A study of the inactivation of trypsin and chymotrypsin by high pressures was reported previously (1). The effects of high pressure on pepsin and chymotrypsinogen, and on the activation of chymotrypsinogen by trypsin, have now been examined.

The effect of high pressure on crystalline pepsin was investigated by Matthews, Dow, and Anderson (2), in particular as affected by magnitude of pressure and duration of pressing. Most of their experiments were carried out at 35.5° and pH 4.8, at one concentration, and with pressing times of 1 to 8 hours. In the present investigation with pepsin, the effect of pH was studied in detail, and the effects of concentration, magnitude of pressure, pressing time, and multiple application of pressure were investigated in solutions in several buffers. The solutions were pressed at 25° in all cases, and generally for only 5 minutes.

Pepsin was found to be considerably more sensitive to pressure than was trypsin or chymotrypsin. It was most stable at pH 3.5 to 4.0. Under a given set of conditions, increasing the concentration of the pepsin generally resulted in a somewhat lower percentage loss of activity, whereas the reverse was true with trypsin. Increase in either pressure or pressing time resulted in further loss of activity of pepsin under all conditions tested; there was no tendency for the loss of activity to reach a maximum short of complete inactivation.

Pressure did not activate chymotrypsinogen. As measured by subsequent activation by trypsin, chymotrypsinogen was found to be somewhat more stable under high pressures than was trypsin or chymotrypsin. The behavior was similar except that chymotrypsinogen was not significantly affected by multiple applications of pressure.

A number of papers have been published on the effect of pressure on reactions catalyzed by enzymes (3–11). Fränkel and Meldolesi (12) reported that the activity of pepsin, trypsin, and diastase was increased by pressures up to 10 atmospheres. Benthaus (13) found that the digestion...
of gelatin by pepsin was retarded by a pressure of 1500 atmospheres (1520 bars), but returned to normal on release of the pressure.

The activation of chymotrypsinogen by trypsin was partly inhibited by pressures as low as 1000 bars; this inhibition was practically complete at 3000 bars. At this point, however, after release of the pressure, the activation proceeded at practically the same rate as in the freshly prepared control. Further increase in pressure resulted in less activation following release of the pressure, until practically no subsequent activation occurred after pressing at 5700 bars.

**EXPERIMENTAL**

*Apparatus and Materials*—The apparatus used for the pressure experiments was that described previously (1). A commercial preparation of crystalline pepsin was used. Crystalline chymotrypsinogen was prepared from beef pancreas by the method of Kunitz and Northrop (14), and was then dialyzed against distilled water until salt-free and finally lyophilized. The crystalline trypsin sample used was obtained from Dr. Moses Kunitz of The Rockefeller Institute for Medical Research.

**Effect of Pressure on Pepsin**

*Procedure*—The procedure employed was similar to that previously described (1). For the experiments on the influence of pH, pepsin was dissolved in distilled water and the pH adjusted to the desired point with dilute acid or alkali. For all other experiments, pepsin was dissolved in one of the following 0.1 M buffers: pH 1.9 chloride, pH 4.0 phthalate, pH 4.0 acetate, or pH 5.9 phosphate. The pressing time was 5 minutes for all experiments except those on the effect of duration of pressing; the temperature of the pressed solutions was 25°. The concentration of pepsin used was 0.05 mg. of protein nitrogen per ml. for all experiments except those on the influence of concentration. Pepsin was assayed by the hemoglobin method of Anson (15).

*Influences of pH and Concentration*—Pepsin was practically completely inactivated at 7600 bars at pH 2.0 to 5.2. A series was then run at 6100 bars over the pH range 1.5 to 6.5. As shown in Fig. 1, the maximum retention of activity was at pH 3.5 to 4.0.

Runs similar to those above were also made in solutions in the four buffers. All of the values except those obtained with phthalate buffer at pH 4.0 were close to the curve in Fig. 1; the value in the latter was only 32 per cent. Results were consistently lower in the phthalate buffer than in the acetate buffer.

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1 Purchased from the Armour Laboratories, Armour and Company, Chicago, Illinois.
Three series of runs, each employing four different concentrations of pepsin, were made as follows: pH 1.9 chloride buffer at 5500 bars, and pH 4.0 acetate and phthalate buffers at 6100 bars. The results as given in Table I show the highest percentage retention of activity in all cases at the highest concentration.

![Graph showing the influence of pH on the effect of pressure on pepsin.](http://www.jbc.org/)

**Fig. 1.** The influence of pH on the effect of pressure on pepsin. Pressure, 6100 bars; time, 5 minutes; concentration, 0.05 mg. of protein nitrogen per ml.

**Table I**

<table>
<thead>
<tr>
<th>Protein nitrogen</th>
<th>Chloride buffer, pH 1.9, 5500 bars</th>
<th>Acetate buffer, pH 4.0, 6100 bars</th>
<th>Phthalate buffer, pH 4.0, 6100 bars</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg. per ml.</td>
<td>Per cent retention of activity</td>
<td>Per cent retention of activity</td>
<td>Per cent retention of activity</td>
</tr>
<tr>
<td>0.15</td>
<td>53</td>
<td>59</td>
<td>45</td>
</tr>
<tr>
<td>0.05</td>
<td>50</td>
<td>51</td>
<td>32</td>
</tr>
<tr>
<td>0.015</td>
<td>36</td>
<td>50</td>
<td>29</td>
</tr>
<tr>
<td>0.005</td>
<td>38</td>
<td>48</td>
<td>33</td>
</tr>
</tbody>
</table>

**Effect of Magnitude of Pressure**—The effect of magnitude of pressure was tested on pepsin solutions in three buffers. The results as given in Fig. 2 show that the curves are roughly parallel, except in buffer of pH 1.9 above 6000 bars. Other than the latter, no tendency was found for a portion of the activity to be resistant to pressure.
**Effects of Duration of Pressing and of Multiple Pressing**—Pressing times of 5, 15, and 60 minutes were compared for pepsin solutions at 5500 bars in chloride buffer at pH 1.9, and at 6100 bars in acetate buffer of pH 4.0. The results as given in Table II show an increase in inactivation with increase in pressing time.

![Graph](chart.png)

**Fig. 2.** The effect of pressure on pepsin. Pressing time, 5 minutes; concentration, 0.05 mg. of protein nitrogen per ml.

**Table II**

 Effects of Duration of Pressing and Multiple Application of Pressure on Pepsin

<table>
<thead>
<tr>
<th>Duration of pressing</th>
<th>Per cent retention of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer, pH 1.9, 5500 bars</td>
</tr>
<tr>
<td>min.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>15</td>
<td>28</td>
</tr>
<tr>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td>5 (3 times)</td>
<td>14</td>
</tr>
<tr>
<td>Calculated</td>
<td>13</td>
</tr>
</tbody>
</table>

Two experiments were performed in which solutions of pepsin in chloride buffer at pH 1.9 and acetate at pH 4.0 were pressed three times for 5 minutes each at 5500 and 6100 bars, respectively, with intervening periods of 5 minutes each when the pressure was lowered to 400 bars. The results (Table II) in both cases were much lower than those for a single 15 minute pressing at the same pressure. On the assumption that the same percentage of pepsin was inactivated during each 5 minute pressing as in the initial 5 minute period, the total amount of inactivation to be
expected was calculated (Table II) and found to be in good agreement with the experimental value.

The solutions at pH values below 4.5 at the higher pressures and concentrations used formed turbidities or precipitates on pressing in all cases. This was not the case at or below pH 4.5.

Effect of Pressure on Chymotrypsinogen

Procedure—The procedure used was essentially that previously described (1), except for the treatment with trypsin before assay. The chymotrypsinogen solutions used generally contained 1 mg. of protein nitrogen per ml. 1 ml. of the pressed solution or control was mixed with 3 ml. of 0.1 M phosphate buffer, pH 7.6, 1 ml. of trypsin solution (containing 0.00067 mg. of protein nitrogen) was added, and the mixture allowed to stand at 25° (14). 1 ml. aliquots were removed for assay after 2 hours, and sometimes also after 24 hours, and added to 4 ml. of 1/15 hydrochloric acid to stop the action of trypsin. Aliquots of this solution were assayed immediately by the method of Anson (15). Under the above conditions the activity produced by trypsin treatment for 2 hours was about half of that for 24 hours. The 2 hour results were used in calculating the percentage retention of activity of the pressed sample compared with the corresponding control.

An experiment was performed in which the trypsin treatment was begun (a) as soon as possible after the pressing ended (about 15 minutes), and (b) after an interval of 2 hours. The results checked closely (69 and 68 per cent, respectively, of the activity of the control), indicating that the change in chymotrypsinogen induced by pressure was permanent.

Non-Activation by Pressure—In a number of experiments, the activities of the pressed and control samples were measured before addition of the trypsin. A very small amount of activity was found in all cases, but there was no significant difference in the activity of the pressed and control samples.

Effects of Duration of Pressing and Magnitude of Pressure—Solutions of chymotrypsinogen (1 mg. of protein nitrogen per ml.) in distilled water were pressed for 5 and 60 minutes, respectively, at 7500 bars. After treatment with trypsin for 2 hours, the pressed samples had 65 and 63 per cent, respectively, as much activity as the corresponding controls.

Solutions of chymotrypsinogen (1 mg. of protein nitrogen per ml.) in distilled water and in 0.1 M phosphate buffer, pH 7.6, were pressed for 5 minutes at pressures of 6700, 7500 and 8400 bars, respectively. In each case the results at 7500 and 8400 bars were in good agreement; the retention of activity at 6700 bars in both cases was considerably higher than those at 7500 bars.

The above results show that with chymotrypsinogen, like chymotrypsin
and trypsin but unlike pepsin, further increases in magnitude or pressing time beyond a certain level have little or no effect.

Effects of Concentration and pH—Solutions of chymotrypsinogen in distilled water containing 0.34, 1.0, 3.3, and 10.2 mg. of protein nitrogen per ml., respectively, were pressed at 7600 bars for 5 minutes. The retention of activity after 2 hour trypsin treatment was 90, 62, 50, and 37 per cent, respectively, showing that an increase in concentration results in a decrease in per cent retention of activity after trypsin treatment.

Three experiments were carried out in which the pH of the solution was 3.1, 5.6, and 7.6, respectively, the concentration of chymotrypsinogen being 1 mg. of protein nitrogen per ml. After pressing 5 minutes at 7600 bars, and subsequent 2 hours trypsin treatment, the per cent retention of activity was 92, 62, and 41, respectively, showing decreasing stability under pressure with increasing pH in the range 3.1 to 7.6.

Effect of Multiple Applications of Pressure—A solution of chymotrypsinogen (1 mg. of protein nitrogen per ml.) in distilled water was pressed successively for 5 minutes each at 7600, 400, 7600, 400, and 7600 bars. After trypsin treatment for 2 hours, the activity was 69 per cent of that of the control. In a similar experiment in which the pressure was applied only once at 7600 bars for 5 minutes, the corresponding value was 68 per cent. Another similar solution was pressed for 5 minutes at 7600 bars, the pressure released, the solution allowed to stand 22 hours at 25°, and then again pressed at 7600 bars for 5 minutes. The activity after 2 hours trypsin treatment was 69 per cent of that of the control.

Similar experiments with solutions of chymotrypsinogen in 0.1 M phosphate buffer, pH 7.6, gave values of 40 and 34 per cent, respectively, for single and triple applications of pressure. Considerable amounts of precipitate formed in the pressed solutions in both cases. The above results show that multiple application of pressure had little or no effect on chymotrypsinogen.

Isolation of Crystalline Chymotrypsinogen after Pressing—A solution of 1 gm. of chymotrypsinogen in 20 ml. of distilled water was pressed for 5 minutes at 5100 bars. The activity after a 2 hour trypsin treatment was 99 per cent of that of the control. On precipitation by ammonium sulfate and recrystallization, very long needles characteristic of chymotrypsinogen were obtained.

In a second similar experiment, on pressing for 5 minutes at 7500 bars some fluorescing material was formed. On attempting to recrystallize the
material, over half of the total protein separated in an amorphous form of low activity after trypsin treatment. A fraction was finally obtained from the filtrate, which on recrystallization formed the very long needles characteristic of chymotrypsinogen. Hence the pressure resulted in a partial denaturation rather than the formation of a new zymogen of lower activity after trypsin treatment.

Effect of Pressure on Activation of Chymotrypsinogen by Trypsin

Procedure—The solutions of chymotrypsinogen and trypsin used contained 1.05 and 0.00067 mg. of protein nitrogen per ml., respectively. 2 ml. of chymotrypsinogen solution were mixed with 6 ml. of 0.1 M phosphate buffer, pH 7.6. 2 ml. of trypsin solution were then added, the time being noted. One-half was introduced into the pressure chamber as rapidly as possible, the other half was held at 25° as a control. The pressure was raised to the desired level, the time again being noted. After exactly 60 minutes the pressure was released. 1 ml. aliquots of both the pressed sample and the control were added to 4 ml. of N/15 hydrochloric acid, the times again being noted, and aliquots were assayed by the method of Anson (15). In some cases aliquots of the pressed solution which had stood for exactly 60 minutes after release of the pressure were assayed as above. The object here was to study the effect of the 1 hour pressing on the rate of activation after release of the pressure.
The activities for the control sample and for the pressed sample were calculated on a 60 minute basis, the actual elapsed time being around 68 minutes. For the pressed sample, the correction for the time between mixing the solution and applying the pressure was calculated from the activity of the corresponding control. The same method was used for the time after release of the pressure until mixing an aliquot with hydrochloric acid, for pressures up to 2700 bars. Above this pressure, the activation proceeded at a slower rate after release of the pressure than initially in the control, and therefore the correction was calculated from this slower rate.

Results—The results are given in Fig. 3. Within the limit of experimental error, no acceleration in the rate of activation of chymotrypsinogen by trypsin under pressure was found. Up to 1000 bars, the pressure had little or no effect, but above this point the per cent activity of the pressed solution decreased rapidly with increase in pressure until it was nearly 0 at 3000 bars.

DISCUSSION

Pepsin—The results obtained with pepsin differ in several important respects from those obtained with trypsin and chymotrypsin (1). The pH optimum for maximum retention of activity was at 3.5 to 4.0, with sharp decreases above or below this range; no decrease was observed in trypsin or chymotrypsin at low pH values. The maximum per cent retention of activity with pepsin was found at the highest concentrations tested, which was the reverse with trypsin and chymotrypsin. With pepsin, increase in pressure or pressing time invariably resulted in a decrease in the activity; there was no tendency for a part of the activity to be very resistant to inactivation by pressure as in the other two enzymes.

As shown above, the results obtained with pepsin differ in several important respects from those obtained with trypsin and chymotrypsin (1). The following simple mechanism, in which P represents pepsin under ordinary conditions and iP pressure-inactivated pepsin, is in accord with most of the pepsin results.

\[ P \rightarrow_p iP \]

However, it can be seen from Table II that three 5 minute pressings produced a considerably greater effect than a single 15 minute pressing. Apparently there is an effect of the application of pressure alone. The following scheme, which is similar to that postulated for trypsin and chymotrypsin, and in which Pa represents reversibly pressure-changed pepsin (folded or aggregated), is more consistent with all of the results.
In the case of pepsin, however, it is postulated that the reaction to form $Pa$ would not go so nearly to completion under pressure, as it would in the case of trypsin or chymotrypsin, and that even under pressure the $Pa$ would revert to $P$ and be continuously converted to $iP$. This scheme is advanced as a tentative hypothesis and not as a final and complete explanation.

The curve (Fig. 1) for the influence of pH on the effect of pressure on pepsin is similar to the corresponding curves for trypsin and chymotrypsin in that retention of activity drops sharply with increase above pH 4. Apparently all three enzymes are quite sensitive to hydroxyl ions. Trypsin and chymotrypsin are most active at about pH 8, and are quite readily inactivated by pressure at this pH. Pepsin, on the other hand, is most active at around pH 2, and we find that with increasing acidity below pH 3.5 pepsin becomes less stable under pressure. Trypsin and chymotrypsin are quite inactive at low pH and are also very little affected by pressure under these conditions. It is probable (1, 10) that when an enzyme is active it is partially unfolded, and when in this condition is much more susceptible to pressure than when it is inactive (but not denatured) and probably in a more folded or globular form.

Chymotrypsinogen—The effect of pressure on chymotrypsinogen is similar in most respects to effects on trypsin and chymotrypsin but it is somewhat more stable under the same conditions. Unlike the two enzymes, chymotrypsinogen was little, if any, further affected by repeated applications of pressure. It appears that under pressure a portion of the chymotrypsinogen is converted to a pressure-resistant form (Xtg)$_a$, still convertible to chymotrypsin, while the remainder is changed to a form not convertible to chymotrypsin (Xtg)$_d$.

Unlike trypsin and chymotrypsin, chymotrypsinogen was little, if any, further affected by repeated applications of pressure. It appears that the pressure-resistant form (Xtg)$_a$ does not readily revert to the original form on release of the pressure, since in one experiment a 22 hour period between two pressings yielded results identical with those obtained in similar experiments with a single 5 minute pressing. It does not appear probable that the high pressure merely converts chymotrypsinogen to another form which reacts more slowly or less completely with trypsin, since in one experiment in which the pressed solution was fractionated, amorphous material of low activity (after trypsin treatment) was obtained, and finally some crystalline material apparently identical with chymotrypsinogen. It appears that during the fractionation, which took place over a period of
several weeks, a considerable part of the \((\text{Xtg})_a\) reverted to the original form.

The accompanying scheme is proposed to account for the observations:

\[
\begin{align*}
\text{Xtg} & \xrightarrow{p} (\text{Xtg})_d \\
(\text{Xtg})_d & \xrightarrow{\text{very slow}} \text{Xtg}
\end{align*}
\]

where \(\text{Xtg}\) is the original chymotrypsinogen, \((\text{Xtg})_a\) is a pressure-resistant form still activatable by trypsin, but only very slowly reverting to \(\text{Xtg}\), and \((\text{Xtg})_d\) is irreversibly denatured chymotrypsinogen not activated by trypsin.

The conversion of chymotrypsinogen to the active enzyme by trypsin is most probably a hydrolytic process. Since Linderstrøm-Lang and Jacobsen (16) have shown that the hydrolysis of proteins is accompanied by a decrease in volume, it might be expected that pressure would favor this conversion. However, no acceleration by pressure was observed, but rather inhibition. It has previously been suggested (1, 10) that an enzyme must be partially unfolded to be active and that pressure opposes the unfolding. In the present case both the enzyme and the substrate (chymotrypsinogen) may need to be partially unfolded. Such a hypothesis is consistent with the results obtained.

**SUMMARY**

The effect of high pressures on pepsin and chymotrypsinogen has been investigated, including the influences of pH, concentration, magnitude of pressure, duration of pressing, and multiple pressing. The results with pepsin differed in several important respects from those previously obtained with trypsin and chymotrypsin. The results with chymotrypsinogen were similar to those with trypsin and chymotrypsin in all respects, except that it was not significantly affected by multiple applications of pressure. Possible explanations of these differences are discussed.

Chymotrypsinogen was not activated by pressure. The activation of chymotrypsinogen by trypsin was not accelerated by pressure; it was appreciably inhibited by a pressure of 1000 bars, and almost completely stopped by a pressure of 3000 bars. At this pressure the activation proceeded at almost the same rate after release of the pressure as in the control. At a pressure of 5700 bars, however, no activation occurred after release of the pressure.

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A. Laurence Curl and Eugene F. Jansen


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