CRYS\,#LLINE TRYPSIN

II. GENERAL PROPERTIES

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The isolation of a crystalline protein having constant properties, including powerful proteolytic activity, was described in the preceding paper (1). Experiments were also described which showed that destruction of the protein by any method tried resulted in a corresponding loss in proteolytic activity. The present paper describes some of the general properties of the preparation.

Extent of Hydrolysis

It was pointed out in discussing the method of preparation, that, as purification is continued, the extent to which the hydrolysis is carried decreases rapidly and in fractions after No. 5 the hydrolysis stops at an increase in formol titration of about 9 ml. $N/50$ per 5 ml. 5 per cent protein solution for casein and 7 ml. for gelatin. This figure is independent of the quantity of the enzyme used and also of the time over which it acts. In other words, it is a final equilibrium value. This figure represents very nearly an increase of 100 per cent in the number of free carboxyl (or amino) groups of casein and of 200 per cent for gelatin and shows that the action of this purified enzyme consists in splitting the molecule once for each free amino (or carboxyl) group already present in casein, and twice in gelatin. The data for an experiment in which this maximum hydrolysis was determined are shown in Fig. 1. It will be noted that this total increase corresponds approximately to that defined by Willstätter (2) as one tryptic unit. It is evident, therefore, that the method of determining tryptic activity used by Willstätter and his collaborators cannot be used for the proteolytic enzyme obtained in the present experiment since the digestion
follows an entirely different course with the purified enzyme from that of the original crude material. This applies to the form of the curve obtained as well as to the final value. This result has previously been reported by Schönfeld-Reiner (3).

Fig. 1 is plotted on a small scale so that the initial stage of the reaction does not appear. In Fig. 2 the first part of the reaction is plotted on a large scale. The figure shows the striking difference in the form of the reaction curve as determined with crystalline or crude trypsin, either by formol titration or the production of non-protein nitrogen. Since the curves obtained with the crude and crystalline fraction cross in two points, it is evident that they cannot be made to coincide throughout. All the curves, however, are straight lines, within the experimental error, up to an increase in formol titration of about 1 ml.
Casein - Non-protein N

Non-protein N per 6 ml 4% Casein

Casein - Non-protein N

Casein Formol

Formed Titrated per 6 ml 4% Casein Solution

Minutes

Fig. 2
or an increase in soluble nitrogen corresponding to an increase of about 5 ml. N/50 alkali, and in this part of the reaction therefore the curves may be superimposed. It follows from this that the curve obtained with the crude material cannot be used to measure the activity of the purified fraction except over this very small initial part of the curve. If the part of the curve beyond this point is used, it will be found that a different figure for the activity of the preparation will be obtained with each different concentration so that the figure is meaningless.

The effect of the protein concentration on the rate of reaction also varies with the purity of the enzyme (Northrop (4)).

The extent of hydrolysis of casein with trypsin-kinase, as reported by Abderhalden and Schwab (5), Waldschmidt-Leitz and Simons (6) is much greater than that caused by the crystalline trypsin and agrees approximately with that found with the crude fractions. This result shows that the enzyme isolated in the course of this work is different from that described by Waldschmidt-Leitz. It is possible that the two enzymes are entirely distinct but it seems more probably that the trypsin-kinase still contains some of the peptonases. The fact that more than half of the original protease activity is contained in the final crystalline fraction shows that the crystalline material represents the principal protase present in the crude material.

The fact that the crude preparation carries the hydrolysis much further than does the purified enzyme might be due to the fact that there is present in the crude material some substance which acts as a coenzyme and which enables the trypsin to attack the primary product resulting from the hydrolysis of the protein. An effect similar to this has been described by Abderhalden (7) who found that enzyme fractions which could not hydrolyze some polypeptides acquired the ability to hydrolyze these substances when some of the decomposition products were added to the digestion mixture. If this mechanism were correct it would be expected that the action of the purified and crude material mixed together would be greater than the sum of the two actions separately and also that the addition of heated crude material to the pure enzyme would allow digestion to proceed further than with the pure enzyme alone, since such coenzymes are usually heat stable. If, on the other hand, the extent of hydrolysis were due to the fact that the crude material contains one or more enzymes
capable of attacking the products formed by the trypsin itself, it would be expected that the action of the purified enzyme should be independent of the presence of the crude fraction. These possibilities were tested by adding crude and crystalline trypsin to a solution of gelatin which had already been digested with the crystalline enzyme, and which had then been boiled to inactivate any enzyme remaining. The results of this experiment are shown in Table I. The experiment shows that the crystalline trypsin is unable to carry digestion any further, even when the inactive, crude preparation is added, and

**TABLE I**

*Extent of Hydrolysis of Gelatin with Mixtures of Crude and Crystalline Trypsin*

<table>
<thead>
<tr>
<th></th>
<th>Crystalline trypsin solution 0.005 mg. protein nitrogen per ml. 0.5 [T. U.] ( ^{49} ) T. U. ( ^{49} ) ml.</th>
<th>Crude trypsin solution 0.025 mg. protein nitrogen per ml. 0.5 [T. U.] ( ^{49} ) T. U. ( ^{49} ) ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin—5 per cent pH 7.6 in M/10 7.6 phosphate buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150 ml gelatin solution + 5 ml. crystalline trypsin solution; 35°C. for 48 hrs.; boiled ( \frac{1}{2} ) hr.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digestion mixture made up as follows:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatin solution (boiled), ml. ........................................</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Crystalline trypsin solution, ml. ...................................</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>Active crude trypsin, ml. .............................................</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Boiled crude trypsin, ml. .............................................</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Formol titration (ml. ( \times 50 ) NaOH) per 5 ml. at 35°C. after 20 hrs., ml. ........................................</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>After 48 hrs. ..............................................................</td>
<td>0.10</td>
<td>0.15</td>
</tr>
</tbody>
</table>

also that the rate at which the digestion proceeds with the crude preparation is not increased by the addition of the crystalline trypsin. The results as a whole, therefore, indicate that the difference in the extent of digestion is due to the presence of several enzymes in the crude material rather than to any activation effect of the crude material on the trypsin itself.

*Hydrolysis by Trypsin Following Pepsin*

The crystalline trypsin hydrolyzes casein less than does pepsin, and gelatin to about the same extent but the hydrolysis occurs at different linkages with the two enzymes. This is shown by the fact
that gelatin or casein solutions, previously digested by pepsin, are hydrolyzed to the same extent, with crystalline trypsin, as are the original protein solutions.

A summary of an experiment in which casein and gelatin were first digested in acid solution with an excess of crystalline pepsin and then digested at pH 7.6 with crystalline trypsin is shown in Table II. The solutions in every case were titrated first to about pH 7.4 and the formol titration determined from this point so that the figures are comparable. The table shows that pepsin or trypsin alone very nearly triples the formol titration of gelatin and also that trypsin digestion following pepsin causes nearly the same increase as when trypsin acts on the original protein solution. Either enzyme, therefore, triples the number of titrable groups present so that there must be two hydrolyses for each original carboxyl group. Since gelatin originally contains about 30 acid groups per molecule, as determined by this method of titration, hydrolysis by either pepsin or trypsin alone must take place in about 60 places in the gelatin molecule. The two enzymes together, therefore, cause hydrolysis at about 120 groups. The formol titration of casein solutions is only doubled by trypsin digestion so that with this protein hydrolysis occurs at about 100 places in the molecule since there are about 100 titrable groups per mole of casein. This is less than the increase caused by pepsin alone, which corresponds to an increase of 200, or 200 per cent in the number of titrable groups. The action of both enzymes on casein liberates about 300 new groups from the casein molecule.

### Table II

Hydrolysis of Gelatin and Casein with Crystalline Pepsin and Trypsin

Formol titration per 5 ml. 5 per cent protein solution, pH 7.0; ml. N/50 sodium hydroxide.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Casein total</th>
<th>Increase</th>
<th>Gelatin total</th>
<th>Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original protein solution</td>
<td>9.0</td>
<td></td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>After pepsin digestion alone</td>
<td>27.0</td>
<td>18.0</td>
<td>11.5</td>
<td>7.5</td>
</tr>
<tr>
<td>After trypsin digestion alone</td>
<td>18.0</td>
<td>9.0</td>
<td>11.0</td>
<td>7.0</td>
</tr>
<tr>
<td>After pepsin digestion followed by trypsin</td>
<td>36.0</td>
<td>27.0</td>
<td>19.0</td>
<td>15.0</td>
</tr>
</tbody>
</table>
Crude trypsin preparations carry the digestion of such peptone solutions at least three times as far as does the crystalline trypsin.

Effect of Enterokinase on the Activity of the Crystalline Trypsin

Trypsin is known to exist in the pancreas in an inactive form, named trypsinogen. The active enzyme appears when the trypsinogen is mixed with intestinal juice or tissue extract. This activating effect of intestinal juice is assumed to be due to the presence of a substance called enterokinase. It is also known that trypsin becomes active if chopped pancreas or pancreatic extract is allowed to stand, especially in slightly acid solution.

The raw material used in the present work was obtained from pancreas which had stood for some time and it is probable that activation had already occurred. No attempts to activate it were made during the activity determinations since this procedure would undoubtedly lead to the isolation of an unknown mixture of active plus inactive enzyme. The activity of the crystalline enzyme is not increased by the addition of enterokinase as is shown by the experiment reported in Table III.
The kinase and the glycerin pancreatic extract were prepared according to Willstätter and Waldschmidt-Leitz (9) and their conditions for activation were used (2). The composition of the solutions for activation is given in the left side of the table. The volume was made up to 5 ml in each case with kinase solution which had been boiled in order to keep conditions as nearly constant as possible. The experiments with glycerin extract show that the kinase was active. Control experiments showed that the boiled kinase did not interfere with the reaction, and also that neither active kinase nor boiled kinase alone has any effect on the methods of determining activity. The activation mixture was kept for ½ hour at 30°C and then analyzed for activity by the usual methods. The resulting specific activity has been expressed as the number of activity units (8) per milligram protein nitrogen of the original enzyme solution. The results show that the kinase brought the specific activity of the glycerin extract up to about that of the pancreatic extract used as a starting point in this work. The specific activity of the crystalline trypsin is not affected by the addition of the kinase.

Since, according to Waldschmidt-Leitz (10) the reaction between kinase and trypsin is stoichiometric, it is necessary to be sure that there is an excess of kinase present since the addition of a small amount of kinase to a mixture already containing a large amount of active enzyme would show no effect. The quantity of active enzyme used in this experiment, therefore, is the minimum quantity which could be detected by the methods used and represents about the same order of activity as that of the activated glycerin extract.

**Effect of pH on the Rate of Digestion of Casein by Trypsin**

The rate of digestion of a 5 per cent casein solution titrated to various pH and at 35.5°C was determined by following the formation of nitrogen not precipitated by 5 per cent trichloracetic acid at different time intervals. The initial rate of the reaction was determined from the slope of these curves. The result of the experiment is shown in Fig. 3 in which the maximum rate observed is taken as 100 per cent. For comparison determinations made previously with crude trypsin preparations are included (11). There is no marked difference between the two curves and both show a rather flat maximum extending from about pH 7.5 to 9.5.
General Protein Tests

A 0.5 per cent solution of the material gave positive tests with Biuret, Millon, xanthoproteic, and Folin's tyrosine reagent (12).

In 0.0005 per cent solution, which is approximately the concentration used for activity determinations, all the protein tests were negative. It is therefore perfectly possible to have active solutions or preparations of the enzyme which give no protein reaction, simply because they are too dilute.

Number of Carboxyl Groups

0.137 gm. of the protein dissolved in 5 ml. water (pH 8.0) required 7.0 ml. N/50 alkali for formol titration. This corresponds to 0.001 equivalents of amino (or carboxyl) groups per gm., or 34 per mole.

Molecular Weight

The molecular weight of the protein was determined by osmotic pressure measurements in \( \frac{1}{4} \) saturated ammonium sulfate and pH 4.0 acetate buffer. The experiments were done with different concentrations of the protein and the pressure was read some time after it remained constant. The results of the experiment are shown in Table IV. The molecular weight is about 34,000.
**CRystalline Trypsin. II**

*Determination of the Diffusion Coefficient*

The determination was carried out as described by Northrop and Anson (13) and the quantity of material diffusing determined both by total nitrogen and by proteolytic activity. In the case of Cell 2 less than 10 per cent of the dissolved protein diffused out during the course of the experiment, while with Cell 4 about 25 per cent diffused out. The results of the experiment are shown in Table V. The value for

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### TABLE IV  
Osmotic Pressure

<table>
<thead>
<tr>
<th>Protein nitrogen</th>
<th>72</th>
<th>71</th>
<th>50</th>
<th>49</th>
<th>22.5</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pressure, mm. Hg</td>
<td>39</td>
<td>38</td>
<td>22.0</td>
<td>21.5</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>~</td>
<td>~</td>
<td>~</td>
<td>~</td>
<td>~</td>
<td>~</td>
</tr>
<tr>
<td>Protein/1</td>
<td>~</td>
<td>~</td>
<td>~</td>
<td>~</td>
<td>~</td>
<td>~</td>
</tr>
</tbody>
</table>

### TABLE V  
Diffusion Constant of Crystalline Trypsin

<table>
<thead>
<tr>
<th>Cell No.</th>
<th>2</th>
<th>4</th>
<th>2</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell constant (hemoglobin standard)</td>
<td>0.054</td>
<td>0.0315</td>
<td>0.054</td>
<td>0.0315</td>
</tr>
<tr>
<td>Solvent</td>
<td>0.5 saturated magnesium sulfate 5°C acetate buffer pH 4.0</td>
<td>0.5 saturated magnesium sulfate 5°C acetate buffer pH 4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sp. g. solvent</td>
<td>0.0303</td>
<td>0.0303</td>
<td>0.0362</td>
<td>0.0362</td>
</tr>
<tr>
<td>Average D cm$^3$ day$^{-1}$</td>
<td>1.115</td>
<td>1.115</td>
<td>1.145</td>
<td>1.145</td>
</tr>
<tr>
<td>Nitrogen activity</td>
<td>0.0207 ±0.001</td>
<td>0.0187 ±0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radius trypsin molecules</td>
<td>$2.72 \times 10^{-7}$ cm.</td>
<td>$2.5 \times 10^{-7}$ cm.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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1 These experiments were carried out by Dr. Henry W. Scherp.
the diffusion coefficient is the same within the experimental error for 
both cells and for both methods of determination.

This is direct experimental evidence that the molecules responsible 
for the proteolytic activity are the same size as those which contain 
the protein nitrogen and that the enzyme molecule cannot be separated 
from the protein molecule by fractional diffusion. The average value 
of the diffusion coefficient is 0.020 ±0.001 cm.² per day, corresponding 
to a molecular radius of about 2.6 × 10⁻⁷ cm.

The volume of 1 mole of (hydrated) protein is therefore (2.6 × 
10⁻⁷)³ × 4/3π × 6.06 × 10²³ = 44,700 cm.³. From the osmotic pressure 
measurements the molecular weight of the anhydrous protein is 34,000. 
If the specific volume of anhydrous protein is assumed to be 0.75 (the 
value generally found for protein), this corresponds to a molecular 
volume of (anhydrous) protein of 26,200 ml. Therefore, each mole 
of protein carried with it about 18,500 gm. of water, which cor-
responds to about 0.53 gm. of water per gm. of anhydrous protein, and 
a molecular weight for the hydrated protein of 53,500.

Hydration from Viscosity Measurements

Hydration of trypsin was determined by measuring the viscosity 
of various concentrations of crystalline trypsin in 0.5 saturated magne-
sium sulfate pH 4.0 at 5°C. The results showed that the trypsin is 
hydrated to the extent of 0.5 gm. of water per gm. of dry trypsin and 
is independent of the concentration of trypsin in the range of 4.0 to 
0.8 gm. of protein per 100 ml. of solution. The hydration value was 
calculated from the specific viscosity by means of Kunitz's formula 
(14). The specific gravity of the anhydrous protein was assumed to 
be 1.33. The value obtained in this way agrees very closely with that 
calculated above from the diffusion and osmotic pressure measurement.

Isoelectric Point

If a solution of the crystalline trypsin is added to boiling M/10 phos-
phate buffer of various pH, a precipitate is formed between pH 7.0 and 
8.0. This precipitate undoubtedly consists of denatured protein and 
the relation of this zone of precipitation of the denatured protein to the 
isoelectric point of the native protein is therefore somewhat doubtful. 
Cataphoreisis measurement showed that the isoelectric point of col-
lodion particles suspended in dilute solutions of trypsin in different buffers is about pH 6.0 in m/50 acetate, about pH 7.0 in m/50 phosphate, and between pH 5.0 and 6.0 in the presence of m/50 ammonium sulfate. These results indicate that the isoelectric point is in the neighborhood of pH 7.0 but its exact position can only be determined by transport measurements. Apparently, however, it is more towards the acid side than that found for crude trypsin preparations by means of the distribution of the enzyme in gelatin particles (Northrop (16)) which indicated an isoelectric point at about pH 10.0. Willstätter (17) also concluded from the behavior with various adsorbents that the enzyme with which he was working had an isoelectric point quite far on the alkaline side. The enzyme described in this paper, however, is undoubtedly different from that with which Willstätter's experiments were done.

**TABLE VI**

*Inactivation of Trypsin Solutions at 30°C. and Various pH*

About 5 per cent solution of crystalline trypsin made up in \(\frac{1}{4}\) saturated ammonium sulfate and increasing amounts of sulfuric acid. pH measured and activity determined after various time intervals at 35°C.

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>N/4</th>
<th>N/8</th>
<th>N/16</th>
<th>N/32</th>
<th>N/64</th>
<th>N/128</th>
<th>N/256</th>
<th>N/512</th>
<th>N/1024</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(_2)SO(_4)</td>
<td>1.5</td>
<td>1.8</td>
<td>2.16</td>
<td>2.42</td>
<td>2.64</td>
<td>2.81</td>
<td>2.95</td>
<td>3.0</td>
<td>3.12</td>
</tr>
<tr>
<td>pH (electrometric)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Time at 30°C. | Per cent of original activity after increasing time at 30°C.

<table>
<thead>
<tr>
<th>hrs.</th>
<th>46</th>
<th>67</th>
<th>81</th>
<th>100</th>
<th>95</th>
<th>80</th>
<th>60</th>
<th>50</th>
<th>48</th>
<th>44</th>
<th>31</th>
</tr>
</thead>
</table>

The effect of pH on the stability of trypsin

The rate of inactivation of a solution of crystalline enzyme in \(\frac{1}{4}\) saturated ammonium sulfate at 30°C. was determined. The activity determinations were made by the gelatin viscosity method. The results of the experiment are shown in Table VI. There is a rather sharp maximum for stability at about pH 2 under these conditions.

The results in general are quite different from those obtained by the writer (26) and by Pace (27) with crude trypsin preparations since the crude preparations were found to be most stable at about pH 6.0.
DISCUSSION

The experiments described in these papers show that a crystalline protein may be isolated from pancreas which has constant physical and chemical properties including intense proteolytic activity. The protein has been studied under a variety of conditions which would be expected to show evidence of mixtures without causing any demonstrable change in its characteristic properties. If the material were other than a protein these experiments would justify the statement that it was a pure substance. Since it is a protein, however, it is quite possible that the material may be a solid solution, as in the case of proteins such solid solutions frequently exist and are extremely difficult to fractionate into their components. The problem is rendered unusually difficult in this case by the extremely unstable nature of the protein. It seems unlikely, however, that the material contains any non-protein molecular species. The constant composition under various conditions of fractionation precludes the possibility of an adsorption compound since it is characteristic of these compounds that their composition varies with the conditions of precipitation.

Even though the crystalline material is a mixture or solid solution and not a pure substance, there seems good reason to believe that the proteolytic activity and the protein properties are attributes of the same molecule. This conclusion is confirmed by a number of experiments in which it was found that any change in the protein properties caused a corresponding decrease in the activity of the solution. Denaturation of the protein by heat, hydrolysis by acid or pepsin or alkali all cause the concentration of native protein in the solution to decrease and this decrease is accompanied by a corresponding decrease in activity. In addition, the denatured, inactive, protein formed by heating the solution reverts to the native condition when the solution is cooled and at the same time the normal specific activity returns (18). In order to account for these results on the assumption that the activity is due to the presence of some non-protein molecule, it is necessary to assume that this hypothetical molecule cannot exist in the absence of the protein and also that it regains its activity under the same conditions as cause the denatured protein to return to the native form. In the absence of positive proof for the existence of such a hypothetical molecule these assumptions seem unlikely. So far as
the writer is aware, there is no positive proof of the existence of such molecules and the assumption that they exist rests merely on the negative fact that most of the attempts to prepare pure substances, i.e. those with constant properties including enzymatic activity, have been unsuccessful. On the other hand it is, of course, impossible to disprove the existence of such molecules. Since nothing is known of the properties of these hypothetical active molecules it would be perfectly logical to assume that they are proteins themselves, especially since the general properties of enzymes such as inactivation by heat, adsorption on surfaces, and destruction by strong acid or alkali are in general those of proteins.

Active enzyme preparations have been obtained which contain very small amounts of protein; on the other hand extremely active preparations of urease, pepsin, and trypsin, and amylase have been obtained which are pure, or nearly pure proteins. If it be assumed that the activity of these protein preparations is due to the presence of some minute amount of a non-protein molecule, it is equally reasonable to assume that the activity of the non-protein preparation is due to the presence of a minute amount of protein.

The fact that in other cases the enzymatic activity may vary independently of the total protein content of the preparation proves only that some of the protein present is inactive but not that all of it is inactive.

Numerous experiments have been reported in the literature in which solutions of pepsin and other enzymes have been found to give negative protein tests although they are active. These experiments are also inconclusive since the activity test is far more delicate than the chemical test for proteins. For instance, a solution of crystalline trypsin or pepsin containing less than 1/1,000,000 of a gram of protein nitrogen per milliliter has an accurately measurable effect on the digestion of casein, while solutions of pepsin containing less than 1/10,000,000 of a gram of nitrogen have a very powerful effect on the coagulation of milk. Such solutions give negative results with protein tests but the dry material from which the solutions are made is practically pure protein. The minimum concentrations of these enzymes which can be detected are at least ten times less than the concentrations mentioned above and are of the same order of magnitude as the concentration of
respiratory ferment in yeast as calculated by Warburg and Kubowitz (19).

It appears to the writers, therefore, that the assumption that enzymes are proteins is in the best accord with the facts up to the present time. Since these proteins possess characteristic enzymatic activity, in addition to the usual properties of proteins, they must possess some characteristic chemical structure which may or may not be an amino acid complex. The problem is the same as in the case of insulin (20). In general, most properties of molecules cannot be considered quantitatively as the sum of the properties of the various groups or atoms of which they are made, but must be considered as properties of the whole molecule. Thus, the optical activity, color, strength of acid groups, etc., of any one molecule depends qualitatively on the presence of a certain group or groups, but quantitatively, the property is affected by any change in the molecule. For instance, to possess optical activity a molecule must contain an asymmetric atom but its specific optical activity will change with any change in the molecule and it is impossible to isolate a group from the compound possessing the optical activity of the whole molecule. The same is true of the color of dyes to a more marked degree.

Hemoglobin presents perhaps the best example. This substance has the general properties of a protein but in addition possesses the remarkable property of combining reversibly with oxygen. It acts as a catalyst in certain oxidation reactions and might, therefore, be considered an enzyme. The property of combining reversibly with oxygen is assumed to be due to the presence of the iron-pyrrol group but denaturation of the protein, a reaction common to all proteins, destroys its power of combining reversibly with oxygen although the denatured protein still contains the prosthetic group.

Krebs (21) has shown that heme itself is a very poor catalyst but when combined with certain nitrogenous groups it forms hemochromogens, some of which are very effective catalysts. Thus the catalytic properties of hemoglobin and these related compounds are all due to the presence of the heme group, but this group when isolated has little or no catalytic activity and the catalytic power of the various heme compounds depends upon the substance with which the heme is combined. It is quite possible that the same general condition
applies to other enzymes and that there are an indefinite number of closely related enzymes depending upon the compound with which the characteristic group is combined. This point of view does not differ very much from that developed by Willstätter (22) and his collaborators except that it regards the various active compounds as definite chemical individuals rather than as adsorption complexes of varying composition.

At the present time, however, there is no direct evidence of the existence of any peculiar prosthetic group not found in other proteins and it is quite possible that their activity depends on some peculiar arrangement of the amino acids, as Jensen (20) has suggested in the case of insulin.

**Mechanism of the Catalytic Effect**

Sørensen (23) has shown that protein solutions in the presence of the solid phase are in true equilibrium and that the system as a whole is a two phase one as defined by the phase rule. The protein solution, therefore, consists of one phase. The solubility experiments with pepsin (24) and trypsin give the same result. These results show that the catalytic reactions caused by pepsin and trypsin in protein solutions are homogeneous rather than heterogeneous.

**SUMMARY**

A method is described for isolating a crystalline protein of high tryptic activity from beef pancreas. The protein has constant proteolytic activity and optical activity under various conditions and no indication of further fractionation could be obtained. The loss in activity corresponds to the decrease in native protein when the protein is denatured by heat, digested by pepsin, or hydrolyzed in dilute alkali.

The enzyme digests casein, gelatin, edestin, and denatured hemoglobin, but not native hemoglobin. It accelerates the coagulation of blood but has little effect on the clotting of milk. It digests peptone prepared by the action of pepsin on casein, edestin or gelatin.

The extent of the digestion of gelatin caused by this enzyme is the same as that caused by crystalline pepsin and is approximately equivalent to tripling the number of carboxyl groups present in the solution.

The activity of the preparation is not increased by enterokinase.
The molecular weight by osmotic pressure measure is about 34,000. The diffusion coefficient in $\frac{1}{2}$ saturated magnesium sulfate at $6^\circ$C. is $0.020 \pm 0.001$ cm.$^2$ per day, corresponding to a molecular radius of $2.6 \times 10^{-7}$ cm.

The isoelectric point is probably between pH 7.0 and pH 8.0. The optimum pH for the digestion of casein is from 8.0–9.0. The optimum stability is at pH 1.8.

REFERENCES