REACTIONS OF TRYPSIN AND CHYMOTRYPSIN WITH 
HEPARIN, TRYPSIN INHIBITOR, AND 
HEXYLRESORCINOL 

By M. K. HORWITT 
(From the Biochemical Research Laboratory, Elgin State Hospital, Elgin, and the 
Department of Biological Chemistry, University of Illinois College of 
Medicine, Chicago) 

(Received for publication, July 24, 1944) 

A study of enzyme inhibitors designed to obtain information on the 
possible physiological significance of blood antiprotease has produced a 
number of simple fundamental reactions which have hitherto not been re-
ported. It is the purpose of this paper to record these reactions for the 
possible benefit of others who may wish to investigate them further. 

Materials—The trypsin, chymotrypsin, and trypsin inhibitor were pre-
pared in crystalline form according to the techniques described by Northrop 
and Kunitz (1). The heparin (110 units per mg.), rennin, and the crystal-
line hexylresorcinol were obtained from the manufacturers of these com-
ounds. 

EXPERIMENTAL 

Important chemical differences between chymotrypsin and trypsin may 
be expected from the fact that their isoelectric points are quite different 
(1). Chymotrypsin has its isoelectric point at pH 5.4, whereas the isoelec-
tric point of trypsin lies between pH 7 and 8. This difference may help 
partially to explain the following observations which indicate that the 
precipitation reactions of these two compounds are unlike. 

Unless otherwise noted, the concentration of the enzyme and inhibitor 
solutions used are 1 mg. of material per ml. of 0.01 M phosphate buffer at 
pH 7.3. 

Experiment 1. Mechanism of Heparin Inhibition of Trypsin—1 ml. of 
trypsin solution added to 1 ml. of heparin solution results in the formation 
of a precipitate. That small amounts of heparin markedly inhibit trypsin 
if the two are permitted to remain in contact with each other for definite 
periods of time before the addition of the protein substrate has already been 
demonstrated by Horwitt (2). If the mixture of the heparin and the tryp-
sin is acidified to pH 3 or less, there is no apparent loss of activity of the 
trypsin after the mixture has stood for 30 minutes at room temperature and 

1 Heparin was obtained from The Connaught Laboratories, Toronto; hexylresor-
cinol from Sharpe and Dohme, Philadelphia; rennin from The Pfanstiehl Chemical 
TRYPSIN AND CHYMOTRYPSIN REACTIONS

after subsequent testing upon a casein substrate. If, however, the trypsin-heparin mixture is permitted to stand for 10 hours at a temperature of 25°, the precipitate gradually disappears. Such a solution has no tryptic activity. If 1 ml. of a fresh solution of trypsin is added to such a mixture, it combines with the heparin to form a precipitate, indicating that the heparin remained unchanged by the reactions involved during the loss of tryptic activity. The amount of the precipitate formed by mixing a trypsin solution with a given amount of heparin is proportional to the activity of the trypsin solution used. This can be proved (a) by mixing different dilutions of active trypsin with a constant amount of heparin to show that the precipitate formed in 1 minute diminishes with the enzyme concentration, and (b) by mixing heparin with a trypsin solution which has been allowed to stand at a temperature of 30° to permit the gradual formation of inactive trypsin (Northrop (1)). Inactive trypsin does not give a precipitate with heparin and the amount of heparin-trypsin precipitate formed in the latter experiment becomes progressively less as heparin is mixed with portions of trypsin solution which has been standing for longer periods of time.

It is apparent from repeated experiments that a mixture of trypsin plus heparin at pH 7.3 loses its tryptic activity on standing at a rate much more rapidly than trypsin would lose its activity in the absence of heparin. This may be due to the fact that the trypsin-heparin complex formed is changed more rapidly than trypsin itself (1).

It should be pointed out that the heparin inhibition of trypsin described by the author (2) may be quite different from that described by Glazko and Ferguson (3) who used relatively enormous amounts of heparin to obtain their inhibitions. Fischer (4) has shown that heparin can combine with casein to shift the isoelectric point of casein to the acid side, and Glazko and Ferguson used sufficient heparin to combine with an appreciable portion of the casein substrate before the protease was added.

Chymotrypsin is not inhibited by heparin (2). Solutions of this enzyme at pH 7.3 remain clear when heparin is added.

Experiment 2. Comparison of Effects of Heparin upon Early Stages of Casein Digestion by Chymotrypsin and Trypsin—It has been shown by the author (5) that chymotrypsin as well as relatively large amounts of trypsin can bring about the formation of a heavy turbidity in a solution of 6 per cent casein after several minutes incubation at a temperature of 50°. A study of the effect of heparin upon this reaction was made and the results are presented in Table I. 10 cc. of a 6 per cent casein solution were used as a substrate. This was prepared by mixing 12 gm. of casein (Hammersten) with 40 ml. of 0.2 N sodium hydroxide. 20 ml. of m/15 phosphate buffer at pH 7.5 plus sufficient water to bring the volume to 200 ml. were
then added. The amounts of (a) chymotrypsin, (b) chymotrypsin and heparin, (c) trypsin, (d) trypsin and heparin, (e) chymotrypsin and trypsin, and (f) chymotrypsin, trypsin, and heparin, indicated in Table I, were mixed in 1 ml. of H₂O and placed in the refrigerator (4°) for 30 minutes. At the end of this period the enzyme and heparin mixtures were warmed in a 50° water bath for 5 minutes, after which they were tipped into the 10 ml. of casein solution which had also been warmed to 50°. Ordinary test-tubes (150 × 18 mm.) were used as containers for the casein digestion. When the digestion tube reached a turbidity equal to that obtained by mixing 3 ml. of a casein solution containing 3.6 mg. with 3 ml. of 1 per cent gum ghatti solution and 4 ml. of 5 per cent sulfosalicylic acid, the time was noted.

### Table I

**Effect of Heparin on Formation of Paracasein A by Trypsin and Chymotrypsin at pH 7.5**

The time required to reach the standard turbidity when chymotrypsin, trypsin, and mixtures of these two enzymes are added to a 6 per cent casein solution at pH 7.5 at a temperature of 50° was determined in the presence and absence of heparin. Experiment a, chymotrypsin; Experiment b, chymotrypsin and heparin; Experiment c, trypsin; Experiment d, trypsin and heparin; Experiment e, chymotrypsin and trypsin; Experiment f, chymotrypsin, trypsin, and heparin.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Enzyme concentration</th>
<th>Time to reach standard turbidity</th>
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</thead>
<tbody>
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<td></td>
<td>mg. N</td>
<td>mg. N</td>
</tr>
<tr>
<td>a and b</td>
<td>0.0288</td>
<td>0.0312</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>0.0480</td>
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</table>

Table I shows that heparin had a slight inhibitory effect on the tryptic reaction rate but none at all on the chymotryptic reaction. The compound formed from casein by the protease at pH 7.3 will hereafter be referred to as paracasein A.

**Experiment 3. Comparison of Effect of Pancreatic Trypsin Inhibitor upon Formation of Paracasein A by Chymotrypsin and Trypsin**—Pancreatic trypsin inhibitor forms a compound with trypsin (Northrop and Kunitz (1)) which has no proteolytic activity. The following experiment was designed to obtain information on the effect of this inhibitor upon chymotrypsin and trypsin by using the change of casein to paracasein A as the criterion of enzymatic action. The procedure used is similar to that described above.
(Experiment 2) except that 0.60 mg. of pancreatic trypsin inhibitor was substituted for 0.60 mg. of heparin. The results are given in Table II. It is apparent from the data in Table II that trypsin is completely inhibited and chymotrypsin considerably inhibited by trypsin inhibitor during the initial stages of casein digestion.

**Experiment 4. Effect of Heparin and Trypsin Inhibitor on Clotting of Milk**—Since the formation of paracasein A may be similar to the production of paracasein from casein by rennet as reported by Bosworth (6) and Van Slyke and Bosworth (7), the effect of these inhibitors on milk clotting was studied. 5 ml. samples of cow’s milk (unbuffered, pH 6.7) were mixed with (a) 2 ml. of a solution containing 1 mg. of rennin, (b) 2 ml. of a solution containing 1.0 mg. of rennin and 2.5 mg. of trypsin inhibitor, and (c) 2 ml. of a solution containing 1.0 mg. of rennin and 2.5 mg. of heparin, respectively, and the time of clotting noted. The addition of these solutions to milk did not have any apparent effect on the pH of the reaction mixture. Rennin alone (a) caused a clot in 7.3 minutes, rennin plus trypsin inhibitor (b) caused a clot in 6.3 minutes, and the solution containing the rennin and the heparin (c) did not clot until 13 minutes had elapsed. Therefore, heparin is an inhibitor of rennin activity. Whether or not this inhibition is related to the known affinity of heparin for casein (4) remains to be investigated.

**Experiment 5. Effect of Hexylresorcinol upon Proteolytic Activity of Trypsin and Chymotrypsin**—That hexylresorcinol serves as a precipitant of trypsin and chymotrypsin can easily be proved by mixing solutions of these

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**Table II**

<table>
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<th>Experiment</th>
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<td></td>
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containing 1.0 mg. of rennin and 2.5 mg. of trypsin inhibitor, and (c) 2 ml. of a solution containing 1.0 mg. of rennin and 2.5 mg. of heparin, respectively, and the time of clotting noted. The addition of these solutions to milk did not have any apparent effect on the pH of the reaction mixture. Rennin alone (a) caused a clot in 7.3 minutes, rennin plus trypsin inhibitor (b) caused a clot in 6.3 minutes, and the solution containing the rennin and the heparin (c) did not clot until 13 minutes had elapsed. Therefore, heparin is an inhibitor of rennin activity. Whether or not this inhibition is related to the known affinity of heparin for casein (4) remains to be investigated.
proteases with hexylresorcinol at pH 7.3. That hexylresorcinol can cause a loss of chymotryptic activity was demonstrated in the preceding paper (5). The following experiment shows the relative effect of hexylresorcinol on the hydrolysis of casein by trypsin and chymotrypsin. The enzyme mixtures contained (a) 0.052 mg. of trypsin nitrogen in 2 ml. of water, (b) 0.042 mg. of chymotrypsin nitrogen in 2 ml., (c) 0.052 mg. of trypsin nitrogen plus 2 mg. of hexylresorcinol in 2 ml., and (d) 0.042 mg. of chymotrypsin nitrogen plus 2 mg. of hexylresorcinol in 2 ml. These enzyme solutions stood for 1 hour in the refrigerator (10°) before they were added to 25 ml. portions of the substrate which was prepared by dissolving 12 gm. of casein in 8.2 ml. of 1 N sodium hydroxide and diluting to a volume of 200 ml. The total volume of the mixture being digested was therefore 27 ml. At definite intervals, samples of this mixture were removed, mixed with 0.5 volume of 40 per cent formaldehyde, and titrated to pH 8.4 with sodium hydroxide. The increase in formol titration expressed in ml. of 0.1 N sodium hydroxide per 27 ml. of digestion mixtures is plotted for the different digestions in Fig. 1. The curves in Fig. 1 show a marked inhibition of the activity of both trypsin and chymotrypsin by hexylresorcinol.

**SUMMARY**

1. A study of precipitation reactions of trypsin and chymotrypsin with heparin, hexylresorcinol, and pancreatic trypsin inhibitor at pH 7.3 shows that trypsin is precipitated by both hexylresorcinol and heparin, that chymotrypsin is precipitated by hexylresorcinol but not by heparin, and that trypsin inhibitor precipitates neither enzyme.

2. The mechanism of trypsin inhibition by heparin is discussed.
3. The formation of paracasein A from casein by chymotrypsin and trypsin is partially inhibited by both heparin and pancreatic trypsin inhibitor.
4. Heparin is an inhibitor of the clotting action of rennin on milk.
5. Hexylresorcinol inhibits the proteolytic action of both trypsin and chymotrypsin.

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