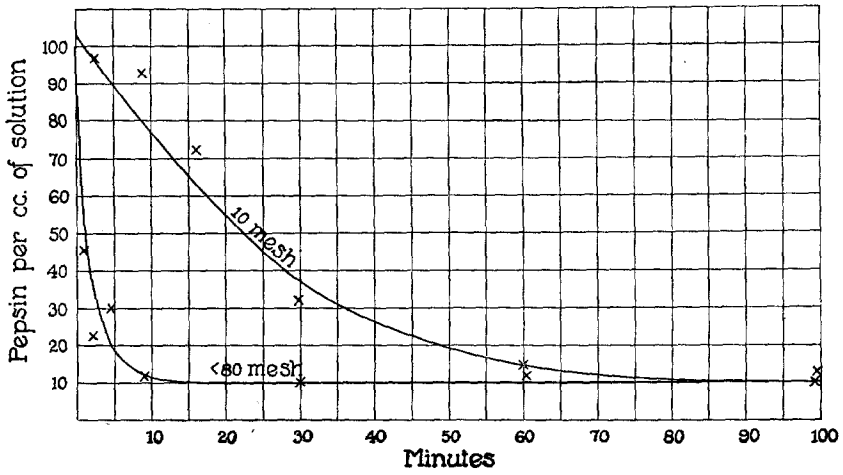


FIG. 2.

*II. The Effect of the Hydrogen Ion Concentration.*

Inasmuch as the activity of pepsin is dependent to a large extent on the hydrogen ion concentration it appeared probable that the combination of pepsin with its substrate would be a function of the same variable. Table V and Fig. 3 show the results of a series of experiments made at different hydrogen ion concentrations. There is a decided optimum for the combination of pepsin with its substrate corresponding (within the limits of error) to the optimum for digestion. These experiments were repeated with casein with approximately the same results. The optimum zone for the digestion of proteins

by pepsin therefore is due to the fact that at this degree of acidity more pepsin combines with the protein than in either a more or less acid solution. Van Slyke and Zacharias,[12] from a study of the constants of their equation for the action of urease, decided that the hydrogen ion concentration affected the *rate* of combination of the

TABLE V.

*Effect of Reaction of Solution on Combination of Pepsin and Coagulated Egg Albumin.*

Experiment A.

Temperature 25°C.

0.5 gm. of egg albumin suspended in 10 cc. of HCl of increasing strength. 1.0 cc. of strong pepsin solution added. Tube shaken, allowed to stand 1 min., and clear liquid pipetted off. pH measured (by gas chain) in part of this sample. 5.0 cc. of remainder brought to same reaction in all tubes by addition of a few drops of strong HCl. All brought to same volume with water and pepsin estimated in 1 cc.

Experiment B.

Same as A, but allowed to stand 2 min.

| pH of solution. | Relative amount of pepsin per cc. | | No albumin. (Control.) | Relative amount of pepsin combined. | |
|---|---|---|---|---|---|
| | Experiment A. | Experiment B. | | Experiment A. | Experiment B. |
| 0.88 | 77 | | 100 | 23 | |
| 1.00 | | 79 | | | 21 |
| 1.24 | 69 | | | 31 | |
| 1.3 | | 68 | | | 32 |
| 2.08 | 60 | | 100 | 40 | |
| 2.2 | | 50 | | | 50 |
| 2.75 | 63 | | | 37 | |
| 2.8 | | 47 | | | 53 |
| 3.5 | 64 | | | 36 | |
| 3.9 | | 68 | | | 32 |
| 4.4 | 77 | 80 | | 23 | 20 |
| 4.9 | 100 | 86 | 100 | 0 | 14 |
| 5.7 | 100 | 94 | 100 | 0 | 6 |

enzyme and substrate. In the case of pepsin, however, it is not the *rate* of combination but the *amount* which is influenced. This is shown by the fact that little or no pepsin is removed from its solution by its substrate at a reaction of 5.0, no matter how long they are

[12] Van Slyke, D. D., and Zacharias, G., *J. Biol. Chem.*, 1914, xix, 181.

left in contact. It seems probable that this is due to a change in the condition of the protein rather than to a change in the enzyme, since, according to Ringer,[13] the optimum reaction is different for different proteins.

The simplest explanation of the above facts would seem to be that the quantity of ionized protein present determines the amount of pepsin which combines with the protein, and hence also determines the rate of digestion. Some direct evidence confirming this hypothesis has been obtained and will be discussed fully later.

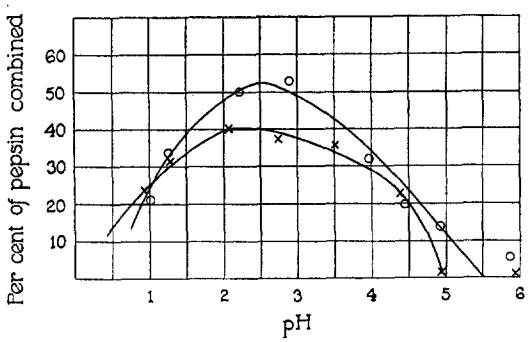


FIG. 3.

## SUMMARY.

1. A quantitative method for the determination of pepsin is described depending on the change in conductivity of a digesting egg albumin solution.

2. The combination of pepsin with an insoluble substrate has been followed by this method.

3. The amount of pepsin removed from solution by a given weight of substrate is independent of the size of the particles of the substrate.

4. There is an optimum zone of hydrogen ion concentration for the combination of enzyme and substrate corresponding to the optimum for digestion.

5. It is suggested that the pepsin combines largely or entirely with the ionized protein.

[13] Ringer, W. E., *Kolloid-Z.*, 1916, xix, 253.