CRYSTALLINE PEPSIN

II. General Properties and Experimental Methods

By JOHN H. NORTHROP

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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The isolation of a crystalline protein having proteolytic activity was described in the preceding paper (1). The results of solubility, inactivation, and diffusion experiments showed that there was reason to believe that the preparation was a pure substance and that the peptic activity was a property of the protein molecule itself. The following paper contains some further properties of the pepsin and a description of the experimental procedure.

Solubility in Various Concentrations of Sodium Sulfate.—The solubility in various concentrations of sodium sulfate is shown graphically in Fig. 1. A concentrated suspension of the crystalline material was made in sodium sulfate of the required concentration made up in 0.001 M sulfuric acid. These suspensions were stirred at 35°C. for 10 minutes and then cooled to 25°C. for 24 hours. The solubility was therefore reached from the supersaturated side. There is a sharp maximum of solubility at about 0.1 M sodium sulfate.

Solubility at Different Temperatures.—The solubility in 0.50 saturated $MgSO_4$ and 0.05 acetate pH 4.65 at various temperatures is shown in Fig. 2. The solubility increases slightly but there are no sharp breaks in the curve and therefore no indication that the material is a double salt, nor that different solid phases appear at different temperatures.

Solubility in Various Concentrations of Hydrochloric or Sulfuric Acid.—These determinations were made by stirring a concentrated suspension with various concentrations of acid as described for the sodium sulfate experiments. The results are given in Fig. 3. There is a minimum of solubility at about pH 2.75 corresponding presumably to the isoelectric point. The solubility increases rapidly on both sides of

this point. The increase in solubility on the acid side cannot be ascribed to salt effect since the series represented by triangles was made with a mixture of hydrochloric acid and sodium chloride in which the

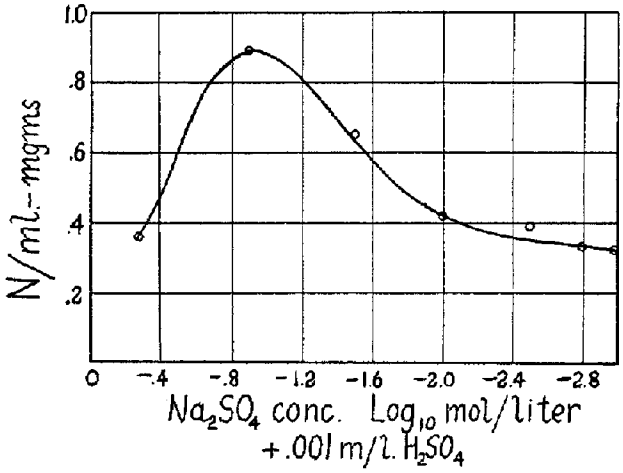

FIG. 1. Solubility of crystalline pepsin in various concentrations of sodium sulfate made up in 0.001 M hydrochloric acid at 25°C.

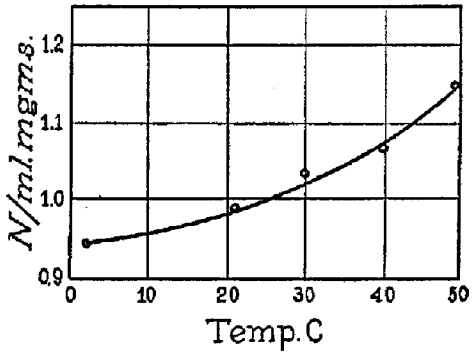

FIG. 2. Solubility of crystalline pepsin in 0.5 saturated magnesium sulfate and 0.05 M acetate at various temperatures.

chloride concentration was kept constant throughout. When the solubility is plotted against the concentration of acid instead of the pH the solubility increases in direct proportion to the addition of acid

and each millimole of acid dissolves about 0.05 mg. of nitrogen, corresponding to 0.33 mg. of pepsin. This is the expected relation for a

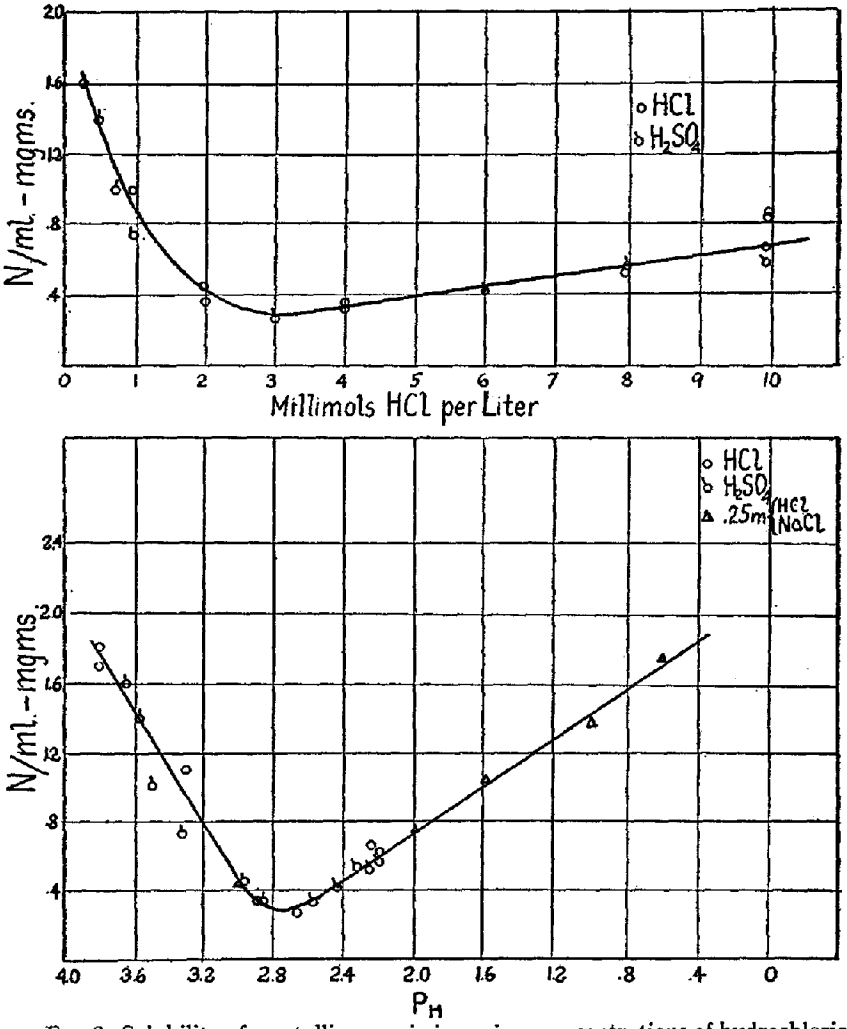

FIG. 3. Solubility of crystalline pepsin in various concentrations of hydrochloric or sulfuric acid.

monovalent base (2). On the alkaline side of the minimum the solubility curve is convex downward. This is the theoretical shape for the solubility curve of a polyvalent acid.

Titration Curve.—The titration curve of the pepsin is shown in Fig. 4. Owing to its insolubility the curve could not be followed on the acid side; but apparently there must be one or more groups with a pK of 1 or less. The curve again indicates an isoelectric point of pH 2.75. Some titrations were made in the presence of solid and indicated that the solid phase appearing on the alkaline side is a salt of the pepsin and acid.

The writer is indebted to Dr. H. S. Simms for the determination of the titration curve.

The Isoelectric Point as Determined by Migration Experiments.—The solubility and titration curves indicate an isoelectric point at about pH

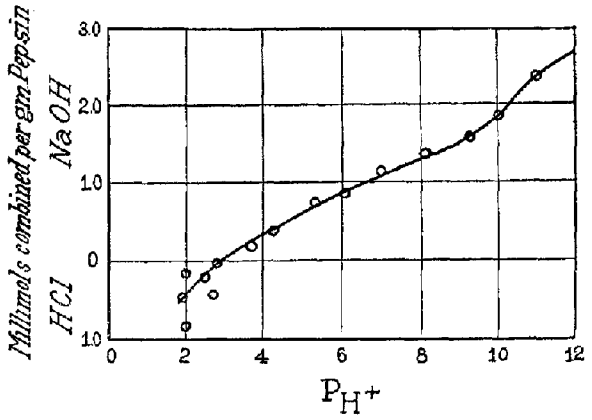

FIG. 4. Titration curve of crystalline pepsin at 22°C. with hydrochloric acid and sodium hydroxide.

2.75. This was confirmed by measurements of the migration of finely ground crystals at various pH as determined microscopically (3). No migration occurred at pH 3. The crystals migrated to the anode on the alkaline side of 3 and to the cathode on the acid side of 3. The isoelectric point of the preparation is therefore probably 2.75, which is not far from that found by Michaelis and Davidson (4) in a study of the migration of crude pepsin preparations. Ringer (5) had, however, stated that some pepsin preparations were always negatively charged and the writer (6) had found that the distribution of pepsin with gelatin particles also indicated that the enzyme was always negatively

charged on the alkaline side of pH 2.0. It does not seem possible at present to account for these discrepancies.

Molecular Weight.—The molecular weight of the pepsin was determined by osmotic pressure measurements in molar sodium acetate buffer at pH 4.60. Since this is quite far from the isoelectric point it is necessary to show that the observed pressure is due to the protein itself and not to a Donnan equilibrium. The effect of the Donnan equilibrium would be slight in molar salt solution and would be expected to increase as the concentration of the protein increased. The experiments, however, show that the calculated molecular weight was constant within the experimental error for a 4 per cent and a 2 per cent

TABLE I

Molecular Weight

Method	Temperature	Concentration of solution	Solvent	M
Osmotic pressure	8°	4 per cent	M/1 acetate pH 4.6	34,000
			" " "	33,000
	8°	2 " "	" " "	36,000
Diffusion coefficient				
Activity	8°	1 " "	" " "	37,000 ± 1,000
Nitrogen			" " "	36,000 ± 1,000
From P content	1 atom P per mole			40,000
Cl "	2 atoms Cl " "			35,000
S "	10 " S " "			36,000

solution. There was probably therefore very little Donnan effect. The molecular weight may also be calculated from the diffusion coefficient by means of Einstein's formula, and also from the percentage of phosphorus, chlorine and sulfur. The results of these calculations are shown in Table I. The various methods all give values for the molecular weight between 33,000 and 38,000.

Experimental Procedure

Material.—The starting point of this work was Parke, Davis pepsin U. S. P. 1:10,000. This material has already been highly concentrated from autolyzed pig stomachs. According to information kindly furnished by Mr. F. O. Taylor, Chief

Chemist of Parke, Davis and Company, the method of purification is in general that of Davis and Merker (7).

Rate of Hydrolysis of Casein, Egg Albumin, Edestin or Gelatin as Determined by the Increase in Carboxyl Groups

The egg albumin had been crystallized three times. Hammersten's casein (Kahlbaum) was used. The edestin was prepared from hemp seed and had been recrystallized twice. The gelatin was prepared from Cooper's powdered gelatin as previously described (8).

The protein solutions were titrated to the required pH with HCl and diluted so as to contain 5 gm. protein per 100 ml. They were then heated to boiling to denature the proteins and stored at 5°C. 5 ml. of the protein solution are pipetted into a series of 50 ml. centrifuge tubes and placed in a water bath at 35.5°C. 1 ml. of the pepsin solution is added to each tube. After about 2, 4, 6 and 8 minutes 5 ml. of 0.10 NaOH and 1 ml. of formalin are added to each tube. They are then titrated to pH 10 with N/50 NaOH (9). The increase in the alkali needed over that used for a control tube containing 1 ml. boiled pepsin solution is the titration. These figures are then plotted against the time of hydrolysis and the increase in the formol titration for the first minute interpolated from the curve. The proteolytic activity per gram is equal to this amount, expressed as milliequivalents divided by the grams dry weight per milliliter of the pepsin solution used.

$$i.e.,\ [PU]_{gm.} = \frac{\text{ml. N/50 NaOH} \times 20 \text{ per minute}}{\text{mg. pepsin per ml.}}$$

The determination is quite accurate with casein or edestin but in the case of egg albumin and gelatin the rate of hydrolysis decreases rapidly after an increase of only 0.3 to 0.5 ml. N/50 NaOH, so that interpolation is difficult unless very small titrations are used and in this case the experimental error of the titration is large.

Determination of Activity by Comparative Methods

The activity determined in this way probably represents the most rational measure of the rate of hydrolysis. For routine comparative purposes, however, it is not convenient since it requires a number of analyses for each determination and since the experimental error is rather large. After the activity in rational units has been determined by this method, however, it is possible to use comparative methods which are more accurate and also more convenient. In order to do this a solution the activity of which has been determined by the previous method is used as a standard. Several comparative methods of this kind have been used.

Increase in Formol Titration after 1 Hour at 35°C. (cas. F.), (gel. F.), etc.—Increasing amounts of the standard pepsin solution containing a known number of PU as determined by the rate of liberation of carboxyl groups as described above, are

added to 5 ml. portions of the various protein solutions and the hydrolysis allowed to proceed for 1 hour at 35.5°C. At the end of this time the increase in the formol titration is determined as described above. This increase is then plotted against the number of proteolytic units added so as to give a standard curve. The activity of an unknown solution is then determined by carrying out the hydrolysis in the same way and interpolating the value of the activity from the standard curve.

Solution of Casein (cas. S.).—1 ml. of various dilutions of the standard pepsin solution is added to 5 ml. of 5 per cent casein pH 2 and kept at 35.5°C. for 21 hours. 5 ml. of this solution is then pipetted into 5 ml. of 20 per cent trichloracetic acid, filtered and the total nitrogen determined in 5 ml. of the filtrate. A standard curve is plotted from these figures and the activity of the unknown solution found by interpolation from this curve. This is the most accurate method, the probable error of a single determination being less than 2 per cent.

Rennet Action (R).—The amount of pepsin solution which is just sufficient to solidify 5 ml. of milk at 35.5°C. in 2 hours is determined. This figure cannot be compared directly with the activity as determined by the liberation of carboxyl groups. For purposes of comparison therefore it is arbitrarily assumed that the activity of the standard solution of crystalline pepsin is the same with respect to rennet action as with respect to the hydrolysis of casein as determined by the rate of liberation of carboxyl groups. 0.085 mg. crystalline pepsin equivalent to 0.008 $[PU]^{cas.\ F.}$ coagulates 5 ml. of milk under the above conditions. 1 ml. of a solution which causes coagulation of 5 ml. of milk in 2 hours therefore contains 0.008 $[PU]^{R}$. The time varies greatly with different samples of milk so that it is necessary to run a known solution simultaneously with the unknown.

Gelatin Liquefaction Method (10) pH 5 (gel. V.).—0.2 ml. of the standard pepsin solution is added to 5 ml. of 2.5 per cent solution of isoelectric gelatin in water and the mixture poured into a viscosimeter at 35.5°C. The change in viscosity is then determined at intervals and the time of outflow after various time intervals plotted against the elapsed time since mixing. The time required to cause a 3 per cent change in the original viscosity is then interpolated from these curves. The time required to cause this change is inversely proportional to the quantity of pepsin so that the activity of an unknown solution in terms of the standard is found by proportion. Since there is no simple relation between the rate of change in viscosity and the liberation of carboxyl groups (11), it is assumed arbitrarily that the activity of the standard crystalline pepsin solution is that determined by the rate of liberation of carboxyl groups from pH 2.5 gelatin. Under these conditions 0.14 mg. of crystalline pepsin equivalent to 2.4×10^{-5} $[PU]^{gel.\ F.}$ cause a 3 per cent change of viscosity in 0.30 hours. Since the activity is inversely proportional to the time

$$[PU]^{gel.\ V.}_{0.2\ ml.} = \frac{0.72 \times 10^{-5}}{t\ \text{hrs.}}$$

and

$$[PU]^{\text{gel v.}}_{\text{ml.}} = \frac{3.6 \times 10^{-5}}{t \text{ hrs.}}$$

where t is the time in hours for 0.2 ml. of the unknown solution to cause a change of 3 per cent in the viscosity of the standard gelatin solution.

Nitrogen Determination.—The nitrogen was determined by a slight modification of the Folin and Farmer (12) micromethod using the following digestion mixture:

2 ml. concentrated H_2SO_4
1.5 gm. K_2SO_4
0.2 gm. mercuric oxide

Digestion is continued 20 minutes after the solution is colorless in 50 ml. Kjeldahl flasks having a constriction half-way up the neck to prevent loss of acid.

35 ml. H_2O, 0.2 gm. sodium hypophosphite and 10 ml. of saturated NaOH are added and the solution distilled through two Kjeldahl traps into standard N/50 acid. 2 to 5 mg. of nitrogen are taken for a determination. The method is accurate to about 0.2 per cent provided the double trap is used. Otherwise there are irregular variations due to spattering of the alkali.

P Determination.—The excellent method described by M. Sørensen (13) was used.

Experimental Methods for the Solubility Determinations

Crystals.—The first attempts to determine the solubility of pepsin were made in the usual way by stirring a suspension of the solid with the liquid and analyzing the supernatant fluid after various times. It was found, however, that equilibrium could not be obtained in this way except when a very large amount of the solid was present. Otherwise, the amount of material dissolved increased rapidly for the first 24 hours and gave what appeared to be an equilibrium value. This value, however, was different with different quantities of precipitate, being higher for the larger quantity. If the stirring were continued, however, it was found that the solubility continued to increase slowly in all the suspensions and this increase continued at a nearly constant rate, apparently indefinitely. At the same time the ratio of activity to nitrogen in the filtrates decreased and a form of nitrogen which could not be coagulated by heat began to appear in the solution. It was evident therefore that this slowly continued increase in soluble protein was not simply a process of solution but was due to the decompostition of the protein. Various methods of stirring combined with low temperatures were tried without success, although it was found that stirring with a glass ball in a test tube completely filled with solution gave much better results.

With large quantities of precipitate constant values for the solubility could be obtained very rapidly by stirring the suspension in a long narrow tube, the stirrer

consisting of a glass rod of about half the diameter of the tube so that there is only a thin layer of suspension between the stirrer and the walls of the tube. The stirrer was rotated at about 500 R.P.M. There is very efficient stirring under these conditions and constant values for the solubility were obtained after about 10 minutes, provided the amount of solid material was more than 3 or 4 times as much as the quantity in solution. The figure for the solubility under these conditions was found to be independent of the quantity of precipitate when relatively large amounts of precipitate were used. However, if the quantity of precipitate were small then equilibrium values could not be obtained before decomposition commenced, so that it was not possible in this way to obtain the part of the curve where there is very little solid. This portion of the curve, however, could be obtained from the supersaturated side. The method finally adopted for solubility measurements may be outlined as follows.

A suspension of freshly recrystallized protein was filtered by suction and washed on the filter 5 or 6 times with the solvent that was to be used for the solubility determinations. About 25 gm. of the filter cake were then suspended in 100 ml. of the solvent and stirred for 15 minutes at the desired temperature, the stirrer being arranged as described above. The suspension was then filtered and the nitrogen content of the filtrate determined. The precipitate was stirred again with 100 ml. of the solvent and this process was repeated until successive determinations gave the same value for the nitrogen in the filtrate. Aliquot portions of the suspension were diluted with solvent so as to give a series of suspensions containing 1/2, 1/4, 1/8 and 1/16 of the concentration of solid present in the original suspension. Each of these suspensions was then stirred for 15 to 20 minutes, filtered and the nitrogen, proteolytic activity and optical rotation of the filtrate determined. These figures represent the solubility as determined from the undersaturated side.

A second series of suspensions was made up in the same way and stirred at 45°C. for 5 to 10 minutes. A small sample of supernatant was taken at 45°C. and analyzed for nitrogen. The figure obtained in this way was always higher than that found at the lower temperature. These suspensions were then cooled to the original temperature and stirred occasionally. After 24 hours they were filtered and the supernatant analyzed as in the first series. This method gives the solubility figure from the supersaturated side. In this way the solubility could be determined for suspensions containing more than twice as much solid protein as dissolved protein. In this range the solubilities determined from the undersaturated side nearly always agreed with those from the supersaturated side showing that they were real equilibrium values. When less solid was present, however, this was not the case. The values from the supersaturated side were always higher than those from the undersaturated side and were frequently higher than those obtained with large quantities of solids from the supersaturated side. In other words, they showed that equilibrium was not obtained when only a small quantity of solid was present. Attempts to obtain equilibrium values by longer stirring resulted only in indefinite increase in solubility accompanied with inacti-

vation. The solubilities at the point where very little solid was present could be obtained, however, by diluting a supersaturated solution and then inoculating with small amounts of solid. About 50 ml. of the original heavy suspension were stirred at 45°C. and filtered at this temperature. The filtrate was then diluted so as to give a series of solutions containing from 0.5 mg. to 3 or 4 mg. of nitrogen per milliliter. A few drops of the heavy suspension were then added to this series of solutions and the solutions cooled to the original temperature. In those solutions containing less than the saturation concentration the added solid dissolved and the solution became perfectly clear. The more concentrated ones remained cloudy and in those which had been diluted very little more solid crystallized out. These solutions were filtered and analyzed after 24 hours in the same way as the other series.

In this way a series of points were obtained showing the solubility up to and just beyond the appearance of the solid phase. The values were reproducible and those obtained when very little solid was present usually agreed well with the values obtained by the methods described for the case when a large amount of solid was present. There still remains a very small portion of the solubility-concentration curve corresponding to the point where the amount of solid was about equal to the amount of material in solution, for which equilibrium values could not be obtained. Values obtained by inoculating the supersaturated solution were usually higher than those found for the rest of the curve. The values obtained from the undersaturated side were usually lower.

Amorphous.—The solubility of the amorphous material as obtained from the undersaturated side was determined in the same way as with the crystals by stirring the precipitate with the solvent. In order to obtain the value from the supersaturated side, however, it was necessary to dissolve the precipitate and then reprecipitate it instead of allowing a supersaturated solution to cool since under these latter conditions crystals appeared. The solvent solution was therefore made up in two parts, in one of which the amorphous material was readily soluble. 20 to 30 gm. of the washed crystals were dissolved in as small a volume as possible of this part of the desired solvent and then precipitated by addition of the other part of the solvent. The solution was stirred during the precipitation and the precipitating solution run in slowly so as to avoid local excess. The amorphous precipitate obtained in this way was redissolved in the first part of the solvent and precipitated again.

This procedure was repeated until the nitrogen content of two or more successive filtrates was the same. This was taken as the value for the solubility from the supersaturated side. The suspension was then filtered and aliquot portions of the precipitate stirred with about 10 ml. of the complete solvent for 10 to 15 minutes. These suspensions were then filtered and analyzed as with the crystalline material. In this way a series of points were obtained showing the solubility obtained with varying amounts of precipitate from the undersaturated side. In order to obtain the value from the supersaturated side with different amounts of solid present the

remainder of the precipitate was dissolved in varying amounts of the first half of the solvent, so as to give a series of solutions containing from 1 to 3 or 4 mg. of nitrogen per milliliter. The second half of the solvent was then added to this series while the solution was being stirred and the suspension filtered at once. If the solutions were filtered immediately these values agreed well with those from the undersaturated side, but if the solutions were allowed to stand the values were consistently lower in those solutions in which only a small amount of solid appeared. This was at first very puzzling and it was thought that it might represent an actual fractionation of the material. However, the properties of the precipitate obtained in this way were precisely the same as that of the original material so that there was no evidence for any fractionation. Microscopic examination of these precipitates, however, showed that they contained some crystalline material, and since the solubility of the crystalline material is much less than that of the amorphous this was evidently the explanation for the low solubility obtained under these conditions. When a large amount of amorphous precipitate is present the material crystallizing out would be immediately replaced by solution of more amorphous so that under these conditions the saturation value of the amorphous precipitate was obtained. When only a small amount of amorphous material was present, however, the loss of material due to crystallization could not be replaced rapidly enough by solution of the amorphous material and so low values for the solubility resulted. In confirmation of this it was found that the values for the solubility obtained by filtering those solutions having very little precipitate immediately after the appearance of the precipitate agreed with those obtained in solutions containing larger amounts of precipitate. It was also found that inoculation of the solutions with a few crystals gave still lower values for the solubility since in this case crystallization proceeded more rapidly.

Composition of the Solutions Used.—In order to carry out the precipitation of the amorphous material as described above, it is necessary to make up the complete solvent by adding together two different solutions. Measured volumes of the two separate parts of the solvent must be used instead of making the mixture up to a constant volume in order to avoid the difficulty of correcting for the volume of the original portion of the concentrated suspension taken. The composition of the solvent is therefore expressed as the ratio of the volume of one portion to the total volume assuming that the volumes are additive. The value given in this way does not correspond strictly with the actual composition of the final solution but does define the composition of the complete solvent. For instance, a solvent designated as half-saturated magnesium sulfate and 0.05 M acetate buffer pH 4.65 refers to a solution prepared by mixing together equal volumes of saturated magnesium sulfate and 0.10 M acetate buffer pH 4.65. The saturated magnesium sulfate was prepared by stirring crystalline magnesium sulfate with water at about 18°C. The supernatant was filtered off and found to have a specific gravity at 22°C. of 1.2955 compared to water at that temperature. The sodium sulfate solution had a specific gravity at 22°C. of 1.120 and was 1.104 molar with respect to Na_2SO_4.

TABLE II

Summary of Properties of Pepsin

Crystalline form. Dihexagonal bipyramids, colorless, 0.01 to 0.1 mm. long. Positive double refraction.
Density. 1.32 gm. per ml.

$[\alpha]_D^{25°}$ (pH 4–5) −70°

Analysis. Per cent of dry weight.	Total N	Amino N	C	H	Cl	S	P	Ash	$SO_4^=$
	15.3	.80	52.4	6.67	.22	.86	.078	.40	00

Xanthoproteic and Biuret test—positive
Molisch test —negative

Diffusion coefficient 8° { M/1 acetate buffer, pH 4.6, Viscosity .017 }
.047 ± .0005 cm.²/day (From N determination)
.0468 + .0005 " " (" activity determination)

Molecular weight from osmotic pressure (M/1 acetate pH 4.6)	35,000 ± 1,000
From diffusion coefficient	36,000 ± 1,000
From P content, 1 atom P per mole	40,000
Cl " 2 atoms Cl per mole	35,000
S " 10 " S " "	36,000

TABLE II (*Continued*)

Solubility	Solvent	.002 HCl	.522 M Na_2SO_4 2.5×10^{-5} N H_2SO_4	.444 M Na_2SO_4 .055 acetate pH 4.65	0 5 sat. $MgSO_4$ .05 acetate pH 4.65	0.5 sat. $MgSO_4$ .01 acetate pH 4.65
Mg. N/ml.	Crystalline	.3 (20°)	.70 (20°)	.43 (20°)	.22 (8°)	
	Amorphous			2.2 (20°)	1.00 (20°)	.65

Velocity constant inactivation

65°— pH 3.0 0.58 Time in hrs. Log_{10}.

1.98 per cent solution in HCl

Activity		
Substrate	pH	Equivalents per mole pepsin per min. 35.5°C.
Edestin	2.0	1,000
Casein	2.0	500
Denatured egg albumin	2.2	320
Gelatin	2.5	6.0

The acetate buffers were made up according to Walpole's curve (14). The pH values given are for solutions corresponding to 0.2 M acetate buffer and do not represent actual pH values of the final solution.

The analytical work and the preparation of most of the crystalline pepsin was carried out by Mr. N. Wuest.

SUMMARY

A number of properties of crystalline pepsin have been determined and are summarized in Table II.

The experimental procedure used is described.

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