THE KINETICS OF TRYSIN DIGESTION.

I. EXPERIMENTAL EVIDENCE CONCERNING THE EXISTENCE OF AN INTERMEDIATE COMPOUND.

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(Received for publication, November 20, 1923.)

The anomalies of enzyme action have frequently been accounted for by the assumption of an intermediate compound between the enzyme and the substrate. It was shown, however, by Nelson and Hitchcock that in the case of invertase the experiments could not be accurately calculated on this basis. The writer found with trypsin that although the effect of varying the substrate concentration could be accounted for, the effect of varying both the substrate and inhibiting substances could not, provided the law of mass action was assumed to hold. The question is one of such importance from the view of all enzyme reactions, however, that it seems important to obtain direct experimental evidence in regard to the existence of such a compound.

The statement is frequently made that the substrate protects the enzyme from heat inactivation and this has been considered as evidence for the existence of a compound between the substrate and enzyme. It has been found, however, that this protective action does not become manifest at once but only after the enzyme and substrate have been in contact, when it becomes more and more pronounced. It can further be shown that the products of digestion afford very marked protection and that this effect is apparent at once. It appears probable, therefore, that the protective action of the substrate solution is not due to the substrate itself but to the products formed by the action of the enzyme and that this is the reason for the delay in the protective action.

A second experimental test is furnished by the action of the enzyme on two substrates at once. If the enzyme is combined with one, it evidently cannot act on the other. This experiment has been performed by the writer on a mixture of casein and gelatin with the result that the rate of reaction of the mixture was the sum of the two independent reactions. In this experiment the sum of the actions was measured which renders the result more complicated. By changing the method, however, it is possible to make a direct experimental comparison and this has been done in the present paper. This is made possible by the fact that casein is insoluble in trichloroacetic acid and may therefore be determined in the presence of gelatin, which is soluble. The experiment, therefore, consists in adding the same amount of trypsin to two solutions each containing 2.5 per cent casein and one of which contains in addition 3 per cent of gelatin. In order to avoid complicating effects due to the retarding action of the products of the reaction, it is necessary to use a large amount of trypsin.

Experiment I. The Effect of Gelatin on the Rate of Hydrolysis of Casein. Casein Solution.—2.5 gm. of casein dissolved in m/10 phosphate buffer pH 7.6, the pH readjusted to 7.6, and the solution made up to 100 cc.

Casein-Gelatin Solution.—2.5 gm. of casein and 2.5 gm. of gelatin dissolved and made up to 100 cc. as above. pH 7.6.

Both solutions brought to 37°C. and 10 cc. of trypsin added to each. 2 cc. samples pipetted into 10 cc. of 5 per cent trichloroacetic acid at time intervals shown in the figure. These solutions were allowed to stand 1 hour, centrifuged, the residue redissolved in alkali, and reprecipitated with trichloroacetic acid. The precipitate was then dissolved in alkali, made up to 10 cc., and 1 cc. analyzed for total nitrogen by the micro Kjeldahl method of Folin and Farmer.5

Experiments in which the concentration of gelatin alone varied show that the amount digested by a given amount of trypsin is practically independent of the gelatin concentration if the latter is greater than 2 per cent. On the intermediate compound theory this would be accounted for by supposing that the enzyme is saturated with substrate. It is evident that if the trypsin is saturated with gelatin, it cannot act on the casein. The experiment showed, however, that the rate of casein digestion is unaffected by the presence of the gelatin.

This experiment shows conclusively that the anomalous results obtained in varying the gelatin concentration cannot be due to the formation of a compound between the enzyme and gelatin, unless it be further assumed that there are present two enzymes, one of which attacks the gelatin and one the casein. The writer has never suc-
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ceeded in obtaining the slightest evidence for the existence of more than one proteolytic enzyme in the trypsin (Fairchild's) used in these experiments.

The activity of the solutions has always been tested by their effect on the viscosity of gelatin and the results obtained in this way have never disagreed with the activity as determined with another protein, although the test has been made a great number of times and under a large variety of conditions. The existence of two such enzymes may, however, be tested for as follows. As was stated above, gelatin or casein solutions do protect trypsin from heat inactiva-

Table I.


1 cc. of 2 per cent dialyzed trypsin added to 10 cc. of m/10 phosphate buffer pH 7.6, containing noted amount of casein or gelatin at 45°C. 1 cc. pipetted into 10 cc. of cold buffer solution after time noted, and gelatin-liquefying power of this solution tested as described in an earlier paper. 6

<table>
<thead>
<tr>
<th>Solution</th>
<th>Control</th>
<th>4 per cent gelatin</th>
<th>4 per cent casein</th>
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<tbody>
<tr>
<td>min.</td>
<td>Relative gelatin-liquefying power after</td>
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<tr>
<td>1</td>
<td>100</td>
<td>100</td>
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<tr>
<td>60</td>
<td>6</td>
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experiment is shown in Tables I and II. The experiments show the peculiarity mentioned at the beginning of this paper; namely, that the protective action is not apparent at once but only after the lapse of a few minutes. In the writer's opinion, it is due not to the substrate but to the products of the reaction. If, however, the existence of an intermediate compound is assumed to account for the anomalies of the concentration effect, then it should logically be used to account for the protective effect. On this basis, the above experiment proves that there is only one enzyme which acts on both products and hence the experiment in which casein and gelatin are digested together cannot be explained by the existence of two enzymes.

**TABLE II.**

*Protective Action of Casein and Gelatin on Casein-Hydrolyzing Power of Trypsin.*

Experimental procedure the same as in Table I, except that the change in viscosity of casein was used as the indicator instead of gelatin.

<table>
<thead>
<tr>
<th>Solution ..........</th>
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<th>4 per cent casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>min.</td>
<td>Relative casein-hydrolyzing power after</td>
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<tr>
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**SUMMARY.**

1. The rate of hydrolysis of a casein solution by trypsin is not affected by the addition of gelatin. The trypsin, therefore, is not combined with the gelatin unless there is a separate enzyme for casein and for gelatin.

2. The presence of casein protects the gelatin-splitting power of trypsin from heat inactivation, and the presence of gelatin protects the casein-splitting power from heat inactivation.

3. It does not seem possible to account for both the above results by the assumption of an intermediate compound between enzyme and substrate, since, in order to account for the first result, a different enzyme must be assumed for each protein, while, to account for the second result, it must be assumed that the same enzyme attacks both.