PURIFICATION AND PROPERTIES OF DESOXYRIBONUCLEASE ISOLATED FROM BEEF PANCREAS*

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The isolation of crystalline ribonuclease by Kunitz (1) provided a specific enzymatic reagent which has been widely used in the study of ribonucleic acid. Our interest in obtaining a purified preparation of the corresponding enzyme acting on nucleic acid of the desoxyribose type arose in connection with studies on the chemical nature of the substance inducing transformation of pneumococcal types. Highly purified preparations of the transforming substance isolated from Pneumococcus Type III have been shown to consist largely, if not exclusively, of desoxyribonucleic acid, and evidence has been obtained which indicates that the nucleic acid is itself responsible for biological activity (2). Since it seemed desirable to provide confirmatory evidence regarding the nature of the transforming substance by the use of specific enzymatic techniques, a study of the enzyme which attacks native desoxyribonucleic acid was undertaken, especially with a view to obtaining the enzyme in purified form.

The enzyme which breaks down highly polymerized desoxyribonucleic acid has been known for many years under various names. In 1903, Araki (3) reported that crude preparations of trypsin and erepsin brought about liquefaction of gels of α-thymonucleate prepared according to the method of Neumann (4). Although Araki interpreted the effect as being proteolytic in nature, his experiments represent the first observations on the enzymatic breakdown of this type of nucleic acid. Two years later Sachs (5) demonstrated that liquefaction of the nucleate gel by pancreatic extracts is caused by an enzyme distinct from trypsin, and further presented evidence indicating that trypsin rapidly inactivates the nuclease. De la Blanchardière (6) confirmed the findings of Sachs, and made several unsuccessful attempts to separate the nuclease from the inactivating tryptic enzymes. This writer also devised a more quantitative method of measuring the nuclease activity by following the fall in viscosity of a dilute solution of thymonucleate during digestion with the enzyme.

All of the early workers were somewhat handicapped by the poorly defined character of the substrate used in the enzyme studies. Neumann's method of obtaining α-thymonucleate from the thymus gland depended on the use of heat and alkali,

* The Bureau of Medicine and Surgery of the U. S. Navy does not necessarily undertake to endorse the views or opinions which are expressed in this paper.

which inevitably resulted in a partial breakdown of the material, so that the final preparation consisted of a mixture of hydrolytic products. Experimental demonstration of this partial destruction has been provided by the ultracentrifuge studies of Schmidt, Pickels, and Levene (7) who showed that nucleic acid of the Neumann type sediments with a very diffuse boundary, and that the estimated particle weight varies from 50,000 to above 1,000,000.

The first studies in which a more suitable substrate was used were those of Greenstein and Jenrette (8) who employed sodium thymonucleate prepared by the method of Hammarsten (9). This method avoids the use of drastic reagents, and all procedures are carried out at 0°C. and at neutrality. The preparations so obtained are considerably more homogeneous and highly polymerized than those obtained by Neumann's method. Greenstein and Jenrette were primarily interested in the comparison of the nuclease activity of normal and cancerous tissues, and they investigated a wide variety of organs for the presence of the enzyme, which they now refer to as desoxyribonucleodepolymerase (10). The activity of the enzyme was determined by measuring the progressive fall in viscosity of the nucleate solution when mixed with tissue extracts. It was shown that the enzyme is present in a large number of organs, as well as in the milk and sera of certain mammalian species.

In the several studies mentioned, as well as in others, such as those of Feulgen (11), in which the enzyme was used in investigating the end products of digestion of nucleic acid, relatively crude tissue extracts were employed as the source of the enzyme. In the present paper a method is described for the purification of this enzyme, which for purposes of convenience will hereinafter be referred to as desoxyribonuclease. In addition, certain observations are reported on the chemical properties and kinetics of action of the purified enzyme. The effect of the purified enzyme on the biological activity of the pneumococcal transforming substance will be the subject of a separate communication (12).

EXPERIMENTAL

Measurement of Desoxyribonuclease Activity

Source of Enzyme.—The results of previous investigators indicate that pancreas is by far the richest source of desoxyribonuclease available. Commercial pancreatin was used early in the present study, and was found to provide highly active enzyme preparations. However, attempted fractionation of pancreatin was not particularly successful, and the use of this type of material was discontinued in favor of fresh beef pancreas.

Substrate.—Sodium desoxyribonucleate was prepared from calf thymus by the method described by Mirsky (13). In this procedure, minced tissue is thoroughly washed with 0.14 M NaCl and then extracted with 1 M NaCl to obtain the nucleohistone. Purification of the nucleohistone is achieved by repeated precipitation at a concentration of 0.14 M NaCl, and resolution in 1 M NaCl. The histone is then removed by shaking the solution with chloroform and amyl alcohol, the process being repeated until no further chloroform-protein gel is formed. Deproteinization is considerably facilitated by first dissociating the nucleic acid and histone by the addi-

tion of alkali to the 1 M NaCl solution (14). The criterion for dissociation is the lack of formation of a fibrous precipitate when a sample of the solution is diluted with six volumes of water. The deproteinized sodium desoxyribonucleate is precipitated by alcohol, washed with absolute alcohol and ether, and dried *in vacuo*.

A characteristic sample of sodium desoxyribonucleate prepared by this method has a phosphorus content of 8.12 per cent and nitrogen content of 13.5 per cent (nitrogen-phosphorus ratio 1.66). The material dissolves readily in water to give clear, highly viscous solutions which may be stored in the refrigerator for long periods of time without loss of viscosity. Even at higher temperatures, *e.g.* 37°C., there is no apparent loss of viscosity over a period of several days. The fact that the nucleate solutions show little evidence of spontaneous depolymerization enhances the reliability of a method of measuring enzymatic breakdown based on decrease in viscosity.

Method of Measuring Enzyme Activity.—Measurement of the progressive fall in viscosity of nucleate solutions during treatment with the enzyme has yielded constant and reproducible results and has proved a reliable method for determining enzymatic activity. The test is carried out as follows: In all the experiments recorded in this paper Ostwald viscosimeters with a uniform capillary size, giving a flow time for water of from 80 to 100 seconds have been used. 4.8 cc. of a 0.05 to 0.1 per cent solution of sodium desoxyribonucleate in veronal buffer pH 7.5 are introduced into the viscosimeter, which is then placed in a water bath at 30°C. The concentration of substrate is selected so as to give an initial viscosity of not over 3 to 4 times that of water, in order that the rate of flow—and thus the time required for each reading—will not be too greatly prolonged. To 4.8 cc. of substrate, 0.2 cc. of the enzyme dilution is added at zero time, thoroughly mixed with the substrate, and readings of viscosity are made at intervals over a period of about 30 minutes. The enzyme solution under test is diluted so as to give a rate of fall in viscosity that is constant for at least 10 minutes.

The use of a constant external pressure in measuring the viscosity of desoxyribonucleate solutions has been emphasized by Greenstein (8) because of the structural viscosity displayed by these solutions. Under the conditions of the present experiments, in which dilute solutions of desoxyribonucleate are used, the application of an external pressure of 16 cm. H_2O was found to have little effect on the slope of the viscosity curves or on the reproducibility of the results, and consequently in the interest of simplicity external pressure was not employed in the routine procedure.

Magnesium Activation.—In the course of preparative procedures it was observed that the activity of desoxyribonuclease is greatly reduced following dialysis, and that original activity can be completely restored by the addition of magnesium ion to the dialyzed material. Thus, the enzyme is apparently among those requiring a metallic activator. Manganese can replace magnesium and is equally effective at an equivalent molar concentration. Zinc also activates slightly but is less effective than magnesium or manganese. Calcium and iron have no activating effect.

The optimum concentration of magnesium ion is in the order of 0.003 M, and as a routine procedure the enzyme dilutions are made in 0.075 M $MgSO_4$ so

that the final concentration in the viscosimeter is 0.003 M (*i.e.*, after adding 0.2 cc. of the enzyme dilution to 4.8 cc. of substrate).

Effect of Varying Enzyme Concentration.—In initial studies it was found that the effect of varying enzyme concentration on the rate of fall in viscosity of the substrate was irregular and unpredictable. Thus, there appeared to be no regular relationship between the concentration of enzyme and the rate of

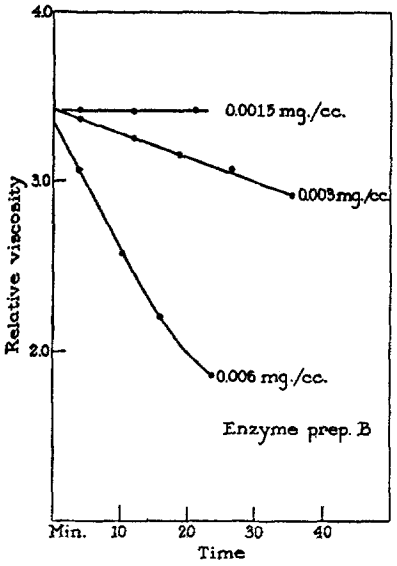

FIG. 1

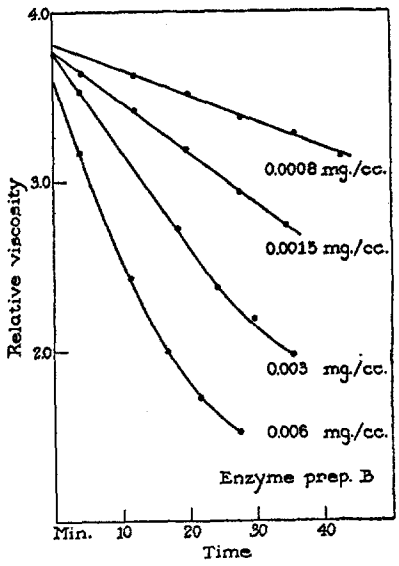

FIG. 2

FIGS. 1 and 2. Effect of varying the enzyme concentration and the use of gelatin as a protective agent. Fig. 1 shows the discrepancy in proportionality between the concentration of enzyme and the rate of fall in viscosity in the absence of a protective agent. Fig. 2 illustrates an experiment carried out under the same conditions, except that gelatin was added to the system in a final concentration of 0.01 per cent. In this case a direct relationship is shown between the concentration of enzyme and the rate of fall in viscosity.

reaction, and it was not possible to relate the activity of one enzyme preparation to another on the basis of rate of fall in viscosity. A typical experiment is illustrated in Fig. 1. Twofold decreases in enzyme concentration were made. In decreasing the final concentration from 0.006 mg. per cc. to 0.003 mg. per cc. there resulted a fivefold decrease in the slope of the viscosity curve, and with a further twofold decrease in enzyme concentration activity was no longer detectable. It was thought that this discrepancy in results was probably explicable on the basis of denaturation of the enzyme protein in dilute solu-

tion, and that the addition of a protective colloid to the system might prevent inactivation of the enzyme. Since gelatin has been used successfully as a protective to prevent or retard loss of activity in the case of other enzymes, such as tyrosinase (15) and ascorbic acid oxidase (16), its effect was tested in the desoxyribonuclease system. An experiment in which gelatin was used as protective agent is recorded in Fig. 2. The enzyme preparation and concentrations were the same as those of the preceding experiment (Fig. 1.) The enzyme diluent contained, in addition to the $MgSO_4$, gelatin in a concentration

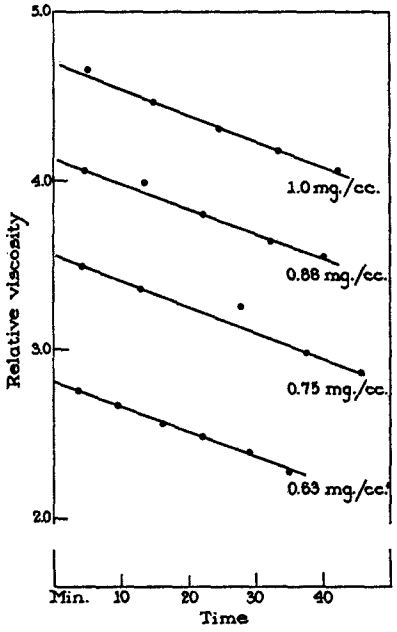

FIG. 3. Effect of varying the concentration of the substrate, sodium desoxyribonucleate.

of 0.25 per cent. The final concentration of gelatin in the viscosimeter was therefore 0.01 per cent, and this amount of gelatin exerted no measurable effect on the viscosity of the substrate. The effect of gelatin in protecting the enzyme was striking. In contrast to measurements carried out without gelatin (Fig. 1), twofold reductions in enzyme concentration resulted in a corresponding twofold decrease in the slope of the viscosity curve (Fig. 2).

Thus in a system containing gelatin the inactivation of the enzyme is sufficiently reduced so that a direct relationship is shown between the concentration of the enzyme and the rate of fall in viscosity, and it is possible to characterize the activity of a given enzyme preparation from the slope of a single curve.

In some later experiments 1 per cent neopeptone has been used in place of gelatin. Identical results are obtained in terms of activity, and indeed very dilute solutions of the enzyme retain activity for longer periods of time in neopeptone than in gelatin. (See below.)

Effect of Varying the Substrate Concentration.—The effect of varying the substrate concentration was studied only within a relatively narrow range in which the viscosity of the solution allowed rapid measurement of enzyme activity. An experiment in which the same enzyme preparation was tested against sodium desoxyribonucleate in concentrations of 0.63, 0.75, 0.88, and 1.0 mg. per cc. is illustrated in Fig. 3. The initial relative viscosity varied from below 3 to almost 5. The slopes of the curves at the various concentrations are identical, showing that the rate of fall in viscosity is independent of the substrate concentration within these narrow limits. Thus, under the conditions of the test, the concentration of the enzyme is the limiting factor in determining the rate of fall in viscosity.

In summary, the activity of the enzyme is tested against a 0.05 to 0.1 per cent solution of sodium desoxyribonucleate in M/40 veronal buffer pH 7.5 at 30°C. in the presence of 0.003 M magnesium and 0.01 per cent gelatin. For the purpose of comparing the activity of various preparations, one unit of the enzyme has been defined as that amount which causes a fall of 1.0 in relative viscosity in 20 minutes under the conditions described.

Isolation and Purification of the Enzyme

The major difficulty encountered in purification of desoxyribonuclease was the marked susceptibility of the enzyme to destruction by the action of proteolytic enzymes in the source material. Thus, pancreatic extracts with extraordinarily high desoxyribonuclease activity were readily obtained by extraction of fresh pancreas with water, but the activity was rapidly lost as the proteolytic enzymes became activated. When fractionation procedures were attempted, solutions of the partially purified material proved to be unstable because of the contaminating tryptic enzymes. Therefore, although pancreas is the richest source of desoxyribonuclease, it has the disadvantage of having at the same time high concentrations of proteolytic enzymes, which in turn digest the active nuclease.

This difficulty has been largely overcome by the use of the acid extract method described by Kunitz and Northrop (17) for the preparation of crystalline chymotrypsin and trypsin from beef pancreas. Fractionation of the acid extract results in more effective separation of the nuclease from the proteolytic enzymes, and a simple procedure for obtaining desoxyribonuclease in a purified form has been devised.

Following the procedure of Kunitz and Northrop, fresh beef pancreas is obtained at the slaughter house and immediately immersed in cold 0.25 N H_2SO_4 to retard

autolytic processes during transportation. The pancreas is passed through a meat grinder, and the ground tissue suspended in two volumes of cold 0.25 N H_2SO_4. Extraction is allowed to proceed in the refrigerator overnight, and the suspension is then filtered through cheesecloth. The residue is reextracted with one volume of 0.25 N H_2SO_4, and immediately filtered through cheesecloth. The combined filtrates are brought to 0.2 saturation with ammonium sulfate by the addition of 114 gm. of solid

TABLE I

Preparation of Desoxyribonuclease

Fraction	Amount	Total activity Units	Units per mg. protein*
Beef pancreas Extracted with 0.25 N H_2SO_4. Filtered through cheesecloth	10 pounds		
Acid extract Brought to 0.2 saturation with solid $(NH_4)_2SO_4$. Filtered with aid of filter cel	9 liters	3×10^7‡	
Filtrate at 0.2 saturation $(NH_4)_2SO_4$ Brought to 0.4 saturation with $(NH_4)_2SO_4$	9 liters	3.5×10^7	450
Precipitate at 0.4 saturation Refractionated twice between 0.17 and 0.3 saturation $(NH_4)_2SO_4$	20 gm. (semidry)	3×10^7	5000
Final precipitate at 0.3 saturation	3–4 gm. (semidry)	2×10^7	7–10,000
Dried enzyme	1–2 gm.	$1–2 \times 10^7$	7–10,000

* Protein estimated colorimetrically with phenol reagent using method of Herriott (18).

‡ The value for total activity of the acid extract is consistently lower than that of the filtrate at 0.2 saturation with ammonium sulfate. This may possibly be explained on the basis of an inhibitor present in the initial extract which is lost on fractionation.

salt per liter of filtrate. Ten gm. of filter cel and 10 gm. of standard super cel are added for each liter of filtrate, and filtration is carried out with suction on a large Büchner funnel. The precipitate is discarded. The clear, straw-colored 0.2 saturated filtrate is brought to 0.4 saturation by the addition of 121 gm. of solid ammonium sulfate per liter, and the resulting precipitate is allowed to settle for 1 or 2 days in the refrigerator. The slightly turbid supernatant is siphoned off as far as possible, and the precipitate recovered on hard filter paper (Schleicher and Schull No. 275). The precipitate at 0.4 saturation contains practically all of the desoxyribonuclease. Chymotrypsinogen, trypsinogen, and ribonuclease are present in the filtrate, which

can be subjected to further fractionation procedures, as described by Kunitz and Northrop (17) and by Kunitz (1) to obtain these enzymes in crystalline form.

The precipitate at 0.4 saturation is redissolved in a small volume of water (50 cc. to each 10 gm. of moist precipitate) and brought to 0.17 saturation by the addition of 20 cc. of saturated ammonium sulfate solution to each 100 cc. of enzyme solution. A small amount of precipitate forms, which is removed by filtration after stirring in 2 gm. of standard super cel for each 100 cc. of solution. The filtrate is brought to 0.3 saturation with ammonium sulfate, and the precipitate formed is recovered on hard paper. This precipitate is dissolved in water and refractionated between 0.17 and 0.3 saturation with ammonium sulfate. Precipitation of the enzyme at the relatively low concentrations of ammonium sulfate used is dependent upon the pH and concentration of the solution. The pH of the solutions from which the enzyme is precipitated at 0.3 saturation is 4.0–4.5, and if the pH is increased to 7 or above, considerably larger amounts of salt are required to bring down the desoxyribonuclease.

The final 0.3 saturated precipitate is dissolved in a small volume of water (10 to 20 cc.) and dialyzed in the cold against 0.002 N H_2SO_4. The dialyzed solution is then dried *in vacuo* from the frozen state. A summary of the method of preparation, giving average values for the amount and activity of the various fractions is presented in Table I.

The yield of dried enzyme from 10 pounds of pancreas is 1 to 2 gm. Numerous attempts have been made to crystallize this purified product, but none has so far been successful.

Properties of the Purified Enzyme

General Properties.—The activity of the dried enzyme is well preserved on storage. The dried material dissolves readily in water to yield clear, colorless solutions with a pH between 4 and 5. The enzyme has its maximun stability in this pH range, so that aqueous solutions are relatively stable. At higher pH values, and especially above pH 7, loss of activity is much more rapid, but this instability may be chiefly the result of the action of small amounts of proteolytic enzymes still present in the purified preparations.

Highly dilute solutions of the enzyme are unstable even in the presence of gelatin, and in order to obtain reproducible results it is necessary to measure activity immediately after preparation of the dilution. Increasing the amount of gelatin has not served to augment significantly the stability of the dilute enzyme solutions, but as mentioned above, the activity of the enzyme is remarkably well preserved in complex mixtures such as neopeptone. Because of its complex nature and variable constitution, peptone cannot be considered ideal for this purpose, but it has proved very useful in those cases where stable dilutions are required.

The results of preliminary studies in the electrophoresis apparatus indicate that the isoelectric point of the enzyme is from pH 5.0 to 5.2. One major

electrophoretic component is present with a small amount of at least one other component.[1]

Specific Activity on Sodium Desoxyribonucleate.—Purified preparations display high activity as measured by the fall in viscosity of desoxyribonucleic acid. As indicated in Table I, the dried material contains approximately 10,000 units per mg. of protein. Thus, 0.1 microgram of the enzyme represents one unit. A definite effect can be demonstrated with enzyme concentrations of less than 0.01 microgram per cc.

The measurement of activity of the dried enzyme is illustrated in Fig. 4. An aqueous solution containing 5 mg. of enzyme per cc. was diluted 100-fold

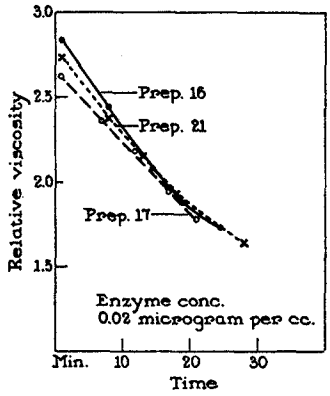

FIG. 4. Activity of purified preparations of desoxyribonuclease.

in water in a volumetric flask. From this dilution a further 100-fold dilution (final 1:10,000) was made in the gelatin-$MgSO_4$ diluent. 0.2 cc. of this dilution (containing 0.1 microgram of enzyme) was mixed with the substrate as described under measurement of activity. The curves obtained with three separate preparations of the enzyme are recorded in Fig. 4, and serve to demonstrate the uniformity in activity of different lots prepared by the method described.

Action of the Purified Enzyme on Substrates Other Than Desoxyribonucleic Acid.—The purified preparations were tested for enzymatic activity on a variety of substrates other than desoxyribonucleic acid in an attempt to obtain information as to the completeness of the separation from other constituents of the pancreatic extracts. Proteolytic activity was measured using gelatin and casein as substrates. The effect on the viscosity of 2.5 per cent

[1] The author is indebted to Lieutenant Commander Vincent P. Dole (MC) USNR, for carrying out the electrophoresis experiments.

gelatin at pH 7.5 was employed as a method of detecting minute amounts of proteolytic enzyme. One to two mg. of purified desoxyribonuclease caused no fall in the viscosity of 5.0 cc. of the gelatin solution, even after attempts to activate the enzyme solution, on the assumption that inactive precursors of the proteolytic enzymes (trypsinogen or chymotrypsinogen) might be present. When 5 mg. of the enzyme were used, a slight effect was noted, corresponding to the effect given by 0.0005 mg. of crystalline trypsin when tested in the same system.

At concentrations of 0.2 to 1.0 mg. per cc. the enzyme also caused definite proteolysis of a 2 per cent solution of casein (pH 7.8) as measured by the increase in tyrosine not precipitable by trichloracetic acid. In general, casein seemed to be much more readily attacked than gelatin. These findings indicate that a small amount of some proteolytic enzyme is present in the purified preparations.

The effect of the enzyme on ribonucleic acid was determined by using the method of Kunitz (1) for measuring ribonuclease activity. A 0.5 per cent solution of yeast nucleic acid was treated for 2 hours (at 37°C. and pH 5.0) with 5 mg. of the enzyme, with no resultant increase in acid-soluble phosphorus. The results indicate that desoxyribonuclease has no action on ribonucleic acid, and this is in conformity with the observations of others on the specificity of ribonuclease, which in turn has no action on desoxyribonucleic acid.

The purified enzyme was also tested for lipase activity using tributyrin as substrate, and for alkaline phosphatase using sodium-β-glycerophosphate. In neither instance was any evidence of splitting detected. It is concluded that the purified preparations do not contain significant amounts of lipase, phosphatase, or ribonuclease.

Optimum pH.—The optimum pH for the action of the enzyme covers a broad range from 6.8 to 8.2. The viscosimetric method is not suitable for measurement of activity below pH 6, because in the acid range non-enzymatic depolymerization of the substrate occurs.

Heat Liability.—In contrast to ribonuclease, which is remarkably heat-stable, desoxyribonuclease is readily inactivated by heat. Experiments carried out with 0.5 per cent solutions of desoxyribonuclease in N/100 HCl, in acetate buffer pH 5.0, and in veronal buffer pH 7.8 gave comparable results. In each case, heating at 55°C. for 15 minutes resulted in a loss of 90 per cent or more of the original activity, and after 1 hour no residual activity was detectable.

Inhibition of Desoxyribonuclease.—In the course of studies on the pneumococcal transforming substance, it was observed that crude preparations of desoxyribonuclease from diverse sources were inhibited by sodium fluoride (2). It is now apparent that fluoride inhibition is probably related to the fact that the enzyme is magnesium-activated, since other magnesium-activated

enzymes have been shown to be inhibited by fluoride. A relatively high concentration of fluoride (0.02 to 0.1 M) is required for effective inhibition of desoxyribonuclease under the conditions of the test, and further study of the inhibition is not readily adaptable to the viscosimetric method because the precipitate of magnesium fluoride which is formed interferes with accurate measurement of viscosity. It has not been determined whether phosphate plays an important rôle in fluoride inhibition as it does in the case of the magnesium-activated enolase studied by Warburg and Christian (19).

Citrate forms a soluble complex with magnesium similar to that which it forms with calcium. Because of this fact sodium citrate was tested for its effect on enzyme activity and was found to be a potent inhibitor. In 0.01 M concentration sodium citrate exerts a profound inhibitory effect on the mag-

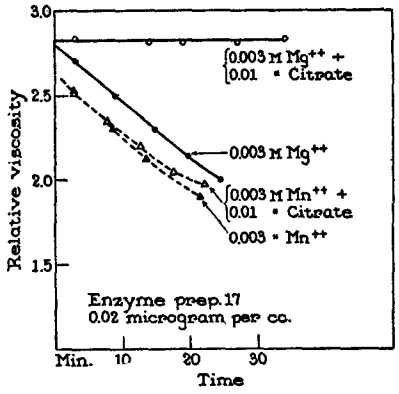

FIG. 5. Effect of citrate on magnesium-activated and manganese-activated desoxyribonuclease.

nesium-activated enzyme even when relatively high concentrations of the enzyme are used. It is of interest, however, that citrate has no such inhibitory effect when manganese is used as the metallic activator. The results of an experiment showing the relationship between the nature of the metallic activator and citrate inhibition are given in Fig. 5. The same enzyme concentration was used in each determination. It is evident that manganese and magnesium ion at 0.003 M concentration activated the enzyme equally. In the presence of 0.01 M sodium citrate, the magnesium-activated enzyme was completely inhibited, while there was no appreciable effect on the manganese-activated enzyme.

Citrate has a similar marked inhibitory action on desoxyribonuclease in crude tissue extracts, and it thus seems likely that magnesium is the naturally occurring activator of the enzyme. In view of this fact, sodium citrate may prove useful as an inhibitor of desoxyribonuclease in biological material. It

has been found, for example, that the enzymatic breakdown of desoxyribonucleic acid which takes place during the autolysis of pneumococci can be completely prevented by citrate without otherwise affecting the course of lysis (20)

Immunological Data.—Attemps were made to prepare antisera to desoxyribonuclease by injecting rabbits intravenously with solutions of the enzyme protein. However, after repeated injections by the intravenous route, the sera of the treated animals showed neither precipitins nor inhibitory action on the enzyme. On the contrary, potent precipitating antisera were obtained by the intraperitoneal injection of alum-precipitated enzyme.

A 1 per cent solution of enzyme (preparation 16) was mixed with an equal volume of 10 per cent potassium alum. The pH of the solution was brought to slightly above 7 with N NaOH with the formation of a voluminous precipitate. The suspended precipitate was injected intraperitoneally without washing or centrifugation. Each of two rabbits received a single injection of 10 mg. of enzyme protein by this route. The sera obtained from these animals 1 week later possessed a high titer of specific precipitins. A second intraperitoneal injection of 50 mg. of alum-precipitated enzyme 2 weeks later resulted in a considerable increase in antibody titer.

In Table II are recorded qualitative precipitin tests with the homologous antigen as well as with crystalline preparations of ribonuclease, chymotrypsin, and trypsin which were prepared from a similar acid extract of beef pancreas.[2] The sera were diluted with 1.5 volumes of saline and mixed with an equal volume of the antigen dilutions in saline. Readings were made after incubation at 37°C. for 2 hours and refrigeration overnight.

The data in Table II show that desoxyribonuclease reacts with its antiserum in dilutions beyond 1:1,000,000, indicating a high degree of serological activity for a protein antigen. On the other hand, the crystalline preparations of ribonuclease, chymotrypsin, and trypsin show negligible cross-reactions with the desoxyribonuclease antiserum. Thus, although the desoxyribonuclease is produced by the same organ that elaborates ribonuclease and tryptic enzymes, there is no evidence from the results of precipitin tests that would suggest a serological relationship on the basis of organ or species specificity. These results are in accord with the findings of TenBroeck (21), who showed that trypsin and chymotrypsin from the same species could be distinguished by means of the anaphylactic test. Thus, the incomplete evidence suggests that each of several enzymes making up a part of the external secretion of the same organ have a distinct serological specificity as well as enzyme specificity.

Evidence for the presence of a species-specific antibody in the desoxyribonuclease antisera is afforded by the fact that precipitates are obtained when beef

[2] The samples of crystalline enzymes were obtained through the courtesy of Dr. John H. Northrop and Dr. M. Kunitz of The Rockefeller Institute for Medical Research, Princeton, New Jersey.

serum is used as antigen. However, the amount of precipitate obtained is relatively small. All the cross-reacting precipitins can be removed by adsorption with beef serum, with only a slight decrease in the titer of antibody specifically reactive with the purified enzyme. It is likely that the beef serum reactive antibody is formed as the result of the presence of extraneous protein in the desoxyribonuclease preparation used as immunizing antigen. In this connection it is of interest that antisera prepared against crystalline ribonuclease by Smolens and Sevag (22) showed no evidence of cross-reaction with beef serum. The possibility of cross-reactivity with trypsin and chymotrypsin was not tested by these authors.

TABLE II

Precipitin Tests with Antidesoxyribonuclease Rabbit Serum

Antigen	Rabbit serum	Final dilution of antigen					Saline
		1:10,000	1:30,000	1:100,000	1:300,000	1:1,000,000	
Desoxyribonuclease (preparation 16)	Normal	−	−	0	0	0	−
	Immune	++++	++++	+++	+++	++	−
Ribonuclease (crystalline)	Normal	−	−	0	0	0	
	Immune	−	−	−	−	−	
Trypsin (crystalline)	Normal	−	−	0	0	0	
	Immune	−	−	Trace	Ft. trace	−	
Chymotrypsin (crystalline)	Normal	−	−	0	0	0	
	Immune	−	−	V. ft. trace	V. ft. trace	−	

++++ = marked precipitation with clear supernatant.

− = no precipitation.

0 = indicates that reactions were not carried out at higher dilutions of antigen with serum of the same rabbit, obtained before immunization was begun.

Effect of Specific Antibody on Enzyme Activity.—The specific antisera were tested for their effect on the ezymatic activity of desoxyribonuclease. The viscosimetric tests for nuclease activity were carried out as previously described, except that in this instance the final dilution of the enzyme was made in the presence of the antiserum under test, rather than in the gelatin diluent.

A 5 mg. per cc. solution of enzyme (preparation 16) was diluted 1:1000 in the gelatin diluent. A further 1:5 dilution of this solution was made in normal and immune rabbit serum in concentrations of 5 and 20 per cent. The serum dilutions contained 0.075 M $MgSO_4$. After 15 minutes at room temperature, 0.2 cc. of the serum-enzyme mixtures was tested for activity in the usual system of buffered desoxyribonucleate with a final volume of 5.0 cc. The final concentrations of serum

were therefore 0.8 per cent and 0.2 per cent, and the final concentration of enzyme was 0.04 micrograms per cc. The tests were carried out in the range of antibody excess, and no visible immune precipitate appeared in either the final enzyme dilution or in the viscosimeter. The results are recorded in Fig. 6.

The presence of 0.8 per cent immune serum resulted in almost complete inhibition of the enzyme as compared with the activity of the same enzyme concentration in the presence of 0.8 per cent normal serum. When the serum concentration was reduced fourfold, the effect was less marked, but about 60 per cent inhibition was obtained. Similar results were obtained on repeated test, and also with serum adsorbed with beef serum, and it would appear that

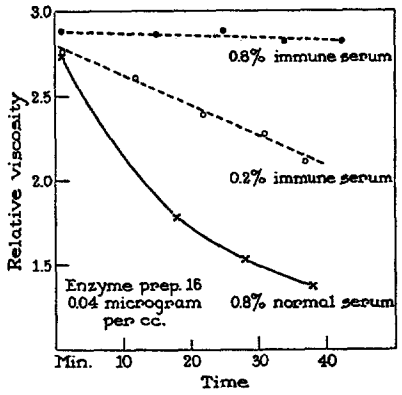

FIG. 6. Effect of specific antibody on the enzymatic activity of desoxyribonuclease.

under the conditions described, desoxyribonuclease falls in that group of enzymes which are inhibited by their specific antibodies.

DISCUSSION

The present paper describes the purification and properties of an enzyme isolated from beef pancreas which attacks native desoxyribonucleic acid. This desoxyribonuclease has been obtained in a highly active and relatively purified form, but has not been crystallized. The enzyme is activated by either magnesium or manganese, and its activity is dependent upon the presence of one or the other of these ions in the system.

Previous workers have characterized the action of the enzyme as a depolymerization, since no release of inorganic phosphorus or other readily dialyzable substance occurs, and the end products of the reaction are apparently relatively large in size. Notwithstanding the fact that the enzyme causes only a limited breakdown of the nucleic acid molecule, its primary action is essential for further degradation of this substance by other enzymes. For example, Schmidt,

Pickels, and Levene (7) showed that the high polymer desoxyribonucleic acid prepared by the Hammarsten method was not dephosphorylated by a purified intestinal nucleophosphatase, whereas the latter enzyme could split off phosphorus from the more degraded form of nucleic acid as prepared by Neumann or Levene. After pretreatment of the Hammarsten nucleic acid with pancreatin, until it no longer gave a fibrous precipitate with alcohol, the material was readily dephosphorylated. Thus, enzymatic depolymerization was required before the dephosphorylating enzyme could act.

More recently Wilson (23) has found that strains of hemolytic streptococcus which require certain purines for growth *in vitro* are unable to utilize highly polymerized desoxyribonucleic acid as a source of these purines. However, the same preparation of nucleic acid after hydrolysis by purified desoxyribonuclease supplies the essential growth factor. The streptococcus apparently lacks an enzyme analogous to desoxyribonuclease, but is capable of metabolizing the units which represent the end products of action of this enzyme.

In the light of these and other similar observations, it seems apparent that desoxyribonuclease is a true "nuclease" in that it splits the intact nucleic acid molecule and must act before further enzymatic degradation can occur. For this reason, and in the interest of brevity, the name desoxyribonuclease has been used in this paper in preference to the term desoxyribonucleodepolymerase suggested by Greenstein.

For the purpose of standardizing the conditions, all the experiments described in the present paper were carried out with the highly polymerized form of desoxyribonucleic acid obtained from a single source, calf thymus. However, the enzyme appears to attack indiscriminately desoxyribonucleic acid from a variety of animal and bacterial cells. Similar preparations of desoxyribonucleic acid from beef spleen, shad sperm, and pneumococci are broken down at the same rate and under the same conditions which obtained in the case of calf thymus nucleic acid.

At present there has been no effective procedure developed for removing the traces of proteolytic enzyme which are present as contaminant in the desoxyribonuclease preparations. Fortunately, the activity of the nuclease is sufficiently great so that it may be diluted far beyond the range of detectable proteolytic activity. Thus, while the proteolytic activity of the best preparations can be demonstrated only when 1 mg. per cc. of the enzyme is used, the desoxyribonuclease is active at concentrations below 0.01 microgram per cc. The disproportion between the two types of enzymatic activity is more than adequate to allow practical use of the nuclease under conditions in which proteolytic activity is totally absent. For example, 0.01 mg. per cc. final concentration of the enzyme causes almost instantaneous loss of viscosity of a desoxyribonucleate solution, but at this concentration none of the ordinary protein substrates is attacked.

Detailed investigations of the enzyme in the electrophoresis apparatus or ultracentrifuge have not been carried out, and it is hoped that greater purification of the enzyme can be accomplished by means of crystallization before study of the physical properties or molecular size is attempted. The degree of purification of desoxyribonuclease achieved in the present work, together with the information obtained concerning the properties of the enzyme, is sufficient to provide the specific enzymatic reagent that was sought for use in the study of the transforming substance of Pneumococcus.

SUMMARY

1. A method is described for the isolation and purification of desoxyribonuclease from a 0.25 N sulfuric acid extract of beef pancreas. The activity of the enzyme is measured by a viscosimetric method using sodium desoxyribonucleate from calf thymus as substrate.

2. The enzyme is highly active, a measurable effect being obtained at concentrations of less than 0.01 microgram per cc. In highly dilute solution the enzyme is rapidly inactivated, and the use of a protective agent such as gelatin or peptone is necessary.

3. The purified material contains traces of a proteolytic enzyme, but displays no ribonuclease, lipase, or phosphatase activity.

4. The enzyme requires activation by magnesium or manganese ion, and citrate serves as a potent inhibitor of the magnesium-activated enzyme.

5. Its enzymatic activity is inhibited by the specific antibody present in the serum of rabbits immunized with enzyme protein.

Addendum.—After the present work was finished and the manuscript completed, a paper came to our attention which describes the purification of the same enzyme under the name thymo-polynucleotidase (Fischer, F. G., Böttger, I., and Lehmann-Echternacht, H., *Z. physiol. Chem.*, 1941, **271,** 246). Although the paper has not been available locally, due to the international situation, a photostatic reproduction has been obtained. The German workers prepared the enzyme from both dried and fresh pancreas. One of the methods described involves ammonium sulfate fractionation of an acid extract of beef pancreas, and in general the procedure is similar to the method presented above, except in the final steps of purification. It is impossible to compare the activity of their preparations with those made in this Laboratory, however, because entirely different methods of measuring activity are employed. The fact that magnesium ion is essential for the action of the enzyme is pointed out, and it is stated that arsenate and arsanilic acid inhibit completely at 10^{-3} M concentration. These workers demonstrated that their preparations had no activity on a variety of substrates other than nucleic acid, including gelatin, ribonucleic acid, nucleotides, nucleosides, and several organic phosphates.

In the light of this article, part of the present paper represents independent confirmation of the work of Fischer *et al.*

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