CRystalline trypsin

IV. Reversibility of the Inactivation and Denaturation of Trypsin by Heat

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It was noted by Mellanby and Wooley (1) that trypsin solutions in dilute acid could be heated nearly to boiling with very little loss in activity, and this observation was confirmed by Eddie (2). At temperatures below 40°C., on the other hand, the enzyme is more stable near pH 4 or 5 than it is in acid solution. This latter result has also been obtained by the writer (3) and by Pace (4). In the course of the preparation of crystalline trypsin by Kunitz and the writer (5) it was found that the further purification proceeded the more heat-stable the preparation became. The final crystalline material may be heated to boiling in dilute solution over the whole range of acidity between pH 1 and pH 7 with little or no loss in activity and apparently without the formation of any denatured protein. This behavior is remarkable, since in general heating denatures proteins and inactivates enzymes. The result is, however, somewhat analogous to that in the case of serum albumin. Spiegel-Adolf (6) found that serum albumin also could be heated in certain pH ranges without the appearance of any denatured protein in the solution after it had been cooled. Spiegel-Adolf, and Anson and Mirsky (7) also showed that the denaturation of serum albumin is easily reversible and it is probable that the fact that no denatured protein is found in the solution after cooling is due to the reformation of native from denatured protein on cooling. It has also been found in the case of crystalline pepsin (8) that the activity is lost when the protein is denatured and regained when the denaturation is reversed. The possibility therefore exists in the case of trypsin that inactivation of the enzyme and denaturation of the protein occur, as
would be expected, at higher temperatures; but that on cooling the
denaturation is reversed and the native, active protein re-formed.

Presence of Denatured Protein in Hot Trypsin Solutions.—If the
protein is actually present in the denatured form at high temperatures, it
would be expected that rapid addition of the hot enzyme solution to
cold salt solution would result in the precipitation of insoluble, de-
natured protein while if the enzyme solution were allowed to cool, as
is usually done, and then added to the cold salt solution, no denatured
protein would be found. If the native protein only is active then it
would also be expected that the filtrate from the denatured protein

TABLE I

Effect of Repeated Heating and Cooling on the Activity and Solubility of Trypsin

0.5 gm. trypsin dissolved in 25 ml. N/20 HCl heated to 70°C. and samples taken
for total and soluble protein and activity. Solution cooled quickly to 20°C. and
sampled in the same way; solution then reheated to 70°C. and another sample
taken.

<table>
<thead>
<tr>
<th>Temp.</th>
<th>Time</th>
<th></th>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>°C.</td>
<td>hrs.</td>
<td>mg.</td>
<td>ml.</td>
<td>ml.</td>
<td>mg.</td>
<td>ml.</td>
<td>ml.</td>
</tr>
<tr>
<td>20</td>
<td>0.10</td>
<td>1.0</td>
<td>102</td>
<td>100</td>
<td>1.0</td>
<td>102</td>
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<tr>
<td>70</td>
<td>0.10</td>
<td>0.9</td>
<td>100</td>
<td>110</td>
<td>Trace</td>
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<td></td>
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<tr>
<td>20</td>
<td>0.10</td>
<td>1.0</td>
<td>108</td>
<td>108</td>
<td>1.0</td>
<td>105</td>
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<tr>
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<td>108</td>
<td>106</td>
<td>Trace</td>
<td>0.1</td>
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</tbody>
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would be inactive while the solution of the enzyme which had been
cooled would be active. This is actually the case as is shown in
Table I.

This experiment shows that when a solution of trypsin in N/20 HCl
at 70°C. is added to one-half saturated ammonium sulfate, without
previous cooling, practically all of the protein precipitates and the
supernatant liquid is inactive. If the enzyme solution is cooled before
being added to the salt solution, on the other hand, no precipitate is
formed and the solution contains its original activity. The heating

1 Denatured protein is defined as that form of the protein which is insoluble at
the isoelectric point or in the presence of strong salt solution but may be soluble
in acid or alkali in the absence of salt.
and cooling may be repeated almost indefinitely without loss of activity provided the solution is not left at 70°C. for too long a time. This observation, in addition to the fact that there is no appreciable change in total protein nitrogen on heating shows that the absence of precipitable protein in the cooled solution is not due to digestion of the protein. The experiment confirms the idea that the protein is denatured in the hot solution but reverts to the native condition on cooling. 

Effect of Temperature on Rate of Reversal.—The experiment may be varied by cooling the enzyme solution very rapidly to zero degree before adding it to the salt solution. It might be expected by analogy with the results with hemoglobin (Mirsky and Anson (9)) that the re-

<table>
<thead>
<tr>
<th>Temperature, °C</th>
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<th>100</th>
<th>0</th>
<th>0</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time, sec.</td>
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<td>5</td>
<td>10</td>
<td>30</td>
<td>600</td>
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<tr>
<td>Soluble [T.  U/ml.</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>1.4</td>
<td>3.7</td>
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<tr>
<td>Insoluble protein</td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>-</td>
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</tbody>
</table>

versal would take place slowly enough at zero degree to be measured. This actually is the case as is shown in Table II. The enzyme protein when cooled very rapidly to zero degree is still in the denatured form and precipitates at first completely with salt. As the solution stands at zero degree the denatured protein changes to the native form and at the same time the activity begins to reappear in the supernatant solution so that after about 10 minutes all the protein has reverted to the native form and the activity is again all found in solution. This experiment rules out any secondary reaction which might be supposed to occur when the hot enzyme solution is added to cold salt solution.

Effect of Temperature on Equilibrium between Native and Denatured Protein.—The preceding experiments show that there is a very rapid change from native to denatured protein at temperatures between zero and 90°C. They indicate that the temperature determines not
only the rate of transformation from native and active to denatured and inactive protein but also the equilibrium between the two forms. In order to determine whether or not this is really the case a trypsin solution was heated rapidly from 20°C to 60°C. and then cooled again to 20°C. Samples were taken and added without previous cooling to cold salt solution at each 10°C temperature interval and the soluble protein and activity determined. The result of this experiment is shown in Table III.

As the temperature rises the quantity of protein soluble in one-quarter saturated ammonium sulfate decreases rapidly and at 60°C there is too little to determine. When the solution cools, soluble protein again appears and the quantity present when cooled to 40°C is nearly the same as that at 40°C when the temperature was raised. The activity of the solution increases and decreases almost exactly in proportion to the quantity of soluble protein present. The experiment shows that equilibrium between native and denatured protein is very rapidly established and is extremely sensitive to changes in temperature so that at 20°C the protein is present almost entirely as native protein while at 60°C it is practically entirely denatured and inactive. It follows that the usual assumption that raising the temperature merely increases the rate of transformation from native to denatured, is not correct in this instance but that the temperature affects the equilibrium as well as the rate of the reaction. This possibility has been con-

### TABLE III

<table>
<thead>
<tr>
<th>Time, min</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature, °C</td>
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<td>40</td>
<td>50</td>
<td>60</td>
<td>50</td>
</tr>
<tr>
<td>[P. N.]_{mg}</td>
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<td>0.16</td>
<td>0.07</td>
<td>0.054</td>
<td>0.12</td>
</tr>
<tr>
<td>[T. U.], ml</td>
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<td>19</td>
<td>6.1</td>
<td>&lt;1</td>
<td>0.6</td>
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<tr>
<td>[T. U.], mg. P.N.</td>
<td>125</td>
<td>120</td>
<td>88</td>
<td>65</td>
<td>100</td>
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</table>
sidered by Anson and Mirsky (10). Somewhat similar results have been noted with other enzymes in that the activity of a solution inactivated by heat has been found to increase on standing at lower temperatures. This result has been described by Bach and Wilenskii (11) in the case of purified peroxidase and has also been noted by Kulikoff and Bobkowa (12) with trypsin and by Anson (13) with pepsin.

Inactivation at Different Temperatures and pH 7.0.—The preceding experiments show directly that the trypsin protein at temperatures above 50°C. is in a form which is insoluble in one-quarter saturated ammonium sulfate, that is to say, it is denatured. They also show directly that the specific activity of that part of the protein which is still native is the same as that of the original preparation. It is not possible to prove from these experiments, however, that the denatured protein is also inactive since, if the precipitate of denatured protein is added to protein solutions the precipitate dissolves and digestion occurs. It can be objected, therefore, that the apparent inactivity of the denatured protein precipitate is simply due to the fact that it is insoluble. It is necessary to show that the enzyme, while still in solution, is inactive at higher temperatures; but this cannot be done in N/15 HCl since trypsin is not active under these conditions at any temperature. The experiments were, therefore, repeated at pH 7, under which conditions the enzyme is active at ordinary temperatures. It was found that the loss in activity when heated to 90°C. at pH 7 was a very sensitive test for the purity of the preparation. Preparations which contain relatively small amounts of inactive protein precipitate when heated at pH 7 and the activity does not return upon cooling. Carefully purified preparations may be heated in solution containing less than 1 mg. protein nitrogen per ml. and less than 0.05 molar salt to 90°C. at pH 7 for 5 to 10 minutes and cooled to 20°C. without any appreciable loss in activity. All preparations tested so far precipitate when heated at pH 7.5 to 9.5 and the activity is not recovered on cooling. Also, solutions which have been heated at pH 1 to 7 remain inactive if added before cooling to pH 8 to 10 buffer solutions. It is necessary at pH 7.0 to add the enzyme solution to the hot phosphate solution rather than to heat the mixture, since as will be seen later, the inactivation at intermediate temperatures of 50–70°C. is not reversible. There is at present no explanation for this peculiar fact, although it
was found that the denatured protein formed at 62°C. was different from that formed at 90°C.

In the following experiments, therefore, 1 ml. of the enzyme solution containing about 5 mg. protein nitrogen was added to 25 ml. of M/20 phosphate solution at the desired temperature. 1 ml. samples were taken at intervals of about 1 minute, added to 10 ml. cold N/10 HCl and this solution analyzed for total activity and total protein nitrogen. Another set of samples was taken and added at once, without cooling, to 5 ml. half saturated ammonium sulfate in M/20 pH 4 acetate buffer. These suspensions were then centrifuged and the supernatant liquid analyzed for activity and for protein nitrogen. This is called soluble activity or soluble protein nitrogen and, presumably, represents the amount of native protein in the heated solution.

Changes in Soluble Protein or Activity.—The results of these experiments are shown in the lower half of Fig. 1. The concentration of total protein, total activity, soluble protein, and soluble activity has been plotted as the log₁₀ of the per cent of the original concentrations. In the unheated solution all the protein and all the activity were present in the soluble form; i.e., no precipitate was obtained when the solution was added to the ammonium sulfate. The higher the temperature the more rapidly the soluble protein or activity disappears until when the temperature is about 50°C. the process becomes so rapid as to render it impossible to make accurate measurements. At any one temperature the percentage loss of soluble protein is nearly the same as the percentage loss in soluble activity, as shown by the fact that the curves for the percentage of those quantities remaining in solution are parallel. In other words, the specific activity of the soluble (native) protein remains practicallly constant. This agrees with the idea that the denatured protein is inactive and that the activity is a property of the native protein molecule.

The experiments are not sufficiently accurate to permit calculation of the exact temperature coefficient, but it is more than two for an interval of 10° and therefore agrees qualitatively with the value usually found for the denaturation of proteins or the inactivation of enzymes. The course of the reaction, as plotted in the figure, is not monomolecular; since the reaction does not go to complete inactivation but stops at an intermediate value, depending on the temperature, the mono-
molecular constants should be calculated using the concentration of soluble protein or activity present at this equilibrium point rather than the total amount present originally, as has been done in the figure. If the reaction velocity is calculated using this equilibrium value it is 

FIG. 1. Decrease in total activity, total protein, soluble activity, and soluble protein concentrations of trypsin solutions at pH 7.0 and various temperatures.
found to be monomolecular within the wide error of the present experiment.

Changes in Total Protein and Total Activity.—The effect of increasing the temperature on the concentration of total protein or total activity, shown in the upper two series of curves, is entirely different from that on the concentration of soluble protein or activity discussed above. At 42°C. the quantity of total protein decreases with time but more slowly than does the soluble protein. At 52°C. it decreases faster than at 42°C. but at 72°C. or 92°C. it decreases much less rapidly than at 52°C. The rate of destruction of the total protein, therefore, goes through a maximum at about 52°C. and then becomes slower. The loss in total activity is nearly parallel to the loss in total protein except that in this case there is very rapid loss at 72°C. At 92°C. however, the activity is destroyed less rapidly than at 42°C. so that there is a general agreement between the decrease in total protein and the decrease in total activity. These results are in qualitative agreement with the idea expressed above, that the denatured, inactive protein found at temperatures above 40°C. reverts to active, native protein when the solution is cooled to 20°C. Since there is a decrease in total protein at 40–60°C., it is necessary to assume in addition that, at this pH, there is some digestion of the denatured protein by the native, active protein. At 42°C., for instance, 10 to 20 per cent of the protein is in the native, active form and, therefore, digests and destroys the denatured form present. The total activity, total protein, soluble activity, and soluble protein, therefore, all decrease.

At 52–62°C. more of the protein is changed rapidly to the denatured, inactive form but the rate of digestion is also increased by the increase in temperature so that this denatured protein is still digested by the small amount of native, active protein present and the total protein and total activity, therefore, still decrease with time. At the higher temperatures the protein is completely and rapidly changed to the denatured form and therefore no digestion occurs since no native, active protein is present. The total protein, therefore, remains constant and reverts to native protein when cooled so that there is no change in the total protein or total activity. The slow decrease in the total activity at 92°C. is probably due to a secondary change occurring in the denatured protein which is not reversible (14).
since all the work on the reversibility of protein denaturation shows that with prolonged heating a form is obtained which does not revert to the native condition. The loss in total activity at 62°C and 72°C without corresponding loss in total protein shows that the protein when heated at these temperatures does not regain its activity on cooling, although it does become soluble again.

**Fig. 2. Rate of digestion of casein or peptone solutions with trypsin at 100°C and 35°C.**

*Trypsin Is Inactive at 100°C.—*It was assumed in accounting for the fact that there was no loss in total protein at 92°C. that the enzyme was inactive at this temperature although, as the experiment shows, it was active in the solution after it had been allowed to cool. This assumption may be directly verified by determining the digestion of casein or of peptone at 100°C and at 35°C. A solution of casein, or of peptone, at pH 7 and 100°C. is not digested by trypsin. If the same solution is

² The rate of hydrolysis of different commercial peptone preparations with the crystalline trypsin varies greatly.
cooled to 35°C. rapid digestion occurs at once. The results of such an experiment are shown in Fig. 2, in which the increase in formol titration in 2 cc. of the digestion mixture has been plotted against the time. Two solutions of casein and of peptone were prepared; one of each was placed at 35°C. and the trypsin added; the other pair was heated to 100°C. and the same quantity of trypsin added. Samples were taken for formol titration at short intervals from all four solutions and after about 5 minutes the solutions at 100°C. were cooled rapidly to 35°C. The results show that no digestion occurred while the solutions were at 100°C. but that as soon as they were cooled to 35°C. rapid digestion commenced and proceeded at the same, or even greater rate,3 than in the control solutions which had not been heated to 100°C. The curves have been plotted so that the time of adding the trypsin to the 35°C. solutions coincides with the time at which the 100°C. solutions were cooled to 35°C.

These experiments show directly that casein and peptone are not acted on by trypsin at 100°C. but are rapidly digested at 35°C. They indicate that the enzyme is inactive at 100°C. but it could be assumed that some reversible change occurs in the substrate at this temperature which renders it resistant to digestion by the enzyme. This appears unlikely and in addition it may be shown directly that the effect is on the enzyme rather than on the substrate by following the digestion of peptone solutions at zero degree upon the addition of hot or cold trypsin solutions.

It was shown before that trypsin solutions heated to 100°C. and cooled very rapidly to zero degree remained in the denatured form for some time as shown by the formation of a precipitate on the addition of ammonium sulfate. If trypsin solution at 100°C. is added rapidly to peptone solution at zero degree, it would be expected that digestion would occur much more slowly than if unheated trypsin were added, or than if the heated trypsin were allowed to cool slowly before being added to the peptone. The result of the experiment done in this way

3 If the experiment is done with gelatin, however, no digestion occurs even after cooling the solution. The gelatin evidently prevents the reactivation of the enzyme and it is possible that some of the split products of trypsin itself act in the same way and that this is the explanation of the failure of the trypsin to reactivate after being heated to 72°C.
is shown in Fig. 3. The experiment shows that the peptone is digested at about the same rate by the trypsin solution before heating or, if it is allowed to cool slowly after heating, but if the hot trypsin solution is added at once to the cold peptone the rate of digestion is much slower. This experiment confirms the assumption made before that the enzyme is inactive at 100°C. and that the failure to digest casein at 100°C. is due to the fact that the enzyme is in an inactive form at this temperature rather than that the casein or peptone is in a resistant form.

_Determination of S-S Groups in the Insoluble Protein._—The conclusion that the insoluble, denatured protein is inactive is further confirmed by determination of the S-S groups in the precipitate formed by the addition of hot trypsin solutions to salt solutions. Mirsky and Anson (15) have shown that denatured proteins differ from native protein in that the S-S groups are free in the denatured form. The writer
is indebted to Drs. Mirsky and Anson for an analysis of the insoluble precipitate obtained by adding hot trypsin solution to cold salt. The analysis showed that the protein contained all the S-S groups in the free form and, therefore, agrees in this respect with other denatured proteins.

**Formation of Native, Active Protein from Denatured Protein.**—The experiments already described show that trypsin at a temperature of 90°C. is inactive and that the trypsin protein precipitates when added to one-quarter saturated ammonium sulfate. This precipitate of denatured protein may be partially reconverted to native, active protein by dissolving in \( \frac{\pi}{50} \) hydrochloric acid. The precipitate dis-

<table>
<thead>
<tr>
<th>No.</th>
<th>Vol.</th>
<th>( \text{U. U.}_\text{ml} )</th>
<th>( \text{U. U.}_\text{total} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 gm. trypsin cake dissolved in 15 ml. ( \text{H}_2\text{O} ).</td>
<td>1</td>
<td>15</td>
<td>200</td>
</tr>
<tr>
<td>Solution 1 poured into 70 ml. boiling pH 7.0 ( \text{M}/10 \text{PO}_4 ), poured into 80 ml. 0.7 saturated ammonium sulfate ( \text{M}/10 \text{pH 4.0 acetate} ), centrifuge supernatant</td>
<td>165</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Precipitate + 75 ml. ( \frac{\pi}{50} \text{HCl} ), stir for 2 hrs. at 35°C</td>
<td>75</td>
<td>14</td>
<td>1050</td>
</tr>
</tbody>
</table>

solves slowly and the solution then contains from 30 to 40 per cent of the original activity. It also contains a soluble protein which is inactive. This soluble, inactive protein is formed from the active trypsin protein on standing at room temperature in dilute acid solution. This reaction was discussed in connection with the preparation of the enzyme (5). The result of this experiment is shown in Table IV.

**DISCUSSION**

Trypsin solutions which have been heated to boiling and then cooled show no loss in activity or formation of denatured protein. The preceding experiments show that these anomalous results are due to the existence of an equilibrium between native, active protein and denatured, inactive protein. Below 30°C. the protein is nearly all in the active and native condition while above 65°C. it is practically all
denatured and inactive. Equilibrium is reached rapidly from either side. The loss in activity is proportional to the formation of denatured protein and when the denatured protein changes back to the native condition the activity is also recovered. This is good evidence that the activity of the preparation is a property of the native protein molecule since, if it be assumed that the activity is due to a special active molecule associated with the protein, it is further necessary to suppose that the conditions for inactivating and reactivating this hypothetical active molecule are quantitatively the same as those for the formation of denatured from native and native from denatured protein.

They agree, therefore, with the results with pepsin denatured by alkali or by heat and it is probable that the same mechanism accounts for the recovery of the activity of other enzyme solutions after heating, recorded in the literature.

They are also very similar to the result with hemoglobin, since this protein when denatured also loses its characteristic property, that of reversible combination with oxygen and recovers this property when the denaturation is reversed (16).

Experimental Procedure

Trypsin.—The trypsin preparation used was crystallized from about 0.7 saturated ammonium sulfate, as described by Northrop and Kunitz (5). It was kept in the form of a moist filter cake at 5°C. The figures given for the weight of trypsin refer to the weight of this filter cake.

Activity Units (T.U.)\(^{16}\text{V}\) — The activity is expressed as per cent change per minute in the viscosity of gelatin solutions. The determination was carried out as previously described (17).

Gelatin.—Gelatin was prepared from Cooper's powdered gelatin as described by Northrop and Kunitz (18).

Casein.—Casein solutions were made up from Kahlbaum's casein "according to Hammarsten."

Peptone.—The preparation used was a sample of Fairchild's peptone which contained more protein nitrogen than usual. Most samples of commercial peptone tried were not digested by the trypsin.

Total Activity.—1 ml. of trypsin solution added to 10 ml. of \(\text{N/20}\) hydrochloric acid and allowed to stand at 20°C. for about \(\frac{1}{2}\) hour and analyzed for activity with standard gelatin solution as usual.

Total Protein Nitrogen.—A volume of solution containing about 1 mg. protein nitrogen is added to an equal volume of 5 per cent trichloracetic acid and warmed
to 70°C. for 5 minutes. The suspension is then cooled, filtered through hardened paper, and the precipitate washed with 2½ per cent trichloracetic acid until the washing gives no test for ammonia. The precipitate is then washed into a Kjeldahl flask and analyzed for total nitrogen.

Soluble Activity and Soluble Protein Nitrogen.—1 ml. of solution is added to 5 ml. half-saturated ammonium sulfate in n/20 pH 4 acetate buffer. The suspension is centrifuged and the supernatant solution analyzed for activity and protein nitrogen as described above.

The analytical determinations were done by Mr. N. Wuest.

SUMMARY

1. If dilute solutions of purified trypsin of low salt concentration at pH from 1 to 7 are heated to 100°C. for 1 to 5 minutes and then cooled to 20°C. there is no loss of activity or formation of denatured protein. If the hot trypsin solution is added directly to cold salt solution, on the other hand, all the protein precipitates and the supernatant solution is inactive.

2. The per cent of the total protein and activity present in the soluble form decreases from 100 per cent to zero as the temperature is raised from 20°C. to 60°C. and increases again from zero to 100 per cent as the solution is cooled from 60°C. to 20°C. The per cent of the total protein present in the soluble (native) form at any one temperature is nearly the same whether the temperature is reached from above or below.

3. If trypsin solutions at pH 7 are heated for increasing lengths of time at various temperatures and analyzed for total activity and total protein nitrogen after cooling, and for soluble activity and soluble (native) protein nitrogen, it is found that the soluble activity and soluble protein nitrogen decrease more and more rapidly as the temperature is raised, in agreement with the usual effects of temperature on the denaturation of protein. The total protein and total activity, on the other hand, decrease more and more rapidly up to about 70°C. but as the temperature is raised above this there is less rapid change in the total protein or total activity and at 92°C. the solutions are much more stable than at 42°C.

4. Casein and peptone are not digested by trypsin at 100°C. but when this digestion mixture is cooled to 35°C. rapid digestion occurs.
A solution of trypsin at 100°C. added to peptone solution at zero degree digests the peptone much less rapidly than it does if the trypsin solution is allowed to cool slowly before adding it to the peptone solution.

5. The precipitate of insoluble protein obtained from adding hot trypsin solutions to cold salt solutions contains the S-S groups in free form as is usual for denatured protein.

6. The results show that there is an equilibrium between native and denatured trypsin protein the extent of which is determined by the temperature. Above 60°C. the protein is in the denatured and inactive form and below 20°C. it is in the native and active form. The equilibrium is attained rapidly. The results also show that the formation of denatured protein is proportional to the loss in activity and that the re-formation of native protein is proportional to the recovery of activity of the enzyme. This is strong evidence for the conclusion that the proteolytic activity of the preparation is a property of the native protein molecule.

REFERENCES

4. Pace, J., *Biochem. J.*, 1930, 24, 606. These results were obtained with crude trypsin preparations. Crystalline trypsin solutions have a maximum stability near pH 4.0 instead of 5.0 (cf. (5)).
14. The loss in activity is proportional to the amount of this irreversible, denatured protein formed (cf. (5)).