CRYSTALLINE RIBONUCLEASE

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(Received for publication, June 3, 1940)

The presence in pancreas of a heat stable enzyme capable of digesting yeast nucleic acid was described by W. Jones in 1920 (1). He found that the digestion was not accompanied by any liberation of free phosphoric acid. Jones' observations were recently confirmed by Dubos (2). Dubos and Thompson (3) carried out a partial purification of the enzyme by means of acetone, and named the enzyme "ribonuclease." Schmidt and Levene (4) considered the name "ribonucleodepolymerase" to be more appropriate for the enzyme since they considered that the enzyme had only a depolymerizing effect on yeast nucleic acid without the production of mononucleotides.

This paper describes the method of preparation as well as some of the properties of a crystalline protein recently isolated by the writer (5) from beef pancreas which acts as a powerful digestive enzyme on yeast nucleic acid. The enzymatic activity of the isolated crystalline protein appears to correspond to the nuclease activity described by the authors mentioned before. The name "ribonuclease" has been provisionally retained for the new crystalline enzyme until definite information becomes available concerning the chemical structure of the split products of digestion of yeast nucleic acid by this enzyme.

Crystalline ribonuclease is a soluble protein of albumin type. Its molecular weight is about 15,000. It contains very little, if any, phosphorus. It yields on hydrolysis tyrosine but not tryptophane. Crystalline ribonuclease is very stable over a wide range of pH. The activity is only very slowly diminished irreversibly when the protein is heated at 100°C. at pH 2.0. Heating at pH 5.0 or higher brings about a gradual denaturation of the protein with a corresponding percentage loss of enzymatic activity.

The digestion of yeast nucleic acid by ribonuclease is accompanied by a gradual formation of free acid groups without any significant liberation of free phosphoric acid. The split products, unlike the undigested yeast

nucleic acid, are not precipitable by glacial acetic acid or by 0.5 M hydrochloric acid. The products of digestion readily diffuse through collodion or cellophane membranes that are impermeable to the undigested yeast nucleic acid. Crystalline ribonuclease does not appear to exert any significant digestive action on thymus nucleic acid.

Dr. R. J. Dubos kindly tested the effect of the new crystalline material on the staining characteristics of two strains of pneumococcus (heat killed). He found "that their staining characteristics are altered after a few hours incubation;" like the material which he described, the new crystalline protein "decreases the affinity of the bacterial cells for basic dyes" (personal communication from Dr. Dubos).

EXPERIMENTAL

I. Method of Isolation of Crystalline Ribonuclease

The method of isolation consists essentially in separating the proteins of an acid extract of fresh beef pancreas by means of fractional precipitation with ammonium sulfate. The bulk of the ribonuclease protein is found in that fraction which is soluble in 0.6 saturated ammonium sulfate and insoluble in 0.8 saturated ammonium sulfate solution. The details of the method are as follows:

1. Preliminary Treatment.—Beef pancreas (about 20 pounds) is removed from the animals immediately after slaughter and immersed at once in enough ice cold 0.25 N sulfuric acid to cover the glands. It can then be stored at 5°C. for a day or so, or worked up immediately. The pancreas is removed from the acid, cleaned of fat and connective tissue, and then minced in a meat chopper. The minced pancreas is suspended in an equal volume of cold 0.25 N sulfuric acid and is stored at about 5°C. for 18–24 hours. It is then strained through cheese cloth. The strained fluid is brought to 0.6 saturation of ammonium sulfate by dissolving 390 gm. of salt in each liter of strained fluid. The mixture is filtered through 50 cm. fluted filter paper (No. 612 Eaton and Dikeman Co., Mt. Holly Springs, Pa., or No. 1450½ Schleicher and Schüll). The clear filtrate (0.6 F) is used for the preparation of ribonuclease while the residue on the paper (0.6 P) can be used for the isolation of chymotrypsinogen, trypsinogen, trypsin, and trypsin inhibitor compound.[1]

The clear filtrate (0.6 F) is brought with solid ammonium sulfate to 0.8 saturation (140 gm. per liter of filtrate) and the precipitate formed is allowed to settle for 2 days in the cold room. The settling is greatly facilitated by occasional stirring and removal of foam during the first day of standing. The clear supernatant fluid is siphoned off and rejected, while the remaining suspension is filtered with suction through hardened paper; yield about 30 gm.

2. Isolation of Ribonuclease Crystals.—Each 10 gm. of the semi-dry precipitate is

[1] The precipitate (0.6 P) is scraped off the filter paper and suspended in about 3 volumes of water. The procedure for further treatment is the same as described by Kunitz and Northrop for the treatment of the original acid extract of fresh beef pancreas in the preparation of chymotrypsinogen etc. (6).

dissolved in 50 ml. distilled water, the pH of the solution is adjusted by means of a few drops of 5 N sodium hydroxide to pH 4.8, and then 50 ml. of saturated ammonium sulfate is added with stirring.[2] The solution is filtered with suction through soft paper with the aid of about 1 gm. of Filter-Cel.[3] The clear filtrate is brought to pH 4.2 (tested with methyl orange) by means of a few drops of 1 N sulfuric acid and then 66 ml. saturated ammonium sulfate is added per 100 ml. of filtrate. The saturated ammonium sulfate is added slowly with stirring. The precipitate formed is filtered with suction through hardened paper; yield about 8 gm. Each 10 gm. of final filter cake is dissolved

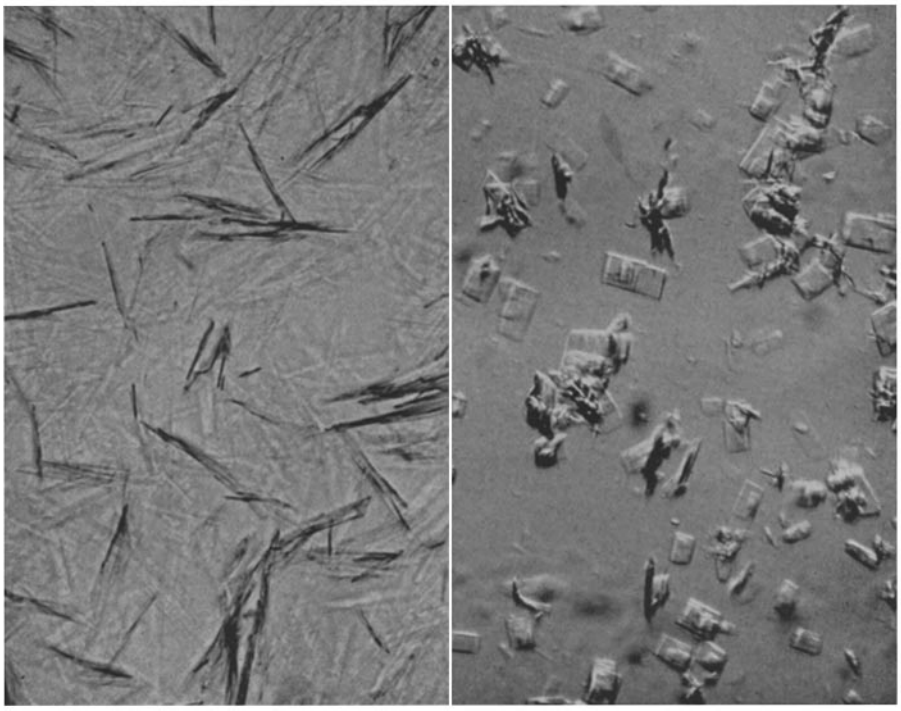

FIG. 1. Crystals of ribonuclease. × 248 and 190

in 10 ml. of water and is refiltered with suction through soft paper on a small Büchner funnel with the aid of 0.5 gm. Filter-Cel. The residue is washed several times with 2–3 ml. water. The combined filtrate and washings are made up with water to a volume of 20 ml. and 7 ml. saturated ammonium sulfate is added with stirring. The clear solution is left at 20–25°C. Crystals of ribonuclease in the form of thin, long plates or fine needles gradually appear (Fig. 1). The crystals are filtered after 2 or 3 days; yield

[2] The pH is determined approximately by mixing on a test plate 1 drop of 0.01 per cent neutralized methyl red (or any other indicator depending on the pH range) with 1 drop of the solution and the color is compared with the color of 1 drop standard buffer solution mixed with the indicator on the plate.

[3] Supplied by Johns-Manville Corporation, New York.

1–2 gm. More saturated ammonium sulfate is added to the filtrate until a slight turbidity is formed. A second crop of crystals appears after several days; yield 2–4 gm.

3. Alternate Method of Isolation of Ribonuclease Crystals.—The following method, described in the preliminary publication, is somewhat simpler in operation but it yields fewer crystals. The 0.25 N acid extract of the minced pancreas is brought to 0.7 saturation with solid ammonium sulfate and filtered. The filtrate is then brought to 0.8 saturation with more ammonium sulfate and is refiltered with suction.

Crystallization.—10 gm. of the semi-dry precipitate is dissolved in about 10 ml. of water. The solution is filtered with the aid of about 0.5 gm. of Filter-Cel through soft filter paper on a small Büchner funnel; the residue on the paper is washed with water. The combined filtrate and washings are brought to a final volume of 20 ml. Saturated ammonium sulfate is then added slowly with stirring until a very faint turbidity appears. The pH of the solution is adjusted first to about pH 5.0 with the aid of a few drops of 1.0 N sodium hydroxide and then to pH 4.2 by means of 1.0 N sulfuric acid. The solution is allowed to stand at about 20°C. An amorphous precipitate rapidly forms. This changes within 1 or 2 days into a mass of fine needles or aggregates of long thin plates. The crystals are filtered after 2 or 3 days. The filtrate on further addition of saturated ammonium sulfate yields more crystals.

4. Recrystallization.—Each 10 gm. of semi-dry filter cake of crystals is dissolved in 20 ml. of water. This solution is filtered with suction through soft paper with the aid of 1 gm. of Filter-Cel. The residue is washed with water. The combined filtrate and washings are made up to 30 ml. with water. 10 ml. saturated ammonium sulfate is added. Rapid crystallization takes place at 20–25°C. The crystals are filtered off after 1 or 2 days; yield about 5 gm. The filtrate on further addition of saturated ammonium sulfate gives more crystals; yield about 2 gm.

5. Recrystallization in Alcohol.—Ribonuclease is readily recrystallizable in dilute alcohol. The material has to be quite pure, however, and salt free. The procedure for crystallization from alcohol is as follows: Ribonuclease is first recrystallized twice by means of ammonium sulfate as described in the preceding section. 10 gm. of the crystal cake from the final crystallization is dissolved in 15 ml. of water and is dialyzed in a collodion bag for 24 hours against cold distilled water by the method of Kunitz and Simms (7). The dialyzed solution is made up with water to 50 ml., is cooled to about 5°C., and then 60 ml. 95 per cent alcohol of the same temperature is added with stirring. A heavy amorphous precipitate is formed which on standing in the cold room changes within several hours into a mass of fine fan shaped rosettes (Fig. 2) of rectangular or needle shaped crystals. The crystals are filtered with suction after 2 days, and washed several times with cold 95 per cent alcohol. They are dried for 24–48 hours in a desiccator over calcium chloride and then in the room for about 24 hours. The dry powder can be stored in a cool place indefinitely; yield is about 3 gm. of dry crystals.

II. Digestion of Yeast Nucleic Acid by Crystalline Ribonuclease

Addition of crystalline ribonuclease to a solution of yeast nucleic acid under appropriate pH and temperature conditions brings about a gradual splitting of the nucleic acid molecules into smaller components. This is shown by an increase in the diffusibility of the nucleic acid. The splitting

of the molecules of yeast nucleic acid by the new enzyme is accompanied by formation of titratable acid groups without the liberation of free phosphoric acid.

1. Diffusion through Collodion or Cellophane Membranes.—The striking difference in the diffusibility through cellophane between digested and undigested yeast nucleic acid is shown in Table I. The split products of digestion diffuse readily through membranes which are practically impermeable to the undigested acid. Similar results were obtained with collodion membranes.

2. Diffusion Measurements.—An approximate estimate of the relative molecular size of the split products as compared with the size of the undigested yeast nucleic acid is conveniently obtained by measuring the diffusion coefficient of the material. The method of Northrop and Anson (8) has been employed for this purpose.

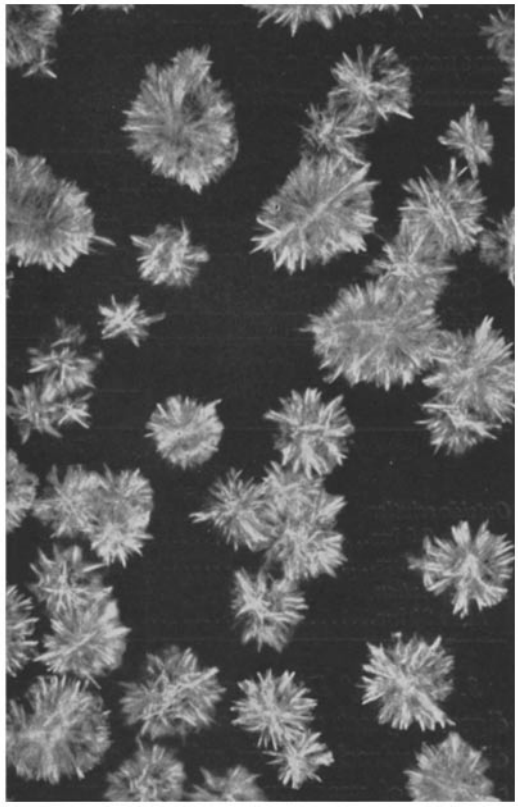

FIG. 2. Crystals of ribonuclease in alcohol. × 190

Experimental Procedure.—50 ml. 6.5 per cent yeast nucleic acid in 0.1 M sodium acetate pH 6.0 and containing 10 mg. of crystalline ribonuclease was left for several days at 5°C. until the maximum amount of digestion was reached. This solution and also a similar solution of nucleic acid but free of ribonuclease were then used for the diffusion experiment. The materials were allowed to diffuse into 20 ml. 0.1 M sodium acetate of pH 6.0 which was changed daily and analyzed for total phosphorus content.

The results are shown graphically in Fig. 3. The data for the diffusion coefficient at 5°C. of the digested as well as of the undigested yeast nucleic acid were plotted against the percentage of the material in the diffusion cell permitted to diffuse into the aqueous solvent. The graphs show that the

diffusion coefficient of the digested nucleic acid is practically constant until 50 per cent of the material has diffused and is numerically twice as great as the diffusion coefficient of the undigested acid.[4] If the assumption is made that the molecules of undigested acid, as well as of the split products, are spherically shaped, then the corresponding molecular volumes are approximately in the ratio of 8:1. The gradual decrease in the diffusion coefficient of the digested nucleic acid shows that the molecules of the split products are not all of the same size. The decrease may be due also to remnants of undigested nucleic acid.

TABLE I

Diffusion through Cellophane

Inside the cellophane bag: About 20 ml. 1.0 per cent yeast nucleic acid in 0.2 M borate buffer pH 7.0 and containing 7 mg. of ribonuclease. Control without enzyme.

Outside the bag: 30 ml. of same buffer solution without nucleic acid.

Left at 20°C. Samples analyzed for total phosphorus concentration.

	Control without enzyme		Digestion mixture	
	a	b	a	b
	Total phosphorus inside originally about 0.7 mg./ml.			
	mg. phosphorus per ml.			
Outside solution				
After 2.5 hrs.	Trace	0.0083	0.166	0.167
After 24 hrs.	0.059	0.089	0.37	0.24
Inside solution				
After 24 hrs.	0.59	0.69	0.32	0.33

3. Effect of Digestion on Precipitation with Glacial Acetic Acid.—Undigested yeast nucleic acid is insoluble in concentrated acetic acid or in dilute hydrochloric or sulfuric acid; hence addition of these acids to a solution of yeast nucleic acid brings about complete precipitation of the nucleic acid. The effect of digestion of yeast nucleic acid by crystalline ribonuclease is to prevent the precipitation by acetic or other acids. This effect is very striking in the case of dilute solutions of yeast nucleic acid. Concentrated solutions of digested nucleic acid continue, however, to give precipitates when mixed with the precipitating reagents even after long digestion with an excess of enzyme. A quantitative study shows that the undigested material which is still precipitable amounts to 10–15 per cent of the total nucleic acid in solution. This may be due to the presence of some modified nucleic acid which cannot be attacked by the enzyme.

[4] Similar results were obtained by Dr. H. S. Loring. Personal communication.

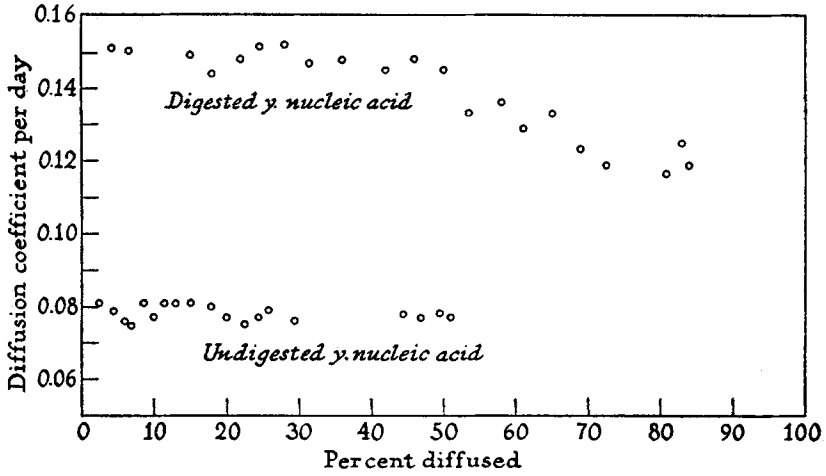

FIG. 3. Diffusion coefficient of digested and undigested yeast nucleic acid in 0.1 M sodium acetate pH 6.0 and 5°C.

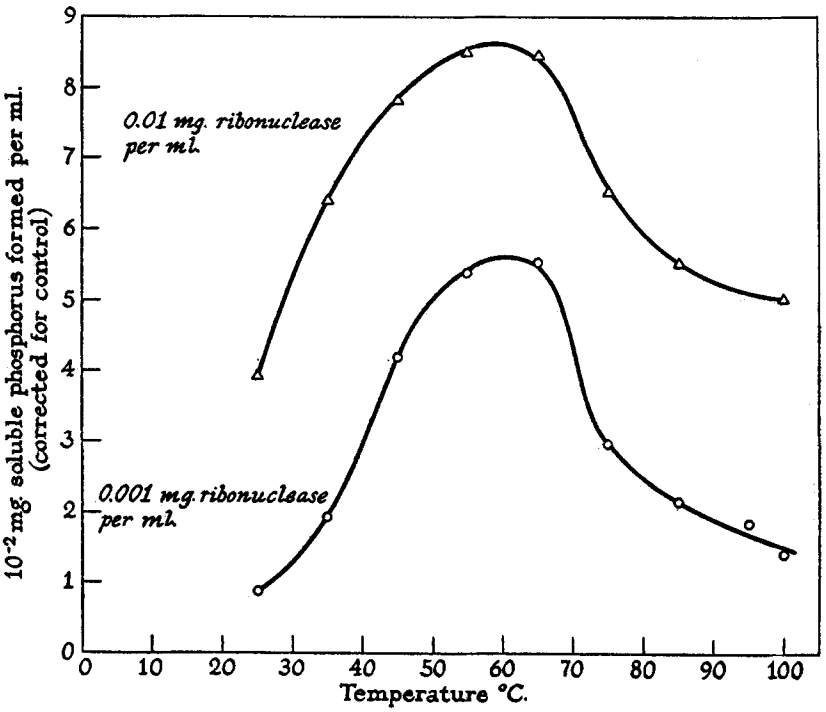

FIG. 4. Effect of temperature on the rate of digestion of yeast nucleic acid by ribonuclease.

(*a*) *Effect of Concentration of Enzyme.*—Tubes containing 0.5 ml. 2 per cent yeast nucleic acid in 0.1 M borate buffer pH 8.0 and 0.5 ml. of various concentrations of ribonuclease in water were placed in a water bath of 25°C. for 10 minutes. 10 ml. of glacial acetic acid was then added to each tube, mixed thoroughly, and filtered after 5 minutes through No. 42 Whatman paper. The total phosphorus per milliliter of filtrate was measured and designated as "soluble phosphorus." The results are given in Table II which shows that addition even of one part of ribonuclease to 2,000 parts of substrate causes formation of soluble phosphorus in 10 minutes at 25°C., equal to 73 per cent of the total phosphorus in the substrate. The ultimate extent of digestion even in the presence of a large excess of enzyme is 87 per cent.

(*b*) *Effect of Temperature.*—The optimum temperature for the rate of digestion as measured by the rate of formation of soluble phosphorus is

TABLE II

Digestion of 1 Per Cent Yeast Nucleic Acid by Crystalline Ribonuclease at 25°C.

Total phosphorus equals 0.75 mg. per ml. digestion mixture.

Mg. ribonuclease per ml. digestion mixture	0	0.005	0.05	0.5	5.0
Mg. "soluble phosphorus" formed in 10 min. per ml.	0.118	0.545	0.605	0.630	0.650
Per cent	16	73	81	84	87

65°C. as shown in Fig. 4. The rapid decline in the rate of digestion at temperatures above 65°C. is probably due to the inactivation of the enzyme.

Experimental Procedure.—Tubes containing 1 ml. yeast nucleic acid (0.5 mg. total phosphorus per milliliter) in 0.1 M acetate buffer pH 5.0 were placed for 3 minutes in water baths of various temperatures, then mixed with 1 ml. ribonuclease solution in water of 25°C. The digestion mixtures were left for 10 minutes at the various temperatures. The digestion was stopped by addition of 2 ml. uranium acetate reagent (see Methods). Control tubes containing mixtures of 1 ml. yeast nucleic acid and 1 ml. water were treated in the same manner as the digestion mixtures.

(*c*) *Effect of pH.*—Fig. 5 shows that ribonuclease acts best in the range of pH 7.0–8.2, the optimum being at pH 7.7.

Experimental Procedure.—1 ml. yeast nucleic acid (0.5 mg. total phosphorus per milliliter) in 0.1 M borate buffer of various pH plus 1 ml. ribonuclease, 0.0013 mg. per ml., in water. Final pH measured by means of a glass electrode. Digested 10 minutes at 25°C., then 2 ml. uranium acetate reagent added.

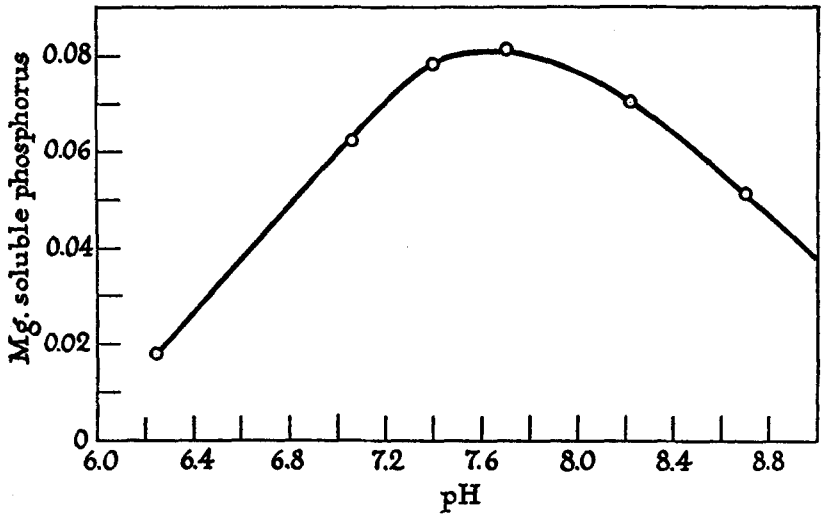

FIG. 5. Effect of pH on the rate of digestion of yeast nucleic acid by ribonuclease

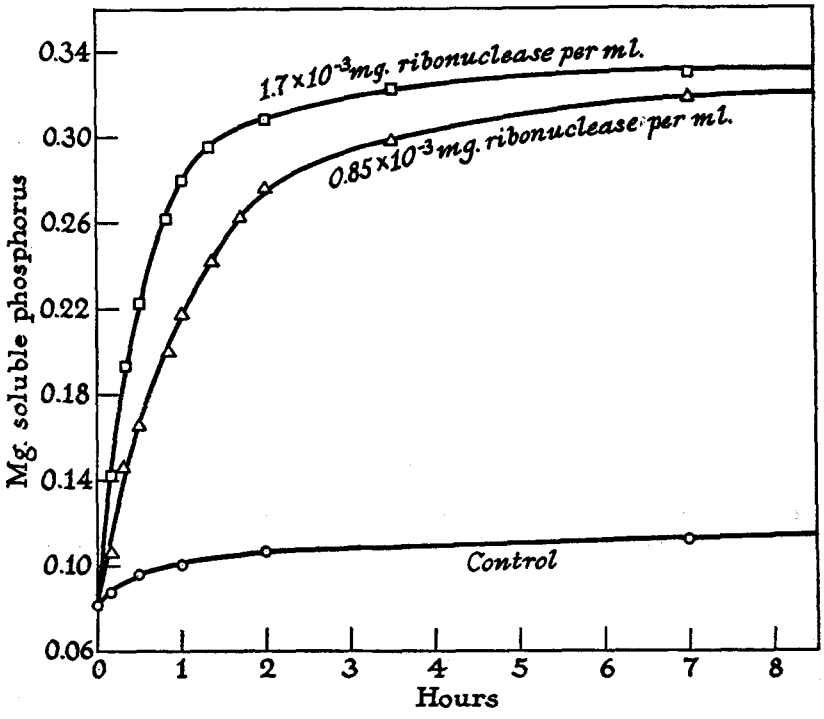

FIG. 6. Effect of concentration of ribonuclease on the rate of digestion of yeast nucleic acid.

(*d*) *Kinetics of the Reaction.*—The digestion of yeast nucleic acid by crystalline ribonuclease when measured by the rate of formation of soluble phosphorus follows the course of a typical enzymatic reaction.

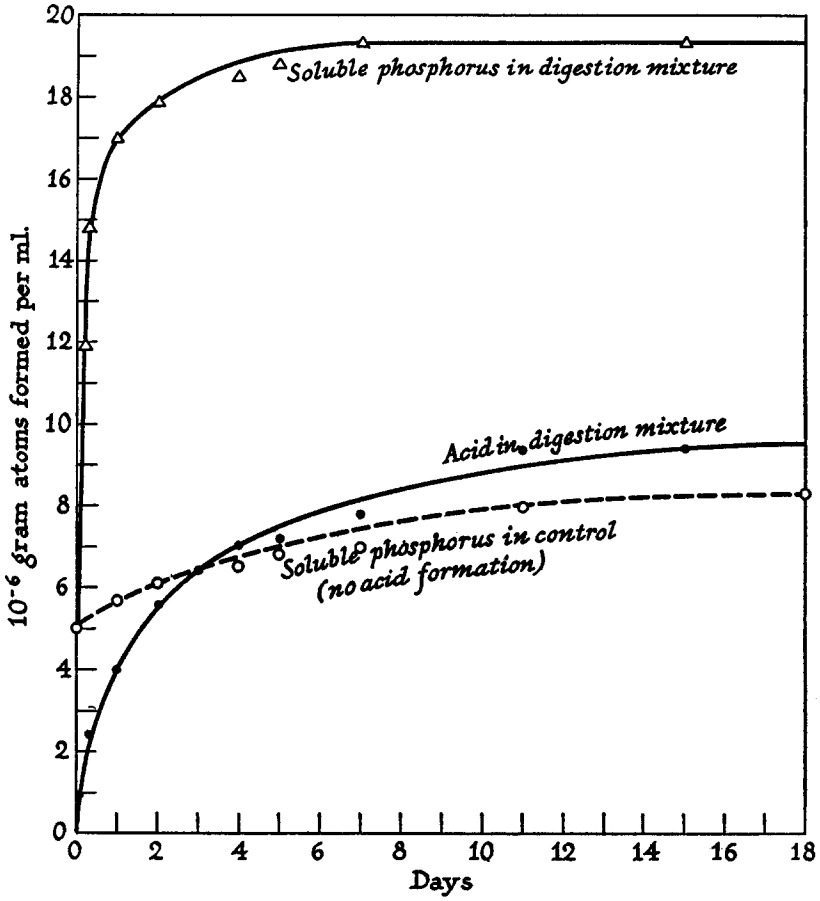

FIG. 7. Rate of formation of free acid

Experimental Procedure.—10 ml. yeast nucleic acid (0.5 mg. total phosphorus per milliliter) in 0.1 M acetate buffer pH 5.0 plus 5 ml. ribonuclease in water. Left at 25°C. Samples of 1 ml. plus 1 ml. 1 M hydrochloric acid, centrifuged after 10 minutes. Total phosphorus in supernatant measured.

Fig. 6 shows the effect of two different concentrations of ribonuclease on the rate of digestion. The time required for any amount of digestion is inversely proportional to the concentration of enzyme in solution while the ultimate amount of digestion is independent of the amount of enzyme used.

A mathematical analysis of the kinetics of the process is complicated by the fact that the enzymatic action is always accompanied by a significant amount of spontaneous hydrolysis of the substrate.

4. Formation of Free Acid.—The digestion of yeast nucleic acid by crystalline ribonuclease is accompanied by a gradual formation of titratable acid groups. The rate of formation of free acid is much slower than the rate of formation of soluble phosphorus as shown in Fig. 7. The ultimate amount of gram atoms of free acid formed is about one-half of the ultimate amount of gram atoms of phosphorus non-precipitable in concentrated acetic acid.

Experimental Procedure.—100 ml. 1 per cent yeast nucleic acid in 0.1 M acetate buffer pH 6.0, plus 2 ml. 0.1 per cent ribonuclease (or 2 ml. water in control). Left at 5°C. Samples of 1 ml. were mixed with 10 ml. glacial acetic acid for measurement of soluble phosphorus. At the same time samples of 5 ml. were pipetted into 50 ml. centrifuge tubes and titrated with 0.02 M sodium hydroxide to a definite pink color using 0.5 ml. 0.1 per cent phenolphthalein as an indicator.

III. Properties of Crystalline Ribonuclease

1. Chemical and Physical Properties.—Table III contains data for the elementary analysis and for other chemical and physical properties of ribonuclease. The material is a protein with a molecular weight of about 15,000.

2. Stability.—An aqueous solution of crystalline ribonuclease is quite stable over a wide range of pH when kept at temperatures below 25°C. Heating to higher temperatures causes gradual loss in enzymatic activity. The rate of inactivation varies, however, with the pH of the solution. The effect of heating solutions of ribonuclease at 100°C. is shown in Table IV. It is evident that ribonuclease is more stable in acid than in neutral or alkaline solutions. The region of maximum stability is between pH 2.0 and 4.5, as shown in Fig. 8.

Experimental Procedure.—Tubes containing 1 ml. 0.01 per cent ribonuclease in water, adjusted with acid or alkali to various pH (measured by a glass electrode) were kept in boiling water. The tubes were removed from the boiling water at various times, plunged for 1 minute in ice water, and left at room temperature for 30 minutes. The cooled solutions were then adjusted by means of acid or alkali to pH 4.0 for activity measurements.

3. Change in Enzymatic Activity with Decrease in Native Protein.—(*a*) *Pepsin Digestion of Ribonuclease.*—Crystalline ribonuclease is readily digestible by pepsin in acid solution.

Experimental Procedure.—Pepsin digestion mixture—0.1 gm. crystalline ribonuclease, plus 9.5 ml. water, plus 0.15 ml. 5 M hydrochloric acid to pH 2.0, plus 1.5 mg. crystalline pepsin; left at 5°C. Samples of 0.5 ml. were brought to pH 9.0 by the addition of 4.5

TABLE III

Chemical and Physical Properties of Crystalline Ribonuclease

Elementary analysis in per cent dry weight (*a*)	C	48.2
	H	6.2
	N	16.1
	S	3.6 (partly inorganic)
	P	Trace
	Residue	0.1
Amino nitrogen as per cent of total nitrogen (*b*)		6.95
Tyrosine equivalent in millimols per mg. total nitrogen (*c*)		5.3×10^{-3}
Tryptophane (*d*)		0
Optical rotation of 5 per cent solution in water $[\alpha]_{28}^{D}$ per mg. nitrogen		−0.47
Molecular weight by osmotic pressure measurement (*e*) at 5°C. of 2.5 per cent solution in 0.5 M and 1 M ammonium sulfate (average of 9 determinations)		15,000 ± 1,000
Diffusion coefficient at 20°C. in 0.5 M ammonium sulfate by the method of Northrop and Anson (8)		0.092 cm.² per day
Molecular volume calculated from diffusion coefficient		14,850
The following measurements were reported by Dr. Rothen (12)		
Isoelectric point by electrophoresis		About pH 8.0
Specific volume at 25°C.		0.707
Sedimentation constant at 25°C. in 0.5 M ammonium sulfate		1.84×10^{-13}
Molecular weight calculated from sedimentation and diffusion data		13,000
Diffusion coefficient in 0.5 M ammonium sulfate at 25°C.		0.116 cm.² per day
Protein tests	Biuret	Positive
	Xanthoproteic	Positive
	Millon	Positive

(*a*) Analyses carried out at the Arlington Laboratories, Arlington, Virginia.

(*b*) Amino nitrogen measured by Van Slyke's manometric method.

(*c*) 1.0 ml. of dialyzed solution containing 0.13 mg. total nitrogen plus 1.0 ml. 1 M hydrochloric acid plus 3.0 ml. water plus 10 ml. 0.5 M sodium hydroxide plus 3.0 ml. of Folin and Ciocalteau's phenol reagent (9) diluted twice with water. Color read after 10 minutes against a similar mixture containing 1×10^{-3} millimols tyrosine.

(*d*) Colorimetric method of R. W. Bates (10).

(*e*) Method of Northrop and Kunitz (11).

ml. 0.2 M borate buffer pH 9.0. This stopped the digestion and also destroyed the peptic activity. The solutions were then analyzed for ribonuclease activity and protein content.

The results, as given in Fig. 9, show that the rate of digestion of ribonuclease protein by pepsin is accompanied by a corresponding percentage loss in the enzymatic activity of the ribonuclease.

(*b*) *Denaturation by Heat.*—The gradual inactivation of ribonuclease when heated at 100°C. is accompanied by gradual denaturation of the protein. The rate of denaturation can be measured by the change in the solubility of the protein in ammonium sulfate solution.

TABLE IV

Inactivation of Ribonuclease at 100°C.

pH.....	2.0 (0.01 M HCl)	3.5	5.8 (0.02 M acetate)	6.6	9.0
Time at 100°C.					
min.			*per cent activity left*		
5	93	95	64	7	0.3
15	87	87	44	2	0
30	78	79	29	1	0

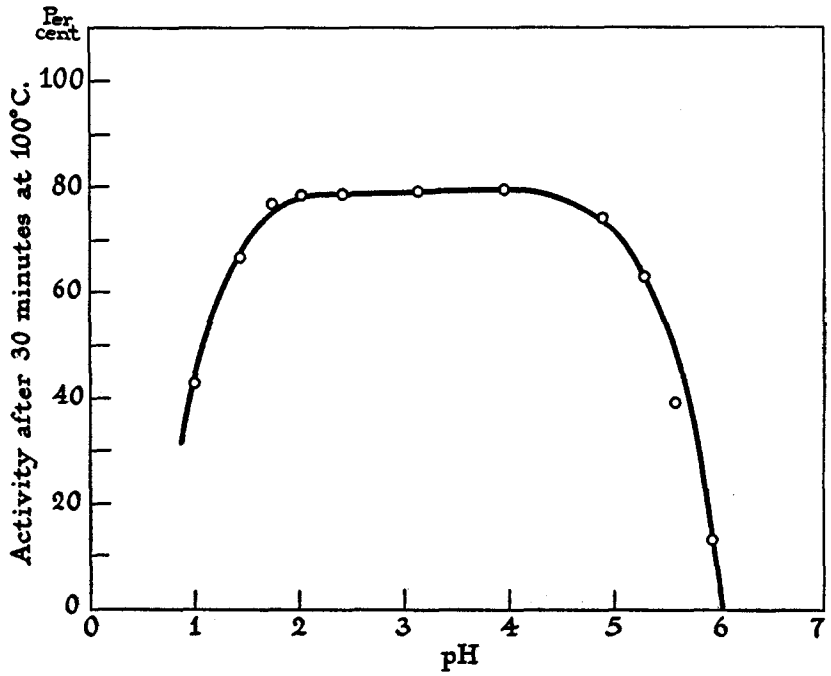

FIG. 8. Effect of pH on stability of ribonuclease at 100°C.

Experimental Procedure.—A series of tubes each containing 4 ml. 0.2 per cent ribonuclease in 0.02 M acetate buffer pH 5.8 was placed in boiling water. The tubes were removed at various intervals of time, cooled rapidly under running cold water, and allowed to stand at 20°C. for 5 minutes. Samples of 1 ml. of the cooled solutions were diluted with 0.01 M acetate buffer pH 4.0 for activity measurement, while samples of 2 ml. of the cooled solutions were mixed with 4 ml. of saturated ammonium sulfate for the

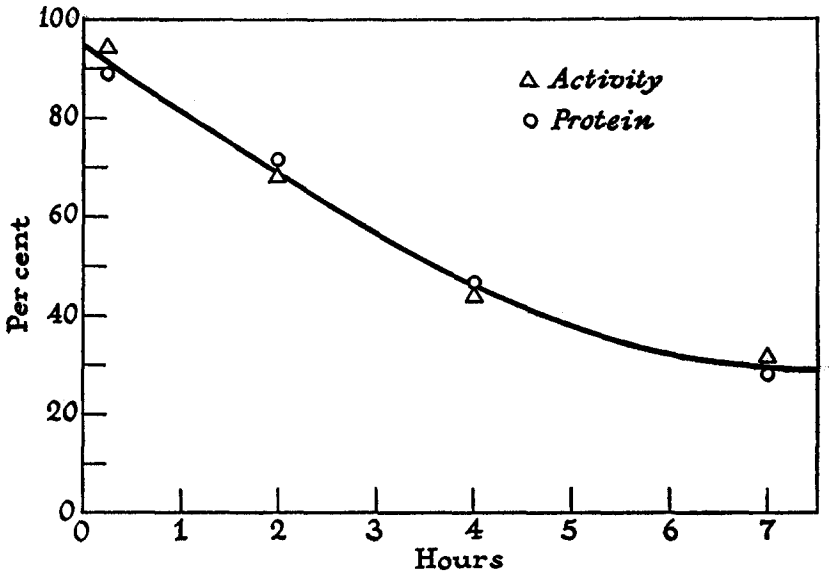

FIG. 9. Digestion of ribonuclease by pepsin

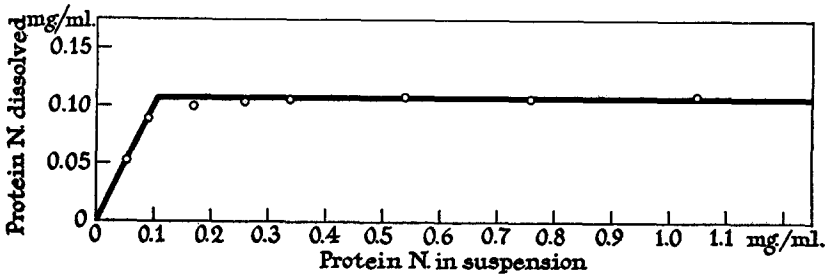

FIG. 10. Solubility of three times crystallized ribonuclease in 0.6 saturated ammonium sulfate pH 4.0 in the presence of increasing quantities of solid phase.

TABLE V

Denaturation of Crystalline Ribonuclease at 100°C.

Time at 100°C.	Activity	Native protein
min.	*per cent*	*per cent*
0	100	100
5	86	83
10	78	77
20	63	61
30	52	48

determination of the amount of denatured protein formed. The unheated sample of ribonuclease did not show any trace of precipitate in the ammonium sulfate mixture while the mixtures of the heated samples with 2 volumes of saturated ammonium sulfate solution gave rise to precipitates of denatured protein. The amount of precipitate formed increased with the time of heating of the sample at 100°C.

The ammonium sulfate mixtures were allowed to stand 5 minutes at 25°C. and then filtered through small No. 42 Whatman filter paper. The concentration of native protein in the filtrates was determined by precipitation in 10 per cent trichloracetic acid.

The results are given in Table V. The experiment shows that the inactivation of ribonuclease at 100°C. is accompanied by a corresponding proportional loss in the concentration of native protein in the ribonuclease solution.

(*c*) *Inactivation by Alkali.*—When ribonuclease is exposed to the action of alkali of pH 12 or higher it gradually loses its enzymatic activity. The loss in activity is also accompanied by a change of the native protein into denatured protein which, like the denatured protein produced by heat, is insoluble in 0.66 saturated ammonium sulfate. Experiments showed repeatedly that the rate of inactivation by alkali is proportional to the rate of change of the native protein into denatured protein.

It is thus evident that changes brought about in the protein molecule by various agents such as heat, alkali, or pepsin, are reflected in every case by a corresponding change in the enzymatic activity of the molecule. This suggests that the enzymatic activity is directly related to the protein molecule.

IV. Tests of Purity of Crystalline Ribonuclease

1. Repeated Crystallization.—Crystalline ribonuclease becomes relatively pure after two or three crystallizations and it retains through further repeated crystallization a constant activity per unit dry weight.

2. Fractional Crystallization.—The material after purification by two or three recrystallizations does not show any difference in the properties of the various crops of crystals obtained through fractional crystallization in various concentrations of ammonium sulfate. The specific activity of the first small crop of crystals does not differ from the specific activity of the succeeding crops and even from the specific activity of the last small amount of material left in solution in the mother liquor.

3. Solubility Test.—The theory as well as the technique of the solubility test for the purity of a protein has been described elsewhere (13). Measurements were made here of the solubility of crystalline ribonuclease in 0.6 saturated ammonium sulfate pH 4.0 in the presence of increasing amounts of crystals of ribonuclease in suspension.

Experimental Procedure.—The material used had been recrystallized three times and then washed several times at 20°C. with 0.6 saturated ammonium sulfate made up in 0.04 M acetate buffer pH 4.0 until the solubility of the crystals in the solvent became constant. Increasing amounts of a concentrated suspension of the crystals in 0.6 saturated ammonium sulfate pH 4.0 were made up in Lusteroid tubes[5] of about 20 ml. capacity, each provided with a Pyrex glass bead, to about 20 ml. with the same solvent. The tubes were stoppered with one-hole rubber stoppers and then plugged with short glass rods so as to remove all the air from the tubes. The suspensions were rocked for 24 hours and then centrifuged in an angular centrifuge[6] for 20 minutes at 3500 R.P.M. All operations, including the centrifuging, were done in a constant temperature room of 20°C. ± 0.5°. Samples of the total suspensions as well as of the clear supernatant solutions were analyzed for activity and protein nitrogen.

The results are shown in Fig. 10. The solid lines represent the theoretical solubility curve of a pure substance. The experimental points fall on the theoretical lines except for one or two points. This indicates the possible presence of a small amount of impurities in the material used. The analytical data for the concentration of protein nitrogen were used. The same result would be obtained if the activity data were used for plotting the curve since the ratio of activity to protein nitrogen was found to be practically constant in all cases.

4. Electrophoresis Test.—Ribonuclease after several recrystallizations does not show the presence of impurities differing in mobility from the bulk of material when tested by electrophoresis (12).

Methods

1. Estimation of Ribonuclease Activity.—Ribonuclease activity is expressed in terms of the rate with which the enzyme changes purified yeast nucleic acid into a form no longer precipitable either by acetic acid, by hydrochloric acid, or by a solution of uranium salt in trichloracetic acid. The last reagent, first suggested by MacFadyen (14), was found to give more reproducible results than acetic or hydrochloric acids. In general the measurements were confined to the initial stage of digestion where the effect is nearly proportional to the concentration of enzyme used. The rate of digestion is determined quantitatively by adding the precipitating agent to samples of the digestion mixture, filtering off the precipitate formed, and finally analyzing the filtrate for total phosphorus.

(*a*) *Precipitation by Uranium Acetate in Trichloracetic Acid.*—Yeast nucleic acid is precipitable from solution when mixed with an equal volume of 0.25 per cent uranium acetate in 2.5 per cent trichloracetic acid. The amount of precipitate is, however, decreased as the nucleic acid is digested by ribonuclease until it reaches a minimum of about 60 per cent in completely digested nucleic acid.

Experimental Procedure.—1 ml. of a solution of yeast nucleic acid (purified as de-

[5] Lusteroid Container Company, South Orange, New Jersey.

[6] Aktiebolaget Winkel Centrifuge, type S. P., Stockholm, Sweden.

scribed later) in 0.1 M acetate buffer pH 5.0 and containing 0.5 mg. total phosphorus is mixed with 1 ml. of ribonuclease solution containing from 0.001 to 0.01 mg. protein nitrogen in 0.01 M acetate buffer pH 4.0. The mixture is left for 10 minutes at 25°C. 2 ml. of uranium acetate is then added from a pipette, thoroughly mixed, left for 30 minutes at 25°C., and filtered through 7 cm. No. 42 Whatman filter paper. 2 ml. of filtrate, which is equivalent to 1 ml. of the original digestion mixture, is analyzed for total phosphorus content. This is designated as soluble phosphorus. The ribonuclease activity unit [N.U.] is defined as the activity which gives rise under these standard conditions to the formation of 1×10^{-3} mg. soluble phosphorus per milliliter of digestion mixture in a range of concentrations of enzyme where the amount of soluble phosphorus formed is proportional to the concentration of enzyme used. For convenience a standard curve is plotted, soluble phosphorus *vs.* [N.U.] from data obtained by measuring the activity of a series of dilutions of ribonuclease of a known enzyme content. The activity of any unknown solution of ribonuclease can then be determined from a single measurement by means of the standard curve. It was found generally that pure ribonuclease contains about 1000 [N.U.] per mg. protein nitrogen. This value varies considerably, however, with the sample of yeast nucleic acid used. The method for the measurement of activity was found also to be very sensitive to slight changes in pH of the substrate, as well as to the age of the solution. Hence, fresh solutions of nucleic acid have to be used and the pH carefully adjusted in order to obtain more or less reproducible results.

(*b*) *Precipitation by Glacial Acetic or Hydrochloric Acid.*—The procedure is the same as described in (*a*) except that 20 ml. of glacial acetic acid or 2 ml. 1.0 M hydrochloric acid instead of 2 ml. of uranium acetate reagent is added for the precipitation and the suspensions filtered after standing 5–10 minutes instead of 30 minutes.

The precipitation by acetic acid or by hydrochloric acid was found to be affected greatly by the presence of traces of ammonium sulfate and, in general, is less reproducible than the precipitation by the uranium acetate reagent.

2. Purified Yeast Nucleic Acid.—Commercial preparations of yeast nucleic acid were purified by reprecipitation with glacial acetic acid.

Experimental Procedure.—A suspension of 100 gm. commercial yeast nucleic acid in 500 ml. water was cooled in an ice water bath to about 2°C. 5 N sodium hydroxide was then added slowly until a clear solution was obtained. Care was taken to keep the solution cold and the pH not in excess of 6.0, as tested colorimetrically on a test plate. The volume of the solution was measured, 5 volumes of glacial acetic acid added, and the whole allowed to stand at 20–25°C. for 10 minutes. The precipitate formed was then filtered with suction on a large funnel, washed twice with about 100 ml. of water, and three times with 95 per cent alcohol. The dry precipitate, about 80 gm., was resuspended in 400 ml. water and treated as in the first precipitation. The final precipitate was washed with water, alcohol, and finally with ether, and dried in the air to constant weight. Final yield about 60 gm.

3. Total Phosphorus.—The colorimetric method of Fiske and SubbaRow (15) as modified by King (16) has been used.

4. Protein Nitrogen.—The ribonuclease protein was precipitated in 10 per cent trichloracetic acid. The amount of protein nitrogen was determined either by the turbidity method (17) or by the Kjeldahl nitrogen method (18).

The writer was assisted by Margaret R. McDonald and Vivian Kaufman.

SUMMARY

1. A crystalline enzyme capable of digesting yeast nucleic acid has been isolated from fresh beef pancreas.

2. The enzyme called "ribonuclease" is a soluble protein of albumin type. Its molecular weight is about 15,000. Its isoelectric point is in the region of pH 8.0.

3. Ribonuclease splits yeast nucleic acid into fragments small enough to diffuse readily through collodion or cellophane membranes.

4. The split products of digestion, unlike the undigested yeast nucleic acid, are not precipitable with glacial acetic acid or dilute hydrochloric acid.

5. The digestion of yeast nucleic acid is accompanied by a gradual formation of free acid groups without any significant liberation of free phosphoric acid.

6. Ribonuclease is stable over a wide range of pH even when heated for a short time at 100°C. Its maximum stability is in the range of pH 2.0 to 4.5.

7. Denaturation of the protein of ribonuclease by heat or alkali, or digestion of the protein by pepsin, causes a corresponding percentage loss in the enzymatic activity of the material.

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