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It has been a well known fact for some time that blood serum has the property of retarding the digestive activity of trypsin. We have, however, no satisfactory information regarding either the nature of the substance which effects the retardation or the type of chemical reaction involved in the process. At one time the inhibitory agent was thought to be an immune antibody and in accordance with custom it was called antitrypsin. There is no evidence to support the idea that the active agent is of the nature of an immune body, so the term antitrypsin is incorrect.

Landsteiner (1) found that the retarding effect was associated with the albumin fraction of serum. Hedin (2, 3) then employed preparations of serum albumin as "antitrypsin" in one of the few experimental inquiries that have been made with the view of studying the nature of the trypsin-antitrypsin reaction (4). While these studies were in progress, Hedin (5) discovered that charcoal adsorbed trypsin and that the equilibrium between these two substances satisfied the conditions demanded by the equation representing adsorption phenomena. A comparison of the experimental results obtained when retardation was effected by charcoal with those of the experiments when serum albumin was used presented so many similarities that Hedin was led to the conclusion that the equilibrium between trypsin and "antitrypsin" (serum albumin) was also governed by adsorption phenomena; that is, the trypsin was adsorbed by the serum albumin as it was by the charcoal. It was, however, not possible to obtain values from the experiments to substitute in the adsorption equation. They seemed plausible, though, because

1Hedin (3), p. 495.
both reactants were known to be colloids and it was the accepted belief that the behavior of colloids in solution is governed chiefly by adsorption phenomena. Also, in accordance with this general idea, adsorption was regarded as an essential factor in all enzyme reactions. However, the correctness of Hedin's interpretation concerning the nature of the equilibrium between serum albumin and trypsin, as well as the fundamental assumptions upon which they are based, are questioned by experimental results recently obtained in a study of the mechanism of the inhibiting action of other substances on trypsin, pepsin, and invertase.

In the experiments referred to, the equilibrium between trypsin and the inhibitory substance formed by the action of the enzyme on protein was studied. Gelatin was hydrolyzed by trypsin until no further increase in formol titration was noted and a solution was made from the products formed. A method was developed by means of which the amount of retardation caused by the inhibiting agent in the solution could be measured quantitatively (6). The retardation effected by the inhibiting solution was accounted for by the conception that the trypsin and the active inhibiting agent combine to form a compound that is inactive. The compound thus formed dissociated so that part of the original trypsin added was combined and inactive and part free or active. In order to subject the reaction between trypsin and the inhibiting substance to analysis it was assumed that the equilibrium for the compound formed by the combination of these substances is governed by the law of mass action and that the rate of hydrolysis is proportional to the concentration of free enzyme. An equation derived from the law of mass action was formulated to express the experimental results to be demanded if the assumptions made were correct. Experiments were made (7) in which each component entering the reaction was varied independently, and the results observed were in such close agreement with those calculated from the equation developed that the assumptions were satisfactorily supported. These results are taken as evidence which supports the view that an enzyme reaction may be explained by conceptions other than those embracing adsorption theories. To assume the equilibrium studied to be governed by surface phenomena, it would be necessary to suppose that the tryp-
sin adsorbed the inhibiting agent, since there is no evidence that this
is present in other than true solution. Moreover, the results would
not lend themselves to mathematical analysis on such an assumption
because the adsorption equation contains no term to represent the
amount of adsorbent combined. There would then be no way to
determine a value for the trypsin combined and for the free trypsin.

The same mechanism described for trypsin was found in the case
of pepsin (8). In this case, the combination was between pepsin
and the digestion products formed during the time pepsin was acting
on the protein. Further evidence that enzyme reactions may con-
form to the same principles is found in the work of von Euler and
Svanberg (4). These investigators have shown this to be so for the
equilibrium between invertase and various crystalloidal substances
which retard its activity. Inasmuch as it is possible to account for
enzyme reactions on the basis of the laws of general chemistry, there
seems to be no theoretical reason to disregard the fact and seek
explanations in the adsorption theories. Further, as to Hedin's
observation regarding the adsorption of trypsin by charcoal, it is
perfectly possible that trypsin may be adsorbed by charcoal and yet
react with other substances according to the principles stated in the
mass action law. Such instances are known in general chemistry.

With the information supplied by these facts, together with theoreti-
cal considerations based on them, it has seemed important to make
further inquiries about the equilibrium concerned in the retardation
of tryptic activity by constituents in the blood. In addition to these
considerations, a better understanding of the nature of this reaction
is of importance in pathology since the inactivating property of
blood serum for tryptic digestion has engaged the interest of several
investigators in this field for a number of years. It has been claimed
that this property of serum varies with respect to certain diseases
and also that it bears an important relation to immune states of the
human body. In this communication we will report the results of
an investigation planned to determine whether or not this inhibiting
effect can be accounted for by the same mechanism as has been found
to hold in the case of the other inactivating substances already
mentioned. Such an investigation is made possible by having at
our disposal a method which permits a quantitative determination
of the amount of trypsin present in a digestive mixture during the course of hydrolysis. This method has been described in a previous paper (9).

In the paper referred to, in which we described the method we employed in the present investigation, it was pointed out that the time required to cause a given change in the viscosity of a gelatin-trypsin solution was inversely proportional to the amount of trypsin present. This fact then permits the use of that value \( \frac{1}{(T \text{ hours})} \) as a measure for the enzyme. For the observations to be reported we chose 20 per cent change in viscosity as the experimental endpoint. As an arbitrary standard for a unit of trypsin we adopted that amount required to change the viscosity of the gelatin solution 20 per cent in 1 hour. The gelatin solutions used in all the experiments contained 3 per cent dry weight of gelatin and were adjusted to pH 7.4. These solutions were heated to 50°C., then placed in a water bath, and kept at 34°C., where they remained until used for an experiment. The interval was about 14 to 16 hours. The trypsin solutions were prepared as previously described (7). We have employed plasma instead of serum as the inhibiting substance. This was done at first because we were using plasma for other experiments and later we found that the inhibiting substance was much more stable in plasma than in serum. This fact will be investigated at another time. The plasma was obtained from rabbits as previously described (10). A 1:10 or a 1:20 dilution of plasma was found to give satisfactory inhibition.

The first experiment was made to determine whether we could measure the amount of retardation quantitatively. In the same experiment we were also able to obtain information regarding the uniformity of the retarding effect of plasma obtained from different animals. 1:20 dilutions of four different samples of plasma were made and equal volumes of these were mixed with equal volumes of a 2:25 dilution of trypsin solution. 0.5 cc. of this mixture was added to 25 cc. of gelatin of concentration and reaction as previously stated, then after thorough mixing, 10 cc. of the mixture were pipetted into separate viscosimeters and observations on the time of outflow were made at frequent intervals. In another viscosimeter there
were 10 cc. of gelatin which contained the same amount of trypsin but with no inhibitor. If the amount of retardation can be measured quantitatively we should find that the inhibition is practically independent of the progress of the change in viscosity over the range selected for our experiments. The results tabulated in Table I show that this is the case within the limits of precision the method

TABLE I.

Effect of Plasma on Retarding the Activity of Trypsin.

<table>
<thead>
<tr>
<th>Change in viscosity</th>
<th>T hrs. for 20 per cent change in viscosity</th>
<th>T inhibitor T control</th>
</tr>
</thead>
<tbody>
<tr>
<td>per cent</td>
<td>Control</td>
<td>Plasma</td>
</tr>
<tr>
<td>5</td>
<td>0.08</td>
<td>19.5</td>
</tr>
<tr>
<td>10</td>
<td>0.18</td>
<td>44.0</td>
</tr>
<tr>
<td>15</td>
<td>0.31</td>
<td>75.0</td>
</tr>
<tr>
<td>20</td>
<td>0.52</td>
<td>115.0</td>
</tr>
</tbody>
</table>

TABLE II.

Effect of Retardation by Plasma from Different Individuals.

25 cc. of 3 per cent gelatin of pH 7.4 + 0.5 cc. of trypsin-inhibitor mixture or trypsin-water mixture for control. 10 cc. pipetted into a viscosimeter.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>T hrs. for 20 per cent change in viscosity.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control: Gelatin { trypsin 2:25 dilution, 0.25 cc. } water, 0.25 cc.</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>2. Plasma 1: Gelatin { trypsin dilution, 0.25 cc. } plasma 1:10 dilution</td>
<td>1.20</td>
<td></td>
</tr>
<tr>
<td>3. &quot; 2: &quot; &quot; &quot;</td>
<td>1.15</td>
<td></td>
</tr>
<tr>
<td>4. &quot; 3: &quot; &quot; &quot;</td>
<td>1.19</td>
<td></td>
</tr>
<tr>
<td>5. &quot; 4: &quot; &quot; &quot;</td>
<td>1.15</td>
<td></td>
</tr>
</tbody>
</table>

allows. In Table II the effect of retardation caused by plasma from different individuals is shown. It will be noted that there is apparently good uniformity.

In the studies concerning the equilibria between the enzymes and inhibitory agents already referred to, it was found in each case that the equilibrium was reached practically instantaneously. Because
EQUILIBRIUM BETWEEN ANTITRYPSIN AND TRYPSIN

of this fact the experimental result was not influenced by the order of mixing the reactants or by the length of time they were allowed to stand. It is important then that we obtain data in this connection for the equilibrium with which our study is concerned; that is, whether there is a difference in the experimental result when we first mix the dilutions of trypsin solution and plasma, then add the mixture to gelatin, or when the diluted plasma is first added to the gelatin, followed by the trypsin solution. If the equilibrium under consideration is similar to the charcoal-trypsin equilibrium, we would expect to find different results since in this case the equilibrium is not reached until some time after the reactants are brought together. It is also

### TABLE III.

**Influence of Order of Mixing.**

<table>
<thead>
<tr>
<th></th>
<th>( T ) hrs. for 20 per cent change in viscosity</th>
<th>( Q - \frac{1}{7} ) hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 cc. gelatin +</td>
<td>(trypsin 2:25 dilution, 0.5 cc.) (control.)</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>water, 0.5 cc.</td>
<td>1.15</td>
</tr>
<tr>
<td>50 cc.</td>
<td>(trypsin 2:25 dilution, 0.5 cc.)</td>
<td>1.30</td>
</tr>
<tr>
<td></td>
<td>plasma 1:20</td>
<td>0.77</td>
</tr>
<tr>
<td>(50 cc. gelatin—</td>
<td>(trypsin 2:25 dilution, 0.5 cc.)</td>
<td>1.36</td>
</tr>
<tr>
<td>plasma 1:20 dilution</td>
<td></td>
<td>0.75</td>
</tr>
<tr>
<td>2:25, 0.5 cc.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

not easily reversible. Hedin, as previously stated, found that the equilibrium between trypsin and "antitrypsin" was influenced by the time of standing, and this result was accepted as evidence to indicate an analogy between the behavior of charcoal and "antitrypsin."

The results of the first experiment made showed a difference when the order of mixing was different. The difference was a little greater than our allowable experimental error. This was true in the second experiment but the difference was reversed. Because of the importance of the information sought, we repeated the experiment several times and while differences were observed, there was no regularity with respect to the order of mixing. Ten such experiments were made and the data obtained from the tenth experiment are given in Table III. It will be noted that within the precision of our method
the order of mixing does not influence the experimental result. However, it is much more difficult to add the plasma and trypsin solutions separately, and for this reason further experiments to be reported were made by first mixing the plasma and trypsin. Table IV shows the results of an experiment made to determine the influence of time of standing on the inhibiting effect. This factor too is shown to be of no importance, since the ratio \( T_{\text{inhibitor}} : T_{\text{control}} \) remains practically constant. Our conclusion then is that the equilibrium under consideration is one that is reached practically instantaneously and is rapidly and completely reversible, as was found to be the case with the equilibria already mentioned.

**TABLE IV.**

*Influence of Time of Standing.*

Equal volumes of trypsin, dilution 2:25, and plasma, dilution 1:20, mixed, then 0.5 cc. of this mixture added to 25 cc. of gelatin at intervals noted.

<table>
<thead>
<tr>
<th>Time to change 15 per cent viscosity.</th>
<th>( T_{\text{plasma}} )</th>
<th>( T_{\text{control}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>After</td>
<td>0 hrs. 2.5 hrs.</td>
<td>0 hrs. 2.5 hrs.</td>
</tr>
<tr>
<td>Control</td>
<td>0.48 0.66</td>
<td>1.37</td>
</tr>
<tr>
<td>Test</td>
<td>0.78 1.03</td>
<td>1.32</td>
</tr>
</tbody>
</table>

This experiment is a critical one for identifying the nature of the reaction since adsorption or other reactions in a two-phase system are as a rule only partially or slowly reversible. On the other hand, reactions in homogeneous solutions are usually easily and rapidly reversible, in accordance with the law of mass action. We shall not attempt to develop a further discussion of the difference between our results and those obtained by Hedin. The experimental conditions in each case are entirely different. For example, Hedin's observations have to do with changes found in equal times, the digestion was allowed to proceed for 24 hours at 37°C., and the enzyme preparations were not the same. This method of procedure leads to confusion in the results with respect to the influence of spontaneous inactivation of the enzyme and of the inhibitive agent (11).
The observations so far stated make it appear that there is striking similarity between the behavior of the inhibiting agent contained in plasma and that contained in the other substances referred to. It now remains for experiments to be made which will further analyze the equilibrium under consideration. Three experiments were planned for this end: namely, the effect of adding increasing amounts of plasma to a constant amount of trypsin; the effect of varying the trypsin while the plasma was kept constant; the effect of varying the volume, i.e. the effect of dilution on the trypsin-plasma mixture.

The results of these experiments are given in Tables V, VI, and VII, and their graphic representation is shown in Figs. 1, 2, 3, and 4.
TABLE VII.

Effect of Diluting a Mixture of Trypsin and Inhibiting Substance.

\[ K = 0.17 \quad i = 70 \quad E = 20 \]

<table>
<thead>
<tr>
<th>Volume.</th>
<th>Time in hrs. for 20 per cent change in viscosity.</th>
<th>Trypsin.</th>
</tr>
</thead>
<tbody>
<tr>
<td>cc.</td>
<td>hrs.</td>
<td>Total.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Per 25.5 cc.</td>
</tr>
<tr>
<td></td>
<td>units</td>
<td>units</td>
</tr>
<tr>
<td>25.5</td>
<td>0.065</td>
<td>15.4</td>
</tr>
<tr>
<td>51.0</td>
<td>0.118</td>
<td>16.94</td>
</tr>
<tr>
<td>102.0</td>
<td>0.220</td>
<td>18.20</td>
</tr>
<tr>
<td>204.0</td>
<td>0.415</td>
<td>19.3</td>
</tr>
</tbody>
</table>

Fig. 1. Inactivation of trypsin caused by increasing quantities of plasma.
The curve in Fig. 1 represents the effect of adding increasing amounts of plasma to a constant quantity of trypsin. In this graph the units of combined trypsin are plotted as ordinates against the cubic centimeters of plasma added as abscissae. The slope of this curve increases very rapidly at first, then approaches an asymptote. This demonstrates the fact that the first unit amount of plasma added has a much greater inhibitive effect than subsequent unit amounts added. These results, though suggestive of reactions in qualitative agreement with the principles of the mass action law, could be explained equally well by other considerations.

In the second experiment where varying amounts of trypsin are added to a constant amount of plasma, we would expect to find either the percentage inactivation equal for each amount of trypsin or increasing with each decrease in the amount of trypsin. The data for this experiment are given in Table VII. The percentage inactivation is observed to increase with smaller amounts of trypsin. These results, together with those of the preceding experiment, suggest that the equilibrium between trypsin and the inhibiting agent in plasma is governed by the principles stated in the law of mass action.

It is possible to obtain additional information to bear on the suggestions stated; namely, to observe the effect of dilution on the dissociation of the trypsin-inhibitor compound. Equal volumes of trypsin solution and plasma were mixed and then this mixture was diluted in the order 1-2-4-8. 0.5 cc. of each dilution was added to gelatin solutions as previously stated. The results of this experiment are shown in Table VII. It will be noted that the total active trypsin does not decrease directly as the total trypsin. This is experimental evidence that the effect of dilution on the trypsin-inhibitor compound is to cause greater dissociation of it and therefore more free trypsin is present in the solution.

In summary, then, it may be said that the results obtained from these three experiments furnish evidence that strengthens the suggestion that the equilibrium between the inhibitory agent in plasma and trypsin is similar in behavior to the equilibria between the enzymes and inhibitory agents mentioned earlier in this paper. It now remains for us to determine how the experimental results can be calculated.
Formulation of Results.

In attempting to calculate the experimental results only one equation was found which would fit the curves for all three experiments. This equation was formulated from the assumption that the results obtained from our experiments indicate that one molecule of trypsin combines with one molecule of inhibiting agent to form one molecule of trypsin-inhibitor compound; that is, the reaction in equilibrium may be represented by the expression

\[ \text{Trypsin} + \text{inhibitor} \rightarrow \text{trypsin-inhibitor} \]

In order to determine the quantitative relation between these reactants in equilibrium the chemical expression can be put into a form for mathematical treatment. This is accomplished in the following equation where the reactants are expressed in terms of their concentrations.

\[
\frac{\text{Concentration of free trypsin} \times \text{concentration of free inhibitor}}{\text{Concentration of trypsin-inhibitor}} = \text{a constant}
\]

If now we let

\[ E = \text{the total trypsin} \]
\[ Q = \text{the free trypsin} \]
\[ I = \text{the units of inhibitor in } P \text{ cc. of plasma} \]

we may substitute these symbols and write the equation

\[
\frac{Q^2}{V} \left[ \frac{PI - (E - Q)}{V} \right] = K
\]

This simplifies to

\[
\frac{Q}{V} \left[ \frac{PI - (E - Q)}{V} \right] = K
\]

Solving this equation for \( Q \) we obtain

\[
Q = \sqrt{\left(\frac{PI + KV - E}{2}\right)^2 + KV} - \frac{PI + KV - E}{2}
\]

We can obtain values from our experimental results for all the terms in equation (1) except \( K \) and \( I \). It is possible, however, to get a solution of approximation by assuming values for \( I \), then solving...
for $K$. When this is done it is found that over a certain range of values $K$ is negative, another range of values gives $K$ positive values, but when these values are substituted in equation (2) the calculated results differ widely from the observed results. There is still another range of values for $I$ that give positive values for $K$ which yield a satisfactory agreement with the observed results. If the same value of $I$ is used for all three experiments one obtains a different $K$ in each instance. These values increase in the order of the experiments. We know that the inhibitive action of plasma decreases with time and this necessarily affects the value of $I$. It follows then that the value of $I$ must be different in each experiment; that is, the change in the inhibitive agent is quantitative rather than qualitative. It would be unreasonable to assume that the value of $K$ would actually vary, and the order of the variation found for the same value of $I$ is what might be expected; namely, that $I$ has the highest value in the first experiment and the lowest value in the third or last experiment. It is necessary, therefore, to take a fixed value for $K$, then substitute in equation (1) and solve for the value of $I$ in the different experiments. We accordingly took one of the values of $K$ determined for the second experiment. The $K$ chosen was that one calculated with an assigned value of $I$ which gave the most constant values of $K$ in the four observations of the experiment. The reason for selecting a $K$ from the second experiment will appear later. The values found for $I$ by this procedure decrease in the order of the experiments as follows: For the first experiment $I = 485$, for the second experiment $I = 275$, and for the third experiment $I = 70$.

At this time we are not prepared to discuss the conditions governing the spontaneous destruction of the inhibitive agent so we are not in a position to comment on the significance of the relative differences stated for $I$. Some indication of these relations can be formed, however, for the first and second experiments. From the curves in Figs. 2 and 3 it will be observed that for two units of trypsin, double the amount of plasma is required in the second experiment to combine the same amount of trypsin. This agrees almost exactly with the relative values of $I$ found by calculation and is strong evidence that the values of $I$ are not mere calculated figures.
The value of $K$ was taken from the second experiment because equation (1) was found to be the only expression that satisfied the curve (Fig. 3) for this experiment. The curves representing Experiments 1 and 3 (Figs. 2 and 4), however, may be satisfied by several equations. This is due to the fact that in these experiments the amount of inhibitor combined is small as compared with the amount that is free. As a consequence the term $(E-Q)$ is negligible with respect to the term $(PI)$. This fact allows the term $(E-Q)$ to be neglected in the numerator of equation (1), and this in turn permits one to obtain reduced forms of the equation which, when solved for $Q$, satisfy the curves for Experiments 1 and 3. The same conditions do not hold in Experiment 2; therefore, this simplification cannot be made.

In presenting the mathematical analysis of the experiments described, we realize that we have employed an equation containing two arbitrary constants. We are cognizant of the bearing this fact might have on the significance of our calculated results.

![Graph](image-url)

**Fig. 2.** Inactivation of trypsin caused by increasing quantities of plasma.
We feel justified, however, in accepting the agreement between the calculated and observed results as significant, since only one equation was found that fits the curves for all three experiments. Also in obtaining the calculations it was necessary to use different values of \( J \) which decrease in order from one experiment to the other, as would be expected from the known fact that the inhibitive action of
plasma decreases with time. Since the equation used is derived from the mathematical expression of the law of mass action, these considerations lead us to conclude that we have presented experimental evidence which, though not final, is more than just suggestive.

![Graph showing effect of dilution on a mixture of trypsin and plasma.]

Fig. 4. Effect of dilution on a mixture of trypsin and plasma.

that the following statements are true: (1) the inhibition of tryptic activity by plasma is effected by an agent which combines with trypsin to form an inactive compound; (2) this compound is dissociated into free trypsin and free inhibitor; (3) the conditions of equilibrium for the reaction are governed by the law of mass action.
CONCLUSIONS.

1. The retarding effect of plasma on the action of trypsin can be measured quantitatively.

2. The nature of the reaction involved in effecting the retardation has been subjected to an experimental study.

3. Evidence is presented which indicates that the equilibrium between the inhibitive agent and trypsin is reached practically instantaneously and is rapidly and completely reversible.

4. This equilibrium has been studied by experiments in which we have observed (1) the effect of adding increasing amounts of plasma to a constant amount of trypsin, (2) the effect of varying the amount of trypsin while the plasma was constant, (3) the effect of dilution on the trypsin-plasma mixture.

5. The results of these experiments are discussed and it is stated that they are in quantitative agreement with the law of mass action.

6. An equation was found which fits the curves for the experiments mentioned in (4). This equation was developed from the assumption that 1 molecule of trypsin combined with 1 molecule of inhibitor to form 1 molecule of trypsin-inhibitor compound. The agreement between the results calculated by this equation and the observed results is satisfactory. It is pointed out that the equation contains two arbitrary constants and the bearing this fact may have on the calculated results is discussed.

7. We conclude from the results of our study that we have adduced evidence which suggests the following statement regarding the so-called "antiptryptic" property of blood. The inhibitive agent and trypsin combine to form an inactive but dissociable compound. The reaction in equilibrium is expressed by the equation

   \[ \text{Trypsin} + \text{inhibitor} \rightleftharpoons \text{trypsin-inhibitor} \]

The conditions of equilibrium are apparently governed by the law of mass action. The behavior of the equilibrium is therefore similar to the behavior of other equilibria between different inhibitive agents and enzymes discussed in the paper.
BIBLIOGRAPHY.